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USAMRMC ltr, 17 Dec 2001

AD_____

GRANT NUMBER DAMD17-94-4313

TITLE: Characterization of CTL Recognized Epitopes on Human Breast Tumors

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REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1996	Annual (19 Aug 95 - 18 Aug 96)

4. TITLE AND SUBTITLE
Characterization of CTL Recognized Epitopes on Human Breast Tumors

5. FUNDING NUMBERS
DAMD17-94-J-4313

6. AUTHOR(S)
Constantin G. Ioannides, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas
Houston, Texas 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Sep 96). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012 (ATTN: MCMR-RMI-S).

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200)*

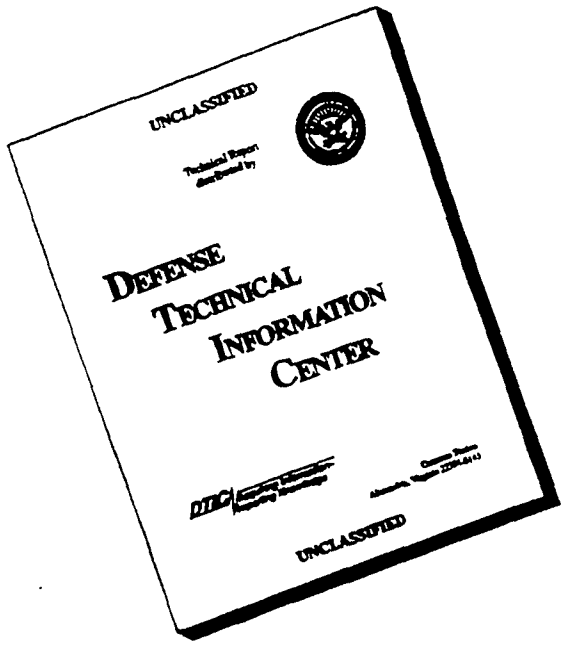
The overall objective of this proposal is to develop novel therapeutic approaches to breast cancer by understanding the molecular and cellular basis of tumor antigen (Ag) recognition by cytotoxic T lymphocytes (CTL). Characterization of HLA-A2 bound peptides by comparison of CTL activity and signal intensity of peptides by mass-spectrometry has lead to identification of a novel tumor Ag, defined by a peptide epitope from the amino-enhancer of split (AES) protein. This protein is part of the "Notch" complex involved in regulation of cell fate during development. This Ag is recognized both by breast and ovarian tumor reactive cell lines. Studies on HER-2 tumor Ag identification and epitope optimization have also led to design of a novel clinical trial with the peptide E75: HER-2 (369 - 377) for patients with breast and ovarian cancer.

To address the significance of these epitopes we are currently exploring the ability of the corresponding peptides to induce tumor specific immunity.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	245
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

DISCLAIMER NOTICE



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Constantin A Joyamides 9/14/1988

PI - Signature Date

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PROGRESS REPORT:

Introduction

Development of biological therapies for cancer in recent years has generated new hopes that improved cancer cure rates can be achieved beyond what is currently obtained with combinations of chemotherapy and radiation therapy. The biological therapies use peptide based tumor Ag and cytokines (or their genes) with the objectives: **(1)** to augment Ag presentation to anti-tumor effectors, **(2)** to ensure full activation of anti-tumor immunity and **(3)** to achieve optimal proliferation and expansion of specific anti-tumor effector T cells (1).

Since tumor antigens are short peptides generated from self-proteins (2), to address the first objective, ongoing studies need to accomplish two major tasks: **(a)** to characterize the tumor Ag either by mapping with synthetic peptides or by sequencing of the naturally processed peptides presented by the tumor (1, 2), and **(b)** to optimize the epitope sequence for binding to the Ag presenting molecules (which are the human histocompatibility antigens A, B, C - HLA, A, B, C), and for enhanced recognition by the corresponding receptor (the T cell antigen receptor, TCR) of the effector cytotoxic T lymphocyte (CTL) (3).

The need for these studies is dictated by a number of factors which are: **(a)** transfer of viable/irradiated tumor cells in a human organism raises serious concerns regarding transfer of genetic material to the recipient. This genetic material (DNA, RNA) contains oncogenes which may induce metaplastic on neoplastic tranformation of healthy tissues. Thus peptide alone, or genes encoding the peptides of interest substituting for the tumor may provide new approaches to immuno- or gene therapy that circumvent this problem. **(b)** therapy with cytokines alone or with live cells transduced with cytokine genes has not been shown to induce systemic anti-tumor immunity (reviewed in 4, 5). Of additional concern is the possibility that systemic administration of cytokines alone may activate primarily auto-immune reactions and processes which may not be directed to the tumor. Effective activation of naive CD8+

cells requires co-stimulation e.g. through the B7-CD28 pathway (6-8). However the co-stimulatory molecules B7.1/B7.2 are up-regulated by cytokines (9, 10). Thus, focused cytokine secretion after specific and well defined cellular interactions may provide a more suitable approach to control the activation of anti-tumor effectors. This can be accomplished by using as "activators" the T helper cells (Th) of Th1 memory phenotype (6). These Th1 cells can recognize the tumor Ag in the context of major histocompatibility Ag class II (i.e. HLA-DR, HLA-DQ) and secrete a specific pattern of cytokines: IFN-γ, IL-3, TNF-β, which are involved in the maturation and effector function of cellular immune cytotoxic effectors such as CTL (11). **(c)** The epitopes defined on tumor Ag are self-peptides of significantly lower binding affinity for both HLA and TCR of the responding CTL. Thus, their efficiency for tumor specific CTL induction will be low.

The need for epitope sequence optimization is dictated by the need to increase the binding affinity to the MHC class I. This will consequently increase the stability of the peptide-MHC complexes with consequent increase of their immunogenicity and their density. Stable, high density peptide-MHC class I complexes are capable of inducing activation of the naive CD8+ CTL without additional co-stimulatory signals (3). This should be important for induction of anti-tumor immunity because: **(a)** epithelial tumors do not express co-stimulatory molecules (i.e of the B7, CD40 families); **(b)** effective expression of co-stimulatory molecules requires an induction process which is dependent on additional complex interactions of antigen presenting cells (APC, i.e. macrophages (Mo) and/or dendritic cells (DC) with Th cells + cytokines) (9).

These studies were performed with model foreign antigens known to be immunogenic in the system to be tested. To address these questions for tumor Ag which are self-antigens, it is of paramount importance to first identify the active epitope. Recently we found an immunodominant epitope from the HER-2/neu (HER-2) proto-oncogene defined by the peptide E75: HER-2 (369-377), which is a target of tumor specific CTL in both breast and ovarian cancer (12, 13) (please see also the previous progress report). The significance of this

epitope was later confirmed in studies by other investigators (14). Those studies utilized transgenic mice to confirm the immunogenicity and test the in vivo stability of the peptide E75. The investigators found that this peptide was immunogenic in vivo, demonstrating that it is both stable (not degraded by proteases before uptake by APC) and non-toxic.

Based on our preliminary work and findings, the peptide E75 will be tested in a first phase Ib clinical trial for an equal number of breast and ovarian cancer patients (please see attachments, Appendix).

The gene for HER-2 is overexpressed in approximately 50% of breast cancer patients. Overexpression of HER-2 provides a mechanism for novel tumor specific epitope expression because both healthy tissues and a number of tumor cells do not overexpress this protein. This raises the need for identification of additional epitopes that can target CTL effectors to HER-2^{lo} tumors or tumor variants that arose from an immunoselection mechanism, i.e. $E75^{-}$ tumor cells can escape the attack of E75 specific CTL, and are at a proliferative advantage. With this objective, studies in the current funding period have advanced the analysis and characterization of the ion 793 (identification reported during the previous period) (15).

The results presented in the following sections **(Body of the Report and Appendix)** show that peptide 793 is part of the AES (Amino enhancer of split) protein (15, 16). This protein is part of the "Notch" complex involved in determination of cell fate during cell and organ differentiation (18). This is important because proteins of the "Notch" complex have been reported to be expressed in breast and ovarian cancer cells (18), and they apparently function by maintaining the undifferentiated state.

Body of the Report

The purpose of the present work continues to be the characterization of the functional significance of the CTL epitopes as potential antigens for targeted immunotherapy. The studies during this granting period focused on the well defined tasks. We developed HLA-A2 transfected breast tumor lines such as SkBr3.A2 (13, 15). This transfected line is currently used for large scale tumor growth and Ag characterization. An important factor in characterization of naturally processed peptides is the definition of conditions for separation of active peptides from the inactive peptides of the same length. To address this issue, we developed a set of successive gradients of acetonitrile (ACN) (19, 20). Thus, peptides extracted from immunoaffinity purified HLA-molecules were separated through a first gradient and the position of the active peaks was identified. A second gradient was modelled for each fraction using a shallow gradient of ACN and a much longer column for high pressure liquid chromatography (HPLC). The successful establishment of these conditions was tested using a freshly isolated ovarian ascitic tumor (because of the larger number of tumor cells that can be recovered compared with primary breast tumors) which we compared with the ovarian cell line SKOV3.A2. This novel methodology was effective in defining the overlapping and non-overlapping epitope repertoire between a tumor line and a freshly isolated tumor of which part is expressed on breast tumor cells (19).

These studies employed contemporary methods for analysis of peptides present in small amounts, i.e. mass spectrometric analysis of the epitope abundance and density. For sequence identification, we employed collision-induced-dissociation (CID) to break the peptide 793 into smaller ions. For sequence analysis we employed the computer program PEPSEQ, as well as comparision of mass-spectra of the unknown peptide 793 with those of synthetic peptides of known sequence that are candidates for reconstitution.

Detailed methods are presented in the attached manuscript (20), and in the attached invention disclosure report.

To accomplish the same proposed tasks and objectives, we have found that optimization of the epitope presentation can be accomplished by sequence optimization(previous year report). This study has now been published in *The Journal of Immunotherapy.* This approach will be used for sequence optimization of both epitopes 793 and E75. In fact, the validity of this approach has been proven during the analysis of the optimal sequence of the epitope 793: GPLTPLPV. We found that introduction of Leu (P2) as the main anchor and extention of the sequence at P1 with Alanine leads to sequences which are recognized by the tumor reactive CTL (15). This article together with recent papers in the field (21) suggest that this novel approach can be used for tumor Ag optimization.

The research performed during the last twelve months has made significant progress towards the goals of this study and the overall goal of developing specific immunotherapy for breast cancer. The results obtained are as follows:

(1) Studies on the immunodominant epitope of HER-2, peptide E75 (369-377) have advanced to the design of a first clinical (phase Ib) study in patients with breast and ovarian cancer. The Chairman of this Study is Dr. James Lee Murray, Professor and Chief of Section of Bioimmunotherapy, Breast Cancer Service, Division of Medicine, M.D. Anderson Cancer Center. Dr. J. Taylor Wharton is co-investigator in this study, while myself am Study Co-Chairman. The protocol has been approved by the Institutional Review Board (IRG) for Clinical Trials in August 1996. A copy of this protocol is attached. Results showing recognition of E75 by breast CTL are enclosed. **Please note that this information is, at this time, confidential and proprietary.** From this study, we expect to learn how the patients respond to vaccination with specific epitope targets of tumor reactive CTL.

(2) Studies on the characterization of the Ag repertoire in breast cancer have lead to identification of a novel tumor Ag. Preliminary results indicated the presence of the ion 793 (please see previous report). We have successfully sequenced this ion and found that it is part of the amino-enhancer of split (AES) protein which is a member of the "Notch" complex involved in determination of cell fate. Detailed description of methodology and sequences is made in the appended Invention Disclosure Report.

This novel Ag provides both a complementary and alternative target to HER-2 for breast cancer therapies. This epitope was recognized to the moment by 4/4 HLA-A2+ breast CD8+CTL lines tested and may reflect an antigen that is more widely expressed than HER-2. Results of this work have been presented at the Annual FASEB Meeting in the Symposium on Tumor Antigens (The corresponding abstract is attached).

The following peptides were found to be recognized (1) G75: GPLTPLPV, (2) G76: LPLTPLPV, (3) G60: ALPLTPLPV, and (4) ALALPLTPL. G75 is the naturally processed peptide, G76, and G60 are the optimized octa- and nona-peptides. It should be noted that G60 binds to HLA-A2 with significantly higher affinity than the other peptides. The fact that G61 is also recognized indicates that CTL recognize the core PLTPL in all the peptides. An invention disclosure report regarding the possible cancer therapies with this Ag has been submitted to the Office of Technology Development of M.D. Anderson. The support from the Breast Army Cancer Program is underscored. A copy of this report is attached. **Because of the proprietary nature of the information in this report regarding methods of procedure, aims and specific sequences (wild-type and optimized) we would appreciate if this information is kept confidential at this time. The abstracted material has been published and can be disseminated.** Because of the regulations regarding patient submission, we are now preparing the manuscript regarding the AES epitope for submission.

(3) Studies reported under the points (1) and (2) above have been performed with breast associated CTL lines using as targets peptides isolated and defined from antigenically similar ovarian tumor lines. To further advance our studies on the nature of the breast cancer antigen new approaches were needed to be developed for immunoaffinity isolation on large scale, HPLC separation, and mass-spectrometric analysis. The objective difficulty encountered in these studies is that breast tumors (especially the primary) are small. This is because, as a result of increased awareness, breast cancer is now detected earlier than in the previous years. To address this question, we developed an alternative approach. This consists of fractionation under strictly defined conditions of the antigenic peptides from ovarian tumors. Detailed methodological information is presented in the two Appended manuscripts No. 3 and No 6. The breast CTL were not stimulated or activated with these target tumors used for fractionation. Thus the Ag (epitope) repertoire of the CTL was not biased. In the second phase both breast and ovarian CTL are used to identify the antigenic epitopes from the fresh tumors. The epitopes are compared (for retention time, shape of the peak of elution, pattern of CTL activity) with the peptides extracted from a tumor line. The overlapping (coincidental) epitopes can be then extracted from the tumor line, which offers an unlimited source of further epitope characterization. We have completed the study of the ovarian indicator SKOV3.A2 cells, and we are now completing study of its breast counterpart, the SKBR3.A2 cell line. The abstracted material is not proprietory and can be disseminated.

(4) The results of the studies on optimization of HER-2 peptide sequences have been published and reprints of this work are attached (J. Immunotherapy).

(5) In addition to the significant effort in the direction of novel antigen identification, we have advanced our studies on the second task of this proposal regarding Ag presentation. During this period we have established

and optimized the approaches for characterization of transporter associated proteins (TAP) and of deficiencies in Ag presentation. These studies used isolation of mRNA (messenger RNA), reverse transcription to complementary cDNA, amplification using polymerase chain reaction (PCR), Southern blotting and computer-assisted scanning densitometry. We have started by comparing the TAP1 and TAP2 expression. To standardize our approaches we have identified a melanoma tumor which shows defective Ag presentation. The SkBr3 ovarian tumor cell line was tested in parallel **(Figure 2)**. This experimental system can determine the presence of peptide transport deficiencies with high sensitivity. Results of this study have been reported. Abstracted material is attached.

(6) Studies performed on the second task have focused on characterization of the effects of a novel multidrug resistance (MDR) reversal agent (N-myristoylated PKC-α pseudosubstrate) peptide on the reversal of MDR. These studies were performed using as targets breast cancer cells - MCF-7 human cancer line. These studies were conducted by Dr. Catherine A. O'Brian who is an investigator on this research project. These studies found that PKC pseudo-substrate peptides inhibit the PKC-α catalyzed P-glycoprotein phosphorylation. These results have been published in *The Journal of Biological Chemistry*. Reprints are attached (Appendix No. 4). This will allow continuation of studies proposed under the second task, i.e. characterization of the effects of drug resistance on the sensitivity to lysis and recognition by tumor specific CTL. We are now selecting the SkBr3.A2 cell line with Adriamycin to generate drug resistant variants of this breast cancer line. A recent study indicated that drug resistance can induce immunological resistance (22).

(7) To address the questions raised under the third task of these studies, we have begun to isolate, expand, and characterize breast reactive CTL clones. To focus the research, studies during this period have isolated CTL clones from the breast tumor associated line BrCTL-10, which recognizes peptides from the

newly identified tumor antigen AES-1/2 (125-137). We have isolated such CD8+ CTL clones by limiting dilution cloning and expansion in vitro. A number of clones preferentially recognize the peptide G60: ALPLTPLPV, while others preferentially recognize the peptide ALALPLTPL **(Figure 3)**. These peptides share the sequence ALPLTPL. Since different clones show different sensitivity to shifting the epitopes, 2 amino acids to N or C-terminal end, this provides an excellent model to investigate the use of the same or different T cell receptors.

Conclusions

The completed research during the last two years has made, we believe, significant contributions to the field of breast cancer immunology. *First*, studies in the first year of award have identified an immunodominat epitope E75 from the HER-2 which is preferentially targeted by the tumor reactive breast CTL. The peptide reconstructing this epitope now forms the basis of a new clinical trial (phase 1b) in patients with breast and ovarian cancer. Thus, the first group of findings reported last year will be soon translated to the clinic. *Second*, our findings on optimization of peptide binding and presentation led to novel approaches for CTL epitope mapping (which were used to map the optimal epitope of the ion 793). This strategy is currently used for induction of tumor specific CTL. It should be mentioned that we have identified an analog of the peptide E75, designated E75-Y8. In this peptide the Phenylalanine at position 8 was replaced by Tyrosine. The analog E75-Y8 is recognized better than E75 by the CTL effectors. Now this peptide is being tested for CTL induction. *Third*, a novel tumor Ag has been found. This epitope is derived from the AES protein, a member of the Notch complex. Characterization of its pattern of recognition by breast CTL is progressing. This epitope will provide both an alternative and complementary strategy for active immunotherapy for patients with breast cancer. This is an important finding, because Muc-1 and HER-2 are as yet the only antigens that have been identified to be recognized by breast tumor reactive CTL. *Fourth*, accomplishment of the second task has established the methodology for

quantitation of TAP1 and TAP2 expression. In addition our collaborators have identified peptides that parttially reverse the multidrug resistance in the breast cancer cells.

Future work will focus on continuing the objectives of this study. An important aspect which arises is the ability of these peptides to induce anti-tumor responses. This will focus on the groups of healthy donors and breast cancer patients with no evidence of disease. This group of patients is of particular interest because after first line surgery, chemotherapy and/or radiotherapy, they have no evidence of tumor. Consequently since it is predictable that over the following years many of these patients will experience relapses, this interval offers a window of opportunity to attempt to induce protective immunity to tumor. This will better address the main problem of developing novel therapies for breast cancer by intervening earlier, and not after it may be too late (metastases). In this direction and particularly to accomplish the second and third task, we will take advantage of the fact that peptides, in contrast with full proteins, need only limited processing (2). Second, we will evaluate the ability of additional co-stimulatory signals to augment the tumor Ag induced CTL responses.

We will investigate the relationship between the activation of CD40 - gp39 (CD40L) pathway and CTL induction. This is an important pathway of co-stimulation because its activation induces the secretion of interleukin-12 (IL-12) (23-24) which is an important cytokine for induction and polarization of Th1 cells. We are negotiating with Immunex Corporation (Dr. Tony Trout) to obtain from them human anti CD40L protein, which they have shown to be stimulatory for CD40+ cells (e.g. B cells, Mo). This approach will address the question whether in addition to the B7-CD28 mediated co-stimulation, the CD40-CD40L mediated co-stimulation is needed for tumor specific CTL induction. In addition to the study of CTL induction by peptides as we described (25), we are currently establishing the approaches for the use of adenovirus and vaccinia based vectors for expression of tumor Ag. The recent

developments in the field make these viruses an important tool for the study of Ag expression and processing in non-proliferating APC (adenovirus) and proliferating APC (retroviruses) respectively. We are now establishing the material transfer agreements (MTA) with Genzyme Corporation (Dr. Bruce Roberts) for transfer of their proprietary recombinant viruses in our laboratory. We anticipate that the use of these approaches will establish the feasibility of epitope specific induction of tumor immunity to breast cancer.

(8) Bibliography

1. Boon, T. and van der Bruggen, P. Human tumor antigens recognized by T lymphocytes. *J. Exp. Med.* 183:725-729, 1996.
2. DeBruijn, M., Jackson, M. R., and Peterson, P. A. Phagocyte-induced antigen-specific activation of unprimed CD8+ T cells in vitro. *Eur. J. Immunol.* 25:1274-1285, 1995.
3. Cai, Z and Sprent, J. Influence of antigen dose and costimulation on the primary response of CD8+ T cells in vitro. *J. Exp. Med.* 183:2247-2257, 1996.
4. Zier, K., Gansbacher, B., Salvadori, S. Preventing abnormalities in signal transduction of T cell in cancer: The promise of cytokine gene therapy. *Immunol. Today* 17:39-45, 1996.
5. Ostrand-Rosenberg, S. Tumor immunotherapy: the tumor cell as an antigen-presenting cell. *Current Opinion in Immunol.* 6:722-727, 1994.
6. Swain, S. L., Croft, M., Dubey, C., Haynes, L., Rogers, P., Zhang, X., Bradley, L. M. From naive to memory T cells. *Immunological Reviews* 150:143-167, 1996.
7. Allison, J. P. CD28-B7 interactions in T-cell activation. *Current Opin Immunol.* 6:414-9, 1994.
8. Leach, D. R., Krummel, M. F., Allison, J. P. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271:1734-1736, 1996.
9. Larsen, C. P., Ritchie, S. C., Hendrix, R., Linsley, P. S., Hathcock, K. S., Hodes, R. J., Lowry, R. P., and Pearson, T. C. Regulation of immunostimulatory function and costimulatory molecule (B7-1 and B7-2) expression on murine dendritic cells. J. Immunol. 152:5208-5219, 1994.
10. Gorelik, L., Bar-Dagan, Y., and Mokyr, M. B. Insight into the mechanism)s) through which TNF promotes the generation of T cell-mediated antitumor cytotoxicity by tumor bearing splenic cells. *J. Immunology* 156:4298-4308, 1996.
11. Durie, F. H., Foy, T. M., Masters, S. R., Laman, J. D., and Noelle, R. J. The role of CD40 in the regulation of humoral and cell-mediated immunity. Immunol. Today 15:406-411, 1994.
12. Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. Identification of an immunodominant peptide of HER-2/neu proto-oncogene recognized by ovarian tumor specific CTL lines. *J. Exp. Med.* 181:2709-2717, 1995.
13. Melichar, B., Hudson, J. M., Honda, T., Tuttle, T., Murray, J. L., Wharton, J. T., Ioannides, C. G. Characterization of antigen recognition in tumor infiltrating lymphocytes in breast cancer. *The FASEB Journal*, 10(5): A1470, 1996.
14. Lustgarten, J. and Sherman, L. Generation of xenogeneic cytotoxic T cells from peptides derived from the HER-2/enu proto-oncogenes. *International Congress of Immunology*, A3935, 662, 1995.
15. Ioannides, C. G., Fisk, B., Melichar, B., Anderson, B., Stifani, S., Papayannopoulos, I., Murray, J. L., Kudelka, A.,Wharton, J. T. Ovarian and breast CTL can recognize peptides from the AES protein of the *Notch* complex. *The FASEB J.* 10: A1437, 1996.

16. Fisk, B., DaGue, B., Seifert, W. E., Lambris, J. D., Papayannopulos, I., Kudelka, A., Wharton, J. T., and Ioannides, C. G. Ovarian cytotoxic T lymphocytes can recognize common peptides presented by HLA-A2+ tumors. *The FASEBJ*, 10: A1467, 1996.
17. Miyasaka, H., Choudhury, B. K., Hou, E. W., Li, S. S. Molecular cloning and expression of mouse and human cDNA encoding AES and ESG proteins with strong similarity to Drosophila enhancer of split groucho proteins. *Eur. J. Biochem.* 216:343-352, 1993.
18. Zagouras, P., Stifani, S., Bleumueller, C.M., Carcangiu, M.L., Artavanis-Tsakonas, S. Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc.Natl.Acad.Sci, USA* 92:6414-8, 1995.
19. Fisk, B., Anderson, B. W., Gravitt, K., O'Brian, C. A., Kudelka, A. P., Murray, J. L., Wharton, J. T. and **Ioannides, C. G.** Identification of naturally processed human ovarian peptides recognized by tumor associated $CD8^+$ CTL. *Cancer Research*, acceptable pending revision, 1996.
20. Fisk, B., DaGue, B., Seifert, W., Melichar, B., Wharton, J.T., Capriolli, R.N., and Ioannides, C.G. Mass-spectrometric analysis of naturally processed peptides recognized by ovarian tumor associated CD8+ CTL. Submitted, 1996.
21. Blake, J., Johnston, J. V., Hellstrom, K. E., Marquardt, H., and Chen, L. Use of combinatorial peptide libraries to construct functional mimics of tumor epitopes recognized by MHC-class I-restricted cytolytic T lymphocytes. *J. Exp. Med.* 184:121-130, 1996.
22. Weisberg, J. H., Curcio, M., Caron, P. C., Raghu, G., Mechetner, E. B., Roepe, P. D., and Scheinberg, D. A. The multidrug resistance phneotype confers immunological resistance. *J. Exp. Med.* 183:2699-2704, 1996.
23. Shu, U., Kiniwa, M., Wu, C. Y., Maliszewski, C., Vezzio, N., Hakimi, J., Gately, M., and Delespesse, G. Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. *Eur. J. Immunol.* 25:1125-1128, 1995.
24. Kennedy, M. K., Picha, K. S., Fanslow, W. C., Grabstein, K. H., Alderson, M. R., Clifford, K. N., Chin, W. A., and Mohler, K. M. CD40/CD40 ligand interactions are required for T cell-dependent productions of interleukin-12 by mouse macrophages. *Eur. J. Immunol.* 26:370-378, 1996.
25. Fisk, B., Chesak, B., Pollack, M., Wharton, J. T., Ioannides, C. G. Oligopeptide induction of a cytotoxic T lymphocyte response to HER-2/neu proto-oncogene in vitro. *Cell. Immunol.* 157:412-427, 1994.
26. Fisk, B., **Ioannides, C.G.,** Aggarwal, S., Wharton, J.T., O'Brian, C.A., Restifo, N and Glisson, B. Differential expression and inducibility of TAP1 and TAP2 genes in drug resistant and sensitive tumor cell lines. *Lymphokine and Cytokine Research*, 13: 125-131, 1994.

(9) Appendices

List of Appended Materials

Manuscripts

1. Fisk, B., Savary, C., Hudson, J.M., O'Brian, C.A., Murray, J. L., Wharton, J.T. and **Ioannides, C.G.** Changes in a HER-2 peptide up-regulating HLA-A2 expression affect both conformational epitopes and CTL recognition. Implications for optimization of antigen presentation and tumor specific CTL induction. *Journal of Immunotherapy* 18, 197-209, 1996.

2. **Ioannides, C.G.**, and Grimm, E.A. **Tumor Immunity**, in *"Principles of Clinical Immunology", First Edition*, Chapter 22, (Rich, R., Shearer, W.T., Strober, W., Fleisher, T.A., and Schwartz, B.D. editors.) Mosby. St. Louis, 333-349, 1995.

3. Fisk, B., Anderson, B. W., Gravitt, K., O'Brian, C. A., Kudelka, A. P., Murray, J. L., Wharton, J. T. and **Ioannides, C. G.** Identification of naturally processed human ovarian peptides recognized by tumor associated $CD8^+$ CTL. *Cancer Research*, acceptable pending revision, 1996.

4. Gupta K.P,Ward,N,E., Gravitt,K.R., Bergman,P. O'Brian,C.A., Partial reversal of MDR in human breast cancer cells by an N-myristoylated PKC-a pseudosubstrate peptide. *J. Biol. Chem*, 271, 2102-11, 1996

5. Fisk, B., DaGue, B., Seifert, W., Kudelka, A., Murray, J.L., Wharton, J.T., and **Ioannides, C.G.** Mass-spectrometric analysis of naturally processed peptides recognized by ovarian tumor associated CD8+ CTL.. Submitted for publication, 1996.

6. Fisk, B., Hudson, J. M., Kavanagh, J., Murray, J. L., Wharton, J. T., **Ioannides, C. G.**, and Kudelka, A. P. Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patient to HER-2 peptides. Submitted for publication, 1996.

Abstracts

1. Melichar, B., Hudson, J. M., Honda, T., Tuttle, T., Murray, J. L., Wharton, J. T., and Ioannides, C. G. Characterization of antigen recognition in tumor infiltrating lymphocytes in breast cancer. *The FASEB Journal*, 10(5): A1470, 1996.

2. **Ioannides, C. G.**, Fisk, B., Melichar, B., Anderson, B., Stifani, S., Papayannopoulos, I., Murray, J. L., Kudelka, A., and Wharton, J. T. Ovarian and breast cytotoxic lymphocytes can recognize peptides from the AES protein of the *Notch* complex. *The FASEB Journal*, 10(5): A1437, 1996.

3. Fisk, B., DaGue, B., Seifert, W. E., Lambris, J. D., Papayannopulos, I., Kudelka, A., Wharton, J. T., and Ioannides, C. G. Ovarian cytotoxic T lymphocytes can recognize common peptides presented by HLA-A2+ tumors. *The FASEB Journal*, 10(5): A1467, 1996.

4. Murray, J. L., Hudson, M. J., Ross, M. I., and **Ioannides, C.G.** Defective TAP1 and TAP2 presentation of melanoma-specific peptide, MART-1. *Annual Conference on Clinical Immunology*, May 31 - June 3, 1996, New Orleans, LA.

5. **Ioannides, C. G.**, Murray, J. L., and Wharton, J. T. Recognition of breast and ovarian cancer antigens by T lymphocytes. *3rd International Conference on Engineered Vaccines for Cancer and AIDS*, October 9-14, 1996, Head Island, South Carolina.

Patent Application

1*. Cancer therapy by proteins by the "Notch" complex. **C. G. Ioannides**, B. Fisk, B. Melichar, B. W. Anderson, J. L. Murray and J.T. Wharton *(Invention Disclosure Report)*, 4/10/96.

Clinical Protocol

1. Murray, J. L. Phase Ib trial of HER-2/neu peptide (E75) vaccine in patients with breast and ovarian cancer. Clinical Protocol, 1996.

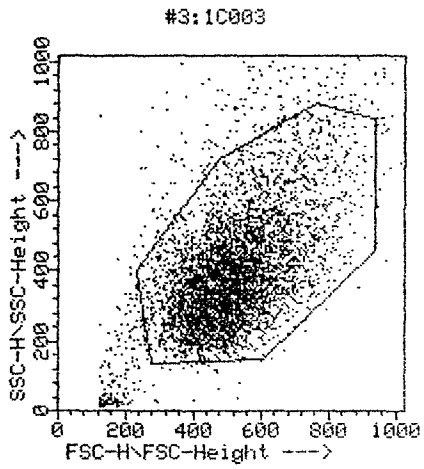
* denotes proprietary information

Legends to the Figures.

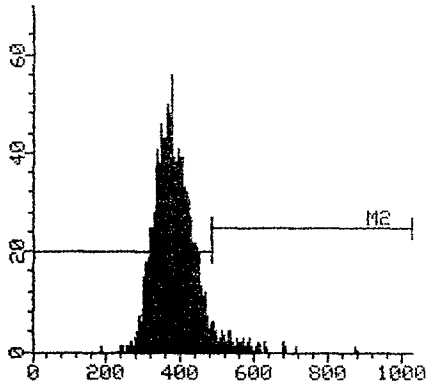
Figure 1. HLA-A2 expression on HER-2^{hi} breast cancer line transfected and expressing the HLA-A2.1 **(A)** SkBr3 cells; **(B)** SkDr3.A2 cells. Detailed description of the Methods for plasmid growth, purification, and transfection are described in the References 12 and 26. The SkBr3.A2 transfected cells are maintained in complete RPMI medium with 200 μg/ml Geneticin (G418). For analysis of HLA-A2 expression, cells were incubated with mAb BB7.2 followed by FITC conjugated secondary antibody and analyzed by flow cytometry.

Figure 2. Polymerase chain reaction (PCR) analysis of the expression of TAP1 and TAP2 in melanoma (lanes 2-3, 5-6) and breast carcinoma SkBr3 cells (lanes 8-9). Lanes 4, 7, and 10 show the comparative levels of expression of β-actin amplified with specific primers in the same experiment. In the lanes 2-3, the melanoma cells were not incubated with IFN-γ. In the lanes 5-6 the melanoma cells were incubated with IFN-γ for 24h before mRNA isolation. In the lanes 8-9, the SkBr3 cells were not incubated with IFN-γ. The results show that SkBr3 cells express both TAP1 and TAP2. The levels of TAP2 (uninduced) are significantly higher than the TAP2 levels induced by IFN-γ. Lanes 1 and 10 show a DNA ladder. The DNA ladder Φ X174 RF DNA/*Hae* III fragments (Gibco, Life Technologies) were 1363, 1078, 872, 603, 310, 281 bp. These bands were visualized by ethidium bromide staining. Amplification of TAP1 and TAP2 used sequence specific primers as described in the Ref 26.

Figure 3. Recognition by $CD8^+$ $CD4^-$ CTL clones derived from the breast TIL line (CTL-BR10) of AES peptides, G60 and G61. Recognition of the immunodominant HER-2 peptide E75 was tested in parallel. Effector:target ration 10:1. The lytic function was determined in a 4 h CTL assay. Using as targets T2 cells pulsed with peptides. Detailed presentation of the Methods is made in Reference 12.



SKBr3.A2



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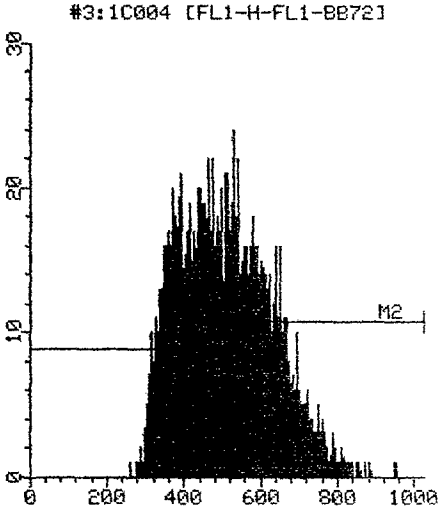
File: #3:1C003 Sample: SKBR3A2C
Date: 8/27/96 Time: 03:48:59
Selected Preferences: Geometric/Channel
Parameter FL1-H FL1-IG Gate G1= R1
Total Events: 5000 Gated Events: 4557

SKBr3.A2
CONTROL

M	Left,Right	Events	% Gate	% Tot	Peak	PkChl	Mean	Median	SD	CV
0	0.00, 1023	4557	100.00	91.14	56	376.00	380.93	374.00	52.61	13.8
1	0.00, 485	4397	96.49	87.94	56	376.00	375.17	372.00	42.68	11.3
2	485, 1023	164	3.60	3.28	6	488.00	537.91	528.00	53.09	9.8



#3:1C004

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File: #3:1C003 Sample: SKBR3A2
Date: 8/27/96 Time: 03:52:55
Selected Preferences: Geometric/Channel
Parameter FL1-H FL1-BB72 Gate G1= R1
Total Events: 5000 Gated Events: 4564

SKBr3.A2

M	Left,Right	Events	% Gate	% Tot	Peak	PkChl	Mean	Median	SD	C
0	0.00, 1023	4564	100.00	91.28	24	529.00	504.77	496.00	113.03	2
1	0.00, 485	2148	47.06	42.96	22	462.00	405.75	407.00	49.06	1
2	485, 1023	2430	53.24	48.60	24	529.00	592.18	582.00	74.58	1

Figure 2

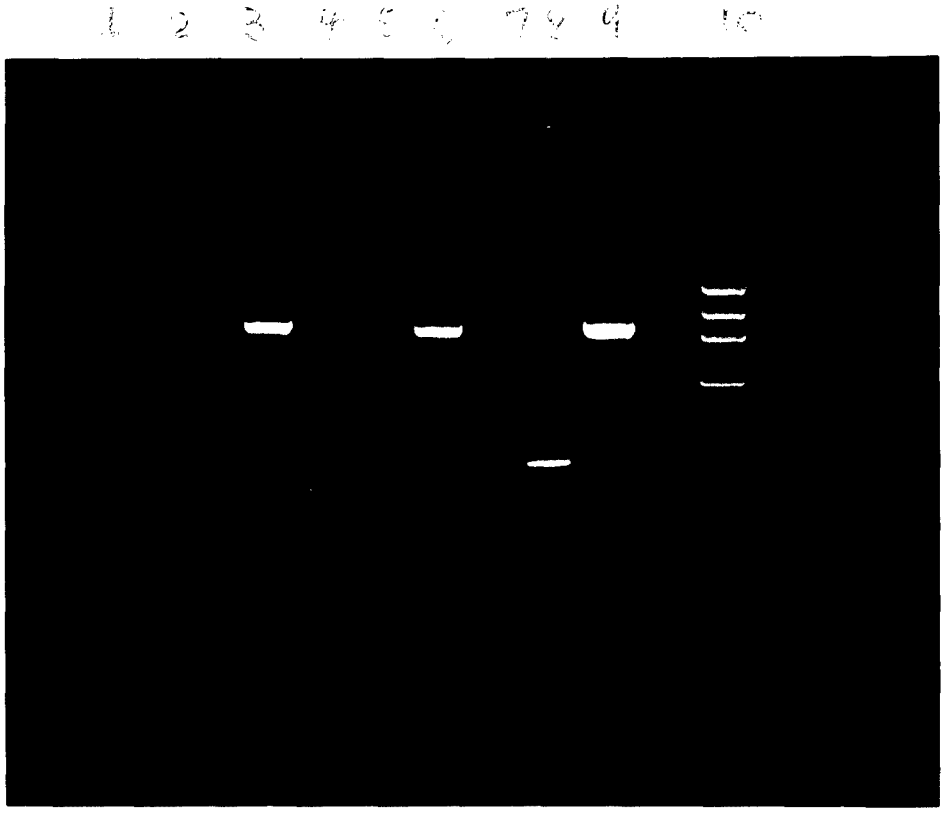
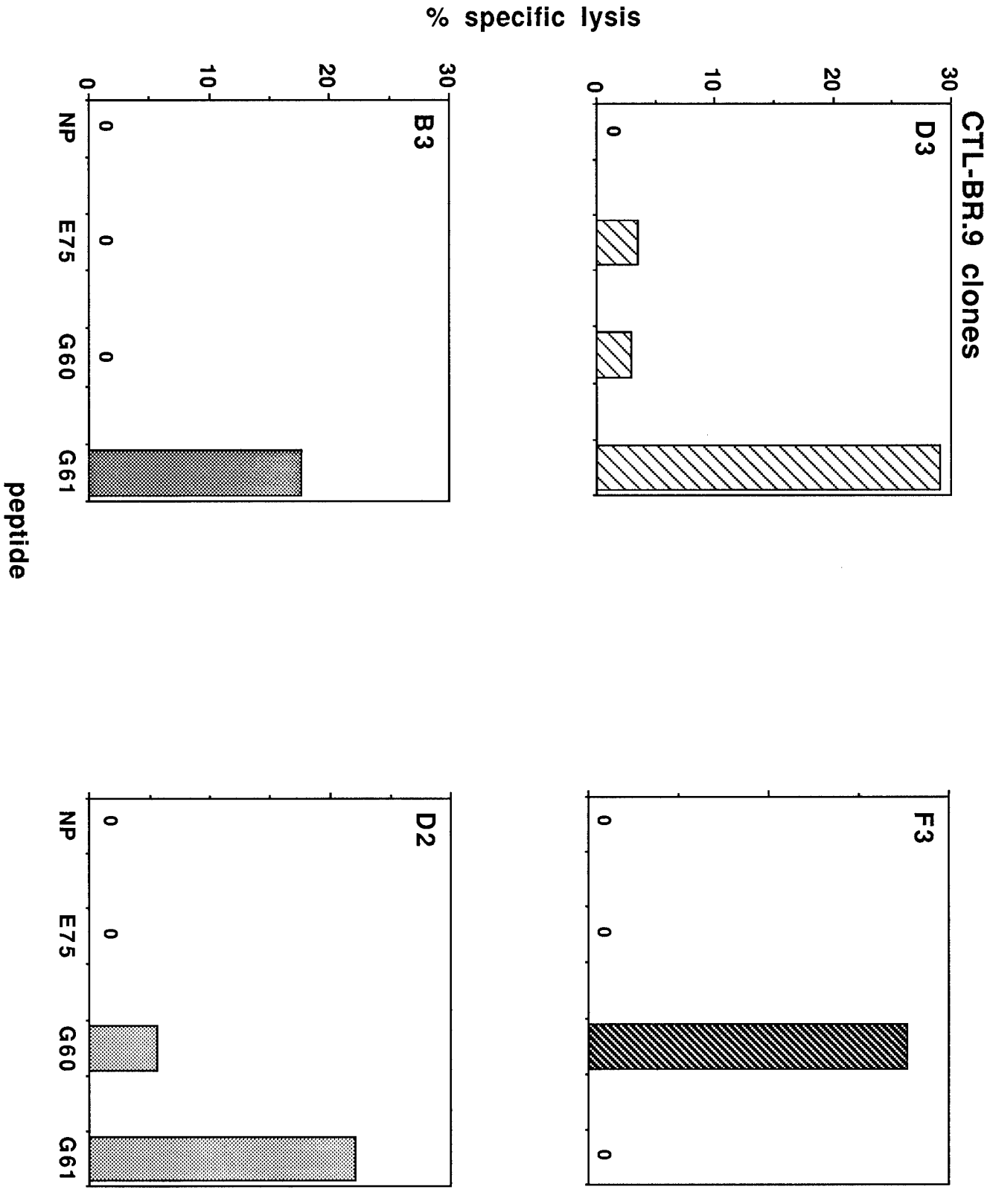


Figure 3


% specific lysis
CTL-BR.9 clones
D3
B3
F3
D2
0
10
20
30
NP
E75
G60
G61
peptide

The FASEB Journal, Volume 10, 1996

2519

OVARIAN AND BREAST CYTOTOXIC LYMPHOCYTES CAN RECOGNIZE PEPTIDES FROM THE AES PROTEIN OF THE NOTCH COMPLEX. C. G. Ioannides, B. Fisk, B. Melichar, B. Anderson, S. Stifani, I. Papayannopoulos, J.L. Murray, A. Kudelka, and J.T. Wharton. M.D.Anderson Cancer Center, Houston, TX, 77030, McGill University, Montreal, Canada, and Biogen, Cambridge, MA 02142.

To date few human tumor antigens (Ag) have been identified in ovarian and breast cancers. We have previously reported that shared tumor Ag in epithelial cancers can be presented by HLA-A2. Thus, they are of particular interest given the high frequency of the HLA-A2. To identify novel tumor Ag, we extracted peptides from immunoaffinity purified HLA-A2 from an established ovarian tumor clone SKOV3.A2.1E4 and separated by two shallow gradients of reverse phase HPLC. A number of candidate epitopes was identified based on the correlation between abundance (ion current) and CTL activity in four consecutive HPLC fractions by two ovarian and one breast CD8+CD4- CTL. Mass spectrometry sequencing and data base search identified a candidate sequence as part of the aminoenhancer of split (AES-1/2; 125 - 133, 136 - 144) of the Notch complex involved in cell differentiation. PCR analysis indicated that transcripts of this gene as well as of a homologous TLE gene are present in both ovarian and breast tumors. Epitope mapping indicated that of the nine overlapping peptides tested AES-1 125 - 133, is recognized with highest affinity. Since the Notch pathway is involved in cell fate determination, this Ag may be of interest for the specific immunotherapy of cancer.

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2707

OVARIAN CYTOTOXIC T LYMPHOCYTES CAN RECOGNIZE COMMON PEPTIDES PRESENTED BY HLA-A2+ TUMORS. B. Fisk, B. DaGue, W. E. Seifert, J. D. Lambris, I. Papayannopoulos, A. Kudelka, J. Taylor Wharton and C. G. Ioannides. M.D. Anderson Cancer Center, and UT Medical School, Houston, TX 77030, Univ. Pennsylvania, Philadelphia, PA and Biogen, Cambridge, MA 02142.

We investigated the pattern of recognition of natural peptides presented by HLA-A2+ ovarian tumors and lines by five ovarian cytotoxic T lymphocytes (OVA-CTL). Four major active fractions were identified in the first dimension HPLC separated peptides eluted from immunoaffinity purified HLA-A2. Three peaks were further separated using a longer C18 column and distinct shallow gradients of acetonitrile (ACN). Comparison of the retention times (Rt) and ACN concentrations for elution of each active peak identified at least 12 distinct peaks of recognition. For a number of peaks the CTL activity correlated with HLA-A2 expression in three distinct samples. However the CTL activity of two major common peaks did not correlate with HLA-A2 expression. Mass-spectrometric analysis revealed a number of ions with similar m/z ratios in a common peak. Ongoing studies aim to define the sequence of the common antigens recognized by OVA-CTL. This suggests that the spectrum of peptides recognized by ovarian CTL is wide and may be significant for identification of novel tumor Ag in ovarian cancers.

The FASEB Journal, Volume 10, 1996

2713

CHARACTERIZATION OF ANTIGEN RECOGNITION IN TUMOR INFILTRATING LYMPHOCYTES IN BREAST CANCER B. Melichar, J.M. Hudson, T. Honda, T. Tuttle, J.L. Murray, J.T. Wharton and C.G. Ioannides. MD Anderson Cancer Center, Houston, TX 77030.

We investigated the proliferation, expansion and recognition of cytotoxic lymphocytes (CTL) from breast tumors (BrCTL). Tumor infiltrating lymphocytes (TIL) isolated from solid tumors and malignant effusions from 15 patients expanded with interleukin-2 between 5-200 fold over 4 weeks as predominantly T cell lines. 6 of 15 TIL lines were predominantly CD8+ while 9 of 15 CTL line were predominantly CD4+. 3 of 5 HLA-A2+ CTL-TIL lines showed specific recognition of autologous tumor. 4 of 6 HLA-A2+ CTL lines recognized an immunodominant epitope of HER-2, 369-377. BrCTL recognized 10-14 peaks of peptides isolated from breast and ovarian tumors and separated by high performance liquid chromatography (HPLC) eluting with distinct retention times in two gradients of acetonitrile. Several peaks were common among freshly isolated tumors and established tumor lines. These results may be of interest for characterization of common tumor antigens and immunotherapy of breast cancer.

Journal of Immunotherapy
18(4):197–209 © 1996 Lippincott–Raven Publishers, Philadelphia

Changes in an HER-2 Peptide Upregulating HLA-A2 Expression Affect Both Conformational Epitopes and CTL Recognition: Implications for Optimization of Antigen Presentation and Tumor-Specific CTL Induction

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Summary: The HER-2/neu protooncogene (HER-2) is overexpressed in a significant number of breast and ovarian tumors. Peptides of HER-2 sequence were recently found to reconstitute recognition of cytotoxic T lymphocytes (CTLs) from tumor-associated (TALs) and tumor-infiltrating (TILs) lymphocytes, indicating that they reconstitute natural epitopes recognized by CTLs on HLA-A2$^+$ tumors. Because HER-2 is an important antigen (Ag) for tumor-specific CTL induction and the immunogenicity of peptides for CTL induction is dependent on their presentation as stable complexes with HLA-A2, we identified peptides of high and low stabilizing activity from the sequence of HER-2 and the folate-binding protein (FBP). Distinct sequence patterns in the region positions (P)3–P5 and P1 were found for peptides with high (HSA) and low (LSA) stabilizing ability. A low-HLA-A2-affinity HER-2 peptide, P1 of the CTL epitope, was found to be permissive to substitutions that enhanced HLA-A2-stabilizing ability and conserved CTL recognition. In contrast, the region P3–P5 was not permissive to sequence changes. We conclude that the selective permissivity of P1 and P9 in the tumor epitope sequence may have important implications for optimization of tumor Ag presentation, and "neo-antigenicity" of self-antigens, aiming toward induction of tumor-reactive CTLs of defined affinity and specificity for target Ags. **Key Words:** HER-2—Presentation—Optimization—CTL—Cancer.

During recent years, studies on human cancer antigens have identified peptides derived from self-proteins recognized by cytotoxic T lymphocytes (CTLs) that can lyse freshly isolated autologous tumors. Most of these peptides from melanoma [e.g., gp100 and MART-1 (1–4)], as well as from ovarian carcinoma [e.g., HER-2; (5,7)], were found to bind major histocompatibility complex (MHC) class I with low affinity, thus forming unstable complexes with the presenting molecule. In CTL reconstitution assays, the low affinity of the peptides for human leukocyte antigen HLA-A2 was compensated for in part by the high concentrations of peptides used in the in vitro assays (2,5–7). Because these peptides are likely candidates for development of tumor vaccines and anti-tumor effector CTLs, this raises the question of how to use low-MHC-affinity peptides for CTL induction both in vitro and in

Received July 26, 1995; accepted November 28, 1995.
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vivo. These low-affinity peptides are expected to compete for the same T-cell receptor (TCR) with other self-peptides of high MHC affinity.

One possible approach to this question is to use for CTL induction high concentrations of peptides delivered either exogenously or endogenously by minigenes or recombinant viral vectors. This may result in sufficiently high numbers of peptide–MHC class I (pep-MHC) complexes to trigger CTL activation. This approach, however, cannot address the question of tumor CTL epitope stability, because low-affinity peptides usually form unstable pep-MHC complexes. Because the stability of pep-MHC complexes has recently been demonstrated to be an important factor in epitope dominance for CTL induction (8), this raises the question of how the stability of the pep-MHC complex can be increased to optimize antigen (Ag) presentation by appropriate changes in the peptide sequence. The second question is whether, for tumor Ags such as HER-2, there are sequence characteristics that associate with MHC stabilizing ability and residue permissive to changes in the sequence whose substitution, while increasing the peptide affinity for HLA, either does not affect or affects only minimally CTL recognition. To address these questions, we used as a model the HER-2 protein and CTL recognizing a low-HLA-A2-affinity epitope from this protein. These Ag and CTL effectors are relevant for immunotherapy of human breast, ovarian, and lung cancers.

The objectives of this study were fourfold: (a) to identify peptides of high (HSA) and low (LSA) stabilizing ability in the HER-2 sequence and determine whether certain sequence characteristics in these peptides associate with HSA and LSA, respectively; (b) to identify specific sequence changes that enhance the stabilizing ability of a low-HLA-A2-affinity peptide; (c) to define the effects of these changes on CTL recognition; and (d) identify residues that, when substituted in the peptide, increase the HLA-A2 affinity but do not affect the specificity of CTL recognition.

Because HER-2 protein is a large molecule (1,255 amino acids), a large number of potential HLA-A2-binding peptides can be identified by using the minimal and extended HLA-A2–binding motifs (>50) (9). Folate-binding protein (FBP) is identical with the human trophoblast antigen recognized by MOv18/MOv19 monoclonal antibody (mAb) and appears to be restricted to ovarian cancer (10). Because of its restricted expression, FBP is also examined as a candidate Ag for anti-tumor cellular responses. For this reason, a number of synthetic peptides corresponding to HLA-A2–binding motifs have been prepared and tested in the same experiments. For this study, we focused on nonapeptides containing mainly Leu and to a lesser extent Ile and Val at position 2 (P2), and Leu/Val and to a lesser extent Met and Ile at P9. The presence of structurally similar side chains at the main anchor positions P2 and P9 facilitates identification of the effects of the other residues in CTL recognition and HLA-A2 stabilization.

Our results identified two groups of peptides designated as having HSA and LSA HLA-A2, on the basis of their ability to enhance HLA-A2 expression on indicator T2 cells. Sequence analysis revealed that the stabilizing ability of HLA-A2 by these peptides was dependent, in many instances, on the nature of residues or groups of residues outside of the main P2 and P9 anchors; residues were identified that occurred with higher frequency in either HSA or LSA peptides.

In HSA peptides, residues with short side chains (Ala/Gly) and positively charged side chains (Arg/Lys/His) were found with increased frequency at P1; hydrophobic aliphatic residues (Leu, Val, Ile, Met) and Gly were found preferentially at P3; and charged residues were found with increased frequency at P4. Replacement of P1 and P9 anchors in an LSA peptide with residues found with higher frequency in HSA peptides significantly increased the stabilizing ability of the resultant hybrid peptides. Of these changes, substitutions of Gly and Phe at P1 led to HSA peptides that were recognized with the same or similar efficiency by peptide-specific CTLs. This approach may be useful for optimization of tumor Ag presentation and development of antigen- and epitope-specific human tumor vaccines.

MATERIALS AND METHODS

Synthetic Peptides

The synthetic peptides used in this study were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center and purified by high-pressure liquid chromatography (HPLC). The purity of the peptides used in this study ranged between 92 and 95%. The purity and amino acid composition of these peptides were established by

amino acid analysis. The peptides synthesized were all nonamers containing L/I/V at P2 and V/L/I/M at P9. Of these peptides, 26 were analogs of HER-2, and five were analogs of FBP.

Monoclonal Antibodies and Immunofluorescence

The hybridomas secreting HLA-A2–specific mAbs BB.7.2 and MA2.1 were obtained from the American Tissue Type Collection (ATTC). Cells were incubated with 1:50 dilution of hybridoma culture supernatant containing BB7.2 and MA2.1 mAb. This supernatant was previously concentrated by filtration through a 50-kDa filter (Amicon, Beverly, MA, U.S.A.). The concentration of BB7.2 and MA2.1 mAbs after concentration was in the range of 200 μg/ml. Serial dilutions of the concentrated BB7.2 and MA2.1 mAbs were tested in parallel, and we established that the same levels of staining were observed with each of these antibodies when used between 1:50 and 1:5 final dilution in the assay. Afterward the cells were washed and incubated with fluorescein isothiocyanate (FITC)-conjugated $F(ab)_2$ fragments of goat anti-mouse immunoglobulin (Ig)G (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.). W6/32 mAb was obtained from DAKO Corporation (Carpinteria, CA, U.S.A.). Cell-surface expression of HLA-A2 on T2 cells was studied with flow cytometry by using an EPICS^{R}V Profile Analyzer (fluorescence-activated cell sorter; FACS; Coulter Corporation, Hialeah, FL, U.S.A.) with a log amplifier.

HLA-A2–Stabilization Assay

T2 cells were pulsed overnight with saturating amounts of individual peptides (100 μg/ml), washed, and then incubated with saturating amounts of MA2.1, BB7.2, or W6/32 mAb, followed by counterstaining with goat–anti-mouse FITC-conjugated antibody. Flow-cytometry analyses were performed on 5,000 T2 cells for each individual peptide. At the same peptide concentration in the assay, the increase in the levels of HLA-A2 expression on T2, as measured by an increase in fluorescence intensity, reflects the ability of a particular peptide to stabilize peptide–HLA-A2 complexes (11). To quantitate the effects of peptides on BB7.2 and W6/32 epitopes expression on HLA-A2 on T2 cells, the channel number corresponding to the peak of fluorescence intensity on the log scale of FACS (mean channel fluorescence, MCF) for each peptide was divided by the value corresponding to the peak of fluorescence intensity for T2 cells cultured in the absence of exogenously added peptide. The resulting value was designated relative mean channel fluorescence (MCF-R) (12).

Cytotoxicity Assays

The CTL line 41 developed by repeated in vitro stimulation of HLA-$A2^+$ peripheral blood mononuclear cells (PBMCs) from a healthy donor with peptide C84, HER-2 (971–979V) and a longer peptide C43, HER-2 (968–981) has been described (13). For these studies, CTL-41s were maintained in culture with monthly restimulation with C84 peptide at 10 μg/ml and autologous or allogeneic HLA-$A2^+$ PBMCs. CTLs used as effectors in these experiments were selected on Ab-coated plates (AIS Micro CELLector, Applied Immune Sciences, Menlo Park, CA, U.S.A.), and were $CD3^+CD4^-CD8^+$. To determine recognition of HER-2 peptides by CTL-41s, ^{51}Cr-labeled T2 cells were preincubated with various concentrations of peptides for 90 min. Effectors were added for an additional 5 h. %Specific lysis was calculated from the formula $(E - S)/(T - S) \times 100$, where E are the counts per min (cpm) of wells containing both effectors and targets, whereas S and T represent the cpm measured for spontaneous and total lysis of targets, respectively, in the absence of effectors but in the presence of the same peptide (6).

Statistical Analysis

MCF values obtained for HSA and LSA groups were examined by Student's t test. Differences were considered significant when $p < 0.05$.

RESULTS

Identification of HER-2 Nonapeptides of High and Low HLA-A2-Stabilizing Abilities

To assess the effects of peptides on HLA-A2 expression and define their stabilizing ability, we examined the role of the peptides listed in Table 1 on the expression of an epitope recognized by the HLA-A2-specific mAb BB7.2 by using the mutant cell line T2. This cell line has a large deletion in the TAP-1 and TAP-2 regions, resulting in very low surface expression of HLA-A2 associated with endogenous signal peptides and undetectable HLA-B5 at

TABLE 1. *Stabilization of BB7.2 epitope by HER-2 and FBP peptides*

Code	Position	Sequence	MCF-R BB7.2	MCF-R W6/32
Peptides with high HLA-A2–stabilizing ability				
F57	435–443	I L H N G A Y S L	6.34	2.35
E75	369–377	K I F G S L A F L	5.01	3.03
E90	789–797	C L T S T V Q L V	4.82	2.99
D113	48–56	H L Y Q G C Q V V	4.56	2.89
F59	447–455	G L G I S W L G L	4.14	1.90
E92	650–658	P L T S I I S A V	3.76	1.89
E38FBP	112–120	N L G P W I Q Q V	3.64	2.42
E93	466–474	A L I H H N T H L	3.32	1.71
E76	402–410	T L E E I T G Y L	3.22	2.08
E88	689–697	R L L Q E T E L V	3.21	2.80
E89	851–859	V L V K S P N H V	2.41	1.81
E91	5–13	A L C R W G L L L	2.40	1.78
Peptides with low HLA-A2–stabilizing ability				
E77	391–399	P L Q P E Q L Q V	1.79	1.26
E78	457–465	S L R E L G S G L	1.76	1.21
F53	654–662	I I S A V V G I L	1.58	1.38
F55	1172–1180	T L S P G K N G V	1.57	1.37
C85	971–979	E L V S E F S R M	1.38	1.13
E39FBP	191–199	E I W T H S T K V	1.33	1.37
F56	411–419	Y I S A W P D S L	1.28	1.37
E71	799–807	Q L M P Y G C L L	1.23	1.00
E41FBP	245–253	L L S L A L M L L	1.20	1.05
E40FBP	247–255	S L A L M L L W L	1.18	1.25
F54	747–755	K I P V A I K V L	1.16	1.12
E37FBP	25–33	R I A W A R T E L	1.13	1.59
F51	160–168	Q L C Y Q D T I L	1.13	1.05
E74	838–846	D V R L V H R D L	1.06	1.18
E70	793–801	T V Q L V T Q L M	1.03	1.00
F52	627–635	P I N C T H S C V	0.99	0.94
F58	442–450	S L T L Q G L G I	0.95	1.26
F50	141–149	Q L R S L T E I L	0.94	0.93
E72	828–836	Q I A K G M S Y L	0.94	0.96
Negative controls				
C61	968–977	R F R E L V S E F S	1.08	1.06
No peptide added			1.00	1.00

HLA, human leukocyte antigen.

HER-2 and FBP peptides were selected from the corresponding protein sequences as previously described (9). FBP after the code (e.g., E38FBP) indicates peptides derived from folate-binding protein. The code for each synthetic peptide was assigned by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center. C61 is a peptide with anchors for HLA-B8 but not HLA-A2. "No peptide added" indicates that T2 cells were not incubated with any of the synthetic peptides listed. Experimental conditions are described in the Materials and Methods section. MCF for C61 was 37 (BB7.2) and 182 (W6/32), and for T2 cells incubated without peptide, was 34 (BB7.2) and 172 (W6/32). MCF for F57 was 216 (BB7.2) and 404 (W6/32).

the cell surface (14). BB7.2 epitope is located on the N-external loop of the α2 domain (including W107) (15–17) and is not expected to interact directly with the specific peptide side chains.

From the values obtained for the MCF-R for all peptides, peptides with MCF-R values of >2.0 were designated as having HSA, whereas peptides with MCF-R <2.0 were designated as having low HLA-A2-stabilizing activity. As seen in Table 1, 12 peptides showed MCF-R of ≥2.40, whereas 19 other peptides had an MCF-R of ≤1.79. These peptides belong to two different groups of HLA-A2–stabilizing ability. The means and SDs of the MCF-R for peptides in the HSA and LSA groups are 3.90 ± 1.4 and 1.24 ± 0.26, respectively ($p < 0.0001$).

To address the question of whether the observed BB7.2 mAb recognition of HLA-A2 reflects stabilization of HLA-A2 on the surface of T2 cells, T2 cells incubated with the same peptides were stained with mAb W6/32, which recognizes a monomorphic determinant on the α3 domain of HLA class I. The specific stabilization of HLA-A2 by the peptides is shown in Table 1. A number of HSA peptides caused a two- to threefold increase in MCF-R for HLA-A2 expression compared with the levels of

HLA-A2 on the T2 cells cultured in the same conditions without exogenous peptides. This was paralleled by similar low levels of stabilization of HLA-A2 on the T2 cell surface by the LSA peptides tested. The means and SDs of MCF-R for W6/32 in the HSA and LSA were 2.26 ± 0.5 and 1.18 ± 0.18, respectively ($p < 0.0001$). The values of MCF-R for W6/32 mAb paralleled the experimentally determined values of BB7.2 for most peptides in the HSA and LSA groups. However, a linear correlation between MCF values for BB7.2 and W6/32 within each group was not found. This suggests that the peptide-induced changes in the BB7.2 epitope expression are the direct result of the increase in the number of HLA-A2 molecules expressed, and they reflect possible conformational changes in the BB7.2 epitope induced by the peptide. This hypothesis can be tested by introducing substitutions in the sequence of peptides (e.g., E88) and determining the W6/32 and BB7.2 epitope expression over a range of peptide concentrations, as previously reported (18,19). If the increase in MCF for W6/32 epitope expression is not paralleled by an increase in BB7.2 epitope expression and vice versa, this would be suggestive of peptide-induced conformational changes in the BB7.2 epitope.

Identification of Specific Sequence Patterns in the Nonapeptides Associated with HLA-A2 Stabilization

In the HLA-A2 system, the main peptide HLA anchors are, in decreasing order of affinity, L > M > I > V,A,T, at position 2 (P2), and V > L > M ≥ I > A at P9 (20–23). For the same molecule, the presence of either negatively or positively charged residues or both at P1, P3, P6, and P7 in the peptide was associated with decreased or no binding, whereas aromatic residues in P1, P3, and P5 were associated with good binding independent of the presence of main anchors in positions 2 and 9 (21). These studies quantitated the ability of a peptide to inhibit binding of a standard radiolabeled peptide to soluble HLA-A2 (21) and established the importance of specific residues in defined positions of peptides from different proteins to influence the peptide affinity for isolated HLA-A2 chains.

In both in vitro and in vivo conditions, the TCR is expected to recognize a cell-bound peptide–HLA complex (24,25). In certain instances, for model peptide CTL epitopes, antigenicity, and immunodominance have been associated with the stability of the peptide–MHC class I complex, which is dependent on the affinity of binding of the peptide to the presenting molecule (8). Recent studies have shown that the presence of certain residues in the nonapeptide sequence affects the affinity of peptide binding and the studies identified such residues (21). However, the distinct sequences of proteins present the case when amino acids expected to confer HSA are absent from the sequence of peptides selected with anchor motifs. In the absence of such residues, predictive motifs need to be experimentally validated. To define the importance of individual residues of HER-2 and FBP peptides for HLA-A2 stabilization, as defined by BB7.2 and W6/32 epitope expression, we calculated the frequency of appearance in the sequence of amino acids in main (dominant) P2 and P9 and secondary (auxiliary) P1, P3–P8 anchor positions. With respect to the main anchors, 11 (91%) of 12 HSA peptides contained L (P2), and one (8.3%) of 12 contained I (P2), whereas of LSA peptides, 10 (52%) of 19 contained L (P2), seven (36.9%) of 19 contained I (P2), and two (10.5%) of 19 contained Val (P2). Nonapeptides with M (P2) were not found in the HER-2 sequence. Of the HSA peptides, six (50%) of 12 contained Val at P9, and six (50%) of 12 contained Leu (P9). Of the LSA peptide, four (21%) of 19 contained Val (P9), 12 (63.2%) of 19 contained Leu (P9), whereas two and one of 19 contained Met and Ile at P9, respectively. These results are in general agreement with the reported association of Leu at P2 and Val/Leu at P9 with high HLA-A2 binding affinity; however, they do not address the fact that at least 10 of 19 peptides that contained Leu (P2) showed LSA, even when either Val or Leu was present at P9. Similarly, a number of peptides that share residues at P1, P2, and P9 (e.g., the pairs E75 and F54, E76 and F55, E92 and E77) had different HLA-A2–stabilizing abilities (Table 1).

These results suggested a significant role for the residues in the central area in HLA-A2 stabilization. To identify the dominance of residues in the nonapeptide sequence, we calculated the frequency of each group of amino acids in each position for the 12 HSA and 19 LSA peptides. Because the sample size was relatively small, amino acids of similar physicochemical characteristics were grouped together (e.g., aromatic, aliphatic nonpolar, negatively charged) following the approach of Ruppert et al. (21). Separate groups were made for G, A, and P because of their side-chain characteristics. The results are shown in Table 2.

A striking trend was found in positions 4 and 5 of

TABLE 2. *Frequencies of amino acid groups at all positions in HSA and LSA peptides*

Residues at positions/ frequencies (%)[a]	1		2		3		4		5		6		7		8		9	
	HSA	LSA	HSA	LSA	HSA	LSA	HSA	LSA	HSA	LSA	HSA	LSA	HSA	LSA	HSA	LSA	HSA	LSA
Y,F,W	00.0	05.3	0		16.7	05.3	00.0	10.6	16.7	10.6	08.3	05.2	08.3	05.2	16.7	16.7	0	
S,T,C	16.7	**26.3**	0		25.0	31.6	16.7	21.0	**33.3**	05.2	25.0	15.8	16.8	**42.0**	08.3	10.2	0	
A	**16.7**	00.0	0		00.0	**15.8**	00.0	10.6	00.0	**15.8**	08.3	00.0	08.3	00.0	08.3	00.0	0	
G	08.3	00.0	0		**16.7**	00.0	08.3	00.0	16.7	10.6	08.3	15.8	08.3	05.2	08.3	15.8	0	
P	08.3	10.5	0		00.0	05.3	08.3	15.8	00.0	00.0	08.3	05.2	00.0	00.0	00.0	00.0	0	
L,V,I,M	16.7	10.5	100	100	**25.0**	10.6	08.3	**31.6**	16.7	**31.6**	33.0	26.0	16.7	21.0	33.0	37.0	100	100
Q,N	08.3	21.0	0		00.0	**15.8**	**25.0**	00.0	00.0	10.6	08.3	05.2	**33.0**	10.0	08.3	05.2	0	
R,H,K	**25.0**	10.5	0		08.3	15.8	**25.0**	05.3	08.2	08.2	00.0	**21.0**	00.0	10.0	16.0	10.0	0	
D,E	00.0	**15.8**	0		08.3	00.0	08.3	05.3	01.0	10.6	00.0	05.2	08.3	10.0	00.0	10.0	0	

FBP, folate-binding protein; HLA, human leukocyte antigen; HSA, high stabilizing ability; LSA, low stabilizing ability.

[a] Thirty-one peptides containing as HLA-A2 anchors L, I, or V at P2 and V, L, M or V at P2 and V, L, M or I at P9 were selected from protein sequences of HER-2 and FBP and tested for induction of expression of the BB7.2 and W6/32 epitopes. From this set, the 12 with HSA and 19 with LSA were compared to determine the percentage of occurrence of a certain group of residues at a certain position (frequency), as described in reference 3.

The frequency of appearance of a certain group of residues at a position in the sequence was considered significant if it exceeded by ≥15% the frequency of occurrence of the same group of amino acids in the other group of peptides (either HSA or LSA). This corresponds to a difference of at least two amino acids of the same group at a given position between HSA and LSA, because we tested 12 HSA peptides, and a difference of at least three amino acids of the same group at a given position between LSA and HSA because we tested 19 LSA peptides.

the nonapeptide sequence. HSA was associated with the presence of predominantly hydrophilic residues Lys, His, Arg, Gln, and Asn at P4, and of Ser, Thr, and Cys at P5, compared with peptides with LSA. In contrast, hydrophobic aliphatic residues dominated both P4 and P5 in LSA peptides. At position 3, only the percentage of hydrophobic residues was found increased, but with the exception of Leu, Val, Ile, Met, and Gly, no defined groups of amino acids were present in a significantly higher percentage in either HSA or LSA peptides. At the other positions, the presence of amino acids was in general agreement with the data reported from previous studies.

At position 1, hydrophilic positively charged residues and Ala were the dominant groups in HSA peptides. In these proteins, the aromatic amino acids (Phe, Tyr, and Trp) that have been reported to be associated with good HLA-A2 binding (21) were not well represented in either HSA or LSA peptides. The group Ser, Thr, and Cys was found with higher frequency in the LSA peptides. At position 6, only the frequency of positively charged residues was increased in LSA peptides compared with HSA peptides, whereas no other group of amino acids was found with significantly higher frequency in any of these peptides. This is also in agreement with previous reports on residues associated with poor HLA-A2 binding (21). There was no significant difference between the presence of aliphatic hydrophobic residues in HSA and LSA groups of peptides. At position 7, Gln and Asn were predominantly found in peptides with HSA, whereas Ser, Thr, and Cys represented 42% of the residues found in peptides with LSA. In contrast, at position 8, no major differences were found between the frequencies of each group of amino acids in HSA and LSA peptides.

These results show an association between high HLA-A2.1–stabilizing ability and the presence at P3 of hydrophobic residues and at P4 of hydrophilic residues. The reverse association at the same positions (P3 and P4) with low HLA-A2 stabilizing ability was observed. Similar results were obtained from the analysis of the percentage expression of hydrophilic residues at each position in the peptides (Fig. 1). The results show a trend of decrease in the percentage of hydrophilic residues from positions 4 to 6 in the HSA group and increase in hydrophilic residues from positions 4 to 6 in the LSA group.

Effects of Substitutions in the Sequence of a Low-HLA-A2-Affinity HER-2 Peptide on Conformational Epitopes Recognition by mAb

To identify residues that positively affect stabilization of the HLA-A2 peptide complex by LSA peptides, we studied the influence of changes in the sequence of HER-2 peptide C85 on the formation of mAb epitopes BB7.2 and MA2.1 compared with W6/32 epitope expression. C85 was chosen because, when presented by T2 cells, it forms an

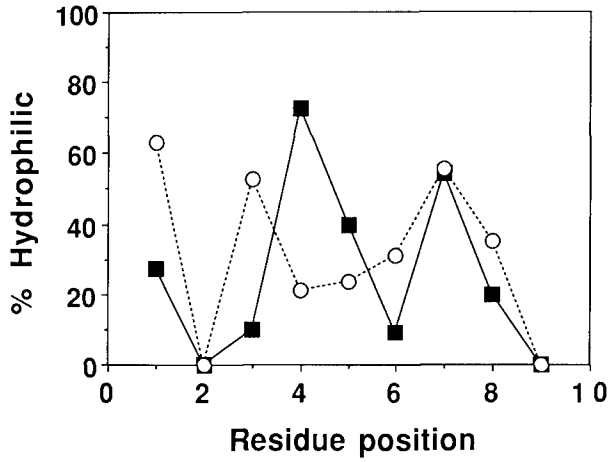

FIG. 1. Frequency of hydrophilic residues at each position in the high stabilizing ability (HSA) and low stabilizing ability (LSA) nonapeptides. Each amino acid was designated either hydrophilic or hydrophobic according to the hydropathy scale of Fauchere-Plitska (32). (-■-), HSA peptides; (○), LSA peptides.

epitope recognized by two ovarian-specific CTL lines (6), raising the possibility that this or a structurally similar peptide–HLA-A2 complex is recognized by the TCR.

Because the LSA of C85 may be due to the presence of Glu at P1 interacting with the negatively charged pocket A of HLA-A2 containing Glu-63 (26), a group of peptides was synthesized containing at P1 either Thr (designated 103T), Gly (103G), Lys (103K), or Phe (103F). The reasons for selecting these amino acids were as follows: (a) aromatic amino acids at P1 were preferentially associated with good binding (21); (b) the presence of Lys at P1 was expected to facilitate the stabilization of the complex through charge interactions with the negatively charged pocket A (26); (c) the small volume and the lack of side chains of Gly will not only avoid steric interactions with mAb MA2.1 but may also provide additional conformational flexibility for the peptide chain (27–29); and (d) the group Ser, Thr, and Cys was found with higher frequency at P1 in our LSA than in HSA HER-2 peptides (Table 2). However, the presence of this group at P1 was associated with a high affinity of other peptides for HLA-A2 (21).

To address the possibility of conformational changes in the "face" (30) of the peptide–HLA-A2 complex, T2 cells were stained with MA2.1 mAb. Because the MA2.1 epitope may be affected by surface/solvent accessible residues, T2 cells incubated with each peptide were also stained with BB7.2 and W6/32 mAb in the same experiment. MCF was calculated for each histogram and is shown in Table 3. For peptides 103T, 103G, and 103F, there was a parallel between the increase in staining by BB7.2 and MA2.1 mAb and the staining by W6/32 mAb, suggesting that these peptides stabilized HLA-class I expression. Peptide 103K showed a different picture. Stabilization of class I expression by this peptide was associated with a different pattern of reactivity by BB7.2 and MA2.1 mAb. For 103K, the reactivity by BB7.2 was essentially unchanged

TABLE 3. *Effects of P1 substitutions in the sequence of HER-2:971–979 peptide on mAb recognition*

Peptide	Amino acid									MCF[a] (Channel no.)		
										W6/32	BB7.2	MA2.1
C85	E	L	V	S	E	F	S	R	M	35.0	29.2	16.8
103T	T	—	—	—	—	—	—	—	—	121.8	47.1	100.8
103G	G	—	—	—	—	—	—	—	—	146.4	64.1	126.8
103K	K	—	—	—	—	—	—	—	—	131.9	30.5	102.2
103F	F	—	—	—	—	—	—	—	—	380.6	131.8	471.4
C84	—	—	—	—	—	—	—	—	V	115.1	110.4	107.6
104T	T	—	—	—	—	—	—	—	V	376.0	112.3	430.5
104G	G	—	—	—	—	—	—	—	V	410.9	128.9	432.4
104K	K	—	—	—	—	—	—	—	V	437.1	133.7	236.9
104F	F	—	—	—	—	—	—	—	V	377.4	189.9	552.8

MCF, mean channel fluorescence; mAb, monoclonal antibody.

[a] The MCF value for each peptide was obtained by subtracting the MCF value for staining by each mAb of T2 cells incubated with a given peptide from the control MCF value for T2 cells incubated without exogenously added peptide and stained with the same mAb. T2 cells were stained with each of W6/32, BB7.2, and MA2.1 mAbs. The results are from one representative experiment of three independently performed. The control values for T2 cells stained with mAb in the absence of peptides were 209 (W6/32), 67 (BB7.2), and 164 (MA2.1).

compared with wild-type peptide C85, whereas for W6/32 and MA2.1, the change in MCF indicated increased HLA-A2 stabilization. When the MCF values for BB7.2 and MA2.1 for the surface expression of class I were corrected for heavy-chain expression (by subtracting the MCF values for W6/32, 18), the results showed that binding of MA2.1 is decreased by a similar number of channels for either C85 (−18), 103T (−21), 103G (−19) and 103K (−29). However, for peptide 103F, the staining by MA2.1 was higher than the staining by W6/32 by 91 channels, suggesting a change in the conformation of the epitope.

We used the same approach to determine the effect of substitution at C-terminal Met → Val (P9) alone, or together with the same substitutions at P1, on the stability of expression and conformation of HLA-A2. C84 increased W6/32 staining by 80 channels compared with wild-type peptide C85. This increase was paralleled by increase in staining by BB7.2 and MA2.1, suggesting that the increase in staining reflects increased expression of HLA-A2 rather than conformational changes detectable by BB7.2 and MA2.1 mAb. Different results were obtained for the double substituted peptides (designated 104, T, G, K, F). For 104G and 104T, the increase in W6/32 staining over C84 and the corresponding single substituted peptides (103G and 103T) was paralleled by the increase in MA2.1 but not in BB7.2 staining. In contrast, the increase in W6/32 staining for 104K was associated with a decrease in reactivity for MA2.1 (−201 channels), whereas for 104F, an increase in staining was observed over C84 for both BB7.2 and MA2.1. These data suggest synergy of F (P1) and V (P9) in the C85 peptide in inducing conformational changes in HLA-A2.

These results show that for the LSA peptide C85, substitution of Met → Val at P9 is likely to enhance the stability of the peptide–HLA-A2 complex and less likely to affect the conformation of MA2.1 and BB7.2 epitopes. Substitutions of Glu → Thr/Gly/Phe at P1 in the same peptides increased the stability of peptide–HLA-A2 complex in parallel with increased expression of MA2.1 epitope and affected the BB7.2 epitope significantly less. Substitutions of Glu → Lys (P1) stabilized the peptide–HLA-A2 complex and apparently induced strong conformational changes in the complex. These results suggest that in certain instances, substitutions at P1 and P9 have an additive/synergistic effect in stabilization of the HLA-A2–peptide complex.

To address the question of the contribution of individual peptide residues from the central peptide area on the peptide–HLA-A2 complex, we investigated the effects of C85 peptide analogs on mAb recognition. The analogs were modeled on the sequence of C84 variant (M → V, P9). We focused mainly on positions 4 and 5, which are considered TCR contacts (26). To establish whether the sequence context of defined residues within the peptide sequence can affect the structural contribution of these residues to pep-MHC conformation, we substituted Glu → Gly (P5) in C84, and Arg (P8) to Lys in its analog C83. The resulting peptides were designated E51 and E54, respectively. Gly was chosen because of its small volume and lack of side chain to minimize the steric and charge interferences with neighboring residues and HLA-A2 helices. As shown in Fig. 2, staining by W6/32 and BB7.2 of T2 cells incubated with E51 was significantly decreased. Staining by the same mAb of T2 cells incubated with E54 was further decreased, suggesting that the substitution Arg → Lys (P8) affected the contribution of Gly (P5) to the stabilization of HLA-A2. The same decrease, but to a lesser extent, was observed for MA2.1 mAb.

Substitution of Ser → Val (P4) in E51 (peptide E52) further diminished mAb binding compared with C84, suggesting, as indicated in Fig. 1, that the presence of hydrophobic aliphatic residues at P4 is associated with LSA peptide. The substitution of Phe → Val (P6) in C83 peptide (C81) increased the binding of all mAbs tested over the levels detected for peptide C83. In this sequence context, when the MCF for all mAbs tested for the peptide pairs C83–E53 and C81–C82 were compared, the substitution of Ser → Lys (P4) affected both the stability of the HLA-A2–peptide complex and possibly its conformation. Substitution Glu → Gly (P5) in C82 led to further reduction of mAbs reacting with T2 cells, confirming the pattern observed with peptides E51 and E54. These results indicate that the magnitude of the structural contribution of residues in the central area to stabilization of the HER-2 peptide–MHC complex is dependent not only on the nature of the residue substituted at each position but also on the sequence context.

Effects of Substitutions in the Sequence of the C85 Epitope on Recognition by CTLs

To define the effects of sequence changes on the recognition by peptide-specific CTLs, we con-

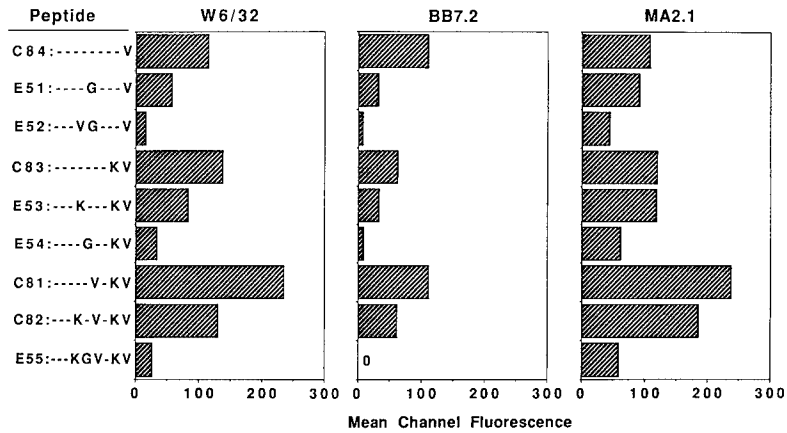

FIG. 2. Stabilization of human leukocyte antigen (HLA)-A2–specific monoclonal antibody (mAb) epitopes by analogs of the HER-2:971–979 peptide substituted at P3, P4, P5, P6, and P8. Stabilization assays were performed as described in Materials and Methods. (–), residues identical to the unsubstituted peptide. Cells were stained by indicated mAb (W6/32, BB7.2, and MA2.1). The antibody specifications are listed in the Results section.

ducted cytotoxicity experiments. CTL-41s induced by peptide C84 (13) were used as indicators, because they were developed against a conservatively substituted C85 peptide. Thus they offered the opportunity of studying the effects of sequence changes on CTL recognition, by excluding the possibility of cross-reactive recognition of the same Ag by CTL of different specificities that may be present in CTL isolated from TILs. Peptides substituted in the central area were recognized significantly less than C84. In contrast with the P1- and P9-substituted peptides, these peptides showed low HLA-A2–stabilizing ability. Because this may result in a small number of peptide–HLA-A2 complexes, we investigated their recognition over a range of concentrations. The results are presented in Fig. 3A. These results show that even at high concentration (20 μM), the P3- and P5-substituted peptides either were not recognized, or their recognition was minimal, indicating that residues in the positions 4 and 5 are part of the epitope recognized by the TCR. C85 at a concentration of 140 nM was needed for induction of the half-maximal cytolytic effect (SD_{50}) of the CTL-41. This value is significantly higher than the amount of C84 (40 pM) giving SD_{50}.

Recognition of P1- and P1P9-substituted peptides by CTL-41 is shown in Fig. 3B. Recognition of all peptides was tested at the same concentration in the assay. The results indicate that the G1-substituted peptide is recognized to a similar extent as C84, whereas T1- and K1-substituted peptides are recognized significantly less. A similar pattern of recognition was observed with peptides substituted at both P1 and P9. Peptides F1M9 and F1V9 were recognized with similar or higher efficiency than C84. Both K1M9 and K1V9 peptides were recognized minimally by CTL-41, suggesting that the observed effect reflects conformational changes in the CTL epitope. These results show that recognition of peptides substituted at P1 is sequence specific. G1M9- and F1M9-substituted peptides were recognized by CTLs with different efficiencies. F1M9 showed significantly higher stabilizing ability than G1M9. However, G1V9 showed equal stabilizing ability with F1V9 but was less recognized by CTL-41. There was no direct correlation between the ability of these peptides to affect either the conformational epitopes BB7.2 or MA2.1 or the magnitude of change in HLA-A2 expression and CTL recognition. All P1P9-substituted peptides showed significantly higher stabilizing ability than C84. The half-maximal stabilization by P1-substituted peptides was observed with 0.5–1.5 μM, whereas for C84, it was observed with 10 μM (Savary et al., unpublished data). This suggests that, compared with C84 or C85 or both, the analogs T1M9, K1M9, and K1V9 are recognized with lower affinity by CTL-41.

DISCUSSION

In this study, we investigated the ability of 26 HER-2 and five FBP peptides (selected using HLA-A2–specific motifs) to stabilize HLA-A2 expression and define peptides of high and low HLA-A2-stabilizing ability. We found that for both proteins, the majority of these peptides are of low binding

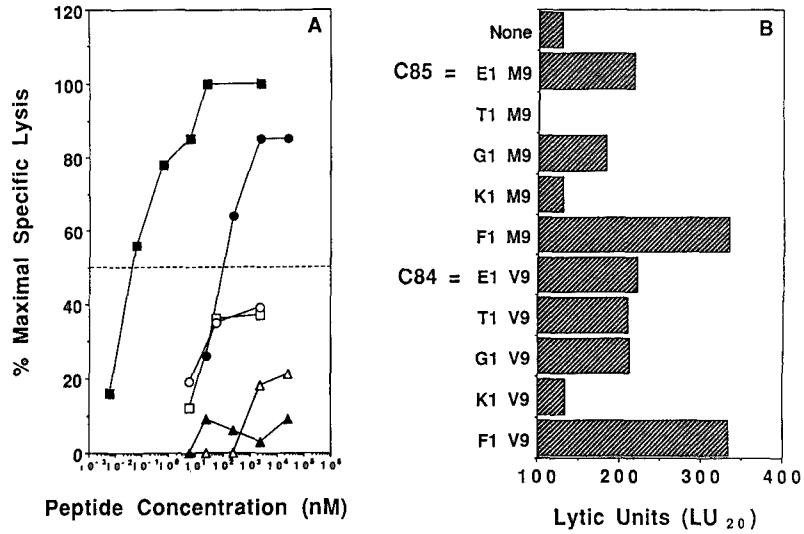

FIG. 3. A: Concentration-dependent recognition of HER-2:971–979 peptide C85 (●) and its substituted analogs in the central area [E51 (△), E52 (▲), E53 (□), E54 (○)] by cytotoxic T lymphocyte (CTL)-41. The sequences of these peptides are presented in Fig. 2. CTL-41 was induced by stimulation with peptide C84 (■). T2 cells were incubated with various concentrations of each peptide for 90 min before being used as targets in the CTL assay. E:T ratio was 20:1. Maximum specific lysis in this experiment for T2 cells pulsed with C84 was 42%. One representative experiment of two performed with similar results is shown. B: Recognition of HER-2:971–979 peptide and its P1- and P9-substituted analogs by CTL-41. T2 cells were incubated with each peptide at 10 μg/ml before being used as targets in CTL assay. CTL-41 were used at three E:T ratios (40, 20, and 10:1). Results are expressed as lytic units ($LU_{20}/10^7$), as we previously described (41). To facilitate comparisons of specificity, the target peptides are presented to indicate the residues that differ in position 1 (e.g., E1, T1, or position M9, V9).

affinity (15 of 26 and four of five, respectively). The amino acids that define these motifs and serve as anchor residues for HLA-A2 were found to differ significantly in the central area of these two groups of peptides (HSA and LSA; residues at P3, P4, and P5), whereas significantly smaller differences were observed for these groups at other positions. These results validate the prominent role of secondary anchor residues for the stability of the entire complex, originally defined from binding measurements with soluble HLA-A2 and various epitopes from various proteins (21). More important, the use for analysis of peptides binding HLA-A2 with high affinity and selected from the same protein allows us to identify the role of the protein sequence in selection of HLA-A2–binding peptides from a tumor protein that is the target of a CTL response. We found that for residue at position 9, replacement of Met with Val enhanced the stability of the HLA-A2–peptide complex. Comparison of the recognition of the conformational epitopes BB7.2 and MA2.1 with monomorphic epitope W6/32 indicated very little change, suggesting that conformational changes induced by the M → V substitution are minimal. The fact that the F1M9 and F1V9 analogs are recognized better than C85 or C84 suggests the possibility of using these modified peptides for CTL induction.

In this context, substitution of negatively charged residues at P1 with residues representative of the major groups of polar, charged, uncharged, and nonpolar amino acids leads to peptides that increase the stability of the HLA-A2–peptide complex. Of the four substitutions tested, three (E → G, E → F, and E → T) showed a parallel increase in both conformational and monomorphic epitope expression, suggesting a stronger stabilizing rather than conformational effect on the peptide–MHC complex. This was equally true for both unsubstituted and P9-substituted peptide C85, suggesting that the contribution of individual residues at P1 and P9 in the epitope sequence to peptide binding may be additive in certain instances. This is of particular interest for use of such peptides for tumor-specific CTL induction, because it may allow sequence optimization for expression, transport, and recognition. The fact that C85-substituted peptides G1V9, F1V9, G1M9, and F1M9, or the wild-type (natural) peptide E1M9 were recognized by CTL induced with the peptide E1V9 raises the possibility that such substituted peptides of high HLA-A2–stabilizing ability can be used as immunogens for induction of tumor-specific CTLs.

These studies were performed with T2 cells as indicators of the ability of HER-2 peptides to stabi-

lize HLA-A2 expression. Although this system allows a fast determination of the binding ability for cell-expressed HLA-A2 as opposed to HLA-A2 in solution, naturally processed peptides associate with nascent class I-β-2 microglobulin complexes. The epitope density is dependent on the levels of MHC class I expression. Both the length and the flanking residues were reported to influence the efficiency of peptide transport and presentation (31,32). These studies provided new insights into the optimization of exogenous antigen presentation. Ongoing studies aim to optimize and quantitate endogenous epitope presentation. APCs [such as B-cells (e.g., C1R.A2) or dendritic cell lines stably transfected with vectors expressing the optimized epitopes] may provide a unique, specific, and renewable source for CTL induction for tumor therapy.

In contrast with the permissivity of P1, substitutions in the central area at P4 and P5 indicated a significant impact of the peptide sequence on the complex stabilization and CTL recognition. The substitutions tested had a rather destabilizing effect on binding, either alone or in combination with conservative substitutions at P8. Furthermore, substituted peptides in the central area P4–P5 were minimally recognized by CTLs. In a similar fashion, investigation of the HLA-A2 stabilization and CTL-3 recognition (6) of peptide E75 (HER-2, 369-377):KIFGSLAFL shows that P1 is permissive to nonconservative changes (e.g., for K → G), whereas P5 is selective. Although at P5, substitutions S → K, S → G, and S → A did not affect HLA-A2 expression, CTL-3 recognition of these three variants was between 20 and 40%, 180 and 200%, and 80 and 90% of the wild-type peptide, respectively. Similar results have been obtained with a breast CTL line (CTL-B5). The variant KIFGGLAFL was better recognized than its wild-type counterpart. Ongoing studies aim to address the effects of changes in P7–P8 in epitope recognition (Fisk et al., unpublished data). The nonpermissivity of the central area of the peptide for substitutions leading to CTL recognition is in agreement with crystallographic models of peptide–MHC class I complexes. Residues at these positions bulge away from the α1 and α2 helices and are likely to be TCR contacts.

Because the low stabilizing ability of the peptides substituted in the central area may lead to an insufficient number of pep-MHC complexes for CTL activation, recognition of these peptides was tested over a range of concentrations. Even at concentrations as high as 20 μg/ml, none of these peptides reached the SD_{50} of CTL-41 for C84. We found that only C85 was recognized by C84-induced CTL at levels comparable with C84, albeit at significantly higher concentrations. Similarly, a number of P1- and P9-substituted peptides were recognized by C84-induced CTLs.

The implications of these findings are twofold: (a) they demonstrate that both P1 and P9 are permissive to substitutions that enhance the stability of the pep-MHC complexes, but the effects of substitutions need to be analyzed for precise prediction of the optimization of HLA-A2 binding and TCR recognition; (b) The selective use of substituted peptides from self-proteins as immunogens leads to CTLs that recognize the natural (wild-type) peptide, although with lower affinity. This finding may have important implications for induction of CTL-recognizing self-proteins. Such HER-2 peptides, as well as the gp 100 peptides, may be expressed at lower amounts on normal tissues than on tumor cells (33). Thus the epitope density may be a limiting factor in epitope recognition (34–36). However, when CTLs are induced by wild-type peptides, recognition of the same epitopes on normal cells may lead to autoimmunity and deviation of the effectors from an antitumor response.

Induction of CTLs by substituted peptides may lead to effectors that recognize the natural peptides with lower affinity than the peptide used as immunogen. Because the low-affinity CTLs need significantly higher epitope density to engage TCRs than high-affinity CTLs (36), effective lysis of tumors overexpressing (e.g., the HER-2 or gp 100 proteins) but not of normal tissues expressing a lower epitope density may be expected. Alternatively, if the tumor epitope is not presented by normal cells, this approach may be used for induction of CTLs recognizing the epitope with higher affinity. Thus variants such as G1V9, F1M9, or F1V9 may be useful for tumor-specific CTL induction. Furthermore, the induced "neo-antigenicity" of the peptides may be beneficial for induction of tumor-reactive CTLs. Although the rules defining CTL cross-reactivity are still unclear, for humoral responses, high-affinity antibodies are more cross-reactive with various antigens than are low-affinity antibodies (37,38).

To date there is little information on induction of CTLs specific for the naturally presented epitope on human tumors using peptides as immunogens. The difficulties in inducing CTLs recognizing these peptides are compounded by the facts that (a) they

are derived from self-proteins, (b) they are present on normal tissues, as demonstrated in melanoma studies (39), and (c) peptide presentation is determined by corresponding HLA molecule coexpression (31). Thus not only induction of CTLs specific for these peptides would require breaking of tolerance in the nonresponder population of patients who express the Ag [e.g., as demonstrated for tyrosinase (3,40)], but induction of CTL-recognizing self-peptides with high affinity also raises concerns about recognition of normal tissues. CTLs that recognize wild-type self-peptides with high affinity need a low epitope density for target lysis. Induction of CTLs that recognize self-peptides with lower affinity may decrease the chances of autoimmunity. However, it raises the question of how it will affect the anti-tumor therapy. Because the density of epitope required for efficient lysis of tumor cells is likely to select TCRs of appropriate affinity, it remains to be seen whether CTLs raised against Ag variants are functional in terms of effective tumor lysis and cytokine production. Thus the use of analogs of tumor peptides for both CTL induction and as MHC/TCR blockers may allow a better defined and controlled approach to immunotherapy of human tumors.

The permissivity of P1 in C85, if confirmed for other peptides as not being essential for CTL recognition, raises the possibility of using P1P9-substituted peptides for tumor specific/reactive CTL induction. These approaches are currently under investigation in our laboratory by using dendritic cells isolated from bone-marrow precursors and T cells from PBMCs from healthy donors and ovarian and breast cancer patients.

Acknowledgment: We thank Dr. David Lawlor (Department of Immunology) for fruitful discussions and Ms. Susan Mondragon for outstanding preparation of this manuscript. This work has been supported by NIH grants. Peptide synthesis was supported in part by core grant 16672.

REFERENCES

1. Kawakami Y, Eliyahu S, Delgado CH, et al. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. *Proc Natl Acad Sci U.S.A.* 1994;91:6458–62.
2. Kawakami Y, Eliyahu S, Jennings C, et al. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *J Immunol* 1995;154:3961–8.
3. Cox AL, Skipper J, Chen Y, et al. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science* 1994;264:716–9.
4. Kawakami Y, Eliyahu S, Sakaguchi K, et al. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J Exp Med* 1994; 180:347–52.
5. Ioannides CG, Fisk B, Fan D, Biddison WA, Wharton JT, O'Brian CA. Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptide derived from the HER-2/neu proto-oncogene. *Cell Immunol* 1993;151:225–34.
6. Fisk B, Blevins TL, Wharton JT, Ioannides CG. Identification of an immunodominant peptide of HER-2/neu proto-oncogene recognized by ovarian tumor specific CTL lines. *J Exp Med* 1995;181:2709–17.
7. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. *Proc Natl Acad Sci USA* 1995;92:432–6.
8. Chen W, Khilko S, Fecondo J, Margulies DH, McCluskey J. Determinant selection of MHC class I-restricted antigenic peptides is explained by class I-peptide affinity and is strongly influenced by non-dominant anchor residues. *J Exp Med* 1994;180:1471–83.
9. Fisk B, Chesak B, Ioannides MG, Wharton JT, Ioannides CG. Sequence motifs of human HER-2 proto-oncogene important for peptide binding to HLA-A2. *Int J Oncol* 1994;5: 51–63.
10. Garin-Chesa P, Campbell I, Saigo PE, Lewis JL Jr, Old LJ, Rettig WJ. Trophoblast and ovarian cancer antigen LK26. Sensitivity and specificity in immunopathology and molecular identification as a folate-binding protein. *Am J Pathol* 1993;142:557–67.
11. Catipovic B, Del Porto J, Mage M, Johansen TE, Schneck JP. Major histocompatibility complex conformational epitopes are peptide specific. *J Exp Med* 1992;176:1611–8.
12. Chen W, Fecondo J, McCluskey J. The structural influence of individual residues located within peptide antigen depends upon their sequence context. *Mol Immunol* 1994;31: 1069–75.
13. Fisk B, Chesak B, Pollack M, Wharton JT, Ioannides CG. Oligopeptide induction of a cytotoxic T lymphocyte response to HER-2/neu proto-oncogene in vitro. *Cell Immunol* 1994;157:412–27.
14. Salter RD, Creswell P. Impaired assembly and transport of HLA-A and -B antigens in a mutant T × B cell hybrid. *EMBO J* 1986;5:943–9.
15. Salter RD, Clayberger C, Lomen CE, et al. In vitro mutagenesis at a single residue introduces B and T cell epitopes into a class I HLA molecule. *J Exp Med* 1987;166:283–8.
16. Santos-Aguado J, Barbosa JA, Biro PA, Strominger JL. Molecular characterization of serologic recognition sites in the human HLA-A2 molecule. *J Immunol* 1988;141:2811–8.
17. Hogan KT, Clayberger C, Bernhard EJ, et al. A panel of unique HLA-A2 mutant molecules define epitopes recognized by HLA-A2 specific antibodies and cytotoxic T lymphocytes. *J Immunol* 1989;142:2097–104.
18. Rohren EM, McCormick DJ, Pease LR. Peptide-induced conformational changes in class I molecules. *J Immunol* 1991;152:5337–43.
19. Bluestone JA, Jameson S, Miller S, Dick R II. Peptide-induced conformational changes in class I heavy chains alter major histocompatibility complex recognition. *J Exp Med* 1992;176:1757–61.
20. Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 1991;351: 290–6.
21. Ruppert J, Sidney J, Celis E, Kubo RT, Grey HM, Sette A. Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. *Cell* 1993;74:929–37.

22. Engelhard VH. Structure of peptides associated with class I and class II MHC molecules. *Annu Rev Immunol* 1994;12: 181–207.
23. Henderson RA, Michel H, Sakaguchi K, et al. HLA-A2.1–associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science* 1992;255:1264–6.
24. Lanzavecchia A, Watts C. Peptide partners call the tune. *Nature* 1994;371:198–9.
25. Townsend A, Elliott T, Cerundolo V, Foster L, Barber B, Tse A. Assembly of MHC class I molecules analyzed in vitro. *Cell* 1990;62:285–95.
26. Madden DR, Garboczi DN, Wiley DC. The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. *Cell* 1993;75:693–708.
27. Collins EJ, Garboczi DN, Wiley DC. Three-dimensional structure of a peptide extending from one end of a class I MHC binding site. *Nature* 1994;371:626–9.
28. Boivier M, Wiley DC. Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules. *Science* 1994;265:398–402.
29. Bednarek MA, Sauma SY, Gammon MC, et al. The minimum peptide epitope from the influenza virus matrix protein: extra and intracellular loading of HLA-A2. *J Immunol* 1991;147:4047–53.
30. Hogquist KA, Grandea AG III, Bevan MJ. Peptide variants reveal how antibodies recognize major histocompatibility complex class I. *Eur J Immunol* 1993;23:2072–7.
31. Griem P, Wallny H-J, Falk K, et al. Uneven tissue distribution of minor histocompatibility proteins versus peptides is caused by MHC expression. *Cell* 1991;65:633–40.
32. Neisig A, Roelse J, Sijts AJ, et al. Major differences in transporter associated with antigen presentation (TAP)-dependent translocation of MHC class I-presentable peptides and the effect of flanking sequences. *J Immunol* 1995; 154:1273–9.
33. Bakker AB, Schreurs MW, de Boer AJ, et al. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. *J Exp Med* 1994; 179:1005–9.
34. Ioannides CG, Ioannides MG, O'Brian CA. T cell recognition of oncogene products, a new strategy for immunotherapy. *Mol Carcinog* 1992;6:77–81.
35. Schild H, Norda M, Deres K, et al. Fine specificity of cytotoxic T lymphocytes primed in vivo either with virus or synthetic lipopeptide vaccine or primed in vitro with peptide. *J Exp Med* 1991;174:1665–8.
36. Kageyama S, Tsomides TJ, Sykulev Y, Eisen H. Variations in the number of peptide-MHC class I complexes required to activate cytotoxic T cell responses. *J Immunol* 1995;154: 567–76.
37. Udaka K, Wiesmuller KH, Kienle S, Jung G, Walden P. Decrypting the structure of major histocompatibility complex class I-restricted cytotoxic T lymphocyte epitopes with complex peptide libraries. *J Exp Med* 1995;181:2097–108.
38. Eisen HN. *General immunology*. Philadelphia: JB Lippincott, 1990.
39. Anichini A, Maccalli C, Mortarini R, et al. Melanoma cells and normal melanocytes share antigens recognized by HLA-A2-restricted cytotoxic T cell clones from melanoma patients. *J Exp Med* 1993;177:989–98.
40. Visseren MJW, van Elsas A, van der Voort EIH, et al. CTL specific for the tyrosinase autoantigen can be induced from healthy donor blood to lyse melanoma cells. *J Immunol* 1995;154:3991–8.
41. Ioannides CG, Platsoucas D, Rashed S, Wharton JT, Edwards CL, Freedman RS. Tumor cytolysis by lymphocytes infiltrating ovarian malignant ascites. *Cancer Res* 1991;51: 4257–66.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 271, No. 4, Issue of January 26, pp. 2102–2111, 1996
Printed in U.S.A.

Partial Reversal of Multidrug Resistance in Human Breast Cancer Cells by an *N*-Myristoylated Protein Kinase C-α Pseudosubstrate Peptide*

(Received for publication, June 13, 1995, and in revised form, October 30, 1995)

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The predominant characteristics of multidrug resistant (MDR) cancer cells are broad spectrum resistance to chemotherapeutic agents and a pronounced defect in intracellular accumulation of the drugs, in association with overexpression of the drug efflux pump P-glycoprotein. Protein kinase C (PKC) phosphorylates the linker region of P-glycoprotein. Evidence has been presented that the isozyme PKC-α may contribute to the drug resistance phenotype of human breast cancer MCF7-MDR cells. PKC-α is markedly overexpressed in MCF7-MDR cells, and artificial overexpression of PKC-α in MCF7 constructs that overexpress P-glycoprotein significantly enhances the MDR phenotype of the cells in association with increased P-glycoprotein phosphorylation. Verapamil, cyclosporin A, and a number of other agents that compete with cytotoxic drugs for binding sites on P-glycoprotein can potently reverse MDR, but this is accompanied by severe toxicity *in vivo*. In this report, we demonstrate that an *N*-myristoylated peptide that contains a sequence corresponding to the pseudosubstrate region of PKC-α (P1) partially reverses multidrug resistance in MCF7-MDR cells by a novel mechanism that involves inhibition of PKC-α. P1 and two related PKC inhibitory *N*-myristoylated peptides restored intracellular accumulation of chemotherapeutic drugs in association with inhibition of the phosphorylation of three PKC-α substrates in MCF7-MDR cells: PKC-α, Raf-1 kinase, and P-glycoprotein. A fourth *N*-myristoylated peptide substrate analog of PKC, P7, did not affect drug accumulation in the MCF7-MDR cells and failed to inhibit the phosphorylation of the PKC-α substrates. The effects of P1 and verapamil on drug accumulation in MCF7-MDR cells were additive. P1 did not affect P-glycoprotein expression. MCF7-MDR cells were not cross-resistant to P1, which suggests that the peptide was not transported by P-glycoprotein. Furthermore, P1 was distinguished from MDR reversal agents such as verapamil and cyclosporin A by its inability to inhibit [^{3}H]azidopine photoaffinity labeling of P-glycoprotein. P1 actually increased [^{3}H]azidopine photoaffinity labeling of P-glycoprotein in MCF7-MDR cells, providing evidence that the effects of P1 on P-glycoprotein in MCF7-MDR cells are not restricted to inhibition of the phosphorylation of the pump. P1 may provide a basis for developing a new generation of MDR reversal agents that function by a novel mechanism that involves inhibition of PKC-α-catalyzed P-glycoprotein phosphorylation.

Resistance to chemotherapy is a major obstacle to successful cancer treatment, and it often accounts for the failure of aggressive chemotherapy to eradicate malignant disease (1). Most metastatic cancers are either innately resistant to chemotherapy or acquire drug resistance during the course of chemotherapy (1). Multidrug resistant (MDR)[1] cancer cells are characterized by broad spectrum resistance to chemotherapeutic drugs, markedly reduced intracellular accumulation of the drugs, and overexpression of the drug efflux pump P-glycoprotein (1, 2). The relevance of MDR to clinical drug resistance in cancer therapy is indicated by the abundant expression of P-glycoprotein and its message *mdr1* in specimens of human cancer that are intrinsically resistant to chemotherapy and in malignant tumors from patients who have relapsed during or after chemotherapy (1, 3, 4).

Protein kinase C (PKC) is an isozyme family with at least ten mammalian members (5). Highly selective phorbol-ester PKC activators induce resistance in cancer cells to multiple cytotoxic drugs that are P-glycoprotein substrates in association with a sharp reduction in the intracellular accumulation of the drugs, providing evidence that PKC activation contributes to MDR. The phorbol-ester effects on chemosensitivity and drug accumulation have been observed in several drug-sensitive and MDR cancer cell lines including human breast cancer MCF7-WT and MCF7-MDR, and the magnitude of the effect is generally a 2–6-fold increase in the IC_{50} values of cytotoxic drugs (6–12). PKC phosphorylates the linker region of P-glycoprotein in MDR human KB-V1 cancer cells (9, 13, 14), and this is tightly coupled to the effects of PKC on intracellular drug accumulation and MDR, providing evidence that PKC regulates the function of P-glycoprotein in this system (11). The isozyme PKC-α has been shown to be overexpressed in several MDR cancer cell lines including MCF7-MDR (8, 15–18). Evidence that PKC-α activation contributes to MDR has been provided by observations that MDR is induced by the selective activation of PKC-α in human colon cancer cells (12) and by transfection of an *mdr1*-transfected human breast cancer MCF7 subline with PKC-α (19).

Verapamil, cyclosporin A, and related MDR reversal agents

* This work was supported by Robert A. Welch Foundation Grant G-1141, National Cancer Institute Grant CA-52460, and an award from The Elsa U. Pardee Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MDR, multidrug-resistant or multidrug resistance; ADR, Adriamycin; 5FU, 5-fluorouracil; PKC, protein kinase C; VLB, vinblastine; VP, verapamil; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; mAb, monoclonal antibody; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

that compete with chemotherapeutic drugs for binding sites on P-glycoprotein (20–23) generally cause severe toxicity *in vivo* at therapeutic concentrations, and this precludes their use in the treatment of drug-resistant cancer (24, 25). In a previous report, we characterized the mechanism of PKC inhibition by an *N*-myristoylated peptide corresponding to the autoinhibitory pseudosubstrate sequence of PKC-α (P1) that had been shown to selectively inhibit PKC in human fibroblasts (26, 27). In this report, we show that the peptide P1 reverses MDR by a novel mechanism that is associated with a sharp increase in intracellular drug accumulation and inhibition of the phosphorylation of P-glycoprotein and two other PKC-α substrates, Raf-1 kinase and PKC-α itself. P1 was distinguished from verapamil and related MDR reversal agents (20–23) in that it did not inhibit photoaffinity labeling of drug-binding sites on P-glycoprotein by [^{3}H]azidopine. P1 did not affect P-glycoprotein expression in MCF7-MDR cells. MCF7-MDR cells were not cross-resistant to P1, providing indirect evidence that the peptide was not transported by P-glycoprotein. The *N*-myristoylated PKC-α pseudosubstrate peptide P1 may be a valuable starting point for developing a new generation of MDR reversal agents that function by a novel mechanism that involves inhibition of PKC-α-catalyzed P-glycoprotein phosphorylation.

MATERIALS AND METHODS

Cell Lines—The drug-sensitive wild-type human breast cancer cell line MCF7-WT and the MDR subline MCF7-MDR were supplied by Dr. Kenneth H. Cowan (National Cancer Institute). The MCF7-MDR cells were derived from the parental line MCF7-WT by serial passage with increasing ADR concentrations and are maintained in the presence of 1 μg/ml ADR. The MCF7-MDR subline is >100-fold resistant to ADR, vincristine, and VLB (18). MCF7-MDR cells were grown in the absence of ADR for at least two passages prior to their use in experiments. Cells were maintained in Eagle's minimum essential medium containing 5% heat-inactivated fetal calf serum, nonessential amino acids, vitamins, sodium pyruvate, L-glutamine, and penicillin-streptomycin.

Synthetic Peptides—The oligopeptides *N*-myristoyl-FARKGALRQ (P1), *N*-myristoyl-YARKGALRQ, FARKGALRQ (P2), *N*-myristoyl-RFARKGALRQKNV(P3), RFARKGALRQKNV(P4), *N*-myristoyl-RKRTLRRL (P5), RKRTLRRL (P6), and *N*-myristoyl-NDSRSSLIRKR (P7) were synthesized using the Vega Coupler 250 peptide synthesizer. The peptides were purified to >98% purity by reverse-phase high pressure liquid chromatography using a Vydac C4 column and an acetonitrile gradient. All peptides were prepared at the M. D. Anderson Cancer Center Synthetic Antigen Facility.

Chemicals and Reagents—[γ-^{32}P]ATP (30 Ci/mmol), [^{32}P]P_i (200 mCi/mmol), [^{14}C]adriamycin (55 mCi/mmol), [^{3}H]azidopine (49 Ci/mmol), and [^{3}H]vinblastine sulfate (11 Ci/mmol) were purchased from Amersham Corp. [^{3}H]5-Fluorouracil (14 Ci/mmol) was obtained from DuPont NEN. The P-glycoprotein monoclonal antibody C219 was obtained from Signet Laboratories (Dedham MA), adriamycin and vinblastine sulfate were from Cetus Laboratories (Emeryville CA), 5-fluorouracil was from SoloPak (Franklin Park, IL), and protein assay solution and SDS-PAGE reagents were from Bio-Rad Laboratories. Tissue culture reagents were purchased from Life Technologies, Inc. Protease inhibitors, tetrazolium, myristic acid, *N,N*-dimethylmyristamide, verapamil, protein A-Sepharose, and all other reagents were obtained from Sigma. Purified rat brain PKC was prepared from rat brains, and its Ca^{2+}- and phosphatidylserine-stimulated histone kinase activity was assayed as described previously (28).

Drug Accumulation and Efflux Assays—The intracellular accumulation of cytotoxic drugs was measured by an established method (10, 12). Stock solutions of 50 μM [^{14}C]ADR, 23 μM [^{3}H]VLB, and 70 μM [^{3}H]5FU were diluted before use in tissue culture medium containing 10 mM HEPES (pH 7.3). Stock solutions of *N*-myristoylated and nonmyristoylated peptides (1 mM) were made in either PBS or water. Cells were plated into 24-well Costar plates (201 mm^2/well) at a density of 5×10^5 cells/well. Following a 20–24-h attachment period at 37 °C, cells were preincubated with the peptides under investigation at indicated concentrations for 30 min at 37 °C. Treatment with buffer alone served as a negative control, and treatment with 10 μM verapamil was used as a positive control. Next, the pretreated cells were incubated at 37 °C with radiolabeled cytotoxic drug ([^{14}C]ADR, [^{3}H]VLB, or [^{3}H]5FU) in the presence of the peptides (or controls) for the duration of the drug accumulation period (5 min to 6 h). The drug accumulation assay was terminated by rapidly washing the cells three times with ice-cold PBS, detaching the cells by a 30-min exposure to trypsin-EDTA at 37 °C, harvesting the cells, and counting them in vials containing 15 ml of scintillation fluid. To measure net drug efflux rates, MCF7-MDR cells were preloaded with 0.2 μM [^{14}C]ADR for 2 h, washed rapidly with ice-cold PBS four times, and incubated at 37 °C in the presence of *N*-myristoylated peptides, verapamil, or medium alone. At the indicated time intervals, the incubation was stopped and the radioactivity released into the medium was counted, as described previously (10).

Growth Inhibition Assay—The reduction in viable cell number affected by a 24-h exposure to cytotoxic drugs (ADR, VLB, and 5FU) was measured as described previously using a tetrazolium assay (10, 12). Cells were harvested in their exponential growth phase, and single cell suspensions with a viability of >95% were seeded into 96-well microculture plates at a density of 3,000 MCF7-WT or 4,500 MCF7-MDR cells/well. Following a 20–24-h attachment period, cells were preincubated for 1 h at 37 °C with either the synthetic peptide under investigation or buffer (which served as a vehicle control). Next, the peptide-containing medium was removed, and the cells were incubated with either medium alone or medium containing cytotoxic drugs (ADR, VLB, and 5FU) for 24 h at 37 °C. Drug-containing medium was then removed from the cells, fresh medium was applied, and the cells were further incubated for 72 h. At the end of the incubation period, 40 μl of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (2.5 mg/ml in PBS) was pipetted into each well and allowed to react with mitochondrial dehydrogenases in viable cells for 2 h at 37 °C. Unreacted dye and medium were removed from the wells, 100 μl of dimethyl sulfoxide was added to each well, and the conversion of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide to formazan by viable cells was quantitated by measuring the absorbance at 570 nm with a microplate-scanning spectrophotometer (10, 12). Direct growth-inhibitory effects of the peptides against MCF7-WT and MCF7-MDR were measured by a modification of this procedure, in which the cells were exposed to the peptides for the entire 96-h growth inhibition assay.

P-glycoprotein, Raf-1 Kinase, and PKC-α Phosphorylation in MCF7 Cells—Effects of *N*-myristoylated and nonmyristoylated synthetic peptides on P-glycoprotein phosphorylation in [^{32}P]P_i-labeled MCF7-MDR cells were determined using established methods (18, 29). Nearly confluent MCF7 cells grown in 75-cm^2 flasks were labeled with [^{32}P]P_i by washing the cells with phosphate-free buffered saline and phosphate-free medium successively and then incubating the cells with 6 ml of phosphate-free medium containing 10 mM HEPES (pH 7.3) and 0.6 mCi of [^{32}P]P_i for 3 h at 37 °C. Where indicated, synthetic peptides were included in the incubation mixtures. All subsequent procedures were done at 4 °C. Labeled cells were washed three times with ice-cold DPBS and scraped from the plates with lysis buffer (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM NaF, 1 mM sodium vanadate, 2 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 200 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride) (1 ml/plate). The resultant cell suspensions were stirred for 15 min, lysates were spun at 13,800 × *g* for 15 min, and supernatants were collected.

The procedures used to immunoprecipitate P-glycoprotein, PKC-α, and Raf-1 kinase were based on previously described methods (18). For P-glycoprotein immunoprecipitation, the supernatant (100 μg of protein) was incubated with 5 μg of C219 monoclonal antibody by rotation for 16 h at 4 °C. In the case of Raf-1 kinase and PKC-α immunoprecipitation, the supernatant was incubated with Raf-1 kinase mAb (5 μg) (Oncogene Science, Uniondale, NY) or PKC-α mAb (5 μg) (Upstate Biotechnology, Inc., Lake Placid, NY) by rotation for 2 h at 4 °C, and rotation was continued in the presence of 5 μg rabbit antimouse IgG for an additional 30 min. at 4 °C. Next, 0.2 ml of a 25% (v/v) suspension of protein A-Sepharose was added to the incubation mixture followed by rotation for 30 min at 4 °C. After a 1-min centrifugation in a microcentrifuge, the beads were recovered as a pellet, and they were washed successively with 1 M NaCl, 1% Nonidet P-40, and lysis buffer containing 1 M urea. To recover immune complexes from the beads, the beads were incubated for 10 min at 30 °C with 0.1 ml of SDS-PAGE sample buffer, and then they were pelleted in a microcentrifuge. The supernatant was recovered and subjected to SDS-PAGE. Gels were silver stained, and autoradiography was done.

P-glycoprotein and Raf-1 Kinase Expression—P-glycoprotein expression was measured by immunoblot analysis of cell lysates using the monoclonal antibody C219 (500 ng/ml) (30). Briefly, cells were exposed to 100 μM P1, P3, P5, or medium alone for 3 h at 37 °C. Cells were washed with PBS and harvested at 4 °C with 1% Triton X-100 in 20 mM Tris-HCl, pH 7.5, 15 mM β-mercaptoethanol, 5 mM EDTA, 5 mM EGTA,

TABLE I
Synthetic peptide substrate analogs of PKC

Name	Structure[a]	IC_{50}[b]
P1	NmFARKGALRQ	16 ± 1 μM (27)
P2	FARKGALRQ	No inhibition (27)
P3	NmRFARKGALRQKNV	3.0 ± 0.6 μM
P4	RFARKGALRQKNV	No inhibition
P5	NmRKRTLRRL	5.0 ± 0.5 μM (33)
P6	RKRTLRRL	No inhibition (33)
P7	NmNDSRSSLIRKR	37 ± 2 μM[c]

[a] Nm = *N*-myristoyl.
[b] Peptide concentration that elicits 50% inhibition of PKC-catalyzed histone phosphorylation.
[c] At concentrations ≤10 μM, P7-stimulated PKC activity up to 2-fold.

0.25 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Immunoblot analysis of cell lysates was done by standard procedures (12), except that samples were not boiled prior to SDS-PAGE (30). The secondary antibody employed was horseradish peroxidase-linked anti-mouse antibody (Amersham Corp.). Immunoreactive bands were detected by enhanced chemiluminescence (Amersham Corp.), and bands were quantitated using a computerized densitometer. For Raf-1 kinase expression, an identical procedure was used except that samples were boiled and the primary antibody employed was a Raf-1 kinase-specific mAb (Oncogene Scis., Uniondale, NY). The mAb concentration was 10 μg/ml. PKC-α immunoblot analysis was done as described in Ref. 12.

Photoaffinity labeling of P-glycoprotein in MCF7-MDR cells—P-glycoprotein was photoaffinity labeled with [^{3}H]azidopine in whole MCF7-MDR cells by a standard method (20, 29). Briefly, 10^7 MCF7-MDR cells suspended in PBS were incubated in the dark with [^{3}H]azidopine (15 μCi) and the peptide under investigation for 1 h at 37 °C. Next, the photoreaction was initiated by irradiation at 365 nm at a distance of 5 cm (4 °C) using a self-filtering lamp. After an irradiation period of 30 min, cells were lysed at 4 °C in 10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10 mM $MgSO_4$, 10 mM $CaCl_2$, and 0.1% Triton X-100. To immunoprecipitate P-glycoprotein, the sample was incubated with 5 μg of C219 mAb, and this was followed by the addition of protein-A Sepharose, as described above. The beads were pelleted and washed once with 1 M NaCl containing 1% Nonidet P-40 and three times with 10 mM Tris-HCl, pH 8.0, containing 150 mM NH_4Cl, 2 mM $MgCl_2$, and 1% CHAPS (20). P-glycoprotein was dissociated from the beads and subjected to SDS-PAGE analysis as described above. Gels were soaked in Amplify solution (Amersham Corp.) for 30 min and dried prior to autoradiography at −70 °C.

Statistical Analysis—For statistical analysis of data, the two-tailed Student's *t* test was performed with Microsoft software using a Dell computer.

RESULTS

Table I shows the structures of the peptides under investigation for modulatory effects on the intracellular accumulation and growth-inhibitory activity of cytotoxic anticancer drugs in human breast cancer MCF7-MDR cells. P2 contains the core sequence of the pseudosubstrate region of PKC-α (PKC[20–28]), P4 contains a more extensive PKC-α pseudosubstrate sequence (PKC[19–31]) (26, 27), and P6 contains the sequence of the PKC phosphorylation site of the epidermal growth factor receptor at Thr^{654} (27). P1, P3, and P5 are *N*-myristoylated analogs of P2, P4, and P6, respectively. Each of these *N*-myristoylated peptides inhibits the histone kinase reaction of purified PKC (Table I); the corresponding nonmyristoylated peptides do not inhibit PKC-catalyzed histone phosphorylation (Table I). P7 is an *N*-myristoylated peptide with a sequence that corresponds to a PKC phosphorylation site in P-glycoprotein (residues 656–666) (13). Although P7 does inhibit the histone kinase activity of PKC, it is weaker than P1, P3, and P5 in this regard, and, at concentrations ≤10 μM, P7 actually stimulates the histone kinase reaction (Table I). In a previous report, we showed that the mechanism of PKC inhibition by several *N*-myristoylated peptide substrate analogs of PKC, including P1 and P5, entailed binding interactions of the inhibitor peptides with the active site of PKC and with phospholipid vesicles (27). In control experiments that monitored the absorbance of the Tyr-containing P1 analog NmYARKGALRQ, we have found that the phospholipid-interacting peptide does not bind to plastic-, glass-, or collagen-coated surfaces (data not shown).

P1 has been shown to inhibit phosphorylation of the PKC substrate MARCKS in intact rat fibroblasts (26), and the truncated P5 analog *N*-myristoyl-KRTLR antagonizes PKC-requiring pathways in T-lymphocytes (31, 32). The demonstrated ability of PKC-inhibitory *N*-myristoylated peptide substrate analogs to inhibit PKC activity in nonpermeabilized mammalian cells (26, 31, 32) provides indirect evidence that the peptides enter mammalian cells, which is consistent with their membrane-active nature (27). P5 partially reverses adriamycin resistance in murine fibrosarcoma cells (33). Specific activation of PKC is correlated with enhancement of MDR in tumor cells, and inhibition of the enzyme is often associated with partial reversal of MDR (10, 11). We hypothesized that P1 and related PKC-inhibitory *N*-myristoylated peptides might antagonize MDR in human breast cancer MCF7-MDR cells.

Fig. 1 shows the effects of the peptides P1–P6 on the intracellular accumulation of [^{14}C]ADR and [^{3}H]VLB, which are P-glycoprotein substrates (1, 2, 10), and [^{3}H] 5FU, which is not a P-glycoprotein substrate (1, 2, 10), in MCF7-MDR cells. All assays of drug accumulation in MCF7-MDR and MCF7-WT cells described in this report were done under conditions where cell viability was >95% at the end of the assay period, according to measurements of trypan blue exclusion. The *N*-myristoylated peptides P1, P3, and P5 markedly and significantly increased the accumulation of [^{14}C]ADR (Fig. 1*A*) and [^{3}H]VLB (Fig. 1*B*) in the MCF7-MDR cells, whereas the nonmyristoylated peptides P2, P4, and P6 had no effect on their accumulation (Fig. 1, *A* and *B*). The *N*-myristoylated peptides enhanced drug accumulation in the MCF7-MDR cells from 3–9-fold, which is comparable with the enhancement of drug accumulation that was achieved by the potent MDR reversal agent verapamil (VP) (Fig. 1, *A* and *B*). For comparison, the levels of intracellular [^{14}C]ADR and [^{3}H]VLB accumulation in MCF7-WT were respectively 5- and 10-fold higher than those in MCF7-MDR. The enhancement of drug uptake was most pronounced when the *N*-myristoylated peptide concentration was 100 μM. However, at a concentration of 50 μM, P1 and P3 each significantly enhanced [^{14}C]ADR uptake, and P1 and P5 significantly enhanced [^{3}H]VLB uptake in the MCF7-MDR cells (Fig. 1, *A* and *B*). In parallel experiments, we found that like VP, P1–P6 did not significantly affect [^{3}H]5FU accumulation in MCF7-MDR cells (Fig. 1*C*). This demonstrates a degree of specificity in the effects of the *N*-myristoylated peptides on drug accumulation in MCF7-MDR cells. [^{14}C]ADR and [^{3}H]VLB accumulation in MCF7-MDR cells were enhanced by less than 5% by myristic acid (25–100 μM) and by *N*,*N*-dimethylmyristamide (25–100 μM), which is a nonpeptidic amide of myristic acid. Thus, specificity is also indicated by the lack of effect of the acyl head group of the peptides on [^{14}C]ADR and [^{3}H]VLB accumulation. In addition, P7 (25–100 μM) failed to enhance [^{14}C]ADR and [^{3}H]VLB accumulation in the cells, indicating that not all *N*-myristoylated cationic peptides can enhance the retention of these cytotoxic drugs in MCF7-MDR cells.

Table II shows that P1–P6 increased [^{14}C]ADR and [^{3}H]VLB accumulation in the drug-sensitive cell line MCF7-WT only marginally (<1.8-fold) (P2, P3, P4) or not at all (P1, P5, P6), and they had no detectable effect on [^{3}H]5FU accumulation in the cells. Of the *N*-myristoylated peptides, only P3 detectably increased [^{14}C]ADR and [^{3}H]VLB accumulation in the MCF7-WT cells (Table II). Thus, the marked enhancement of [^{14}C]ADR and [^{3}H]VLB uptake achieved by the *N*-myristoy-

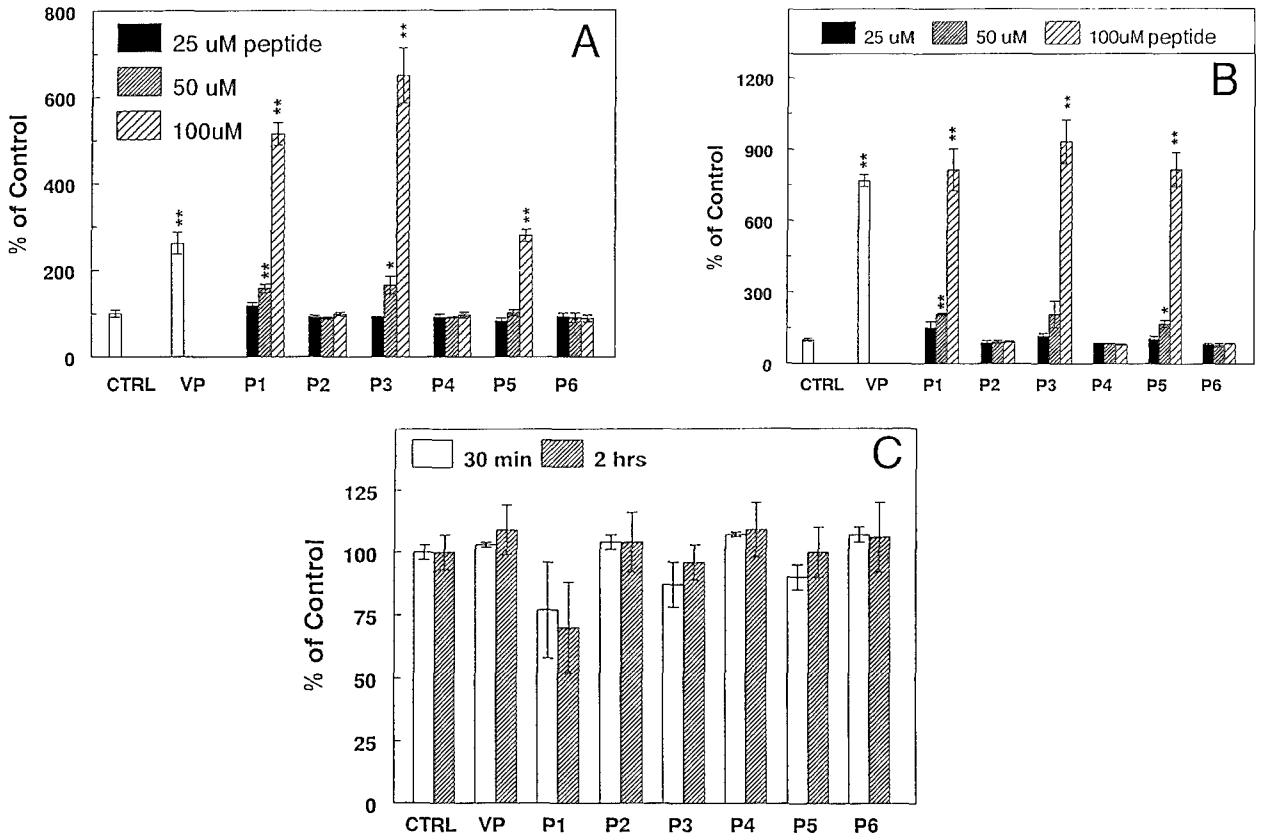

FIG. 1. **Effects of synthetic peptide substrate analogs of PKC on cytotoxic drug accumulation in MCF7-MDR cells.** Effects of the *N*-myristoylated peptides P1, P3, and P5 and the corresponding nonmyristoylated peptides P2, P4, and P6 at concentrations of 25, 50, and 100 μM on drug accumulation in MCF7-MDR cells are shown. Drug accumulation was assayed as described under "Materials and Methods." Under each experimental condition examined in *A–C*, >95% cell viability was observed at the end of the drug accumulation period by measurements of trypan blue exclusion. Each experimental value shown is the average value from three experiments that were done in triplicate. Values that differ significantly from the untreated control are identified by *asterisks*. **, $p < 0.015$; *, $p < 0.05$. *A*, [^{14}C]ADR accumulation in MCF7-MDR cells is shown. *% of Control*, the amount of [^{14}C]ADR accumulation observed in the presence of the indicated peptide expressed as a percentage of [^{14}C]ADR accumulation observed in untreated control (*CTRL*) MCF7-MDR cells. Treatment with 10 μM VP served as a positive control. The [^{14}C]ADR concentration was 0.1 μM, and accumulation was measured after a 2-h incubation period. Untreated cells retained 2.2 ± 0.2 pmol [^{14}C]ADR/10^6 cells. *B*, [^{3}H]VLB accumulation in MCF7-MDR cells is shown. Assays were done with 10 nM [^{3}H]VLB, and accumulation was measured after incubating the cells with drugs for 30 min. Untreated MCF7-MDR cells retained 0.066 ± 0.005 pmol [^{3}H]VLB/10^6 cells. For other details, see the description of *A*. *C*, [^{3}H]5FU accumulation in MCF7-MDR cells is shown. Where indicated, peptides (P1–P6) were present at 100 μM; [^{3}H]5FU was at 10 nM. Drug accumulation was measured after 30 min and 2 h, as shown. [^{3}H]5FU accumulation in control (*CTRL*) cells was 0.058 ± 0.002 (30 min) and 0.058 ± 0.004 pmol [^{3}H] 5FU/10^6 cells (2 h). For other details, see the description of *A*.

TABLE II
Effects of synthetic peptide substrate analogs of PKC on cytotoxic drug accumulation in MCF7-WT cells

	Drug uptake[a]		
	[^{14}C]ADR	[^{3}H]VLB	[^{3}H]5FU
VP	93 ± 13	124 ± 13	97 ± 12
P1	99 ± 14	115 ± 13	92 ± 5
P2	95 ± 5	160 ± 9	97 ± 7
P3	173 ± 13	153 ± 6	102 ± 1
P4	136 ± 20	137 ± 24	103 ± 6
P5	104 ± 6	107 ± 10	97 ± 2
P6	98 ± 3	121 ± 10	93 ± 2

[a] Drug uptake is expressed as a percentage of the uptake observed in untreated MCF7-WT cells. Drug uptake was measured as described under "Materials and Methods" and the legend to Fig. 1. Peptides were present where indicated at 100 μM, and VP was employed at a concentration of 10 μM. Under each set of conditions, the cells were determined to be >95% viable at the end of the drug accumulation assay by trypan blue exclusion. Each experimental value represents an average of triplicate determinations. The results shown were determined to be reproducible in a duplicate experiment.

lated peptides P1, P3, and P5 but not by their nonmyristoylated counterparts (Fig. 1) were specific to the MDR subline.

Having established that the *N*-myristoylated peptides significantly increase [^{14}C]ADR and [^{3}H]VLB accumulation in MCF7-MDR cells at 2 h and 30 min, respectively (Fig. 1), we next examined the effects of P1–P6 on the accumulation of [^{14}C]ADR and [^{3}H]VLB in MCF7-MDR cells over a 6-h time course. In these experiments, cells were pretreated with peptide for 30 min, and the time course was initiated by the addition of drug, as described under "Materials and Methods." The enhancement of drug accumulation by the *N*-myristoylated peptides (P1, P3, and P5) was observed within 5 and 15 min, respectively, for [^{3}H]VLB (Fig. 2, *C* and *D*) and [^{14}C]ADR (Fig. 2, *A* and *B*). The enhancement of [^{14}C]ADR accumulation was sustained at a maximal or nearly maximal level over a 6-h time course in each case, as was the enhancement of [^{3}H]VLB accumulation by P1 (Fig. 2, *A–C*), but the effects of P3 and P5 on [^{3}H]VLB accumulation peaked within 2 h and then gradually declined throughout the remainder of the time course (Fig. 2*D*). No enhancement of [^{14}C]ADR or [^{3}H]VLB accumulation was observed with the nonmyristoylated peptides (P2, P4, and P6) (Fig. 2). The relatively slow uptake of [^{14}C]ADR induced by the *N*-myristoylated peptides allowed estimation of the effects of the peptides on the [^{14}C]ADR efflux rate of MCF7-MDR cells by measuring the net efflux of [^{14}C]ADR from preloaded cells. Across a 30-min time course, a decline in the net [^{14}C]ADR efflux rate was observed in MCF7-MDR cells with each *N*-myristoylated peptide (100 μM P1, P3, and P5), and the mean value of the decline achieved by the peptides was 13 ± 4%,

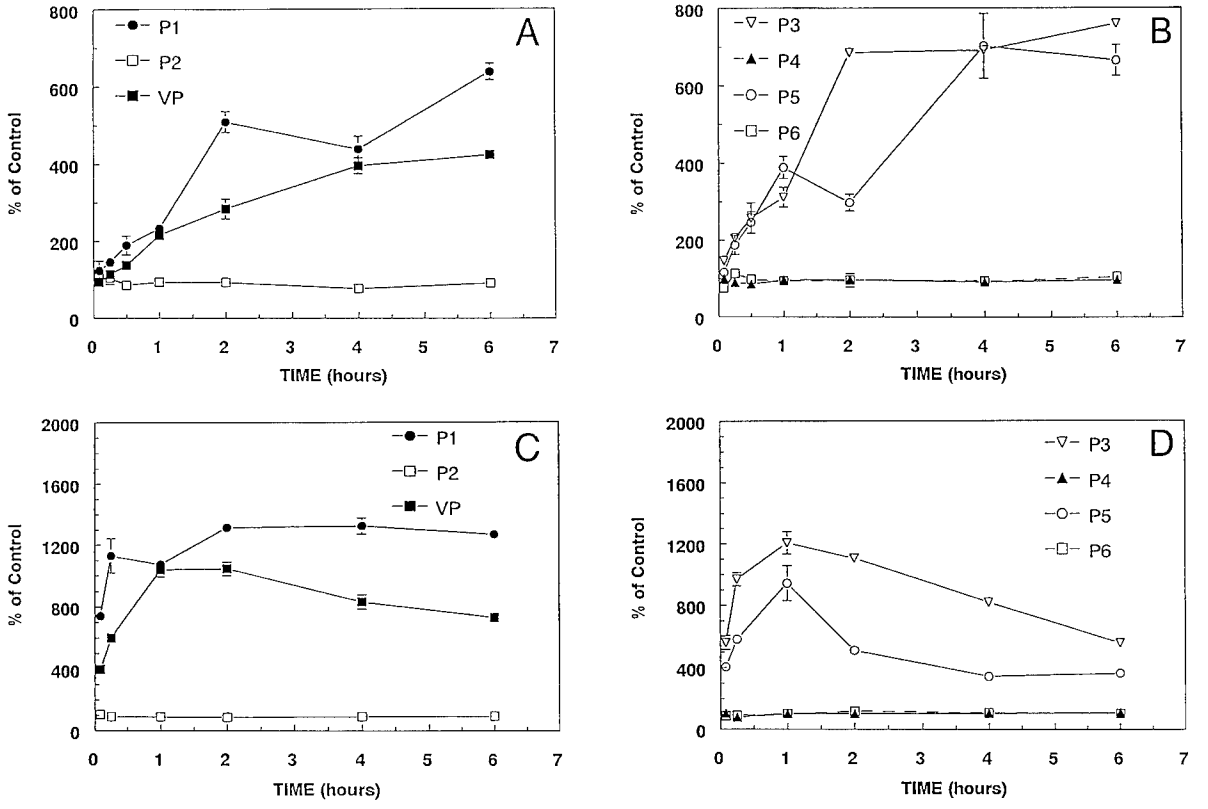

FIG. 2. **Effects of synthetic peptide substrate analogs of PKC on the kinetics of net drug uptake in MCF7-MDR cells.** Intracellular accumulation of [^{14}C]ADR (*A* and *B*) and [^{3}H]VLB (*C* and *D*) was measured in MCF7-MDR cells at the indicated time intervals. Where specified, assays were done in the presence of 100 μM peptide (*A* and *C*, P1 and P2; *B* and *D*, P3, P4, P5, and P6) or 10 μM VP (*A* and *C*). In these experiments, cell viability was >95% according to trypan blue exclusion measurements. For definition of *% of Control*, assay conditions, and other experimental details, see the legend to Fig. 1 and "Materials and Methods." Each experimental value represents an average of triplicate determinations, and the results shown were determined to be reproducible in a duplicate experiment.

which was comparable with the decline of 19 ± 2% observed with 10 μM verapamil.

At a concentration of 50 μM, the *N*-myristoylated pseudosubstrate peptide P1 significantly enhanced [^{14}C]ADR and [^{3}H]VLB accumulation in the MCF7-MDR cells, but in each case the degree of enhancement was very modest when compared with 10 μM VP (Fig. 1). Likewise, at concentrations of ≤2.5 μM, VP had little effect on [^{14}C]ADR accumulation (Table III). In an attempt to demonstrate efficacy of P1 at concentrations of ≤50 μM, we measured the enhancement of [^{14}C]ADR uptake in MCF7-MDR cells by combinations of P1 (≤50 μM) and VP (≤2.5 μM). We found that 2.5 μM VP in combination with 25 μM P1 enhanced [^{14}C]ADR accumulation in MCF7-MDR cells approximately as effectively as 10 μM VP, and the degree of enhancement was significantly greater than that achieved by either 25 μM P1 or 2.5 μM VP alone (Table III). We observed a similar but more marked statistically significant effect by 50 μM P1 in combination with 2.5 μM VP (Table III). These results demonstrate that the effects of P1 and VP on drug accumulation in MCF7-MDR cells are additive, and they show that P1 can modulate drug accumulation in the MDR breast cancer cells at concentrations as low as 25 μM. (5 μM P1 was ineffective in combination with VP in enhancing [^{14}C]ADR accumulation) (data not shown).

The restoration of [^{14}C]ADR and [^{3}H]VLB accumulation by the *N*-myristoylated pseudosubstrate peptide P1 in MCF7-MDR cells suggested that the peptide might also sensitize MCF7-MDR cells to the cytotoxic drugs. To test this, we measured the effect of a 1-h exposure to P1 on MCF7-MDR cell growth using a 96-h assay system and found that under these conditions, P1 was not growth-inhibitory (Fig. 3). Thus, P1 could be tested for MDR reversal activity in this system under conditions where it potently stimulated drug accumulation in MCF7-MDR cells (Fig. 2, *A* and *C*). Under these conditions, P1 significantly reduced the ADR concentration required for 50% MCF7-MDR cell growth inhibition (IC_{50}) approximately 2-fold, from a value of 26.33 ± 0.40 μg/ml to 14.11 ± 0.41 μg/ml ($p < 0.001$, $n = 6$). Representative results obtained in one experiment are shown in Fig. 3*A*. Similarly, the IC_{50} of VLB was reduced by P1 approximately 2-fold in MCF7-MDR cells, from

TABLE III
Additive induction of [^{14}C]ADR uptake in MCF7-MDR by P1 and VP

[P1]	[VP]	[^{14}C]ADR uptake[a]	Statistical significance[b]
μM	μM		
25	0.5	145 ± 21	n.s.[c]
25	2.5	212 ± 12	$p < 0.01$
50	0.5	189 ± 31	n.s.
50	2.5	317 ± 15	$p < 0.01$
25	0	131 ± 15	n.a.[d]
50	0	153 ± 4	n.a.
0	0.5	107 ± 10	n.a.
0	2.5	136 ± 15	n.a.
0	10	236 ± 16	n.a.
0	0	100 ± 8	n.a.

[a] [^{14}C]ADR uptake is expressed as the percentage of Control, *i.e.* as the percentage of [^{14}C]ADR uptake observed in untreated MCF7-MDR cells. For experimental details, see "Materials and Methods" and the legend to Fig. 1.

[b] P values are indicated wherever the [^{14}C]ADR uptake observed with a combination of P1 and VP was significantly different from the uptake observed both with P1 alone and with VP alone at equivalent concentrations.

[c] n.s., not significant.

[d] n.a., not applicable. The results shown are an average of four experiments done in triplicate.

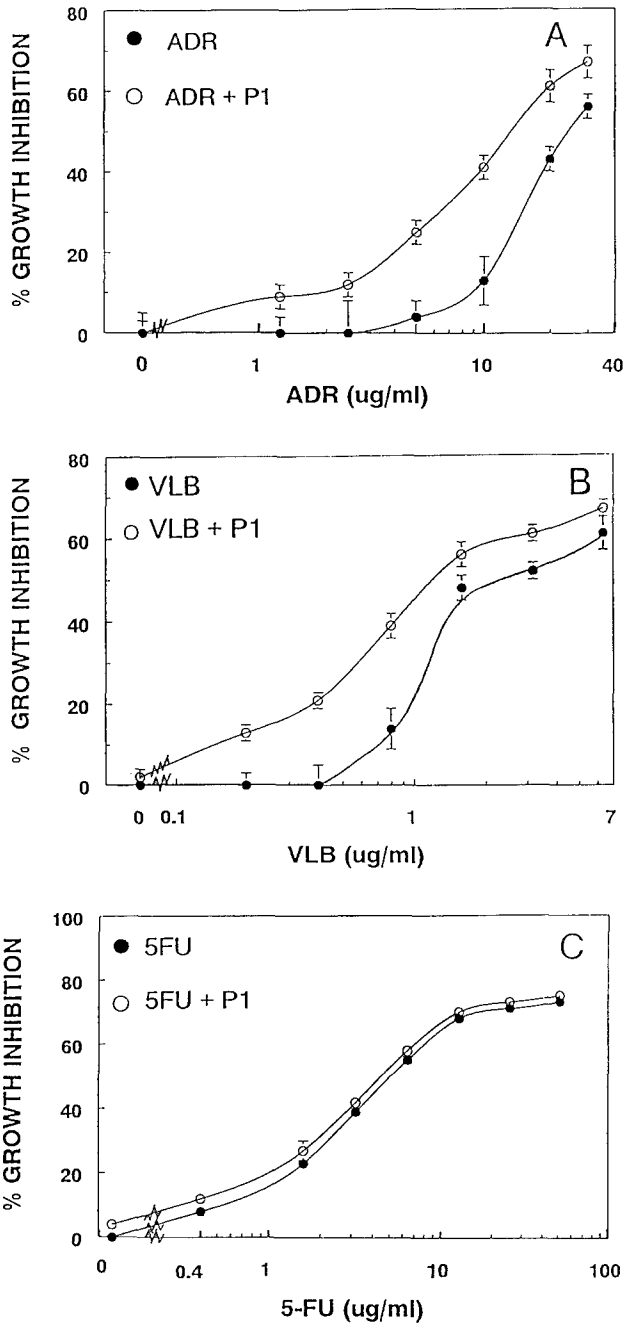

FIG. 3. **Chemosensitization of MCF7-MDR cells by the *N*-myristoylated PKC-α pseudosubstrate peptide P1.** Effects of 100 μM P1 on the growth-inhibitory activity of ADR (*A*), VLB (*B*), and 5FU (*C*) in MCF7-MDR cells are shown. In these experiments, cells were treated with P1 for 1 h followed by a 96-h exposure to the cytotoxic drug. Under these conditions, the direct growth-inhibitory effects of P1 were negligible. For other experimental details, see "Materials and Methods." In *A* and *B*, results of a single experiment are shown; each point represents an average of eight determinations. In *C*, the data shown are an average of three experiments, in which each data point was an average of eight determinations.

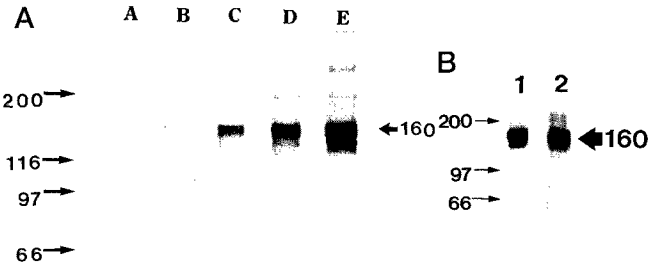

FIG. 4. **Inhibition of P-glycoprotein phosphorylation in MCF7-MDR cells by the *N*-myristoylated PKC-α pseudosubstrate peptide P1.** *A*, effects of P1 on P-glycoprotein phosphorylation in MCF7-MDR cells were determined by exposing the cells to P1 during a 3-h [^{32}P]P_i-labeling period and recovering ^{32}P-labeled P-glycoprotein from lysates of P1-treated cells by immunoprecipitation with the mAb C219. The immunoprecipitated protein was subjected to SDS-PAGE analysis followed by autoradiography of gels, as described under "Materials and Methods." MCF7-WT (*lane A*), MCF7-MDR treated with 100 μM P1 (*lane B*), 50 μM P1 (*lane C*), 25 μM P1 (*lane D*), and 0 μM P1 (*lane E*) are shown. P-glycoprotein is the 160-kDa band. *B*, the effect of 100 μM P1 on P-glycoprotein expression in MCF7-MDR cells was measured under the conditions of the P-glycoprotein phosphorylation experiments by immunoblot analysis of cell lysates with the mAb C219 (500 ng/ml), as described under "Materials and Methods." Immunoblots corresponding to untreated (*lane 1*) and P1-treated MCF7-MDR cells (*lane 2*) (25 μg of protein/lane) are shown. P-glycoprotein is the band migrating at 160 kDa.

2.56 ± 0.32 μg/ml to 1.18 ± 0.08 μg/ml VLB ($p < 0.02$, $n = 5$); representative data are shown in Fig. 3*B*. In contrast, P1 was without effect on 5FU cytotoxicity in MCF7-MDR cells. Fig. 3*C* shows that the percentage of MCF7-MDR cell growth inhibition achieved by 5FU was approximately the same in the presence (*open circles*) and in the absence of P1 (*closed circles*); the results shown are an average of three experiments. Taken together with the effects of P1 on drug accumulation in MCF7-MDR cells (Figs. 1 and 2), these results provide evidence that P1-mediated chemosensitization of MCF7-MDR cells involves inhibition of P-glycoprotein-mediated drug transport.

We next tested whether restoration of drug accumulation in MCF7-MDR cells by P1 and related *N*-myristoylated PKC-inhibitory peptides was associated with inhibition of P-glycoprotein phosphorylation. Fig. 4*A* shows that exposure of MCF7-MDR cells to P1 (25–100 μM) during a 3-h ^{32}P-labeling period was associated with a concentration-dependent inhibition of P-glycoprotein phosphorylation; maximal inhibition was achieved with 100 μM P1. Similarly, P3 and P5 inhibited P-glycoprotein phosphorylation in MCF7-MDR cells, although P5 was considerably less effective than either P1 or P3 in this respect (Table IV). In contrast, the effects of the nonmyristoylated peptides P2, P4, P6, and the *N*-myristoylated peptide P7 on P-glycoprotein phosphorylation in MCF7-MDR cells were negligible (Table IV). In control experiments, we determined by immunoblot analysis of P-glycoprotein with C219 mAb that P1, P3, and P5 had only minor or negligible effects on P-glycoprotein expression under the conditions of the P-glycoprotein phosphorylation experiments (Fig. 4*B*; Table IV).

To determine whether the PKC-inhibitory effects of the *N*-myristoylated peptides in MCF7-MDR cells were restricted to integral membrane protein PKC substrates such as P-glycoprotein, we examined the phosphorylation state of the PKC substrate Raf-1 kinase (34), which shuttles between the cytoplasmic compartment and the plasma membrane of mammalian cells (35). Raf-1 kinase has been implicated in MDR (36), and it plays a pivotal role in PKC-α-mediated signal transduction (34). Activation of cellular PKC-α triggers a protein kinase cascade that begins with PKC-α-catalyzed phosphorylation and activation of Raf-1 kinase and ultimately results in the phosphorylation of nuclear proteins (37). Raf-1 kinase phosphorylation was analyzed by immunoprecipitation of the protein from lysates of ^{32}P-labeled cells with a Raf-1 kinase-specific mAb. We found that the phosphorylation of Raf-1 kinase was at least 10 times greater in the MCF7-MDR line compared with the drug-sensitive line MCF7-WT (Fig. 5*A*, *lanes F* and *G*), when the phosphorylation data in Fig. 5*A* were subjected to densitometric analysis and then normalized for the approximately 2-fold increase in Raf-1 kinase expression that we observed in

TABLE IV
Inhibition of P-glycoprotein phosphorylation by synthetic peptide substrate analogs of PKC

Peptide[a]	% P-glycoprotein phosphorylation[b]	% P-glycoprotein expression[b]
None	100	100
P1	15 ± 3	100 ± 3
P2	79 ± 4	n.d.
P3	17 ± 4	79 ± 5
P4	114 ± 14	n.d.
P5	59 ± 2	86 ± 6
P6	115 ± 1	n.d.
P7	94 ± 23	n.d.
MCF-7 WT cells	<5	<5

[a] All peptides were present at 100 μM.

[b] Unless otherwise indicated, the cell line employed was MCF7-MDR. Conditions for exposure of MCF7-MDR cells to peptides were the same for measurements of P-glycoprotein phosphorylation and P-glycoprotein expression. Immunoblot analysis of P-glycoprotein expression was done with 500 ng/ml C219 mAb, and the resultant band at 160 kDa corresponding to P-glycoprotein was quantitated by computerized densitometric analysis. For further details, see "Materials and Methods" and the legend to Fig. 4.

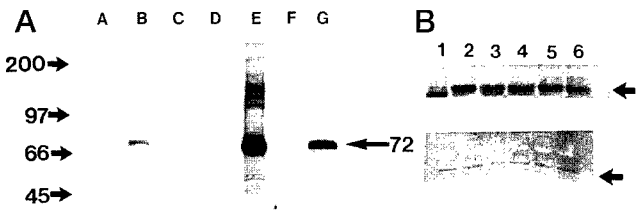

FIG. 5. **Inhibition of Raf-1 kinase phosphorylation in MCF7-MDR cells by *N*-myristoylated synthetic peptide substrate analogs of PKC.** *A*, MCF7-WT or MCF7-MDR cells were labeled with ^{32}P for 3 h prior to detergent lysis of cells and recovery of Raf-1 kinase by immunoprecipitation with a Raf-1 kinase mAb as described under "Materials and Methods." Where indicated, MCF7-MDR cells were treated with *N*-myristoylated synthetic peptide substrate analogs of PKC for the duration of the 3-h labeling period. Immunoprecipitated Raf-1 kinase was analyzed by SDS-PAGE followed by autoradiography. The same exposure period was used for each lane. MCF7-MDR cells were treated with 200 μM P1 (*lane A*), 100 μM P1 (*lane B*), 100 μM P3 (*lane C*), 100 μM P5 (*lane D*), or 100 μM P7 (*lane E*). In control lanes, untreated MCF7-WT (*lane F*) and MCF7-MDR (*lane G*) cells are shown. Raf-1 kinase migrated as a 72-kDa band. *B*, immunoblot analysis of Raf-1 kinase expression in MCF7 cell lysates (50 μg of cell lysate protein/lane) using 10 μg/ml Raf-1 kinase monoclonal antibody as the primary antibody is shown in the *upper panel*; the *lower panel* shows the results of a control immunoblot analysis done in the absence of primary antibody. The *arrows* indicate the positions of MCF7-MDR Raf-1 kinase (72 kDa); MCF7-WT Raf-1 kinase migrated slightly more rapidly (68 kDa). Cells were treated with peptides as described for *A*. *Lane 1*, MCF7-WT; *lane 2*, MCF7-MDR; *lane 3*, P1-treated MCF7-MDR; *lane 4*, P3-treated MCF7-MDR; *lane 5*, P5-treated MCF7-MDR; *lane 6*, P7-treated MCF7-MDR. Peptides were present at 100 μM. The results shown in *A* and *B* were reproducible in separate experiments.

the MDR line by Western analysis (Fig. 5*B*). This increase in the phosphorylation of the PKC-α substrate Raf-1 kinase in MCF7-MDR is consistent with the reported 30-fold overexpression of PKC-α in the cells (18). As in the case of P-glycoprotein phosphorylation, 100 μM P1, P3, and P5 each potently inhibited Raf-1 kinase phosphorylation in the MCF7-MDR cells, whereas 100 μM P7 was noninhibitory (Fig. 5*A*, *lanes B–E*). In contrast, the peptides had little or no effect on Raf-1 kinase expression in MCF7-MDR cells, according to immunoblot analysis of cell lysates (Fig. 5*B*). Densitometric analysis revealed that 100 μM P3 and P5 each inhibited Raf-1 kinase phosphorylation in MCF7-MDR cells by more than 95% (Fig. 5*A*, *lanes C* and *D*), and 200 μM P1 achieved a similar degree of inhibition (Fig. 5*A*, *lane A*). The inhibition of Raf-1 kinase phosphorylation achieved by 100 μM P1 was approximately 80% (Fig. 5*A*, *lane B*).

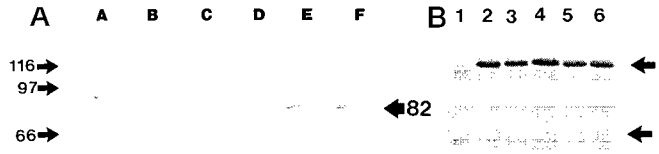

FIG. 6. **Inhibition of PKC-α phosphorylation in MCF7-MDR cells by *N*-myristoylated synthetic peptide-substrate analogs of PKC.** *A*, MCF7-WT or MCF7-MDR cells were incubated with $[^{32}P]P_i$ in phosphate-free medium for 3 h as described under "Materials and Methods." Where indicated, MCF7-MDR cells were treated with synthetic peptides (P1, P3, P5, and P7) for the duration of the labeling period. ^{32}P-labeled cells were lysed with detergent, and PKC-α was immunoprecipitated from the cell lysates, as described under "Materials and Methods." Immunoprecipitated PKC-α was detected by SDS-PAGE and autoradiography of the gel; the autoradiogram was subjected to densitometric analysis. Results are shown for MCF7-WT (*lane A*), MCF7-MDR treated with 100 μM P1 (*lane B*), 100 μM P3 (*lane C*), 100 μM P5 (*lane D*), 100 μM P7 (*lane E*), and untreated MCF7-MDR (*lane F*). PKC-α is the radiolabeled band migrating at 82 kDa. *B*, immunoblot analysis of PKC-α expression in MCF7 cell lysates (50 μg of protein/lane) using 1 μg/ml PKC-α monoclonal antibody as the primary antibody (12) is shown in the *upper panel*; a control analysis done in the absence of the primary antibody is shown in the *lower panel*. The *arrows* indicate the positions of PKC-α (82 kDa). Cells were treated with peptides as described for *A*. *Lane 1*, MCF7-WT; *lane 2*, MCF7-MDR; *lane 3*, P1-treated MCF7-MDR; *lane 4*, P3-treated MCF7-MDR; *lane 5*, P5-treated MCF7-MDR; *lane 6*, P7-treated MCF7-MDR. Peptides were present at 100 μM. The results shown in *A* and *B* were reproducible in separate experiments.

PKC-α-catalyzed Raf-1 kinase phosphorylation is preceded by PKC-α activation and autophosphorylation (38, 39). To test whether the alterations in Raf-1 kinase phosphorylation shown in Fig. 5*A* were reflective of changes in PKC-α activity, we analyzed the MCF7-WT and MDR cells for comparable changes in PKC-α phosphorylation. Like Raf-1 kinase, PKC-α is localized in the plasma membrane and the cytoplasmic compartment of mammalian cells (17, 40). Following a 3-h labeling period, a major 82-kDa radiolabeled band corresponding to phosphorylated PKC-α was detected in ^{32}P-labeled MCF7-MDR but not in MCF7-WT by immunoprecipitation of the protein from the cell lysates with a PKC-α-specific mAb (Fig. 6*A*, *lanes A* and *F*). Similarly, PKC-α was readily detected in an MCF7-MDR cell lysate but not in an MCF7-WT cell lysate by immunoblot analysis (Fig. 6*B*, *lanes 1* and *2*). Densitometric analysis of Fig. 6*A* showed that as in the case of Raf-1 kinase phosphorylation, PKC-α phosphorylation was inhibited in MCF7-MDR cells >95% by 100 μM P3 (*lane C*) and 100 μM P5 (*lane D*) and approximately 80% by 100 μM P1 (*lane B*); 100 μM P7 affected <25% inhibition of PKC-α phosphorylation (*lane E*). The peptides had little or no effect on the expression of PKC-α in MCF7-MDR cells under these experimental conditions according to immunoblot analysis of cell lysates (Fig. 6*B*). At 200 μM, P1 inhibited PKC-α phosphorylation in MCF7-MDR cells >95%, but P7 still achieved <25% inhibition (data not shown). Thus, the *N*-myristoylated peptides P1, P3, and P5 potently inhibited the phosphorylation of three PKC-α substrates (P-glycoprotein, Raf-1 kinase, and PKC-α) in MCF7-MDR cells under conditions where the peptides restored intracellular drug accumulation, whereas the *N*-myristoylated peptide P7 inhibited the phosphorylation of these PKC-α substrates very weakly or not at all and was without effect on the uptake of cytotoxic drugs in the MDR breast cancer cells. In most cases, the peptide concentration required for potent induction of drug uptake by P1, P3, and P5 (100 μM) (Fig. 1) caused ≥80% inhibition of the phosphorylation of P-glycoprotein, Raf-1-kinase, and PKC-α in the MCF7-MDR cells (Figs. 4–6; Table IV), suggesting that nearly complete inhibition of PKC-α catalysis may be required for substantial reversal of MDR by the peptides.

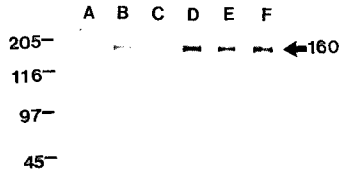

FIG. 7. **Effects of *N*-myristoylated synthetic peptide substrate analogs of PKC on photoaffinity labeling of P-glycoprotein by [^{3}H] azidopine in MCF7-MDR cells.** Photoaffinity labeling of P-glycoprotein in MCF7-MDR cells was done following preincubation of the cells with [^{3}H]azidopine and the peptide under investigation in the dark, as described under "Materials and Methods." Peptides were employed at a concentration of 100 μM. Photoaffinity labeling was followed by extraction and immunoprecipitation of P-glycoprotein and SDS-PAGE analysis. [^{3}H]Azidopine-labeled P-glycoprotein, which migrated as a 160-kDa band, was quantitated by autoradiography and computerized densitometry. Samples were untreated MCF7-WT (*lane A*), untreated MCF7-MDR (*lane B*), MCF7-MDR treated with 30 μM VLB (*lane C*), 100 μM P1 (*lane D*), 100 μM P3 (*lane E*), and 100 μM P5 (*lane F*).

Prenylcysteine methyl esters and several cyclic peptides have been shown to compete for drug-binding sites on P-glycoprotein in studies of photoaffinity labeling of the transporter with [^{3}H]azidopine (41, 42), and several hydrophobic linear peptides have been reported to serve as P-glycoprotein substrates (42–45). In contrast, the amphiphilic linear peptides melittin and alamethicin do not interact with P-glycoprotein (42). To test whether the mechanism of MDR reversal by the amphiphilic peptide P1 could involve direct interactions between P1 and drug-binding sites of P-glycoprotein in addition to inhibition of P-glycoprotein phosphorylation, we analyzed the effects of P1 and related peptides on the photoaffinity labeling of P-glycoprotein with [^{3}H]azidopine in MCF7-MDR cells. Results of photoaffinity labeling experiments done in whole MCF7 cells are shown in Fig. 7. A single prominent [^{3}H]azidopine-labeled band that corresponded to P-glycoprotein (160 kDa) was observed in the lane corresponding to untreated MCF7-MDR cells (*lane B*); the absence of this band in the MCF7-WT sample (*lane A*) confirmed its identity as P-glycoprotein. Exposure of the MCF7-MDR cells to vinblastine achieved about 50% inhibition of the labeling of P-glycoprotein according to densitometric analysis (*lane C*). Exposure to 100 μM P1, P3, and P5 (*lanes D–F*) had no inhibitory effect on photoaffinity labeling of P-glycoprotein in MCF7-MDR cells. In fact, the peptides actually enhanced the labeling of P-glycoprotein by [^{3}H]azidopine.

Comparisons of the chemosensitivities of MCF7-MDR and MCF7-WT cells to cytotoxic drugs have shown that the relative resistances of MCF7-MDR cells to the P-glycoprotein substrates ADR and VLB are, respectively, 610- and 360-fold (18). As a test of whether P1 could serve as a P-glycoprotein substrate in MCF7-MDR cells, we analyzed the MCF7-MDR cells for cross-resistance to P1 by comparing the growth-inhibitory activity of P1 and related peptides against MCF7-WT and MCF7-MDR cells. In these experiments, cells were exposed to the *N*-myristoylated peptides during the entire growth inhibition assay period (96 h; the 1-h exposure period employed in Fig. 3 was not used because it would have required very high peptide concentrations to achieve >50% cell growth inhibition). Although statistically significant cross-resistance was observed in MCF7-MDR cells with each peptide, the very modest degree of cross-resistance to the pseudosubstrate peptides P1 and P3 (<1.5-fold) (Table V) provided evidence that P1 and P3 did not serve effectively as P-glycoprotein substrates in the cells. In contrast, the cross-resistance of the cells to P5, which contains a sequence that corresponds to a PKC phosphorylation site in the EGF receptor, was pronounced (10-fold) (Table V), providing evidence that P5 may be transported by P-glycoprotein. Ideally, an MDR reversal agent should be equipotent against drug-sensitive and MDR cancer cells in its direct growth-inhibitory effects, because cross-resistance of the MDR cells to the agent could necessitate its use at concentrations that are directly toxic to nontransformed cells to accomplish reversal of MDR in cancer cells *in vivo*. Thus, the minor degree of cross-resistance of the MCF7-MDR cells to the *N*-myristoylated pseudosubstrate peptides P1 and P3 (Table V) further indicates the potential value of the pseudosubstrate peptides as MDR reversal agents. In contrast, the pronounced cross-resistance of the MCF7-MDR cells to the EGF receptor-related peptide P5 indicates that it is not appropriate for MDR reversal.

TABLE V
Growth-inhibitory activity of N-myristoylated synthetic peptide substrate analogs of PKC against wild-type and multidrug-resistant MCF7 cells

IC_{50} values are shown for the peptides P1, P3, and P5 in the growth inhibition of MCF7-WT and MCF7-MDR cells. Cells were exposed to the peptides for the entire 96-h cell growth inhibition assay period. For peptide structures, see Table I. For experimental details, see "Materials and Methods." Each IC_{50} shown is the average value of three experiments. In each experiment, data points were calculated as the average of eight determinations. *P* values describe the significance of the difference between the IC_{50} values obtained in MCF7-WT and MCF7-MDR cells (n = 3).

	IC_{50}		Fold resistance	*P* value
	MCF7-WT	MCF7-MDR		
	μM			
P1	29 ± 1	42 ± 3	1.45	<0.05
P3	48 ± 1	62 ± 3	1.29	<0.05
P5	5.9 ± 0.3	63 ± 3	10.68	<0.01

DISCUSSION

Previous reports have shown that the isozyme PKC-α is selectively overexpressed in human breast cancer MCF7-MDR cells (18), and artificial overexpression of PKC-α in MCF7 constructs that overexpress P-glycoprotein increases the drug resistance of the cells in association with increased P-glycoprotein phosphorylation (19), providing evidence that PKC-α-catalyzed P-glycoprotein phosphorylation may contribute to MDR in MCF7 cells. In this report, we demonstrate that an *N*-myristoylated PKC-α pseudosubstrate peptide, *N*-myristoyl-FARKGALRQ(P1), partially reverses drug resistance in MCF7-MDR by a novel mechanism that involves PKC-α inhibition. P1 induced cytotoxic drug accumulation in MCF7-MDR cells just as effectively as the potent MDR reversal agent verapamil (Fig. 1) in association with potent inhibition of P-glycoprotein phosphorylation (Fig. 4). P1 also inhibited the phosphorylation of two other PKC-α substrates in MCF7-MDR cells, Raf-1 kinase (Fig. 5), and PKC-α (Fig. 6) under these conditions. Thus, induction of drug accumulation by P1 was associated with PKC-α inhibition in MCF7-MDR cells. Based on the evidence described above (18, 19) that PKC-α-catalyzed P-glycoprotein phosphorylation may be a contributing factor in the MDR phenotype of MCF7-MDR cells, it is evident that the mechanism of MDR reversal by P1 most likely involves inhibition of P-glycoprotein phosphorylation.

The mechanism of P1-mediated MDR reversal clearly does not involve competitive binding at [^{3}H]azidopine binding sites on P-glycoprotein (Fig. 7). This distinguishes P1 from MDR reversal agents such as PKC-inhibitory staurosporines (47, 48), verapamil, and cyclosporin A, which are highly effective MDR reversal agents *in vitro* but cannot be used to reverse MDR *in vivo* due to severe toxic effects at therapeutic concentrations (24, 47). It is also evident that MDR reversal by P1 does not involve altered P-glycoprotein expression and that it is not compromised by cross-resistance in the MDR cells (Fig. 4B, Table V). It should be noted that modulation of the ATPase

activity of isolated P-glycoprotein and the [^{3}H]VLB binding activity of P-glycoprotein-containing membrane vesicles by phospholipid-interacting peptides, such as melittin (42) and the *N*-myristoylated peptides described here (27), cannot be used to characterize interactions between the peptides and P-glycoprotein because of the pronounced nonspecific effects of phospholipid-interacting peptides in these assay systems (42). It is also worthwhile to note that because treatment of breast cancer patients with tamoxifen, which is PKC-inhibitory at therapeutic concentrations (49), is associated with little toxicity, it appears that PKC-inhibitory MDR reversal agents such as P1 could potentially give rise to a new generation of MDR reversal agents that are associated with acceptably low toxicity.

The inability of P1 to antagonize [^{3}H]azidopine labeling of P-glycoprotein in MCF7-MDR cells and the lack of cross-resistance of MCF7-MDR cells to P1 suggest that P1 is not a P-glycoprotein substrate. However, these results do not exclude the possibility that linear myristoylated peptides such as P1 may interact with a P-glycoprotein site that is nonoverlapping and distinct from the azidopine binding site. In fact, the enhancement of [^{3}H]azidopine labeling of P-glycoprotein by P1 is suggestive of such interactions. The enhanced labeling affected by P1 cannot be explained simply by the inhibitory activity of P1 against P-glycoprotein phosphorylation, because PKC-α-catalyzed P-glycoprotein phosphorylation increases azidopine-labeling of the pump (46). Nor can it be explained by the amphiphilicity of P1, because other linear amphiphilic peptides do not enhance azidopine labeling of P-glycoprotein (42).

In this study, we compared the effects of P1 and other *N*-myristoylated peptide-substrate analogs of PKC in MCF7-MDR cells. In general, the ability of the *N*-myristoylated peptide-substrate analogs to inhibit the phosphorylation of endogenous PKC-α substrates (P-glycoprotein, Raf-1 kinase, and PKC-α) in MCF7-MDR cells correlated with restoration of drug uptake in the cells by the peptides, *i.e.* P1, P3, and P5 were active, whereas P7 was inactive. Discrepancies within this general trend may be due to the pronounced cross-resistance of the MCF7-MDR cells to P5, effects of the *N*-myristoylated peptides on P-glycoprotein in addition to inhibition of the phosphorylation of the pump (these putative effects are inferred from the enhancement of photoaffinity labeling of P-glycoprotein by the peptides as discussed above), differences among the amphiphilic peptides in their interactions with cell membranes, etc. Furthermore, because P1 and P3 contain PKC-α pseudophosphorylation sequences (26, 27), they are likely to be recognized not only by PKC-α but also by other proteins that interact with naturally occurring PKC-α phosphorylation sites, *e.g.* protein phosphatases. It is also important to note that because of the overlapping substrate specificities of PKC isozymes (50), P1 and P3 may also antagonize the function of several other PKC isozymes in MCF7-MDR cells, and these inhibitory effects may contribute to their MDR reversal activity. Finally, complexity is also introduced by the existence of multiple mechanisms of drug resistance in drug-selected MDR cancer cells that overexpress P-glycoprotein, such as MCF7-MDR. Contributing factors to MDR can include multidrug resistance protein (MRP), glutathione *S*-transferase, topoisomerases, etc. (25), and some of these drug resistance mechanisms might also be affected by the peptides.

Studies of the inhibition of purified PKC by *N*-myristoylated peptide substrate analogs demonstrated that the *N*-myristoylated peptides interacted with the phospholipid cofactor, whereas their nonmyristoylated counterparts did not (27). The membrane-active nature of the *N*-myristoylated peptides most likely accounts for their ability to access cellular PKC (26, 31, 33). As the phospholipid cofactor concentration is increased in the PKC assay system, the inhibitory potency of the *N*-myristoylated peptides declines (27), providing evidence that their inhibitory effects are subject to surface dilution. This may account for the sharp increase in the potencies of P1, P3, and P5 as inducers of drug uptake in MCF7-MDR cells when the bulk peptide concentration was increased from 50 to 100 μM.

Bioactive peptides such as P1 are subject to proteolytic degradation, which often limits their potency. This may explain the superior potency of P1 in inducing drug uptake in short-term assays (≤6 h) compared with its potency in reversing MDR over a 96-h time course. In some cases, this problem can be overcome by designing retro-inverso analogs of the peptides. In a retro-inverso analog, the sequence of the parent peptide is reversed, and the residues are replaced by the corresponding D-enantiomers (51). The side chain surfaces of parent and retro-inverso peptides are similar or identical, but the topologies of their backbones differ (51). Thus, superior bioactivity can be achieved by retro-inverso analogs, when the side chain surface prevails in the interaction of the bioactive peptide with its target (52). A retro-inverso analog of a PKC-α pseudosubstrate peptide has been shown to potently inhibit phosphorylation of a synthetic peptide-substrate by PKC (53). Efforts are now underway to design retro-inverso analogs of P1 and related *N*-myristoylated peptides that are superior to the parent peptides in the reversal of MDR in human breast cancer cells. In a recent report, computer-based algorithms were successfully used to predict MDR reversal activity based on structural features of compounds entered into the data base (54); this type of strategy may also be useful for designing peptidic or peptidomimetic P1 analogs with optimized MDR reversal activity.

Acknowledgment—We thank Patherine Greenwood for expert preparation of the manuscript.

REFERENCES

1. Gottesman, M. M., and Pastan, I. (1993) *Annu. Rev. Biochem.* **62,** 385–427
2. Endicott, J. A., and Ling, V. (1989) *Annu. Rev. Biochem.* **58,** 137–171
3. Goldstein, L. J., Galski, H., Fojo, A., Willingham, M., Lai, S.-L., Gazdar, A., Pirker, R., Green, A., Crist, W., Brodeur, G. M., Lieber, M., Cossman, J., Gottesman, M. M., and Pastan, I. (1989) *J. Natl. Cancer Inst.* **81,** 116–124
4. Weinstein, R. S., Jakate, S. M., Dominguez, J. M., Lebovitz., M. D., Koukoulis, G. K., Kuszak, J. R., Klusens, L. F., Grogan, T. M., Saclarides, T. J., Roninson, I. B., and Coon, J. S. (1991) *Cancer Res.* **51,** 2720–2726
5. Asaoka, Y., Nakamura, S., Yoshida, K., and Nishizuka, Y. (1992) *Trends Biochem. Sci.* **17,** 414–417
6. Ferguson, P. J., and Cheng, Y. (1987) *Cancer Res.* **47,** 433–441
7. Fine, R. L., Patel, J., and Chabner, B. A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 582–586
8. O'Brian, C. A., Fan, D., Ward, N. E., Dong, Z., Iwamoto, L., Gupta, K . P., Earnest, L. E., and Fidler, I. J. (1991) *Biochem. Pharmacol.* **41,** 797–806
9. Chambers, T. C., McAvoy, E. M., Jacobs, J. W., and Eilon, G. (1990) *J. Biol. Chem.* **265,** 7679–7686
10. Dong, Z., Ward, N. E., Fan, D., Gupta, K. P., and O'Brian, C. A. (1991) *Mol. Pharmacol.* **39,** 563–569
11. Chambers, T. C., Zheng, B., and Kuo, J. F. (1992) *Mol. Pharmacol.* **41,** 1008–1015
12. Gravitt, K. R., Ward, N. E., Fan, D., Skibber, J. M., Levin, B., and O'Brian, C. A. (1994) *Biochem. Pharmacol.* **48,** 375–381
13. Chambers, T. C., Pohl, J., Raynor, R. L., and Kuo, J. F. (1993) *J. Biol. Chem.* **268,** 4592–4595
14. Orr, G. A., Han, E. K. H., Browne, P. C., Nieves, E., O'Conner, B. M., Yang, C. P. H., and Horwitz, S. B. (1993) *J. Biol. Chem.* **268,** 25054–25062
15. O'Brian, C. A., Fan, D., Ward, N. E., Seid, C., and Fidler, I. J. (1989) *FEBS Lett.* **246,** 78–82
16. Posada, J. A., McKeegan, E. M., Worthington, K. F., Morin, M. J., Jaken, S., and Tritton, T. R. (1989) *Cancer Commun.* **1,** 285–292
17. Lee, S. A., Karaszkiewicz, J. W., and Anderson, W. B. (1992) *Cancer Res.* **52,** 3750–3759
18. Blobe, G. C., Sachs, C. W., Khan, W. A., Fabbro, D., Stabel, S., Wetsel, W. C., Obeid, L. M., Fine, R. L., and Hannun, Y. A. (1993) *J. Biol. Chem.* **268,** 658–664
19. Yu, G., Ahmad, S., Aquino, A., Fairchild, C. R., Trepel, J. B., Ohno, S., Suzuki, K., Tsuruo, T., Cowan, K. H., and Glazer, R. I. (1991) *Cancer Commun.* **3,** 181–189
20. Kang, Y., and Perry, R. R. (1994) *Cancer Res.* **54,** 2952–2958
21. Chen, G., Ramachandran, C., and Krishan, A. (1993) *Cancer Res.* **53,** 2544–2547
22. Tamai, I., and Safa, A. R. (1991) *J. Biol. Chem.* **266,** 16796–16800
23. Kiue, A., Sano, T., Suzuki, K., Inada, H., Okumura, M., Kikuchi, J., Sato, S.,

Kohno, K., and Kuwano, M. (1990) *Cancer Res.* **50,** 310–317
24. Dalton, W., and Sikic, B. I. (1994) *J. NIH Res.* **6,** 54–57
25. O'Brian, C. A., Ward, N. E., Gupta, K. P., and Gravitt, K. R. (1995) in *Alternative Mechanisms of Multidrug Resistance in Cancer* (Kellen, J., ed) pp. 173–190, Birkhaueser Publishers, New York
26. Eichholtz, T., de Bont, D. B., de Widt, J., Liskamp, R. M., and Ploegh, H. L. (1993) *J. Biol. Chem.* **268,** 1982–1986
27. Ward, N. E., and O'Brian, C. A. (1993) *Biochemistry* **32,** 11903–11909
28. O'Brian, C. A., and Ward, N. E. (1990) *Biochemistry* **29,** 4278–4282
29. Bates, S. E., Lee, J. S., Dickstein, B., Spolyar, M., and Fojo, A. T. (1993) *Biochemistry* **32,** 9156–9164
30. Zheng, B., Chambers, T. C., Raynor, R. L., Markham, P. N., Gebel, H. M., Vogler, W. R., and Kuo, J. F. (1994) *J. Biol. Chem.* **269,** 12332–12338
31. Ioannides, C. G., Freedman, R. S., Liskamp, R. M., Ward, N. E., and O'Brian, C. A. (1990) *Cell Immunol.* **131,** 242–252
32. Barja, P., Alavi-Nassab, A., Turck, C. W., and Freire-Moar, J. (1994) *Cell Immunol.* **153,** 28–38
33. O'Brian, C. A., Ward, N. E., Liskamp, R. M., de Bont, D. B., Earnest, L. E., van Boom, J. H., and Fan, D. (1991) *Invest. New Drugs* **9,** 169–179
34. Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U. R. (1993) *Nature* **364,** 249–252
35. Leevers, S. J., Paterson, H. F., and Marshall, C. J. (1994) *Nature* **369,** 411–414
36. Cornwell, M. M., and Smith, D. E. (1993) *J. Biol. Chem.* **268,** 15347–15350
37. Egan, S. E., and Weinberg, R. A. (1993) *Nature* **365,** 781–783
38. Huang, K. P., Nakabayashi, H., and Huang, F. L. (1986) *Proc. Natl. Acad. Sci.* **83,** 8535–8539
39. Dutil, E. M., Keranen, L. M., DePaoli-Roach, A. A., and Newton, A. C. (1994) *J. Biol. Chem.* **269,** 29359–29362
40. Epand, R. M., and Lester, D. S. (1990) *Trends Pharmacol. Sci.* **11,** 317–320
41. Zhang, L., Sachs, C. W., Fine, R. L., and Casey, P. J. (1994) *J. Biol. Chem.* **269,** 15973–15976
42. Sharom, F. J., DiDiodato, G., Yu, X., and Ashbourne, K. J. D. (1995) *J. Biol. Chem.* **270,** 10334–10341
43. Raymond, M., Gros, P., Whiteway, M., and Thomas, D. Y. (1992) *Science* **256,** 232–234
44. Sharma, R. C., Inoue, S., Roitelman, J., Schimke, R. T., and Simoni, R. D. (1992) *J. Biol. Chem.* **267,** 5731–5734
45. Sarkadi, B., Muller, M., Homolya, L., Hollo, Z., Seprodi, J., Germann, U. A., Gottesman, M. M., Price, E. M., and Boucher, R. C. (1994) *FASEB J.* **8,** 766–770
46. Ahmad, S., Safa, A. R., and Glazer, R. I. (1994) *Biochemistry* **33,** 10313–10318
47. Miyamoto, K., Inoko, K., Wakusawa, S., Kajita, S., Hasegawa, T., Takagi, K., and Koyama, M. (1993) *Cancer Res.* **53,** 1555–1559
48. Sato, W., Yusa, K., Naito, M., and Tsuruo, T. (1990) *Biochem. Biophys. Res. Commun.* **173,** 1252–1257
49. O'Brian, C. A., Liskamp, R. M., Solomon, D. H., and Weinstein, I. B. (1985) *Cancer Res.* **45,** 2462–2465
50. Dekker, L. V., and Parker, P. J. (1994) *Trends Biochem. Sci.* **19,** 73–77
51. Brady, L., and Dodson, G. (1994) *Nature* **368,** 692–693
52. Jameson, B. A., McDonnell, J. M., Marini, J. C., and Korngold, R. (1994) *Nature* **368,** 744–746
53. Ricouart, A., Tartar, A., Sergheraert, C. (1989) *Biochem. Biophys. Res. Commun.* **165,** 1382–1390
54. Klopman, G., Srivastava, S., Kolossvary, I., Epand, R. F., Ahmed, N., and Epand, R. M. (1992) *Cancer Res.* **52,** 4121–4129

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August 20, 1996

William J. Doty
Director, Office of Technology and Development
The University of Texas
M.D. Anderson Cancer Center
1515 Holcombe Blvd, Box 510
Houston, Texas 77030

Dear Mr. Doty:

I am enclosing for your consideration a complete invention disclosure report entitled *"Cancer therapies by proteins of the "Notch" complex."*

This is novel information and has not been published before. Only an abstract reporting laboratory findings has been submitted and it is in press in the FASEB Journal, 1996. A draft of this report was submitted to your office on April 1996. Dr. DeJaeger from RGene has expressed interest in this application.

I would also appreciate an update on the status of my other application: SN 08/403, 459, which I understand your office has assigned to Corixa Corporation.

If you have any questions or comments, please do not hesitate to contact me at 792-2849, FAX 792-7586 or via interoffice mail, box 67.

Thank you very much.

Sincerely,

Constantin G. Ioannides

Constantin G. Ioannides, Ph.D.
Associate Professor of Immunology
(Gynecology)

CGI/swm

Enclosure

THE UNIVERSITY OF TEXAS M.D. ANDERSON CANCER CENTER
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1 a Description/Title of Invention:

1.a. Cancer therapies by proteins members of the "Notch" complex

b. Key word Descriptions of Invention (max.five):

1.b. (Notch, AES, TLE) code names for proteins, induction of tumor reactive T cells,

2. Name of Inventor(s):

	Name	Div/Dept/ Phone	% Creative Contribution	Employer/ Address		
a.						
b.	1. Constantin G. Ioannides,Ph.D.	Assoc. Professor		Gyn.Oncol.	22849	100
	2. Bryan Fisk, M.Sc.	Res. Asst. II		Gyn.Oncol	22849	100
c.	3. Bohuslav Melichar, M.D.	Grad.Std.		Gyn.Oncol.	22849	100
d.	4. Brett Anderson, B.Sc.	Res.Asst.I		Gyn.Oncol.	22849	100
	5. James Murray, M.D.	Professor		Med.Oncol.	24571	100
e.	6. J. Taylor Wharton, M.D.	Professor		Gyn.Oncol.	28628	100
f.						

3. Contribution: briefly describe each inventors' association with the invention, i.e. exactly what creative action/activity he/she contributed to the invention (you may attach an extra sheet if you need more space).

a.

b. see attached

c.

d.

e.

f.

Post-it® Fax Note 7671	Date 04/17/96	# of pages ▶ 4
To Dr. Ioannides	From Paul Lin	
Co./Dept.	Co.	
Phone #	Phone #	
Fax # 2-7586	Fax #	

4. a. Date the invention was conceived (when you first thought of it or made the key observation):

b. Is the invention described in a meeting, poster session or seminar, or in a published paper or abstract (or submitted for publication)?

Yes X No _____ If Yes, please provide the following information:

i) Name of Journal/Meeting: ~~FASEB~~, 1996, June 1996
ii) Date of submission (unless already published): 1/31/96
iii) Date of Publication (estimate if only submitted): 5/1/96
iv) Date of Electronic Publication (in full or abstract) on Internet, World-Wide-Web, Gopher Site, Subscriber Service etc. (if you're not sure, contact the publiser or meeting site):
iv) Was the disclosure sufficient to allow someone else to duplicate the invention? ✓ Yes _____ No

c. Date you made the first drawing, design, formulation, construction or model, if applicable: Aug - Sept. 1995

d. Date of the first use of the invention, if applicable:

5. Was the development of the invention (or any of the inventors) supported IN ANY WAY (equipment, funding, materials, etc.) by a Gift, Grant or Contract?

a. Yes ✓ No _____ If Yes, complete b and c:

b. Gift/Grant Information (Company, Institution, Government Agency, or Private Foundation): See attached

Funding Source_____
Project Title: _____
Number _____ MDA Acct No. _____

c. Contract Information (Private Industry Material Transfer and/or Sponsored Research):

Sponsor Name _____
Project Title _____
Contract No._____
Contact Name _____Phone : _____

Please continue on a separate sheet if you used more than one funding source, or if you used a funding source other than provided for here.

6. How much money to date has been spent on the development of this technology?

6. At least $50,000. This includes $5-10,000 for supplies for cell growth only, $5,000 for HPLC and sequencing, immunochemistry plus salaries.

7. Please provide names of M. D. Anderson faculty/staff who have sufficient technical knowledge in this field to serve as a scientific reviewer of your invention.

7. 1. Dr. Ralph B. Arlinghaus, Chairman and Professor, Molecular Pathology
Dr. Gary Gallick, Professor of Tumor Biology
Dr. Elizabeth Grimm, Professor of Tumor Biology
Dr. Gabriel Lopez-Berestein, Professor of Immunobiology and Drug Carriers

8. **Please respond to items 8 a through k on a separate attachment:**

a. Please explain what the invention does or how it could be used.

b. Please explain how the invention works.

c. Please explain how the invention is an improvement over the way things were done before (better results, easier to use, etc.) or whether it permits something completely new to be done.

d. Please summarize the results of your in vitro and in vivo experiments and note to what extent the experiments have been repeated.

e. If your invention is an experimental observation (a new biological effect, a purified protein, a DNA sequence, a new therapeutic or diagnostic method, etc.) please attach your experimental procedures and data. For example, you may attach a manuscript in preparation.

f. If your invention is a device, please include a sketch, drawing, circuit diagram, photograph, etc. Does a prototype exist? ___ yes ___ no

g. Please describe the products or services that could be sold based upon your invention.

h. **Please explain your Clinical Development Plans:** What are the steps that must be taken to complete the basic research and pre-clinical research (toxicology, pharmacokinetics, tissue distribution, animal efficacy, etc.)? Which steps will you perform and which steps will be done by collaborators and who are they?

i. Please prepare a budget (as your would in a grant application) with your best estimate of the time and cost (salaries, fringe benefits, materials and supplies, equipment and travel) to complete the research and get this invention to the first phase of clinical testing.

j. Please suggest companies that might be interested in producing products or services using your invention; if known, please provide the name, address, and phone number of a contact person at each company. See attached

k. Please attach a bibliography of the references that you are aware of that are relevant to your invention, including all relevant articles by any of the inventors (PLEASE ENCLOSE COPIES OF EACH REFERENCE). **Please explain how each reference is similar to or different from your invention.**

SIGNATURE OF INVENTOR(S)

C. G. Ioannides

CONSTANTIN G. IOANNIDES — Date 8/6/96

Date 8/8/96

J. TAYLOR WHARTON — Date

Date 8/21/96

JAMES LEE MURRAY — Date

Date

g. Please describe the products or services that could be sold based upon your invention.

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SIGNATURE OF INVENTOR(S)

Brett Wayne Anderson _____ _____

Brett Anders _____ Date 4-18-96

_____ Date

_____ Date

Bryan Fisk _____

Bryan Fisk _____ Date 4-18-96

_____ Date

_____ Date

g. Please describe the products or services that could be sold based upon your invention.

h. **Please explain your Clinical Development Plans**: What are the steps that must be taken to complete the basic research and pre-clinical research (toxicology, pharmacokinetics, tissue distribution, animal efficacy, etc.)? Which steps will you perform and which steps will be done by collaborators and who are they?

i. Please prepare a budget (as your would in a grant application) with your best estimate of the time and cost (salaries, fringe benefits, materials and supplies, equipment and travel) to complete the research and get this invention to the first phase of clinical testing.

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k. Please attach a bibliography of the references that you are aware of that are relevant to your invention, including all relevant articles by any of the inventors (PLEASE ENCLOSE COPIES OF EACH REFERENCE). **Please explain how each reference is similar to or different from your invention.**

SIGNATURE OF INVENTOR(S)

BOHUSLAV MELICHAR	4-18-96
	Date
	Date
	Date
	Date
	Date
	Date

1.a. Cancer therapies by proteins members of the "Notch" complex
1.b. (Notch, AES, TLE) code names for proteins, induction of tumor reactive T cells, (CTL) and eventually $CD4^+$ cells, immunotherapy, gene therapy

2.

1. Constantin G. Ioannides,Ph.D. 100	Assoc. Professor	Gyn.Oncol.	22849
2. Bryan Fisk, M.Sc. 100	Res. Asst. II	Gyn.Oncol	22849
3. Bohuslav Melichar, M.D. 100	Grad.Std.	Gyn.Oncol.	22849
4. Brett Anderson, B.Sc. 100	Res.Asst.I	Gyn.Oncol.	22849
5. James Murray, M.D. 100	Professor	Med.Oncol.	24571
6. J. Taylor Wharton, M.D. 100	Professor	Gyn.Oncol.	28628

3. 1. Conception and development of experimental design
2. Experimental design and execution of experiment, computer analysis
3. Development of experimental design, execution of experiments, computer analysis
4. Execution of experiments
5. Conception and development
6. Conception and development

4. 100%

5. a. August 1995
b. yes
(i) Annual Meeting of the Federation of the American Societies for Experimental Biology, June 1996 New Orleans
(ii) January 30, 1996
(iii) May 1996
c. August - September 1995. After comparing sequencing data by mass spectrometry of an ion isolated from immunoaffinity separated HLA-A2 molecules from the ovarian tumor line and Gene Bank sequences.
d. n/a

a yes

1. b NCI-RO1 CA 57293
T cell epitopes on ovarian tumors

2. b DOD Grant
Characterization of T cell epitopes on breast tumors

3. M.D. Anderson Cancer Center funds for support in part of the salary of Dr. Ioannides

c. This research was not supported by contract or other funds of such type.

6. At least $50,000. This includes $5-10,000 for supplies for cell growth only, $5,000 for HPLC and sequencing, immunochemistry plus salaries.

7. 1. Dr. Ralph B. Arlinghaus, Chairman and Professor, Molecular Pathology
Dr. Gary Gallick, Professor of Tumor Biology
Dr. Elizabeth Grimm, Professor of Tumor Biology
Dr. Gabriel Lopez-Berestein, Professor, Immunobiology and Drug Carriers

8. PATENT DISCLOSURE

8a. Breast and ovarian cancer affect a large number of women. Disease progression even after conventional therapies requires development of novel approaches based on the understanding of the molecular interactions that control tumor development and progression. A number of factors have been associated with neoplastic transformation, including but not limited to genetic, environmental, developmental and immunologic. Human adult (normal) cells are continuously renewed to maintain the integrity and function of the mature healthy and functional organs. A number of genes are activated/silenced during development. Receptors involved transduce environmental signals to the cells, activating pre-programmed pathways that determine the cell fate. The fact that many cancer cells express or secrete proteins normally found in earlier developmental stages may be indicative of the fact that they are arrested in less mature stages of development. Maintenance, progression or death of a cell in a certain developmental stage also depends on its ability to interpret the signals received from the environment, using signal tranducing elements (1). One of this signal transduction complexes is defined as the "Notch" complex (2-5).

The Notch gene itself encodes a membrane bound receptor that suppresses the differentiation. Several other genes are further involved in the Notch induced suppression. Interestingly this suppression is initiated by synthesis of a group of repressors of transcription encoded by the Enhancer of split genes *[E (spl)]* (4). Synthesis and expression of these suppressor proteins may reflect expression of novel genes products during neoplastic transformation. An alternative possibility is that the synthesis lead to overexpression of the *E (spl)* protein which otherwise are expressed at significantly lower levels. Thus such proteins can be used as targets for development of novel therapeutic approaches in cancer.

There is little information about the expression and function of the proteins of the "Notch" complex in humans. A group has named some of them as TLE, (transducin-like enhancer of split) (6). Another group has identified human and mouse TLE like proteins representing the N-terminal region of the TLE and named them AES 1/2 (amino-enhancer of split) (7), AES 1 and AES 2 shows certain homology with TLE (7). Furthermore another group has reported the same sequence as AES of a Grg, a mouse gene elated to the *groucho* transcript of the Drosophila, Enhancer of split (8). This group has also proposed a cytoplasmic localization for the Grg/AES protein based on the fact that the mouse (as well as the human Grg/AES) do not contain consensus sequences for nuclear localization or CkII and cdc 2 kinase sites (8). Thus it was proposed that Grg may interact with TLE in the cytoplasm and inhibit their function (8).

Based on these hypotheses, with the caveat, that there is little direct information on the functional aspects of the E (spl) proteins in human cancers, it is possible that of "Notch" (receptor) represents the signal transduction element, TLE, 1-4 members of the *E (spl)* complex have

nuclear effector function, while the AES/Grg may be activated, and overexpressed in tumors in an effort to maintain the undifferentiated state. This raises the questions as to whether (1) such proteins or fragments of such proteins can be processed and presented to immune effectors, and whether (2) CTL can recognize sequences from this protein. An answer to these questions indicating that these hypotheses may be valid was obtained from two ongoing studies in our laboratory.

(1) In the first group of studies we have grown in large numbers cells of the ovarian tumor line SKOV3.transfected and expressing these gene for the HLA-A2. Similarly we have grown in the equivalent large numbers (1 x 10^{10}) cells of the C1R.A2 cell line transfected with and expressing the gene product of the HER-2 gene. Thus of the MHC-class I genes C1R.A2 cells express only HLA-A2.

The HLA-A2 was isolated by immunoaffinity from tumor cell lysates. Peptides bound were eluted and separated by two rounds of HPLC chromatography. Specific experimental and technological details on immunoaffinity, HPLC and CTL assay as described in detail in the attached manuscript (9). This manuscript is being submitted as of 4/12/96 for publication. To identify candidate peptides tumor Ag we (a) analyzed the ion composition of four consecutive samples from CTL activity peak; (b) correlated the abundance of the ions with the CTL activity profile. We found a number of ions whose abundance matched the CTL activity profile. One of them with a mass of 793 Da, was sequenced. The sequence obtained was interpreted using: (1) the computer program PEPSEQ version 1.2. Several candidate sequences were obtained; (2) by following overlapping a, b, and y ions resulted in the C.I.D. (collision-induced dissociation spectrum; (3) a third verification was made independently from sequencing data by Dr. Ioannis Papayanopoulos from Biogen Corporation. The interpretation of these data is consistent with the presence in this peak of several ions of distinct chemical nature such as phospholipids and peptides.

The reconstituted sequence of the 793 ion matched 7/8 amino-acids **PLTPLPV** in the sequence of AES 1/2. The AES sequence is **LPLTPLPV**, while our deduced sequence was **GPLTPLPV**. Because of the possible loss of N terminal ions during purification procedures, by the action of aminopetidases, or during ES-MS/MS-MS procedures we cannot ascertain with precision that the N terminal sequence of the peptide corresponded to a nonapeptide **AGPLTPLPV**, which would represent a mutated AES peptide, or whether the signal corresponding to the N-terminal residue is derived from the same or another ion.

It should be noted that (a) the human AES was sequenced only a few times. Thus the presence of allotypes or mutated forms of these genes are not known. (b) Our sensitivity to date is comparable with that of other studies in determining the identity of naturally processed peptides (6/8 matches) (10). These results indicated the likely possibility that a peptide

derived from the AES protein of the Notch complex can be naturally processed and presented.

B. To address the question whether the AES protein is present the SKOV3 tumor line, we selected primers from the areas adjacent to the AES 1/2 125-135. We amplified by PCR the cDNA prepared from mRNA isolated from the SKOV3 ovarian cell line and the SkBr3 breast cell line. The TLE 1 protein was amplified in the same experiment. The results of hybridization with sequences specific probes for TLE-1 and AES-1 for the sequences of interest showed that the transcripts for both TLE and AES are present in both SKOV3 and SkBr3 cells.

C. To address the question whether ovarian and breast CTL recognize AES peptides, from this area a number of peptides corresponding to overlapping sequences were prepared.

We prepared overlapping peptides from the area: AES-1:125-137. This area is identical in the AES-2 and the Grg, but the numbers are shifted (7, 8). The MS/MS spectra of the synthetic peptide G57: **GPLTPLPV** were determined and they showed at least 6/8 matches in the 6 ions with the natural product.

These peptides were also tested in CTL assays for recognition by breast and ovarian CTL lines. The peptides of sequence **ALPLTPLPV** corresponding to AES-1 (127-135) and overlapping **ALALPLTPL** AES-1 (125 - 133) were recognized with the highest efficacy among the AES peptides tested by the breast and ovarian CTL tested. These results indicate that AES protein is a target of tumor reactive CTL.

Based on these results we are not only patenting the sequences of these peptides as targets of tumor reactive CTL, but we are patenting the use and the targeting of the Notch proteins as immunotherapeutic approaches. These approaches are based on induction by these proteins of peptide specific and tumor reacting T cells as well as CD8+ CTL and CD4+ T helper cell responses. Furthermore, targeting of the Notch receptor by antibodies may inhibit or suppress the Notch activation pathways. This may lead to cancer cell death. We are patenting the concept of developing drugs based on or focused on the proteins of the Notch complex: (a) such as Ab to the extracellular receptors; (b) anti-sense technology directed to the Notch complex; (c) peptide induced CTL.

References

1. Zagouras, P., Stifani, S., Blaumueller, C.M., Carcangiu, M.L., and Artavanis-Tsakonas, S. Alternations in Notch signaling in neoplastic lesions of the human cervix. *Proc.Natl.Acad.Sci* 92:6414-6418, 1995.

2. Kopan, R., and Weintraub, H. Mouse notch: expression in hair follicles correlates with cell fate determination. *J. Cell Biology* 121:631-641, 1993.

3. Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M.E. Notch signaling. Science 268:225-232, 1995.

4. Goodbourn, S. Notch takes a short cut. *Nature* 377:288-289, 1995.

5. Hartley, D.A., Preiss, A., and Artavanis-Tsakonas, S. A deduced gene product from the Drophophila neurogenic locus, *Enhancer of split,* Shows homology to mammalian G-Protein b subunit. *Cell* 55:785-795, 1996.

6. Stifani, S., Blaumueller, C.M., Redhead, N.J., Hill, R.E., and Artavanis-Tsakonas, S. Human homologs of a *Drosophila Enhancer of Split* gene product define a novel family of nuclear proteins. *Nature (Genetics)* 2:119-127, 1992.

7. Miyasaka, H., Choudhury, B.K., Hou, E.W., and Li, S-L. Molecular cloning and expression of mouse and human cDNA encoding AES and ESG proteins with strong similarity to *Drosophila* enhancer of split groucho protein. *Eur. J. Biochem.* 270:343-352, 1993.

8. Mallo, M., Franco del Amo, F., and Gridley, T. Cloning and developmental expression of *Grg*, a mouse gene related to the *groucho* transcript of the *Drosphila Enhancer of split* complex. *Mechanisms of Development* 42:67-76, 1993.

9. Mallo, M., Steingrimsson, E., Copeland, N.G., Jenkins, N.A., and Gridley, T. Genomic organization, alternative polyadenylation, and chromosomal localization of *Grg*, a mouse gene related to the *groucho* transcript of the *Drosphila enhancer of split* complex. *Genomics* 21:194-201, 1994.

10. Fisk, B., DaGue, B., Seifert Jr., W.E., Hudson, J.M., Kudelka, A.P., Wharton, J.T., Murrary, J.L. and Ioannides, C.G. Mass-spectrometric analysis of naturally processed peptides recognized by ovarian tumor associated CD8+ CTL. (submitted for publication) 1996.

11. den Haan, J. M.D., Sherman, N.E., Blokland, E., Huczko, E., Koning, F., Drijfhout, J.W., Skipper, J., Shabanowitx, J., Hunt, D.F., Engelhard, V.H., and Goulmy, E. Identification of a graft versus host disease-associated human minor histocompatibility antigen. *Science* 268:1476-1480, 1995.

Abbreviations: HER-2, HER-2/neu proto-oncogene; HPLC, reverse phase high pressure liquid chromatography; MS, mass spectrometry; ESI/MS, electrospray-ionization-tandem mass-spectrometry; TFA, trifluoroacetic acid; ACN, acetonitrile; Ag, antigen; TCR, T cell receptor; Rt, Retention time; Fxn, Fraction; m/z;, mass - to - charge ratio; Da, mass unit; ESI, electrospray ionization; CID, collision-induced dissociation.

Key Words: natural peptides, CTL epitopes, ovarian cancer, AES, Notch

INTRODUCTION

The development of rational immunotherapy approaches for human cancers depends on a detailed understanding and quantitation of the host anti-tumor responses involving recognition by CTL of specific epitopes on various normal and malignant tissues. CTL epitopes are short peptides (8-10 amino acids long) that are presented on the cell surface by MHC class I molecules (1). This recognition is also dependent on the density of the peptide and affinity of TCR (2,3).

This is of particular importance because, first, natural peptides produced by tumor Ag processing machinery may not be entirely identical to the synthetic peptides defining CTL epitopes. The latter are usually defined with HLA-class I anchor motifs or genetic approaches (4). Second, for human cancers a number of peptides derived from self-proteins were found to reconstitute the lytic function of tumor reactive CTL. Thus, with few exceptions, evidence to date reflects the immune repertoire rather than a specific and selective response to the tumor (5). Third, it is still unknown whether the lack of recognition of a particular tumor by its autologous CTL reflect either the lack of presentation of the tumor Ag, or tolerance to the particular epitope. Tumor antigens may also be presented to T cells by professional antigen presenting cells (APC), which can process particulate antigens or damaged cells for MHC-class I presentation (6-8). Thus identification of the natural peptides presented in association with MHC molecules on human tumors should be important for identification of tumor Ag with consequent development of novel approaches for tumor specific CTL induction.

To date, with the few exceptions of melanoma gp100 and MART-1 CTL epitopes (9-10) there is limited information on the identity and density of the peptides presented by most human solid tumors. Ovarian CTL lines were

recently reported to recognize a number of HER-2/neu (HER-2) peptides (11-14) but the nature of peptides presented by the tumor is still unknown. To address these questions for Ag that are relevant to breast and ovarian cancer, we have investigated the identity of peptides presented by HLA-A2 on targets recognized by HLA-A2$^+$ CD8$^+$ CTL lines isolated from patients with ovarian cancer. We used a cloned ovarian tumor line (SKOV3.clone 1E4) and the cloned C1R.A2 line (clone HER-2.J) transfected with HLA-A2 and HER-2 respectively. Since these cells express defined levels of MHC antigens, adhesion molecules and HER-2 quantitation of HLA-A2 bound peptide expression can provide a reference point for analysis of peptides from freshly isolated tumors.

We fractionated HLA-A2 bound peptides using two dimensions of reverse phase HPLC (HPLC). Reconstitution profiles of CTL activity indicate at least five peaks of peptides (designated as *1, 2A, 2B, 2C* and *3*) eluting with distinct retention times (Rt). Mass spectrometric (MS) analysis of peak *2B* which corresponded to the Rt of the immunodominant epitope HER-2 (369-377), revealed a large number of ions with m/z in the mass range of peptides having 8 - 11 amino acids. Comparison of the presence and abundance of ions in four consecutive fractions of the peak *2B* identified several peptides whose abundance correlated with CTL activity. One of these ions (m/z = 793) was sequenced by MS-MS. Peptides corresponding to reconstituted sequence were recognized by tumor associated CTL.

MATERIALS AND METHODS

Cell Lines

The following human tumor lines were used in these experiments: *(1)* ovarian tumor line SKOV3 (HLA-A3, A28, B18, B35, Cw5) stably transfected with the gene for HLA-A2. The gene for HLA-A2.1 was kindly provided by Dr. William E. Biddison (NIAID, NIH). A tumor clone, SKOV3.A2.1E4, expressing

high levels of both HER-2 and HLA-A2 has been designated as 1E4 and was selected for expansion in large numbers and peptide fractionation experiments; *(2)* C1R:A2 cells (a kind gift from Dr. Biddison), which express the product of the same HLA-A2 gene on the surface. These cells were transfected with the gene encoding for the HER-2 proto-oncogene (plasmid pCMV.HER-2$^+$ encoding a full length HER-2.cDNA) and co-transfected with the plasmid SV2.Hygro (ATCC). These plasmids were a kind gift of Dr. Mien-Chie Hung, Department of Tumor Biology (MDACC) After selection with Hygromycin B, C1R.A2.HER-2$^+$ cells were cloned by stringent limiting dilution. At least twenty C1R.A2.HER-2$^+$ clones (designated HER-2.A-T) were isolated expressing variable levels of HER-2 receptor on their surface. A clone of C1R:A2.HER-2 cells designated here as HER-2.J was selected for expansion in large numbers and biochemical fractionation of HLA-A2 bound peptides. Cell lines were grown in RPMI 1640 medium containing 10% FCS supplemented with 2 mM L-glutamine, and 100 μg/ml gentamycin (complete RPMI medium). HLA-A2 transfected 1E4 and C1R:A2 cells were selected with 250 μg/ml G418 (GIBCO, BRL, Gaithersburg, MD). Both SKOV3 and C1R.A2 express the same HLA-A2 gene. HER-2.J cells were selected with 50 μg/ml of Hygromycin B. This concentration was found to result in the death of over 50% of untransfected C1R:A2 cells within 4 days in parallel experiments. Effector CTL-OVA3 used in these studies has been previously reported (14). CTL-OVA5 was also obtained from tumor associated lymphocytes (TAL) from another patient with adenocarcinoma of the ovary. The corresponding tumor (OVA-5) was HLA-A2$^+$ and HER-2^{high}. CD8$^+$ cells were isolated from cultured TAL on Ab coated plates AIS MICRO^{CELLECTOR} (Applied Immune Sciences). Both CTL lines were CD3$^+$ CD8$^+$. CD4$^+$ cells represented $\leq$ 5% of total cell populations.

Flow cytometry.

Target cells were tested in fluorescence experiments to confirm the expression of HLA-A2, MHC class I, and HER-2, as previously described (14). mAb BB7.2 and MA2.1 were obtained from ATCC. In brief, 1E4 and HER-2J cells were incubated with mAb BB7.2 (a2 domain), W6/32 (HLA-A,B,C, a3 domain, monomorphic) (Dako-Dakopatts, Denmark) and *Ab2* specific for the extracellular domain of HER-2 (Oncogene Science) followed by FITC conjugated goat-anti-mouse IgG. Surface antigen expression was determined by flow-cytometry using a fluorescence activated cell sorter, FACSscan (Beckton-Dickinson) with a log amplifier. CD3, CD4 and CD8 antigen expression on the effectors was determined by immunofluorescence with corresponding FITC conjugated mAb. C1R.A2 clones were designated as HER-2^{high} when MCF for HER-2 expression was above 200, and HER-2^{low} when MCF was below 40. Expression of CD18, CD11a (LFA-1), CD54 (ICAM-1) and CD58 (LFA-3) antigens was also tested with corresponding antibodies (Beckton Dickinson).

Peptide extraction and fractionation.

1E4 and HER-2.J cells were grown in 10 chamber cell factories (Nunc, Thousand Oaks, CA) in complete RPMI 1640 medium. Between 1.0 - 1.5. x 10^9 cells were obtained from one cell factory. Cells were collected and washed three times with cold PBS. Further, cells were lysed using a buffer (15) containing protease inhibitors (aprotinin, leupeptin, pepstatin, PMSF, iodacetamide), in PBS with the difference that CHAPS 0.5% was used as lysing agent to minimize binding to C18 columns (V. Engelhard, personal communication). This buffer is designated here as lysis buffer. MgCl2 at 6 mM and Glycerol at 20% final concentration were added in the lysis buffer to minimize the denaturation of extracted proteins. Detergent-solubilized extracts of 1E4 and HER-2.J cells were obtained after centrifugation at 40,000g for 2h. HLA-A2.1 was isolated from the supernatants of centrifugation by affinity chromatography on Protein A-Sepharose prebound with mAb BB7.2, as

described (15) except that the cell extracts were pre-absorbed on Protein-A Sepharose (Sigma Chemical Co., St. Louis, MO) to minimize non-specific binding. The column was washed with PBS containing 0.25M NaCl with monitoring of the OD210 nm (for peptide bond), then eluted with 0.2M acetic acid. The eluate was boiled for 5 min to allow dissociation of peptides from HLA-A2, centrifuged through an Ultrafree CL unit (3-kDa cut-off) (Millipore) and lyophilized. For these studies, at least 2×10^{10} cells of each 1E4 and HER-2.J. were grown in batches of $1.5 - 2.0 \times 10^{9}$ cells. Peptides with masses < 3kDa were pooled, lyophilized and separated by reverse phase-high pressure liquid chromatography (HPLC).

HPLC fractionation of HLA-A2 bound peptides.

Tumor peptides extracted from HLA-A2.1 molecules of both 1E4 and HER-2.J cells were separated in the first dimension on a Brownlee C18 Aquapore column (2.1 x 30 mm, pore size, 300 A; particle size, 7μm, Applied Biosystems, Perkin-Elmer Corporation) and eluted with a 60 min gradient of 0-60% (vol/vol) acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA), (Gradient I) at a flow rate of 200 μl/min using an HPLC system model 1090 (Applied Biosystems) as previously described (15-18). For the second dimension separation, pooled fractions from the first dimension corresponding to the peak of elution of E75 and the corresponding peak of CTL activity were injected into a Brownlee C18 Aquapore column of 2.1 x 220 mm, (300A, 7μm) and eluted with a shallower gradient: 0-5 min, 0-15% ACN in 0.1% TFA, 5-45 min 15-35% ACN in 0.1% TFA and 45-60 min, 35-60% ACN in 0.1% TFA (Gradient II). The flow rate was 200 μl/min and fractions were collected at 1 min intervals.

Synthetic peptides were prepared by the Synthetic Antigen Laboratory at the M.D. Anderson Cancer Center using a solid phase method and purified by HPLC. Identity of peptides was established by amino acid analysis. The purity

of peptides was more than 97%. HER-2 peptides, E75 (369-377, KIFGSLAFL), C85 (971-979, ELVSEFSRM), E90 (789-797, CLTSTVQLV) and E89 (851-859, VLVKSPNHV) identified in the previous studies to be recognized by ovarian CTL-OVA3 line were separated in the same conditions using Gradients I and II. Their peaks of elution and retention times (Rt) were determined.

CTL assays.

Reconstitution of CTL epitopes was performed using HPLC fractions from the first and second RP-HPLC dimensions (Gradients I + II). 50ml aliquots of each fraction were concentrated by vacuum centrifugation (Speed Vac) to approximately 1/10 of its original volume to remove TFA and ACN, then reconstituted with RPMI medium to the initial volume and added to ^{51}Cr labeled T2 cells in V bottom microtiter plates. After pre-incubation with peptides for 90 min, effectors were added at various E:T ratios and a standard CTL assay was performed for 5h as described (14). Control wells were made either with T2 cells incubated with HPLC fractionated peptides without CTL, or with CTL without adding HPLC separated peptides.

Percentage of specific lysis was determined from the equation (A-B)/(C-B) x 100, where A is lysis of T2 cells by effectors in the presence of a peptide or an HPLC fraction, B is spontaneous release from T2 cells in the presence of the same peptide but in the absence of effectors, and C is the maximum ^{51}Cr release. The experiments were performed in triplicate, and the mean $\pm$ SD values were calculated from at least two separate experiments. Since even one amino acid change in peptide length at the COOH-terminus can have dramatic effects on peptide recognition, concentration of natural peptides in the sample is unknown, and identification of CTL epitopes is performed with a mixture of active and inactive peptides, cytotoxicity values were considered to indicate significant recognition of a peptide when the differences between mean $\pm$ SD values for specific lysis of T2 cells preincubated with a given fraction of peptide

or medium were >10%, at an E/T ratio of 20:1 and statistically significant ($P < 0.05$) (19).

Mass-Spectrometry.

Electrospray mass spectrometric (ES-MS) analyses were performed on a Finnigan MAT TSQ70 triple-quadrupole instrument upgraded with TSQ700 software and a 20 kV conversion dynode electron multiplier. The Vestec ES source (PerSeptive Biosystems, Vestec Products, Cambridge, MA) was modified as previously described by Emmett and Caprioli (20). Typical ES operating parameters were as follows: needle voltage, 3.5 kV; nozzle voltage, 250 V; repeller voltage, 10-12 V; source block temperature, 240 ± 5 C. Standard peptides were dissolved at 1-2 nmol/ml in water or 0.5% aqueous acetic acid (V%), diluted to 10 pmol/ml with 100:100:0.5 (V:V:V) methanol:water:acetic acid (ES-MS solvent), and introduced into the MS by infusion at 0.82 µl/min. HPLC fractions obtained from biological samples were first lyophilized to dryness in polypropylene micro-centrifuge tubes. The dried samples were then reconstituted in either 10 ml 0.5% acetic acid and a portion mixed with an equal volume of methanol, or in 20 ml ES-MS solvent. One-microliter samples were then introduced into the MS by injection into the infused ES-MS solvent via a Rheodyne 8125 injector fitted with a 5-µl sample loop.

Collision-induced-dissociation mass spectra (MS/MS) were obtained with argon collision gas at a pressure of 1-2.5 mTorr and a collision offset potential of -6 to -20 eV. Spectra were acquired at the rate of one scan/second (or approximately 1000 Da/s, depending on the mass range needed) with a running average of 16 scans. For daughter-ion scans, the resolution on the first quadrupole (Q1) was adjusted to allow transmission of ±2 Da from the center of the mass of interest. A peak width of 1000 mDa was used for post-acquisition spectral averaging and for quantitation by manual integration of selected ion

chromatograms from the injection analyses. Data analyses were performed using the Finnigan MAT ICIS software.

In certain instances samples containing hydrophobic material were first reconstituted with 0.5% acetic acid, then mixed with an equal volume of methanol. Peptide standards (des-Tyr^{1}-Leu-Enk, m/z 393.2 $(M + H)^{+}$ substance P, 1-11 m/z 674.4 $(M + 2H)^{+2}$, b-casomorphin 790.4D $(M + H)^{+}$ and bovine insulin m/z 956.6 $(M + 6H)^{6+}$ were analyzed to verify the sensitivity of detection. Each sample was analyzed at least twice. Sensitivity of detection was at the level of 25 fmols/ml sample injected. Variability between determinations did not exceed 2-4% in two independently performed experiments (data not shown). Background samples were run in parallel to establish that ions identified are not derived from background signals or peptide contamination. Qualitatively similar profiles were obtained from reconstituted samples stored at -65°C for up to one month.

Synthetic HER-2 peptides, E75, C85, and E92 (14) were sequenced by ES/MS/MS. To facilitate interpretation of results, in certain instances, the MS/MS spectrum was compared with that recorded for the corresponding methyl ester. In the spectrum of the methyl ester, signals containing the C-terminus and Asp and Glu groups shift by increments of 14 Da. Methyl esterification of carboxyl groups was performed using acetyl chloride in methanol as the methylating agent. Data analysis for sequence reconstitution was performed using the program PEPSEQ version 1.2. software (21). This program identifies candidate sequences based on the concordance of determined and predicted peak values of ions in a candidate sequence. The lowest deviation of the experimentally determined versus theoretical values for the respective ions, was defined as the lowest score/peak ratio (sc/p).

RESULTS

Definition of CTL epitopes recognized by CTL-OVA3 .

To estimate the pattern of recognition of CTL-OVA3 for antigens (Ag) presented by HLA-A2, peptides were extracted from immunoaffinity purified HLA-A2 molecules from 10^9 of each 1E4 and HER-2.J cells. The presence of common peptides recognized by CTL in the peaks of HPLC fractions with the same Rt it is likely to indicate common CTL epitopes (16-18, 22).

Definition of the epitopes recognized by CTL-OVA3 was performed with peptides fractionated by HPLC using Gradient I. CTL recognition was demonstrated by three distinct peaks of 1E4 peptides (designated as ***1***, ***2***, ***3***) as indicated by lysis of T2 cells incubated with equal volumes of each fraction (Fxn) eluting in Gradient I (**Figure 2**). Of the HER-2 peptides, C85 (971-979) eluted in the Fxn 22-23 (Rt = 22.8 min) while E75 (369-377) eluted in the fractions 29-30 (Rt = 29.8 min) suggesting that peptide(s) of similar retention time with E75 are present in the peak *2* of 1E4 peptides. Similar patterns of activity with the same HPLC fractions separated in Gradient I were observed for two other ovarian CTL lines, CTL-OVA4 and CTL-OVA5. The position of peak *2* (Fxn 28-32) was identical for CTL-OVA3 , CTL-OVA4 and CTL-OVA5. The position of the peak *3* (Fxn 43-50) was the same only for CTL-OVA3 and CTL-OVA5 (data not shown).

Since these results may be suggestive of presence in these HLA-A2 patients of CTL of common specificity we focused first on the bioactive peak *2* because it was recognized by 3/3 ovarian CTL lines. Since each fraction is expected to contain a large number of peptides, the pooled active fractions 27-32 from peak 2 corresponding to the peak of elution of E75 $\pm$ 2 min were re-chromatographed on a longer C18 column of 220 mm containing particles of the same size and pore size. We used a shallower and thus more resolving Gradient II (0.5% ACN/min, between 15-35% ACN) (17,18). Fractions with

higher and lower retention times than E75 were included in this separation to avoid missing shorter or longer peptides and simultaneously to minimize losses in the material due to absorption of peptides at low concentration.

Rechromatography of peak *2* (pooled fractions 27-32) using Gradient II conditions, resolved into three distinct peaks of CTL-OVA3 activity. These peaks were designated as *2A, 2B, 2C*. Peak *2A* (fractions 32-34) appeared to show a double peak of activity. CTL activity was detected in the area of peak *2B*, surrounding the Rt expected for elution of E75 (**Figure 2C**). To determine whether active peptides are presented by HER-2.J cells, we tested recognition of Fxn 14 - 33 eluting from HPLC using Gradient I conditions. CTL-OVA3 recognition of peptides in these fractions resulted in two peaks of activity (***1*** and ***2***) eluting with similar Rt as peptides from 1E4. Fractions 27-32 from the peak 2 were pooled and separated using Gradient II conditions. The results in **Figure 2D** show a similar pattern of recognition by CTL-OVA3 for HER-2.J peptides as for 1E4 peptides peaks *2A, 2B, 2C*. The retention times of E75 and of the homologous peptide from epidermal growth factor receptor (EGF-R), 364-372: SISGDLHIL (designated as F48) were compared under the same HPLC conditions. F48 eluted in fraction 38. E75 co-eluted with the peak of CTL activity with retention time of 41.0 ± 0.5 min. Both E75 and F48 recovered from the HPLC column were tested for recognition by CTL. E75 was active between 5-50 pmoles, while F48 was inactive even at a nanomolar concentration. Identical position of peaks of recognition of peptides from the same Gradient II fractions: *2A* (Fxn. 33-36), *2B* (Fxn. 39-41) and *2C* (Fxn. 43-48) were observed with CTL-OVA5 (data not shown).

These results indicate that at least three peaks of activity from both 1E4 and HER-2.J cells elute in Fxn 27-32 under Gradient I conditions. These data are consistent with the presence of peptides in peak *2* that reconstitute three peaks of activity *2A, 2B, 2C* for the CTL-OVA3 . Of these, the peptides in the

peak *2B* have a similar retention time with E75, suggesting that natural peptides of similar sequence may be presented by these cells.

Fractionation of 1E4 and HER-2.J peptides.

To identify peptides presented by HLA-A2 by mass-spectrometry (MS) equivalent samples (in terms of cell numbers) were prepared from 1E4 and HER-2.J. cells. Peptides were extracted from immunoaffinity isolated HLA-A2 molecules as described in the Methods Section. For these experiments 20 x 10^9 1E4 cells and 10 x 10^9 HER-2.J cells were used. The peptide material separated through 3kDa filters was chromatographed consecutively through the two C18 columns of 30 and 220 mm length using Gradients I and II respectively. Peak *2B* from each sample was recovered in four fractions designated 39, 40, 41 and 42 as shown in **Figure 2.**

Activity was found in Fxn 40 and 41 (designated as active fractions) in agreement with the data shown in **Figure 2**. The activity of Fxn 39 and 42 was minimal (thus, designated as inactive fractions). For MS, peptide material from each of Fxn 39 - 42 was lyophilized and reconstituted to 20 ml final volume. Each determination was made from 1 µl sample, representing 2.5% of material. This represents 0.5 x 10^9 cell equivalent/injection.

Mass spectrometric analysis of peptides from the HPLC fractions.

To identify and define peptides whose abundance match the activity profile of the CTL-OVA3 sensitizing activity, each of the peak *2B* fractions 39-42 of 1E4 and HER-2.J peptides were analyzed by ES-MS. The rationale for this approach was that correlations between presence and abundance of defined ions in consecutive fractions and the patterns of CTL recognition can focus the search for tumor CTL epitopes. For example, if an ion is present in two consecutive fractions one of which is active and the other inactive, it is less likely to be recognized by CTL if its abundance is higher in the inactive fraction.

Conversly if the abundance of an ion in an active fraction is below the lower limits for binding to HLA-A2 in a T2 assay it is less likely to sensitize CTL. These limits were recently reported to be in the 10^{-12} - 10^{-13} M range (2).

To address these questions we compared the identity and abundance of ions in the active fractions 40-41 with the ions in the inactive fractions 39 and 42 of 1E4 and HER-2.J. Total ion signal from equivalent samples of 1E4 and HER-2.J cells was obtained by scanning the masses corresponding to mass-to-charge ratio *(m/z)* between 200 and 1500 are then summing the obtained spectra. At each voltage, the limit of detection was defined at a signal to noise (S/N) ratio of two (24).

To illustrate the ions present in each fraction and their relative amounts (defined as signal intensity) the mass spectra of Fxn.40 - 41 of 1E4 in the m/z range of 700 - 1300 are shown in **Figures 3A, and 3C**. Expansion of areas from each spectrum revealed additional ions present in lower amounts. This is shown in **Figure 3B** for the area 870 - 1100 of Fxn.40. Similar pattern of ions distribution and abundance were found in peak *2B* of HER-2.J cells. This is shown for Fxn. 41 **(Figure 3D)**.

Seven 1E4 ions with mass-to-charge ratio *(m/z)*, 777, 793, 807, 818, 834, 905 (actual m/z = 904.3) and 934 and most likely singly charged were found in both Fxn 40 and 41 **(Figure 3A,B,C)**. With the exception of the ion at m/z 934 which was present in Fxn 39, these ions were not detected in Fxn 39 and 42. The ion at m/z 934 was more abundant in Fxn 39 than in Fxn 40-41. Since Fxn 39 was inactive in the CTL-OVA3 assay, this suggests that the m/z 934 species is not recognized by CTL-OVA3 . The ions at m/z 994, 1008 and 1017 were present in Fxn 40 **(Figure 3B)**, but they were not detectable above the S/N >2 level in Fxn 41. With respect to HER-2.J peptides, five singly charged ions with *m/z* 781, 793, 906 (actual m/z, 905.6), 955 and 1038 were found to be most abundant in both Fxn 40 and 41 compared with the other ions. Their signal

intensity was comparable with that of the ions detected in Fxn 40-41 of 1E4 compare (**Figure 3A** and **3C** with **Figure 3D**).

Comparison with β-casomorphin standards of similar mass 790 (+1), suggests that in 1E4, the ion 793 is present at approximately 650 fmoles/μl injected sample in Fxn 40, at 330 fmoles/μl in Fxn 41 and at 100 fmoles/μl on corresponding samples from HER-2.J cells. The single-charged ion at m/z 497.3 **(Figure 4)** was a major component of Fxn 40 - 41 from both 1E4 and HER-2.J cells but was absent from Fxn 39 and 42. The mass of this ion is within 1 mn of the mass of the double charged ion of the peptide E75: HER-2 (369-377). In some samples additional smaller peaks of close m/z: 497.6, 498.3, were found (data not shown). The peak 498.3 may also correspond to an isotopic form of peak 497.3 Da at +1 charge state.

Correlations between signal intensity and CTL activity of fractions in peak 2B.

The CTL-OVA3 and CTL-OVA5 activity profile for the peptide fractions extracted from 1E4 is shown **(Figure 5)**. Comparison of the activity profiles for CTL-OVA3 and CTL-OVA5 with the signal intensity of ions detected by MS indicated that the pattern of T2 lysis by CTL-OVA3 correlated with the presence in Fxn.39 - 42 of 1E4 ions: 497.3, and 793 (+1) **(Figure 5A, B)**. The ion of m/z 934 was found in significantly higher concentration (2000-3000 fmoles) in Fxn 39 than in Fxn 40 and 41 in both cell lines. For CTL-OVA3 Fxn 39 is essentially inactive, but CTL-OVA5 shows low level recognition of this fraction. These results indicate that additional CTL epitopes may be antigenic in different individuals.

Sequence reconstitution of MS-MS sequenced ions. The sequence reconstitution approach, of peptide 793, using PEPSEQ program, revealed three possible octapeptides, excluding permutations, within a mass tolerance of 0.02%. These sequence combinations contained the N- terminal tripeptide

GPX, GPV or GPS. It should be noted that the last two C-terminal residues determined by MS-MS sequencing may appear reverted. Thus both sequences **GPLTPLVP** (designated G57) and **GPLTPLPV** (designated G76) are possible. Comparison with the theoretical values of the masses of B ions for the peptides **GPLTPLPV** and **GPLTPLVP** showed good correlation with experimentally determined and deduced B ions (at least 6 of 8) and Bo ions (5 of 8) from 793. The same good correlation was found between the 793 daughter ions and synthetic peptide G57 daughter ions sequenced under the same conditions, 6 of 8 B ions and 5 of 8 Bo ions respectively (Diagrams A - E).

The search of DNA and protein sequences identified a distinct coding sequence LPLTPLPV matching peptide GPLTPLPV at seven of eight residues and peptide GPLTPLVP, at five of eight residues (underlined) but not for the other sequences containing Val or Ser at P3. No significant matches were found in the Gene Bank data bases for the same peptides containing Ile instead of Leu. The entire central area PLTPL of peptide 793 matched with the sequence encoded by the AES-1/2 protein positions 125-135: ALALPLTPLPV. This area contains three nested nonapeptides ALALPLTPL, ALPLTPLPV and PLTPLPVGL (26) containing canonical HLA-A2 anchors. The N-terminal Gly does not match the published AES 1/2 sequence. It is unknown whether the resulting peptide corresponds to a mutant of AES protein, on the strong Gly signal is a contaminant present in the peak m/z 793 from another peptide.

Only few members of the AES gene family have been sequenced, thus at this moment we cannot establish whether the sequence identified is derived from an yet uncharacterized human protein, a mutant AES, or represents a fragment of a longer AES peptide. The corresponding AES gene or an allelic form of an AES protein is a member of the so-called "Notch" pathway (26-27). In Drosophila, proteins of the "Notch" group have been implicated in corrections of developmental choices for cells that have not acquired their final

developmental stage. Of interest only testes and ovaries express high levels of Notch proteins in adult organisms (28).

We synthesized both peptides G76: GPLTPLPV corresponding to the MS deduced sequence and G60: ALPLTPLPV, corresponding to the w.t. nonapeptide AES published sequence. Both peptides were tested in the same experiment, for their ability to sensitize targets for lysis. The results of experiments performed with two breast $CD8^+$ CTL lines are shown in Figure 6. These CTL lines were isolated from breast tumors. They were HLA-$A2^+$. $CD8^+$ cells recognized both G76 and G60 peptides. Peptide G60 was recognized significantly better than G76, suggesting that when bound on HLA-A2 of T2 cells from the epitope that is recognized by these CTL. To characterize their binding affinity, both peptides were tested in HLA-A2 stabilization assays. We observed a strong increase in the mean channel fluorescence intensity (MCF) when T2 cells were incubated with peptide G60. The stabilizing ability of HLA-A2 by these peptides decreased in the order: G60 > G61 > G75 > G76 (Table 1). Of interest at the same concentration, G76 decreased the MCF for both BB7.2 and MA2.1 mAb recognized epitopes suggesting that this peptide in addition to its low binding affinity induced conformational changes in the HLA-A2 molecules.

CONCLUSIONS

A peptide, 793, isolated from HLA-A2 of 1E4 cells (m/z = 792.8) whose abundance matched the CTL activity in the HPLC second dimension peaks 39 - 42 was sequenced by CID. A peptide with similar mass (793.2) was found in the corresponding fractions from HER-2.J cells, and it is likely, pending sequencing, that peptides of the same or similar sequence are present in both cell types. A good match (7 of 8) for peptide 793 was found in the AES protein which is a member of the Notch group involved in cell differentiation. At this moment is unknown whether peptide 793 corresponds to a wild-type AES-

protein with Gly (P1) derived from a contaminant or from a mutant Notch protein. Of interest, chromosome translocations in leukemia predict truncated mutant Notch proteins (29). Abnormally expressed Notch-like proteins appear to be present and involved in epithelial cancers as recently reported (28). Since these proteins inhibit differentiation it has been proposed that in tumors they maintain the undifferentiated state.

Of interest, nonapeptides corresponding to the wild-type sequence of AES are recognized better than the octapeptide 793 and they have signficantly higher binding affinity to HLA-A2. Furthermore, cold-target inhibition experiments showed that peptides G60 and G61 inhibit lysis of ovarian tumor line SKOV3.A2 and breast tumor line SkBr3.A2. This inhibition appears to be specific for G60 and G61 since irrelevant or mutated peptides (D132 and F46) do not significantly inhibit lysis. Possible implications of these findings are that: (a) the octapeptide is part of a nonapeptide which is proteolytically trimmed during processing; (b) the mutant peptide sequenced may represent an analog of the wild-type epitope, whose recognition may be ineffective or impaired.

LEGENDS TO THE FIGURES

Figure 1. CTL-OVA3 and CTL-OVA5 mediated lysis of SKOV3 and C1R.A2 cells. Effector:target ratio was 10:1. **(A)**. CTL-OVA3 ; **(B)**. CTL-OVA5.

Figure 2. Reconstitution of CTL-OVA3 epitopes with HPLC fractions from naturally processed peptides extracted from HLA-A2.1 chains of SKOV3 clone 1E4 **(A, C)** and HER-2.J cells **(B, D)**. Experimental conditions for peptide extraction as described in the Materials and Methods. **(A)** peptides extracted from 10^9 1E4 cells separated by HPLC Gradient I. **(B)** peptides extracted from 10^9 HER-2.J cells separated in Gradient I using a C18 column of 21 x 30 mm. **(C)** and **(D),** pooled fractions 27-32 from the Gradient I shown in A and B were separated in Gradient II using a C18 column (220 x 30 mm). Equal amounts of peptides E75 and F48 were chromatographed either individually or mixed and separated in the same conditions as 1E4 and HER-2.J fractions 27-32 using Gradient II. F48 and E75 eluted with Rt of 38.0 and 41.0 min respectively, indicating that this HPLC approach can separate mutated HER-2 peptides. The Rt of the other HER-2 peptides were as follows: Gradient I, E89, 14.8 min; E90, 22.4 min, GP2 (HER-2, 654-662, IISAVVGIL), 35.5 min. E92, HER-2, 650-658, PLTSIISAV; 26.7 min, E91, HER-2, 5-13, ALCRWGLLL, 38.4 min; Gradient II, E90, 25.4 min, and E92, 32.8 min. Effector:target ratio was 20:1. (■-■), % Specific lysis; (---), OD21O nm.

Figure 3. Ion composition of peak *2B* fractions. 100 µl each of the fractions 39 - 42 (i.e. 50%) was vacuum dried, and reconstituted in 20 µl of ESI buffer. 1 µl volumes (2.5% of each sample) were injected in the electrospray ionization source and the mass-spectra of the peptides collected in each fraction were recorded on a triple quadrupole mass spectrometer (Finnigan). Chromatograms of 1E4 peak *2B* ions: **(A)** fraction 40 ions, with mass to charge ratio (m/z) between 750 - 1100; **(B)** Expanded presentation of the Fxn 40 area

m/z 860 - 1100; **(C)** Fraction 41 ions, m/z, 700 - 1300; **(D)** Mass-spectra of the Fxn 41 (HER-2.J) showing the ions m/z 775 - 1400. Note the presence and abundance of ions at m/z 497 (panel D), 793 (panels A and B), and 906 (A, B, D). All determinations at a multiplier setting of 1600 eV. Total ion current is 50, 52, 31, 5.5 x 10^7 for the panels A - D, respectively.

Figure 4. A - D. Mass spectra of the peak *2B* ions from Fxn 40 - 42 of HER-2.J cells. Ion chromatograms over the m/z range 350 - 600: **(A)** Fxn 40; **(B)** Fxn 41; **(C)** Fxn 42. The ion at m/z 497.3 gives a saturated signal in Fxn 40. The ion 453 is present in Fxn 40, 41. The ion 519.3 likely corresponds to an $(M+H+Na)^+$ ion of 497.3 (sodium adduct). The ion 497.3 is absent from the Fxn 39 and 42. Multiplier setting at 800 eV. x axis = m/z ratio. y axis = % relative abundance to the total ion current. This was 7.5, 0.25 and 0.13 x 10^7 for the panels A - C respectively.

Figure 5. Identification of candidate CTL epitopes by mass-spectrometry combined with CTL assay. Each of fraction 39 - 42 of peptides from 5 x 10^9 1E4 cells eluting from a C18 column (220 x 2.1 mm) in Gradient II (as described in the Materials and Methods) was used for cytotoxicity determinations (■ - ■). Ion abundance is shown for ions with m/z of 793 (□) and 934 (○). The ion abundance is shown as signal intensity (ion - current). Scale is 10^8. **(A)** CTL-OVA3 , **(B)** CTL-OVA5. Effector - target ratio was 20:1.

Figure 6. Recognition of peptides G60 and G76 by CTL-Br9 **(A)** and CTL-Br10 **(B)**. Effector to target ration, 20:1.

Figure 7. Cold-target inhibition of lysis of tumor cell line SKOV3.A2 by CTL-BR1.0 and CTL-BR.11 by AES peptides G60 and G61. NP, indicates no peptide in the assay. D132 and F42 are control irrelevant peptides. The percent inhibition is indicated in parentheses.

DIAGRAMS

Sequencing and reconstitution results for the ion 793. A1. Sequence reconstitution of the ion 793 using the program PEPSEQ. Candidate peptides corresponding to the sequence of the ion 793 with the highest degree of tolerance. **B.** Predicted values of b and y ions from the sequence of the peptide G76: GPLTPLPV. **C.** Predicted daughter ions of peptide G57: GPLTPLVP. **D.** Resulting B and Y ions from sequencing of synthetic peptide G57. **E.1**. MS-MS analysis of the ion 793, (m/z scans 0 - 800). Expanded presentation of the ions resulting from CID sequencing of the ion 793: **E.2.** m/z 0 - 500; The ion 496 which likely corresponds to an impurity (phospholipid) it is not shown since his signal may suppress detection of the other ions. **E.3.** m/z 500 - 800).

REFERENCES

1. Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammense, H-G. (1991) Allele specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* **351**:290-296.

2. Kageyama, S., Tsomides, T.J., Sykulev, Y., and Eisen, H. (1995) Variations in the number of peptide-MHC class I complexes required to activate cytotoxic T cell responses. *J.Immunol.* **154**:567-576.

3. Sykulev, Y., Brunsmark, A., Tsomides, T.J., Kageyama, S., Jackson, M., Peterson, P.A., and Eisen, H.N. (1994)High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogenic and syngeneic major histocompatibility complex class I proteins. *Proc.Natl.Acad.Sci.* **91**:11487-11491.

4. Tsomides, T.J., Aldovini, A., Johnson, R.P., Walker, B.D., Young, R.A., and Eisen, H.M. (1994) Naturally processed viral peptides recognized by cytotoxic T lymphocytes on cells chronically infected by HIV type I. *J.Exp.Med.* **180**:1283-1293.

5. Houghton, A.N. (1994) Cancer Antigens: Immune recognition of self and altered self. *J.Exp.Med.* **180**:1-4.

6. Bevan, M.J. (1995) Antigen presentation to cytotoxic T lymphocytes in vivo. *J. Exp. Med.* 182:639-641.

7. Huang, A.Y.C., Golumbek, P., Ahmadzadeh, M., Jaffe, E., Pardoll, D., and Levitsky, H. (1994) Role of bone-marrow derived cells in presenting MHC class I-restricted tumor antigens. *Science* **264**:961-965.

8. Kovacsovics-Bankowski, M. and Rock, K.L. (1995) A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* **267**:243-246.

9. Cox, A.L., Skipper, J., Chen, Y., Henderson, R.A., Darrow, T.L., Shabanowitz, J., Engelhard, V.H., Hunt, D.F., and Slingluff, C.L. (1994) Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science* **264**:716-719.

10. Castelli, C., Storkus, W.J., Maeurer, M.J., Martin, D.M., Huang, E.C., Pramanik, B.N., Nagabhushan, T.L., Parmiani, G., and Lotze, M.T. (1995) Mass spectrometric identification of a naturally processed melanoma peptide recognized by $CD8^+$ CTL. *J.Exp.Med.* **181**:363-368,.

11. Yoshino, I., Goedegeboure, P.S., Peoples, G.E., Parikh, A.S., DiMain, J.M., Lyerly, H.K., Gazdar, A.F., and Eberlein, T.J. (1994) HER2/neu-derived peptides are shared antigens among human non-small cell lung cancer and ovarian cancer. *Cancer Res.* **54**:3387-3390.

12. Peoples, G.E., Goedegebuure, P.S., Smith, R., Linehan, D.C., Yoshino, I., and Eberlein, T.J. (1995) Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. *Proc.Natl.Acad.Sci, USA* **92**:432-436.

13. Ioannides, C. G., Fisk, B., Fan, D., Biddison, W. A., Wharton, J. T., and O'Brian, C. A. (1993) Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptide derived from the HER-2/neu proto-oncogene. *Cell. Immunol.* 151:225-234..

14. Fisk,B., Blevins,T. L., Wharton, J. T., and Ioannides C. G. (1995) Identification of an immunodominant peptide of HER-2/neu proto-oncogene recognized by ovarian tumor specific CTL lines. *J.Exp. Med.* **181**:2709-2717.

15. Slingluff, Jr., C.L., Cox, A.L., Henderson, R.A., Hunt, D.F., and Engelhard, V.H. (1993) Recognition of human melanoma cells by HLA-A2.1-restricted cytotoxic T lymphocytes is mediated by at least six shared peptide epitopes. *J. Immunol.* **150**:2955-2963.

16. Tsomides, T.J., Walker, B.D., and Eisen, H.N. (1991) An optimal viral peptide recognized by $CD8^+$ T cells binds very tightly to the restricting class I major histocompatibility complex protein on intact cells but not to the purified class I protein. *Proc.Natl.Acad.Sci. USA* **88**:11276-11280.

17. Udaka, K., Tsomides, T.J., and Eisen, H.N. (1992) A naturally occurring peptide recognized by alloreactive $CD8^+$ cytotoxic T lymphocytes in association with a class I MHC protein. *Cell* **69**:989-998.

18. Wu, M.X., Tsomides, T.J., and Eisen, H.N. (1995) Tissue distribution of natural peptides derived from ubiquitous dehydrogenase, including a novel liver-specific peptide that demonstrates the pronounced specificity of low affinity T cell reactions. *J.Immunol.* **154**:4495-4502,.

19. Kawakami, Y., Eliyahu, S., Sakaguchi, S., Sakaguchi, K., Robbins, P.F., Rivoltini, L., Yanelli, E., Appella, E. and Rosenberg, S.A. (1994) Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J. Exp, Med.* **180**:347-352,.

20. Emmett, M.R., and Caprioli, R.M. (1994) Micro-electrospray mass spectrometry: ultra-high-sensitivity analysis of peptides and protein. *J.Am.Soc.Mass Spectrom.* **5**:605-613.

21. Samson, I., Kerremans, L., Rozenski, J., Samyn, B., Van Beeumen, J., Van Aerschot, A., and Herdewijn, P. (1995) Identification of a peptide inhibitor against glycosomal phosphoglycerate kinase of Trypanosoma brucei by a synthetic peptide library approach. *Bioorganic and Medicinal Chemistry* **3**:257-65,.

22. Griem, P., Wallny, H-J., Falk, K., Rotzschke, O., Arnold, B., Schonrich, G., Hammerling, G., and Rammensee, H-G. (1991) Uneven tissue distribution of minor histocompatibility proteins versus peptides is caused by MHC expression. *Cell*, **65**:633-640.

23. Fisk, B., Chesak, B., Pollack, Wharton, J.T., and Ioannides, C.G. (1994) Oligopeptide induction of a cytotoxic T lymphocyte response to HER-2/neu proto-oncogene *in vitro. Cell. Immunol.* **157**:412-427.

24. Hunt, D.F., Henderson, R.A., Shabanowitz, J., Sakaguchi, K., Michel, H., Sevilir, N., Cox, A.L., Appella, E., and Engelhard, V.H. (1992) Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* **255**:1261-1263.

25. Christinck, E.R., Luscher, M.A., Barber, B.H., and Williams, D.B. (1991) Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. *Nature* **352**:67-70.

26. Miyasaka, H., Choudhury, B.K., Hou, E.W., Li, S.S. Molecular cloning and expression of mouse and human cDNA encoding AES and ESG

proteins with strong similarity to Drosophila enhancer of split groucho proteins. *Eur.J.Biochem.* 216:343-352, 1993.

27. Artavanis-Tsakonas, S., Matsuno, K., Fortini, M.E. Notch signaling. *Science* 268:225-232, 1995.

28. Zagouras, P., Stifani, S., Blaumueller, C.M., Carcangiu, M.L., Artavanis-Tsakonas, S. Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc.Natl.Acad.Sci.USA* 92:6414-6418, 1995.

29. Ellisen, L.F., Bird, J., West, D.C., Soreng, A.L., Reynolds, T.C., Smith, S.D., and Skiar, J. *TAN-1*, the human homolog of the drosophila *Notch* gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 66:649-661, 1991.

Figure 1

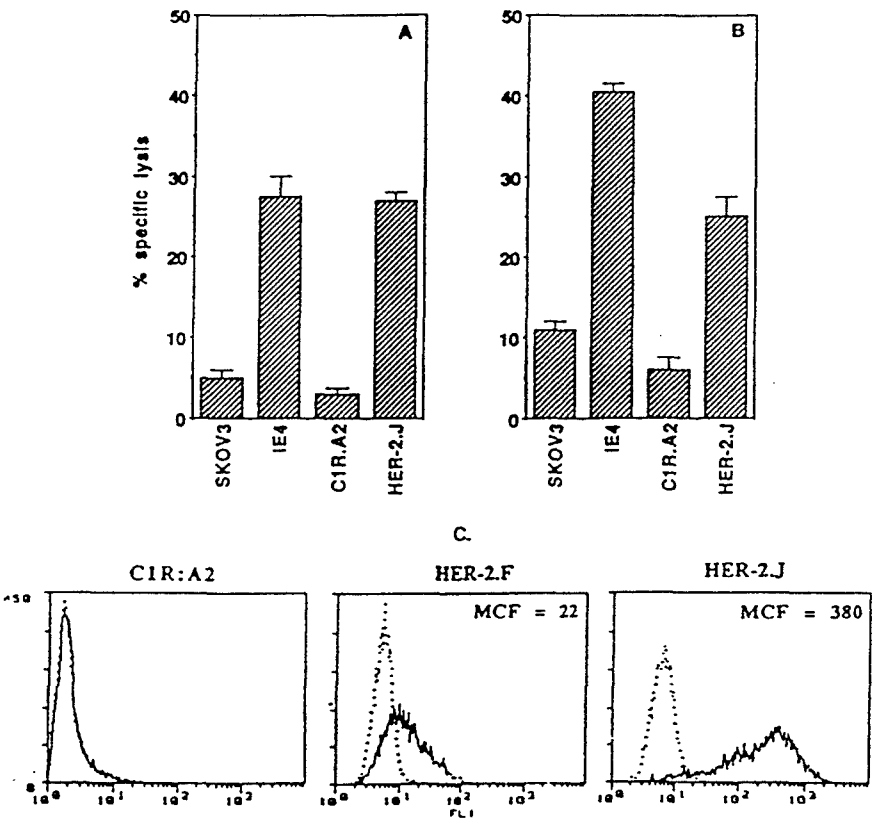

A
B
% specific lysis
50
40
30
20
10
0
SKOV3
IE4
C1R.A2
HER-2.J
C.
C1R:A2
HER-2.F
HER-2.J
MCF = 22
MCF = 380
FL1

Figure 2

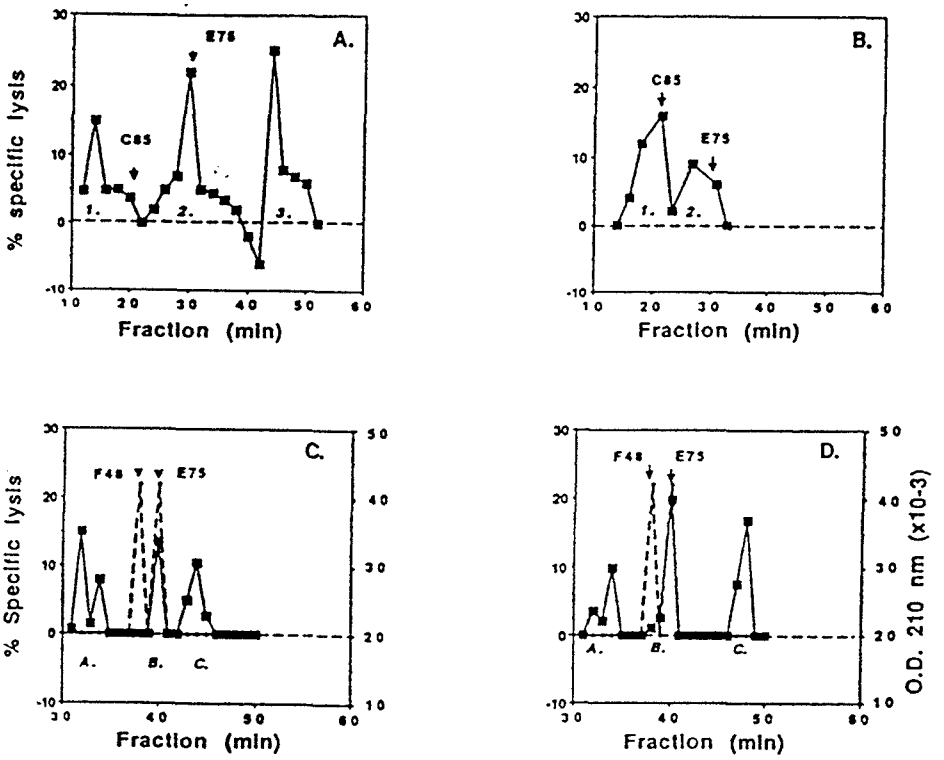

A.
B.
C.
D.
E75
C85
F48
1.
2.
3.
A.
B.
C.
% specific lysis
% Specific lysis
Fraction (min)
O.D. 210 nm (x10-3)

Figure 3

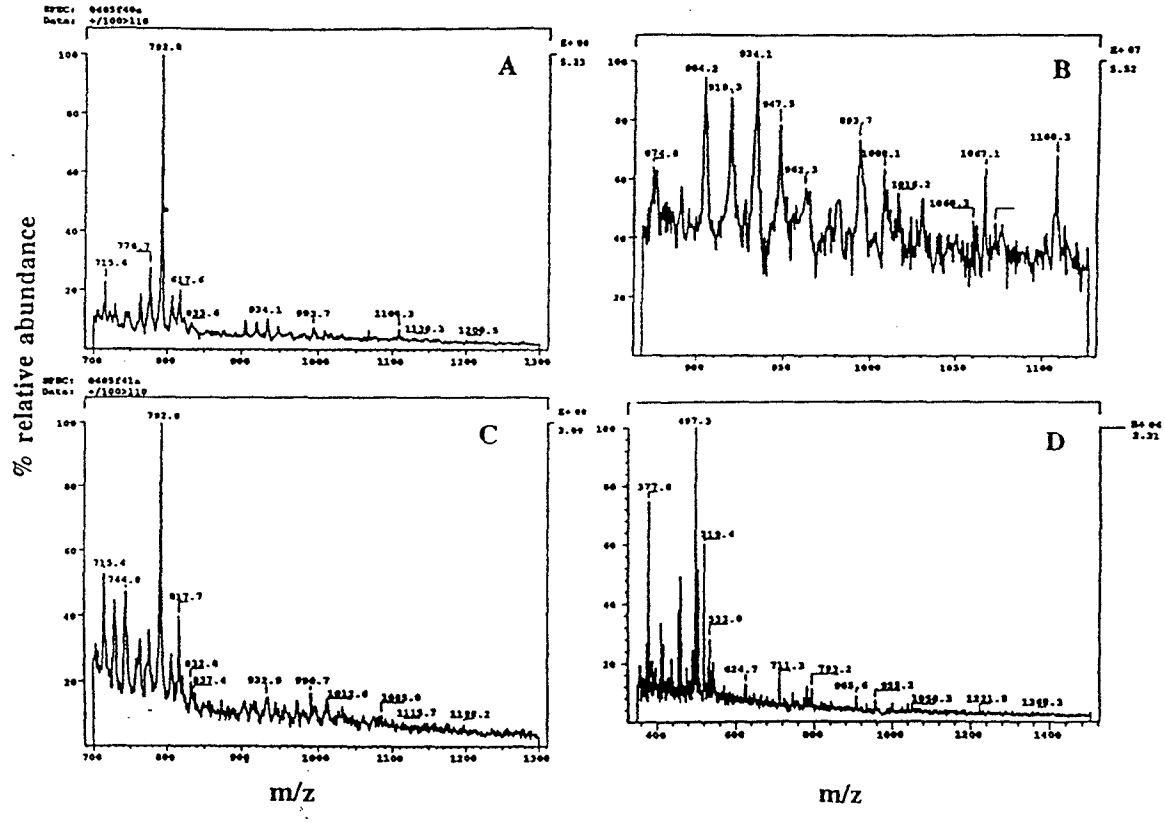


Figure 4

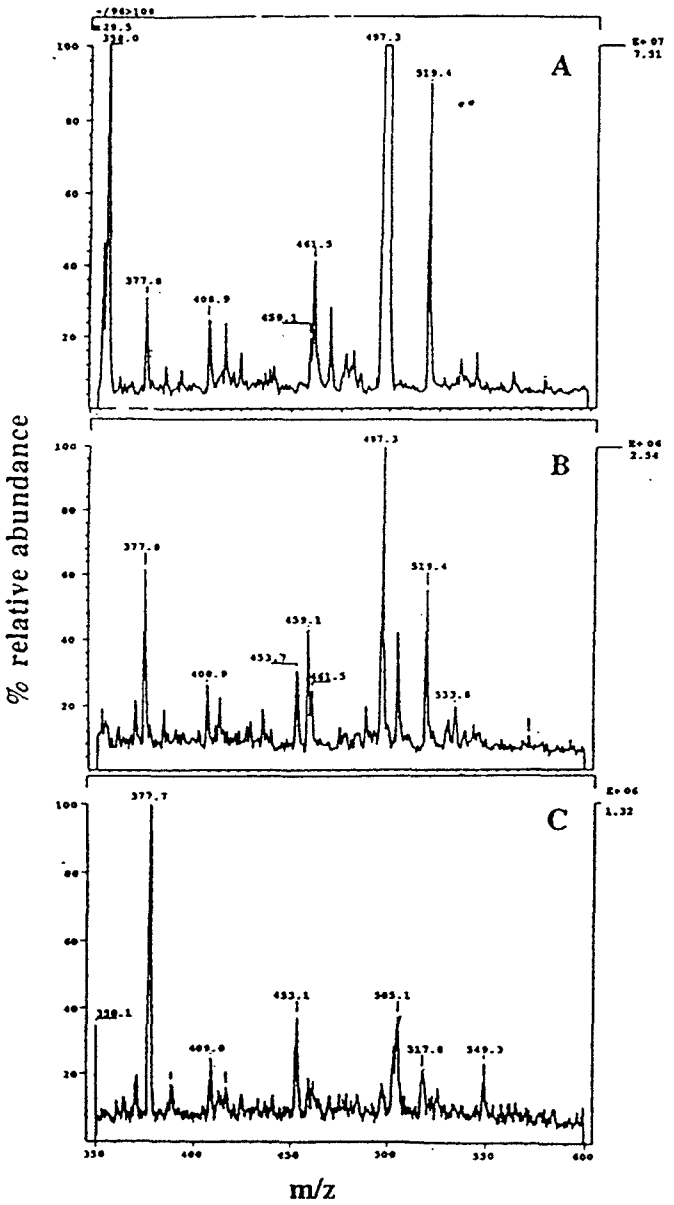


Figure 5

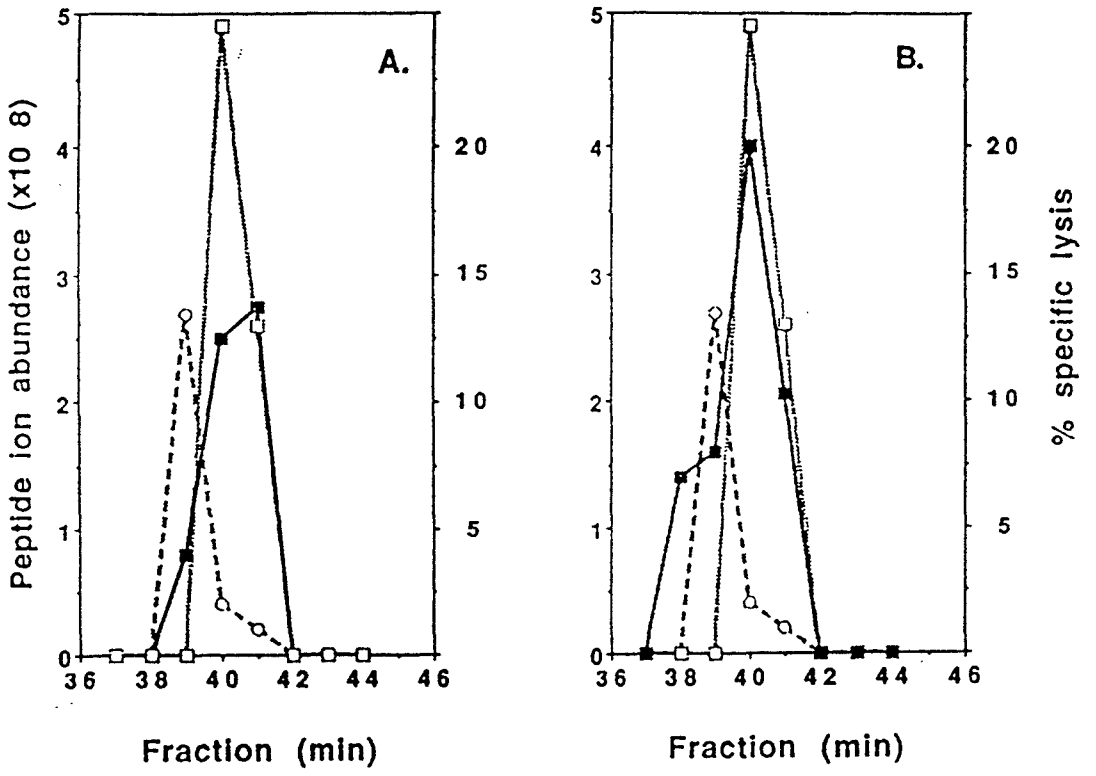
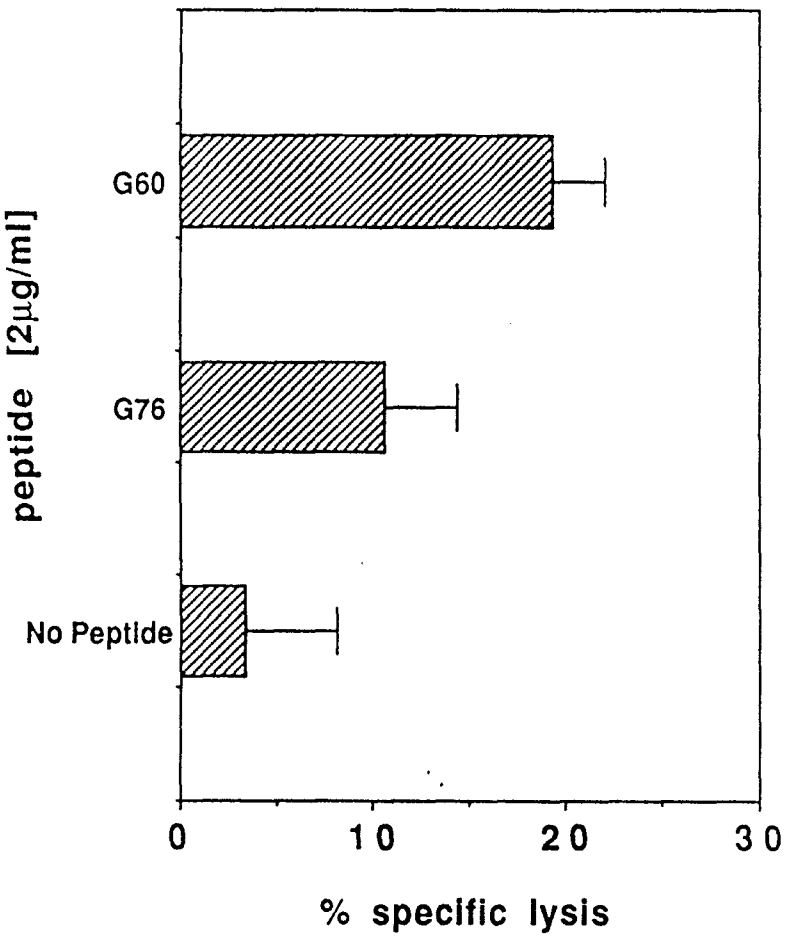
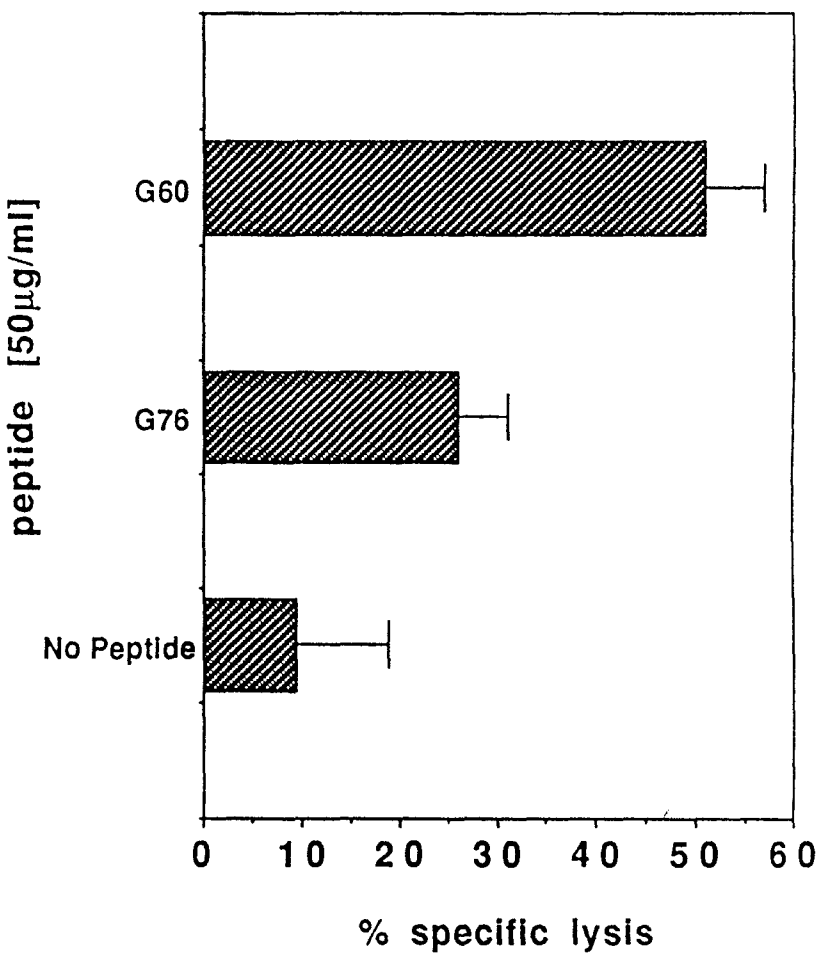

A.
B.
Peptide ion abundance (x10 8)
% specific lysis
Fraction (min)
Fraction (min)
0
1
2
3
4
5
10
15
20
36
38
40
42
44
46

Figure 6



G60 ALPLTPLPV
G76 GPLTPLPV



G60 ALPLTPLPV
G76 GPLTPLPV

Figure 7

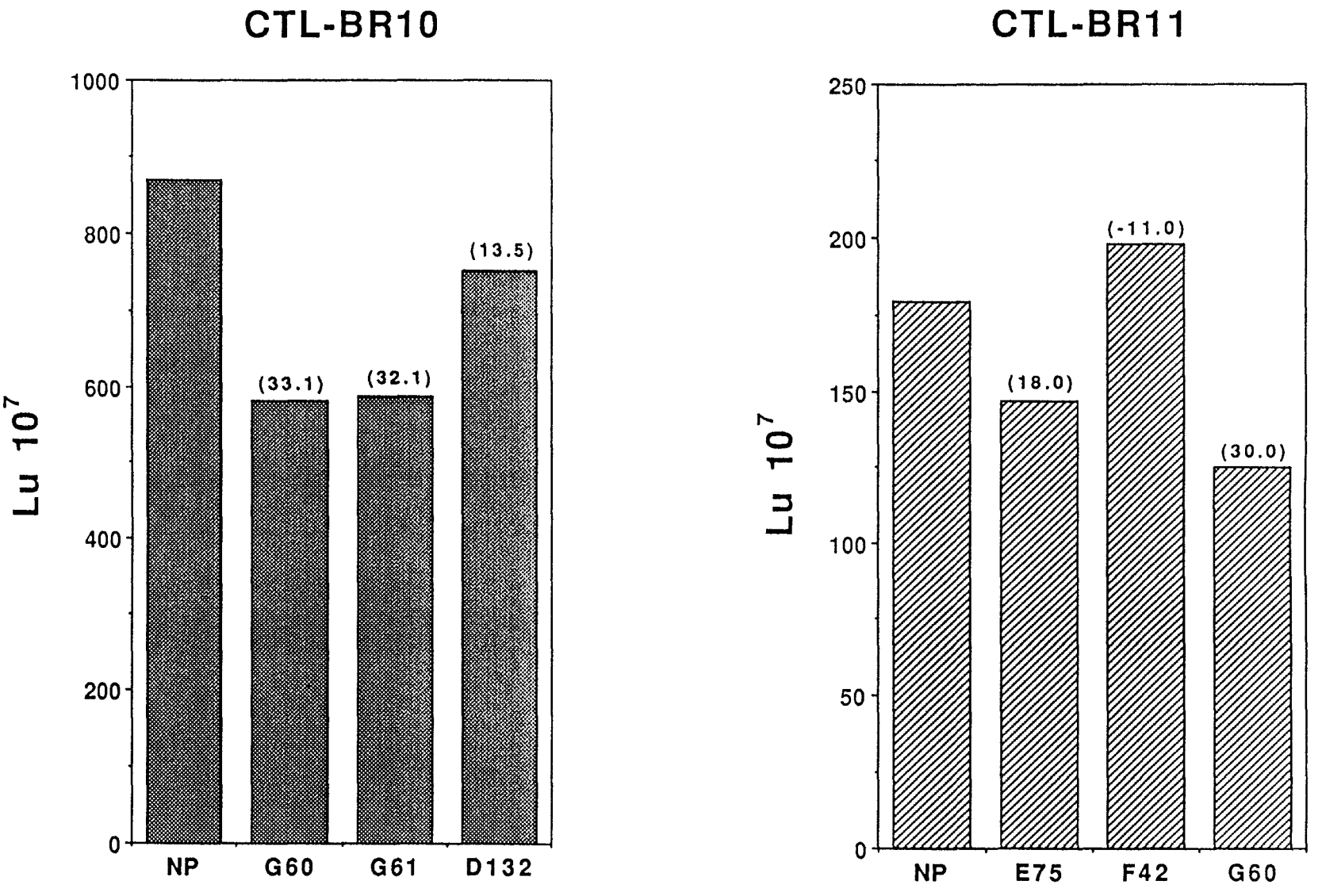

CTL-BR10
Lu 10^7
1000
800
600
400
200
0
(33.1)
(32.1)
(13.5)
NP
G60
G61
D132
CTL-BR11
Lu 10^7
250
200
150
100
50
0
(-11.0)
(18.0)
(30.0)
NP
E75
F42
G60

Table I. Stabilization of HLA-A2 expression by AES peptides.

Peptide Code		Sequence	Conc (μg/ml)	MCF BB7.2	MCF MA2.1
G75	AES-1 (128-135) AES-2 (139-146)	L P L T P L P V	0	60.4	51.0
			50	74.7	
			100	91.8	
G76		G P L T P L P V	50	52.3	42.9
			100	62.2	
G61	AES-1 (125-133)	A L A L P L T P L	100	160.9	
G78	AES-1 (128-137) AES-2 (139-148)	L P L T P L P V G L	0	77.4	
			50	70.7	
			100	92.6	
G77		G P L T P L P V G L	50	69.1	42.7
			100	75.3	
G60	AES-1 (129-135) AES-2 (138-146)	A L P L T P L P V	0	721	51.1
			25	1955	208.3
G61	AES-1 (125-133) AES-2 (136-144)	A L A L P L T P L	25	1213	107.6

Diagram A.1. Results of sequence reconstitution using the PEPSEQ Program.

```
eptide 7

AA  =              9      MOLW  =   791.9922    DATA =      PEPSEQ.DAT
INA =              8      MOLH  =   793.0000    INPT =      PEPSEQ.INP
INP =              5      TOLE  =   (0.7000)    REPO =        #QMB/PC
AXS =    9999.0000        NEND  =     1.0073    IONT :             B:
XP  =    9999.0000        CEND  =    17.0000
   =        1.0000        MAXN  =        999

G:   57.0000   V:   99.0000   X.   113.0000   S.   87.0000   T:   101.0000
P    71.0000   P:   97.0000   A:    71.0070

1:   24.9000   2:   58.0000   3:   123.2000   4   140.5000   5:   155.2000
6:  261.5000   7.  341.9000   8:   351 1000   9   365.9000  10:   465.5000
1:  479.4000  12:  496.7000  13:   513.2000  14:  678.9000  15    775.0000
:   792 7000

IT#                  MOLW        SC      SC.P    

  1  GPVPTXXP   792.0078     1.30     0 26     5   -58.01  -155.01   254.01  -351 01   4
.01  565.01 -678.01   736.02   639.02   540.02   443.02  -342.02   229.02   115.02
  2  GPPVTXXP   792.0078     1.30     0.26     5   -58.01  -153.01   252.01  -351.01   4
.01  565.01 -678.01   736 02   639.02   542.02   443.02  -542.02   229.02   118.02
  3  GPTXPVXP   792.0078     1.71     0.34     5   -58.01  -155.01   256.01  -369.01  -4
 01  565.01 -678.01   736.02   639.02   538.02   425.02   328.02   229.02   115 02
 (4) GPXTPVXP   792 0078     1.71     0.34     5   -58.01  -155.01   268.01  -369.01  -4
.01  565 01 -678.01   736.02   639.02   526.02   425.02   338.02   229.02   115 02
  5  GPTXPXVP   792.0078     1.71     0.34     5   -58.01  -155.01   256.01  -369.01   4
.01  579 01 -678.01   736.02   639.02   538.02   425.02   328.02   215.02   116.02
 (6) GPXTPXVP   792.0078     1.71     0.34     5   -58.01  -155.01   268.01  -369.01  -4
.01  579.01 -678.01   736.02   639.02   526.02   425.02   328.02   215.02   116.02

ECKED  -       33142
T                  6
```

\+ GPVPTXXP
(+) GPPVTXXP
— GPTXPVXP
o GPXTPVXP
— GPTXPXVP
o GPXTPXVP

X = Leu/Ile.

— GPTX P V/X X/V P — Group 1

o GPX T P V/X X/V P — Group 2

(+) GP V/P P/V TXXP — Group 3

Diagram B. Predicted daughter ions of peptide G76: GPLTPLPV.

Seq Name : None
Composition: C38 H64 N8 O10 (G76)
Exact Mass : 792.47461
Bull&Breese: -3460 Charge: 1
Mass Modify: 0.0
N-terminal : Free Amino

Seq Length : 8(0)
Avg Mass : 792.97473
HPLC Index : 53.4
Derivatize : None
C-terminal : Free Acid

No.	Seq	A	B	Bo	D	Y"	Yo	No.	
1	Gly	30.0	58.0 +	40.0 -		75.1	793.5	775.5 +	8
2	Pro	127.1	155.1 +	137.1 +/-		172.1	736.5	718.5 +/-	7
3	Leu	240.2	268.2	250.2 +		285.2	639.4	621.4 +	6
4	Thr	341.2 +	369.2	351.2 +		386.2	526.3	508.3 +	5
5	Pro	438.3	466.3	448.3 +/-		483.3	425.3	407.3	4
6	Leu	551.4	579.4 +/-	561.3 +		596.4	328.2	310.2	3
7	Pro	648.4 +/-	676.4 +	658.4 +		693.4	215.1	197.1 +/-	2
8	Val	747.5 +	775.5 +	757.5 +		792.5	118.1	100.1	1
		2/8 + 1/8 +/-	6/8 + 1/8 ±	5/8 + 2/8 +/-			3/8 + 1/8 ±	3/8 + 2/8 +/-	

Daughter ion matches between predicted values G57, determined values for synthetic peptide G76 and ion 793.

A = 2/8 (+), 1/8 (±)
B = 6/8 (+), 1/8 (±)
B_o = 5/8 (+), 2/8 (±)
Y" = 3/8 (+), 1/8 (±)
Y_o = 3/8 (+), 2/8 (±)

Diagram C. Predicted daughter ions of peptide G57: GPLTPLVP.

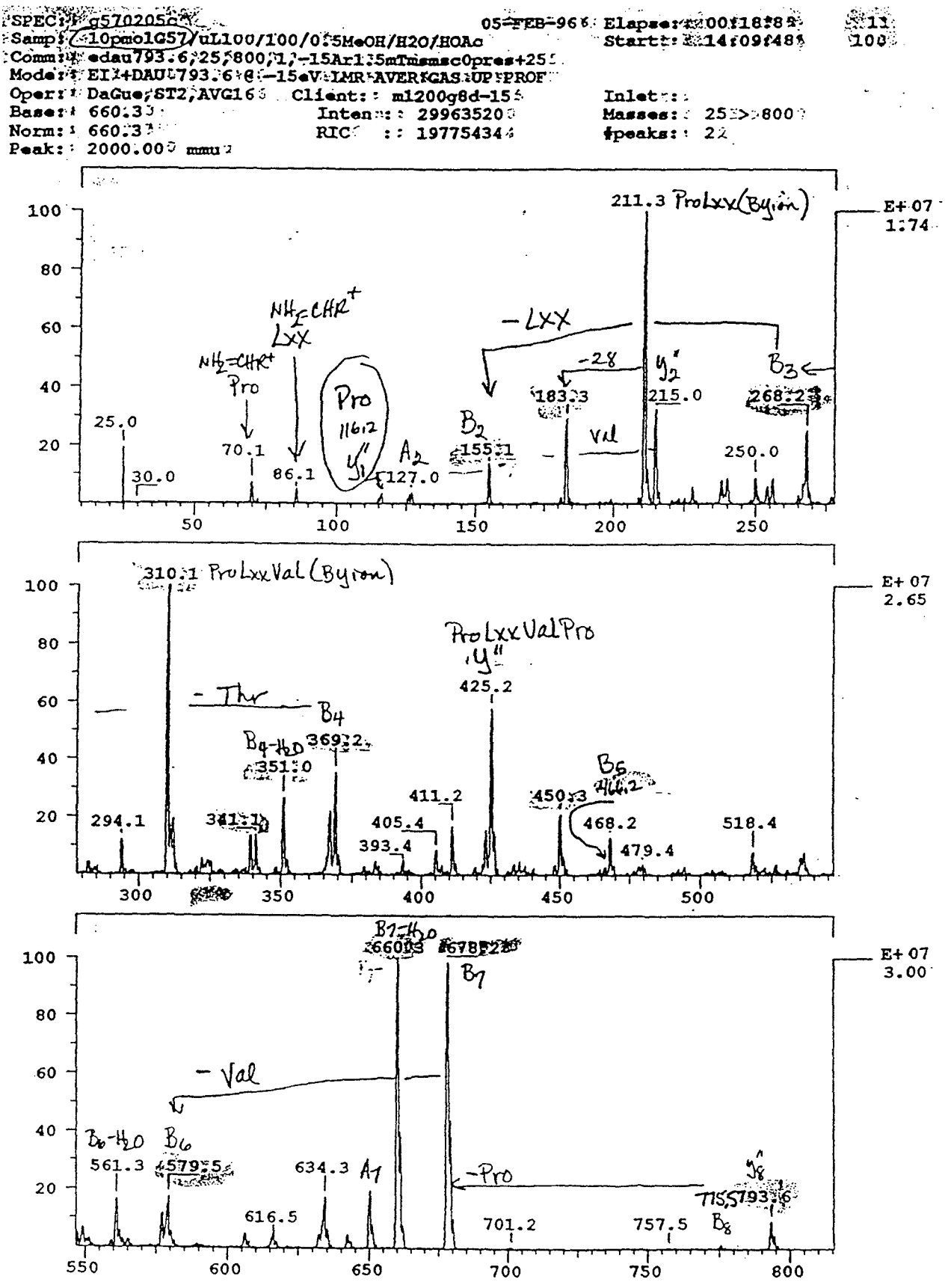
Seq Name :	None	Seq Length :	8 (0)
Composition:	C38 H64 N8 O10		
Exact Mass :	792.47461	Avg Mass :	792.97473
Bull&Breese:	-3460	Charge: 1 HPLC Index :	53.4
Mass Modify:	0.0	Derivatize :	None
N-terminal :	Free Amino	C-terminal :	Free Acid

No.	Seq	A	B	Bo	D	Y"	Yo	No	
1	Gly	30.0	58.0	40.0	75.1	793.5	775.5		8
2	Pro	127.1	155.1	137.1	172.1	736.5	718.5		7
3	Leu	240.2	268.2	250.2	285.2	639.4	621.4		6
4	Thr	341.2	369.2	351.2	386.2	526.3	508.3		5
5	Pro	438.3	466.3	448.3	483.3	425.3	407.3		4
6	Leu	551.4	579.4	561.3	596.4	328.2	310.2		3
7	Val	650.4	678.4	660.4	695.4	215.1	197.1		2
8	Pro	747.5	775.5	757.5	792.5	116.1	98.1		1

58+
155
—
369
466
678
(-) 775
—
6/8

793
—
639
526 —
425 —
328 —
215 +
116 —
—

Diagram D. Resulting B and Y ions from sequencing of synthetic peptide G57.



SPEC: 0605f40c 05-JUN-95 DERIVED SPECTRUM #9
Samp: Ioannides Fr40 rep Start : 13:21:30 192
Comm: 100/100/0.5MeOH/H2O/HOAc, manifold@80,dres ltadd+10V,recal dcal
Mode: EI +DAU 792.5 @-15eV LMR AVER GAS UP PROF
Oper: DaGue,ST1,AVG16 Client: m1600g8d-15 Inlet :
Base: 496.7 Inten : 190319344 Masses: 25 > 1500
Norm: 496.7 RIC : 1370856857 #peaks: 2457
Peak: 1000.00 mmu
Data: +/80>90

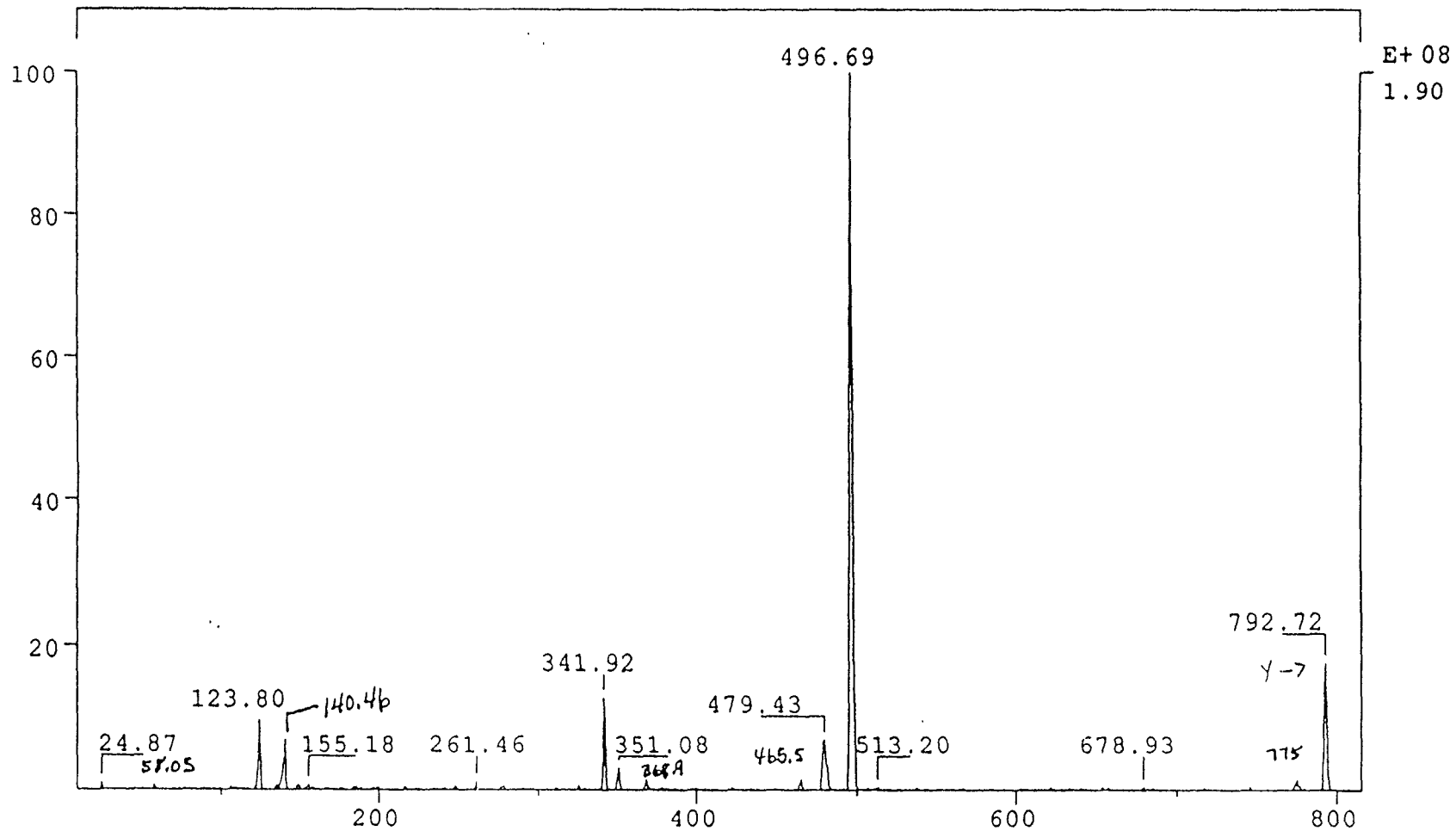
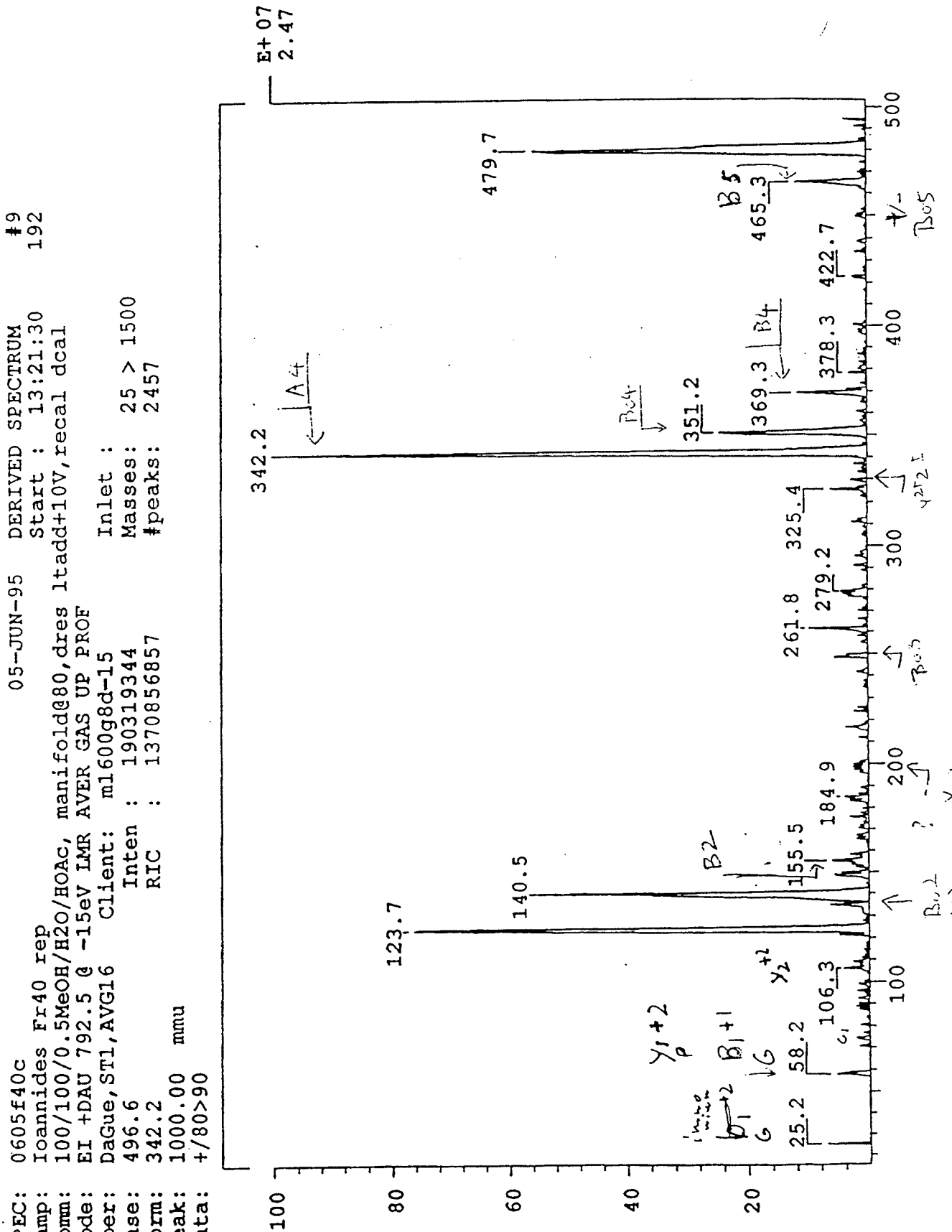


Diagram E.1. MS-MS analysis of ion 793.

E (2)

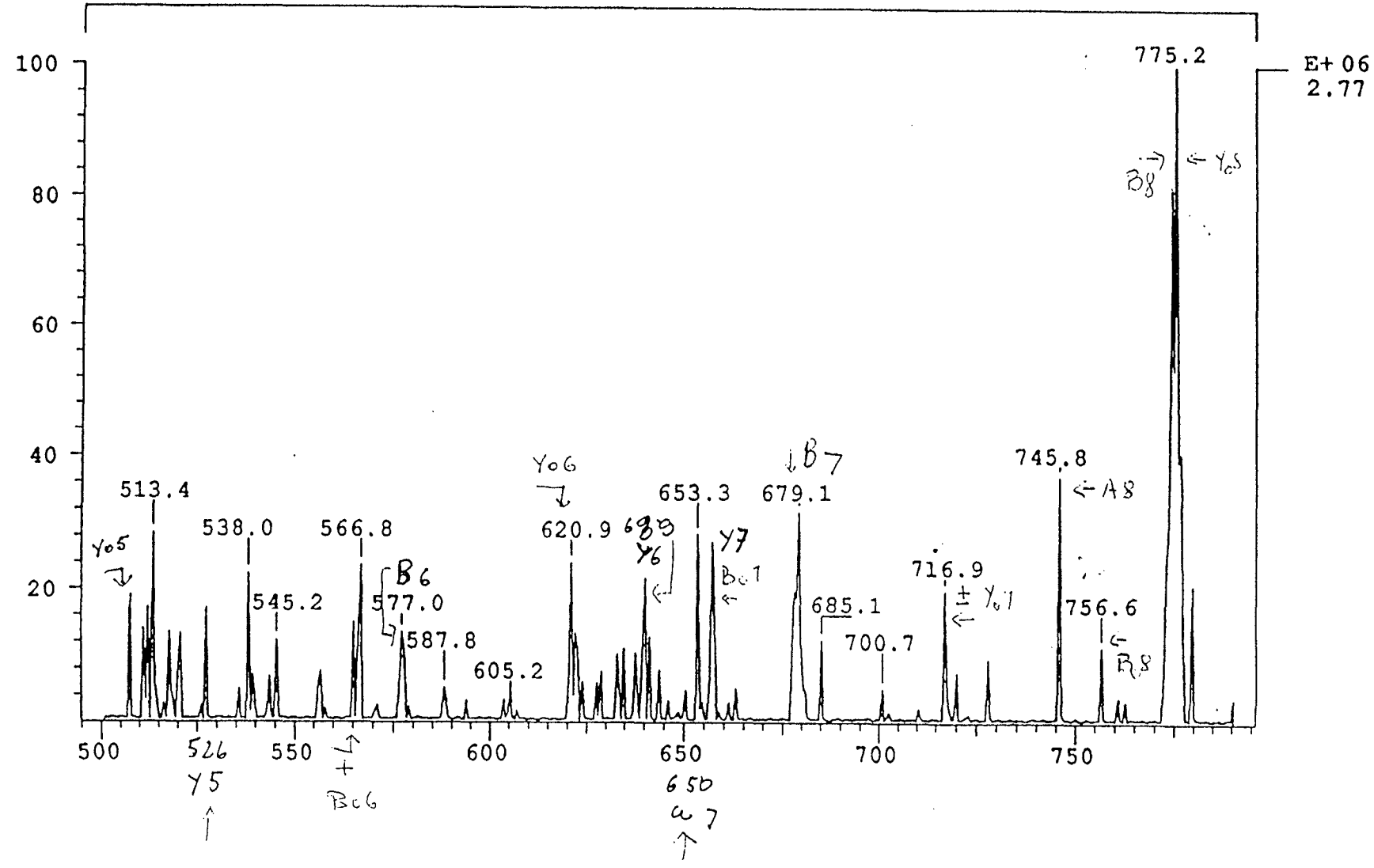
Diagram E.2.



E (3)

Diagram E.3.

SPEC: 0605f40c 05-JUN-95 DERIVED SPECTRUM #9
Samp: Ioannides Fr40 rep Start : 13:21:30 192
Comm: 100/100/0.5MeOH/H2O/HOAc, manifold@80,dres 1tadd+10V,recal dcal
Mode: EI +DAU 792.5 @ -15eV LMR AVER GAS UP PROF
Oper: DaGue,ST1,AVG16 Client: m1600g8d-15 Inlet :
Base: 496.6 Inten : 190319344 Masses: 25 > 1500
Norm: 775.2 RIC : 1370856857 #peaks: 2457
Peak: 1000.00 mmu
Data: +/80>90



Phase Ib Trial of HER-2/*neu* Peptide (E75) Vaccine in Patients with Breast and Ovarian Cancer

STUDY DIRECTOR: Dr. James L. Murray

SPONSOR: Kenneth Grabstein, Ph.D.
Vice President, Director of Immunology
Corixa Corporation
1124 Columbia Street, Suite 464
Seattle, Washington 98104

STUDY SITE: M.D. Anderson Cancer Center, Houston, Texas

Phase Ib Trial of HER-2/*neu* Peptide (E75) Vaccine in Patients with Breast and Ovarian Cancer

THE UNIVERSITY OF TEXAS
M. D. ANDERSON CANCER CENTER
DIVISION OF MEDICINE

STUDY CHAIRMAN: James L. Murray, M.D.

1.0 OBJECTIVES
2.0 BACKGROUND
3.0 PRODUCT INFORMATION
4.0 PATIENT SELECTION
5.0 STUDY PLAN
6.0 PATIENT MONITORING
7.0 TOXICITY EVALUATION
8.0 DETERMINATION OF IMMUNOLOGIC ENDPOINTS AND OPTIMAL BIOLOGIC DOSE (OBD)
9.0 RESPONSE CRITERIA
10.0 CRITERIA FOR REMOVAL FROM STUDY
11.0 STATISTICAL ANALYSIS
12.0 REFERENCES

Appendix A:
Appendix B:
Appendix C:
Appendix D:

STUDY CHAIRMAN:

_____
James Lee Murray, M.D.

STUDY CO-CHAIRMEN:

Dr. Constantin G. Ioannides

Dr. Dena Rahman

COLLABORATORS:

Dr. Gabriel N. Hortobagyi

Dr. John Kavanagh

Dr. Aman Buzdar

Dr. J. Taylor Wharton

Dr. Daniel Booser

Dr. Andrew Kudelka

Dr. Robert Bast

Dr. Claire Verschraegen

Dr. Ronald Walters

Corixa Coporation

Dr. Kenneth Grabstein

Protocol Abstract

Phase Ib Trial of HER-2/*neu* Peptide (E75) Vaccine in Patients with Breast and Ovarian Cancer

STUDY CHAIRMAN

James Murray, M.D.

OBJECTIVES

1. The main objective is to perform a Phase Ib feasibility trial, of HER-2/neu peptide, vaccine E75 combined with GM-CSF in ovarian and breast cancer patients with HER-2+, HLA-A2+ tumors. The objectives of this trial are:
   a. To assess safety and immunological efficacy of synthetic HER-2 peptide E75 plus GM-CSF. Immunological parameters include:
      1. Measurement of MHC restricted (CTL) T-cell generation prior to and following vaccination.
      2. Measurement of antibodies to E75.
      3. Measure T-cell proliferation in response to peptide.
      4. Measurement of delayed type hypersensitivity (DTH) responses to peptide.
      5. Th1 and Th2 cytokine profiles in culture supernatant (i.e. IFNγ, TNFα, IL4, IL10).
   b. To document the local and systemic toxicity of E75 + GM-CSF.
   c. To determine the maximum tolerated dose (MTD) and/or optimal biologic dose. (OBD)to peptide, if feasible.
   d. To evaluate anti-tumor activity, if any,

In this initial trial we have selected GM-CSF because of the preclinical as well as clinical evidence that this cytokine can augment immune responses to vaccines (see Background and Preliminary Results). GM-CSF has also been shown to promote expansion and differentiation of dendritic cells and to enhance presentation of Class I antigens and accessory cells on tumors.

RATIONALE

Previous in vitro and in vivo studies have shown that peptides derived from the intra and extracellular domains of HER-2/neu are immunogenic. Recent studies using peptides combined with GM-CSF have shown promise. Hence, there is a need to determine whether E75, a high affinity HER-2/neu peptide combined with GM-CSF is immunogenic in man.

PATIENT ELIGIBILITY

Patients are eligible for enrollment into this study if they meet the following criteria:

1. Patients who have HER-2/neu, HL-A2+ histologically proven breast or ovarian cancer who have not received more than two prior chemotherapy or immunotherapy regimens excluding (adjuvant chemotherapy) for metastatic disease.
2. Patients who have not received interventional chemotherapy or immunotherapy within four weeks prior to entry into the study.
3. Patients must have a performance status of ≤ 1 on the ECOG scale or >70% on the Karnofsky scale (APPENDIX D).
4. Patients must have reached 18 years of age.
5. Patients must have an expected survival of at least 3 months.
6. Patients must have evaluable or measurable disease defined as bidimensionally measurable on radiological examination such as CT or MRI or clinically measurable by two examiners independently.
7. Patients must either:
   i. Have evidence of loco-regional relapse either at the primary site, or on the adjacent chest wall or in regional lymph nodes, (breast or peritoneum ovary). OR
   ii. Have measurable (clinically or radiologically) small volume metastatic disease at other sites (e.g., a solitary pulmonary metastasis). Ovarian cancer patients are also eligible if they have non-measurable disease with elevated CA125 level.

TREATMENT PLAN

Provided patients are immunocompetent and have tumors which are HER-2/*neu*+ and HLA-A2+ they will be scheduled to receive vaccine. Prior to starting therapy, patients will also have the following tests performed.

1. CBC, platelet count, urinalysis, blood for HIV testing, blood chemistries.
2. Documentation of metastatic disease. Patients with recurrent skin lesions will have photographs taken to monitor response.
3. Immunologic tests: in addition to screening tests mentioned above, patients will have skin tests to > recall antigens (Merieaux multitest) as well as to peptide E75 (see Appendix). Sixty ml of peripheral blood will be drawn, peripheral blood mononuclear cells will be separated by Hypaque-gradients and frozen for later antigen proliferation assays and cytotoxicity tests. Ten cc of serum will also be saved for analysis of HER-2/*neu* specific antibody titers.

STATISTICAL CONSIDERATIONS

Groups of 3 patients will be treated with various dose levels of vaccine mixed with GM-CSF. If one instance of Grade 3 or 4 toxicity are observed at a given dose level a total of six patients will be placed on that dose level. If three or more instances on Grade 3 or 4 toxicity are observed during the course of treating patients on a dose level accrual onto the trial will be terminated and next lower dose will be declared the MTD. In all other cases, dose escalation will proceed. It is estimated that from 9-15 patients will be entered on study. Based on an accrual rate of 1 patient per month (based on a 30%-50% predicted ineligibility for patients who are HER-2, HLA-A2 negative), the study should take from 9-18 months to complete. If there are no immunologic responses detected in 15 patients then the study will be terminated

STATISTICAL CONSIDERATIONS (cont)

and we will conclude with 95% power and 95% confidence that the vaccine is able to produce appropriate responses in 20% of patients. Differences in pre and post-immunization studies as well as differences between dose levels will be determine by ANOVA, CH1- square analysis as well as paired t-tests and t-test for independent variables. Appropriate regression analyses may be used to study the relationship, if any between immunologic findings and clinical outcomes. It is possible that the MTD or OBD will not be determined in this trial.

PATIENT EVALUATION

Patients will have toxicity assessment and physical exam 24 hours after injection. CBC, platelet count, serum chemistries, will be performed weekly x 4 then monthly to assess toxicity. Patients will have PE, x-rays or scans to document disease status at 8 weeks.

Immunologic Monitoring

At one month and 2 months after beginning treatment patients will have from 60-80 cc of blood drawn for measurement of PBMC proliferation to vaccine, CTL responses to peptides, and measurement of TH_1 and TH_2 cytokines in supernatants (see appendix for details). In addition, 10 cc of serum will be stored for later analysis of anti-HER-2 antibodies. Patients will have recall skin tests as well as skin testing for E75 performed at 8 weeks.

Selected patients with ≥ 1 cm tumor nodules accessible for biopsy will have nodules removed, processed; and analyzed for presence of peptide using chromatography and mass spectroscopy.

ESTIMATED ACCRUAL

It is estimated that from 9-15 patients will be studied in this trial.

SITE OF STUDY

M.D. Anderson Cancer Center

LENGTH OF STAY

N/A

RETURN VISITS

One weekly x 4 weeks, then monthly.

HOME CARE

N/A

WHERE WILL STUDY BE CONDUCTED

M.D. Anderson Cancer Center

NAME OF SPONSOR/FUNDING SOURCE

Corxia Corporation

COMPETING PROTOCOLS

a) Phase I trial of E1A gene therapy
b) Chemotherapy protocols

NAME OF RESEARCH NURSE

Hannah Brewer, RN

1.0 *OBJECTIVES*

1.1 The main objective is to perform a Phase Ib feasibility trial of HER-2/neu peptide vaccine E75 combined with GM-CSF in ovarian and breast cancer patients with HER-2+, HLA-A2+ tumors. The specific objectives of this trial are:

a. To assess safety and immunological efficacy of synthetic HER-2 peptide E75 plus GM-CSF. Immunological parameters include:

1. Measurement of MHC restricted cytotoxic T-cell (CTL) T-cell generation prior to and following vaccination.
2. Measurement of antibodies to E75.
3. Measure T-cell proliferation in response to peptide.
4. Measurement of delayed type hypersensitivity (DTH) responses to peptide.
5. Th1 and Th2 cytokine profiles in culture supernatant (i.e. IFNγ, TNFα, IL4, IL10).

b. To document the local and systemic toxicity of E75 + GM-CSF.

c. To determine the maximum tolerated dose (MTD) and/or optimal biologic dose (OBD) to peptide ± GM-CSF, if feasible.

d. To evaluate anti-tumor activity, if any. In this initial trial we have selected GM-CSF because of the preclinical as well as clinical evidence that this cytokine can augment immune responses to peptide vaccines (see below). GM-CSF has also been shown to promote expansion and differentiation of dendritic cells and to enhance presentation of Class I antigens and accessory cells on tumors.

2.0 BACKGROUND

2.1 Patient Population

Breast cancer is the most common malignancy in females in this country and accounts for approximately 18% of all cancers in women. It is also the second most common cause in women with an estimated 43,000 deaths per year. Although survival figures appear to have improved for patients with localized disease at presentation with the advent of adjutant chemotherapy, the long-term outlook for patients with advanced, recurrent, or metastatic disease remains poor. Indeed, no curative treatment regimens have been developed for these types of patients. With this in mind, there is an obvious need for newer modalities of treatment to be used separately from or in combination with current modalities.

Treatment of ovarian cancer, the third most common cancer in women, is also problematic. In the United States, 20,000 women present annually with ovarian cancer. Of these, 12,500 will eventually die from cancer. The diagnosis, unfortunately, is usually made late in the course of the disease and the current chemotherapy following cytoreductive surgery is inadequate to totally eradicate tumor in the majority of patients. With the exception of recent data suggesting a survival advantage of a combination of platinum and Taxol chemotherapy (1), the incorporation of cisplatinum and other platinum analogues with other agents has increased the response rate but has not significantly influenced long term survival.

Although many strategies for salvage therapy following platinum based treatment have been utilized, none has demonstrated substantial response rates or significant prolonged duration of response. Hence, it is critical to test other agents in this disease particularly in the minimal disease and adjuvant setting.

2.2 Tumor Specific Vaccines

2.2.1. Recently there has been considerable interest in the development of tumor specific vaccines (2-5). This resurgence of interest has been mostly due to a better understanding of the immune system along with the identification of T-cell specific antigens in several cancers. The identification of specific tumor associated antigens/peptides associated with malignant transformation have been defined or are currently being defined using immunologic and molecular biology techniques.

The pioneering studies of Boon and colleagues in identifying the MAGE series of T-cell specific tumor antigens recognized in melanoma and breast cancer (6) has generated excitement and given impetus to defining strategies to identify and clone novel peptide antigens in melanoma (7) and other tumors (8,9). These studies have been possible either through identification of target genes encoding for specific epitopes from which candidate peptides can be synthesized and tested for CTL response (6) or through biochemical procedures such as acid elution of peptides from MHC Class I (10,11) followed by reverse phase HPLC fractionation and sequencing (12,13). In order to be recognized by the immune system, antigens must be discriminated from self. In this context, the majority of immunogenic proteins/peptides identified have either been products of mutated oncogenes or suppressor genes, (i.e. ras, p53) (14), developmental antigens reexpressed during tumorigenesis (i.e. MAGE 1,2,3) (6) oncofetal proteins or proto-oncogenes which are presumably recognized because of their overexpression concentration on tumor compared to normal tissues (CEA, HER-2) (15,16) or lineage specific differentiation antigens (Tyrosinase, gp100, MART-1 or Melan-A) (17-22). Since the above are found in much lower concentrations on normal tissues, it must be presumed that a state of tolerance existed at some point which, through mechanisms which are currently unclear, could be broken in the presence of sufficient concentrations of antigen. Alternatively, epitopes presented when the total protein concentration in tumor is between 40-200 fold above the levels of expression in normal tissues may present novel epitopes that are not expended on normal cells, and/or "cryptic"

epitopes which may be presented by CTL by antigen presenting cells (APC) after phagocytosis of tumor particles (23). Indeed, immune recognition for melanocyte differentiation antigens has been confirmed by the prevalence of autoreactive, high affinity IgG antibodies against gp75, the antigen homologue to the Brown locus in melanoma (24) and the discovery of peripheral blood or tumor infiltrating lymphocytes from responding melanoma patients which demonstrated CTL activity against allogeneic tumors transfected with either Melan-A or gp100 genes (18,25). The discovery of specific CTL activity against normal differentiation antigens without definite evidence of autoimmunity against normal tissues has generated a host of questions regarding the nature of tolerance and the mechanisms responsible for CTL induction against a number of these antigens in different tumors.

2.2.2. Evidence for Existent Immunity Against the HER-2/neu Proto-Oncogene

Studies have shown that malignant transformation results from mutations of genes related to normal cell growth or overexpression of oncoproteins through abnormal concentration of "wild type" genes. An example of the latter is the proto-oncogene HER-2/neu, which is expressed to a varying extent on epithelial malignancies (ovary, breast, lung, colon, and squamous cell carcinoma). Studies from Dr. Martin Cheever's group as well as Dr. Ioannides and others have demonstrated preexisting T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer (26,27) and ovarian cancer (9) . The HER-2/neu proto-oncogene (HER-2) encodes for a 185 KD transmembrane receptor which has homology with the epidermal growth factor receptor (28). In the rat, HER-2 is activated by a transforming point mutation in the transmembrane domain (28). In human breast cancer, no point mutations of the gene have been found, implying that transforming activity is related to overexpression of protein. Overexpression of protein has been identified in numerous epithelial neoplasms and has been associated with poor prognosis in breast cancer patients (29). The selective overexpression of HER-2 by malignant cells coupled with the evidence for existing immunity against the protein make it a possible target for T-cell and antibody-mediated immunotherapy approaches (30).

Ioannides et al. (16) and subsequently Yoshino et al. (31) were the first to demonstrate the association of HER-2 expression with sensitivity to tumor specific CTL in ovarian cancer. Ioannides went one step further by demonstrating that CTL expanded from tumor associated lymphocytes (TAL) from HLA-A2+ and HER-2/neu+ tumors could specifically recognize synthetic peptides corresponding with amino acids 971-980 of the HER-2/neu protein (32). The sequences recognized contained a potential amphipathic area which contained both Rothbards's epitope motifs (34) and HLA-A2 anchor residues (35). These studies were verified by additional data from Cheever's group (36), who

synthesized 4 peptides with amino acid motifs similar to that recognized by HLA-A2- binding peptides. Two of the peptides were shown to elicit peptide specific CTL by primary immunization in a culture system using peripheral blood mononuclear cells (PBMC) from a *normal* individual homozygous for A2. This has also been confirmed by Ioannides' group, in which peptide (CBS/HER-2, 971-979) induced CTL from normal individuals recognized and lysed HER-2 ^{high} tumors (37). Studies by Lustgarten in mice transgenic for HLA-A2 confirms that HER-2/neu specific peptide E75 is recognized (38). Moreover, CTL clones isolated from ovarian cancer patients have demonstrated significant CTL activity against other HER-2/neu expressing tumors (39). These data give credence for the use of specific HER-2 peptides as cancer vaccines to induce tumor specific HLA-A2 restricted CTL in patients with HER-2+ tumors.

2.2.3. Identification of an Immunodominant Peptide (E75) of HER-2/neu Proto-oncogene in Ovarian and Breast Cancer

Previous (32) as well as ongoing studies have identified a nanopeptide E75 (KIFGSLAFL) which was efficient in sensitizing T2 cells for lysis by 6/7 ovarian carcinoma lines as well as 3/3 breast cancer lines. HER-2 and control peptides were synthesized using the synthetic antigen laboratory at the M.D. Anderson Cancer Center using a solid phase method and purified by HPLC. For E75 mass spectrometry (MS) was employed to identify the unique mass numbers of the single charge (1^+) ions (995.4) and double charged (2+) ions (M/2 ratio: 498.4) and the pattern of b and y ions. Final peptide structure was identified by amino acid analyses. Nineteen peptides were identified from the HER-2 sequence which contained specific HLA-A2 anchor motifs (Leu/Ile at position 2(P2); Val/Leu/Met at positive 9 (P9). Peptides were selected from signal, extracellular, transmembrane and cytoplasmic domains of HER-2. Priority was given to peptides from regions 364-474 and 781-859 because they contain the highest density of continuous and overlapping epitopes with HLA-A2.1 binding motifs.

Peptides were selected based on their ability to bind to MHC Class I in an HLA-A2 stabilization assay (40) using T2 cells defective in the normal antigen processing pathway, thereby expressing HLA-A2 binding to signal peptides (41). Following addition of peptide to T2 cells they were stained with FITC-conjugated W6/32 (anti-Class I monomorphic) and BB7.2 (anti-HLA-A2 α-2 domain) Mab and analyzed for mean channel fluorescence (MCF) using flow cytometry.

Based on the above tests, 9 of 19 nonamers induced a greater than two-fold increase in MCF for HLA-A2 expression, using BB7.2 Mab compared to a negative control peptide C61. Three peptides (E75, E90 & E89) demonstrated MCF values of 131, 164 and 82 respectively,

with a range of peptide concentrations from 1-50 μg/ml. Ten other peptides had low stabilizing ability for HLA-A2 (Table 1).

Table 1. *Cell surface expression of BB7.2 epitope on HLA-A2.1 of T2 cells by HER-2 peptides.*

Code	Position	Sequence (1 2 3 4 5 6 7 8 9 10)	BB7.2 MCF*	BB7.2 Rank+	W6/32 MCF	W6/32 Rank
HER-2 PEPTIDES						
E91	5-13	A L C R W G L L L	82	9	306	8
D97	42-49	H L D M L R H L	52	12	167	18
D113	48-56	H L Y Q G C Q V V	155	2	496	2
E75	369-377	K I F G S L A F L	131	3	474	4
E77	391-399	P L Q P E Q L Q V	61	10	216	11
E76	402-410	T L E E I T G Y L	109	6	358	5
E78	457-465	S L R E L G S G L	60	11	208	12
E93	466-474	A L I H H N T H L	113	5	293	9
E92	650-658	P L T S I I S A V	128	4	324	6
E88	689-697	R L L Q E T E L V	109	7	481	3
E70	793-801	T V Q L V T Q L M	35	18	172	17
E90	789-797	C L T S T V Q L V	164	1	515	1
E71	799-807	Q L M P Y G C L L	42	16	173	15
E72	828-836	Q I A K G M S Y L	32	19	166	19
E73	835-842	Y L E D V R L V	51	13	234	10
E74	838-846	D V R L V H R D L	36	17	203	13
E89	851-859	V L V K S P N H V	82	8	310	7
C85	971-979	E L V S E F S R M	47	14	194	14
D99	1089-1098	D L G M G A A K G L	44	15	172	16
CONTROL PEPTIDES††						
HER-2						
C81	971-979	E L V S E V S K V	76		261	
C61	968-977	R F R E L V S E F S	37		182	
FOLATE BINDING PROTEIN						
E38	112-120	N L G P W I Q Q V	77		N.D.	
E37	25-33	R I A W A R T E L	34		N.D.	
E41	245-253	L L S L A L M L L	38		N.D.	
NO PEPTIDE			34		172	

* Mean channel fluorescence (MCF) corresponding to the peak of fluorescence for T2 cells preincubated with 50μ g/ml of each peptide was determined for all peptides in the same experiment as described in the Materials and Methods. MCF for both W6/32 and BB7.2 are presented and compared to confirm the increase in MHC class I heavy chain expression.

\+ Peptides are ranked in decreasing order of their ability to increase HLA-A2.1 expression.

†† The variant peptide of C85 containing three substituted residues F->V(P6), R->K(P8), and M->V(P9) was used as positive control because the resulting variant (C81), contains four dominant and strong anchors residues (10,11) reported to favorize binding to HLA-A2. The peptide C61 (HER-2: 968-977) contains HLA-B8 anchors and was used as negative control.

Folate binding protein (FBP) peptides, were selected from the FBP sequence based on the concordance of T cell epitopes predicted by the computer program, ANT.Find.M (3), and the presence HLA-A2 specific anchor motifs (10).

2.2.3.1. Recognition of E75 Peptide by Ovarian and Breast Tumor Reactive CTL

Seven $CD8^+$ CTL lines generated from ovarian tumor associated lymphocytes (TAL). TAL were tested for cytotoxic activity against HLA-A2+ autologous tumors. As shown in Table 2, 4 HLA-A2+ CTL lysed their respective HLA-A2+ tumors (autologous and allogeneic), but not A2 negative tumors or K562 (NK sensitive). To determine which peptides are recognized by CTL ^{51}Cr-labeled T2 cells were incubated with each of the 19 peptides and $CD3^+$ $CD8^+$ CD4- CTL were added. Percentage of specific lysis for 4/10 HLA-A2+ CTLs is shown in Figure 1A. As noted, E75 peptide was immunodominant, resulting in significant lysis by all CTL's tested. Similar results were seen with 2 breast cancer tumor (Figure 1B). Cytotoxicity was peptide concentration dependent. (Figure 1C).

Table 2. *Recognition of HLA-A2+ Tumors by Ovarian Specific CTL Lines.*

	% Specific Lysis*			
Targets†	CTL-1	CTL-2	CTL-3	CTL-4
Auto-T (HER-2+,A2+)	47	65	28	41
Allo-T (HER-2+,A2+)	40	41	14	38
SKOV3.A2.1E4 (HER-2+,A2+)	45	39	42	84
Allo-T (HER-2-,A2-)	5	15	N.T.	3
2774 (HER-2-,A2-)	0	0	0	4
K562 (HER-2-,A2-)	1	3	3	4

* Percent specific lysis is shown for an effector to target ratio of 20:1. Target lysis was determined in a 5h ^{51}Cr release assay.

† Auto-T and Allo-T represent autologous and allogeneic freshly isolated ovarian tumors. SKOV3.A2.1E4 is an ovarian tumor clone expressing HLA-A2. 2774 is a human ovarian tumor line. N.T. not tested.

Figure 1 A

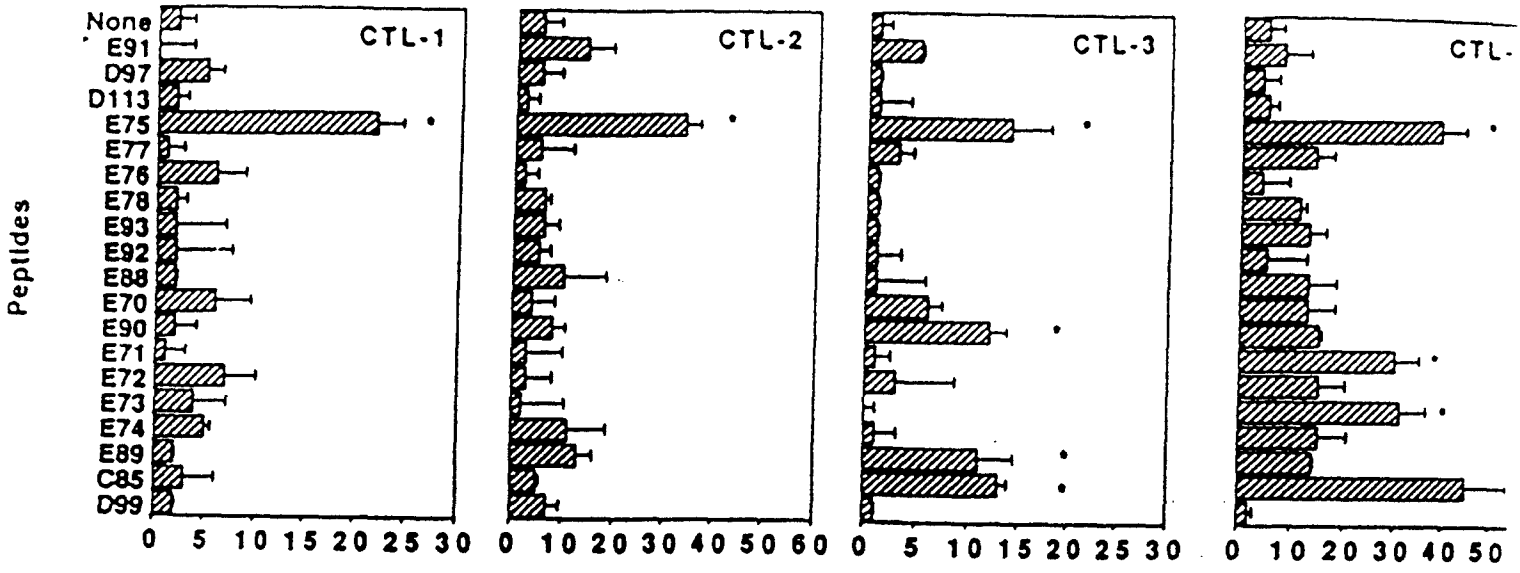


Figure 1A Recognition of HER-2 peptides by $CD3^+CD8^+CD4^-$ CTL isolated from four different ovarian cancer patients. Cytotoxicity was determined using T2 cells preincubated for 60 min with each peptide at 25 μg/ml in a 5h ^{51}Cr release assay. % specific lysis is shown for all CTL lines for an effector: target ratio of 20:1 % specific lysis was calculated as described in Materials and Methods. *Indicates mean cytotoxicity values which are least 10 percentage points greater than mean values for the lysis of T2 in the absence of peptide and are also significantly different by Student t-test ($P < 0.05$).

Figure 1B

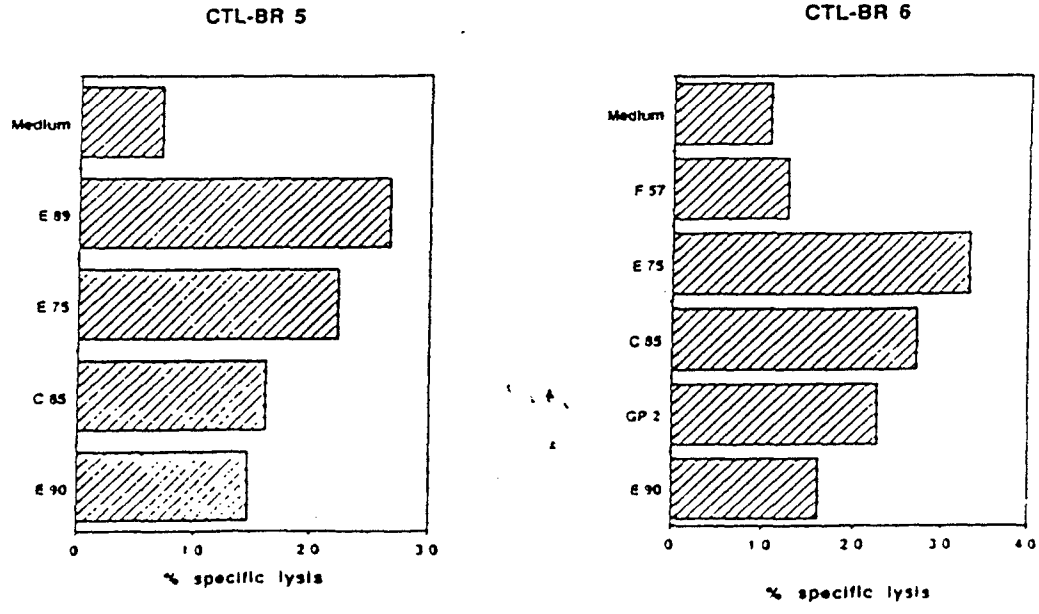


Figure 1B
Same as for breast cancer CTL (BR5 and 6).

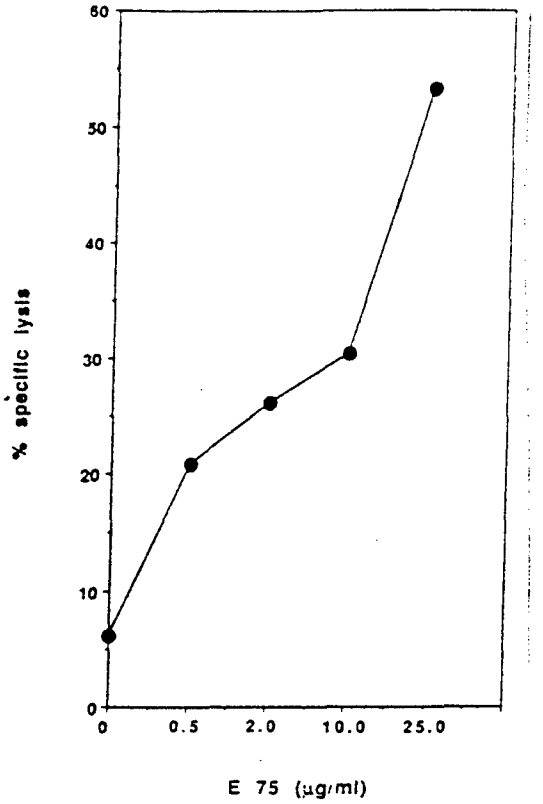


Figure 1C
E75 concentration dependent lysis by CTL-BR6

To determine whether reactivity to E75 was a property of distinct non-cross reactive clones from a line of multiple HER-2 specificity's, and to establish whether these clones recognize E75 in a peptide concentration-dependent and specific manner, clones were developed from CTL-3 by limiting dilution and expanded in culture.

C1R cells (a human B-cell line) and SKOV3 (ovarian cancer line) were transfected with the HLA-A2 Class I gene and tested for lysis by CTL-3. T2 cells were pulsed with HER-2 specific peptides E75, E90, and E89, as well as Folate-binding peptides (FBP) E38 and E48 and MUC-1 derived peptides D125 and D132. As shown in Figure 2A, CTL-3 effectively lysed T2 cells pulsed with HER-2 peptides but not T2 cells pulsed with FBP and MUC-1 derived peptides. C1R, and C1R-A2 cells were not killed suggesting that CTL-3 clone did not recognize endogenous HLA-A2 Class I peptides on these cells, and that CTL-3 recognized only HER-2 derived peptides. Lysis was dependent on peptide concentration (Figure 2B). E75 was also recognized by two separate CTL clones (Figures 2C&D) where as T2 cells pulsed with low binding peptide E91 were not.

Peptide E75 was also recognized by clones 3C4F and 3B4E in a dose dependent fashion (Figure 3A). Moreover, specific inhibition of lysis of ovarian cell line OVA-1 and HLA-A2 transfected SKOV3. A2. IE4 by autologous CTL-1 (which also recognizes E75) occurred when T2 cells pulsed with E75 were added (Figures 3B&C). Inhibition was Class I but not Class II specific (Figure 3D).

Figure 2

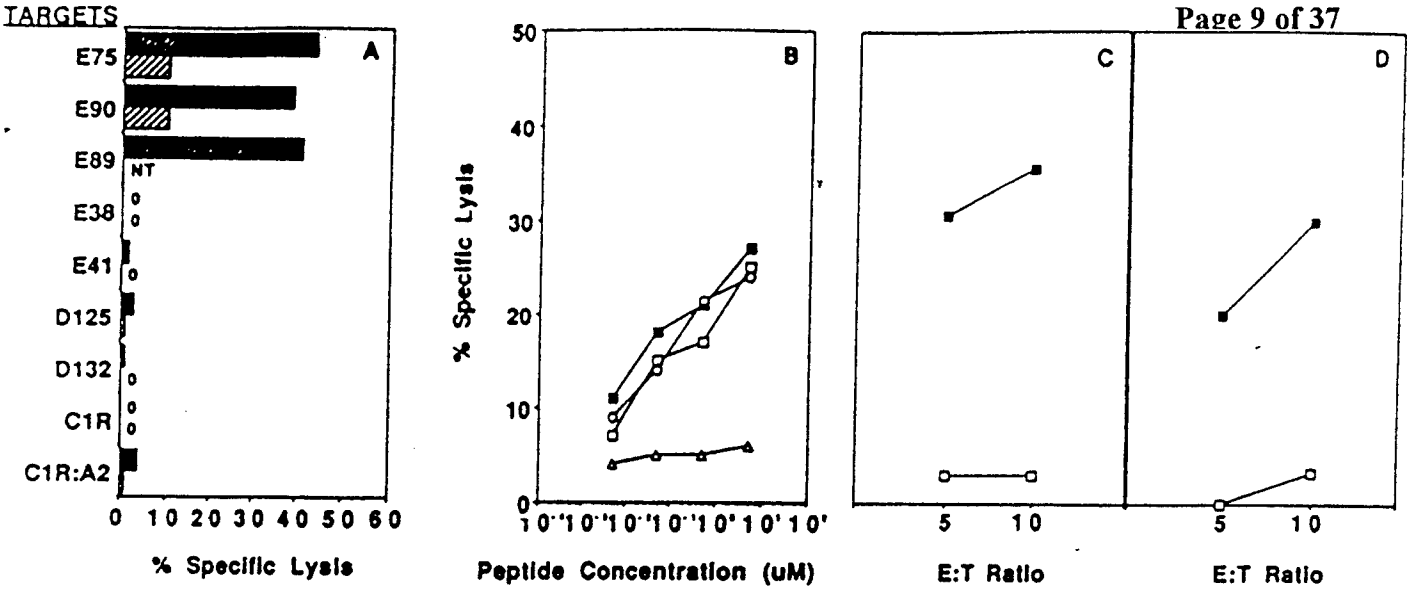


Figure 2. Recognition of E75 by CTL-3 clones. **A.** Lysis by the CTL-3 line. 3000 ^{51}Cr labeled T2 cells wer incubated with HER-2 peptides E75, E89, and E90 FBP peptides E38 and E41, and variant Muc-1 peptides D125 an D132 at a final concentration of 25μM for 60 min before effectors were added. Supernatant was collected and counte after 5 h. Effector to target ratios were 10:1 (■) and 5:1 (▨). Results are presented as % specific lysis by effectors c T2 cells pulsed with peptides. The same numbers (3000) of C1R and C1R:A2 cells were used as targets. **B.** Concentration dependent recognition of E75 (■), E90 (□), and C85 (O), and E92 (Δ) by CTL-3 line at an effector target ratio of 20:1. **C, D.** Lysis by clones 3C4F (C), and 3B4E (D) of E75 (■) and E90 (□) pulsed T2 cells. Lysi of T2 cells incubated with E89 at 25 μM was 7% by clone 3C4F, and 5% by clone 3B4E at 10:1 effector : targe ratios.

Figure 3

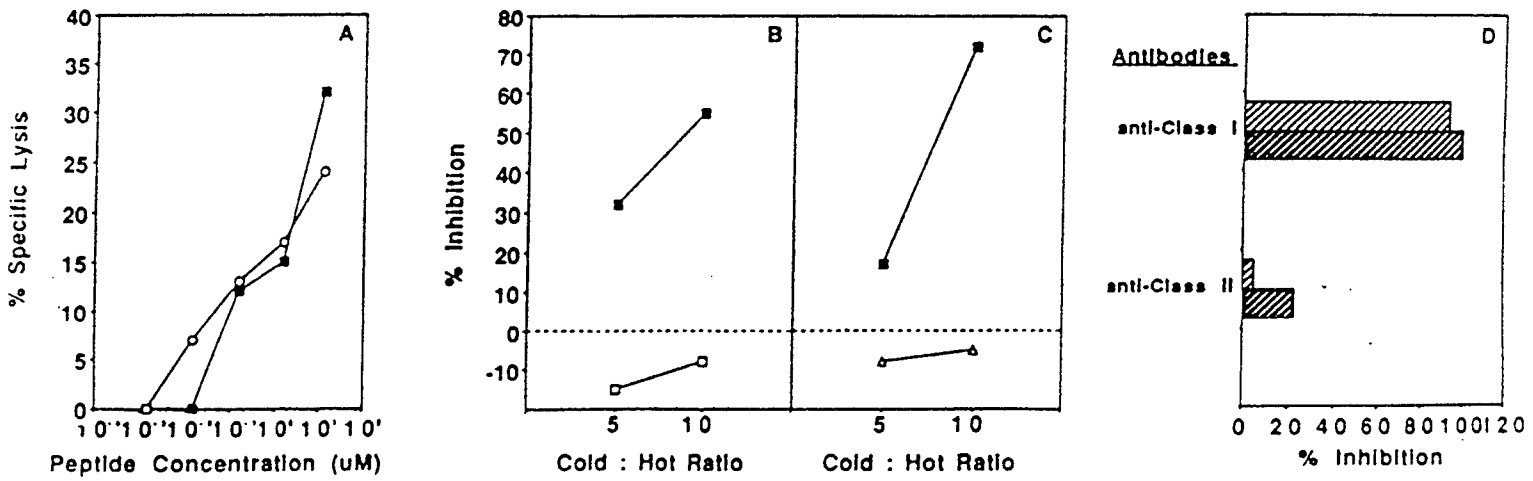


Figure 3. Inhibition of ovarian tumors recognition by clone CTL-3C4F by T cell epitope E75. **A.** Dose-respons recognition of peptide E75 by clones 3C4F (■) and 3B4E (O). Serial dilutions of peptide E75 were incubated wit 3000 T2 cells for 60 min. CTL were added at a E:T ratio of 4:1 and a standard 5h cytotoxicity assay was performed Lysis of T2 cells pre-incubated with E90 at 10 μM was <5% by both CTL clones. **B.C.** Cold-target inhibition o lysis of freshly isolated ovarian tumor OVA-1 (B), and ovarian tumor clone SKOV3.A2.1E4 (C) by T2 cells pre incubated with peptides E75 (■), E90 (□), and D132 (Δ). The effector (CTL-3C4F clone) : hot target ratio was 10:1 Peptide pulsed T2 cells (cold targets) were added in the assay at 5:1 and 10:1 cold:hot target ratios. Inhibition of lysis was determined in a 5h ^{51}Cr release assay. Results are presented as percentages of inhibition of tumor target lysis by clone 3C4F which was 46% for OVA-1 (B), and 28% for SKOV3.A2.1E4 (C). Lysis of parental control targets SKOV3 (HLA-A2$^-$) was 6% and of D132 pulsed T2 cells was 5% at the same E:T ratio. **D.** Lysis of SKOV3.A2.1E4 was inhibited by anti-HLA-class I (W6/32 mAb) but not by anti-HLA-DR (L243 mAb), at both 10:1 (▨) and 5:1 (□) effector to target ratios.

In summary, E75 is a high binding HER-2 peptide which is recognized by a majority of ovarian and breast cancer CTLs studied to date.

2.3 Enhanced Delayed Type Hypersensititivy Responses to Mixtures of HER-2/*neu* Administered with Peptides in a GM-CSF Rat Model

GM-CSF is a pleiotropic growth factor which has multiple actions in differentiation and immunity. Recombinant rhGM-CSF has been shown to enhance monocyte as well as neutrophil cytotoxicity against melanoma tumor cells and to enhance activity-dependent cellular cytotoxicity of monocytes and neutrophils against targets coated with the anti-ganglioside antibodies 14G2A and A108 (42,43). Macrophages derived after rhGM-CSF treatment were found to express enhanced levels of tumor necrosis factor and IL-IB, and had increased superoxide production (44).

With respect to cellular vaccines, Dranoff et al (45) has demonstrated that immunization with rhGM-CSF-transduced tumor cells produced the greatest degree of systemic immunity compared to tumor cells transfected with other cytokines and irradiated, non-transduced tumors. Immunity was dependent on both CD4+ and CD8+ T-cells despite the fact that the tumors were MHC Class II negative. One explanation for GM-CSF's local effect may relate to its unique ability to differentiate hematopoietic precursors to dendritic cells which are the most potent antigen presenting cells (APC) for helper T-cells (46,47).

Disis, in Dr. Martin Cheever's laboratory, has tested the immunogenicity of several HER-2/neu peptides derived from the external domain of the Her-2/neu receptor (48) in rats. A rat model was used since rat new protein is 89% homologous to human HER-2/neu protein and has a similar time distribution and expression level. Rats immunized with either human or rat neu protein did not develop antibody or T-cell responses; however, immunization of rats with individual peptides derived from the intracellular (ICD) and extracellular domain (EcD) of HER-2 evoked significant antibody and T-cell responses. Of major importance was the fact that peptide-sensitized T-cells were also capable of recognizing native protein. This data implied that native protein was taken up by APC and processed such that the natural peptide isotope was presented to the Class II MHC molecules in a configuration similar to that of the immunizing peptides. No evidence of autoimmunity was seen in any of the immunized mice.

In another set of experiments, rats were immunized using 3 or 6 in vivo injections of a mixture of ICD or ECD HER-2/neu peptides. Freund's complete followed by incomplete adjuvant (CFA/IFA) was used along with the peptide mixture and compared to immunizations of peptide mixture plus rhGM-CSF. Animals were sacrificed at one month following the last immunization and tissues were examined for toxic effects. Prior to

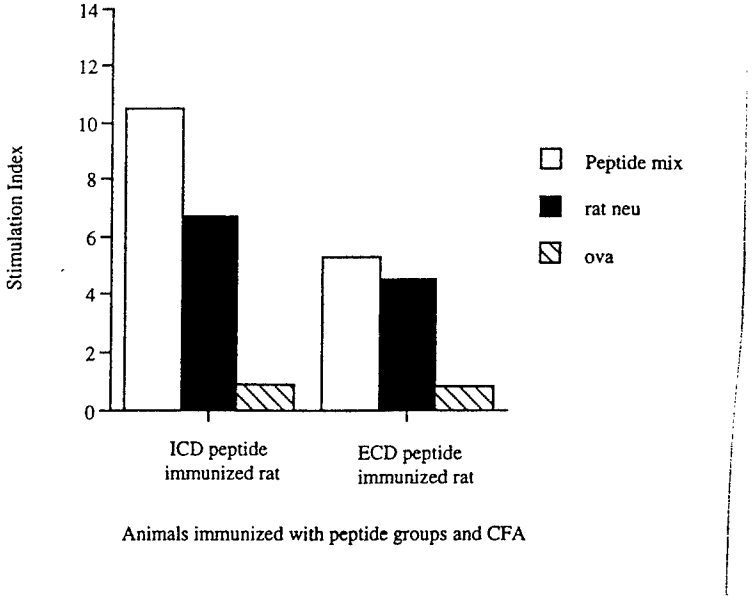
sacrifice, animals were tested for DTH responses or for the ability of rat spleen cells to be stimulated with peptide in vitro. These data are shown for 3,4, and 6 immunizations respectively in Figures 4-7. In summary, 1) ICD peptides in CFA were more immunogenic then ECD peptides (Figure 4), 2) DTH responses were induced with peptides plus rhGM-CSF (Figure 5), and were greater than peptides mixed with CFA (Figures 6 & 7).

Examination of multiple tissues revealed no evidence of end organ toxicity in animals receiving 3,4 or 6 injections. Resected skin at the injection site revealed accumulations of lymphoid cells and histiocytes. Minor membranous changes were noted in rat glomeruli which were thought to be related to a spontaneous lesion commonly seen in rats.

2.4 Rationale for E75 Immunization with rhGM-CSF

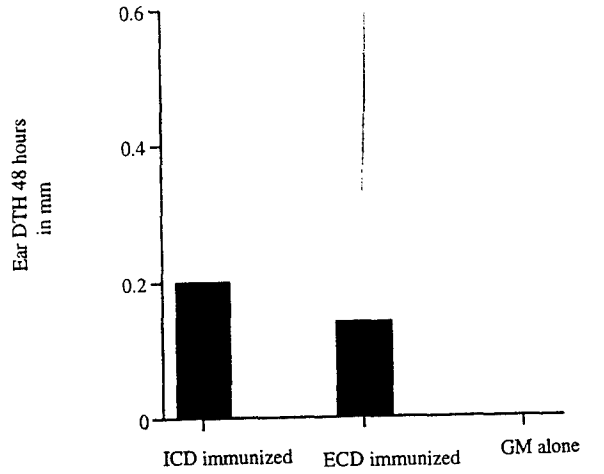
Based on the above experiments, it was noted by Cheever that peptides which elicited the highest immune response should be based in vaccines along with rhGM-CSF. Since E75 is a high affinity peptide which has been shown to elicit significant CTL responses in beast and ovarian cancer patients, we have selected it as the peptide of choice for this study.

Figure 4



Experiment: Animals immunized 1/95. Underwent 3 in vivo immunizations with either ICD or ECD peptide mix. Inoculations were 1 month apart. Animals sacrificed 1 month after the final immunization.
Clinical Observations: no abnormalities, weight loss, or signs of distress
Histopathology: (95-1816) both rats had normal histological surveys- no evidence of autoimmune disease

Figure 5



Experiment: Animals immunized 5/95. Underwent 3 in vivo immunizations with either ICD or ECD peptide mix. GM-CSF 5 ug used as adjuvant each immunization. Inoculations were 1 month apart. Animals sacrificed 1 month after the final immunization.
Clinical Observations: no abnormalities, weight loss, or signs of distress
Histopathology: (#95-2577)
ICD rat: Liver, kidney, small and large intestine, pancreas, myocardium, and skin: histologically normal.
ECD rat: Kidney, small and large intestine, pancreas, liver, and myocardium all histologically normal.. There is a very small accumulation of lymphoid cells and a few histiocytes surrounding or adjacent to several myocytes in the subcutis. (cellulitis, focal, non-suppurative, very mild- subcutis)

DTH Responses to Rat Neu in Animals Immunized with ECD Vaccine: Monthly Boosters for 6 Months

Figure 6

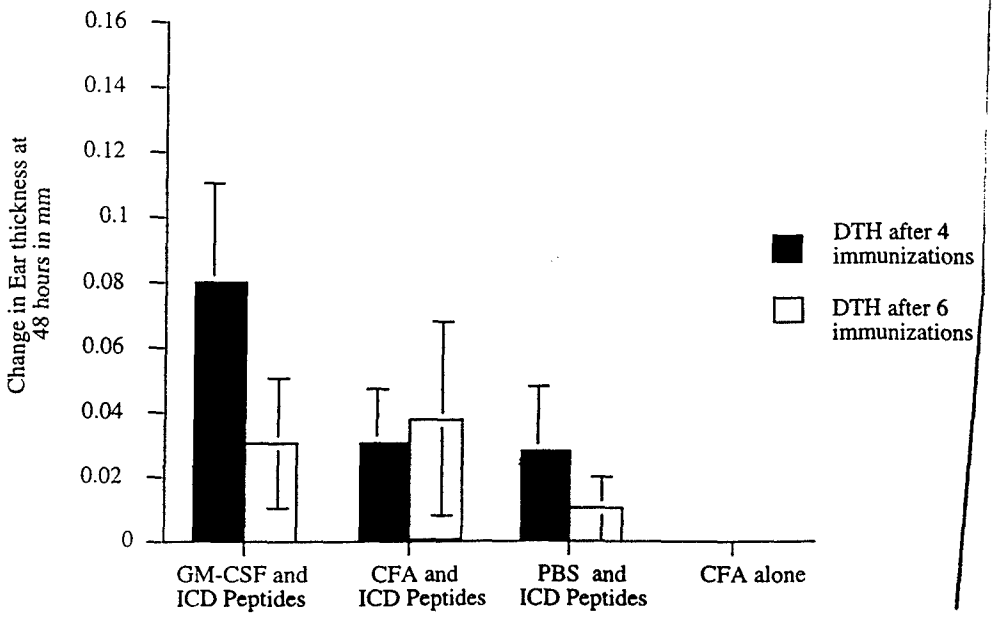
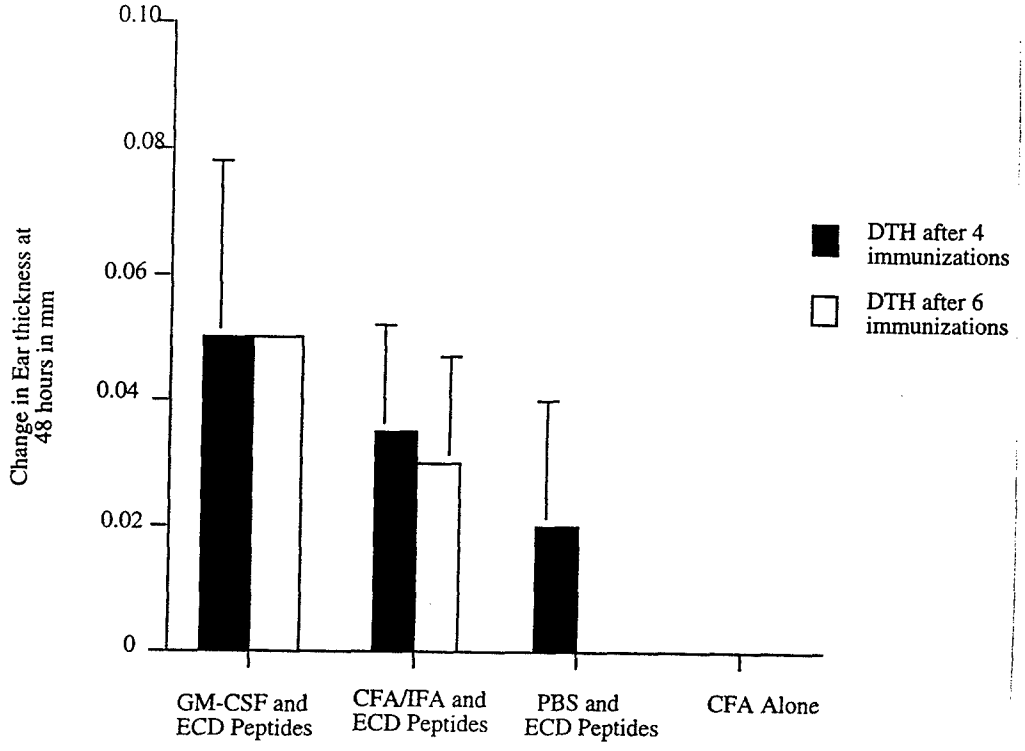


Figure 7

DTH Responses to Rat Neu in Animals Immunized with ECD Vaccine: Monthly Boosters for 6 Months



Animal Groups: 4 Rats/Group

3.0 PRODUCT INFORMATION

3.1 Peptide E75 will be synthesized and purified at Corixa Corporation according to GM P specifications. E75 peptide will be synthesized on a will be synthesized on a Millipore 9050 peptide synthesizer using HBTU (O-Benzotriazole-N,N,N^1, N^1-tetramethyluronium hexafluorophosphate) activation. Cleavage of the peptides from the solid support is carried out using the following cleavage mixture: trifluoroacetic acid:ethanedithiol:thioanisole:water: phenol (40:1:2:2:3). After cleaving for two hours, the peptide will be precipitated in cold ether. The peptide pellets are then dissolved in 10% v/v acetic acid and lyophilized prior to purification by C18 reverse phase HPLC. A gradient of 0-60% acetonitrile (containing 0.1% TFA) in water (containing 0.1% TFA) will be used to elute the peptide. Following purification, the peptide will be converted to their acetate salt using Dowex ion exchange resin.

The purity of the peptide will be verified by HPLC and mass spectrometry and the amino acid content is to be determined by amino acid analysis. E75 will be

purified to >95% before use. The peptide content of these samples is typically 70-80% with the remainder being water and acetate salts.

Peptide for skin testing will be dissolved in phosphate buffered saline and sterile filtered prior to vialing. The vialed material will be tested for pyrogenicity using the LAL assay, while sterility and general safety testing will be carried out by Microbiological Associates Inc., (Rockville, MD). Vialed peptides are to be stored at -20°C prior to use, and the stability of these formulations will be checked on a weekly basis to ensure that the samples remain stable over the period of the study. Prior to study, peptide will be reconstituted at various mcg amounts in sterile saline for injection along with 250 µg rhGM-CSF.

3.2 rhGM-CSF Sargramostim

rhGM-CSF sargramostim will be supplied as a sterile, white, preservative-free lyophilized powder in vials containing 500 mcg rhGM-CSF protein. Each vial also contains the following inactive ingredients: 40 mg mannitol, USP: 10 mg sucrose, NF; 1.2 mg tromethamine, USP.

3.2.1. Stability

Vials should be stored refrigerated at 2-8 C. Do not freeze. Each lot of material undergoes periodic shelf-life monitoring; vials do not bear an expiration date. Investigators will be notified by the sponsor when any lot of rhGM-CSF is found to have exceeded its useful shelf life.

3.2.2. Reconstitution

Each 500 mcg vial should be reconstituted with 1.0 ml of either sterile water for injection, USP or bacteriostatic water for injection (containing 0.9% benzyl alcohol). To avoid excess foaming, direct the diluent against the side of the vial. Gently rotate the vial to dissolve powder; avoid excess or vigorous agitation. Reconstituted rhGM-CSF is clear, colorless and isotonic, with a pH of 7.1 - 7.7. rhGM-CSF reconstituted with sterile water for injection should be used within 24 hours. When reconstituted with bacteriostatic water for injection, rhGM-CSF may be administered immediately or held at 2-8 C in the original vial or a syringe for up to 7 days. Do not freeze after reconstitution. Do not filter solution during preparation or administration.

3.2.3 Dilution for use

The reconstituted product should be diluted with 0.9% sodium chloride injection, USP. If the rhGM-CSF is diluted to less than 10 mcg/ml, 0.1% albumin (human) should be added to the saline solution before the rhGM-CSF is added.

3.2.4. How Supplied

Investigational rhGM-CSF will be supplied by Corixa Corporation, Seattle, Washington.

4.0 *PATIENT SELECTION*

4.1 Patients are eligible for enrollment into this study if they meet the following criteria:

1. Patients who have HER-2/neu, HL-A2+ histologically proven breast or ovarian cancer who have not received more than two prior chemotherapy or immunotherapy regimens excluding (adjuvant chemotherapy) for metastatic disease.
2. Patients who have not received interventional chemotherapy or immunotherapy within four weeks prior to entry into the study.
3. Patients must have a performance status of ≤ 1 on the ECOG scale or >70% on the Karnofsky scale (APPENDIX D).
4. Patients must have reached 18 years of age.
5. Patients must have an expected survival of at least 3 months.
6. Patients must have evaluable or measurable disease defined as bidimensionally measurable on radiological examination such as CT or MRI or clinically measurable by two examiners independently.
7. Patients must either:

   i. Have evidence of loco-regional relapse either at the primary site, or on the adjacent chest wall or in regional lymph nodes, (breast or peritoneum ovary).

   OR

   ii. Have measurable (clinically or radiologically) small volume metastatic disease at other sites (e.g., a solitary pulmonary metastasis). Ovarian cancer patients are also eligible if they have non-measurable disease with elevated CA125 level.

4.2 Exclusion Criteria

Patients will be excluded from this study if they meet any of the following criteria:

1. Patients who have received chemotherapy or radiotherapy within the previous 4 weeks, or hormonal therapy within the previous 2 weeks, or will likely require any one of these treatments for symptoms due to cancer or for rapidly progressive disease.
2. Patients who have received more than two regimens of chemotherapy or three regimens including adjuvant chemotherapy.

3. Patients who have more than 3 liver metastatic lesions or patients with liver metastases greater than 2 cm.
4. Patients who have known bone marrow involvement.
5. Patients who have known (current) brain metastases.
6. Patients who have large pleural or peritoneal effusions. (Patients with small effusions must have at least one site of measurable disease.)
7. Patients who have received immunotherapy (interferons, tumor necrosis factor, other cytokines [e.g., interleukins] or biological response modifiers, monoclonal antibodies or BCG vaccines) within the previous 4 weeks. (Patients who have had diagnostic studies with radiolabelled murine monoclonal antibodies are acceptable).
8. Patients with serum calcium >12.0 mg/dL.
9. Patients who have compromised hematopoietic function (hemoglobin <8.0 g/dL; lymphocyte count <1000/mm^3; neutrophil count <1500/mm^3; platelet count <100,000/mm^3).
10. Patients with significant hepatic dysfunction in the absence of liver metastases (bilirubin or ALT >2 times or alkaline phosphatase >3 times the upper normal limit).
11. Patients with renal dysfunction (serum creatinine >1.8 mg/dL).
12. Patients who are pregnant. Patients whom the investigator considers may be at risk of pregnancy will have a pregnancy [HCG] test and will be using a medically approved contraceptive method. Patients who are breast feeding are also excluded.
13. Patients who have an active autoimmune disease (e.g., rheumatoid arthritis; SLE; ulcerative colitis; Crohn's Disease, MS, ankylosing spondylitis).
14. Patients being treated with immunosuppressive drugs such as cyclosporine, ACTH or corticosteroids. Ideally, patients should not be treated with methylxanthine derivatives (pentoxifylline, theophylline, aminophylline, oxtriphylline) while on active specific immunotherapy.
15. Patients with an active infection causing fever.
16. Patients with a recognized immunodeficiency disease including cellular immunodeficiencies, hypogammaglobulinemia or dysgammaglobulinemia; patients who have hereditary or congenital immunodeficiencies.
17. Patients with significant cardiovascular abnormalities (clinically apparent hypotension, severe hypertension, CHF (NYHA Classes II-IV), CAD [including uncontrolled angina], or uncontrolled arrhythmias).
18. Patients who have an intercurrent illness or chronically taking medication (e.g. antihistamines) which would confound the results of the study, preclude the patient from completing the study, or mask an adverse reaction.
19. Patients who have a concurrent malignancy except cutaneous malignancy (excluding melanoma), *in situ* carcinoma of cervix, or other malignancy unless the patient has been disease free for ≥5 years.
20. Patients receiving other investigational drugs.
21. Inability to understand and sign a written consent form which must be obtained prior to treatment.

5.0 *STUDY PLAN*

5.1 All patients will be registered with the protocol Data Management office Extension 2-2933.

5.2 Prior to vaccination, all patients will either have biopsies of accessible lesions or previous tissue blocks obtained for staining for HER-2/*neu* and HLA-A2 by immunohistochemistry using monoclonal antibodies A3 and BB7.1, respectively. To qualify for study, patients must have at least 10% of tumor cells positive for both receptors.

5.3 Immunologic Tests - Immune Status

Prior to treatment, patients will also have 60cc of peripheral blood drawn for HLA-A2 phenotyping of mononuclear cells using antibody BB7.2, and for proliferative response to Tetanus toxoid (TT) and PHA mitogen. To be eligible for study all patients must have significant proliferative responses to PHA and TT and have cells expressing HLA-A2 MHC (see schema ,5.53). (See Appendix for discussion of TT and PHA assays). All assays will be performed in the laboratory of Dr. C. Ioannides. All patients must have positive tests to qualify for vaccination.

5.4 Treatment Plan

Provided patients are immunocompetent and have tumors which are HER-2/*neu*+ and HLA-A2+ they will be scheduled to receive vaccine. Prior to starting therapy, patients will also have the following tests performed.

1. CBC, platelet count, urinalysis, blood for HIV testing, blood chemistries.
2. Documentation of metastatic disease. Patients with recurrent skin lesions will have photographs taken to monitor response.
3. Immunologic tests: in addition to screening tests mentioned above, patients will have skin tests to > recall antigens (Merieaux multitest) as well as to peptide E75 (see Appendix). Sixty ml of peripheral blood will be drawn, peripheral blood mononuclear cells will be separated by Hypaque-gradients and frozen for later antigen proliferation assays and cytotoxicity tests. Ten cc of serum will also be saved for analysis of HER-2/*neu* specific antibody titers.

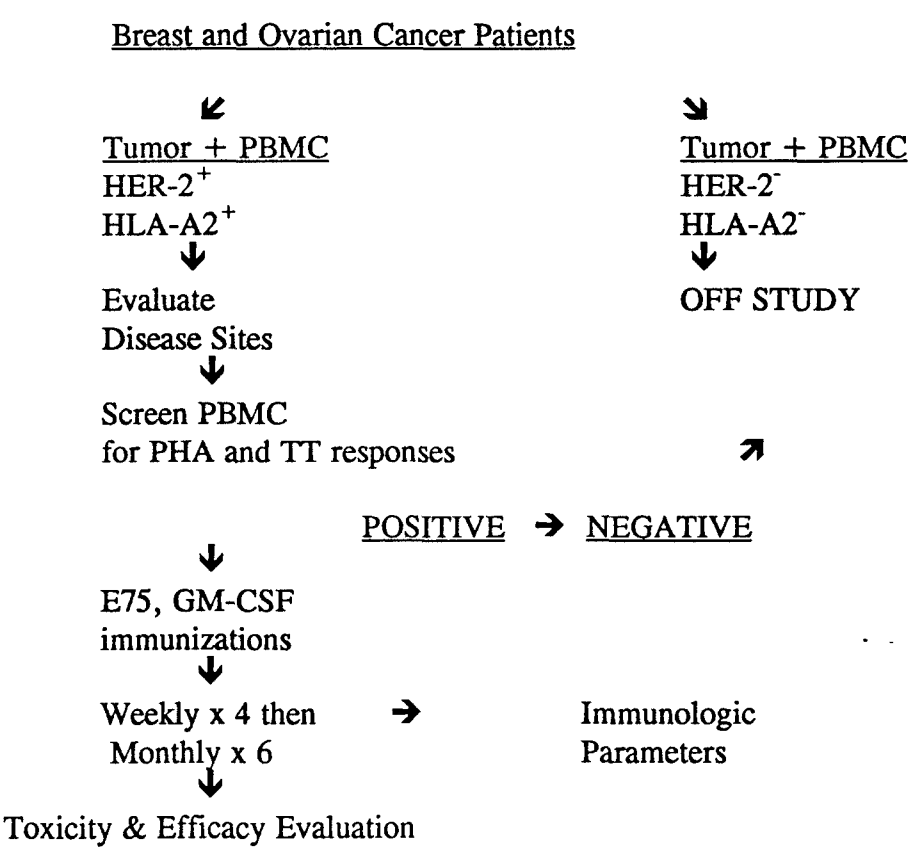
5.5 Three patients per group will receive escalating doses of E75 (100, 500 or 1,000mcg) mixed with 250 mcg of rhGM-CSF in a total volume of no more than 1 ml by intradermal injection weekly for 4 weeks and then monthly for a total of 10 injections. All injections will be given in the Ambulatory Treatment Center - Chair Unit. Vital signs will be monitored for ½ hour following each injection and at 24 hours when patients will be seen by a physician. At that time, all adverse effects will be recorded. All patients will receive a minimum of 5 injections over 8 weeks at which time they will be evaluated for response.

5.5.1 Any patient achieving a response or who has stable disease at 8 weeks may continue to receive monthly injections of vaccine for up to 6 months at the discretion of the investigator.

5.5.2 The following are excluded during treatment:

1. Aspirin or NSAID's
2. Corticosteroids, antihistamines
3. Immunotherapy, hormonal therapy, chemotherapy or radiotherapy
4. Other investigational drugs

5.5.3 Schema Summary



6.0 *PATIENT MONITORING*

6.1 Patients will have toxicity assessment and physical exam 24 hours after injection.

6.2 CBC, platelet count, serum chemistries, will be performed weekly x 4 then monthly to assess toxicity.

6.3 Patients will have PE, x-rays or scans to document disease status at 8 weeks.

6.4 Immunologic Monitoring

At one month and 2 months after beginning treatment patients will have from 60-80 cc of blood drawn for measurement of PBMC proliferation to vaccine, CTL responses to peptides, and measurement of TH_1 and TH_2 cytokines in supernatants (see appendix for details). In addition, 10 cc of serum will be stored for later analysis of anti-HER-2 antibodies. Patients will have recall skin tests as well as skin testing for E75 performed at 8 weeks.

6.5 Selected patients with ≥ 1 cm tumor nodules accessible for biopsy will have nodules removed, processed; and analyzed for presence of peptide using chromatography and mass spectroscopy (see Appendix).

7.0 *TOXICITY EVALUATION*

The Graded Toxicity Scale (Appendix D) will be used in this study. A scale for local toxicity is also presented.

7.1 For Grade I or II toxicity, the patient may continue the treatment at the discretion of the Investigator.

7.2 For Grade III toxicity, the patient's dose will be held until a return to Grade 0 toxicity at which point the patient may resume therapy at next lower dose level. If Grade III toxicity occurs at dose level I, patients will resume at 50% of that dose, after return to Grade 0 toxicity.

7.3 For Grade IV toxicity, the patient will be removed from the study. If an instance of Grade IV toxicity, other than idiosyncratic allergic reactions secondary to the antibody is observed, accrual of patients at the dose level resulting in Grade IV toxicity will be held and an additional three patients will be entered at the next lowest dose level, or in the event of Grade IV toxicity at level I, 50% of dose level I. Further accrual at higher dose levels will be carried out only after consultation with the protocol's sponsor.

7.4 If one instance of Grade III toxicity is observed during the course of treating patients on a dose level, a total of six patients will be placed on that dose level.

7.5 If three or more instances of Grade III toxicity are observed during the course of treating patients at a dose level, accrual onto that level will be terminated and the next lowest dose will be declared the MTD. The MTD will be defined as the highest dose which does not produce ≥ Grade III toxicity in greater than two patients.

7.5.1 All life-threatening events (Grade IV toxicity) which may be due to the vaccine, all fatal events and first occurrence of any toxicity, regardless of grade,

will be reported to the Protocol Review Office, the sponsor, and FDA within 24 hours.

7.5.2 Written report of all adverse drug reactions will be filed within ten working days with the sponsor, FDA and Protocol Review Office.

8.0 *DETERMINATION OF IMMUNOLOGIC ENDPOINTS AND OPTIMAL BIOLOGIC DOSE (OBD)*

8.1 Immunologic Endpoints

Since a major goal of this trial is to see whether vaccination can induce specific immune responses against E75 and/or HER-2/*neu* protein, immunologic endpoints have been established using the following criteria below. Patients will be considered to have a positive immune response if 2 out of 3 of the following criteria are met.

8.1.1. CTL Response:

Depending on whether there is detectable pre-immunization specific killing, a 2 fold increase the pre-immunization percent killing will be considered a positive response. If there is no detectable lysis post-immunization, a post-immunization specific lysis of $\geq$ 10% will be considered positive.

8.8.2. Proliferation Index

As with CTL, a positive response is that in which the post-vaccination response (stimulation index) is 2 fold greater than the preimmunization response.

8.8.3. Gamma-Interferon Levels

If there is a detectable (20pg/ml) pre-immunization peptide specific IFN-γ response; a $\geq$ 2 fold increase over the pre-immunization level will be considered positive. For no detectable pre-immunization response a positive post-immunization response will be that which is > 3 standard deviations above the mean response of unimmunized control donors to the peptide.

8.2 Determination of optimal biologic dose (OBD). The OBD will be defined at the highest dose in which 2/6 patients (one third) meet immunologic response criteria as defined above. If the MTD is not achieved prior to determination of the OBD, additional patients may be added.

9.0 *RESPONSE CRITERIA*

Although this is mainly a feasibility study, responses will be evaluated as follows:

9.1 Complete Response (CR):

Complete disappearance of all clinical and laboratory signs and symptoms of active disease for a minimum of four weeks.

9.2 Partial Response (PR):

A minimum of a 50% reduction in the size of measurable lesions as determined by the product of the longest perpendicular lesion diameters (width in cm. X length in cm.) for a minimum of four weeks. Every lesion need not regress for qualification as partial response. However, if any lesion progresses or if new lesions appear, the response cannot be classified as a partial response.

9.3 Stable disease (SD)

No change in measurable disease or increase in lesion size of less than 25%.

9.4 Progressive Disease (PD):

Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease will also include those instances where new lesions have appeared.

10.0 *CRITERIA FOR REMOVAL FROM THE STUDY*

Patients may withdraw or be removed from the study in the following instances:

10.1 Intercurrent illness which would, in the judgment of the Investigator, affect assessments of clinical status to a significant degree, requires discontinuation of drug, or both.

10.2 Unacceptable toxicity as defined in Section 7.0.

10.3 Disease progression.

10.4 Patient noncompliance, or request to withdraw.

11.0 *STATISTICAL ANALYSES*

11.1 MTD Determination

Groups of 3 patients will be treated with various dose levels of vaccine mixed with GM-CSF. If one instance of Grade 3 or 4 toxicity are observed at a given dose level a total of six patients will be placed on that dose level. If three or more instances on Grade 3 or 4 toxicity are observed during the course of treating patients on a dose level accrual onto the trial will be terminated and next lower dose will be declared the MTD. In all other cases, dose escalation will proceed. It is estimated that from 9-15 patients will be entered on study. Based on an accrual rate of 1 patient per month (based on a 30%-50% predicted ineligibility for patients who are HER-2, HLA-A2 negative), the study should take from 9-18 months to complete. If there are no immunologic responses detected in 15 patients then the study will be terminated and we will conclude with 95% power and 95% confidence that the vaccine is able to produce appropriate responses in 20% of patients. Differences in pre and post-immunization studies as well as differences between dose levels will be determine by ANOVA, CH1- square analysis as well as paired t-tests and t-test for independent variables. Appropriate regression analyses may be used to study the relationship, if any between immunologic findings and clinical outcomes. It is possible that the MTD or OBD will not be determined in this trial.

12.0 *REFERENCES*

1. McGuire WP, Hoskins WJ, Brady MF, Kucera PR, Partridge EE, Look KY, Clarke-Pearson DL, Davidson M: Cyclophosphamide and cisplatin compared with paciltaxel and cisplatin in patients with stage III and stage IV ovarian cancer. N Engl J Med 334:1-6, 1996.

2. Cohen J: Cancer vaccines get a shot in the arm. Science 262: 841-843, 1993.

3. Greenberg PD, Riddell SR: Tumor-Specific T-cell immunity: ready for prime time. J Natl Can Inst 84:1059-1061.

4. Lanzavecchia A: Identifying strategies for immune intervention. Science 260:937-944, 1993.

5. Ioannides CG, Grimm EA: Tumor Immunity: In: Principles of Clinical Immunology, First Edition, Chapter 22, (Rich R., Shearer WT, Strober W, Fleisher TA, and Schwartz BD (eds) Mosby. St. Louis, 333-349, 1995.

6. Van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254:1643-1647, 1991.

7. Pardoll DM: Tumor Antigens - A new look for the 1990s. Nature 369:357-358, 1994.

8. Jerome KR, Barnd DL, Bendt KM, Boyer CM, Taylor-Papadimitriou J, McKenzie IF, Bast RC Jr, Finn OJ. Cytotoxic T lymphocytes derived from patients with breast adenocarcinoma recognize an epitope present on the protein core of a mucin molecule preferentially expressed by malignant cells. Can Res 51:2908-2916, 1991.

9. Ioannides CG, Fisk B, Jerome KR, Irimura T, Wharton JT, Finn OJ. Cytotoxic T cells from ovarian malignant tumor can recognize polymorphic epithelial mucin peptides. J Immunol 151:3693-3703, 1993.

10. Cox AL, Skipper J, Chen Y, Henerson RA, Darrow TL, Shabanowitz J, Engelhard VH, Hunt DF, Slingluff CL Jr. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. Science 264:716-719, 1994.

11. Mandelboim O, Berke G, Fridkin M, Feldman M, Eisenstein M, Eisenbach L. CTL induction by a tumour-associated antigen octapeptide derived from a murine lung carcinoma. Nature 369:67-71, 1994.

12. Rotzschke O, Falk K, Deres K, Schild H, Norda M, Metzger J, Jung G, Rammensee HG. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. Nature 348:252-254, 1990.

13. Van Bleek G, Nathenson SG. Isolation of an endogenously processed immunodominant viral peptide from the Class I H-2Kb molecule. Nature 348:213-216, 1990.

14. Yanuck M, Carbone DP, Pendleton CD, Tsukui T, Winter SF, Minna JD, Berzofsky JA: A mutant p53 tumor supressor protein is a target for peptide-induced $CD8^+$ cytotoxic T-cells. Can Res 53:3257-3261, 1993.

15. Kantor J, Irvine K, Abrams S, Kaufman H, DiPietro J, Schlom J: Antitumor activity and immune responses induced by a recombinant CEA-vaccinia virus vaccine. J Natl Can Inst 84:1084-1091, 1992.

16. Fisk B, Chesak B, Pollack M, Wharton JT, Ioannides CG. Oligopeptide induction of a cytotoxic T lymphocyte response to HER-2/neu proto-oncogene *in vitro*. Cell Immunol 157:412-427, 1994.

17. Bakker ABH, Schreurs MW, de Boer AJ, Kawakami Y, Rosenberg SA, Adema GJ, Figdor CG. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor infiltrating lymphocytes. J Exp Med 179:1005-1009, 1994.

18. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Sakaguchi K, Appella E, Yannelli JR, Adema GJ, Miki T, Rosenberg SA. Identification of a human

melanoma antigen recognized by tumor-infiltrating lymphocytes associated with *in vivo* tumor rejection. Proc Natl Acad Sci USA 91:6458-6462, 1994.

19. Coulie PG, Brichard V, Van Pel A, Wolfel T, Schneider J, Traversari C, Mattei S, De Plaen E, Lurquin C, Szikora JP, Renauld JC, Boon T. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanoma. J Exp Med 180:35-42, 1994.

20. Kawakami Y, Eliyahu S, Sakaguchi K, Robbins PF, Rivoltini L, Yannellli JR, Appella E, Rosenberg SA. Identification of the immunodominant peptides of MART-1 human melanoma antigen recognized by the majority of HLA-A2 restricted tumor infiltrating lymphocytes. J Exp Med 180:347-352, 1994.

21. Brichard V, Van Pel A, Wolfel T, Wolfel C, De Plaen E, Lethe B, Coulie P, Boon T. The tryosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J Exp Med 178:489-495, 1993.

22. Topalian S, Rivoltini L, Mancini M, Markus NR, Robbins PF, Kawakami Y, Rosenberg SA. Human $CD4^+$ T cells specifically recognized a shared melanoma-associated antigen encoded by the tyrosinase gene. Proc Natl Acad Sci USA 91:9461-9465, 1994.

23. Kovascovics-Bankowski M, Rock KL. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC Class I molecules. Science 267:243-245,1995.

24. Vijayasaradhi S, Bouchard B, Houghton AN. The melanoma antigen gp75 is the human homologue of the mouse *b (brown)* locus gene product. J Exp Med 171:1375-1380, 1990.

25. Rivoltini L, Kawakami Y, Sakaguchi K, Southwood S, Sette A, Robbins PF, Marincola FM, Salgaller M, Yannelli JR, Appella E and Rosenberg SA. Induction of tumor reactive CTL from peripheral blood and tumor-infiltrating lymphocytes of melanoma patients by *in vitro* stimulation with an immunodominant peptide of the human melanoma antigen MART-1. J Immunol 154:2257-2265, 1995.

26. Disis ML, Calenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, Jeschke M, Lydon N, McGlynn E, Livingston RB, Moe R, Cheever MA. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Can Res 54:16-19, 1994.

27. Pupa SM, Menard S, Andreola S, Colnaghi MI. Antibody response against the c-*erb* B-2 oncoprotein in breast carinoma patients. Can Res 53:5864-5666, 1993.

28. Bargmann C, Hung M, Weinbert R. The *neu* oncogene encodes an epidermal growth factor receptor-related protein. Nature 319:226-230, 1986.

29. Slamon D, Clark G, Wong S, Levin W, Ullrich A, McGuire W. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177-182, 1987.

30. Ioannides CG, Ioannides MG, O'Brian CA. T-cell recognition of oncogene products: A new strategy for immunotherapy. Molecular Carcinogenesis 6:77-82, 1992.

31. Yoshino I, Peoples GE, Goedegebuure PS, Maziarz R, Eberlein TJ. Association of HER-2/neu expression with sensitivity to tumor-specific CTL in human ovarian cancer. J Immunol 152:2393-2400, 1994.

32. Fisk B, Blevins TL, Wharton JT, Ioannides CG. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med 181:2109-2117, 1995.

33. DeLisi C, Berzofsky JA. T-cell antigenic sites tend to be amphipathic structures. Proc Natl Acad Sci USA 82:7048-7052, 1985.

34. Rothbard JB, Taylor WR. A sequence pattern common to T-cell epitopes. EMBO J 7:93-102, 1988.

35. Engelhard VH. Structure of peptides associated with Class I and Class II MHC molecules. Annual Rev Immunol 12:181-207, 1994.

36. Disis ML, Smith JW, Murphy AE, Chen W, Cheever MA. *In vivo* generation of human cytolytic T-cells specific for peptides derived from the HER-2/neu protooncogene protein. Can Res 54:1071-1076, 1994.

37. Fisk B, Chesak B, Pollack MS, Wharton JT, Ioannides CG. Oligopeptide induction of a cytotoxic T lymphocyte response to HER-2/neu protooncogene *in vitro*. Submitted

38. Lustgarten J, Sherman L. Generation of xenogeneic cytotoxic T cells from peptides derived from the HER-2/neu protoonocogene. Presented at the 9th International Congress of Immunology: Abstract 3935:663, 1995.

39. Ioannides CG, Fisk B, Pollack MS, Frazier ML, Taylor Wharton J, Freedman RS. Cytotoxic T cell clones isolated from ovarian tumors infiltrating lymphocytes recognize common determinants on non-ovarian tumor clones. Scand J Immunol 37:413-24, 1993.

40. Leong SPL, Enders-Zohr P, Zhou YM, Allen RE. GM-CSF and autologous melanoma vaccine mediate tumor regression in patients with metastatic melanoma. J Immunotherapy 18:2;132 Abstract.

41. Houbiers JGS, Nijman HW, Van der Burg SH, Drokfhout JW, Kenemans P, van de Velde CJH, Brand A, Momberg F, Kast WM, Melief CJM. *In vitro* induction of human cytotoxic T lymphocyte responses against peptides of mutant and wild-type p53. Eur J Immunol 23:2072-2077, 1993.

42. Murray JL, Kleinerman ES, Jia S-F, Rosenblum MG, Eton O, Buzaid A, Legha S, Ross MI, Thompson L, Mujoo K, Reiger PT, Saleh M, Khazaeli MB, Vadhan-Raj S: Phase Ia/Ib trial of anti GD2 chimeric monoclonal antibody 14.18 (ch14.18) and recombinant hman granulocyte-macrophage colony-stimulating factor (rhGM-CSF) in metastatic melanoma. In press.

43. Kushner BH, Cheung NKV: GM-CSF enhances 3F8 monoclonal antibody-dependent cellular cytotoxicity against human melanoma and neuroblastoma. Blood 1989;73:1936-1941.

44. Perkins RC, Vadhan-Raj S, Scheule RK, Hamilton R, Holian A: Effects of continuous high dose rhGM-CSF infusion on human monocyte activity. Am J Heamatol 1993;43:279-285.

45. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Pardoll D, Mulligan RC. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc Natl Acad Sci USA 90:3539-3943, 1993.

46. Inaba K, Steinman RM, Pack MW, Aya H, Inaba M, Sudo T, Wolpe S, Schuler: Identification of proliferating dendritic cell precursors in mouse blood. J Exp Med 175:1157-1167, 1992.

47. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM: Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 176:1693-1702, 1992.

48. Disis ML, Gralow JR, Bernhard H, Hand SL, Rubin WD, Cheever MA. Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, and oncogenic self-protein. J Immunol 156:3151-3158, 1996.

APPENDIX A

STUDY FLOW CHART

	PRE STUDY	TREATMENT (VACCINATIONS)							
TIME (Weeks)	**-1 to -4**	**1**	**2**	**3**	**4**	**8**	**12**	**16**	**Etc.**
Visit No.	1								
History	X					X	X	X	
Physical	X					X	X	X	
Vital Signs	X	X	X	X	X	X	X	X	
Tumor Imaging	X					X		X	
Treatment Site & Inspection		X[a]	X	X	X	X	X	X	
EKOG/Karnovsky Status	X					X	X	X	
Laboratory Tests									
Hematology	X	X	X	X	X	X	X	X	
Chemistries	X	X	X	X	X	X	X	X	
UA	X					X	X	X	
Immunology							X		
IHC for HLA & HER-1[b]	X								
Serum Antibodies	X				X	X	X	X	
PHA + TT STIM	X								
Skin Tests (DTH)	X					X			
Cytokine Assays	X				X	X	X	X	
Cytotoxicity	X				X	X	X	X	
Proliferation	X				X	X	X	X	
Tumor Biopsies[c]						X			

a. 24 hour after injections
b. Fresh frozen or blocks
c. If indicated for E75 purification & HLA-A2 expression.

APPENDIX B

Immunologic Tests

a. Immunohistochemistry Procedures

Tumor blocks or fresh frozen tumor specimens from patients will be analyzed for HER-2, HLA-A2.1 and B7 using monoclonal antibodies Ab-3 (Oncogene Sciences, Uniondale, NY), BB7.2, (Dako) and anti-CD80 (B7) (Becton Dickinson) respectively.

For paraffin sections, the tissues are deparaffinized in xylene and rehydrated in graded alcohols. Serial slides of each specimen are cut at 5 μm thickness and mounted on Vectabond-coated slides. Endogenous peroxidase activity is quenched by incubating the slides with 0.3% hydrogen peroxide in methanol for 15 minutes followed by 3 PBS rinses. The slides are then incubated for 30 minutes in 10% normal horse serum to reduce any nonspecific staining and then incubated overnight at 4°C with the primary antibody. All slides are washed 3 times in PBS, incubated with the second antibody, biotinylated mouse-antihuman tissues. IgG (Vector Labs, Burlingame, CA), diluted 1:200 for 60 minutes, washed again 3 times in PBS and then incubated with the ABC reagent (Vector Labs) for 30 minutes. The peroxidase activity is developed with freshly prepared 0.06% 3', 3'-diaminobenzidine (Sigma Chemical Co, St. Louis, MO), containing 0.1% hydrogen peroxide. Methyl green (fresh tissue) or hematoxylin (paraffin blocks) are used as counterstain.

Each section can be qualitatively evaluated for the presence of epithelial intracellular brown diaminobenzidine precipitate indicative of Mab binding. Sections are scored + for weakly positive, ++ for positive and +++ for strongly positive. The sum of + to +++ immunoreactivities is used for total cellular reactivity. The approximate percentage of positive carcinoma cells can be assigned according to the number of carcinoma cells positive with a given Mab divided by the total number of carcinoma cells present x 100. To be eligible for study patients must have at least 10% of cells expressing membrane staining for HER-2/neu.

b. Determination of HLA-A2 Positivity of PBMC in Patients

From 5-10 ml heparinized blood will be drawn prior to study. PBMCs will be separated on Ficoll-Hypaque cushions and $1x10^6$ cells in 0.5 ml PBS/0.1% BSA incubated with BB7.2, a mouse Mab specific for HLA-A2.1 for 1 hour at 4°C. Following incubation FITC-goat anti-mouse Ig is added and incubated for 30 minutes at 4°C. Cells are washed and examined for the percentage of cells binding Mab and mean cell fluorescence (MCF) using a FAC Scan flow cytometer (Becton-Dickinson). Percentage of fluorescent cells must be 2 x background for a patient to be eligible.

c. Measurement of CTLs Reactive Against E75

From 50-60 cc of blood will be drawn prior to study and at 1 month, 2 months, and following the last vaccination. PBMCs will be separated using Ficoll Hypaque gradients. Adherent cells will be separated from T-enriched cells by adherence to plastic. T cells will be enriched for $CD8^+$ cells by "Panning" using plates coated with anti-CD8 and anti-CD4 antibodies (AIS Microcellector ™, Applied Immune Sciences, Mehlo Park, CA) (positive and negative selection). Adherent cells and CD8 enriched T cells will be collected in 24 well plates ($1x10^6$ cells per well) at a responder to stimulator ratio of 10:1 in RPMI 1640 medium supplemented with 10% FCS, 100 mg/ml L-glutamine, and 100mg/ml gentamicin (complete RPMI medium) in the presence of 50 µg/ml peptide. After 48 hours 25U/ml IL-2 (Chiron) and 25 µg/ml of IL-12 (Genetics Institute, Cambridge, MA) are added to culture. Cells are cultured for 1 week after which they will be washed and used as effector cells against ^{51}Cr labeled T2 cells, pulsed with E75 and C1R:A2 cells transfected with the gene for HER-2. Controls will include an HLA-A2 negative ovarian cell line, T2 cells pulsed with irrelevant peptide E91, and an HLA-A2+ melanoma cell line, A375. In selected patients with accessible tumors, CTL activity against *autologous tumor* will also be tested. After 4-5 hr incubation at 37°C, 100 ul of supernatant will be collected and radioactivity quantitated. Percentage lysis will be calculated using the formula: 100x [(E-S)/(T-S)], where E=experimental release, S=release in the absence of CTL, and T=release in 2M Hcl.

d. Antibody Response to E75

Antibody levels from sera collected and frozen at the time of the cytotoxicity assay will be assessed by RIA. Polystyrene beads (Precision Plastic Bell, Chicago, IL) are coated with purified E75 (2µg/bead in PBS), washed x 3 with PBS containing 1% BSA and stored in wash buffer at 4°C until use. Twenty ul patient sera diluted to 100 ul in PBS is incubated with a single bead for 2 hours on a laboratory shaker at room temperature, washed in PBS and incubated with 100 ul ^{125}I-labeled peptide (10^6 cpm/ug) at 2 ug/ml for 1 hr. Bead is rewashed in PBS and cpm determined using a gamma counter. Background nonspecific binding of 1% of the available ^{125}I-labeled peptide is subtracted from cpm bound and the ng peptide bound to the bead/ml sera was calculated from the known specific activity of the ^{125}I-labeled E75. A positive response will be defined as a value exceeding 2x the standard deviations above the mean value of 10 normal human sera.

e. DTH Responses to Peptide

Prior to study and at 2 months patients will be tested for *in vivo* DTH responses to peptide. Briefly, 100ul of E75 (50ug) will be injected intradermally on the volar surface of the forearm. At 48 h bidimensional measurements of erythema

and induration are recorded and compared to a standard skin test battery (Multitest™, Merieaux, Paris, FR) consisting of 7 recall antigens. Mean diameters measured prior to study will be compared to those obtained after 2 months of vaccination.

f. T-Cell Proliferative Responses

T-cell proliferative responses to peptide as well as PHA and tetanus toxoid (TT) will be performed as follows:

Patients' PBMC obtained prior to study and monthly x 2 will be frozen in FCS + 10% DMSO. At the time of testing PBMC will be thawed and washed in complete RPMI media; 2 x 10^5 cells will be placed in each of triplicate wells of 96-well round-bottomed microtiter plates (Corning, Corning, NY) in complete RPMI media. Experimental groups will consist of wells incubated with PBMC and no peptide, 50ug/ml peptide, 1 ug/ml PHA, and 5 ug/ml tetanus toxoid. Plates will be incubated at 37°C for 4 days following which wells are pulsed with 1 μCi of [^{3}H] thymidine for 6-8 hours, harvested and counted. Results are expressed as a stimulation index which is: Experimental cpm (i.e. E75) - cpm obtained from wells containing PBMC and no peptide/cpm from cells pulsed with irrelevant peptide-control wells PBMC alone.

g. Measurement of Th1 and Th2 Cytokine Production

Culture supernatants obtained 48 hours after peptide stimulation (see CTL assay) will be harvested and stored at -70°C until assayed. Interferon-γ, TNFα and IL-10 levels will be measured in titrated amounts of culture supernatant using commercially available ELISA test kits (Endogen, Boston, MA) (Genzyme, Cambridge, MA). These data will be correlated with CTL lysis because $CD8^+$ cells secreting TH2 cytokines (i.e IL-10) have been reported to show reduced cytolytic potential.

Tumor Peptide Extraction and Fractionation

Ovarian and breast tissue cells are obtained following enzymatic digestion (in the case of solid tumor masses) followed by separation from TIL cells by differential centrifugation on Ficoll-Hypague gradients. Tumor cells obtained from ovarian cancer patient ascitic fluid do not undergo enzymatic digestion prior to separation from TIL's. From 1 to 1.5x10^9 cells are collected and washed three times in cold PBS. Cells are lysed using the buffer previously described by Engelhard, Slingluff, and collaborators containing protease inhibitors (aprotinin, leupeptin, pepstatin, PMSF, iodacetamide), in PBS with the difference that CHAPS 0.5% was used as lysing agent to minimize binding to C18 columns (V. Engelhard, personal communication). This solution is designated here as lysis buffer. $MgCl_2$ at 6 mM and Glycerol at 20% final concentration was added in the lysis buffer to minimize the denaturation of extracted proteins. Detergent-solubilized extracts of tumor cells are obtained after centrifugation

at 40,000g for 2 hours. HLA-A2.1 is isolated from the supernatants of centrifugation by affinity chromatography on Protein A-Sepharose prebound with Mab BB7.2 except that the cell extracts will be pre-absorbed on Protein-A Sepharose (Sigma Chemical Co., St. Louis, MO) to minimize non-specific binding. The column was washed with PBS containing 0.25M NaCl with monitoring of the OD210 nm (for peptide bond), then eluted with 0.2M acetic acid. The eluate is boiled for 5 min to allow dissociation of peptides from HLA-A2, centrifuged through an Ultrafree CL unit (3-kd cut-off) (Millipore) and lyophilized. For these studies, at least 10^{10} tumor are grown in batches of 1.5 - 2.0 x 10^{9} cells. Peptides with masses <3Kd are pooled, lyophilized and separated by reverse phase-high pressure liquid chromatography (RP-HPLC).

Fractionation of HLA-A2 Bound Peptides

Tumor peptides extracted from HLA-A2.1 molecules of from tumor cells are separated in the first dimension on a Brownlee C18 Aquapore column (2.1 x 30mm, pore size, 300 A; particle size, 7μm Applied Biosystems, Perkin-Elmer Corporation) and eluted with a 60 min gradient of 0.60% (vol/vol) acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 200μl/min using an HPLC system model 1090 (Applied Biosystems) as previously described. This gradient is designated as Gradient I. HER-2 peptide E75 identified in previous studies to be recognized by ovarian CTL-3 line is separated in the same conditions and the retention times (Rt) determined. For the second dimension separation, pooled fractions from the first dimension corresponding to the peak of elution of E75 are injected into a Brownlee C18 Aquapore column of 2.1 x 200 mm, (300A, 7μm) and eluted with a shallower gradient: 0-5 min, 0-15% ACN in 0.1% TFA, 5-45 min 15-35% ACN in 0.1% TFA and 45-60 min, 35-60% ACN in 0.1% TFA, designated as Gradient II here. The flow rate was 200μl/min and fractions were collected at 1 min intervals. Peaks for peptides eluted from tumor are compared to the elution time for E75. Peptides with similar retention times to E75 suggest that natural peptides of similar sequence may be presented by the cells. In addition, mass and charge of the respective peptides will be checked by mass spectrometry.

APPENDIX C
EVALUATION OF PERFORMANCE STATUS

Description	Karnofsky* Scale (%)	ECOG Scale
Normal, no complaints, no evidence of Disease.	100	0
Able to carry on normal activity, minor symptoms or signs of disease.	90	1
Normal activity with effort, some signs of symptoms of disease.	80	
Cares for self, unable to carry on normal activity or to do active work.	70	2
Requires occasional assistance, but is able to care for most needs.	60	
Requires considerable assistance and frequent medical care.	50	3
Disabled, requires special care and assistance.	40	
Severely disabled, hospitalization is indicated although death is not imminent.	30	4
Very sick, hospitalization necessary, active supportive treatment necessary.	20	
Moribund, fatal processes progressing rapidly.	10	5
Dead	0	

* Karnofsky DA, Abelman WH, Craver LF, Burchenal JH. The use of nitrogen mustards in the palliative treatment of carcinoma. cancer 1948;1:634-656

¶Oken M, Creech RH, Tormey DC, Horton J, Davis TE, McFadden E, Carbone PP. Toxicity and response Criteria of the Eastern Cooperative Oncology Group.
AM J Clin Oncol (CCT) 1982;5:649-655.

APPENDIX D

Toxicity	0 None	1 (Mild)	2 (Moderate)	3 (Severe)	4 (Maximal/Life Threatening)
TENDERNESS	None	Pain elicited on deep palpation	Painful to touch or light palpation	Severe pain to touch or light palpation	Excruciating
PAIN (AT INJECTION SITE)	None	Noticeable but no effect on activity	Impairs activity, may require mild analgesics	Severe pain, requires narcotic analgesic	Incapacitating, requires repeated narcotics
PRURITUS	None	Noticeable but no effect on activity	Impairs activity	Impairs function and requires steroids	Requires hospitalization
LOCAL REACTION	None	Erythema, slight induration	Marked induration; vesiculation	Ulceration, may require debridement	Exfoliation, necrosis, requires plastic surgery

WHO TOXICITY GUIDELINES

CALGB Expanded Common Toxicity Criteria

(Adapted from Common Toxicity Criteria, SWOG Toxicity Criteria, CALGB Toxicity Grading)

TOXICITY	GRADE 0	1	2	3	4
Hematologic					
WBC	≤4.0	3.0-3.9	2.0-2.9	1.0-1.9	<1.0
PLT	WNL	75.0-normal	50.0-74.9	25.0-49.9	<25.0
Hgb	WNL	10.0-normal	8.0-10.0	6.5-7.9	<6.5
Granulocytes/bands	≥2.0	1.5-1.9	1.0-1.4	0.5-0.9	<0.5
Lymphocytes	≥2.0	1.5-1.9	1.0-1.4	0.5-0.9	<0.5
Hematologic-Other		Mild	Moderate	Severe	Life-threatening
Hemorrhage (clinical)	None	Mild, no transfusion	Gross, 1-2 units transfusion per episode	Gross, 3-4units transfusion per episode	Massive, >4 units transfusion per episode
Infection	None	Mild - no active treatment (e.g., viral syndrome)	Moderate - requires outpatient PO antibiotic	Severe - requires IV antibiotic or antifungal or hospitalization	Life-threatening e.g. septic shock
Gastro-intestinal					
Nausea	None	Able to eat reasonable intake	Intake significantly decreased but can eat	No significant intake	
Vomiting	None	1 episode in 24 hours	2-5 episodes in 24 hours	6-10 episodes in 24 hours	>10 episodes in 24 hr or requiring parenteral support
Diarrhea	None	Increase of 2-3 stools/day over pre-Rx	Increase of 4-6 stools/day, or nocturnal stools, or moderate cramping	Increase of 7-9 stools/day, or incontinence, or severe cramping	Increase of ≥10 stools/day or grossly bloody diarrhea, or need for parenteral support
Stomatitis	None	Painless ulcers, erythema, or mild soreness	Painful erythema, edema, or ulcers, but can eat	Painful erythema, edema, or ulcers, and cannot eat	Requires parenteral or enteral support
Esophagitis/ dysphagia	None	Painless ulcers, erythema, mild soreness or mild dysplasia	Painful erythema, edema or ulcers or moderate dysphagia but can eat without narcotics	Cannot eat solids or requires narcotics to eat	Requires parenteral or enteral support or complete obstruction or perforation
Anorexia	None	Mild	Moderate	Severe	Life-threatening
Gastritis/ ulcer	No	Antacid	Requires vigorous medical management or nonsurgical treatment	Uncontrolled by medical management; requires surgery for GI ulceration	Perforation or bleeding
Small bowel obstruction	No	-	Intermittent, no intervention	Requires intervention	Requires operation
Intestinal fistula	No	-	-	Yes	-
GI - other	-	Mild	Moderate	Severe	Life-threatening
Other Mucosal	None	Erythema, or mild pain not requiring treatment	Patchy and produces serosanguinous discharge or requires non-narcotic for pain	Confluent fibrinous mucositis or requires narcotic for pain or ulceration	Necrosis
Liver					
Bilirubin	WNL	-	<1.5 x N	1.5-3.0 x N	>3 x N
Trans-aminase (SGOT,SGPT)	WNL	≤2.5 x N	2.6-5.0 x N	5.1-20.0 x N	>20.0 x N
Alk Phos or 5' nucleotidase	WNL	≤2.5 x N	2.6-5.0 x N	5.1-20.0 x N	>20 x N
Liver- clinical	No change from baseline	-	-	Precoma	Hepatic coma
Liver - other	-	Mild	Moderate	Severe	Life-threatening
Weight gain	<5%	5.0-9.9%	10.0-19.9%	≥20.0%	-
Weight loss	<5%	5.0-9.9%	10.0-19.9%	≤20.0%	-

TOXICITY	0	1	GRADE 2	3	4
Kidney, Bladder					
Creatinine	WNL	<1.5 x N	1.5-3.0 x N	3.1-6.0 x N	>6.0 x N
Proteinuria	No change	1+ or <0.3 g/100 mL or <3 g/l	2-3+ or 0.3-1.0 g/100 mL or 3-10 g/l	4+ or >1.0 g/100 mL or >10 g/l	Nephrotic syndrome
Hematuria	Neg	Micro only	Gross, no clots	Gross + clots	Requires transfusion
BUN mg %	WNL,<20	21-30	31-50	>50	-
Hemorrhagic cystitis	None	Blood on microscopic exam	Frank blood no treatment required	Bladder irrigation required	Requires cystectomy or transfusion
Renal Failure	-	-	-	-	Dialysis required
Other Kidney /Bladder:					
Incontinence	Normal	With coughing, sneezing,etc.	Spontaneous some control	No control	-
Dysuria	None	Mild pain	Painful or burning urination, controlled by pyridium	Not controlled by pyridium	-
Urinary retention	None	Urinary residual >100 cc or occasionally requires catheter or difficulty initiating urinary stream	Self catheterization always required for voiding	Surgical procedure required (TUR or dilation)	-
Increased frequency/ urgency	No change	Increase in frequency or nocturia up to 2x normal	Increased >2x normal, but < hourly	With urgency and hourly or more or requires catheter	-
Bladder cramps	None	-	Yes	-	-
Ureteral obstruction	None	Unilateral, no surgery required	Bilateral, no surgery required	Not complete bilateral, but stents, nephrostomy tubes or surgery required	Completed bilateral obstruction
GU Fistula	None	-	-	Yes	-
Kidney/ Bladder-other	-	Mild	Moderate	Severe	Life-threatening
Alopecia	No loss	Mild hair loss	Pronounced or total hair loss		
Pulmonary					
Dyspnea	None or no change	Asympto-matic with abnormality in PFT's	Dyspnea or significant exertion	Dyspnea at normal level of activity	Dyspnea at rest
PO_2/PCO_2	No change or PO_2 >85 and PCO_2 ≤40	PO_2 >70 and PCO_2 ≤50, but not grade 0	PO_2 >60 and PCO_2 ≤60, but not grade 0-1	PO_2 >50 and PCO_2 ≤70 but not 0-2	PO_2 ≤50 or PCO_2 >70
Carbon Monoxide diffusion capacity (DLCO)	>90% of pretreatment value	Decrease to 76-90% of pretreatment	Decrease to 51-75% of pretreatment	Decrease to 26-50% pretreatment	Decrease to ≤ 25% of pretreatment
Pulmonary fibrosis	Normal	Radiographic changes, no symptoms	-	Changes with symptoms	
Pulmonary edema	None	-	-	Radiographic changes and diuretics required	Requires intubation
Pneumonitis (non-infectious)	Normal	Radiographic changes, symptoms do not require steroids	Steroids required	Oxygen required	Requires assisted ventilation
Pleural effusion	None	Present	-	-	-
Adult respiratory distress syndrome (ARDS)	None	Mild	Moderate	Severe	Life-threatening
Cough	No change	Mild, relieved by OTC meds	Requires narcotic antitussive	Uncongrolled coughing spasms	-
Pulmonary - other	-	Mild	Moderate	Severe	Life-threatening

TOXICITY	GRADE 0	1	2	3	4
Heart					
Cardiac dys-rhythmias	None	Asymptomatic, transient, requiring no therapy	Recurrent or persistent, no therapy required	Requires treatment	Requires monitoring, or hypotension, or ventricular tachycardia, or fibrillation
Cardiac function	None	Asymptomatic, decline of resting ejection fraction by less than 20% of baseline value	Asymptomatic, decline of resting ejection fraction by more than 20% of baseline value	Mild CHF, responsive to therapy	Severe or refractory CHF
Cardiac-ischemia	None	Nonspecific T wave flattening	Asymptomatic ST and T wave changes suggesting ischemia	Angina without evidence for infarction	Acute myocardial infarction
Cardiac - pericardial	None	Asymptomatic effusion, no intervention required	Pericarditis (rub, chest pain, ECG changes)	Symptomatic effusion; drainage required	Tamponade; drainage urgently required
Heart - other		Mild	Moderate	Severe	Life-threatening
Circulatory					
Hypertension	None or no change	Asymptomatic, transient increase by greater than 20 mm Hg (D) or to >150/100 if previously WNL. No treatment required	Recurrent or persistent increase by greater than 20 mm Hg (D) or to >150/100 if peviously WNL. No treatment required	Requires therapy	Hypertensive crisis
Hypotension	None or no change	Changes requiring no therapy (including transient orthostatic hypotension)	Requires fluid replacement or other therapy but not hospitalization	Requires therapy and hospitalization; resolves within 48 hrs of stopping the agent	Requires therapy and hospitalization for >48 hr after stopping the agent
Phlebitis/ thrombosis/ embolism	-	-	Superficial phlebitis (not local)	Deep vein thrombosis	Major event (cerebral/ hepatic/ pulmonary/ other infarction) or pulmonary embolism
Edema	None	1+ or dependent throughout in evening only	2+ or dependent throughout day	3+	4+, generalized anasarca
Dermatologic					
Skin	None or no change	Scattered macular or papular eruption or erythema than is asymptomatic	Scattered macular or papular eruption or erythema with pruritis or other associated symptoms	Generalized symptomatic macular, papular, or vesicular eruption	Exfoliative dermatitis or ulcerating dermatitis
Local	None	Pain	Pain and swelling with inflammation or phlebitis	Ulceration	Plastic surgery indicated
Neurologic					
Neurosensory	None or no change	Mild paresthesias, loss of deep tendon reflexes	Mild or moderate objective sensory loss; moderate paresthesias	Severe objective, sensory loss or paresthesias that interfere with function	-
Neuromotor	None or no change	Subjective weakness; no objective findings	Mild objective weakness without significant impairment of function	Objective weakness with impairment of function	Paralysis
Neurocortical	None	Mild somnolence or agitation	Moderate somnolence or agitation	Severe: somnolence or agitation or confusion or disorientation or hallucinations or aphasia, or severe difficulty communicating	Coma, seizures, toxic psychosis
Neurocere-bellar	None	Slight incoordination, dysdiadokinesis	Intention tremor, dysmetria, slurred speech, nystagmus	Locomotor ataxia	Cerebellar necrosis
Neurologic - Other		Mild	Moderate	Severe	Life-threatening

TOXICITY	GRADE 0	1	2	3	4
Allergy	None	Transient rash, drug fever <38C, 100.4F	Urticaria, drug fever ≥38C, 100.4F, mild bronchospasm	Serum sickness, bronchospasm, req. parenteral meds	Anaphylaxis
Flu-like symptoms					
Fever in absence of infection	None	37.1-38.0C 98.7-100.4F	38.1-40.0C 100.5-104.0F	>40.0C >104.0F for less than 24 hours	>40.0C (104.0F) for more than 24 hrs or fever accompanied by hypotension
Chills	None	Mild or brief	Pronounced and prolonged	-	-
Myalgia/ arthralgia	Normal	Mild	Decrease in ability to move	Disabled	-
Sweats	Normal	Mild and occasional	Frequent or drenching	-	-
Malaise/ fatigue	None	Mild, able to continue normal activities (PS1)	Impairment of normal daily activity or bed rest <50% of waking hours (PS2)	In bed or chair >50% of waking hrs (PS3)	Bedridden or unable to care for self (PS4)
Flu-like symptoms -other	-	Mild	Moderate	Severe	Life-threatening
Metabolic					
Hyperglycemia	<116	116-160	161-250	251-500	>500 or ketoacidosis
Hypoglycemia	>64	55-64	40-54	30-39	<30
Amylase	WNL	<1.5 x N	1.5-2.0 x N	2.1-5.0 x N	>5.1 x N
Hypercalcemia	<10.6	10.6-11.5	11.6-12.5	12.6-13.5	≥13.5
Hypocalcemia	>8.4	8.4-7.8	7.7-7.0	6.9-6.1	≤6.0
Hypomagnesemia	>1.4	1.4-1.2	1.1-0.9	0.8-0.6	≤0.5
Hyponatremia	No change or >135	131-135	126-130	121-125	≤120
Hypokalemia	No change or >3.5	3.1-3.5	2.6-3.0	2.1-2.5	≤2.0
Metabolic - other	-	Mild	Moderate	Severe	Life-threatening
Coagulation					
Fibrogen	WNL	0.99-0.75 x N	0.74-0.50 x N	0.49-0.25 x N	≤0.24 x N
Prothrombin time	WNL	1.01-1.25 x N	1.26-1.50 x N	1.51-2.00 x N	>2.00 x N
Partial thrombo-plastin time	WNL	1.01-1.66 x N	1.67-2.33 x N	2.34-3.00 x N	>3.00 x N
Coagulation - other	-	Mild	Moderate	Severe	Life-threatening
Endocrine					
Impotence/ libido	Normal	Decrease in normal function	-	Absence of function	-
Sterility	-	-	-	Yes	-
Amenorrhea	No	yes	-	-	-
Other Endocrine:					
Gynecomastia	Normal	Mild	Pronounced or painful	-	-
Hot flashes	None	Mild or <1/day	Moderate and ≥1/d	Frequent and interferes with normal function	-
Cushingoid	Normal	Mild	Pronounced	-	-
Endocrine - other	-	Mild	Moderate	Severe	Life-threatening
Eye					
Conjunctivitis/ keratitis	None	Erythema or chemosis not requiring steroids or antibiotics	Requires treatment with steroids or antibiotics	Corneal ulceration or visible opacification	
Dry eye	Normal	-	Requires artificial tears	-	Requires enucleation
Glaucoma	No change	-	-	Yes	-
Eye - other	-	Mild	Moderate	Severe	Life-threatening

THE UNIVERSITY OF TEXAS
M.D. ANDERSON CANCER CENTER

INFORMED CONSENT

PROTOCOL TITLE: **Phase Ib Trial of HER-2/*neu* Peptide (E75) Vaccine in Patients with Breast and Ovarian Cancer**

1. _____ _____
Participant's Name I. D. Number

You have the right to know about the procedures that are to be used in your participation in clinical research so as to afford you an opportunity to make the decision whether or not to undergo the procedure after knowing the risks and hazards involved. This disclosure is not meant to frighten or alarm you; it is simply an effort to make you better informed so you may give or withhold your consent to participate in clinical research. This informed consent does not supersede other informed consents you may have signed.

2. PURPOSE OF STUDY

The purpose of this clinical research study is to determine whether E75, a small peptide vaccine derived from a larger protein called HER-2/neu found on approximately 30-50% of breast and ovarian cancers, can stimulate anti-tumor immunity. To better stimulate immunity, the peptide will be combined with a recombinant protein, granulocyte-macrophage colony stimulating factor (rhGM-CSF). Whether any anti-tumor effects occur from vaccination will also be determined. E75 and rhGM-CSF are investigational agents.

OPTIONAL PROCEDURES

In selected patients, we will also determine whether E75 peptide is found on tumor cells after treatment.

3. DESCRIPTION OF RESEARCH

Prior to study, all patients will have routine scans and x-rays performed to document disease. Patients with ovarian cancer with a specific blood test elevated CA125 levels but without measurable tumors are also eligible. Prior to treatment, all

patients will have tumor analyzed for expression of the HER-2/neu receptor and another molecule, HLA-A2. Ideally, small biopsies of accessible tumors will be performed, or, if not feasible, old tissue blocks will be used. In addition, patients will have 10cc of blood drawn to test for the presence of the HLA-A2 molecule and to determine whether the lymphocytes respond to an immune stimulus. If all the above tests are positive, patients will be eligible to receive intradermal (into the skin) injections of E75 plus rhGM-CSF. Injections will be given weekly for 4 weeks and then monthly for up to 6 months provided the treatment is well tolerated and tumors remain stable or regress. Patients whose cancers significantly increase in size in spite of treatment at 8 weeks may have vaccine discontinued at the discretion of the investigator. Sixty mls of blood (approximately 1 cupful) will be obtained at monthly intervals in order to test for specific immune responses to the vaccine. In addition, prior to beginning injections and at 8 weeks, patients will be skin tested (have the skin pricked) for recall antigens (such as tetanus) and E75 peptide. At 8 weeks patients will have repeat x-rays and scans to determine tumor response. Approximately 9-15 patients will be treated on this study. Patients must be willing to remain in Houston for 24 hours after each injection to determine toxicity and local reactions to vaccine.

OPTIONAL PROCEDURES

Selected patients with more than two accessible tumors greater than 1 cm in size may have an excisional biopsy before and following treatment to determine if E75 peptide is found on tumor cells and to determine if patient's immune lymphocytes are capable of killing their own tumor.

4. RISKS, SIDE EFFECTS AND DISCOMFORTS TO PARTICIPANTS

Similar vaccines have been given to many other cancer patients over the past 2-3 years; in general, injections have been well tolerated without life threatening side effects.

Possible reactions to E75 peptide include, pain and inflammation at the injection site, fever, chills, and muscle aches. It is also possible that certain allergic reactions could occur including joint swelling and/or inflammation, skin rash, and rarely, inflammation of blood vessels in liver or kidney.

Side effects with rhGM-CSF include bone pain, skin rash, inflammation of the veins and fluid retention. There is also the possibility of additional as well as, unknown

side effects from the combination of peptide and rhGM-CSF. Blood drawing could cause pain and swelling at the injection site.

4a. This clinical research study may involve unforeseeable risks to the participant. This clinical research may involve unforeseeable risks to unborn children; therefore, the participants should practice adequate methods of birth control throughout the period of their involvement in the clinical study if they are sexually active. To help prevent injury to children, the female participants should refrain from breast feeding during participation in the clinical research study.

OPTIONAL PROCEDURES

Excisional biopsy of tumors may result in bleeding infection and discomfort.

5. POTENTIAL BENEFITS

Treatment: Since this is a Phase I study, the benefits for the individual may be minimal. However, if anti-tumor effects are seen, patients may have a prolongation of survival.

OPTIONAL PROCEDURES

There are no potential benefits for the individual patients undergoing the optional procedures. Future patients may benefit from the information gathered from this clinical research study. This information may or may not be useful for the participants but will enable physicians to learn more about the use of this and other vaccines in cancer treatment.

6. ALTERNATE PROCEDURES OR TREATMENTS

Treatment: Alternate forms of treatment may include chemotherapy, radiation and/or surgery or other investigational agents..

OPTIONAL PROCEDURES

Treatment with vaccine may be given without giving consent to for tumor biopsy.

UNDERSTANDING OF PARTICIPANTS

7. I have been given an opportunity to ask any questions concerning the treatment involved and the investigator has been willing to reply to my inquiries. This treatment will be administered under the above numbered, titled, and described clinical research protocol at this institution. I hereby authorize Dr. _____, the attending physician/investigator, and designated associates to administer the treatment.

8. I have been told and understand that my participation in this clinical research study is voluntary. I may decide not to participate, or withdraw my consent and discontinue my participation at any time. Such action will be without prejudice and there shall be no penalty or loss of benefits to which I may otherwise be entitled, and I will continue to receive treatment by my physician at this institution.

   Should I decide not to participate or withdraw by consent from participation in this clinical research, I have been advised that I should discuss the consequences or effects of my decision with my physician.

   In addition, I understand that the investigator may discontinue the clinical research study if, in the sole opinion and discretion of the investigator, the study or treatment offers me little or no future benefit, or the supply of medication ceases to be available, or other causes prevent continuation of the clinical research study. The investigator will notify me should such circumstances arise and my physician will advise me about available treatments which may be of benefit at that time.

   I will be informed of any new findings developed during the course of this clinical research study, which may relate to my willingness to continue participation in the study.

9. I have been assured that confidentiality will be preserved except that, if applicable, qualified monitors from the Food and Drug Administration (FDA), may review my records where appropriate and necessary. Qualified monitors shall include assignees authorized by Surveillance Committee of this institution provided that confidentiality is assured and preserved. My name will not be revealed in any reports or publications resulting from this study, without my expressed consent. In special circumstances, the FDA might be required to reveal the names of participants.

10. I have been informed that should I suffer any injury as a result of my participation in this research activity, medical facilities are available for treatment at this institution. I understand however that I cannot expect to receive any credit, reimbursement for expenses from this institution or any financial compensation from this institution for such injury of financial compensation for such an injury.

11. I have been informed that I should inquire of the attending physician whether or not there are any services, investigational agents or devices, and/or medications being offered by the sponsor of this clinical research project at a reduced cost or without cost. Should the investigational agent become commercially available during the course of the study, I understand that I may be required to cover the cost of the subsequent doses.

    Costs related to my medical care including expensive drugs, tests or procedures that my be specifically required by this clinical research study shall be my responsibility unless the sponsor or other agencies contribute toward said costs. I have been given the opportunity to discuss the expenses or costs associated with my participation in this research activity.

12. It is possible that this research project will result in the development of beneficial treatments, devices, new drugs, or possible patentable procedures, in which event I understand that I cannot expect to receive any compensation or benefits from the subsequent use of information acquired and developed through my participation in this research project.

13. I understand that refraining from breast feeding and practicing effective contraception are medically necessary and a prerequisite for my participation in this clinical research study. Should contraception be interrupted or if there is any suspicion of pregnancy, my participation in this clinical research study will be terminated at the sole discretion of the investigator.

14. I may discuss question or problems during or after this study with Dr. James L. Murray at (713) 792-4561. In addition, I may discuss any problems I may have or any questions regarding my rights during or after this study with the Chairman of the Surveillance Committee at (713) 792-2933 and may in the event any problem arises during this clinical research, contact the parties named above.

CONSENT

Based upon the above, I consent to undergo the described procedure, and have received a copy of the consent form.

Date	Signature Of Participant
Witness Other Than Physician Or Investigator	Signature Of Person Responsible And Relationship

I have discussed this clinical research study with the participant and/or his or her authorized representative, using a language which is understandable and appropriate. I believe that I have fully informed this participant of the nature of this study and its possible benefits and risks and I believe the participant understood this explanation.

Physician/Investigator

Identification of Naturally Processed Human Ovarian Peptides Recognized by Tumor Associated CD8+ CTL

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[2]*Abbreviations*: antigen, Ag, HER-2/neu proto-oncogene, HER-2; reverse phase high pressure liquid chromatography, HPLC; mass spectrometry, MS; trifluoroacetic acid, TFA; acetonitrile, ACN;; T cell receptor, TCR, Retention time, Rt; Fraction, Fxn., Antigen presenting cell, APC.

Key Words: natural peptides, CTL epitopes, ovarian cancer.

Running title: HLA-A2 associated peptides on ovarian tumors

Acknowledgments. The authors wish to thank Ms. Susan Mondragon for outstanding editorial assistance. The authors also wish to thank Dr. Victor Engelhard and Dr. John Lambris for fruitful discussions and advice and Dr. William E. Seifert for mass-spectrometric analysis.

Abstract

Identification of naturally processed peptides recognized by tumor specific CTL may lead to epitope specific tumor vaccines. Since these epitopes may be differently expressed on epithelial tumors and may differ in their ability to induce CTL in vivo, we have isolated the HLA-A2-peptide complexes by immunoaffinity from an established ovarian tumor line transfected with and expressing HLA-A2 gene. HPLC fractionated peptides were used to reconstitute epitopes recognized on HLA-A2 by three HLA-A2+ CD8+ CTL lines. These lines recognized at least three of the same groups of fractions (designated SKOV3.A, B, and C) but showed differences in the pattern of recognition of other fractions. To gain insight in the epitope distribution by freshly isolated ovarian tumors we compared the recognition of peaks SKOV3.B and C with the corresponding peaks from an ovarian tumor (OVA-6) which expressed similar levels of HLA-A2, using one of these lines (CTL-OVA-5) as indicator. CTL-OVA-5 recognized a large number of epitopes from peaks B and C rechromatographed on more resolving HPLC gradients. While a number of peaks appeared to be coincident on both SKOV3 and OVA-6 an even higher number appeared either not to, or to only partially overlap. These findings, which represent the first analysis of the epitopes presented by a patient tumor, suggest that the use of tumor lines derived peptides for vaccination may require selection of the epitopes corresponding to the ones presented by freshly isolated human tumors.

Introduction

The development of rational immunotherapy approaches for human cancers will depend on a detailed understanding and quantitation of the host anti-tumor responses. These responses involve, (a) recognition by effector CTL of specific epitopes on malignant tissues and (b) immunogenic epitopes, i.e. peptides that can induce in vitro and in vivo anti-tumor response. CTL epitopes are short peptides (8-10 amino acids long) that are presented on the cell surface by MHC class I molecules (1). Since peptides of distinct sequences can be distinguished by their physicochemical characteristics (2), elution under defined pH and concentration conditions, proffers an approach to map the universe of epitopes recognized by tumor-reactive CTL. This may allow us to establish which peptides (or groups of peptides) correspond to common dominant epitopes recognized, by ex vivo induced tumor-reactive CTL. Due to TCR plasticity the common epitopes can be reconstituted by either unique or cross-reactive peptides (3, 4). Furthermore, for CTL induction, tumor antigens may be presented to T cells by professional APC (5), which can process particulate antigens for MHC-class I presentation (6, 7). Thus, identification of the natural peptides presented in association with MHC molecules on human tumors should be important in the development of novel approaches for tumor specific CTL induction.

To date, with the few exceptions of melanoma Ag^2, gp100 and MART-1 (8-10), there is limited information on the identity and density of antigenic peptides and CTL epitopes presented by human solid tumors. The presence, distribution and density of these epitopes on freshly isolated epithelial tumors is still unknown. Our studies as well as research from other laboratories have shown that multiple epitopes can be recognized on epithelial tumors (i.e. ovarian, colon, pancreatic, breast and lung) (11-13). Since acid-eluted peptides have been shown to be immunogenic, by inducing anti-tumor immunity to established model tumors (14, 15), this raises the need for adequate sources of tumor epitopes for human studies. This can be accomplished with freshly isolated primary tumors only rarely because the small amount of peptides recovered preclude peptide sequence analysis. An approach to overcome these limitations is to use ovarian and breast tumor lines expressing the MHC-class I molecule of interest to address the question whether the patterns of epitope recognition by tumor-reactive CTL on the lines and freshly isolated human tumor are similar.

To address these questions, we have investigated the identity of epitopes presented by HLA-A2 on tumor targets recognized by HLA-A2$^+$ CD8$^+$ CD4$^-$ CTL lines isolated from patients with ovarian cancer. In this report we characterized the pattern of CTL epitopes extracted from a freshly isolated ovarian tumor and HPLC-separated by two consecutive gradients of acetonitrile (ACN), and we identified common and distinct epitopes between this tumor and HLA-A2 bound peptides fractionated from an established ovarian tumor line SKOV3. Although recognition of a number of epitopes defined as bioactive peaks eluting with distinct retention times appeared to correlate with the cell number used and the levels of HLA-A2, on these tumors for some common epitopes such correlation was not observed suggesting that MHC class I expression is not the only determining factor in tumor Ag expression.

Materials and Methods

Tumor Cells and Cell Lines. The following human tumor lines were used in these experiments: *(1)* ovarian tumor line SKOV3 (HLA-A3, A28, B18, B35, Cw5) stably transfected with the gene for HLA-A2 (16). The gene for HLA-A2 was kindly provided by Dr. William E. Biddison (NIAID, NIH). A tumor clone, SKOV3.A2.1E4, expressing high levels of both HER-2 and HLA-A2 has been designated as 1E4 and was selected for expansion in large numbers and peptide fractionation experiments; and *(2)* C1R:A2 cells (a kind gift from Dr. Biddison), which express only HLA-A2 on the surface, stably transfected in our laboratory with the gene encoding for the HER-2 proto-oncogene (plasmid pCMV.HER-2 encoding a full length HER-2.cDNA). This plasmid was a kind gift of Dr. Mien-Chie Hung, Department of Tumor Biology (MDACC). C1R.A2 cells were co-transfected with the plasmid SV2.Hygro (ATCC). HLA-A2 transfected SKOV3 and C1R:A2 cells were selected with 250 μg/ml G418 (GIBCO, BRL, Gaithersburg, MD). C1R.A2.HER-2 cells were also selected with 50 μg/ml of Hygromycin B in addition to G418. This concentration was found to result in the death of over 50% of untransfected C1R:A2 cells within 4 days in parallel experiments. After selection with Hygromycin B, C1R.A2.HER-2 cells were cloned by stringent limiting dilution. At least twenty C1R.A2.HER-2 clones (designated HER-2.A - T) were isolated expressing variable levels of HER-2 receptor on their surface. A clone of C1R:A2.HER-2 cells designated here as HER-2.J was selected for further studies.

Ovarian tumor cells were collected from the ascitic fluid of a patient with epithelial ovarian cancer and separated from debris and lymphocytes by centrifugation over two gradients of Ficoll as we have previously described (17, 18). They were HLA-A2$^+$ and expressed high levels of HER-2 (HER-2^{hi}). These tumor cells were designated as OVA-6.

Effector CTL lines. T cell lines from tumor associated lymphocytes (TAL) were grown in RPMI 1640 medium containing 10% FCS and supplemented with 2 mM L-glutamine, and 100 μg/ml gentamycin (complete RPMI medium) and 50 - 100 U/ml of IL-2 (Cetus). Most effectors expanded as T cell lines contained >95% CD3$^+$ cells and variable proportions of CD8+ and CD4+ cells. For these experiments, CD8$^+$ cells were selected on mAb coated plates as we described (16). The resulting cells were >95% CD3$^+$ CD8$^+$ cells. Effector CTL-3 used in these studies has been previously reported (16). CTL-4 and CTL-5 were also

obtained from TAL from other patients with adenocarcinoma of the ovary. The corresponding tumors were HLA-A2^{+} and overexpressed HER-2 (HER-2^{hi}).

Immunofluorescence. Target cells were tested in fluorescence experiments to confirm the expression of HLA-A2, MHC class I, and HER-2, as previously described (15). Hybridomas secreting mAb BB7.2, MA2.1 and GAR-3 (HLA-A3 specific) were obtained from ATCC. In brief, OVA-6, SKOV3, and HER-2.J cells were incubated with mAb specific for MHC-class I and *Ab2* specific for the extracellular domain of HER-2 (Oncogene Science) followed by FITC conjugated goat-anti-mouse IgG. Surface antigen expression was determined by FACS using a FACSscan (Beckton-Dickinson) with a log amplifier. CD3, CD4 and CD8 antigen expression on the effectors was determined by immunofluorescence with corresponding mAb. C1R.A2 clones were designated as HER-2^{hi} when MCF for HER-2 expression was $\geq$40. Expression of CD18, CD11a (LFA-1), CD54 (ICAM-1) and CD58 (LFA-3) was also tested the corresponding antibodies (Beckton Dickinson).

Tumor peptide extraction. SKOV3.A2.1E4 cells were grown in 10 chamber cell factories (Nunc, Thousand Oaks, CA) in complete RPMI medium. Between 1.0 - 1.5. x 10^9 cells were obtained from one cell factory. For these studies, at least 10^{10} cells of the SKOV3.A2.1E4 cloned line were grown in batches of 1.5 – 2.0 x 10^9 cells. Cells were collected and washed three times with cold PBS. Further, cells were lysed using the buffer previously described by Engelhard, Slingluff, and collaborators (19) containing protease inhibitors (aprotinin, leupeptin, pepstatin, PMSF, iodacetamide), in PBS with the difference that CHAPS 0.5% was used as lysing agent to minimize binding to C18 columns (V. Engelhard, personal communication). This solution is designated here as lysis buffer. MgCL2 at 6 mM and Glycerol at 20% final concentration were included in the lysis buffer to minimize denaturation of extracted proteins. Detergent-solubilized extracts of SKOV3.A2 and OVA-6 cells were obtained after centrifugation at 40,000g for 2h. HLA-A2.1 was isolated from the supernatant obtained from SKOV3.A2 cells by affinity chromatography on Protein A-Sepharose prebound with mAb BB7.2, as described (19) except that the cell extracts were pre-absorbed on Protein-A Sepharose (Sigma Chemical Co., St. Louis, MO) to minimize non-specific binding. The affinity column was washed with PBS containing 0.25M NaCl with monitoring of the OD210 nm (for peptide bond), then eluted with 0.2M acetic acid in 1.0 ml fractions. Fractions containing material absorbing at 210 nm were boiled for 5 min to allow

dissociation of peptides from HLA-A2, centrifuged through an Ultrafree CL unit (3-kD cut-off) (Millipore) and lyophilized. Peptides with masses < 3kD were pooled, lyophilized and separated by reverse phase-high pressure liquid chromatography (HPLC). This approach yields primarily HLA-A2 bound peptides and to a much lesser extent peptides associated with other MHC-class I molecules.

Since freshly isolated ovarian tumors are unique specimens and the corresponding MHC-class I associated peptides are important for identification of epitopes presented by other HLA-molecules, peptides were extracted from OVA-6 centrifuged lysates with 0.1% TFA following the approaches of Eisen and collaborators (20, 21). This approach has the advantage of eluting peptides with affinity high for MHC-class I. Peptides of mass of 3kD or less were isolated by centrifugation through filters with a cut-off of 3kD, lyophilized and separated by HPLC chromatography.

Fractionation of HLA-A2 bound peptides. Tumor peptides extracted from HLA-A2.1 molecules of SKOV3.A2 cells were separated in the first dimension on a Brownlee C18 Aquapore column of 2.1 x 30 mm, pore size, 300 A; particle size, 7µm, (Applied Biosystems, Perkin-Elmer Corporation) and eluted with a 60 min gradient of 0-60% (vol/vol) acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 200 µl/min using an HPLC system Model 1090 (Applied Biosystems). HER-2 peptides E75 (369-377), C85 (971-979), E90 (789-797) and E89 (851-859) identified in the previous studies to be recognized by ovarian CTL-3 line were separated under the same conditions. Their retention times (Rt) were determined, and their resolution by these HPLC conditions was established.

For the second dimension HPLC separation, fractions from the first dimension including the peaks of CTL activity were pooled in two groups B (Fxn 27 - 33), and C (Fxn 37 - 45). Each group was lyophilized and reconstituted in the elution buffer corresponding to the gradient used in the second dimension (described below). Then, peptides were injected into a Brownlee C18 Aquapore column of 2.1 x 220 mm, (300A, 7µm) and eluted with shallower ACN gradients. The flow rate was 200 µl/min and fractions were collected at 1 min intervals. Peak B (Fxn 27 - 33) were separated with a gradient of 0.1% TFA in H20, 0 - 5 min, 6 - 15 min 0.1% TFA in 0 - 20% ACN (2%/min), and 16 - 55 min, 0.1% TFA in 20 - 40% ACN (0.5% increment/min)

56 - 60 min. 0.1% TFA in 40% ACN (designated as Gradient II). Peak C (Fxn 37 - 45) were separated in a linear gradient of 0 - 60 min of 30 - 50% ACN in 0.1% TFA thus, with an increment of ACN concentration of 0.3%/min (designated as Gradient III). These gradients were selected to allow better resolution of peptides eluting in the corresponding concentration of ACN in the first dimension. For example peptides eluting in the first dimension between 37 - 45% ACN over 8 min, were separated with a gradient of 30 - 50% ACN over 60 min. Peptides extracted from the freshly isolated tumor OVA-6 were separated by two rounds of HPLC under identical conditions as SKOV3 peptides, to allow comparison of biological activity.

Antigens. Synthetic HER-2 peptides E75, G89, C85, E90, E91, E92, and the EGF-R peptide F49 (22) were prepared by the Synthetic Antigen Laboratory at the M.D. Anderson Cancer Center using a solid phase method and purified by HPLC as described (23). Identity of peptides was established by amino acid analysis. The purity of peptides was more than 97%.

CTL epitopes reconstitution. Reconstitution of CTL epitopes was performed using HPLC fractions from the first and second HPLC dimensions. 50µl aliquots of each fraction were concentrated by vacuum centrifugation (Speed Vac) to approximately 1/10 of its original volume to remove TFA and ACN, reconstituted with water, re-concentrated, then reconstituted with RPMI medium to the initial volume and added to ^{51}Cr labeled T2 cells in V bottom microtiter plates. After pre-incubation with peptides for 90 min, effectors were added at 10:1 - 20:1 E:T ratios and a standard CTL assay was performed for 5h as described (15). Control wells were made with T2 cells incubated with equal volumes of the same fraction of HPLC separated peptides without CTL, to account for direct cytotoxicity of HPLC fraction themselves.

Percentage of specific lysis was determined from the equation (A-B)/(C-B) x 100, where A is the ^{51}Cr release from T2 cells by effectors in the presence of a peptide fraction, B is the release from T2 cells in the presence of the same volume of the same HPLC fraction but in the absence of effectors, and C is the maximum ^{51}Cr release. At least two determinations were made from each fraction.

Results

Similar patterns of HLA-A2 associated recognition for peptides isolated from an established line and a freshly isolated ovarian tumor. To evaluate the role of the transfected HLA-A2 as a presenting molecule for the ovarian $CD8^+$ CTL, we tested recognition by the ovarian CTL lines CTL-OVA-3 and CTL-OVA-5 of the SKOV3, SKOV3.A2 (1E4), C1R.A2 and C1R.A2.HER-2.J cells in parallel with OVA-6. SKOV3 and SKOV3.A2. cells are of identical origin. Introduction of the HLA-A2 gene is expected to allow presentation of a number of endogenously processed peptides. Similarly, C1R.A2 and C1R.A2.HER-2.J cells are of identical origin. C1R.A2 cells express a complete and functional antigen-processing and transport (TAP) system. In these cells introduction of the HER-2 gene is expected to lead to presentation of a number of peptides derived either from HER-2 or from proteins subject to upregulation or stabilization of expression by HER-2 overexpression (24). Since these CTL lines were previously shown to recognize HER-2 peptides (16), failure of the effectors to lyse C1R.A2.HER-2 cells would suggest that peptides recognized are not presented, and/or other peptides derived from HER-2 (which are not recognized) are processed and presented by the C1R.A2.HER-2 cells.

The results in **Figure 1, A, B** show that target recognition by CTL-OVA-3 and CTL-OVA-5 is associated with HLA-A2 expression. Similarly, both CTL-OVA-3 and CTL-OVA-5 recognized OVA-6 **(Figure 1).** Recognition of CTL-OVA-3 and CTL-OVA-5 was inhibited by antibodies to MHC-class I but not to MHC-class II (data not shown).

For comparative analysis of epitope recognition, peptides were extracted from a freshly isolated ovarian tumor, OVA-6 and the clone SKOV3.A2.1E4 (designated SKOV3.A2). Both tumors expressed similar levels of HLA-A2 (SKOV3.A2, 80% $HLA\text{-}A2^+$ cells, mean channel fluorescence (MCF) = 144, OVA-6, 75% $HLA\text{-}A2^+$ cells, MCF = 175). The levels of HER-2 were significantly higher on SKOV3.A2 cells (MCF = 324) compared with OVA-6 (MCF = 160).

To establish whether HLA-A2 bound bioactive peptides extracted from SKOV3, can be detected, we compared the recognition of SKOV3.A2 peptides by two ovarian CTL-lines, CTL-OVA-3 and CTL-OVA-4 **(Figure 2A, B)**. Equal amounts of SKOV3 peptides separated in the first dimension HPLC were used for both CTL lines. Analysis of recognition of SKOV3.A2 (Fxn) fractions 10 -

34, by each of the CTL lines revealed four major peaks of biological activity. Two of three peaks, SKOV3 (22 -27) and SKOV3 (29 - 32) appeared to coincide, while the third peak SKOV3 (18 - 21) to partially overlap. The positions and heights of the peaks of more hydrophilic peptides eluting with smaller retention times (Rt) were different between CTL-OVA-3 and CTL-OVA-4. This may suggest that different antigenic specificities are present in each CTL population. Furthermore, differences in the magnitude of response of CTL-OVA-3 and CTL-OVA-4 to peptides in peaks SKOV3 (22 - 27) and SKOV3 (29 - 32) were observed, suggesting not only the presence of common tumor Ag in the SKOV3 cells, but also differences in the frequency of CTL clones reacting with these peptides. The range of CTL activity observed was similar with the levels reported in recent studies for melanoma (10), and ovarian carcinoma (13).

The ascites which were the source of OVA-6 cells contained mainly tumor cells and very few lymphocytes. These lymphocytes could not be grown in sufficient numbers to perform the CTL assays. The use of high concentrations of Il-2 to promote growth lead to MHC-unrestricted lysis (data not shown). Thus the analysis of OVA-6 peptides used established CTL lines.

Because of the limited amount of fresh tumor material, identification of bioactive peaks was made only with CTL-OVA-5. For epitope reconstitution studies we used peptides extracted from different numbers of SKOV3.A2 and OVA-6 cells. The rationale of this approach is that if an epitope is expressed at similar densities in both tumors as shown by Rammensee and collaborators, the use of different amounts of peptides for epitope reconstitution would be detected as a proportional change in the CTL activity (26). SKOV3 peptides from 9.5 x 10^7 cell equivalents were added in each well in the CTL assay. For OVA-6 the equivalent number was 5.5 x 10^7 cells, resulting in a ratio of 1.8:1.0 between SKOV3 and OVA-6 peptides. This ratio was also maintained and for determination of recognition of peptides separated in the second dimension. Indeed, the cytotoxicity levels detected with CTL-OVA-5 were in most instances higher with SKOV3 than with OVA-6 peptides **(Figures 2 and 3)**, suggesting that the epitopes detected using cell lines are the most stimulatory ones.

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This should be important for peptide quantitation and epitope identification studies since differences in CTL epitopes expression have been correlated in some (26, 27) but not in other studies (3) with different levels of MHC-class I expression. To determine whether a similar pattern of bioactive

peptides is expressed by the freshly isolated ovarian tumor, OVA-6, recognition of OVA-6 peptides separated in the first dimension HPLC, and of SKOV3.A2 peptides was tested in parallel using as effectors CTL-OVA-5.

We focused on fractions with higher retention times in OVA-6, i.e., the elution positions associated with a potential common tumor Ag in Figure 2, and assayed every fraction in this region to optimize resolution of bioactive peaks. The results in **Figure 2C, D** show that a broad peak SKOV3 (20 - 25) corresponds to a double peak of OVA-6 (22 - 26); a broad peak of SKOV3 (28 - 34) corresponds to two resolved peaks OVA-6 (28 - 29) and OVA--6 (31 - 32). A third broad peak of SKOV3 (39 - 44) corresponds to peak OVA-6 (38 - 41). These results indicate that CTL-OVA-5 recognize peptides present in overlapping peaks of activity with CTL-OVA-3 and CTL-OVA-4. These peaks are designated as A, B and C in the **Figure 2C, D**. Furthermore identification of activity in OVA-6 peptide peaks eluting in the same positions with peaks A, B, C with a tolerance of $\pm$1 min (1% ACN difference), suggests the possibility that a number of active tumor peptides may be common between the freshly isolated tumor OVA-6 and the SKOV3.A2 tumor line.

Multiple epitopes recognized by CTL are presented by HLA-A2 on ovarian tumors. Results presented above show that a number of bioactive peaks corresponding to tumor peptides are recognized by ovarian CTL lines. To address the question of whether the epitope repertoire is composed of a limited number of peptides, fractions corresponding to bioactive peaks, A, B, and C from SKOV3.A2 and OVA-6 were subjected to an additional round of HPLC separation. To improve resolution, a longer HPLC column (of 220 mm length) of the same diameter and pore size as the one used in the first dimension was employed. Fractions separated in the first dimension HPLC usually contain a large number of peptides (25). Since not every peptide is active, it is possible that a number of inactive peptides of higher affinity for HLA-A2 compete with the active peptide; thus the observed CTL activity can appear lower than the activity of the fractions subjected to additional rounds of purification (25). The analysis of CTL activity presented here was focused primarily on the peptides of higher Rt eluting in peaks B and C. To afford comparisions, the CTL-OVA-5 was used as indicator in all the assays.

With regard to peak B, which eluted as a broad peak of peptides from SKOV3.A2, and a double peak from OVA-6 **(Figure 2C, D)**, fractions 27 - 33,

were pooled, lyophilized, fractionated using a shallower ACN gradient with increments of 0.5% per min, and subjected to CTL analysis. We focused on the Fxn 30 - 47, because they eluted at ACN concentration ranging between 27 - 35%, i.e. the range where peak B eluted during fractionation in the first dimension. The results are shown in **Figure 3A - B**. SKOV3 peptides eluted in three major peaks of activity designated as: 1 (Fxn. 31 - 36), 2 (Fxn 38 - 42) and 3 (Fxn 43 - 47). OVA-6 peptides eluted in three broad peaks designated as 1 (Fxn 35 - 40), 2 (Fxn 40 - 43) and 3 (Fxn 43 - 47). They were preceeded by a small peak (Fxn 32 - 35) which overlapped with SKOV3, peak B.1. Peak B3 was almost coincident in SKOV3.A2 and OVA-6. The peak CTL values and the shape of peaks B1 and B2 of CTL activity in OVA-6 were different from SKOV3. This suggests the possibility that peptides in peaks B1 and B2 quantitatively and/or qualitatively differ in SKOV3 and OVA-6. The fact that peptides in the peak SKOV3.B1 are more stimulatory than OVA-6 peptides eluting in the same positions, suggest the possibility that these peptides may have been presented and were immunogenic in an earlier stage tumor.

Peak C which eluted as a single peak in both OVA-6 and SKOV3.A2 fractions, and likely contains more hydrophobic peptides than peaks A and B was fractionated using a shallower (0.3% increments/min) and thus more resolving ACN gradient. The results are shown in **Figure 3C, D**. Under these separation conditions peak C resolved in at least 8 distinct peaks of both SKOV3 and OVA-6 peptides. Comparison of the plotted CTL activity over retention time shows that peaks 2 (Fxn 16 - 19) and 3 (Fxn 20 - 23) are almost coincident. These peaks eluted at ACN concentrations ranging between 35.3 - 37.7%. In addition peak 4 is maximal at 24 min with a broad shoulder at 25 - 27 min (38% ACN), but is poorly separated (or recovered) in SKOV3. This suggests that several peptides with very similar retention time endowed with biological activity are present in both OVA-6 and SKOV3.A2 cells.

The shape and peak values of the other peptides, or groups of peptides corresponding to peaks, 1, 5, 6, 7 and 8 show differences between OVA-6 and SKOV3.A2. SKOV3.C.1 (13 - 14) and OVA-6.C.1 (11 - 14) elute at similar positions, but the maximal value of SKOV3.C.1 corresponds to the shoulder of peak OVA-6.C1. Similarly SKOV3.C5 (25 - 29) and OVA-6.C5 (28 - 30) partially overlap. Based on comparison of cytotoxicity values peptides isolated from ovarian cell line appear to be more stimulatory then the peptides isolated from the fresh tumor. The reasons for the observed differences are currently under

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investigation. First it should be noted that under these gradient conditions ±1 min differences in the retention times of peaks of elution reflect differences of only ±0.3% in ACN concentration. Second, the amount of HLA-A2 bound peptides isolated from SKOV3.A2 and OVA-6 cells was different because of different amounts of starting material. Although we isolated a large number of OVA-6 cells the number of OVA-6 cells was significantly lower than the number of SKOV3.A2 cells used in these studies.

To gain insight into the relationship between HLA-A2 expression and epitope density, we compared the cytotoxicity values in the bioactive peaks of SKOV3.A2 and OVA-6 which showed the highest levels of coincidence in their retention times (±1.0 min tolerance). The results are presented in **Table I.** These results show that for peaks C2 and C3 the activity in the SKOV3 fractions was significantly higher then in the corresponding fractions from OVA-6. Particularly for the peak SKOV3.C2 the activity was higher than the one expected from an 1.8:1.0 ratio (tumor line:fresh tumor). The same pattern was observed for the peaks SKOV3.B1, and the partially overlapping peaks B2, C5, C6 and C7. For peak C4, the CTL activity was significantly higher in the OVA-6 fractions. Similarly, the levels of CTL activity in OVA-6 fractions were the same or higher in the peak B3 than the levels observed with SKOV3.A2 fractions. This suggests that if for a number of tumor epitopes the density may correlate with the cell number (and with the levels of HLA expression) for others this does not apply suggesting that they may be preferentially processed and presented by tumor cells.

DISCUSSION

In this report we present evidence that peptides extracted from an established ovarian tumor line and from a freshly isolated ovarian tumor can reconstitute the lytic activity of ovarian CTL isolated from malignant ascites of patients with ovarian cancer. Both ovarian tumors and the effectors shared HLA-A2. Assessment of the number of epitopes recognized suggests that OVA-6 and SKOV3 can present at least 11 distinct epitopes to an ovarian CTL line. Of these eleven epitopes four, one separated in the Gradient II (B3), and three separated in the Gradient III (C2, C3, C4), appear to be shared between SKOV3 and OVA-6, using as comparison factors the retention times and the shapes of the peaks. The number of shared epitopes it is likely to be higher if at least some of the peptides present in the partially overlapping peaks B2, C1, C5 and

C6 are identical in both samples. The retention times of synthetic peptides used as markers, (Figure 2 and 3) show that both Gradients II and III are quite resolving since they can separate the HER-2 peptide: E75 (369 - 377) from the corresponding mutated EGF-R (356 - 364) peptide. Similarly mass-spectrometric analysis of ions presumed to be peptides in peaks of Gradient II and III, show that each ion was present mainly in two and no more then three consecutive HPLC fractions (Dr. W. E. Seifert and B. DaGue, preliminary studies). Thus it is likely that most of the non-overlapping peaks of activity correspond to different epitopes.

The total number of distinct epitopes it is also likely higher, since only the epitopes eluted in two major first dimension HPLC peaks of peptides were analyzed. Indeed preliminary studies in our laboratory indicate that peak A can be resolved in at least five peaks of biological activity from both OVA-6 and SKOV3.A2 cells. A recent study using cultured breast and ovarian tumors had reached similar conclusions regarding the potential number of CTL epitopes in breast and ovarian cancer (28). HER-2 peptides found to be active in previous studies of mapping CTL epitopes with synthetic peptides appeared to co-elute with the major peaks of CTL activity. Preliminary analysis also indicate that the immunodominant HER-2 peptide (369 - 377) co-eluted with the peak B2 in the gradient II. Since these CTL lines were previously shown to recognize E75 it is likely that a peptide with similar retention time forming a similar epitope on T2 cells is presented by both the established line SKOV3 and the freshly isolated ovarian tumor OVA-6.

The epitope repertoire identified is only partially overlapping. The fact that none of the effectors CTL was stimulated with either tumor suggests that the effector repertoire was not altered by the tumor epitope recognition. Two categories of epitopes have been identified: **(a)** overlapping (shared), best illustrated by peaks B2, C2, C3 and C4; and **(b)** non-overlapping, illustrated by peak B1. With respect the overlapping epitopes, for some the cytotoxicity values appear to reflect differences in the cell numbers of SKOV3 and OVA-6 tumors of similar levels of MHC-class I expression, for others the distribution is uneven suggesting that they may be preferentially processed and presented by each tumor. The latter possibility is supported by recent studies indicating that preferential expression of CTL epitopes from the ubiquitous dehydrogenase in different tissues does not correlate with the levels of MHC-class I expression (3).

C. 2

The variable ability of CTL-OVA5 to recognize these epitopes may derive from differential expression of the precursor of these epitopes in the fresh tumors and tumor line (10). An alternative possibility is that the decreased stimulatory ability of the fresh tumor epitopes such as peaks C2, C5, C6 may reflect altered rates of processing of the same precursor (10, 29). This may reduce epitope generation, leading to decreased recognition by the immune system (29). The presence of non-overlapping epitopes such as B1 is of particular interest. The fact that SKOV3.B1 is recognized suggests that such an epitope was present and stimulatory on the autologous tumor. The absence from the fresh tumor may suggest immunoselection against those cells expressing SKOV3.B1.

C. 2

This study which represents the first comparative analysis of the epitope repertoire presented by a cloned established ovarian tumor line (SKOV3.1E4) and a freshly isolated ascitic ovarian tumor, indicates that a significant number of epitopes presented by the ovarian tumor can be detected in the established tumor line SKOV3. Furthermore a significant number of the peaks of peptides eluted from SKOV3 and OVA-6 are recognized by a human breast CTL line isolated from an HLA-$A2^+$ donor suggesting that at least some of these epitopes are shared by the breast and ovarian tumors (Melichar et. at. Manuscript in preparation). Thus, with the possible limitations due to the use of allogeneic effectors, these results show that the potential number of CTL epitopes on breast and ovarian tumors is high, and their identification deserves further investigation. This should be of interest for identification and characterization of tumor Ag in ovarian and breast cancer. Freshly isolated ovarian tumors are difficult to grow and their establishment in long term culture, to achieve the desired cell number for this type of studies usually requires specific culture conditions which involve the use of growth factors and stimulation. Thus the comparative analysis of the epitopes using defined numbers of tumor cells fractionated under defined HPLC conditions should allow identification of bioactive peptides for tumor associated CTL and characterization of tumor Ag.

The possiblity of characterizing tumor peptides in human ovarian and breast cancer may have important implications for understanding tumor immunity and development of epitope specific cancer vaccines. In contrast with melanoma where a large number of tumor Ag have been identified

(reviewed by Boon, 30), the number of tumor Ag found in breast and ovarian cancer is significantly smaller. It comprises Muc-1 (31), HER-2 (32), the AES protein of the Notch complex (33) and possibly the folate binding protein (Anderson, Fisk et. al preliminary studies). Identification of additional Ag may allow development of polyvalent tumor vaccines directed to several tumor epitopes. This can minimize the escape of tumor variants and establishment of metastases. Second, the use of HPLC fractionated peptides from established tumor lines may allow us to focus the therapy on the immunogenic epitopes. The active peptide fractions represented less than 10% of the total peptide material eluting from each HPLC column. Their use for vaccination studies will circumvent the blocking of the presenting molecules by inactive peptides. This should increase the Ag density on APC a factor that is critical for CTL induction. Third, since the presence of CTL lacking specificity in the OVA-6 tumor infiltrate, as well as of large numbers of tumor cells suggest that this CTL response is ineffective at this tumor stage, vaccine strategies could be developed using these peptides for therapy of earlier stage tumors of smaller size. Epitopes from tumor lines that are recognized by CTL associated with fresh tumors may provide an unlimited source of material for induction of a therapeutic response, bypassing the limitations imposed by the small amounts of fresh tumor cells. In fact a recent study has demonstrated the ability of acid eluted peptides to induce curative tumor immunity (14).

e.2

Legends to the Figures

Figure 1. Recognition by ovarian CD8+CD4- CTL lines CTL-OVA-3 and CTL-OVA-5 of the ovarian tumors SKOV3 and OVA-6 and C1R.A2 cells expressing a transfected HLA-A2 and HER-2 genes. Effector to target ratio was 20:1. Results were obtained in a 5 h CTL assay.

Figure 2A, B. Recognition by ovarian CTL lines, CTL-OVA-3 and CTL-OVA-4 of HLA-A2 bound peptides from the SKOV3.A2 cell line fractionated in the first dimension HPLC. Recognition by ovarian CTL-line CTL-OVA-5, of HLA-A2 bound peptides from SKOV3.A2 cells **(C)** and of TFA-extracted peptides from the freshly isolated ovarian tumor OVA-6 **(D)**. Both fractionations employed first dimension HPLC conditions as described in the Materials and Methods section. Effector-to-target ratio was 20:1. %Specific lysis of T2 cells that were not incubated with HPLC fractions was 2% for CTL-OVA-3 and 4% for CTL-OVA-4. These values were not substracted from the cytotoxicity values induced by peptide fractions, because the control T2 cells were not incubated with column fractions. cpm ^{51}Cr release by T2 cells incubated with HPLC fractions in the absence of effectors were in most instances lower than cpm ^{51}Cr release of T2 incubated without HPLC fractions. Details are presented in the Materials and Methods. The retention times of the HER-2 peptides separated under the same conditions were: C85 (HER-2, 971 - 979): 23.7 min, E89 (HER-2, 851 - 859): 13.4 min, E75 (HER-2, 369 - 377): 31.5 min, and control hydrophobic signal peptide E91 (HER-2, 5 - 13): 38.4 min.

Figure 3. Recognition by CTL-OVA-5 of second dimension HPLC fractions corresponding to the peaks B of activity in the first dimension shown in Figure 2. **(A), (C)** SKOV3.A2 peptides, **(B), (D)** OVA-6 peptides. Dashed areas indicate coincident peaks in the B3 peak of activity. The positions of elution of the HER-2 peptides used as markers for these HPLC conditions were: E90 (HER-2, 789 - 797): 25.4 min, E92 (HER-2, 650 - 658): 32.4 min, and E75 (40.5 min). The Rt of a EGF-R peptide: F49 (EGF-R, 356 - 364) KILGNLDFL was 38.5 min. Underlined residues indicate mutations in the E75 sequence. Lysis of T2 cells incubated with CTL-5 was as follows: no peptide (3 $\pm$ 1%), E75 at 1μg/ml (14 $\pm$ 2%) and F49 at 5 μg/ml (2 $\pm$ 1%). In the same experiment lysis of T2 cells by TAL-OVA-6 (autologous with OVA-6) was: no peptide (71 $\pm$ 10%), E75 at 1 μg/ml (73 + 7%) TAL-OVA-6 showed slow growth. The use of high

concentrations of IL-2 to enhance proliferation lead to high non-specific cytotoxicity levels.

(C, D). Recognition by CTL-OVA-5 of second dimension HPLC fractions corresponding to the peak C of activity in the first dimension shown in the **Figure 2C, D**. Dashed areas indicate coincident peaks C2, C3, and C4. There is no lysis by peptide only if compared with control T2 cells, because the cpm released in this group were the same or lower than spontaneous release by T2 cells. The elution positions of the HER-2 peptides used as markers to verify the capacity of separation in the Gradient III, were F57 (HER-2, 435 - 443): 14.7 min, E90: 16.1 min, F49: 18.5 min, E75: 19.9 min, and E91: 29.7 min.

REFERENCES

1. Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., Rammense, H-G. Allele specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature *351*:290-296, 1991.

2. Tsomides, T.J., Aldovini, A., Johnson, R.P., Walker, B.D., Young, R.A., and Eisen, H.M. Naturally processed viral peptides recognized by cytotoxic T lymphocytes on cells chronically infected by HIV type I. J.Exp.Med. *180*:1283-1293, 1994.

3. Wu, M.X., Tsomides, T.J., and Eisen, H.N. Tissue distribution of natural peptides derived from ubiquitous dehydrogenase, including a novel liver-specific peptide that demonstrates the pronounced specificity of low affinity T cell reactions. J.Immunol. *154*:4495-4502, 1995.

4. Udaka, K., Wiesmuller, K-H., Kienle, S., Jung, G., and Walden, P. Decrypting the structure of major histocompatibility complex class I-restricted cytotoxic T lymphocyte epitopes with complex peptide libraries. J. Exp. Med., *181*:2097-2108, 1995.

5. Huang, A.Y.C., Golumbek, P., Ahmadzadeh, M., Jaffe, E., Pardoll, D., and Levitsky, H. Role of bone-marrow derived cells in presenting MHC class I-restricted tumor antigens. Science *264*:961-965, 1994.

6. Bevan, M.J. Antigen presentation to cytotoxic T lymphocytes in vivo. J. Exp. Med. *182*:639-641, 1995.

7. Kovacsovics-Bankowski, M. and Rock, K.L. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science *267*:243-246, 1995.

8. Cox, A.L., Skipper, J., Chen, Y., Henderson, R.A., Darrow, T.L., Shabanowitz, J., Engelhard, V.H., Hunt, D.F., Slingluff, C.L. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. Science *264*:716-719, 1994.

9. Castelli, C., Storkus, W.J., Maeurer, M.J., Martin, D.M., Huang, E.C., Pramanik, B.N., Nagabhushan, T.L., Parmiani, G., Lotze, M.T. Mass spectrometric identification of a naturally processed melanoma peptide recognized by $CD8^+$ CTL. J.Exp.Med. *181*:363-368, 1995.

10. Storkus, W.J., Zeh III, H.J., Marurer, M.J., Salter, R.D., and Lotze, M.T. Identification of human melanoma peptides recognized by Class I restricted tumor infiltrating T lymphocytes. J.Immunol. *151*:3719-3727, 1993.

11. Ioannides, C. G., Fisk, B., Pollack, M., Frazier, M. L., Wharton, J.T., and Freedman, R. S. Cytotoxic T cell clones isolated from ovarian tumor infiltrating lymphocytes recognize common determinants on allogeneic tumors. Implication for identification of TIL defined Ag on tumors. Scandinavian Journal of Immunology, *37*:413-424, 1993.

12. Yoshino, I., Goedegeboure, P.S., Peoples, G.E., Parikh, A.S., DiMain, J.M., Lyerly, H.K., Gazdar, A.F., and Eberlein, T.J. HER2/neu-derived peptides are shared antigens among human non-small cell lung cancer and ovarian cancer. Cancer Res. *54*:3387-3390, 1994.

13. Peoples, G.E., Goedegebuure, P.S., Smith, R., Linehan, D.C., Yoshino, I., and Eberlein, T.J. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc.Natl.Acad.Sci, USA *92*:432-436, 1995.

14. Zitvogel, L., Mayordomo, J. I., Tjandrawan, T., DeLeo, A. B., Clarke, M. R., Lotze, M. T., and Storkus, W. J. Therapy of murine tumors with tumor peptide-pulsed dendritic cells: Dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J. Exp. Med. 183*:87-97, 1996.

15. Celluzzi, C. M., Mayordomo, J. I., Storkus, W. J., Lotze, M. T., and Falo, J. Peptide-pulsed dendritic cells induce antigen-specific, CTL-mediated protective tumor immunity. J. Exp. Med. *183*:283-287, 1996.

16. Fisk,B., Blevins,T. L., Wharton, J. T., Ioannides C. G. Identification of an immunodominant peptide of HER-2/neu proto-oncogene recognized by ovarian tumor specific CTL lines. J.Exp. Med. *181*:2709-2717, 1995.

17. Ioannides, C.G., Platsoucas, C.D., Rashed, S., Wharton, J.T., Edwards, C. L., and Freedman, R.S. Tumor cytolysis by lymphocytes infiltrating ovarian malignant ascites. Cancer Research *51*:4257-4265, 1991.

18. Ioannides, C.G., Freedman, R.S., Rashed, S., Platsoucas, C.D., and Kim, Y-P. Cytotoxic T cell clones isolated from ovarian tumor infiltrating lymphocytes recognize multiple antigenic epitopes on autologous tumor cells. The Journal of Immunology *146*:1700-1707, 1991.

19. Slingluff, Jr., C.L., Cox, A.L., Henderson, R.A., Hunt, D.F., and Engelhard, V.H. Recognition of human melanoma cells by HLA-A2.1-restricted cytotoxic T lymphocytes is mediated by at least six shared peptide epitopes. J. Immunol. *150*:2955-2963, 1993.

20. Tsomides, T.J., Walker, B.D., and Eisen, H.N. An optimal viral peptide recognized by $CD8^+$ T cells binds very tightly to the restricting class I major histocompatibility complex protein on intact cells but not to the purified class I protein. Proc.Natl.Acad.Sci. USA *88*:11276-11280, 1991.

21. Sykulev, Y., Brunsmark, A., Tsomides, T. J., Kageyama, S., Jackson, M., Peterson, P. A., and Eisen, H. N. High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogenic and syngeneic major histocompatibility comples class I proteins. Proc. Natl. Acad. Sci. *91*:11487-11491, 1994.

22. Fisk, B., Savary, C., Hudson, J.M., O'Brian, C.A., Murray, J. L., Wharton, J.T. and Ioannides, C.G. Changes in a HER-2 peptide up-regulating HLA-A2 expression affect both conformational epitopes and CTL recognition. Implications for optimization of antigen presentation

and tumor specific CTL induction. Journal of Immunotherapy *18*, 197-209, 1996.

23. Ioannides, C. G., Fisk, B., Fan, D., Biddison, W. A., Wharton, J. T., O'Brian, C. A. Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptide derived from the HER-2/neu proto-oncogene. Cell. Immunol. *151*:225-234. 1993.

24. Yoshino, I., Peoples, G.E., Goedegebuure, P.S., Maziarz, R., Eberlein, T.J. Association of HER-2/neu expression with sensitivity to tumor-specific CTL in human ovarian cancer. J.Immunol. *152*:2393-2400, 1994.

25. Udaka, K., Tsomides, T.J., and Eisen, H.N. A naturally occurring peptide recognized by alloreactive $CD8^+$ cytotoxic T lymphocytes in association with a class I MHC protein. Cell *69*:989-998, 1992.

26. Griem, P., Wallny, H-J., Falk, K., Rotzschke, O., Arnold, B., Schonrich, G., Hammerling, G., and Rammensee, H-G. Uneven tissue distribution of minor histocompatibility proteins versus peptides is caused by MHC expression. Cell, *65*:633-640, 1991.

27. Rivoltini, L., Barracchini, K.C., Viggiano, V., Kawakami, Y., Smith, A., Mixon, A., Restifo, N.P., Topalian, S.L., Simonis, T.B., Rosenberg, S.A., and Marincola, F.M. Quantitative correlation between HLA class I allele expression and recognition of melanoma cells by antigen-specific cytotoxic T lymphocytes. Cancer Research *55*:3149-3157, 1995.

28. Peoples, G.E., Smith, R.C., Linnehan, D.C., Yoshino, I., Goedegebuure, P.S., and Eberlein, T.J. Shared T cell epitopes in epithelial tumors. Cellular Immunology *164*:279-286, 1995.

29. Rotem-Yehudar R, Groettrup M, Soza A, Kloetzel PM, and Ehrlich R. LMP-associated proteolytic activities and TAP-dependent peptide transport for class I MHC molecules are suppressed in cell lines transformed by the highly oncogenic adenovirus 12. J. Exp. Med. 183:499-514, 1996.

30. Boon, T. and van der Bruggen, P. Human tumor antigens recognized by T lymphocytes. J. Exp. Med. *183*:725-729, 1996.

31. Jerome, K.R., Domenech, N., Finn, O.J. Tumor-specific CTL clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin cDNA. J. Immunol. *151*:1653-1662, 1993.

32. Ioannides, C. G., Ioannides, M. G., O'Brian, C. A. T-cell recognition of oncogene products: a new strategy for immunotherapy. Molec. Carcinogen. *6*:77-81, 1992.

33. Ioannides, C. G., Fisk, B., Melichar, B., Anderson, B., Stifani, S., Papayannopoulos, I., Murray, J. L., Kudelka, A.,Wharton, J. T. Ovarian and breast CTL can recognize peptides from the AES protein of the *Notch* complex. The FASEB J. *10*: A1437, 1996.

Table I. Common epitopes on SKOV3.A2 and OVA-6 recognized by CTL-OVA-5*

Peak Designation (Fxn. No.)		Peptides* SKOV3.A2 %specific lysis				OVA-6 %specific lysis			
B3	(43 - 46)	18	21	5	12	16	20	19	12
C2	(16 - 19)	8	13	14	12	2	8	6	0
C3	(21 - 23)	16	22	1		8	9	4	
C4	(24 - 25)	6	5			17	7		

*Numbers indicate %specific lysis by CTL-OVA-5 of T2 cells incubated with equal volumes of the same HPLC fractions as described (26).

Figure 1

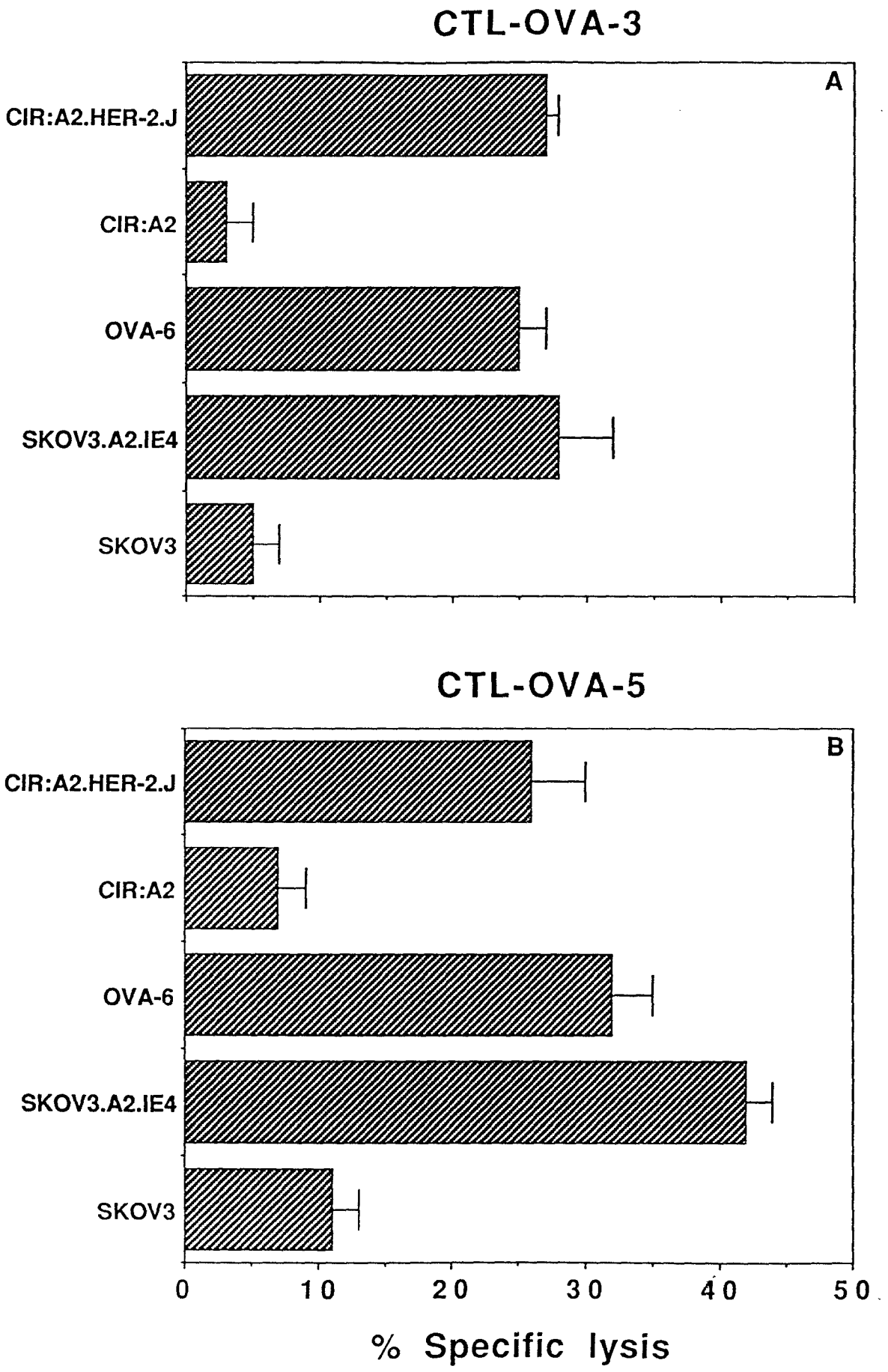
CTL-OVA-3
A
CIR:A2.HER-2.J
CIR:A2
OVA-6
SKOV3.A2.IE4
SKOV3
CTL-OVA-5
B
CIR:A2.HER-2.J
CIR:A2
OVA-6
SKOV3.A2.IE4
SKOV3
0
10
20
30
40
50
% Specific lysis

Figure 2

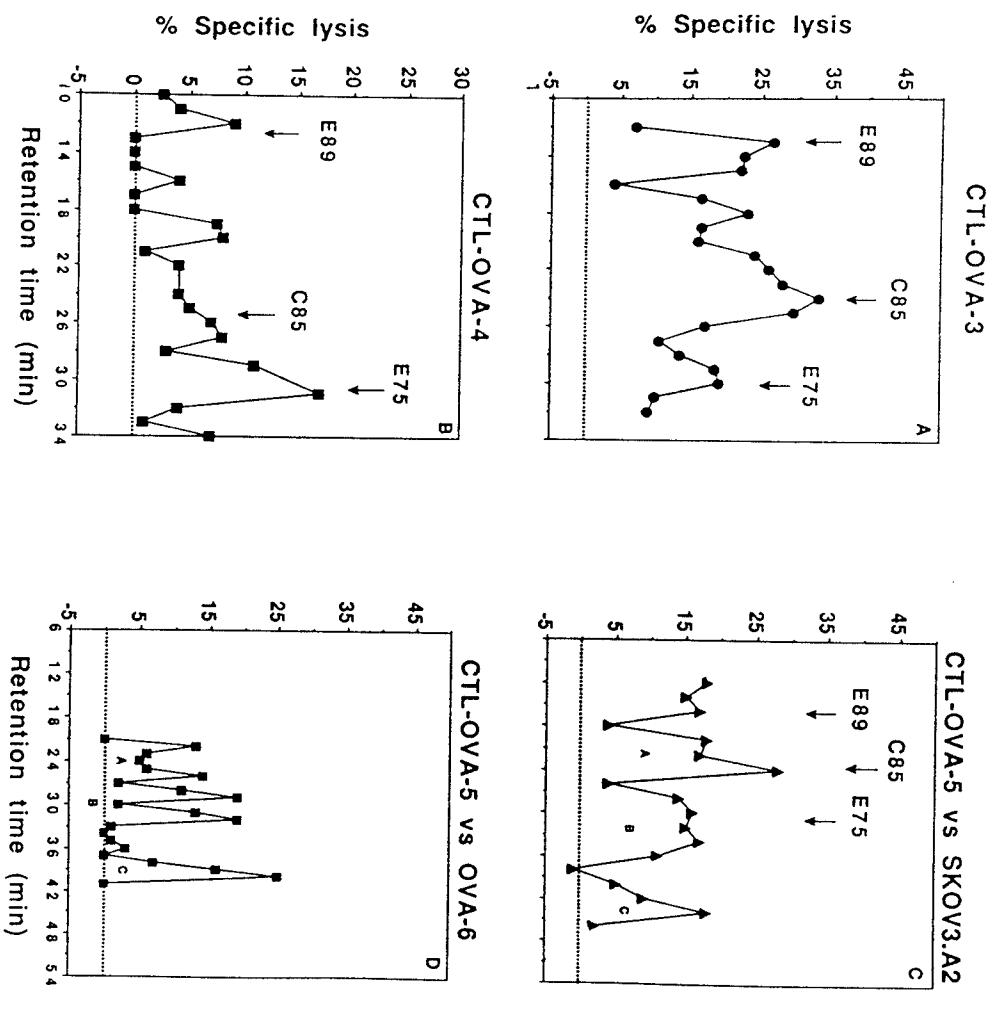
CTL-OVA-3
% Specific lysis
E89
C85
E75
A
CTL-OVA-4
% Specific lysis
E89
C85
E75
B
Retention time (min)
CTL-OVA-5 vs SKOV3.A2
E89
C85
E75
A
B
C
C
CTL-OVA-5 vs OVA-6
A
B
C
D
Retention time (min)

Figure 3

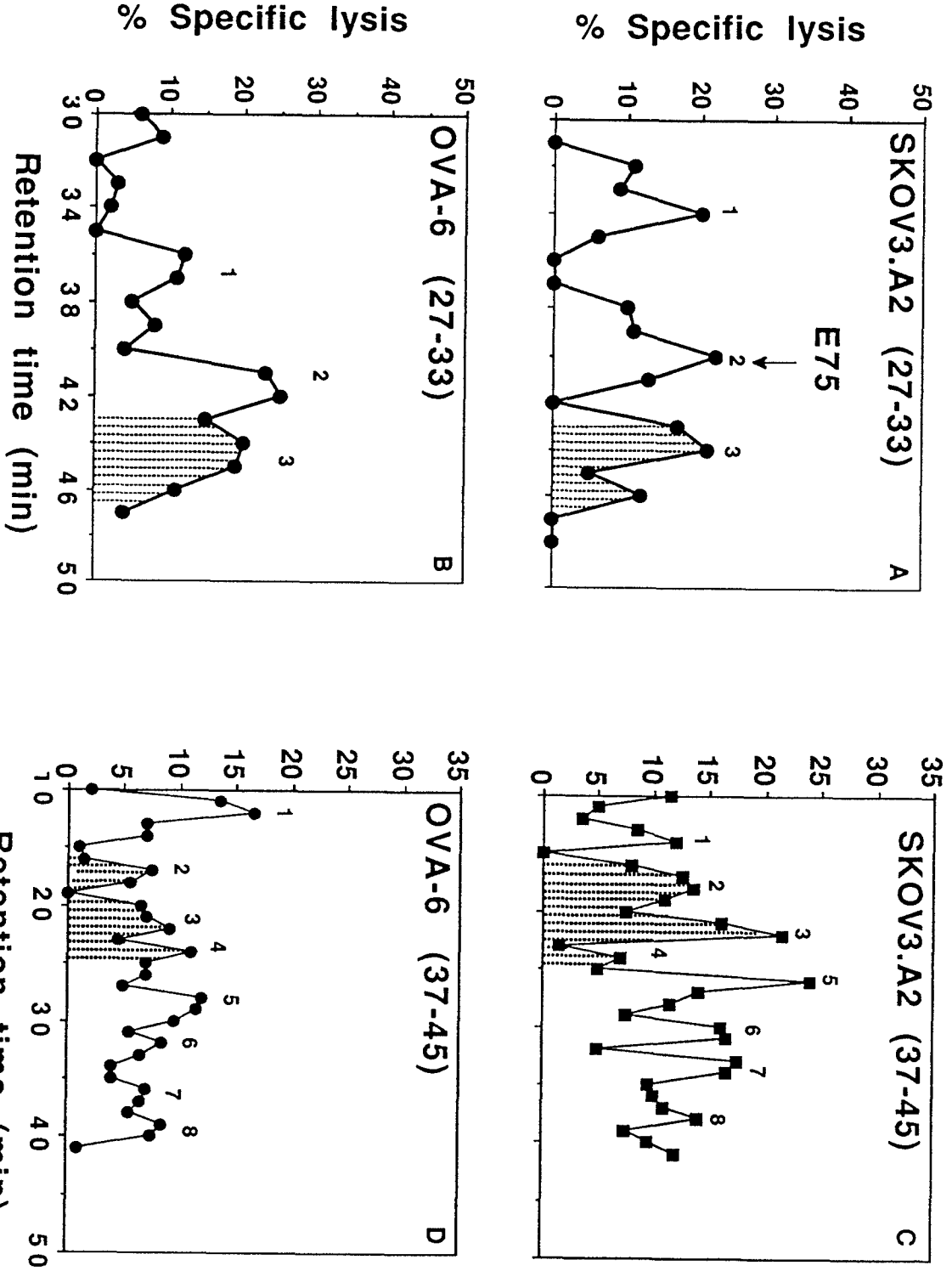
% Specific lysis
SKOV3.A2 (27-33)
E75
A
% Specific lysis
OVA-6 (27-33)
B
Retention time (min)
SKOV3.A2 (37-45)
C
OVA-6 (37-45)
D
Retention time (min)

Table I. Common epitopes on SKOV3.A2 and OVA-6 recognized by CTL-OVA-5*

		Peptides*							
Peak Designation		SKOV3.A2				OVA-6			
(Fxn. No.)		%specific lysis							
B3	(43 - 46)	18	21	5	12	16	20	19	12
C2	(16 - 19)	8	13	14	12	2	8	6	0
C3	(21 - 23)	16	22	1		8	9	4	
C4	(24 - 25)	6	5			17	7		

*Numbers indicate %specific lysis by CTL-OVA-5 of T2 cells incubated with equal volumes of the same HPLC fractions as described (26).

Existent Proliferative Responses of Peripheral Blood Mononuclear Cells from Healthy Donors and Ovarian Cancer Patients to HER-2 Peptides[1].

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Key words: HER-2, $CD4^+$, epitope, ovarian cancer, Th1, Th2, cytokines.

Running title: Proliferative responses to HER-2 peptides

[2]*Abbreviations used in this paper:* Cytotoxic T Lymphocytes, CTL, Position, P; T cell receptor, TCR; HER-2/neu proto-oncogene, HER-2; stimulation index, S.I.; standard deviation, SD, TT, tetanous toxoid

[1]*Acknowledgments.* We thank Ms. Rosario Mante for collecting specimens and Ms. Susan Mondragon for the outstanding preparation of this manuscript. Peptide synthesis was supported in part by core grant 16672.

ABSTRACT

Identifying target antigens for tumor-reactive T cells is important for understanding the mechanisms of tumor escape and developing novel anticancer therapies. To date, mainly CTL responses from tumor infiltrating/associated lymphocytes (TIL/TAL) to peptide antigens have been investigated in ovarian cancer. In the present study, the ability of self-peptides derived from HER-2/neu proto-oncogene product (HER-2) to stimulate proliferation of PBMC from healthy donors and ovarian cancer patients has been assessed. Peptide sequences from HER-2 containing anchors for major human MHC-class II molecules have been identified. These peptides induced proliferative and cytokine responses at higher frequency in healthy donors than ovarian cancer patients. Four HER-2 peptides corresponding to positions: 396 - 406, 474 - 487, 776 - 789, and 884 - 899 were able to stimulate proliferation of a larger number of healthy donors than three other distinct HER-2 peptides 449 - 465, 975 - 987 and 1086 - 1098. The pattern of responses of twenty five ovarian cancer patients was different from that in healthy donors. T cell lines were developed by stimulation with peptides from PBMC of an ovarian cancer patient who showed a stable response to all four HER-2 peptides for over six months. Each T cell line was different in its ability to secrete IFN-γ and IL-10. These results demonstrate (a) that self-peptides from HER-2 can stimulate expansion of T cells in both healthy donors and ovarian cancer patients, and (b) the ability of different peptides to stimulate secretion of different cytokines from lymphocytes of ovarian cancer patients. These results may be important for understanding the mechanisms of tolerance and autoimmunity in human cancers.

INTRODUCTION

The HER-2/neu proto-oncogene product (HER-2) is the target of autoantibodies in breast cancer (1) and of cytotoxic T lymphocytes (CTL) in ovarian, breast, and lung cancer (2-5). Since these auto-antibodies are specific for the native conformation of the HER-2, they must be induced by the native molecule. HER-2 is present in both healthy individuals and cancer patients. Similarly HER-2-reactive CTL are specific for a number of epitopes (2-5) of which one HER-2, 369-377 was found to be immunodominant in our studies (5). These CTL were isolated from lymphocytes associated with ovarian tumors in patients with advanced disease after culture in the presence of IL-2. This suggests that $CD4^+$ T cells capable of helping either B cells, or $CD8^+$ CTL, or both may be present in the cancer patients. This raises the question as to whether $CD4^+$ T cells capable of recognizing epitopes from human HER-2 are also present. Previous studies have shown that $CD4^+$ T cells with specificity for HER-2 can be identified in breast cancer patients (1). The extent and the existence of autoreactive T cell repertoire to HER-2 in both healthy humans and ovarian cancer patients has not been previously identified. To assess the specificity of these T cells and identify potential targets for epitope-specific immunotherapy, we investigated the responses to HER-2 of a group of patients with ovarian cancer subsequent to chemotherapy and a group of healthy individuals.

To characterize the T cell response to HER-2 we assessed a number of T cell epitopes of HER-2 with a set of synthetic peptides based on the HER-2 sequence. We wanted to identify a set of such peptides to which healthy donors and ovarian cancer patients respond by proliferation and determine the frequency of these responses. We stimulated PBMC from twenty five ovarian cancer patients and fourteen healthy donors with synthetic HER-2 peptides. PBMC from each donor were stimulated individually with each peptide, but not with pooled peptides. The general pattern of response was characterized by a group of four HER-2 peptides designated as D122:HER-2,396 - 406, F7:HER-2:776 - 789,

F13:HER-2,884 - 899, F14:HER-2, 474 - 487 which induced a significantly higher frequency of responses than the other three HER-2 peptides designated as F10:HER-2,975 - 997, F12:449 - 465, D100:HER-2,1086 - 1098 in both healthy donors and ovarian cancer patients. The frequency of responses to most HER-2 peptides was significantly lower in ovarian cancer patients who had received chemotherapy than in healthy donors.

T cell lines were raised against individual HER-2 epitopes represented by peptides F7, F13, F14 and D122 from PBMC of an ovarian cancer patient by restimulation with HER-2 peptides and expansion in IL-2. These T cell lines showed a different pattern of IFN-γ and IL-10 production. F13 induced T cells secreted significantly higher amounts of IFN-γ than IL-10 while F7 and F14 induced T cells secreted significantly higher levels of IL-10 than F13 and D122 induced cells.

MATERIALS AND METHODS

Cells. Peripheral blood nononuclear cells (PBMC) were obtained from fourteen healthy donors and twenty five ovarian cancer patients. All patients had advanced disease. After initial surgery, they were treated with platinum (cisplatin or carboplatin). One patient was receiving primary platinum based chemotherapy. The other twenty four patients had received additional chemotherapy. The latter was either carboplatin reinduction, salvage therapy with paclitaxel or experimental therapy with several different drugs. Blood collection was made at least three weeks after the last chemotherapy administration. PBMC were isolated from heparinized peripheral blood as described (1,6). At the time of the assay the patients were not receiving chemotherapy.

HER-2 epitopes selection. Peptides to be tested in the proliferation assays were selected based on the T cell sites in HER-2 predicted by the computer program

ANT.FIND.M, the general binding motif for human class MHC-II antigens, and the presence in the sequence of anchors for a number of MHC-class II antigens: HLA-DR1, HLA-DR3, HLA-DR4, HLA-DR11, and HLA-DQ7 (7-13). The sum of the allelic frequencies of these antigens cover approximately 100% of the Caucasian and Hispanic populations and between 75 - 92% of the African American and Asian populations. For example HLA-DQ7 is present at 28% (Caucasians) 23% (African Americans), and at 43% (Hispanics). Similarly, each of HLA-DR1, DR3, DR4 and DR11 is present between 17 - 20% in each of the major population groups. The general binding motif for various human MHC-class II molecules consist of a position 1 (P1) anchor, i.e. an aromatic or large aliphatic residue in the first 3 - 5 amino acids close to the N-terminus, and other major but less essential anchors at positions 4, 5 - 7, and 9 counting from the P1 anchor (13). A large number of "promiscuous" peptides are capable of binding to many different MHC-class II molecules (13, 14), because their sequences contain overlapping binding motifs for MHC-class II molecules (13, 14). The search for specific anchors for these major MHC-class II antigens in the HER-2 sequence indicated significantly more candidate epitopes for binding to HLA-DR1 (n = >20) than for HLA-DR3 (n = 11), than for binding to HLA-DR4, HLA-DQ7, and HLA-DR11.

Ten HER-2 peptides 11 - 22 residues long **(Table I)** were synthesized by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center, using a solid phase method as previously described (2). The identity of peptides was determined by amino acid analysis. The purity of peptides ranged between 93 - 97% as determined by HPLC. Peptides were dissolved in PBS, aliquoted at 2 mg/ml and stored frozen at -20°C until use. The codes used to identify these HER-2 peptides in this paper were assigned by the Synthetic Ag Laboratory. To ensure better representation of different binding motifs for these MHC class II antigens, at least two peptides were synthesized containing anchors for each of HLA-DR1, -DR3, and -DQ7. When possible the sequences were selected to contain anchors for two MHC class II antigens **(Table I)**. Each of the peptides synthesized

contained at least two of three anchors for each HLA-DR antigen, as shown in the **Table I**, and the main P1 anchors for most class II alleles. In peptides D122, F12, F7, F6, F8 and F13, positions P4 and P5 are occupied by hydrophobic, aromatic followed by aliphatic residues in that order to facilitate peptide binding in different frames.

The binding motifs of synthetic peptides for MHC-class II molecules may differ from natural ligands because the latter incorporate constraints of processing in addition to binding requirements. For these reasons, peptides were synthesized by following, when possible, the common motifs for all MHC-class II molecules defined by pool sequencing of naturally processed peptides (7). This was possible for four peptides, F6, F7, F13 and F14. In these peptides, the sequence was extended to include Pro (P) N-terminal to either the Tyr (Y), which is the P1 anchor for HLA-DR1, DR3, DR4, and DQ7, or Trp (W) which is reported by the P1 anchor for HLA-DR4 and DR11. Peptides F6 and F7 overlap in the first thirteen residues. In F6 the sequence was extended at its C-terminal to incorporate a region 783-797 previously reported to induce proliferation of PBMC from breast cancer patients. The sequence of F14 was also extended to include Pro at the C terminus, after Arg (R), the third anchor in the correct position for HLA-DR3 and HLA-DR11. Sequences were also extended at the N- and C-termini. This was made to facilitate the natural proteolytic trimming of peptides since most aminopeptidases stop cutting one residue before reaching a Pro residue (7).

Stimulation and propagation of T cells. Freshly separated PBMC from healthy donors and ovarian cancer patients were stimulated with each HER-2 peptide at a final concentration of 50 μg/ml and cultured at 1 x 10^6 cells/ml in RPMI 1640 medium (GIBCO) with 5% pooled human AB serum and antibiotics (complete RPMI medium) (1). After 3 - 4 days of stimulation with each peptide, cultures were expanded with IL-2 (20 - 40 U/ml) for the following week (15, 16). To induce antigen specific T cells, the cells were then "rested" for 3 - 4 days by culture in the absence of IL-2. Then, the cells were stimulated at a 1:1

(stimulator:responder ratio) with irradiated (10.000 Rad) autologous PBMC, which had been first stimulated with PHA, expanded in IL-2, and then were pulsed with individual peptides for at least 90 min at 37°C before addition to the cultures as described (16). Control cultures were stimulated with the same number of IL-2 expanded PBMC in the absence of peptides. For further expansion, four to five days later 20 - 40 U/ml IL-2 was added to the cultures for seven additional days.

Proliferation assays. The proliferation assay was done by culturing 2 x 10^5 PBMC from each donor in quadruplicate in a 96 well plate in 200 µl with each peptide at 50 µg/ml, with tetanus toxoid at 5 µg/ml and PHA (GIBCO) at a final concentration 1:100 for 96 h as described (1, 16). For the last 16 h, 1 µCi [^{3}H]-Tdr was added to the cultures. Afterwards, the cells were harvested and the radioactivity counted in a Beckman LS3501 liquid scintillation counter as previously described (6). A significant proliferative response was defined as a statistically significant increase in the cpm proliferation in the tetraplicate cultures stimulated with any of the peptides, PHA, or TT, above that in cultures from the same donor that received peptide. Values obtained for cpm (^{3}H-Tdr incorporation) by the PBMC incubated with PBS, PHA or synthetic HER-2 peptides were examined by the Student's *t* test. Differences were considered significant when the P values were < 0.05.

Flow cytometry. T cells were tested in fluorescence experiments to determine the surface antigen expression as previously described (2, 6). Surface antigen expression was determined by FACS analysis using a FACSscan (Beckton-Dickinson, Sunnyvale, CA)) with a log amplifier. CD3, CD4 and CD8 antigen expression on T cell cultures was determined by immunofluorescence with corresponding mAb FITC-conjugated (Beckton Dickinson).

Cytokine production. The ability of PBMC to produce antigen induced IFN-γ and IL-10 was determined by culturing the PBMC either as unstimulated or stimulated with the corresponding peptides or PHA, (GIBCO) diluted 1:100, or tetanus toxoid. Supernatants were collected after 48h and stored frozen at -20°C until assayed for cytokine level. Cytokine containing supernatants from the T cell lines were generated by adding OKT3 and phorbol myristate acetate (PMA) to the cells to 96 well plate for 18h as described (17, 18). Afterwards, supernatants were collected for measurement of IFN-γ and IL-10 levels. IFN-γ and IL-10 were measured by double sandwitch-ELISA using the corresponding kits provided by BioSource International (Camariyo, CA). Supernatants from tetraplicate wells were pooled and tested in triplicates. The cytokine assays were calibrated with human recombinant IFN-γ and IL-10 to detect each cytokine in the range of 10-1000 pg/ml.

RESULTS

Proliferative responses of PBMC to HER-2 peptides. To map the HER-2 peptides most frequently recognized and to identify potentially immunodominant epitopes, we determined the responses to individual peptides of PBMC from twenty five ovarian cancer patients. Patients previously treated with chemotherapy were allowed on this study, since immunotherapy approaches to ovarian cancer are usually initiated after conventional therapies thus making the responses of these donors more likely to reflect the responder status of candidates for tumor-vaccine therapies. Responses of PBMC from healthy donors to the same peptides were tested in parallel in the same experiment. Depending on the number of PBMC available from each ovarian cancer patient a minimum of four peptides and PHA were tested in the same experiment. Results are summarized in **Figure 1**. Peptides F11 (HER-2, 5 - 19) (which corresponds to the highly hydrophobic signal area) F6 (HER-2, 776 - 797) and F8 (HER-2, 832 - 851) were not easily solubilized in PBS, thus their use for stimulation was discontinued after several assays. PBMC from most donors were tested with at least six peptides in each experiment. Each donor responded to some peptides but not to others. The lack of a common response it is not entirely suggesting that these peptides do not bind MHC molecules. A negative response of any of these peptides could also reflect T cell unresponsiveness (tolerance) to this epitope.

To ensure that the lack of responsiveness of PBMC to HER-2 peptides did not reflect a generalized suppression of responses to antigen or mitogen, all patients' lymphocytes were tested for their ability to respond to PHA. Of 29 patients tested, 28 showed significant responses to PHA compared with unstimulated cultures. All PBMC samples from healthy donors showed significant responses to PHA (data not shown). Together these results indicated that most PBMC from the ovarian cancer patients with advanced disease respond to PHA after chemotherapy and that their ability or failure to respond to HER-2 peptides did not reflect their ability to respond to a T-cell mitogen.

The frequency of responses of healthy donor PBMC to four peptides: D122, F7, F13 and F14 was higher than the frequency of responses to three other peptides, F10, F12, and D100. The same pattern of preferential responses to F7 and F13 was seen in PBMC from ovarian cancer patients **(Figure 1B)**. The results also indicate that PBMC from ovarian cancer patients responded at a significantly lower frequency than healthy donors to F7, F13, F14 and D122. However, the decrease was not the same for all peptides. For peptides D122, F13, and F14 the decrease in responses in PBMC from patients versus healthy donors was in the range of 70%, while for peptide F7 the decrease was only 50%. No significant responses to peptides D100 (1086 - 1098), F10 (975 - 987), and F12 (449 - 465) were observed. Representative results from PBMC of four healthy donors (two responders and two non-responders) are shown in **Figure 2**. Donors A and B were considered responders based on the ability of their PBMC to proliferate to at least one of the eight HER-2 peptides tested. These responses were significantly higher than responses by PBMC that have not been pulsed with peptides. In both responders and non-responders, the responses to HER-2 peptides did not correlate with the ability of the same PBMC to respond or with the magnitude of response to TT **(Figures 2 and 3)**. Of twenty five ovarian cancer patients tested, seven responded to F7 with statistically significant differences in cpm proliferation between peptide-induced and control cultures. Of seven F7 responding patients three responded to F13 two responded to F7, F13 and F14; one to F7 and F14; and four only to F7. Of five F13 responding patients two did not respond to F7 or F14. Only two patients responded to all four peptides: F7, F13, F14 and D122. There was no significant difference in the magnitude of their proliferative responses to these four peptides.

For comparison, the pattern and the magnitude of the proliferative responses to HER-2 peptides of PBMC one of these patients (designated as Patient A) is shown in **Figure 3** together with those of PBMC from another healthy donor tested in the same experiment. Both donors responded to D122, F7, F13 and F14 but failed to respond to

D100 and F12. To establish whether the pattern of responses to the HER-2 peptides in PBMC from ovarian cancer patients changed over time, responses to the same peptides were determined at two additional time points over five months when the patients were not receiving chemotherapy. PBMC from Patient A showed a constant high level of responses to at least two of the four peptides tested, but PBMC from a nonresponding patient failed to proliferate above the control levels. The same PBMC showed significant responses to PHA. It should be mentioned that sixteen months after the first determination, PBMC from the same Patient A responded primarily to F13 also less strongly to F7 (data not shown). PBMC from the same responding patients were tested again after several months when the disease progressed. At this time significant proliferative responses to HER-2 peptides were not observed. All three patients that responded to both F7 and F13 had stable disease. This suggests that disease progression affects the ability of PBMC from cancer patients to respond to HER-2 peptides.

In vitro expansion and cytokine production by HER-2 peptide stimulated T cells. To establish whether HER-2 peptide stimulated lymphocytes can be expanded in culture as T-cell lines, PBMC from Patient A were selected for these experiments. These PBMC were chosen because they showed a stable and significant proliferative response to at least four HER-2 peptides of distinct sequence. Primary cultures with F7, D122, F13 and F14 were initiated for four days, after which IL-2 (40 U/ml) was added for four more days. Afterwards, IL-2 was removed and the cells were "rested" in complete RPMI medium in the absence of IL-2 for four days. Afterwards, each peptide initiated culture was restimulated with autologous T cells from PHA-stimulated PBMC expanded in IL-2 prepulsed with the corresponding peptide. Control cultures were then restimulated with PHA blasts in the absence of peptides. After four days, IL-2 (20 U/ml) was added to the cultures for 48 h. The S.I. were determined by comparing the ^{3}H-Tdr incorporation during the last 16 h. As shown in **Figure 4A** all peptide stimulated cultures showed an increase in S.I. of >2.0 over the control cultures. Similarly, cultures stimulated initially with TT, PHA and OKT3 in

the presence of IL-2 showed a significant increase in proliferation over the peptide-stimulated cultures.

To address whether T cells induced by one of the peptides can be stimulated to proliferate by another HER-2 peptide, we investigated the response of F13-induced T cells to F13, D122, F14, and F7. The results are shown in **Figure 4B**. PHA and OKT3 mAb were used for stimulation as positive controls. Cells were counted after four days. A significant increase in number over control cultures was observed in F13 induced cultures restimulated with F13. In contrast, F13-induced cultures showed no significant proliferation in response to F14, and F7 while in response to D122 the viable cell number actually decreased. These results indicate that F13-induced T cells preferentially proliferate in response to F13 and are only minimally cross-stimulated to proliferate by other HER-2 peptides.

To define the T cell phenotype, the resulting PBMC cultures, were analyzed for CD4 and CD8 antigen expression. Analysis of the phenotype of the control cultures was performed in parallel. The results are shown in the **Figure 4C**. Most cells in the primary stimulated cultures were $CD4^+$ T cells, however there were some significant differences between cultures. F7- and F14-stimulated cultures contained 12 and 16% $CD8^+$ cells respectively, while D122 and F13-stimulated cultures had a significantly higher proportion of $CD8^+$ cells (40 - 44%). Control cultures which were stimulated only with autologous PBMC and IL-2 were of predominantly of the CD4 phenotype while in PHA and OKT3 stimulated cultures CD8+ cells were in majority. These results indicate that, for the same donor, stimulation with each peptide affected in a different way the proliferation of either CD4 or $CD8^+$ cells or both. This does not reflect the ability of one or another subset to proliferate better in the presence of IL-2. Control cultures which were not stimulated with exogenously added peptides, and TT-stimulated cultures showed a predominantly $CD4^+$ phenotype. Inhibition studies using anti-HLA-mAb indicated that HER-2 peptides induced

proliferation was inhibited by anti-MHC class II and at lesser extent by anti-MHC-class I Abs suggesting that the responder cells are T cells (data not shown).

Each set of HER-2 stimulated lymphocytes showed different proportions of $CD4^+$ and $CD8^+$ cells. Since this may reflect the ability of each peptide to induce cytokines, which can affect the proliferation of each T cell subset, the capacity of each HER-2 induced T-cell line to secrete IFN-γ and IL-10 was determined. These cytokines have been associated with Th1 and Th2 types of responses, respectively (19-21). Since cells cultured in IL-2 usually produce background levels of IFN-γ, all peptide-stimulated T-cell lines and control lines were washed and cultured in complete RPMI medium without IL-2 for two days, before being stimulated with OKT3 and PMA. The results of one representative experiment (of two experiments performed) are presented in **Figure 5.**

T-cell lines stimulated by F7, F13, and F14 produced IFN-γ at higher levels than observed in control cultures stimulated with autologous PBMC but not with peptides. The highest levels were observed with F13 and were similar to the levels induced by TT-induced T cell lines. Interestingly, a different pattern of IL-10 secretion was observed. IL-10 secretion by F13-stimulated T cells was only slightly above the control levels. However, high levels of IL-10 were found in the supernatants from F7- and F14-stimulated T cells. The levels of IL-10 were almost half the level of IL-10 produced by TT-induced T cells. In contrast, while the levels of IFN-γ produced by D122-induced line were higher than those produced by the control cultures, the levels of IL-10 produced by the D122-induced line were minimal. These results show a good correlation between the IL-10 secretion and the $CD4^+/CD8^+$ ratio of T cells from HER-2 peptide induced T cell lines. F7-, F14- and TT-induced T-cell lines secreted high levels of both IFN-γ and IL-10. Conversely, D122- and F13-stimulated cultures secreted different amounts of IFN-γ but low amounts of IL-10.

This may be suggestive of a Th1 function for the F13 peptide in this patient and for a Th2 function for the F7 peptide in the same patient.

DISCUSSION

Recognition of HER-2 epitopes by CD8+ cytotoxic T lymphocytes has been extensively documented (2-5). However significantly less information is available about the recognition of HER-2 epitopes by CD4+ cells. Although CD4+ cells may not be always involved in tumor lysis in breast and ovarian cancer, helper T cells may be essential for initiating, sustaining and amplifying an anti-tumor response. CTL induced by stimulation with Ag in the presence of co-stimulation with B7 may become exhausted by the interaction with B7- tumor cells. The presence of Ag specific $CD4^+$ T cells may provide the "self-help" needed to sustain CTL responses (22, 23). Thus Th1 cells recognizing peptides derived from the processing of HER-2 may produce cytokines (IFN-γ, TNF-α/β) that are thought to provide help for CTL function. The same HER-2 epitopes may produce Th2 cytokines in association with other MHC-class II types.

The objectives of this study were to (a) determine whether HER-2 peptide recognition occurs in healthy donors and in ovarian cancer patients with advanced disease and (b) whether distinct HER-2 peptides differ in their ability to modulate the cytokine secretion potential of the T cells from the same donor. In this report we present evidence that T cells responsive to multiple epitopes on a self-protein, HER-2, exist *in vivo* in healthy donors as well as in ovarian cancer patients. These cells can be stimulated to proliferate and expand *in vitro* and can secrete Th1 and Th2 cytokines. The observed *in vitro* responses of normal T cells to multiple peptides derived from HER-2 cannot be attributed to mitogenic effects by a particular peptide since (a) different peptides elicited PBMC proliferation in different donors and (b) three peptides containing the same P1 anchor for the same MHC-class II molecules failed to induce proliferative responses with the same frequency as four other peptides. Our analysis revealed that PBMC from ovarian cancer

patients after chemotherapy responded less frequently than PBMC from healthy donors to the same peptides.

The reasons for the reduced frequency of responses in PBMC from ovarian cancer patients are still unknown. One possibility, to be addressed in future studies, is whether the ability of T cells from patients to respond is affected by chemotherapy. Chemotherapy can eliminate proliferating reacting clones to HER-2 peptides. An alternative is that individuals susceptible to ovarian cancer may be less responsive to self-antigen. This hypothesis could be tested in future studies using HLA-typed ovarian cancer patients. At this moment there is unknown whether there is a disproportionate expression of MHC-class II allele in the cancer patients that may account for the different response pattern to the peptide antigens. Other possibilities currently under investigation are whether (a) $CD4^+$ T cells from these patients are anergic to these peptides or (b) their response is suppressed (1, 22). Suppressive effects due to disease progression may account for the lack of responses to these peptides at this stage. Although the group of patients with stable disease is relatively small to allow conclusive comparisions to be made, three of seven patients with stable disease responded to F13 while only two of eighteen patients with progressive disease (11%) responded to F13. In several cases when both PBMC and TAL were available from the same patient, we found that only PBMC responded to these peptides, suggesting the presence in situ of potentially negative regulatory mechanisms. Our preliminary results on the pattern of cytokine responses to F7 and F13 show that for a number of patients IL-10 was detectable at 48h in the peptide induced cultures but the levels of IFN-γ were below the levels of detection of the assay (10 pg/ml). PHA stimulated PBMC from the same donors secreted both IFN-γ and IL-10. In other patients, only F7-induced PBMC secreted TNF-α and/or IFN-γ (Melichar et. al. manuscript in preparation).

In our study, PBMC from ovarian cancer patients responded less frequently to F13 than did PBMC from healthy donors. The frequency of responses to F7 and F13 in healthy donors (54 and 62%, respectively) does not correlate with the frequency of expression of HLA-DR4 (25%) in the human population, suggesting that these peptides can be presented by other class II molecules.

Our current study also sought to elucidate the ability of HER-2-peptide stimulated T cells to expand and secrete cytokines. In this case we studies PBMC from a patient who had shown a stable response to several HER-2 peptides over a six month period. T cell lines of predominantly $CD4^+$ phenotype were readily expanded by restimulation with these peptides and low concentrations of IL-2. Interestingly, the resulting T cell lines differed in their proportions of $CD4^+$ and $CD8^+$ cells in their pattern of IFN-γ and IL-10 secretion.

One possible explanation for these observed differences is that peptides F13 and F7 function as Th1 and Th2 epitopes respectively in association with certain MHC-class II molecules. Both F7 and F13 contain a set of P1-P4-P6 anchors for HLA-DR4, though these sequences differ in charge at the P4 anchor: R (782) in F7 and E (892) in F13 correspond to the motifs for peptide binding sites to the DRB1*0402 and DRB1*0401/0404 alleles (12, 23). Phenotypic analysis of cells in the T cell lines stimulated by these peptides revealed a significantly larger population of $CD8^+$ cells in F13- than in F7- stimulated T cell lines from this donor. Furthermore, a T cell response to the epitope HER-2:783-797 mapped with the peptide SRLLGICLTSTVQ was detected in a breast cancer patient with high level of HER-2 auto-antibodies (1). F7, HER-2 (776-789) overlaps with HER-2:783-797 in the area 776 - 783. The possibility that T cells stimulated by F7 can provide help for Ig synthesis deserves further consideration.

The fact that T cells from healthy donors and ovarian cancer patients respond to HER-2 peptides, indicates that tolerance to some of these self-epitope is not induced.

Ongoing studies aim to determine whether T cells induced by these peptides recognize the HER-2 protein, the restriction element and the dominant epitopes for induction of a Th1 response. The implications of the observed responses in immunity to, or progression of ovarian cancer deserve further consideration as to whether the responses to these peptides correlate with HER-2 expression, stage and clinical outcome. Such studies are currently in progress in our laboratory. The results presented in this study should be useful for investigation of the mechanisms of Ag specific immunity, auto immunity, tolerance and design of epitope specific tumor vaccines.

LEGENDS TO THE FIGURES

Figure 1. Histograms of positive blastogenic responses of PBMC from **(A)** 14 healthy donors and **(B)** 25 ovarian cancer patients to seven HER-2 peptides of the sequences listed in Table I. The Y axis indicates the fraction of donors with a positive response to each peptide. A blastogenic response was considered significant when the p values for peptide stimulated vs. control cultures were <0.05. Stimulation indexes (S.I.) were obtained by dividing the means of cpm proliferation of PBMC in the presence of peptides with the means of cpm proliferation in the absence of peptide. S. I. for the peptides inducing significant proliferation ranged between 1.5 – 3.0, while for the peptides that did not induce significant proliferation ranged between 0.9 – 1.2. Proliferation values (cpm + SD) are shown in Figures 2 and 3. Peptides F7 and F13 elicited the most positive responses in PBMC from both healthy donors and patients (at least 5 donors positive in each group). In **(B)**, **(*)** the frequency of responses to F10, F12, and D100 respectively were as follows: F10: 1/25, F12, 1/25 and D100: 2/25.

Figure 2. Proliferative responses to HER-2 peptides by PBMC from four healthy donors **A, B,C,D** determined in the same experiment. Two responded to peptides as follows: **Donor A** to D100, D122, F13 and F14; **Donor B** to F7 and F13. Two were non-responders **(panels**

C, D). Results indicate cpm $\pm$ SD. Dotted lines indicate proliferation corresponding to S.I. of 1.0 and 2.0 respectively. *cpm proliferation to TT not on scale.

Figure 3. Proliferative responses to HER-2 peptides of PBMC determined in the same experiment. **(A)** ovarian cancer patient; **(B)** healthy donor. Significant differences between peptide stimulated and no peptide cultures were observed in **(A)** for D122, F7 and F13 and in **(B)** for D122, F13 and F14. Dotted lines indicate proliferation corresponding to S.I. of 2.0.

Figure 4. **(A)** Proliferative responses to HER-2 peptides F7, F13, F14, and D122 by T-cell cultures induced with the corresponding peptide and expanded in IL-2. **(B)** Increase in cell number of F13 induced T cells described in (A) after restimulation with either no peptides (control), each of the peptides D122, F14, F7 and F13; or PHA and OKT3 mAb. Experimental conditions were as described in the Materials and Methods. **(C)** Cell surface phenotypes of peptide-induced cultures described in (A), after restimulation with each peptide and expansion in IL-2 ❑ $CD4^+$ cells, ❑ $CD8^+$ cells.

Figure 5. Cytokine secretion by HER-2 peptides-induced T-cell lines. Equal numbers of cells (10^5) from each culture were plated in 96-well plates and incubated with PMA and OKT3 mAb as described in Materials and Methods. Cytokine expression was determined by ELISA. The concentrations were calculated by comparison with standard plots of IL-10 and IFN-γ in the same assays.

Table I. Sequences of HER-2 peptides

Peptide Code	Position	Sequence[a]
F11	5 – 19	A L C R W G L L L A L L P P G
D122	396 – 406	Q L Q V F E T L E E T
F12	449 – 465	G I S *W* L G **L** R S **R** E **L** G S G L
F14	474 – 487	T V P *W* D Q *L* F *R* N P H Q A
F7	776 – 789	G S P *Y* V S **R** L **L** G I C L
F6	776 – 797	G S P *Y* V S **R** L **L** G I C L T S T V Q
F8	832 – 851	G M S *Y* L E D V R L V H R D L A A R N
F10	975 – 997	F S R *M* A R S P Q R *F* V V **I** Q N **E** D **L** G
D100	1086 – 1098	F D G D L M G A A K G L
F13	884 – 899	V P I K *W* **M** A L **E** S **I** L R R R F

[a]Potential DR4 anchors that distinguish between DRB1*0401/0404 and DRB1*0402 are shown in bold. Tyr (Y) and Trp (W) residues characteristic of the P1 anchors for DRB1*0401 and DRB*0101 binding motifs (21) are italicized. Prolines for protection from proteolysis are underlined. Peptide F10 extends a potential helper epitope after the CTL epitope C85 (the sequence is underlined). Both DR4 allotypes (DR4.1 and DR4.2) accept peptides with Leu, Ile, Met, Phe, and Val as P1 anchor residues (19).

REFERENCES

1. Disis ML, Callenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, Jeschke M, Lydon N, McGlynn E, Livingston RB, Moe R, and Cheever MA: Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res. *54*:16-20, 1994

2. Ioannides CG, Fisk B, Fan D, Biddison WA, Wharton JT, and O'Brian CA. Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptides derived from the HER-2/neu proto-oncogene. Cell. Immunol. *151*:225-234, 1993.

3. Yoshino I, Goedegeboure PS, Peoples GE, Parikh AS, DiMain JM, Lyerly HK, Gazdar AF, and Eberlein TJ: HER-2/neu derived peptides are shared antigens among human non-small cell lung cancer and ovarian cancer. Cancer Res. 54:3387-3390, 1994.

4. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, and Eberlein TJ: Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER-2/neu-derived peptide. Proc.Natl.Acad.Sci, USA *92*:432-436, 1995.

5. Fisk B, Blevins TL, Wharton JT, and Ioannides CG: Identification of an immunodominant peptide of HER-2/neu-proto-oncogene recognized by ovarian tumor specific CTL lines. J.Exp. Med. *181*:2709-2717, 1995.

6. Ioannides CG, Platsoucas CD, and Freedman RS: Immunological effects of tumor vaccines: II. T cell responses directed against cellular antigens in the viral oncolysates. In Vivo 4:17-24.1990

7. Falk K, Rotzschke O, Stevanovic S, Jung G, and Rammensee H-G: Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constrainsts of processing, and general rules. Immunogenetics *39*:230-242,1994

8. Kropshofer H, Max H, Muller CA, Hesse F, Stevanovic S, Jung G, and Kalbacher H: Self-peptide released from class II HLA-DR1 exhibits a hydrophobic two-residue contact motif. J.Exp.Med. *175*:1799-1803, 1992

9. Malcherek G, Falk K, Rotzschke O, Rammensee H-G, Stevanovic S, Gnau V, Jung G, and Melms A: Natural peptide ligand motifs of two HLA molecules associated with myastenia gravis. Int. Immunol. *5*:1229-1237, 1993.

10. Sette A, Sidney J, Oseroff C, del Guercio M-F, Southwood S, Arrheuius T, Powell MF, Colon SM, Graeta FCA, and Grey HG: HLA Drw4-binding motifs illustrate the biochemical basis of degeneracy and specificity in peptide-DR interactions. J. Immunol. *151*:3136-3170, 1993.

11. Hammer J, Valsasnini P, Tolba K, Bolin D, Higelin J, Takacs B, and Sinigaglia F: Promiscuous and allele-specific anchors in HLA-DR binding peptides. Cell. *74*:197-203, 1993.

12. Hammer J, Gallazzi F, Bono E, Karr RW, Guenot J, Valsasnini P, Nagy ZA, and Sinigaglia F: Peptide binding specificity of HLA-DR4 molecules: Correlation with rheumatoid arthritis association. J. Exp. Med. *181*:1847-1855, 1995.

13. Sinigaglia F, and Hammer J: Motifs and supermotifs for MHC class II binding peptides. J. Exp. Med. *181*:449-451, 1995.

14. Marshall KW, Wilson KJ, Liang J, Woods A, Zaller D, and Rothbard JB: Prediction of peptide affinity to HLA-DRB1*0401. J. Immunol. *154*:5927-5933, 1995.

15. Barker RN, and Elson CJ: Multiple self epitopes on the Rhesus polypeptides stimulate immunologically ignorant human T cells. Eur. J. Immunol. *24*:1578-1582,1994

16. Elson CJ, Barker RN, Thompson SJ, and Williams NA: Immunologically ignorant autoreactive T cells, epitope spreading and repertoire limitation. Immunol. Today *16*:71-76, 1995.

17. Barker, E., Mackewicz, C. E. and Levy, J. A. Effects of Th1 and Th2 cytokines on CD8+ cell response against human immunodeficiency virus: Implication for long-term survival. Proc. Natl. Acad. Sci, USA *92*:11135-11139, 1995.

18. Tsuchida T, Parker KC, Turner RV, McFarland HF, Coligan JE, and Biddison WE: Autoreactive CD8+ T-cell responses to human myelin. Proc. Natl. Acad. Sci. USA *91*:10859-10863, 1994.

19. Salgame P, Abrams JS, Clayberger C, Goldstein H, Convit J, Modlin RL, and Bloom BR: Differing lymophokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science (Wash. DC) *254*:279-282, 1991.

20. Matsuda M, Salazer F, Petersson M, Masucci G, Hansson J, Pisa P., Zhang, Q. J., Masucci, M. G. and Kiessling, R. Interleukin 10 pretreatment protects target cells from tumor- and allele-specific cytotoxic T cells and downregulates HLA class I expression. J. Exp. Med. *180*:2371-2376, 1994.

21. Croft M, Certer L, Swain SL, and Dutton RW: Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. J.Exp.Med. *180*:1715-1728, 1994.

22. Allison JP, Hurwitz AA, and Leach DR: Manipulation of costimulatory signals to enahnce antitumor T-cell responses. Curr. Opin. Immunol. 7:682-686, 1995.

23. Wu T-C, Guarnieri FG, Staveley-O'Carroll KF, Viscidi RP, Levistsky HI, Hedrick L, Cho KR, August JT, and Pardoll DM. Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens. Proc. Natl. Acad. Sci, USA *92*:11671-11675, 1995.

24. Topalian SL, Rivoltini L, Mancini M, Markus NR, Robbins PF, Kawakami Y, and Rosenberg SA: Human CD4+ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. Proc. Natl. Acad. Sci. USA *91*:9461-9465, 1994..

25. Nanda NK and Sercarz EE: Induction of anti-self-immunity to cure cancer. Cell *82*:13-17, 1995.

26. Wucherpfennig KW, and Strominger JL: Selective binding of self peptides to disease-associated major histocompatibility complex (MHC) molecules: a mechanism for MHC-linked susceptibility to human autoimmune diseases. J.Exp.Med. *181*:1597-1601, 1995.

27. Pfeiffler C, Stein J, Southwood S, Ketelaar H, Sette A, and Bottomly K: Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. J. Exp. Med. *181*:1569-1574, 1995.

28. Kumar V, Bheardwaj V, Soares L, Alexander J, Sette A, and Sercarz E: MHC binding affinity of an antigenic determinant is crucial for the differential secretion of IL4/5 or IFN γ by T cells. Proc.Natl.Acad.Sci, USA *92*:9510-9514, 1995.

29. Wucherpfennig KW and Strominger JL: Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Cell *80*:695-705, 1995.

30. Silver RF, Wallis RS, and Ellner JJ: Mapping of T cell epitopes of the 30-kDa α antigen of *mycobacterium bovis* strain bacillus Calmette-Guerin in purified protein derivative (PPD)-positive individuals. J. Immunol. *154*:4665-4674, 1995.

31. Clerici M, and Shearer GM. The Th1 - Th2 hypothesis of HIV infection: new insights. Immunology Today *15*:575-581, 1994.

33. Sheen-Chen S-M, Chou F-F, Eng H-L, and Chen W-J: An evaluation of the prognostic significance of HLA-DR expression in axillary-node-negative breast cancer. Surgery *116*:510-515, 1994.

34. Iarygin LM, Malyshev VS, Polianskaia IS, Denisov LE, Zimin II, Alekseeva PL, and Khotchenknoa NV. The clinical significance of determining the HLA-DR4 antigen in patients with breast cancer. Voprosy Onkologii *37*:796-799, 1991.

35. Casoli C, Zanelli P, Adorni A, Starcich BR, and Neri, T: Serological and molecular study on the HLA phenotype of female breast cancer patients. Eur. J. Cancer *30*:1207-1208, 1994.

36. Lee JE, Reveille JD, Ross MI, and Platsoucas CD: HLA-DQB1*0301 association with increased cutaneous melanoma risk. Int. J. Cancer *59*:510-513, 1994.

37. Apple RJ, Erlich HA, Klitz W, Manos MM, Becker TM, Wheeler CM: HLA DR-DQ association with cervical carcinoma show papillomavirus-type specificity. Nature Genetics *6*:157-162, 1994.

38. Takahashi T, Chapman PB, Yang SY, Hara I, Vijayasandhi S, and Houghton AN: Reactivity of autologous CD4+ T lymphocytes against human melanoma. J. Immunol. *154*:772-779, 1995.

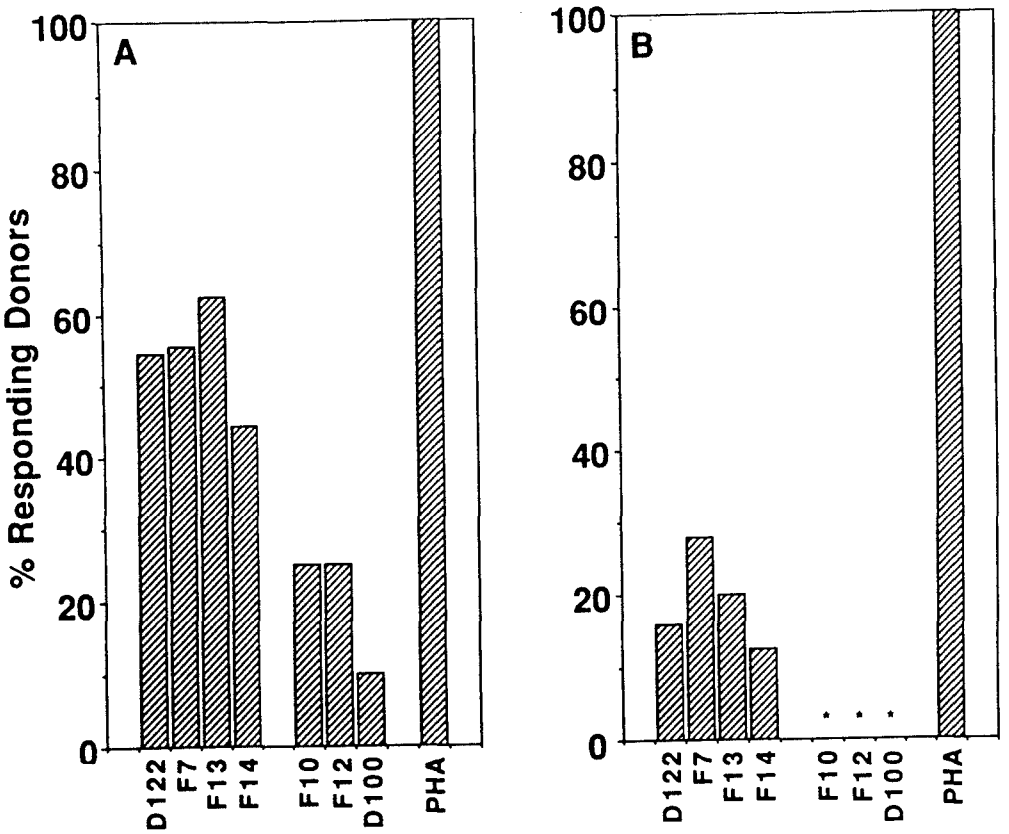
39. Topalian SL, Rivoltini L, Mancini M: Human CD4+ T cells specifically recognize a shared melanomas-associated antigen encoded by the tyrosinase gene. Proc. Natl. Acad. Sci. USA *91*:9461-9465, 1994.

40. Becker JC, Brabletz T, Czerny C, Termeer C, and Brocker EB: Tumor escape mechanisms from immunosurveillance: induction of unresponsiveness in a specific MHC-restricted CD4+ human T cell clone by the autologous MHC class II+ melanoma. International Immunology *5*:1501-1508, 1993.

41. Matsuo H, Batocchi A-P, Hawke S, Nicolle M, Jacobson L, Vincent A, Newsom-Davis J, and Willcox N: Peptide-selected T cell lines from mysastenia gravis patients and controls recognize epitopes that are not processed from whole acetylcholine receptor. J. Immunol. *155*:3683-3692.1995.

42. Ioannides CG, and Freedman RS: T cell responses to ovarian tumor vaccines. International Reviews of Immunology *7*:349-364, 1991.

43. Dong R-P, Kamikawaji N, Toida N, Fujita Y, Kimura A, and Sasazuki T. Characterization of T cell epitopes restricted by HLA-DP9 in streptococcal M12 protein. J. Immunol. *154*:4536-4545, 1995.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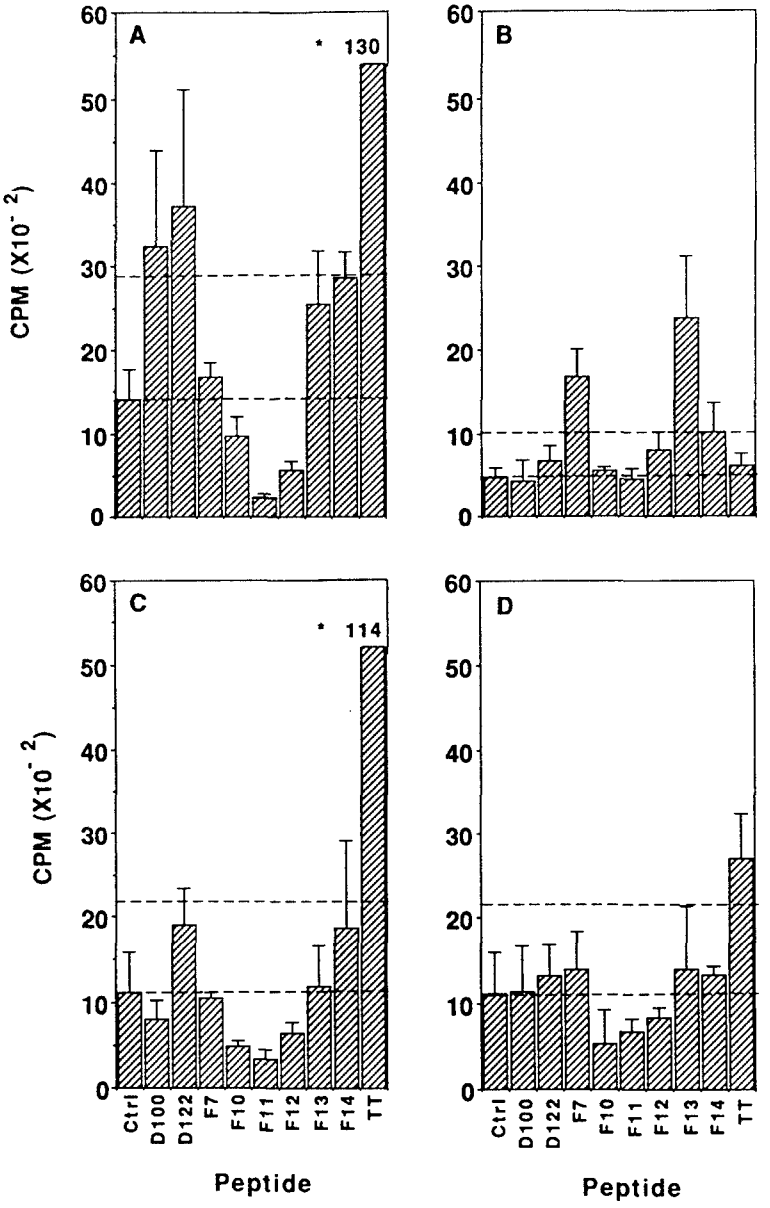
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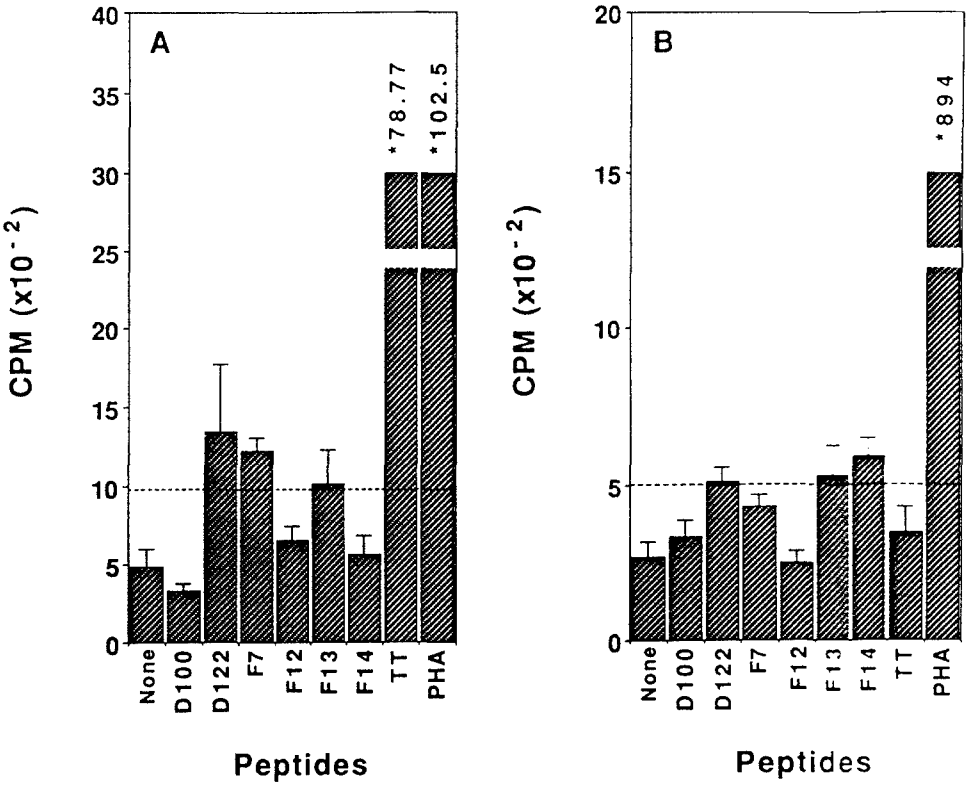
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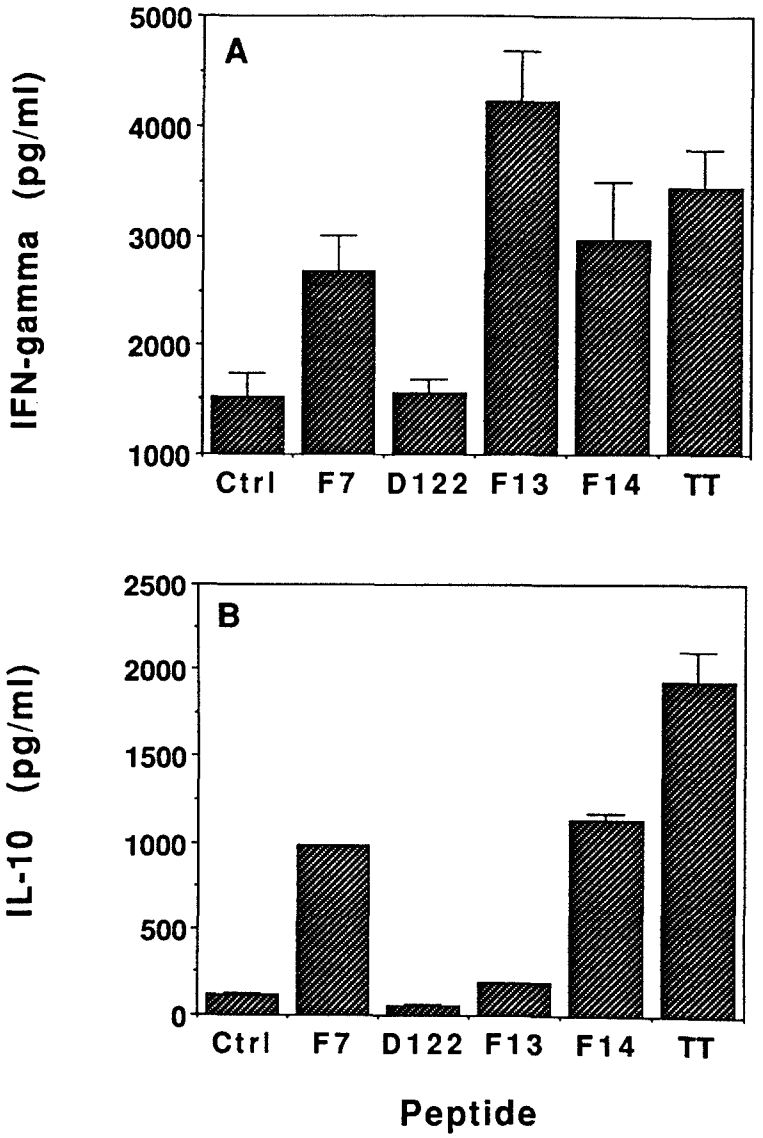
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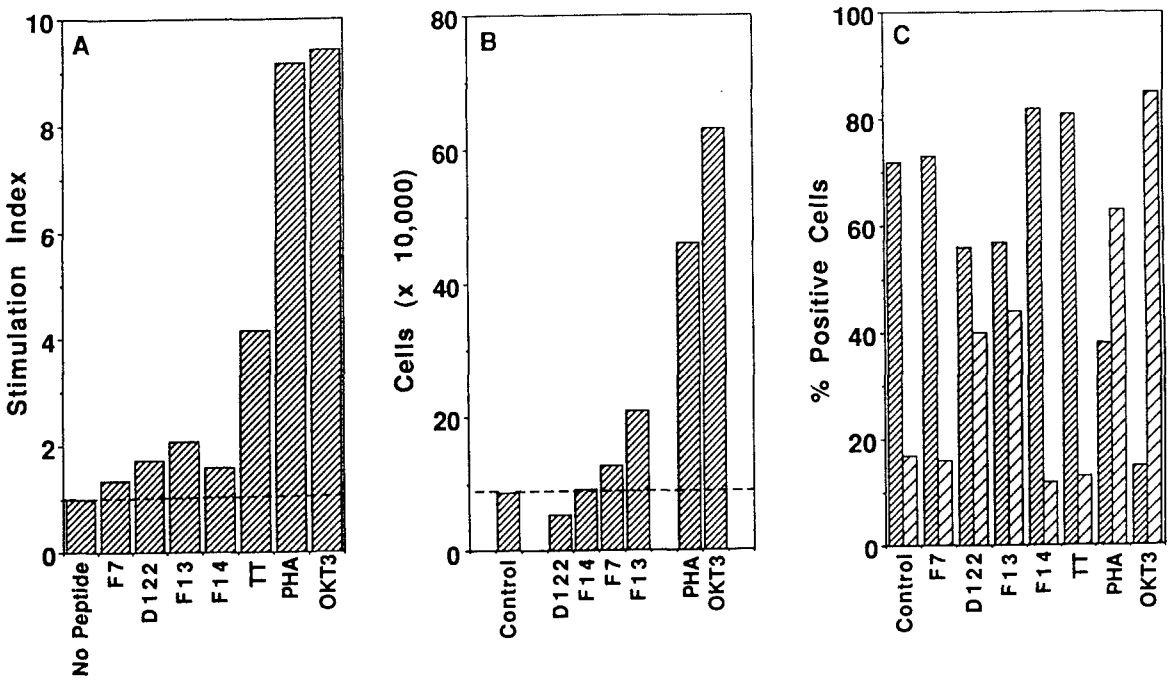
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MASS-SPECTROMETRIC ANALYSIS OF NATURALLY PROCESSED PEPTIDES RECOGNIZED BY OVARIAN TUMOR-ASSOCIATED CD8+ CTL

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Abbreviations: *HER-2*, HER-2/neu proto-oncogene; *HPLC*, reverse phase high pressure liquid chromatography; *MS*, mass spectrometry; *ES-MS*, electrospray-ionization mass-spectrometry; *TFA*, trifluoroacetic acid; *ACN*, acetonitrile; *Ag*, antigen; *TCR*, T cell receptor; *Rt*, Retention time; *Fxn*, Fraction; *m/z*;, mass - to - charge ratio; *Da*, mass unit; *ESI*, electrospray ionization; *CID*, collision-induced dissociation.

Key Words: natural peptides, CTL epitopes, ovarian cancer.

Running title: HLA-A2-associated peptides on ovarian tumors

Acknowledgments. The first two authors (B.F, and B.D.) equally contributed to this paper. We thank Dr. Richard M. Caprioli (University of Texas Medical School) for fruitful discussions, Dr. Ioannis Papayannopoulos (Biogen Corporation, Boston) and Dr. Bohuslav Melichar (M.D. Anderson Cancer Center) for interpretation of CID spectra, and assistance with data analysis and Dr. James Briggs (University of California, San Diego) for providing the PEPSEQ software. The authors also wish to thank Ms. Susan Mondragon for her outstanding editorial assistance.

ABSTRACT

Antigens recognized by cytotoxic T cells (CTL) are expressed as peptides presented by MHC class I molecules. To isolate peptides from the MHC molecule HLA-A2.1 and identify epitopes that define the activity profile of ovarian $CD8^+$ CTL. Peptides were separated by reverse-phase high-pressure liquid chromatography (HPLC), and analyzed by electrospray ionization-tandem mass spectrometry (ES-MS). HLA-A2.1-bound peptides were extracted from the ovarian tumor line SKOV3 transfected with the HLA-A2.1 (clone 1E4) and the C1R.A2 cells transfected with HLA-A2.1 and HER-2 (clone HER-2.J) by immunoaffinity chromatography. At least five peaks of distinct retention times (termed *1*, *2A*, *2B*, *2C*, and *3*) were recognized by an ovarian HER-2^{high} (HER-2^{hi}) tumor-associated HLA-$A2^+$, $CD8^+$ CTL line. ES-MS analysis was performed for peak *2B* peptides from both cells. In the four consecutive fractions of peak 2B, at least 27 and 16 ion species of mass-to-charge *(m/z)* ratio between 760 - 1300 were detected in 1E4 and HER-2.J cells, respectively. The abundance of four 1E4 and six HER-2.J ions believed to be peptides in four consecutive HPLC fractions in this peak matched the CTL activity profile. Of these, two ions with actual *m/z* ratios 497.3-498.4 and 792.8-793.2, were found in the peak *2B* from both cells. Since little is known about the tumor Ag recognized in human cancers, characterization of these ions may lead to identification of novel tumor Ag in breast and ovarian cancers. This may also be useful in developing quantitative approaches to the identification of tumor Ag and the determination of epitope density on tumor and normal cells. This may help characterize the relationship between tumor immunity and epitope tolerance in human epithelial cancers.

INTRODUCTION

The development of rational immunotherapies for human cancers depends on a detailed understanding and quantitation of the host anti-tumor responses that involve recognition by CTL of specific epitopes on various normal and malignant tissues. CTL epitopes are short peptides (8-10 amino acids long) presented on the cell surface by MHC class I molecules (1). In considering peptide recognition by CTL, it is important to learn how much this recognition depends on the Ag (peptide) density on the target and on the affinity of the T-cell receptor (TCR) for the particular epitope (2, 3). To address the question of epitope density, it is first necessary to identify precisely the tumor peptide involved.

This is of particular importance for several reasons. First, natural peptides produced by tumor Ag processing machinery may not be entirely identical to the synthetic peptides used to define CTL epitopes. The latter are usually defined with HLA-class I anchor motifs or via genetic approaches (4). Second, for human cancers, a number of peptides derived from self-proteins have been found to reconstitute the lytic function of tumor reactive CTL. Thus, with few exceptions, evidence to date reflects an immune repertoire rather than a specific and selective response to tumor (5). Third, since a number of these epitopes are derived from structurally similar hydrophobic areas (e.g. signal and transmembrane domains) of various proteins (6-8), this raises the question whether the epitope(s) defined by synthetic peptides are identical or cross-reactive with the peptides presented by the tumor. Fourth, it is still unknown whether the lack of recognition of a particular tumor by its autologous CTL reflects the lack of presentation of the tumor Ag or tolerance of the particular epitope. Tumor antigens may also be presented to T cells by professional antigen-presenting cells (APC) (9), which can process particulate antigens or damaged cells for MHC class I presentation (10-11). Thus, identification of the natural peptides presented in association with MHC molecules on human tumors should

be important for the identification of tumor Ag and consequent development of novel approaches to tumor-specific CTL induction.

To date, with the few exceptions of melanoma gp100 and MART-1 CTL epitopes (12, 13) there is little information on the identity and density of the peptides presented by most human solid tumors. Ovarian CTL lines were recently reported to recognize a number of HER-2/neu (HER-2) peptides (7, 14, 15), but the nature of peptides presented by the tumor is still unknown. To address the nature of Ag relevant to CTL targeting in breast and ovarian cancer, we have investigated the identity of peptides presented by HLA-A2 on targets recognized by HLA-A2$^+$ CD8$^+$ CTL lines isolated from patients with ovarian cancer. We used a cloned ovarian tumor line (SKOV3.clone 1E4) and the cloned C1R.A2 line (clone HER-2.J) transfected with HLA-A2 and HER-2, respectively. Since these cells express defined levels of MHC antigens, adhesion molecules, and HER-2, quantitation of HLA-A2-bound peptides expression can provide a reference point for analysis of peptides from freshly isolated tumors.

MATERIALS AND METHODS

Cell lines.

The ovarian tumor line SKOV3 (HLA-A3, A28, B18, B35, Cw5) was stably transfected with the gene for HLA-A2. The gene for HLA-A2.1 was kindly provided by Dr. William E. Biddison (NIAID, NIH). A tumor clone, SKOV3.A2.1E4, expressing high levels of both HER-2 and HLA-A2 and designated 1E4 was selected for expansion in large numbers and for peptide fractionation experiments. C1R:A2 cells (a kind gift from Dr. Biddison), which express the product of the same HLA-A2 gene on their surface as SKOV3.A2 cells were transfected with the gene encoding for the HER-2 protooncogene (plasmid pCMV.HER-2 encoding a full-length HER-2.cDNA) and co-transfected with the plasmid SV2.Hygro (ATCC). These plasmids

were a kind gift of Dr. Mien-Chie Hung, Department of Tumor Biology (M. D. Anderson Cancer Center). After selection with hygromycin B, C1R.A2.HER-2^{+} cells were cloned by stringent limiting dilution. At least 20 C1R.A2.HER-2^{+} clones (designated HER-2.A-T) expressing variable levels of HER-2 receptor on their surface were isolated. A clone of C1R:A2.HER-2 cells designated HER-2.J was selected for expansion in large numbers and for biochemical fractionation of HLA-A2-bound peptides.

Cell lines were grown in RPMI 1640 medium containing 10% FCS supplemented with 2 mM L-glutamine and 100 μg/ml Gentamicin (complete RPMI medium). HLA-A2-transfected 1E4 and C1R:A2 cells were selected with 250 μg/ml G418 (GIBCO, BRL, Gaithersburg, MD). Both SKOV3 and C1R.A2 express the same HLA-A2 gene. HER-2.J cells were selected with 50 μg/ml of hygromycin B. CTL-OVA3 effector cells used in these studies were previously described. The corresponding HLA-A2^{+} tumor overexpressed HER-2 (HER-2^{hi}) (15). CD8^{+} cells were isolated from cultured TAL on Ab coated plates in an AIS MICRO^{CELLECTOR} (Applied Immune Sciences). CTL-OVA3 line was CD3^{+} CD8^{+}. CD4^{+} cells represented $\leq 5\%$ of total cell population.

Flow Cytometry

Target cells were tested in fluorescence experiments to confirm the expression of HLA-A2, MHC class I, and HER-2, as previously described (15). Hybridomas producing mAb BB7.2 and MA2.1 were obtained from the American Tissue Culture Collection (ATCC). Surface antigen expression was determined by flow cytometry using a fluorescence-activated cell sorter FACSscan (Beckton-Dickinson) with a log amplifier. CD3, CD4, and CD8 antigen expression on the T cell lines were determined by immunofluorescence with corresponding FITC-conjugated mAb. C1R.A2 clones were designated as HER-2^{hi} when MCF for HER-2 expression was above 200 and HER-2^{low} when MCF was below 40. Expression of CD18,

CD11a (LFA-1), CD54 (ICAM-1) and CD58 (LFA-3) antigens was also tested with corresponding antibodies (Beckton-Dickinson).

Peptide extraction.

1E4 and HER-2.J cells were grown in complete RPMI 1640 medium in 10-chamber cell factories (Nunc, Thousand Oaks, CA). Afterwards, cells were collected and washed three times with cold PBS. Between 1.0 and 1.5 x 10^9 cells were obtained from each cell factory. Cells were then lysed using a buffer containing protease inhibitors (aprotinin, leupeptin, pepstatin, PMSF, iodacetamide) in PBS (16). 0.5% CHAPS was used as lysing agent to minimize binding to C18 columns. MgCl2 and glycerol at final concentrations of 6mM and 20% were added in the lysis buffer to minimize the denaturation of extracted proteins. Detergent-solubilized extracts of 1E4 and HER-2.J cells were obtained after centrifugation at 40,000 g for 2 h. HLA-A2.1 was isolated from the centrifugation supernatants by affinity chromatography on Protein A-Sepharose prebound with mAb BB7.2 as described (16), except that the extracts were preabsorbed on Protein-A Sepharose (Sigma Chemical Co., St. Louis, MO) to minimize nonspecific binding. The column was washed with PBS containing 0.25M NaCl. The OD_{210} nm (for peptide bond) of the eluate was monitored. The column was then eluted with 0.2 M acetic acid. The eluate was boiled for 5 min to allow dissociation of peptides from HLA-A2, centrifuged through an Ultrafree CL unit (3-kDa cut-off) (Millipore), and lyophilized. For these studies, at least 2 x 10^{10} cells each of 1E4 and HER-2.J were grown in batches of 1.5–2.0 x 10^9 cells. Peptides with masses < 3 kDa were pooled, lyophilized, and separated by HPLC.

HPLC fractionation of HLA-A2-bound peptides.

Tumor peptides extracted from HLA-A2.1 molecules of both 1E4 and HER-2.J cells were separated in the first dimension on a Brownlee C18 Aquapore column (2.1 x 30 mm; pore size, 300 A; particle size, 7μm; Applied Biosystems, Perkin-Elmer Corporation). They were eluted with a 60-min gradient of 0-60% (vol/vol) acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA)

(Gradient I) at a flow rate of 200 µl/min using Model 1090 HPLC System (Applied Biosystems) as previously described (16-18). For separation in the second dimension, pooled fractions from the first dimension corresponding to the peak of elution of peptide E75 and the corresponding peak of CTL activity were injected into a Brownlee C18 Aquapore column of (2.1 x 220 mm 300A, 7 µm) and eluted on a shallower gradient: 0-5 min, 0-15% ACN in 0.1% TFA; 5-45 min, 15-35% ACN in 0.1% TFA; and 45-60 min, 35-60% ACN in 0.1% TFA (Gradient II). The flow rate was 200 µl/min, and fractions were collected at 1-min intervals.

Synthetic HER-2 peptides were prepared by the Synthetic Antigen Laboratory at M. D. Anderson Cancer Center using a solid-phase method and then purified by HPLC. The identity of peptides was established by amino acid analysis. The purity of peptides was more than 97%. HER-2 peptides E75 (369-377, KIFGSLAFL), C85 (971-979, ELVSEFSRM), and E91 (5 - 13, ALCRWGLLL) were separated under the same conditions using Gradients I and II. Their elution peaks and retention times (Rt) were determined.

CTL assays.

CTL epitopes were reconstituted from HPLC fractions obtained in the first and second RP-HPLC dimensions (Gradients I + II). Aliquots of each fraction (50 ul) were concentrated by vacuum, reconstituted with RPMI medium to the initial volume, and then added to ^{51}Cr-labeled T2 cells in V-bottom microtiter plates. After incubation with peptides for 90 min, effectors were added at various effector/target (E/T) ratios, and a standard CTL assay was performed for 5 h as previously described (15).

Mass spectrometry.

Electrospray-ionization-mass-spectrometric (ES-MS) analyses were performed on a Finnigan MAT TSQ70 triple-quadrupole instrument upgraded with TSQ700 software and a 20-kV conversion dynode electron multiplier. The Vestec ES source (PerSeptive Biosystems,

Vestec Products, Cambridge, MA) was modified as previously described by Emmett and Caprioli (20). Typical ES operating parameters were as follows: needle voltage, 3.5 kV; nozzle voltage, 250 V; repeller voltage, 10-12 V; source block temperature, 240 ± 5°C. Standard peptides were dissolved at 1-2 nmol/μl in water or 0.5% aqueous acetic acid (V%), diluted to 10 pmol/μl with 100:100:0.5 (vol/vol/vol) methanol/water/acetic acid (ES-MS solvent), and introduced into the MS by infusion at 0.82 μl/min. HPLC fractions obtained from biological samples were first lyophilized to dryness in polypropylene microcentrifuge tubes. The dried samples were then reconstituted in either 10 μl 0.5% acetic acid and an equal volume of methanol or in 20 μl ES-MS solvent. One-microliter samples were then introduced into the mass-spectrometer by injection into the infused ES-MS solvent via a Rheodyne 8125 injector fitted with a 5-μl sample loop.

Collision-induced dissociation (CID) mass spectra (MS–MS) were obtained with argon collision gas at a pressure of 1-2.5 mTorr and a collision offset potential of -6 to -20 eV. Spectra were acquired at the rate of 1 scan/second (or approximately 1000 Da/s, depending on the mass range needed) with a running average of 16 scans. For daughter-ion scans, the resolution on the first quadrupole (Q1) was adjusted to allow transmission of ±2 Da from the center of the mass of interest. A peak width of 1 Da was used for postacquisition spectral averaging and for quantitation by manual integration of selected ion chromatograms from the injection analyses. Data analyses were performed using Finnigan MAT ICIS software.

In certain instances, samples containing hydrophobic material were first reconstituted with 0.5% acetic acid, and then mixed with an equal volume of methanol. Peptide standards (des-Tyr^1-Leu-Enk, *m/z* 393.2 $(M + H)^+$, substance P, 1-11 *m/z* 674.4 $(M + 2H)^{+2}$; β-casomorphin m/z 790.4 $(M + H)^+$; and bovine insulin, *m/z* 956.6 $(M + 6H)^{6+}$ were analyzed to verify the sensitivity of detection. Each sample was analyzed at least twice. Sensitivity of

detection was at the level of 25 fmol/μl sample injected. Determinations did not vary more than 2-4% in two independently performed experiments (data not shown).

Synthetic HER-2 peptides E75, C85, and E91 (15) were sequenced by MS–MS. To facilitate the interpretation of results, the CID spectrum in certain instances was compared with that recorded for the corresponding methyl ester. In the spectrum of the methyl ester, signals containing the C-terminus and Asp and Glu groups shift by increments of 14 Da. Methyl esterification of carboxyl groups was performed using acetyl chloride in methanol as the methylating agent. Data for sequence reconstitution were analyzed using the program PEPSEQ version 1.2 software (21). This program identifies candidate sequences based on the concordance of determined and predicted peak values of ions in a candidate sequence. The lowest deviation between the experimentally determined versus theoretical values for the respective ions was defined as the lowest score/peak ratio (sc/p).

RESULTS

Definition of CTL epitopes recognized by CTL-OVA3 .

In our previous studies we found that CTL associated with ovarian tumors recognize peptides of distinct sequence. In addition to autologous tumors, these CTL were found to specifically recognize ovarian tumor lines that expressed the Ag presenting molecule HLA-A2 (15). CTL-OVA3 recognized several HER-2 peptides including E75 as previously reported (15). Furthermore, both HLA-A2-transfected SKOV3 cells and HER-2 transfected C1R.A2 cells were lysed by cloned CTL from a $CD8^+$ $CD4^-$ line (CTL-41) that was induced by stimulation of $HLA\text{-}A2^+$ PBMC with a variant of the peptide C85 (22). This suggests that some of the epitopes recognized on these targets by CTL are similar. SKOV3 cells constitutively overexpress HER-2 but do not express HLA-A2. In contrast, C1R.A2 cells expressed only the product of transfected HLA-A2 gene. Experiments on lysis of HER-2-transfected clones of C1R:A2 such as HER-2.B, HER-2.L, and HER-2.J by highly purified natural killer (NK) cells were performed in parallel, and results indicated an inverse correlation between HER-2 expression and susceptibility to lysis by NK cells (data not shown). These results suggest that within the CTL-OVA3 line there exists T-cell populations that recognize, (a) HER-2 peptides presented by HLA-A2 on the targets, (b) epitopes derived from proteins associated with HER-2 overexpression, (c) epitopes from proteins associated with tumor cell growth.

Recently we found that three of three ovarian CTL lines recognized naturally processed peptides eluted from HLA-A2 of the SKOV3.A2 cells. These CTL also recognized C1R.A2.HER-2 lymphoblastoid line. Some of these peptides eluted with similar retention times with peptides eluted from a freshly isolated ovarian tumor line (23). These studies raised the possibility that common naturally processed peptides are presented by these cells. These findings raised novel questions on the composition of the active peaks regarding the peptide size, abundance, and identity.

To define the pattern of recognition of CTL-OVA3 for antigens (Ag) presented by HLA-A2, peptides were extracted from immunoaffinity purified HLA-A2 molecules from 1 x 10^9 1E4 and HER-2.J cells each. The presence in the peaks of HPLC fractions of peptides with the same Rt likely indicated common CTL epitopes (16-18, 22). Peptides were fractionated by HPLC on Gradient I using a C18 column (2.1 x 30 mm). CTL recognition was demonstrated by three distinct peaks of 1E4 peptides (designated ***1, 2, and 3***), respectively as indicated by lysis of T2 cells incubated with equal volumes of each fraction eluting on Gradient I (**Figure 1**). Of the HER-2 peptides, C85 (971-979), eluted in Fxn 22-23 (Rt = 22.8 min), while E75 (369-377) eluted in Fxn 29-30 (Rt = 29.8 min), suggesting that peptide(s) of similar Rt with E75 were present in peak *2* of 1E4 peptides. Similar patterns of activity with the same HPLC fractions separated on Gradient I were observed for two other ovarian CTL lines, CTL-OVA4 and CTL-OVA5. The position of peak *2* (Fxn 28-32) was identical for CTL-OVA3, CTL-OVA4, and CTL-OVA5 cells (23).

These results suggest the presence in these HLA-$A2^+$ patients of CTL of common specificity. We focused first on the bioactive peak *2* because it was recognized by all three of our ovarian CTL lines. Since each fraction was assumed to contain a large number of peptides, the pooled active Fxn 27-32 from peak *2* corresponding to the peak of elution of E75 $\pm$ 2 min were rechromatographed on a longer C18 column of 220 mm containing particles of the same size as in the shorter column. We used a shallower and thus more resolving Gradient II (increments of 0.5% ACN/min, between 15-35% ACN) (17,18). Fractions with higher and lower retention times than E75 were included in this separation to avoid missing shorter or longer peptides and to minimize losses in the material due to absorption of peptides at low concentrations. The focus on peptides eluting in the area corresponding to the peak of elution of E75 was dictated by the fact that E75 was found to be immunodominant among the HER-2

peptides tested (15). Furthermore, two CTL lines recently isolated from breast tumors of HLA-A2^{+}, HER-2^{hi} phenotype (CTL-BR5 and CTL-BR6) also recognized E75 in a concentration-dependent fashion (Melichar B, et.al., manuscript in preparation).

Rechromatography of peak *2* (pooled Fxn 27-32) under Gradient II conditions resulted in three distinct peaks of CTL-OVA3 activity. These peaks were designated *2A, 2B, and 2C,* respectively. Peak *2A* (Fxn 32-34) appeared as a double peak of activity. The peak *2B* (Fxn 39-42) eluted around the Rt expected for E75 (**Figure 1C**). To determine whether active peptides are presented by HER-2.J cells, we tested recognition of Fxn 14-33 eluting under Gradient I HPLC conditions. Recognition of peptides in these fractions by CTL-OVA3 resulted in two peaks of activity (***1*** and ***2***) **(Figure 1B)** eluting with similar Rt as peptides from 1E4. Fxn 27-32 from peak *2* were pooled and separated under Gradient II conditions. The results in **Figure 1D** show a similar pattern of recognition by CTL-OVA3 for HER-2.J peptides as for 1E4 peptides peaks *2A, 2B, and 2C.* The Rt of E75 and of the homologous peptide from epidermal growth factor receptor (EGF-R 364-372: SISGDLHIL, designated F48) were compared under the same HPLC conditions. F48 eluted in fraction 38. E75 coeluted with the peak of CTL activity with an Rt of 41.0 $\pm$ 0.5 min suggesting that natural peptides of similar sequence may be presented by these cells. Both E75 and F48 peptides recovered from the HPLC column were tested for recognition by CTL. E75 was active between 5-50 pmol, while F48 was inactive even at nanomolar concentrations (data not shown). These data are consistent with the presence of peptides from both 1E4 and HER-2.J cells that reconstituted three peaks of activity *(2A, 2B, and 2C)* for CTL-OVA3

Mass-spectrometric analysis of peptides from HPLC fractions.

To identify peptides presented by HLA-A2 by mass-spectrometry (MS), equivalent samples (in terms of cell numbers) were prepared from 1E4 and HER-2.J. cells. The starting

material consisted of 20 x 10^9 1E4 cells and 10 x 10^9 HER-2.J cells respectively. Peptides were extracted from immunoaffinity-isolated HLA-A2 molecules using BB7.2 mAb. To maximize peptide recovery, BB7.2 mAb was added to and incubated with the eluate from the BB7.2-protein A-Sepharose column for an additional 24 h, and the immunocomplexes were extracted after binding on Protein A-Sepharose. The recovered cell lysate was subjected to a third round of extraction using goat anti-mouse Sepharose to recover HLA-A2-BB7.2 complexes that did not bind to Protein A. However, no material absorbing at 210 nM could be recovered after this step. The peptide material separated through 3kDa filters was chromatographed consecutively through two C18 columns (30 mm and 220 mm long, respectively) using Gradients I and II, respectively. Peak *2B* from each sample was recovered in four fractions designated 39, 40, 41 and 42 **(Figure 1).** Activity was found in Fxn 40 and 41 (designated active fractions) in agreement with the data shown in **Figure 1**. The activities of Fxn 39 and 42 were minimal, so they were designated inactive fractions.

To establish the yields of recovery of the HPLC separation, defined amounts of peptide E75 were chromatographed through the 220-mm C18 column under Gradient II conditions and then evaluated by ES-MS. E75 recovery was estimated by quantitating the peak at *m/z* 498.3, which corresponds to the $(M + 2H)^{+2}$ ion of E75, using the plot established with known amounts of E75 (**Figure 2**). The yields of recovery were 88% and 83% at column loads of 5000 pmol and 100 pmol of E75, respectively (data not shown). Since both 1E4 and HER-2.J expressed high levels of HLA-A2, the total amount of peptides bound on 20 x 10^9 cells (assuming a minimum of 1-2 x 10^5 HLA-A2 molecules/cell), should be at least 3200 - 6400 pmol. Based on the integration of the area of peak *2* (Fxn 27-32) of the plot of absorbance at 210 nM, this peak contained approximately 10% of the peptide material collected under Gradient I conditions (data not shown). This corresponds to 320-640 pmol. This amount is higher than the amount of E75 loaded on the long HPLC column. Since the shorter HPLC

column is expected to absorb less material than the longer column, we assume that the yields of recovery from the Gradient I were at least the same with Gradient II. Thus, the recovery rate of E75 at 100 pmol is indicative of the peptide recovery in Gradient II. Therefore, we tentatively estimated the yields of HPLC fractionation at ~68% (0.83 x 0.83).

To obtain some indication of the abundance of unknown peptides in the HPLC fractions, known amounts of synthetic peptides were analyzed by MS, and the signal intensity was recorded (**Table I**). For peptides present in the range of 25-100 fmol/μl sample, better results were obtained at a multiplier setting of 1600 eV. To avoid carryover, the standards included peptides of sequences less common to epithelial cells. Several peptides were tested since the same amounts of peptides of different sequences give signals of different intensities in the ES-MS spectrum. However similar masses does not mean similar sequences, and consequently similar ionization intensities. For this reason we tested several HER-2 peptides of known mass and sequence (15). Although their signal intensities were different, the differences did not exceed one order of magnitude. Similarly the differences in signal intensities recordered for two control peptides, β-casomorphin and LVV.hemorphin 7 did not exceed one order of magnitude. These peptides are rich in hydrophobic aliphatic residues in a similar fashion with reported CTL epitopes.

For a given peptide (e.g. E75), the signal intensity was linearly proportional to concentration (**Figure 2**). Fluctuations in the linear relationship between concentration and signal intensity were observed when the concentration of E75 was high (0.5-1.0 pmol/μl). At a multiplier setting of 1600 eV, the signal intensity for 100 fmol of standard peptides was in the range of 9-20 x 10^7, while for 50 fmol it was 3-6 x 10^7 (**Table I**). For determinations at lower multiplier settings (800-1100 eV), we observed that for each increase in voltage of 100 eV, the

signal intensity increased by a factor of 2. For analysis of unknown peptides from these samples, we used a multiplier setting of 1600 eV in most experiments.

Each of the peak *2B* Fxn 39-42 of 1E4 and HER-2.J peptides were analyzed by ES-MS. For MS, 25% of 1E4 and 50% of HER-2 material from each of Fxn 39-42 was lyophilized and reconstituted to a final volume of 20 µl. Each determination was made from a 1-µl sample representing 2.5% of material. This would represent 5 x 10^8 cell equivalents/injection. The total ion signal from equivalent samples of 1E4 and HER-2.J cells was obtained by scanning the masses corresponding to *m/z* between 200 and 1500 and then summing the obtained spectra. At each voltage, the limit of detection was defined at a signal-to-noise ratio (S/N) of 2 (24). To determine the ions present and their relative amounts (defined as signal intensity), the mass spectra of Fxn 40 - 41 of 1E4 in the *m/z* range of 700-1300 were determined **(Figure 3A, C)**. Expansion of areas from each spectrum revealed smaller amounts of additional ions. This is shown in **Figure 3B** for the area 870-1100 of Fraction 40. Similar patterns of ion distribution and abundance were found in peak 2*B* of HER-2.J cells. This is shown for Fraction 41 **(Figure 3D)**. The ions shown in **Figure 3** and **Table II** represent an average of 2% of the total ion current, as follows: Fraction 39 (2.59%), Fraction 40 (2.17%), Fraction 41 (1.60), Fraction 42 (0.46%). Similar results were obtained for the HER-2.J peptides. The remaining current was distributed among less abundant material, which may not be composed entirely of peptides.

Twenty-seven ion species with *m/z* ratios corresponding to peptides longer than seven amino acids and a S/N > 2 were found to be the most abundant in the four 1E4 fractions **(Table II)**. Seven 1E4 ions with ratio *m/z*, of 777, 793, 807, 818, 834, 904 (actual m/z = 903.5 - 904.3), and 934, respectively and most likely singly charged were found in both Fxn 40 and 41 **(Figure 3A, C)**. With the exception of the ion at *m/z* 934, which was present in Fraction 39, these ions were not detected in Fxn 39 and 42. The ion at *m/z* 934 was more abundant in

Fraction 39 than in Fxn 40-41. The ions at *m/z* 994, 1008 and 1017 were present in Fraction 40 **(Figure 3B)**, but they were not detectable above a S/N > 2 in Fraction 41. Twenty other ions not shown here were found primarily in each of Fxn 39 and 42.

Sixteen ion species of comparable size with 1E4 ions of *m/z* > 750 were found in Fxn 39-42 of HER-2.J. Of these, five singly charged ions with *m/z* 781, 793, 906 (actual *m/z*, 905.0-905.6), 955 and 1038, respectively, were found to be most abundant in both Fxn 40 and 41 when compared with the other ions. The ions detected in Fxn 41 are shown in **Figure 3C.** Their signal intensities were comparable with those of the ions detected in Fxn 40 and 41 of 1E4. The ion 454 (2+) (actual *m/z* 453.1 - 453.7) was found in both Fxn.41 and 42 **(Figure 4C,D)** but not in Fxn 40, and may be the corresponding double-charged form of ion 906. The ion 497.3 was present in both Fxn 40 - 41 but absent from Fxn 42 **(Figure 4A-C)**. Thiss ion was present in the same fractions from 1E4 cells.

Since the sequence of the ions detected was not known, a first estimation of their abundance was made by comparing their signal intensity with β-casomorphin. Thus the ion abundance listed below represents molar equivalents of β-casomorphin with the caveat that peptides with different sequences show variable ionization efficiencies **(Table I)**. Thus the real abundance of these ions may be one order of magnitude higher or lower than the one determined by comparison with β-casomorphin. Peptides corresponding to ions 807, 818, 834, 994, 1008, and 1017 detected at a similar signal intensity were present at approximately 25 fmol/μl. This equals 1000 fmol/5 x 10^9 cells or an average of 240 complexes/cell. Ion 817 was present at 65 fmol/μl in Fxn 40 and 73 fmol/μl in Fxn 41. Based on other reports, this amount should be sufficient for T-cell activation (2, 25). Comparison with β-casomorphin standards of similar mass 790 (+1), suggests that in 1E4, the ion 793 (**Table II**) was present at approximately 650 fmol/μl injected sample in Fraction 40, at 330 fmol/μl in Fxn 41 and at 100 fmol/μl on

corresponding samples from HER-2.J cells. If the overall fractionation yield is ~50%, this represents for 5 x 10^9 HER-2.J cells, 960 HLA-A2.1-793 complexes/cell. For the same number of 1E4 cells, this represents at least 5600 HLA.A2.1-793 complexes. The singly charged ion at *m/z* 497.3 was a major component of Fxn 40 and 41 from both 1E4 and HER-2.J cells but was absent from Fxn 39 and 42. Its signal intensity determined in the same experiment at 800 eV multiplier setting was for 1E4 cells 4.74 and 1.21 x 10^{10} (Fxn 40 and 41 respectively) and for HER-2.J cells 1.2 x 10^{10} and 8.2 x 10^8 respectively. In a number of samples tested in separate experiments, additional peaks of similar *m/z* (i.e. 497.6 and 498.3), were found (data not shown). Because of the closeness of ion 497.3, we could not clearly identify ion 498.3 (2+) which would correspond to the peptide E75. The mass of the E75 synthetic peptide (498.3) is within 1 mu tolerance of the mass of the naturally presented ion (497.3). Peak 498.3, may also correspond to an isotopic form of peak 497.3 at a +1 charge state. For these reasons, the exclusive assignment of any of ions 497.3, 497.6, and 498.3 to E75, another peptide, or a lipid cannot be made based only on the *m/z* values.

Correlations between signal intensity and CTL activity of fractions in peak 2B.

We compared the identity and abundance of ions in active Fxn 40 and 41 with the ions in inactive Fxn 39 and 42 of 1E4 and HER-2.J. The rationale for this approach was that correlations between presence and abundance of defined ions in consecutive fractions and the patterns of CTL recognition can focus the search for tumor CTL epitopes. For example, if an ion is present in two consecutive fractions, one active and the other inactive, the ion is less likely to be recognized by CTL if it is more abundant in the inactive fraction. Conversely, if the abundance of an ion in an active fraction is below the lower limits for binding to HLA-A2 in a T2 assay, (i.e.10^{-12} to 10^{-13} M) it is less likely to sensitize CTL (2).

For determination of CTL activity 10% of Fraction 40 was placed in a well of 200 µl. Of the same fraction 2.5% was used for MS. Thus, for peptides detected at 25 and 100 fmol (as β-casomorphin equivalents), respectively, this corresponded to 100 and 400 fmol/well (0.10 and 0.40 ng/well, assuming an average mass of 1000 Da for a nonapeptide) or to 0.5 and 2.0 nM, respectively. Naturally processed peptides have been reported to sensitize targets for half-maximal lysis at concentrations ranging between 0.01 - 50 nM (17, 26, 27). If any of the peptides detected at low amounts in Fxn 40 and 41 of 1E4 cells (see **Figure 3**) are recognized by CTL, the TCR affinity for these epitopes should be high. This suggests that even if the actual concentration of peptides corresponding to more abundant ions (such as 497.3 and 793) is one order of magnitude lower than the one estimated by comparison with β-casomorphin, they can be recognized by CTL with both high and low affinity for Ag.

Comparison of the activity profiles for CTL-OVA3 and CTL-OVA5 with the signal intensity of ions detected by MS indicated that the pattern of T2 lysis by CTL-OVA3 correlated with the presence in Fxn 39-42 of 1E4 ions: 497.3, 777, 904 and 793 (**Figure 5A, B**). For HER-2.J peptides, the pattern of T2 lysis correlated with the presence of ions 497.3, 781, 793, 906, 955, and 1038 (data not shown). Ions of *m/z* 784, 914, and 1028 were found in both Fxn 39 and 40 but not in Fxn 41. Since their signal intensity was several-fold higher in Fxn 39 than in Fxn 40, and since the peak of CTL-OVA3 activity corresponds to Fxn 40, it is likely that peptides corresponding to these ions were not recognized by CTL-OVA3. The ion 934 was found in a significantly higher concentration (2000-3000 fmol) in Fraction 39 than in Fxn 40 and 41 in both cell lines. CTL-OVA3 showed essentially no recognition of fraction 39, but CTL-OVA5 showed low-level recognition of this fraction. Therefore, of the ion species present in Fxn 39-42 of peak *2B* of 1E4 and HER-2.J, the ions 497.3, and 793 appeared to be common among SKOV3.A2 and C1R.A2.HER-2 cells. However, the CTL-OVA3 and CTL-OVA5 activity profiles for the peptide fractions in peak *2B* extracted from 5 x 10^9 cells, did not

strictly follow only the abundance of ions 497.3 and 793. This suggests that in addition to 497.3 and 793, several other peptides may be candidate epitopes for CTL-OVA3 and CTL-OVA5 and may be antigenic in different individuals.

DISCUSSION

In this study, we used immunoaffinity, HPLC separation, ES-MS, and CTL epitope reconstitution to define the pattern of possible antigenic peptides recognized by $CD8^+$ CTL in the context of HLA-A2 on ovarian tumors associated with HER-2 overexpression. Using an ovarian tumor associated HLA-$A2^+$ $CD8^+$ CTL line we established that $CD8^+$ CTL recognized at least five peaks of HLA-A2-bound peptides derived from an ovarian tumor line overexpressing HER-2. Similar peaks of activity were observed using the same effectors in C1R.A2.HER-2-derived peptides. Together these data indicate that a number of naturally processed peptides presented by HLA-A2 may be derived either from HER-2 or from proteins associated with HER-2 overexpression as previously suggested (26, 27) or from proteins involved in maintenance of the transformed state. Since CTL-OVA3 cells recognize at least five distinct peaks of SKOV3 peptides, the number of CTL epitopes on ovarian tumors is likely to be higher. Indeed, ongoing fractionation studies have used peptides extracted from a freshly isolated HLA-$A2^+$ HER-2^{high} ovarian tumor (OVA-6) (23). In these studies, HPLC on three shallow gradients of ACN in the second dimension for each of the peaks 1, 2, and 3 in the first dimension indicated at least 10 peaks of bioactive peptides of distinct Rt. A number of these epitopes appear to be common for breast CTL (Melichar, et. al. manuscript in preparation). Together, these results are in agreement with recent reports on the high number of distinct peaks of tumor peptides (in melanoma, breast, and ovarian carcinoma) recognized by CTL/TIL in the context of HLA-A2 (8, 16, 28).

In this study, we also established the pattern of distribution and abundance of naturally processed peptides in active peak *2B* from both target cloned cell lines. With the exception of three to four common ions of similar *m/z* ratios in each bioactive Fxn 40 and 41 of Gradient II from both cell lines, all other ions were of distinct masses. A number of peptides isolated from C1R.A2.HER-2 cells had masses identical to those of the previously reported peptides from C1R.A2 cells (24). Since the most abundant ions of mass 750-1100 in each fraction represented an average of 2% of the total ion current, this suggested that for ovarian tumor cell line SKOV3, HLA-A2 associates with a large number of different species of endogenous peptides.

In ongoing studies, we are attempting to resolve the ions in the peak *2B* by determining the collision-induced dissociation (CID) spectrum and interpreting possible sequences using the PEPSEQ software. It should be noted that for all these ions, the CID spectra indicated the presence of certain impurities likely phospholipids that tend to associate with hydrophobic peptides, (I. Papayannopoulos and B. DaGue, personal observations). The fragment distribution is inconsistent with the existence of a single ion in this peak (either peptide or lipid) but rather with the presence of several ions in this peak. This adds to the complexity of analysis and interpretation of data toward precise sequence identification. 6 of 9 matches within 1 Da were found for b and y ions of 497 with the values determined for the daughters ions of E75. Several possible peptide sequences were obtained with the PEPSEQ software from comparison of peak values. One of these corresponds to a peptide of sequence KXFGSXAFX (X = Leu/Ile), although the order of the amino acids in the sequence GSX could be reversed. A similar approach is taken for sequencing of the ion 793. CID spectra of the ions 454, 1008 and 1017 suggest that they represent peptides. Ions of *m/z* 1008 and 1017 corresponding to peptides 8-11 amino acids long have been detected in these fractions at significantly lower levels (1000 fmol/5 x 10^9 cells) under these experimental conditions.

Ongoing studies use microcapillary HPLC and MS/MS sequencing to resolve the components of the peak *2B*. Synthetic peptides corresponding to the possible reconstituted sequences of ions 793, 1017 and 1008 are being prepared to verify the CID spectra and to determine whether these peptides are being recognized by CTL.

This raises the question as to whether peptides expressed on lower numbers of freshly isolated ovarian and breast tumors can be identified and quantitated. Although high numbers of tumor cells ($\geq 10^9$) are obtained from ascitic or advanced primary solid ovarian tumors, the amount of material available from small breast primary tumors (< 2 cm), is much smaller. Since each MS determination was made on 2.5% of a sample of 5 x 10^9 cells, it is likely that individual peptides of known sequence and signal in the ES-MS present at the same density, can also be detected. Therefore, peptides recovered at 100 fmol/10^8 cells can now be identified by MS, as demonstrated by previous studies with C1R.A2 cells (24) and this study (i.e., ions 1008 and 1017).

A second issue raised by identification of the naturally processed peptides is whether the amount of peptide recovered from a tumor sample is sufficient to bind HLA-A2 on T2 cells and to sensitize CTL for lysis in reconstitution assays. In other words, can the assay detect the presence of the peptide? If 10 fmol of peptides recovered are used to sensitize 5 x 10^4 T2 cells in 1.0 ml (i.e., 10 0.1-ml wells, controls and samples included), this corresponds to a concentration of 10 x 10^{-12} M. Although the SD_{50} and equilibrium constant (Ka) for binding to HLA-A2 may differ among peptides, these values are in agreement with recent estimates for the minimum concentration of exogenous peptide needed to bind HLA-A2 (10^{-12}-10^{-13}M) and the number of peptides-MHC complexes required to activate CTL (2, 25). Consequently, unless the peptide affinity for HLA class I and TCR is extremely high, which is the case with viral but

not tumor CTL epitopes, the amount of material available is a major limiting factor in epitope identification.

Several ways to overcome these limitations are now being explored. Although direct sequencing by tandem MS of a peptide present at a level below 100 copies/cell in 10^8 cells or less may prove difficult, a novel technology for detection peptides of known sequence at low to subfemtomole levels is available. It has been shown by Caprioli and coworkers (20, 29) that by using nanoliter flow-rate desalting and preconcentration techniques with a micro-electrospray ionization source, a sample of less than 5 amol of neurotensin. Methionine enkephalin can be detected using similar techniques that should allow detection and quantitating, peptides of known sequence and mass at low to subfemtomole level.

The correlations between the peptide abundance and the shape of the plot of CTL activity in consecutive fractions, does not necessarily imply that all these ions form epitopes recognized by CTL. In fact if they are of peptide nature, most ions with *m/z* 700 - 800 correspond to hepta- or octapeptides. Hepta and octapeptides were found less frequently than nonapeptides to reconstitute CTL epitopes. With respect to heptapeptides, one possibility that cannot be excluded is that they are derived from longer peptides trimmed at their ends. Another possibility is that they derive from the intracellular pool of peptides which bind a number of free HLA-A2 molecules during the immunoaffinity purification (Sette, A., personal communication). Finally a last possibility that needs to be addressed is that changes in the function of the proteasome LMP2 and LMP7 proteins may lead to qualitative differences in peptide production between tumor and normal cells (30).

Identification and characterization of peptides bound to MHC class I molecules may have important implications for understanding the presentation and recognition of or tolerance

to tumor Ag in human cancers. Selection of a tumor epitope for immunotherapy or gene therapy should address a number of concerns. (a) Is the epitope presented by the tumor? (b) Is its density on the tumor sufficiently high to activate effector CTL? (c) If present on normal tissues, as suggested by melanoma studies (31), is its density lower than on tumor so as to avoid CTL activation with consequent refocusing of effectors and autoimmune damage. While questions on HLA-A2 binding and TCR affinity for an epitope can be addressed with synthetic peptides and by determination of corresponding Ka and SD_{50} values, the answers to these concerns require that the epitope be identified from among MHC class I presented peptides and then quantitated. The use of immunoaffinity would limit significantly the interference from incompletely processed epitopes (32). Therefore, approaches developed in this study should be useful for the identification and quantitation of CTL epitopes from tumors.

LEGENDS TO THE FIGURES

Figure 1. Reconstitution of epitopes for CTL-OVA3 from HPLC fractions containing naturally processed peptides extracted from HLA-A2.1 chains of SKOV3 clone 1E4 cells **(A, C)** and HER-2.J cells **(B, D)**. Peptides were extracted as described in Materials and Methods. **(A)** Peptides extracted from 10^9 1E4 cells separated on HPLC Gradient I using a C18 column (21 x 30 mm). **(B)** Peptides extracted from 10^9 HER-2.J cells separated on Gradient I using the same column. Peptides C85 and E75 eluted with Rt of 22.8 and 29.8 min, respectively. **(C, D)** Pooled Fxn 27-32 from Gradient I shown in A and B, respectively, were separated on Gradient II using a C18 column (220 x 30 mm). Equal amounts of peptides E75 and F48 were chromatographed individually or mixed under the same conditions as 1E4 and HER-2.J Fxn 27-32 on Gradient II using a C18 column (220 x 30 mm). Peptides F48 and E75 eluted with Rt of 38.0 and 41.0 min, respectively, indicating that this HPLC approach can separate mutated peptides of the same length. Effector/target ratio 20:1. (■-■) % specific lysis; (---) OD_{210} nm.

Figure 2. **(A)** Concentration-dependent ES-MS responses of peptide E75. Synthetic peptide E75 was analyzed by ES-MS as described in Materials and Methods at a multiplier setting of 1600 eV. Results are presented for the $[M+2H]^{+2}$ ion at *m/z* 498.3. In the inset, the plot signal-peptide concentration in the range 10-200 fmol/1 µl sample is shown. **(B)** Mass spectra of peptide E75 showing the double-charged $[M+2H]^{+2}$ ion at *m/z* 498.4, the singly charged $[M+H]^{+}$ ion at *m/z* 995.5, and a number of daughter y and b ions (multiplier setting 1100 eV).

Figure 3. Identification of ion composition of peak *2B* fractions by mass spectrometry. 100 µl of the Gradient II Fxn 39-42 (i.e. 50%) was vacuum dried and reconstituted in 20 µl of ES-MS buffer. Next, 1-µl volumes were injected in the electrospray ionization source. The

mass-spectra of the peptides collected in each fraction were then recorded on a triple quadrupole mass spectrometer (Finnigan). Shown are chromatograms of 1E4 peptide peak *2B* ions as follows: **(A)** fraction 40, *m/z* 750-1100; **(B)** expanded presentation of fraction 40 areas, *m/z* 860-1100; **(C)** fraction 41; All determinations at a multiplier setting of 1600 eV. Total ion current is 52, 31, and 5.5 x 10^7 for panels **(A-C)**, respectively. **(D)** Mass spectra of fraction 41 (HER-2.J) showing the ions with *m/z* 775-1400 (multiplier setting of 800 eV). Note the presence and abundance of ions with *m/z* 497, 793, and 906.

Figure 4. (A - C). Mass spectra of peak *2B* peptides from Fxn 40-42 of HER-2.J cells. Ion chromatograms over the *m/z* range 350-600: **(A)** fraction 40; **(B)** fraction 41; **(C)** fraction 42. The ion with *m/z* 497.3 gave a saturated signal in fraction 40. Note the presence of ion with *m/z* 453 in Fxn 41 and 42. The ion with *m/z* 519.3 likely corresponds to an $(M+H+Na)^+$ ion of *m/z* 497.3 (sodium adduct). Note the absence of the ion of *m/z* 497.3 from fraction 42. Mass spectra were recorded on 2.5% of the material. Determinations were made at a multiplier setting of 800 eV. x axis = *m/z* ratio, y axis = relative abundance to the total ion current, which was 7.5, 0.25, and 0.13 x 10^7 respectively.

Figure 5. Identification of candidate CTL epitopes by correlation of mass spectrometry and CTL assay. Each of Fxn 39 - 42 contained peptides from 5 x 10^9 1E4 cells eluting from a C18 column (220 x 2.1 mm) on Gradient II (as described in Materials and Methods). Ten percent of each sample was used in each well for cytotoxicity determinations (■-■). Ion abundance is shown for ions with *m/z* of 793 (❐-❐) and 934 (○-○). Ion abundance is shown as signal intensity (ion - current). Scale is 10^8. **(A)** CTL-OVA3, **(B)** CTL-OVA5. Effector/target ratio, 20:1.

REFERENCES

1. Falk K, Rotzschke O, Stevanovic S, Jung G, and Rammense H-G. Allele specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 351:290-296, 1991.

2. Kageyama S, Tsomides TJ, Sykulev Y, and Eisen H. Variations in the number of peptide-MHC class I complexes required to activate cytotoxic T cell responses. J.Immunol. 154:567-576, 1995.

3. Sykulev Y, Brunsmark A, Tsomides TJ, Kageyama S, Jackson M, Peterson PA, and Eisen HN. High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogenic and syngeneic major histocompatibility complex class I proteins. Proc.Natl.Acad.Sci. 91:11487-11491, 1994.

4. Tsomides TJ, Aldovini A, Johnson RP, Walker BD, Young RA, and Eisen HM. Naturally processed viral peptides recognized by cytotoxic T lymphocytes on cells chronically infected by HIV type I. J.Exp.Med. 180:1283-1293, 1994.

5. Houghton AN. Cancer Antigens: Immune recognition of self and altered self. J.Exp.Med. 180:1-4, 1994.

6. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Sakaguchi K, Appella E, Yannelli JR, Adema GJ, Miki T, and Rosenberg SA. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc.Natl.Acad.Sci. USA 91:6458-6462, 1994.

7. Yoshino I, Goedegeboure PS, Peoples GE, Parikh AS, DiMain JM, Lyerly HK, Gazdar AF, and Eberlein TJ. HER2/neu-derived peptides are shared antigens among human non-small cell lung cancer and ovarian cancer. Cancer Res. 54:3387-3390, 1994.

8. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, and Eberlein TJ. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc.Natl.Acad.Sci, USA 92:432-436, 1995.

9. Bevan MJ. Antigen presentation to cytotoxic T lymphocytes in vivo. J. Exp. Med. 182:639-641, 1995.

10. Huang AYC, Golumbek P, Ahmadzadeh M, Jaffe E, Pardoll D, and Levitsky H. Role of bone-marrow derived cells in presenting MHC class I-restricted tumor antigens. Science 264:961-965, 1994.

11. Kovacsovics-Bankowski M. and Rock KL. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science 267:243-246, 1995.

12. Cox AL, Skipper J, Chen Y, Henderson RA, Darrow TL, Shabanowitz J, Engelhard VH, Hunt DF, and Slingluff CL. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. Science 264:716-719, 1994.

13. Castelli C, Storkus WJ, Maeurer MJ, Martin DM, Huang EC, Pramanik BN, Nagabhushan TL, Parmiani G, and Lotze MT. Mass spectrometric identification of a naturally

processed melanoma peptide recognized by CD8+ CTL. J.Exp.Med. 181:363-368, 1995.

14. Ioannides CG, Fisk B, Fan D, Biddison WA, Wharton JT, and O'Brian CA. Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptide derived from the HER-2/neu proto-oncogene. Cell. Immunol. 151:225-234, 1993.

15. Fisk B, Blevins TL, Wharton JT, and Ioannides CG. Identification of an immunodominant peptide of HER-2/neu proto-oncogene recognized by ovarian tumor specific CTL lines. J.Exp. Med. 181:2709-2717, 1995.

16. Slingluff Jr, CL, Cox AL, Henderson RA, Hunt DF, and Engelhard VH. Recognition of human melanoma cells by HLA-A2.1-restricted cytotoxic T lymphocytes is mediated by at least six shared peptide epitopes. J. Immunol. 150:2955-2963, 1993.

17. Udaka K, Tsomides TJ, and Eisen HN. A naturally occurring peptide recognized by alloreactive CD8+ cytotoxic T lymphocytes in association with a class I MHC protein. Cell 69:989-998, 1992.

18. Wu MX, Tsomides TJ, and Eisen HN. Tissue distribution of natural peptides derived from ubiquitous dehydrogenase, including a novel liver-specific peptide that demonstrates the pronounced specificity of low affinity T cell reactions. J.Immunol. 154:4495-4502, 1995.

19. Kawakami Y, Eliyahu S, Sakaguchi S, Sakaguchi K, Robbins PF, Rivoltini L, Yanelli E, Appella E. and Rosenberg SA. Identification of the immunodominant peptides of the

MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J. Exp, Med. 180:347-352, 1994.

20. Emmett MR, and Caprioli RM. Micro-electrospray mass spectrometry: ultra-high-sensitivity analysis of peptides and protein. J.Am.Soc.Mass Spectrom. 5:605-613, 1994.

21. Samson I, Kerremans L, Rozenski J, Samyn B, Van Beeumen J, Van Aerschot A, and Herdewijn P. Identification of a peptide inhibitor against glycosomal phosphoglycerate kinase of Trypanosoma brucei by a synthetic peptide library approach. Bioorganic and Medicinal Chemistry 3:257-65, 1995.

22. Fisk B, Chesak B, Pollack C. Wharton JT, and Ioannides CG. Oligopeptide induction of a cytotoxic T lymphocyte response to HER-2/neu proto-oncogene *in vitro.* Cell. Immunol. 157:412-427, 1994.

23. Fisk B, Anderson BW, Gravitt K, O'Brian CA, Kudelka AP, Murray JL, Wharton JT, and Ioannides CG. Identification of naturally processed human ovarian peptides recognized by tumor associated $CD8^+$ CTL. (submitted for publication), 1996.

24. Hunt DF, Henderson RA, Shabanowitz J, Sakaguchi K, Michel H, Sevilir N, Cox AL, Appella E, and Engelhard VH. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science 255:1261-1263, 1992.

25. Christinck ER, Luscher MA, Barber BH, and Williams DB. Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. Nature 352:67-70, 1991.

26. Ioannides CG, Ioannides MG, and O'Brian CA. T-cell recognition of oncogene products: a new strategy for immunotherapy. Molec. Carcinogen. 6:77-81, 1992.

27. Yoshino I, Peoples GE, Goedegebuure PS, Maziarz R, and Eberlei TJ. Association of HER-2/neu expression with sensitivity to tumor-specific CTL in human ovarian cancer. J.Immunol. 152:2393-2400, 1994.

28. Storkus WJ, Zeh III HJ, Marurer MJ, Salter RD, and Lotze MT. Identification of human melanoma peptides recognized by Class I restricted tumor infiltrating T lymphocytes. J.Immunol. 151:3719-3727, 1993.

29. Andren PE, Emmett MR, and Caprioli RM. Micro-electrospray: Zeptomole/attomole per microliter sensitivity for peptide. J.Am.Soc.Mass Spectrom. 5:867-869, 1994.

30. Rotem-Yehudar R, Groettrup M, Soza A, Kloetzel PM, and Ehrlich R. LMP-associated proteolytic activities and TAP-dependent peptide transport for class I MHC molecules are suppressed in cell lines transformed by the highly oncogenic adenovirus 12. J. Exp. Med. 183:499-514, 1996.

31. Anichini A, Maccalli C, Mortarini R, Salvi S, Mazzocchi A, Squarcina P, Herlyn M, and Parmiani G. Melanoma cells and normal melanocytes share antigens recognized by HLA-A2-restricted cytotoxic T cell clones from melanoma patients. J.Exp.Med. 177:989-998, 1993.

32. Dick LR, Aldrich C, Jameson SC, Moomaw CR, Pramanik BC, Doyle CK, DeMartino GN, Bevan MJ, Forman JM. and Slaughter CA. Proteolytic processing of ovalbumin and β-galactosidase by the proteasome to yield antigenic peptides. J.Immunol. 152:3884, 1994.

Figure 1

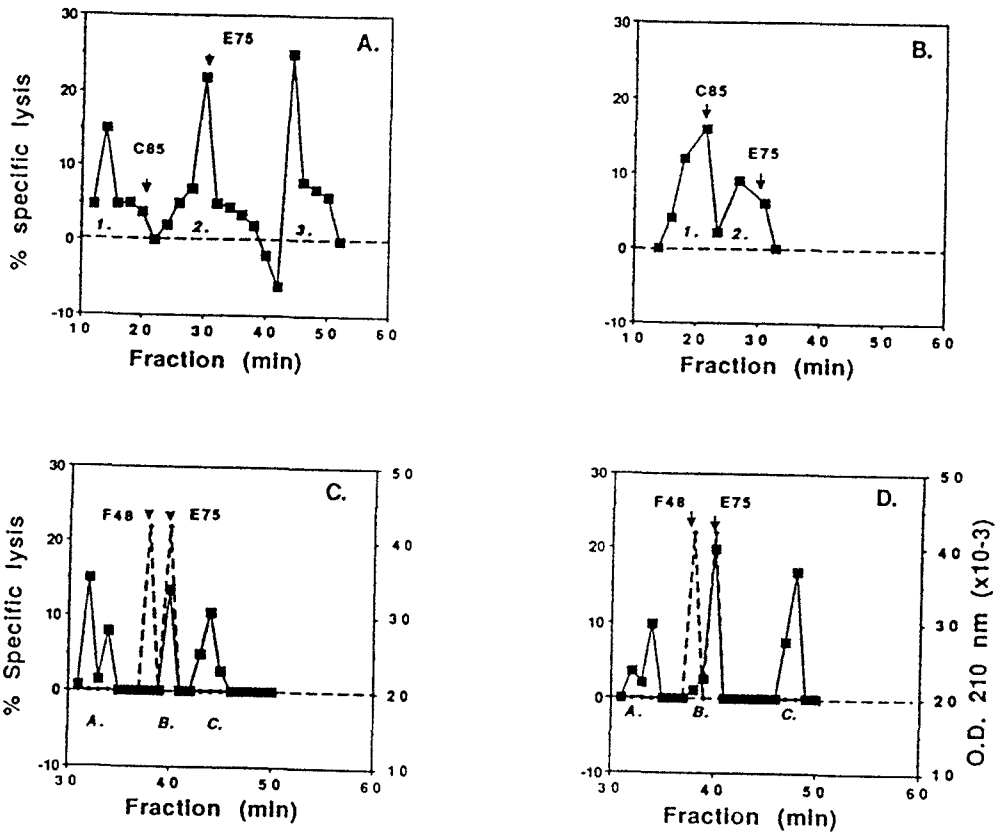
A.
E75
C85
1.
2.
3.
% specific lysis
Fraction (min)
B.
C85
E75
1.
2.
Fraction (min)
C.
F48
E75
A.
B.
C.
% Specific lysis
Fraction (min)
D.
F48
E75
A.
B.
C.
O.D. 210 nm (x10-3)
Fraction (min)

Figure 2

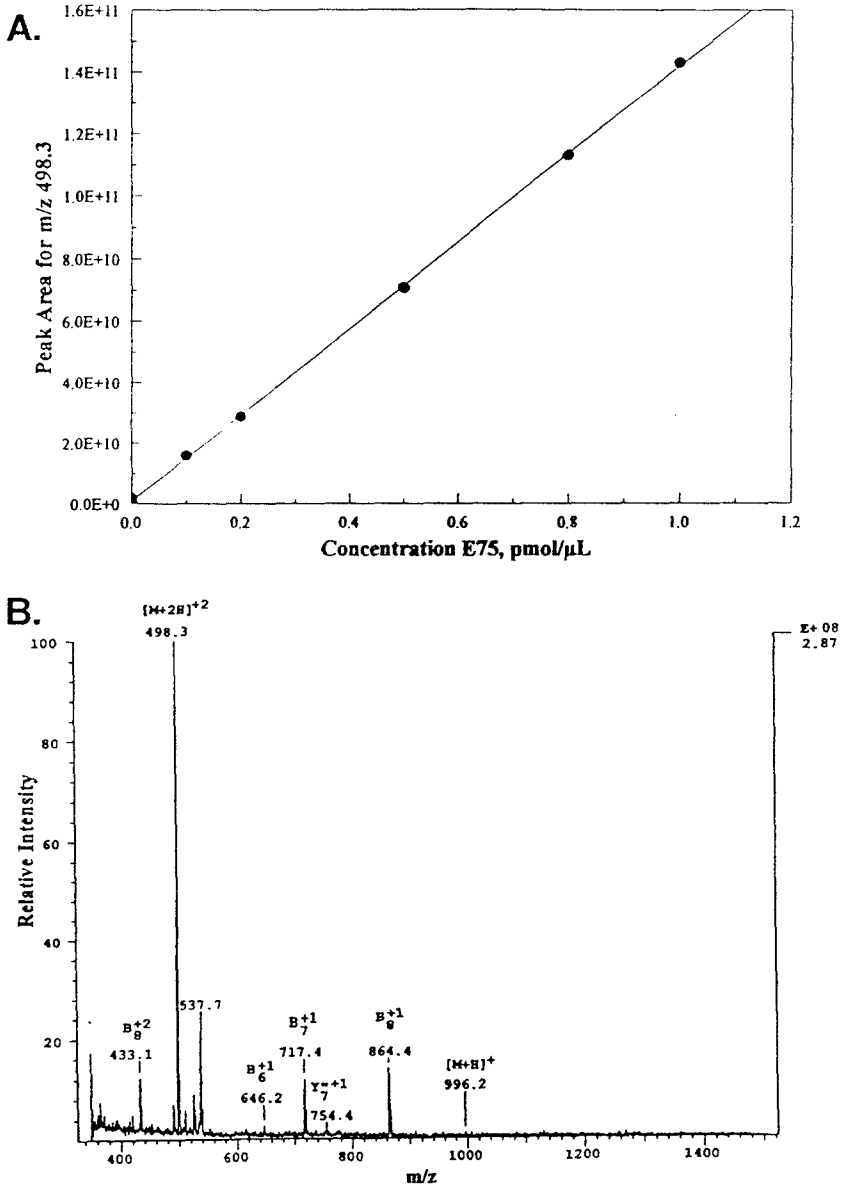
A.
1.6E+11
1.4E+11
1.2E+11
1.0E+11
8.0E+10
6.0E+10
4.0E+10
2.0E+10
0.0E+0
Peak Area for m/z 498.3
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Concentration E75, pmol/µL
B.
[M+2H]+2
498.3
E+08
2.87
100
80
60
40
20
Relative Intensity
537.7
B8+2
433.1
B7+1
717.4
B9+1
864.4
B6+1
646.2
Y7*+1
754.4
[M+H]+
996.2
400
600
800
1000
1200
1400
m/z

Figure 3

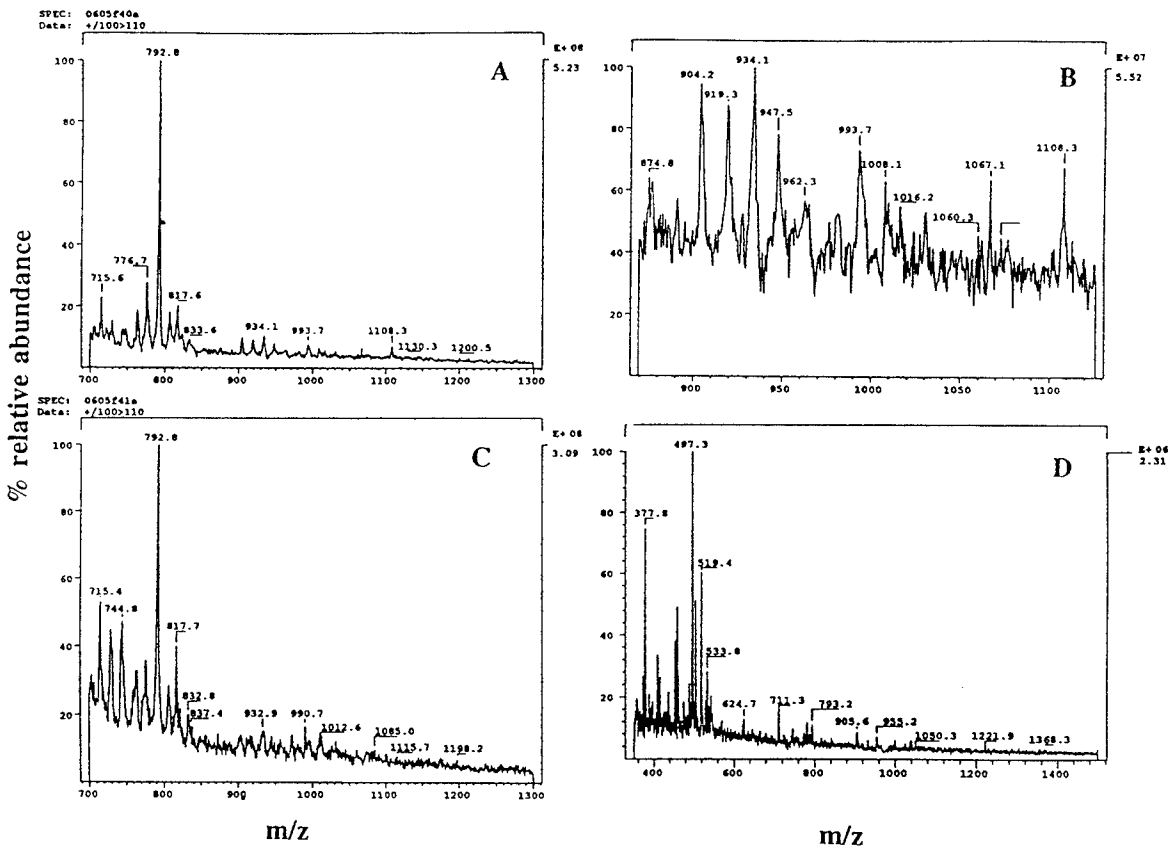
SPEC: 0605f40a
Data: +/100>110
A
792.8
776.7
715.6
817.6
833.6
934.1
993.7
1108.3
1139.3
1200.5
E+ 08
5.23
B
934.1
904.2
919.3
947.5
993.7
874.8
962.3
1008.1
1016.2
1060.3
1067.1
1108.3
E+ 07
5.52
SPEC: 0605f41a
Data: +/100>110
C
792.8
715.4
744.8
817.7
832.8
837.4
932.9
990.7
1012.6
1085.0
1115.7
1198.2
E+ 08
3.09
D
487.3
372.8
519.4
533.8
624.7
711.3
793.2
905.6
955.2
1050.3
1221.9
1368.3
E+ 06
2.31
% relative abundance
m/z
m/z

Figure 4

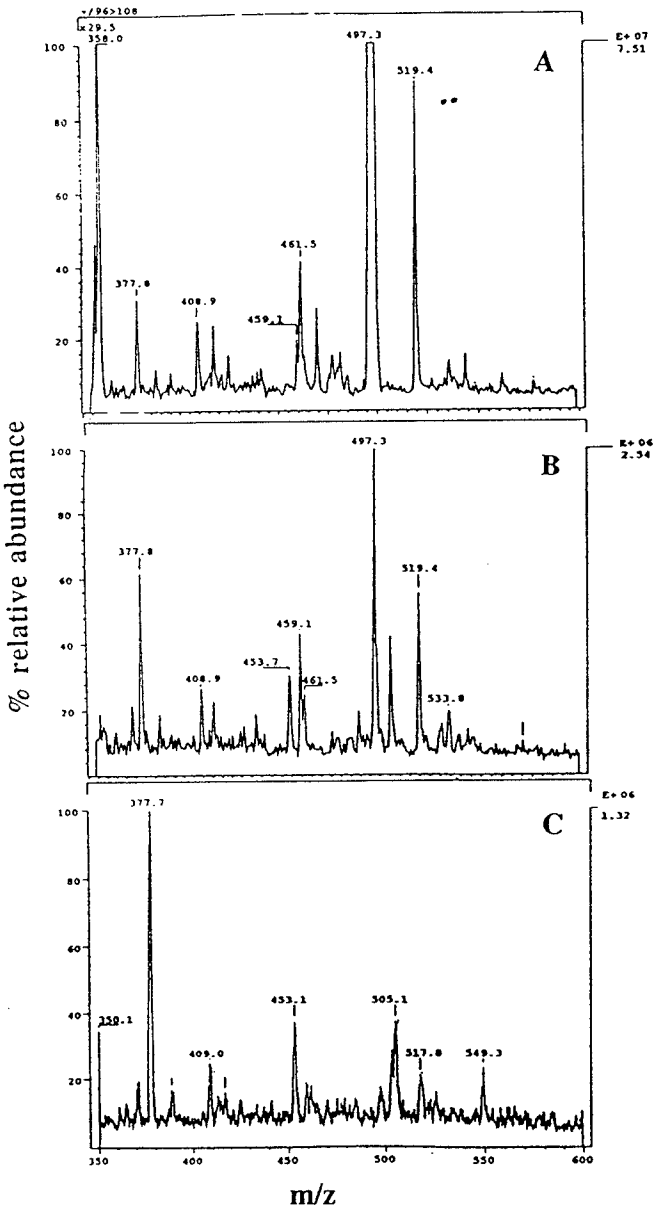
A
B
C
% relative abundance
m/z
497.3
519.4
461.5
377.8
408.9
459.1
453.7
533.8
377.7
453.1
505.1
409.0
549.3
350
400
450
500
550
600

Figure 5

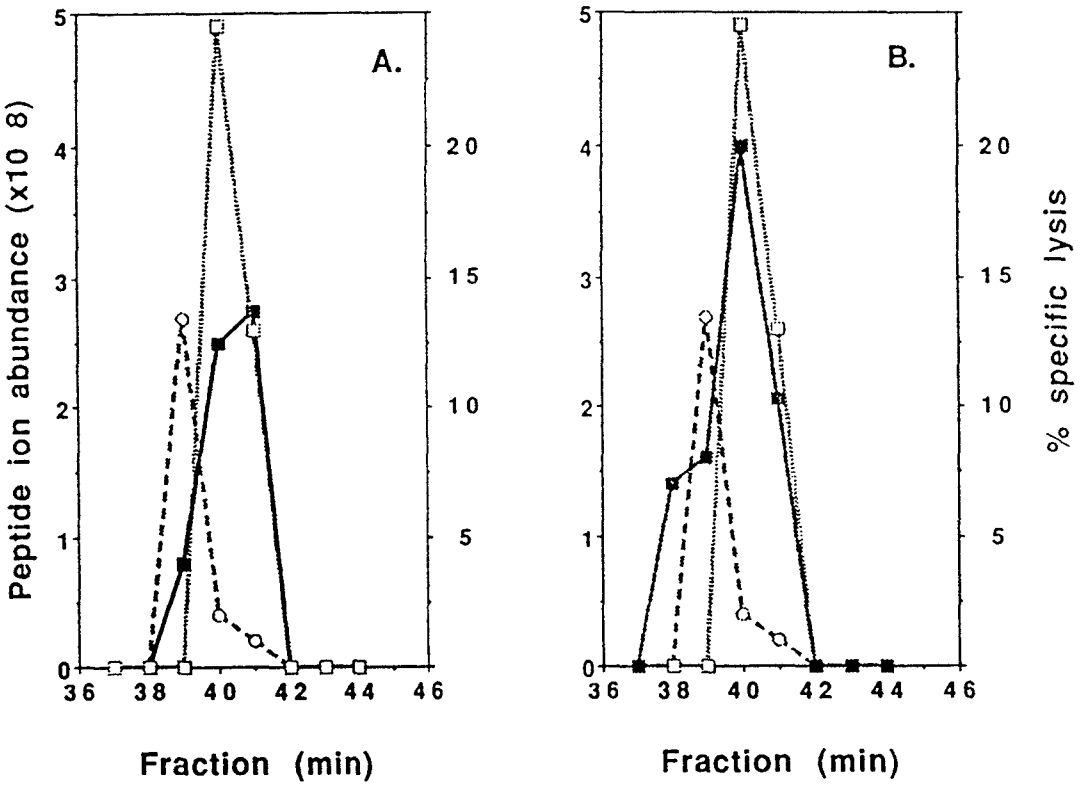
A.
B.
Peptide ion abundance (x10 8)
% specific lysis
Fraction (min)
Fraction (min)
0
1
2
3
4
5
10
15
20
36
38
40
42
44
46

Table I. Comparision between the amount and signal intensity of standard peptides in ES/MS.

Peptide*	Amount (pM/µl)	Multiplier Setting (eV)	Charge State (M + H)	Signal (ion-current) ($x10^7$)
E75	10.0	900	+1	0.01
E75	10.0	900	+2	7.5
C85	10.0	900	+1	0.16
C85	10.0	900	+2	12.0
E91	10.0	900	+1	3.8
E91	10.0	900	+2	11.0
β-casomorphin	0.100	1600	+1	3.8
Substance P	0.100	1600	+2	11.0
Lvv-hemorphin 7	0.100	800	+1	0.22
Lvv-hemorphin 7	0.250	1600	+1	15.0
Lvv-hemorphin 7[+]	0.025	1600	+1	5.0

Peptide standards are: *E75, C85, and E91 are synthetic HER-2 peptides (15). β-casomorphin (YPFPGPI), substance P (RPKPQQFFGLM), and LVV-hemorphin 7 (LVVYPWTQRF) are peptide standards.

[+]Determined in a separate experiment

Table II. Signal strength of peptides bound to HLA-A2 from SKOV3.A2 1E4 cells in HPLC fractions 39-42

	Signal Intensity ($x10^7$)*/Fraction No.			
m/z	39	40	41	42
767	15.6	–	–	–
772	6.9	–		–
788	–	–	–	1.9
793	–	49.3	26.0	–
817	–	8.2	9.2	–
833	–	2.5	2.5	–
873	–	–	–	1.7
884	3.1	–	–	–
904	–	3.0	1.2	1.1
934	26.5	4.1	1.8	–
943	87.4	–	–	–
950	50.0	–	–	–
991	–	–	2.2	–
994	–	2.4	–	–
1010	11.4	–		–
1011	–	–	2.2	–
1017	–	2.1	–	–
1045	–	–	–	1.2
1060	–	–	0.7	–
1075	6.7	–	–	–
1094	–	–	–	0.9
1197	–	–	–	1.5
1230	34.3	–	–	–
1249	85.3	–	–	–
1382	9.4	–	–	–

*The signal intensity for these ions was determined at a multiplier setting of 1600 eV. The ion at m/z 787 was identified at 800 eV in a different determination. Its signal intensity in Fxn 40 and 41 was 3.42×10^7 and 7.28×10^6 respectively. Similarly, the ion at m/z 1008 was identified at 800 eV; its signal intensity in Fxn 40 and 41 was 2.4×10^7 and 6.6×10^6, respectively.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Dec 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-94-J-4313. Request the limited distribution statement for Accession Document Number ADB218882 be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management