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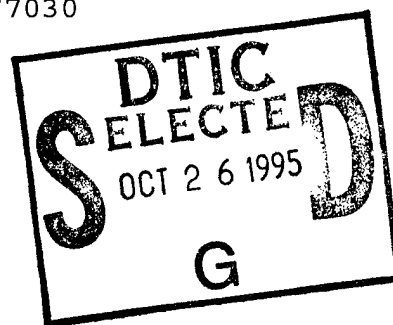
TITLE: Characterization of CTL Recognized Epitopes on Human Breast Tumors

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*Christopher G. Frammides* 9/15/1995  
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## (5). Introduction

Development of resistance and the de novo resistance to anti-cancer drugs of tumor cell populations represents a major obstacle to successful cancer therapy of many malignancies including gynecologic cancers such as breast, which are a major cause of cancer mortality in our society (1). It has been proposed that anti-cancer drugs can potentate antitumor immunity that emerges after chemotherapy by various mechanisms (2-5). Current gene therapy of cancer has progressed to clinical testing being focused on the following three functions (6): **(a)** marking of cells used for immunotherapy (e.g. TIL); **(b)** enhancing the immune response to tumor cells (e.g. by introduction of lymphokine genes), and **(c)** inducing cancer cell death through toxic metabolic compounds. In parallel, tumor vaccines using either autologous or genetically modified tumor cells have shown certain clinical responses (7). **Therefore, the majority of modern molecular therapy approaches focus on modifications of tumors or specific immune effectors function to achieve improve cancer cure rates.** Specific cell-mediated eradication of human tumors requires that: **(1)** tumor targets express Ag recognized by T cells (8) and **(2)** effectors T cells expressing this function can be induced and expand in large number to allow clinical trials (9-12). **(3)** An additional requirement arises from the fact that in both adoptive immuno- and in gene therapy approaches (a,b above) clonality markers are needed to help identify specific effectors, since TIL marking with neoR is indiscriminate. Characterization of T cell receptor (TCR) V $\beta$  and V $\alpha$  usage and repertoire of tumor reactive T cells become imperative.

Studies in rodents have demonstrated that T cells recognize tumor Ag (13-17). In humans, melanoma (18-21) and ovarian CTL-TIL in our studies (22-24), were found at clonal level to recognize private and common Ag on tumor cells (22-30). Although recent technological advances in growth and propagation in vitro of T cells of defined specificity (rIL-2, bioractors) have shown that an answer to the second requirement is feasible by demonstrating large-scale and long-term propagation of tumor specific T cells isolated from tumor infiltrating lymphocytes (TIL) (31), **the key questions regarding the nature of Ag**

**recognized by T cells and the TCR effector phenotypes still remain to be answered.** Ag recognized by CTL are short peptides formed by intracellular degradation of exogenous and/or endogenous proteins (32034). Due to polymorphic nature of both Ag and MHC molecules, different Ag bind to different MHC class I/II molecules (35). The Ag (peptide) is believed to be positioned between two parallel  $\alpha$ -helices connected underneath by a  $\beta$ -sheet plate (Ag binding groove) (36-39). TCR co-recognize epitopes (40) on Ag+MHC, reacting with both Ag (peptide) and the  $\alpha$ -helical side chains of MHC, a phenomenon named MHC restriction (34,41). Therefore T cell epitopes, by virtue of their position are central to the specific immune system. Studies with viral (42-47), parasitic (48-49), bacterial (50) Ag have clearly defined helper and cytotoxic epitopes in protein Ag only when associated with particular MHC molecules. Regardless of the progress made in identification of T cell epitopes, very little is known about the nature of epitopes recognized by T cells on human tumors, constituting a major barrier to development of cell-mediated specific immunotherapy.

Recently, we (51) and independent others (52) have proposed that tumor-associate Ag such as HER-2/neu (HER2) can be targets for specific therapy either through immunotherapy with cells or vaccines or gene therapy. This hypothesis was confirmed by our findings (26) as well as by characterization of T cell epitopes on MAGE, and tyrosinase in melanoma (53-55) and ability of p97 specific T cells to eradicate experimental melanoma (56-57). Evidence for the ability of *ex vivo* stimulated CTL cells to recognize epitopes on such proteins was provided by our studies on HER2 in ovarian cancer (26,51). Amplification and overexpression of HER2 proto-oncogene (58-60) in a significant part of breast (46%) and ovarian (28%) tumors (61-62), correlates with resistance to TNF- $\alpha$  and LAK cells (63), is associated with poor prognosis and survival (58-59). The p185HER2/Neu (pHER2) is a large (185kDa) transmembrane protein with significant homology with EGF-R (64-66) that contains epitopes capable of inducing an immune response, as suggested by animal studies (67). pHER2 contain B cell epitopes capable of inducing a humoral response (67-68). **No information is available regarding CTL responses to pHER2 in breast cancer patients.** In

contrast with rodents (69-70), human pHER2 appears to be seldomly expressed at detectable levels either at the intrauterine stages or in adult tissues of various embryonic origin (61,71). These features make pHER2 an attractive target for study of T cell immunity to oncogene encoded proteins in gynecologic malignancies, since it may provide a *modus operandi* for elimination of oncogene driven tumor cells (27). While the correlation between HER2/neu expression and cancer survival has received great attention, little is known regarding its ability to induce cellular responses *in situ*. Recently, evidence for a relationship between tumor specific CTL-TIL and pHER2 has been provided by our studies on the recognition of pHER2 derived peptides by CTL-TIL in a MHC class I (HLA-A2) and TCR dependent fashion (26,51) on ovarian tumors expressing pHER2.

We initiated studies (22-26) to define Ag recognition by T cells on human epithelial tumors. Ovarian CTL-TIL lines of predominantly CD3<sup>+</sup>CD8<sup>+</sup>CD4<sup>-</sup> phenotype exhibited specific killing and proliferative responses primarily MHC class I restricted, and at least *in vitro* killing involves a TCR dependent recognition mechanisms (22,23). An alternative mechanism may involve production of lymphokines by the CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells (23,31) which may result either directly in lysis of the tumor cells or may induce differentiation of pre-CTL to effector CTL. They also may induce/enhance TAP and MHC class I expression on tumor targets. Our study of Ag specificity of CTL-TIL clones has demonstrated, that multiple Ag epitopes are expressed on the same tumors, and common (shared) and private epitopes can be distinguished on human epithelial tumor cells and lines using CTL-TIL clones (22-24). We found that ovarian tumor reactive CTL recognize breast cancer (Muc-1) core peptides (25). Muc-1 is also an important target Ag in breast cancer (25). Similarly, we found several epitopes on HER-2/neu recognized by ovarian CTL-TIL. HER-2/Neu is overexpressed in a larger number of breast than ovarian tumors with poor prognosis. An important step towards molecular biotherapies of cancer targeting HER-2 was our development of approaches for *in vitro* stimulation of PBMC by HER-2 peptide analogs, yielding CD8<sup>+</sup> lines that specifically

lyse tumors overexpressing HER-2/neu. Specificity of recognition was demonstrated by peptide specific inhibition of HER2<sup>+</sup> tumor lysis (73).

#### **(6). Body: Experimental Methods and Results**

Studies during this granting period were focussed on (a) epitope analysis and definition of immunodominant epitopes on HER-2 using synthetic peptide analogs and tumor reactive CTL from breast and ovarian cancer; (b) optimization of epitope recognition by introduction of specific sequence changes to enhance Ag binding and recognition of CTL; (c) Induction of tumor Ag specific CTL using dendritic cells (DC) as antigen presenting cells (APC); (d) fractionation, biochemical characterization and mass-spectrometric analysis of peptides presented by breast and ovarian tumors.

(a) Studies on epitope analysis and definition of immunodominant epitopes recognized by CTL have revealed that of the 19 HER-2 peptides containing HLA-A2 anchors, one designated as E75 HER-2: CD3<sup>+</sup>CD8<sup>+</sup>CD4<sup>-</sup> CTL (J.Exp.Med. 181,2109, 1995). The same peptide was recognized by breast CD3<sup>+</sup>CD8<sup>+</sup> CTL in a concentration dependent fashion (**Figure 1**). These results indicate that both breast and ovarian CTL can recognize the same antigenic peptide from HER-2. This may be important for design of Ag and tumor specific vaccines<sup>a</sup>.

(b) Studies on optimization of epitopes presentation were focussed on identification of structural features in the sequence of HER-2 peptides that bind with high and low affinity to HLA-A2. Since HER-2 is an important antigen for tumor specific CTL induction and since 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 ability. Distinct sequence patterns in positions (P3-P5) and P1 were found for peptides with high (HSA) and low (LSA) stabilizing ability. In HER-2 peptides with low HLA-A2 affinity (CTL epitopes) P1 was found to permit substitution that enhanced HLA-A2 stabilizing ability and conserved CTL recognition. In contrast, the region P3-P5 was not permissive of sequence changes. We concluded that the selective permissivity of P1 and P9 in the tumor epitope sequence may



have important implications for the optimization of tumor Ag presentation of tumor Ag presentation and for the "neo-antigenicity" of self-antigens ultimately leading towards induction of tumor-reactive CTL of defined affinity and specificity for target Ag<sup>b</sup>.

(c) Dendritic cells (DC) constitutively express HLA-class II and CD80/CD86 and are considered the most efficient antigen-presenting cells. DC are present in very low frequency in tissues so that significant efforts should be made to propagate these cells *in vitro*. We found that cells with the morphology and phenotype of DC (CD33<sup>+</sup>) can be generated in relatively large numbers from CD34<sup>+</sup> bone marrow progenitors of normal donors and cancer patients after 7-10 days culture with IL-2 and stem cells factor (SCF). Important, these DC were efficient in inducing CD3<sup>+</sup>CD8<sup>+</sup> CTL specifically recognizing the immunodominant HER-2 nonapeptide E75 (369-377). This is of importance for specific cellular therapy of breast cancer because may allow to induce *in vitro* Ag specific CTL using autologous-bone marrow. These CTL can be further expanded *in vitro* by different means and used for treatment of patients with tumors<sup>c</sup>.

(d) To define the universe of naturally processed peptides associated with HER-2 overexpression which are presented by the HLA-A2 antigens and are recognized by tumor reactive CTL we have extracted peptides from immunoaffinity separated HLA-A2 molecules of the breast cell line SkBr3 of a lymphoblastoid cell line (C1R:A2) transfected with and expressing HER-2 and of the ovarian tumor cell line SKOV3 transfected with and expressing HLA-A2. Our preliminary results show that a large number of short peptides are bound and presented by HLA-A2. Mass-spectrometric analysis of these peptides is performed in collaboration with Dr. Capriolli (U.T. Medical School, Analytical Chemistry Center). At the moment we have identified ions of 497.3 – 498.4 (2+) which likely correspond to the double charged ion of the E75 peptide. Another peptide with mass-to-charge ratio (m/z) of 793 (actual mass 792.8) was also found, both in tumor cells and C1R:A2 cells transfected with HER-2 suggesting that these peptides are associated with HER-2 overexpression. The importance of this study is underscored

by the fact that the majority of peptides presented by the tumors are derived from self-antigens. Thus identification of the peptides presented as well as of the peptides recognized can define the active Ag that can become targets of a therapeutic response in breast and ovarian cancer. Towards this goal we are generating a large collection of breast tumors and corresponding associated/infiltrating lymphocytes.

At least 10 T cell lines have been established during the previous research period and are currently used for characterization of the spectrum of Ag recognized by CTL in breast cancers.

## **(7) Conclusions**

Studies during the first year of this grant have been focused on the accomplishment of the specific aims. Towards these goals we have identified an immuno-dominant peptide of HER-2 which is a common antigen for both ovarian and breast CTL. We have also identified that for optimization of peptide binding to HLA-A2, the area P1-P3 is permissive to changes while the area P5-P8 is not permissive to substitutions. Furthermore we have established the ability of dendritic cells isolated from the bone-marrow of breast cancer patients to present the immunodominant HER-2 peptide to T cells and induce antigen specific CTL. These results are very important for development of antigen epitope specific therapies for breast cancer. In addition we have established the ability of breast derived T cells and CTL to grow in large numbers and for long periods of time. The objective of this investigation for the next year are unchanged. We plan to continue this investigation towards elucidation of the structure and function of naturally processed peptides recognized by the CTL, the nature of the receptors employed for breast tumor recognition.

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## APPENDIX

The following appended publications and manuscripts describes in detail our results during this granting period, and the methodology used for this purpose.

- (a) 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. *The Journal of Experimental Medicine*, 181: 2109-2117, June, 1995.
- (b) Fisk, B., Savary, C., Hudson, J.M., O'Brian, C.A., 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* 1995 (submitted for publication).
- (c) Savary, S.A., Woodside, D.G., Hudson, J.M., Fisk, B., McIntyre, B., and **Ioannides, C.G.** Generation of tumor-binding dendritic celss from C34<sup>+</sup> progenitors cultured with IL-2 and stem cell factor (SCF). (submitted for publication), 1995.
- (d) Fisk, B. DaGue, B., Seifert, Jr., W.E., Melichar, B., Kudelka, A.P., Wharton, J.T., Capriolli, R.M. and **Ioannides, C.G.** Mass-spectrometric identification of naturally processed peptides presented by breast and ovarian tumors. (Manuscript in preparation), 1995.
- (e) **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.
- (f) O'Brian, C.A., Gravitt, K.R., Ward, N.E., Gupta, K.P., Bergman, P.J., and **Ioannides, C.G.** Protein kinase C activation and the

intrinsic drug resistance of human colon cancer. (*Drug Resistance in Oncology*), 1995, (in press).

## **APPENDIX: Legends to the Figures**

**Figure 1.** Concentration dependent recognition of the immunodominant peptide E75 by breast CTL-6B line from an HLA-A2<sup>+</sup> patient with a HER-2<sup>high</sup> breast tumor. Effector to target ratio was 20:1. Experimental details are described in the attached publication.

**Figure 2.** Recognition of HER-2 peptides by HLA-A2<sup>+</sup> breast CTL-6B. Effector to target ratio was 20:1. Experimental details are described in the attached publications. C85 = HER-2:971-979, F53 = HER-2:648-654, E75 = HER-2:369-377, F125 (gp100): YLEPGPVTA. The gp100 peptide from melanoma that is not recognized was a negative control peptide.

**Figure 3.** Recognition of reverse-phase HPLC separated peptide fractions by breast and ovarian CTL from HLA-A2<sup>+</sup>, HER-2<sup>high</sup> tumors. Both ovarian and breast CD8<sup>+</sup> CTL recognize a number of common peaks of peptides eluting with similar retention times. CTL-6B is isolated from an HLA-A2<sup>+</sup> breast cancer patient. CTL-5 is isolated from an HLA-A2<sup>+</sup> ovarian cancer patient.

*Tumor 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  $1.0 - 1.5 \times 10^9$  cells were obtained from one cell factory. Cells were collected and washed three times with cold PBS. Further, cells were lysed using the buffer previously described by Engelhard, Slingluff, and collaborators (74) 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<sub>2</sub> 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 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 (75) excepting 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 OD<sub>210</sub> 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-kd cut-off) (Millipore) and lyophilized. For these studies, at least  $10^{10}$  cells of each 1E4 and HER-2.J. lines were grown in batches of  $1.5 - 2.0 \times 10^9$  cells. Peptides with masses < 3Kd were 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 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 Å; 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 as previously described using an HPLC system model 1090 (Applied Biosystems). This gradient is designated as Gradient I here. 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 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 and the corresponding peak of CTL activity were injected into a Brownlee C18 Aquapore column of 2.1 x 220 mm, (300Å, 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 45-50 min, 35-60% ACN in 0.1% TFA, designated as Gradient II here. 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%. Synthetic peptides E75, C85, E90 and E89 were separated in the same

conditions of Gradient II and the peaks of elution and retention times were determined.

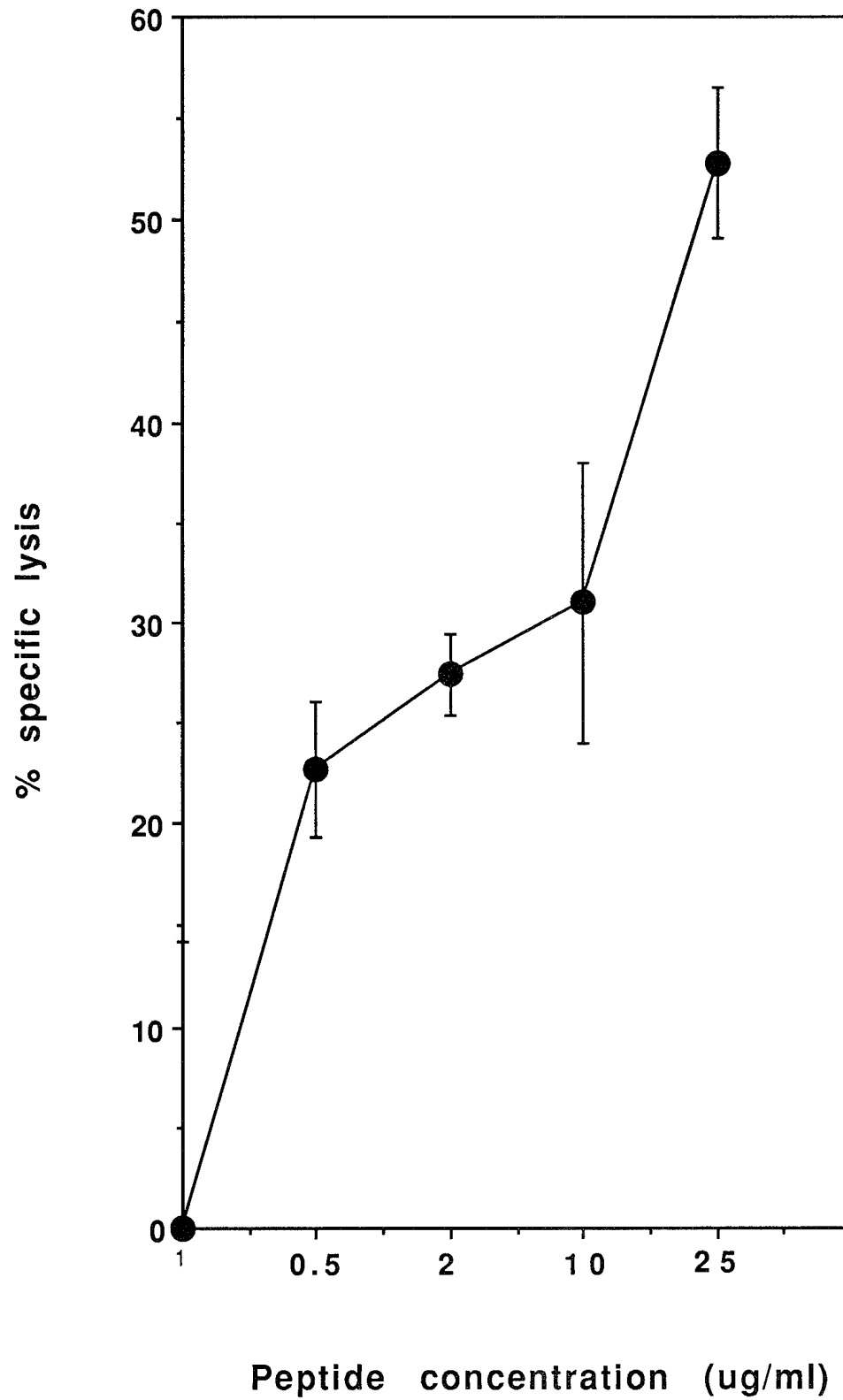
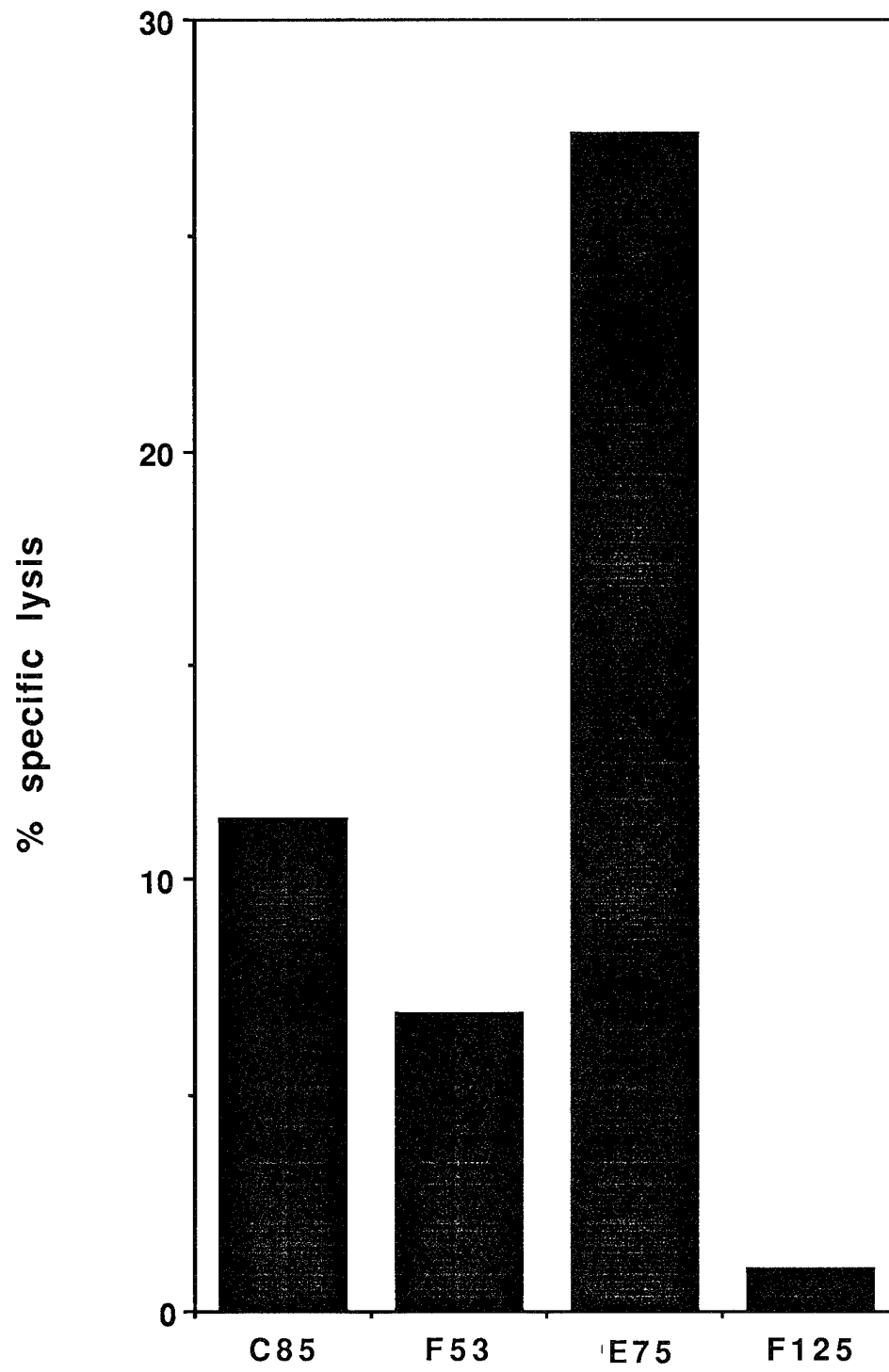
**Figure 1.**

Figure 2.







# Identification of an Immunodominant Peptide of HER-2/neu Protooncogene Recognized by Ovarian Tumor-specific Cytotoxic T Lymphocyte Lines

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## Summary

Synthetic peptide analogues of sequences in the HER-2 protooncogene (HER-2) were selected based on the presence of HLA-A2.1 anchor motifs to identify the epitopes on HER-2 recognized by ovarian tumor-reactive CTL. 19 synthetic peptides were evaluated for recognition by four HLA-A2<sup>+</sup> ovarian-specific cytotoxic T lymphocyte (CTL) lines obtained from leukocytes associated with ovarian tumors. The nonapeptide E75 (HER-2, 369-377:KIFGSLAFL) was efficient in sensitizing T2 cells for lysis by all four CTL lines. This peptide was specifically recognized by cloned CD8<sup>+</sup> CTL isolated from one of the ovarian-specific CTL lines. E75-pulsed T2 cells inhibited lysis by the same CTL clone of both an HLA-A2<sup>+</sup> HER-2<sup>high</sup> ovarian tumor and a HER-2<sup>high</sup> cloned ovarian tumor line transfected with HLA-A2, suggesting that this or a structurally similar epitope may be specifically recognized by these CTL on ovarian tumors. Several other HER-2 peptides were recognized preferentially by one or two CTL lines, suggesting that both common and private HER-2 epitopes may be immunogenic in patients with ovarian tumors. Since HER-2 is a self-antigen, these peptides may be useful for understanding mechanisms of tumor recognition by T cells, immunological tolerance to tumor, and structural characterization of tumor antigens.

The existence of CTLs in the leukocyte infiltrations of ovarian tumors, that when expanded in culture in the presence of IL-2 are capable of recognizing autologous, and HLA-matched allogeneic tumors provides strong support to the hypotheses that these CTL recognize multiple private and/or common Ag on tumor and that these Ag can induce T cell responses (1, 2). A critical step towards testing this model is the identification of tumor-specific T cell epitopes. This goal is highly significant because it may lead to an understanding of the immune responses to tumors, the reasons for failure of such responses to control tumor growth in vivo, and development of novel strategies for cancer therapy. Processing of tumor cellular proteins may result in CTL epitopes. In general, the ability of peptide ligand to compete for receptor binding improves as its concentration increases, and the distinction between tumor and normal tissue reactivity may be predicated on the ability of peptides from an overexpressed protein to occupy a significant number of MHC molecules in competition with other peptides according to the laws of mass action (3). Based on these considerations, we proposed the HER-2/neu protooncogene (HER-2)<sup>1</sup> as a

potential target for a T cell response against epithelial tumors such as those in breast and ovary, because in a number of tumors the concentration of this protein is increased by up to 100-200-fold over normal tissues. Processing of this overexpressed protein may result in increased peptide supply, which may activate/reactivate an immune response against tumor (3). In support of this hypothesis, evidence from a large case analysis in breast cancer indicates that HER-2 overexpression correlates with a favorable prognosis in patients with breast cancer having a high density of local lymphocyte infiltration (4).

The importance of HER-2 in the recognition of ovarian and breast tumors by CTL in vitro and in vivo has not yet been elucidated, nor have the common epitopes of HER-2 recognized by CD8<sup>+</sup> CTL lines from different donors and cloned CD3<sup>+</sup>CD8<sup>+</sup>CD4<sup>-</sup> CTL been identified. In this study, we have identified common immunogenic epitopes of HER-2 recognized by four out of four and two out of four CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> ovarian-specific CTL lines that were isolated from tumor-associated lymphocytes (TAL) from HLA-A2<sup>+</sup> ovarian cancer patients. CTL clones isolated from one of these lines confirmed recognition of one common HER-2 epitope, and they suggest that a peptide with an identical or cross-reactive sequence is recognized by tumor-reactive CTL on ovarian tumors. Identification of common antigenic CTL epitopes of HER-2 may help to develop targeted immunother-

<sup>1</sup> Abbreviations used in this paper: FBP, folate-binding protein; HER-2, HER-2/neu protooncogene; MCF, mean channel fluorescence number; TAL, tumor-associated lymphocytes; TAP, peptide transporter-associated proteins; (TAP1 and TAP2); TIL, tumor-infiltrating lymphocytes.

apeutic strategies for breast and ovarian cancer and to elucidate the mechanisms of tolerance towards these epitopes.

## Materials and Methods

**Synthetic Peptides and Monoclonal Antibodies.** HER-2 and control peptides were synthesized by the Synthetic Antigen Laboratory at the M.D. Anderson Cancer Center using a solid-phase method and purified by HPLC. Identity of the final peptides was established by amino acid analysis. The purity of the peptides used in these experiments was  $\geq 97\%$ . mAb to CD3 (OKT3-FITC), CD4 (OKT4-FITC), and CD8 (OKT8-FITC) were obtained from Ortho Diagnostic (Raritan, NJ), mAb W6/32 (anti-HLA-A, -B, -C) from Dako (Dakopatts, Denmark), and anti-HLA-A2 mAb BB7.2 (anti- $\alpha$ -2 domain) and MA2.1 (anti- $\alpha$ -1 domain) from American Tissue Culture Collection (ATCC, Rockville, MD). mAb Ab2 against HER-2 was obtained from Oncogene Science (Manhasset, NY).

**Target Cells and Cell Lines.** The human cell line 174CEM.T2 (T2) was a kind gift from Dr. Peter Creswell (Yale University, New Haven, CT). These cells are defective in the normal antigen processing pathway and they express HLA-A2.1 occupied only by signal peptides (5). C1R transfectants C1R:A2 and C1R:A1 cells express HLA-A2.1 and HLA-A1, respectively. Parental Hmy2.C1R (class I reduced) cell line does not express any HLA-A, but expresses low HLA-B35. These cells were a generous gift from Dr. William E. Biddison (National Institute of Neurological Disorders, Bethesda, MD). Tumor lines and leukocytes of the donors of ovarian malignant ascites were phenotyped for HLA-A, -B, and -C antigens by the Blood Bank at the M.D. Anderson Cancer Center. Expression of HLA-A2 on freshly isolated ovarian tumors and transfectants was confirmed by immunofluorescence using MA2.1 mAb. Ovarian tumor lines of known HLA phenotypes, MDAH 2774 (HLA-A3, 24, B45, w57) and SKOV3 (HLA-A3, 28, B18(w6), 35(w6), Cw5), were also used as targets in these experiments.

SKOV3 cells were transfected with the HLA-A2 expression vector RSV.5-neo containing the same full-length HLA-A2.1 cDNA expressed in C1R:A2 cells (6) (kindly provided by Dr. W. Biddison), using the Lipofectin reagent and procedure as described by the manufacturer (Gibco Life Technologies, Grand Island, NY). After selection with G418, clones that expressed high levels of HLA-A2 and HER-2 (as determined by immunofluorescence with MA2.1 and Ab2 mAbs) were selected for further experiments.

**HER-2 Peptide Binding to HLA-A2.1** To establish the ability of HER-2 peptides to stabilize HLA-A2 expression, the T2 MHC class I peptide stabilization assay was performed as described (7). T2 cells were incubated overnight with saturating amounts of all of the selected HER-2 peptides, as well as with positive and negative control peptides at the same concentration (50  $\mu$ g/ml). Cells were then washed, stained with BB7.2 and W6/32 mAbs, and analyzed by flow cytometry as described (2, 7, 8). Fluorescence intensity and positions of the peaks were determined using an Epics<sup>®</sup> V profile analyzer with a log amplifier (Coulter Electronics, Hialeah, FL). Results are expressed as the mean channel fluorescence number (MCF) on a logarithmic scale corresponding to the peak of fluorescence for HLA-A2 (8).

**Generation of Ovarian-specific CTL Lines and Clones.** CTL were generated by culturing freshly isolated tumor-associated lymphocytes (TAL) from ovarian malignant ascites in complete RPMI medium in the initial presence of autologous ovarian tumor, 25–50 U/ml of IL-2 (Cetus Corp., Emeryville, CA), and 250 U/ml of TNF- $\alpha$  (Genentech, South San Francisco, CA), for 2 wk, followed

by selection of CD8<sup>+</sup> cells on anti-CD8 mAb-coated culture flasks (AIS Micro CELLector<sup>™</sup>; Applied Immune Sciences, Menlo Park, CA) and negative selection on anti-CD4 mAb coated flasks as described (9). Isolated CD8<sup>+</sup>CD4<sup>-</sup> cell lines designated CTLs 1–4 were propagated in culture in complete RPMI medium supplemented with IL-2. CD3<sup>+</sup>CD8<sup>+</sup>CD4<sup>-</sup> clones were established by limiting dilution from CTL-3 line as we described (1, 2).

**Identification of Antigenic Peptides.** To identify the antigenic HER-2 peptides, CTL lysis of T2 cells preincubated for 60 min with each peptide was measured in a <sup>51</sup>Cr release cytotoxicity assay (1, 7). For titration of HER-2 peptides for recognition by CD8<sup>+</sup> CTL, T2 cells were incubated with varied concentrations of purified HER-2 peptides. For antibody inhibition experiments, targets were preincubated with the appropriate antibody as described (2), then washed and incubated with effectors. Percentage of specific lysis was determined from the equation  $(A - B)/(C - B) \times 100$ , where A is lysis of T2 cells by effectors in the presence of a peptide, 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 <sup>51</sup>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-terminal end can have dramatic effects on peptide recognition (10), and identification of CTL epitopes is performed with synthetic peptides, cytotoxicity values were considered to indicate significant recognition of a peptide when the differences between mean  $\pm$  SD values for percent of specific lysis of T2 cells preincubated with a peptide or medium were  $\geq 10\%$ , at an E/T ratio of 20:1 (10) and statistically significant ( $p < 0.05$ ). Cold target inhibition of cytotoxicity was performed using <sup>51</sup>Cr-labeled ovarian tumors, OVA-1 (autologous with CTL-1), as well as SKOV3.A2.1E4 transfectants as "hot" targets and T2 cells pulsed with peptides as "cold" targets. T2 cells were preincubated with HER-2 or control peptides (50  $\mu$ g/ml) overnight, then washed and admixed with <sup>51</sup>Cr-labeled targets at 5:1 and 10:1 (cold/hot) target ratios.

**Statistical Analysis.** Values obtained for percent of specific lysis by the same effectors of T2 cells preincubated with HER-2 peptides and percentage of specific lysis of T2 cells in the absence of exogenous peptides were examined by the Student's *t* test. Differences were considered significant when the  $p < 0.05$ .

## Results

**Identification of HER-2 Peptides That Stabilize HLA-A2.1 Expression.** To identify the HER-2 epitopes recognized by these CTL, 19 peptides were selected from the HER-2 sequence based on the HLA-A2 anchor motifs based on the presence of Leu/Ile at position 2 (P2) and Val/Leu/Met at P9 (11, 12). The majority (16 out of 19) peptides selected for this study were nonamers. Two octamers were included because they were part of overlapping epitopes. 15 out of 19 peptides contained Leu (P2), while two peptides contained Ile (P2) and two peptides contained Val (P2). Only octa and decamers were found in the HER-2 sequence to contain Met at P2, and consequently these peptides were not included in the present study. Peptides were selected from signal, extracellular, transmembrane, and cytoplasmic domains of HER-2 (13). Priority was given to peptides from the 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 also selected from the signal and transmembrane domains of HER-2 because hydrophobic, Leu-, Ile-, and Val-rich peptides were found bound on HLA-A2 of both T2 and C1R:A2 cells (5, 14), were described as CTL epitopes (10), and may be bound to HLA-A2 in transporter-associated proteins (TAP)-deficient targets (5). The peptides selected for this study represent more than 50% of all nonamers that are potential HER-2 epitopes with HLA-A2 anchor motifs (11, 12).

Since both P2 and P9 anchors and residues from the central area of peptide contact HLA-A2 and define the affinity of a peptide to the presenting molecule (12), to determine the binding ability of HER-2 peptide analogues, expression of HLA-A2 on T2 cells was determined in the presence of each HER-2 peptide and the corresponding control peptides. All peptides were tested for binding to the MHC class I in the HLA-A2 stabilization assay using T2 as indicator cells (7, 8). To establish that the results reflect MHC class I heavy chain expression indicative of absolute binding and not only the effects of peptide-induced conformational changes (8) that may affect the reactivity of mAb with the peptide-binding pocket, T2 cells were stained with both W6/32 (anti-class I monomorphic) and BB7.2 (anti-HLA-A2  $\alpha$ -2 domain) mAb. As shown in Table 1, 9 out of 19 HER-2 peptides (ranked 1-9 based on their HLA-A2-stabilizing ability) induced a greater than twofold increase in MCF for HLA-A2 expression, compared to negative control peptide C61, as determined with BB7.2 mAb. Similar results were obtained after staining with MA2.1 mAb (data not shown). HLA-A2 stabilization for E75, E90, and E89 was peptide concentration dependent in the range 1-50  $\mu$ g/ml (not shown). This suggests that these peptides have higher stabilizing ability of both conformational epitope BB7.2, as well as HLA-A2 molecule expression than the other 10 peptides (ranked 10-19), which were designated as peptides with low stabilizing ability for HLA-A2.1. Five other peptides, E70, E71, E72, D97, and D99, did not affect serological epitope W6/32 expression, suggesting that they bound poorly to HLA-A2. Of these peptides, the octamer D97 induced a significant increase in BB.7.2 epitope expression, suggesting induction of a conformational epitope rather than stabilization of HLA-A2 expression. In contrast, BB.7.2 bound poorly to T2 cells pulsed with peptide E74 compared with W6/32. The implication of this serological analysis is that HER-2 peptides, in addition to having binding and stabilizing effects on HLA-A2 expression, may lead to conformational changes in the Ag-binding pocket.

**Recognition of HER-2 Peptides by Ovarian Tumor-reactive CTL.** To identify HER-2 peptides recognized by ovarian tumor-reactive CTL lines, four CD8<sup>+</sup> CTL lines designated CTLs 1-4 were generated from cultured TAL from four different HLA-A2<sup>+</sup> donors after CD8<sup>+</sup> cell selection on anti-CD8 antibody-coated plates. These CTL lines were 100% CD3<sup>+</sup>, 100% CD8<sup>+</sup>, and 0-2% CD4<sup>+</sup>. This approach was considered necessary because the Ag specificity of CD3<sup>+</sup> CD8<sup>+</sup> CD4<sup>-</sup> CTL isolated from tumor-infiltrating lymphocytes (TIL)/TAL, which have been in culture for 2 wk, will not be diluted or masked by the overgrowth of CD4<sup>+</sup> cells,

which is encountered in long-term TIL/TAL cultures. To avoid changes in the Ag specificity, the isolated CD3<sup>+</sup> CD8<sup>+</sup> CD4<sup>-</sup> lines were not restimulated with autologous or allogeneic HLA-A2<sup>+</sup> tumors. These CTL lines recognized autologous and allogeneic HLA-A2<sup>+</sup> ovarian tumors, but not HLA-A2<sup>-</sup> ovarian tumors or lines, as illustrated in Table 2. Since freshly isolated ovarian tumors from different donors may be antigenically heterogeneous or may express variable levels of HER-2, we needed as target a cloned ovarian tumor of high and stable HER-2 protein expression and known HLA-phenotype having HLA-A2 in common with the effectors. Tumor cells of the SKOV3 line, which overexpresses HER-2 protein (15), were transfected with the HLA-A2 gene. TAP1 and TAP2 message expression in SKOV3 is increased in parallel with HLA class I by IFN- $\gamma$  treatment (16), suggesting unimpaired Ag-presenting ability. A clone SKOV3.A2.1E4 expressing high and stable levels of HER-2 protein and HLA-A2 was used as a target in these experiments. Four HLA-A2<sup>+</sup> ovarian CD8<sup>+</sup> CD4<sup>-</sup> CTL lines lysed SKOV3.A2.1E4 clone in addition to autologous and allogeneic HER-2<sup>high</sup> HLA-A2<sup>+</sup> tumors. They did not recognize HLA-A2<sup>+</sup>, HER-2<sup>low</sup> ovarian cell lines. Furthermore, they did not lyse K562 cells, indicating that they did not express NK or LAK activity (Table 2). Autologous tumor lysis was inhibited by mAb to CD3 TCR (OKT3) and HLA-A2 (MA.2.1), but not by anti-HLA-DR mAb, suggesting that they can recognize Ag presented by HLA-A2 (data not shown).

To evaluate whether these CD8<sup>+</sup> CTL recognized the same or different HER-2 peptides, lysis of T2 cells preincubated with each peptide was tested with all CTL lines. Both high and low affinity peptides were tested in the same experiment since it has been reported that a melanoma CTL epitope is derived from low affinity HLA-A2-binding peptides (17). For increased stringency in epitope identification, recognition of an HER-2 peptide was considered significant based on convergence of results of statistical analysis of differences in cytotoxicity data (18, 19) and assigning a cut-off value of at least 10% for the differences between recognition of T2 cells exogenously loaded with HER-2 peptides and T2 cells presenting only endogenous peptides. This approach was necessary because we wanted to identify peptides that, based on the levels of observed lysis, are either recognized with higher affinity than others or their recognition reflects the presence of a higher percentage of specific reactive clones.

Based on comparison of cytotoxicity values for T2 cells, lysis by CD8<sup>+</sup> CTL1-4 in the presence and absence of HER-2 peptides, CTL1 and CTL2 recognized mainly peptide E75 (Fig. 1). CTL3 recognized, in addition to E75, three other peptides (E90, E89, and C85), but it did not recognize the remaining 15 peptides. CTL4 recognized four of the peptides tested, including E75 and C85. CTL4 recognized, at a lesser extent than E75 and C85, two other HER-2 peptides residues, 799-807 (E71) and 835-842 (E73). Peptides E71 and E73 were not recognized by the other three CTL lines, even when reconstitution of the epitopes was attempted at either higher peptide concentrations or higher E/T ratios, and may represent private epitopes for CTL4 (Fig. 1). Since CTL4

Table 1. Cell Surface Expression of BB7.2 Epitope on HLA-A2.1 of T2 Cells by HER-2 Peptides

Code	Position	1	2	3	4	5	6	7	8	9	10	BB7.2		W6/32	
												MCF*	Rank†	MCF	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 50 µg/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 anchor residues (11, 12) reported to favor 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 of HLA-A2-specific anchor motifs (11).

was isolated from the ascites corresponding to previously reported TAL-24, these results confirm the recognition of a peptide from the area 968-984 (C85) as a potential T cell epitope derived from HER-2 or a structurally similar peptide HLA-A2 complex (20). Peptide E75 was recognized by all four CTL lines, C85 by two out of four CTL lines, while peptides E89 and E90 were recognized by CTL-3, and E71

and E73 by CTL-4. All four CTL lines failed to specifically recognize a number of HER-2 peptides presented by T2 cells with canonical HLA-A2 anchors at P2 and P9 and different central sequences, including D113, which was reported to bind HLA-A2 with high affinity (12). All CTL lines showed low levels of lysis of T2 cells without exogenous peptides (T2 cells present a number of signal peptides) (5), comparable

**Table 2.** Recognition of HLA-A2<sup>+</sup> Tumors by Ovarian-specific CTL Lines

Targets <sup>†</sup>	Percent of specific lysis*			
	CTL-1	CTL-2	CTL-3	CTL-4
Auto-T (HER-2 <sup>+</sup> , A2 <sup>+</sup> )	47	65	28	41
Allo-T (HER-2 <sup>+</sup> , A2 <sup>+</sup> )	40	41	14	38
SKOV3.A2.1E4				
(HER-2 <sup>+</sup> , A2 <sup>+</sup> )	45	39	42	84
Allo-T (HER-2 <sup>-</sup> , A2 <sup>-</sup> )	5	15	NT	3
2774 (HER-2 <sup>-</sup> , A2 <sup>-</sup> )	0	0	0	4
K562 (HER-2 <sup>-</sup> , A2 <sup>-</sup> )	1	3	3	4

\* Percent specific lysis is shown for an effector to target ratio of 20:1. Target lysis was determined in a 5-h <sup>51</sup>Cr release assay. NT, not tested.

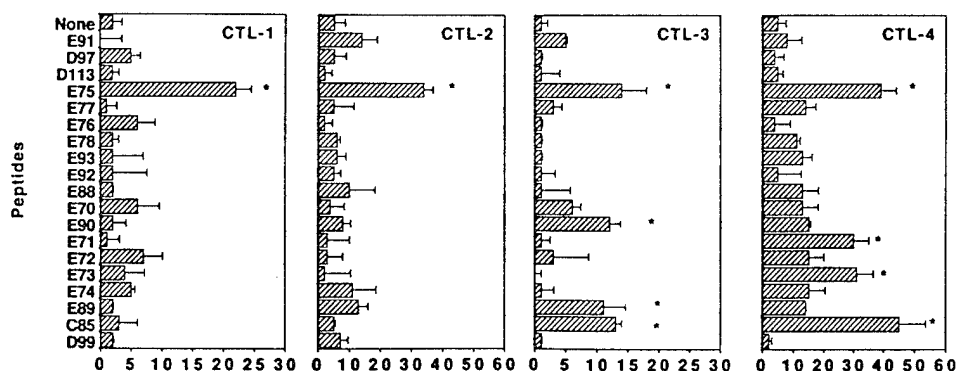
<sup>†</sup> Auto-T and Allo-T represent autologous and allogeneic freshly isolated ovarian tumors. SKOV3.A2.1E4 is an ovarian tumor clone expressing HLA-A2. 2774 is a human ovarian tumor line.

to lysis of the NK targets, K562 cells. These results show that ovarian tumor-reactive CTL can recognize common HER-2 epitopes, although the pattern of peptide recognition is different for each line.

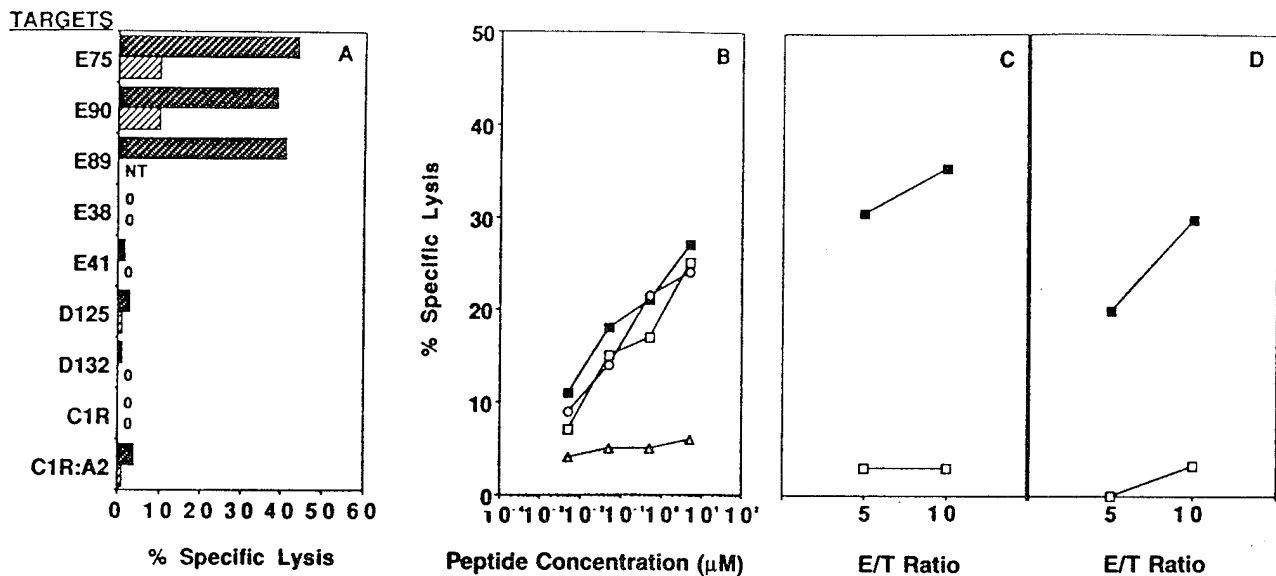
**Recognition of E75 by CTL3 Clones.** Results presented above are suggestive of common HER-2 peptide recognition by four distinct CTL lines. We wanted to establish whether E75 is specifically recognized by cloned CTL, and whether it may correspond to an epitope recognized by the same CTL clone on ovarian tumors that overexpress HER-2. CTL-1 and, to a lesser extent, CTL-2, appear to be highly restricted in their recognition of E75. This can cause the results of peptide specificity experiments with cloned CTL-1 and -2 to ap-

pear biased in the favor of E75. To address the question of whether reactivity to E75 was a property of distinct non-cross-reactive clones from a line of multiple HER-2 specificities, and to establish whether these clones recognize E75 in a peptide concentration-dependent and -specific manner, clones were developed from CTL-3 by stringent limiting dilution and further expanded in culture.

CTL-3 line did not lyse the C1R or C1R:A2 cells (Fig. 2 A). Since C1R:A2 and SKOV3.A2.1E4 were transfected with the same plasmid carrying the same HLA-A2 gene, this suggests that the endogenous peptides recognized on SKOV3.A2.1E4 by CTL-3 may be different from the ones presented by C1R:A2 cells. Both E75 and C85 were recognized by CTL-3 and CTL-4 when presented by C1R:A2 but not by C1R:A1 transfectants (data not shown). Concentration-dependent recognition of E75, C85, and E90 was observed with CTL-3 (Fig. 2 B). This recognition was confirmed in independently performed experiments. Documentation of restricted expression of FBP on ovarian tumors suggests the possibility of cellular immune recognition of FBP peptides (21). CTL-3 did not recognize two peptide analogues of FBP, residues 112-120 (E38) and residues 245-253 (E41) (Fig. 2 A). FBP peptides were selected to include HLA-A2-binding anchors and to exhibit high (E38) and low (E41) HLA-A2-binding affinity, respectively (Table 1). Similarly, CTL-3 did not recognize two peptide analogues of the Muc-1 core peptide (22), D125 and D132. Recognition by cultured ovarian TAL of Muc-1 core exposed on ovarian tumors and of Muc-1 gene transfected and expressed by EBV-B cell lines has been suggested (23). Muc-1 core sequence lacks canonical HLA-A2 anchors at correct distances to allow binding to the main HLA-A2 pockets (21), but its recognition has been described mainly as non-MHC restricted (24). Therefore, in both Muc-1 peptides, GLTSAPDTRV (D125) and SLADPAHGV (D132), HLA-A2 anchors were introduced (underlined) to engage binding and to present the intervening sequence to TCRs. These results, together with the results presented in Fig. 1, suggest that



**Figure 1.** Recognition of HER-2 peptides by CD3<sup>+</sup>CD8<sup>+</sup>CD4<sup>-</sup> CTL isolated from four different ovarian cancer patients. Cytotoxicity was determined using T2 cells preincubated for 60 min with each peptide at 25  $\mu$ g/ml in a 5-h <sup>51</sup>Cr release assay. Percentage of specific lysis is shown for all CTL lines for an E/T ratio of 20:1. Percentage of specific lysis was calculated as described in Materials and Methods. Asterisk indicates mean cytotoxicity values that are at 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's *t* test (*p* < 0.05).

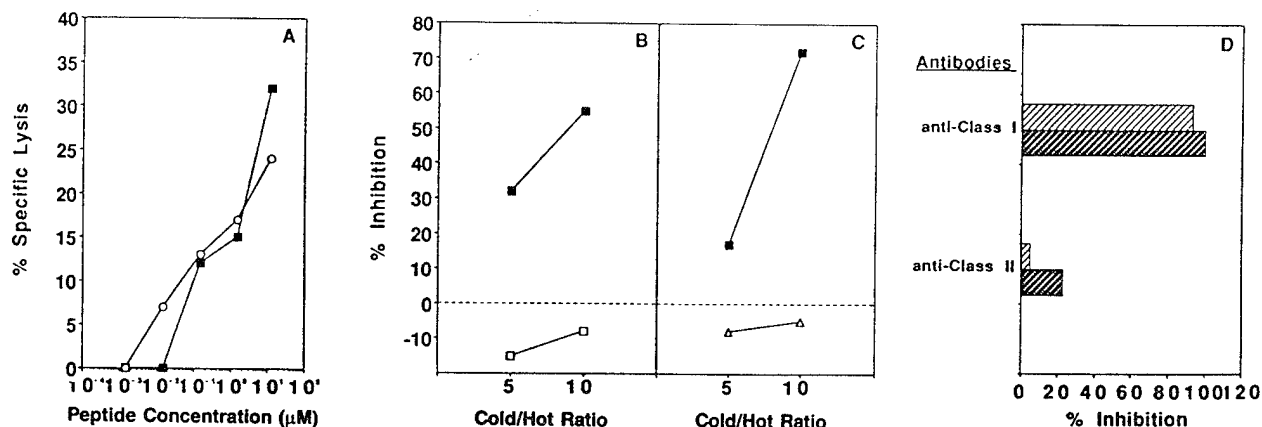


**Figure 2.** Recognition of E75 by CTL-3 clones. (A) Lysis by the CTL-3 line. 3000 <sup>51</sup>Cr-labeled T2 cells were incubated with HER-2 peptides E75, E89, and E90, FBP peptides E38 and E41, and variant Muc-1 peptides D125 and D132 at a final concentration of 25 μM for 60 min before effectors were added. Supernatant was collected and counted after 5 h. E/T ratios were 10:1 (□) and 5:1 (■). Results are presented as the percentage of specific lysis by effectors of T2 cells pulsed with peptides. The same numbers (3,000) of C1R and C1R:A2 cells were used as targets. (B) Concentration-dependent recognition of E75 (■), E90 (□), C85 (○), and E92 (Δ) by CTL-3 line at an E/T ratio of 20:1. (C and D) Lysis by clones 3C4F (C) and 3B4E (D) of E75- (■) and E90- (□) pulsed T2 cells. Lysis of T2 cells incubated with E89 at 25 μM was 7% by clone 3C4F and 5% by clone 3B4E at 10:1 E/T ratios.

CTL-3 line contains clones that are specific for particular peptide epitopes.

Two clones, 3C4F and 3B4E, isolated from CTL-3 line that recognized E75 but not E90 or E89 peptides presented by T2 cells, are shown in Fig. 2 (C and D). Recognition of T2 cells incubated with the same concentration of E75 suggest that clones 3C4F and 3B4E are specific for peptide E75 (Fig.

2, C and D). Recognition of E75 by these two clones was compared over a range of concentrations (10 nM–10 μM) (Fig. 3 A). At an E/T ratio as low as 4:1, peptide E75 reconstituted T cell recognition by clone 3C4F at a concentration (100 nM) similar to that reported for an HLA-A2-restricted epitope gp100 and an HLA-A1-restricted epitope from MAGE-3 recognized by melanoma-specific CTL (25, 26), but at higher



**Figure 3.** Inhibition of ovarian tumor recognition by clone CTL-3C4F by T cell epitope E75. (A) Dose-response recognition of peptide E75 by clones 3C4F (■) and 3B4E (○). Serial dilutions of peptide E75 were incubated with 3,000 T2 cells for 60 min. CTL were added at a E/T ratio of 4:1, and a standard 5-h cytotoxicity assay was performed. Lysis of T2 cells preincubated with E90 at 10 μM was <5% by both CTL clones. (B and C) Cold-target inhibition of lysis of freshly isolated ovarian tumor OVA-1 (B), and ovarian tumor clone SKOV3.A2.1E4 (C) by T2 cells preincubated 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 5-h <sup>51</sup>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 (■) E/T ratios.

E/T ratios. These peptide concentrations are, for MAGE-3, gp100 and HER-2, significantly higher by at least two orders of magnitude than those of HLA-A2.1-restricted viral proteins (27).

To confirm that clone 3C4F recognizes a natural epitope associated with HLA-A2 on ovarian tumors, we examined the ability of E75 and E90 pulsed T2 cells to inhibit lysis of freshly isolated OVA-1 because CTL-1, autologous with this tumor, recognize only E75. Significant inhibition of lysis was observed by T2 cells pulsed with E75 but not with E90 (Fig. 3 B). Since antigen expression on freshly isolated ovarian tumors can be heterogeneous, to confirm that E75 represents an epitope presented by HLA-A2 on an ovarian tumor clone, the ability of E75 pulsed T2 cells to inhibit lysis of clone SKOV3.A2.1E4 was examined. Both OVA-1 and SKOV3.A2 transfectants share only HLA-A2 with effectors and express HER-2 on the surface, OVA-1: 79% HER-2<sup>+</sup> cells, MCF = 29; SKOV3 (positive control): 100% HER-2<sup>+</sup> cells, MCF = 40; SKOV3.A2.1E4: 100% HER-2<sup>+</sup> cells, MCF = 40; C1R:A2 cells (negative control) MCF = 0.7. Ovarian clone SKOV3.A2.1E4 lysis by 3C4F clone was inhibited by anti-MHC class I (W6/32) but not by anti-MHC class II mAb (Fig. 3 D). Again, significant inhibition of lysis by clone 3C4F was observed in the presence of E75. Control peptide D132, which was not recognized when pulsed on T2 cells, failed to redirect clone 3C4F lysis (Fig. 3 C). Therefore, E75, which is recognized by four CTL lines and cloned CTL isolated from one of these lines, and which specifically inhibits recognition of ovarian tumors, may be a natural common HER-2 epitope recognized by ovarian-specific CTL.

## Discussion

In this study, we have investigated recognition of synthetic peptide analogues of HER-2 epitopes containing HLA-A2-binding motifs by CD8<sup>+</sup>CD4<sup>-</sup> CTL lines and clones isolated from TAL with ovarian tumors. We have identified one common epitope (E75) that is dominantly recognized by four out of four CTL lines. Of 19 peptides tested, another common epitope, C85, is recognized by two out of four lines. Several other epitopes, E89, E90, E71, and E73, are recognized only by one of the four CTL lines used, suggesting that they may be either private epitopes for these CTL or clones recognizing these epitopes are present with low frequency in the other CTL lines. The second possibility is more likely because the pattern of concentration-dependent recognition for E90 is similar with that of E75 and C85. In certain experiments, statistical analysis found that recognition of E89 and E90 by CTL-2 and CTL-4, and E91 by CTL-2 was significantly different from control targets, but the levels of recognition were lower (5–7%) than the cut-off value. We have observed that some ovarian CTL cultures lose the ability to recognize a number of these peptides over time probably because of gradual loss of lytic function or overgrowth of CTL of different specificities (Ioannides, C. G., and B. Fisk, unpublished data). We confirmed the specificity of E75 recognition by using two clones isolated from one of the CTL

lines. E75 effectively inhibited lysis by CTL clones of both a freshly isolated ovarian tumor and an ovarian tumor line transfected with HLA-A2, indicating that the epitope recognized is not a culture artifact. Control peptides containing HLA-A2 anchor motifs (11, 12) but different intervening sequences failed to inhibit lysis, suggesting that a natural peptide with an identical or cross-reactive sequence is immunogenic in HLA-A2 ovarian cancer patients and may be presented on ovarian tumors.

Both E75 and C85 were recognized with different efficiencies by CTL1-4 at the same peptide concentration. This may be caused by the existence of clones in these CTL lines that recognize other as yet unknown antigens. The existence of multiple distinct ovarian Ag expressed simultaneously on the same tumor clone has been shown by analyzing recognition of ovarian clones by CTL isolated from TAL (1). Ovarian-specific CTL lines restricted by HLA-A2 recognize common epitopes present on allogeneic HLA-A2<sup>+</sup> ovarian tumors or lines, but not on HLA-A2<sup>+</sup> melanomas. Individual ovarian-specific CTL lines were found to recognize multiple Ag epitopes. Some of the common determinants may be expressed on other HLA-A2<sup>+</sup> epithelial tumors (2, 3). We have previously shown that ovarian TAL can recognize Muc-1 core peptides and HER-2, 968-984, a longer analogue of C85 peptide (HER-2, 971-979) (20). CTL-4 was isolated from TAL-24 used in these studies (20, 23). Recognition of Muc-1 by at least some of the clones derived from the CTL lines used in this study is likely. Although the percentage of tumor cells expressing Muc-1 in a tumor sample is variable and its expression is heterogeneous, most ovarian tumors (>80% of serous adenocarcinomas) have been reported to express Muc-1 (23). Another HER-2 peptide, 654-662, derived from the transmembrane domain, was found to be recognized by TIL isolated from non-small cell lung cancer and developed by different methods (19). HER-2 is expressed in ~30% of ovarian and breast carcinomas. However, its expression is relatively stable over time through the clinical course of invasive breast cancer, it is relatively congruent at all metastatic sites, and it is not affected by tumor heterogeneity (28). This has potential clinical applications because it may allow development of therapies based on HER-2 targeting (28).

Previous studies have shown a direct relationship between HER-2 overexpression and sensitivity to CTL of ovarian tumors (18). Since overexpression of HER-2 may induce expression of other proteins that can provide peptides (18) with the same or cross-reactive sequences, gene and protein sequence databases were searched for homologous sequences. 100% matches for both C85 and E75 were not found. For E75 only EGF-R (HER-1), HER-3, and HER-4 gave matches for the main HLA-A2 anchors at P2, P6, and P9, but nonconservative changes (underlined) were found in positions 1, 3, 5, and 7 (EGF-R, residues 364-372: SISGDLHIL, HER-3, residues 356-364: KILGNLDFL, HER-4: KINGNLIFL). Central positions are expected to be contact points for TCR (15, 29). Matches for peptide C85 appear in EGF-R, ERB.B3, DNA-directed RNA polymerase (RPB-1), and in an unknown nuclear protein (UL2-1). Nonconservative changes in the se-

quence are dominant at positions expected to be TCR contacts such as P4 and P7 in RPB-1 and P5 in UL2-1. Based on recent crystallography data, the peptide termini are bound to HLA-A2 similarly but the central area of the peptide adopts different conformations that represent the epitopes recognized by TCR (29). Therefore, the nonconservative changes in the sequence of the homologous peptides from the other members of the HER family may affect epitope conformation and if these peptides are processed, presented, and recognized by TCR may constitute the equivalents of variants of peptides derived from the HER-2 protein.

As for the other tumor Ags (10, 25, 26), validation of HER-2 epitopes requires identification and quantitation of peptides bound to HLA class I on ovarian tumors. Since E75 lacks charged residues in the central area, it will be important to determine whether the same or conservatively substituted peptides from other proteins are naturally processed and presented to CTL. With the exception of E75 recognized by all four CD8<sup>+</sup> CTL, and in part of C85, which confirms our previous findings with unseparated CTL-TAL (20), three CTL recognized distinct HER-2 peptides at low level. These peptides were different in each system and their HLA-A2-stabilizing ability was variable over a wide range of concentrations. E89 binding affinity to HLA-A2 is at least three to four orders of magnitude lower than of naturally processed viral peptides (12), suggesting that the affinity of TCR for E89-HLA-A2 complexes may be high. This may also be true for C85. The affinity of a peptide for HLA-A2 is not the determining factor for the abundance of particular peptide presented by HLA-A2 (30). Other important factors are protein concentration and the processing efficiency of an antigenic peptide (30).

The mechanisms of HER-2 overexpression reflect gene amplification and upregulation of transcription (31). The involvement of translational, posttranslational mechanisms, or reduced rate of HER-2 turnover in HER-2 overexpression in cancer cells are still unclear (31). At this time, there is no simple explanation for the distinct pattern of peptide recognition between these lines. All tumors autologous with these CTL overexpressed HER-2 protein at similar levels that were consistent with HER-2 receptor overexpression when analyzed using monoclonal antibody Ab2 that is specific for the extracellular domain of HER-2 (e.g., the levels of HER-2 expression were similar for OVA-1 and OVA-4 tumors [autologous with CTL-1 and CTL-4]: OVA-1, 79% HER-2<sup>+</sup> cells, MFC = 29, OVA-4, 100% HER-2<sup>+</sup> cells, MFC = 34, control OVA-17, 21% HER-2<sup>+</sup> cells, MFC = 4). However, CTL-4 associated with OVA-4 recognized in addition to E75 three other peptides. It is possible that these peptides if presented are processed by the tumor with different efficiencies (30). It is also possible that while for self-proteins the tolerance is not absolute, as shown for melanoma TIL specific for either MART-1 or gp100 (10, 25), elimination of high affinity T cells for a number of epitopes by tolerance may affect CTL with distinct specificities in each individual (10, 25).

Processing of overexpressed HER-2 in cancer cells may lead to peptides that differ in quantity from the HER-2 epitopes found on normal cells. Since HER-2 is present in normal epithelial tissues at lower levels and the protein concentration may be a limiting factor in epitope presentation, it will be important to determine how widely CTL-mediated HER-2 recognition is observed in ovarian and breast cancer patients, and whether such CTL can cause tumor rejection and show toxicity towards normal tissues.

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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.

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*Abbreviations used in this paper: Cytotoxic T Lymphocytes, CTL, Position, P; high  
stabilizing ability, HSA; low stabilizing ability, LSA; T cell receptor, TCR; HER-2/neu  
proto-oncogene, HER-2; folate binding protein, FBP, standard deviation, SD; peptide-  
MHC class I, pep-MHC; mean channel fluorescence, MCF.*

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**Summary**

The HER-2/neu proto-oncogene (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 CTL from tumor associated (TAL) and infiltrating (TIL) lymphocytes, indicating that they reconstitute natural epitopes recognized by CTL on HLA-A2+ tumors. Since HER-2 is an important 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. For a low HLA-A2 affinity HER-2 peptide, CTL epitope, P1 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-antigenity" of self-antigens, aiming towards induction of tumor reactive CTL of defined affinity and specificity for target Ag.

## Introduction

During the recent years, studies on human cancer antigens have identified peptides derived from self-proteins recognized by cytotoxic T lymphocytes that can lyse freshly isolated autologous tumors. Most of these peptides from melanoma, e.g. gp100 and MART-1 (1-3,4) as well as from ovarian carcinoma (e.g. HER-2), (5,7), were found to bind 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 HLA-A2 was compensated in part by the high concentrations of peptides used in the *in vitro* assays (2,5-7). Since these peptides are likely candidates for development of tumor vaccines and of anti-tumor effector CTL, this raises the question of how to utilize low MHC affinity peptides for CTL induction both *in vitro* and *in 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 utilize 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. Since 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 Ag presentation by appropriate changes in the peptide sequence. The second question is whether for tumor Ag such as HER-2 there are sequence characteristics which associate with MHC stabilizing ability and which are residues permissive to changes in the sequence whose substitution while increasing the peptide affinity for HLA either do not affect or affect only minimally CTL recognition. To address these questions we utilized as a model the HER-2 protein and CTL recognizing a low HLA-A2 affinity epitope from this protein. This Ag and CTL effectors are relevant for immunotherapy of human breast, ovarian and lung cancers.

The objectives of this study were three-fold: (1) to identify in the HER-2 sequence, peptides of high and low stabilizing ability, and determine whether there are certain sequence characteristics in these peptides which associate with high and low stabilizing activity respectively; (2) to identify specific sequence changes that

enhance the stabilizing ability of a low HLA-A2 affinity peptide; (3) to define the effects of these changes on CTL recognition, and identify residues that when substituted in the peptide increase the HLA-A2 affinity but do not affect the specificity of CTL recognition.

Since HER-2 is a large molecule (1255 amino acids), a large number of potential HLA-A2 binding peptides can be identified using the minimal and extended HLA-A2 binding motifs (>50) (9). 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 high (HSA) and low (LSA) stabilizing ability based on 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 the main P2 and P9 anchors, and identified residues which occurred with higher frequency in the peptides with either high and low stabilizing activity.

In peptides with high HLA-A2 stabilizing ability, 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, while charged residues were found with increased frequency at P4. Replacement of P1 and P9 anchors in a low-stabilizing ability peptide with residues found with higher frequency in HSA peptides significantly increased the stabilizing ability of hybrid peptides. Of these changes, substitutions of Gly and Phe at P1 lead to HSA peptides that were recognized with the same or similar efficiency by peptide specific CTL. This approach may be useful for optimization of tumor antigen 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 HPLC. The purity of the peptides used in this study ranged between 92-95%. The purity and amino acid composition of these peptides were established by amino acid

analysis. The peptides synthesized were all nanomers containing L/I/V at P2 and V/L/I/M at P9. Of these peptides 26 were analogs of HER-2, while 5 were analogs of folate binding protein (FBP).

**Monoclonal antibodies and immunofluorescence** The HLA-A2 specific mAb BB.7.2 and MA2.1 were obtained from the American Tissue Type Collection (ATTC). W6/32 mAb was obtained from DAKO-(Dakopatts, Denmark). Cell surface expression of HLA-A2 on T2 cells was studied by flow cytometry using an EPICS<sup>R</sup> Profile Analyzer (FACS) with a log amplifier.

**HLA-A2 stabilization assay.** T2 cells were pulsed overnight with saturating amounts of individual peptides (100µg/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 5000 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 (10-11). 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 as relative mean channel fluorescence (MCF-R) (12).

**Cytotoxicity assays.** The CTL line 41 developed by repeated in vitro stimulation of HLA-A2<sup>+</sup> PBMC 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-41 were maintained in culture with monthly restimulation with C84 peptide at 10 µg/ml and autologous or allogeneic HLA-A2<sup>+</sup> PBMC. CTL used as effectors in these experiments were selected on Ab coated plates (AIS Micro CELLector<sup>TM</sup>, Applied Immune Sciences, Menlo Park, CA), and were CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup>. To determine recognition of these peptides by CTL-41, <sup>51</sup>Cr labelled T2 cells were pre-incubated with various concentrations of peptides for 90 min. Effectors were added for an additional 5 hrs. %specific lysis was calculated from the formula (E-S)/(T-S) x 100 where E are the cpm obtained from wells containing both effectors and targets,

while S and T represent the cpm measured from spontaneous and total lysis of targets in the absence of effectors and presence of the same peptide (6). *Statistical Analysis.* MCF values obtained for HSA and LSA groups were examined by the 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 I on the expression of an epitope recognized by the HLA-A2 specific mAb BB7.2 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 the cell surface (14). BB7.2 epitope is located on the N-external loop of the  $\alpha 2$  domain (including W107) (15-17) and is not expected to directly interact 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 high stabilizing activity (HSA) while peptides with MCF-R  $< 2.0$  were designated as having low HLA-A2 stabilizing activity (LSA). As seen in the **Table I**, twelve peptides showed MCF-R of 2.40 or higher while 19 other peptides an MCF-R of 1.79 or lower. These peptides belong to two different groups of HLA-A2 stabilizing ability. The means and SD of the MCF-R for peptides in the groups of high stabilizing ability and low stabilizing ability are  $3.90 \pm 1.4$  and  $1.24 \pm 0.26$  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  $\alpha 3$  domain of HLA-class I. The specific stabilization of HLA-A2 by the peptides is shown in **Table I**. A number of HSA peptides caused a 2 - 3 fold increase in MCF-R for HLA-A2 expression compared to 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 SD of MCF-R for W6/32 in the HSA and LSA were:  $2.26 \pm 0.5$  and  $1.18 \pm 0.18$  respectively ( $p < 0.0001$ ). The values for 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 suggesting that peptides induced changes in the BB7.2 epitope which are not a direct result of an increase in the number of HLA-A2 molecules expressed on the surface of T2 cells (18,19).

*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 negatively and/or positively charged residues at P1, P3, P6 and P7 in the peptide was associated with decreased or lack of binding, while 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). Furthermore 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 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, 11/12 (91%) HSA peptides contained L (P2) and 1/12 (8.3%) contained I(P2), while of LSA peptides 10/19 (52%) contained L(P2), 7/19 (36.9%) contained I (P2) and 2/19 (10.5%) contained Val (P2). Nonapeptides with M (P2) were not found in the HER-2 sequence. Of the HSA peptides 6/12 (50%) contained Val at P9, and 6/12 (50%) contained Leu (P9), while of the low stabilizing ability 4/19 (21%) contained Val (P9), 12/19 (63.2%) contained Leu (P9) while 2/19 and 1/19 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 low stabilizing affinity even when either Val or Leu were 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 I**).

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 nanopptide sequence we calculated the frequency of each group of amino acids in each position for the 12 HSA peptides and 19 LSA peptides. Since the sample size is relatively small, amino acids of similar physicochemical characteristics were grouped together (e.g. aromatic, aliphatic nonpolar, negatively charged, etc.) 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 II**.

A striking trend was found in positions 4 and 5 of the nonapeptide sequence. HSA was associated with the presence of predominantly hydrophilic residues Lys, His, Arg, Gln, 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 excepting Leu, Val, Ile, Met, and Gly no defined groups of amino acids were present in 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, Cys was found with higher frequency in the LSA peptides. At position 6, only the frequency of positively charged residues was increased in peptides with LSA compared with peptides with HSA, while no other group of amino acids was found with significantly higher frequency in any of these groups of peptides. This is also in agreement with previous reports on residues associated with poor HLA-A2 binding (21). Interestingly, there was no significant difference between the presence of aliphatic hydrophobic residues in each of HSA and LSA groups of peptides. At position 7, Gln and Asn were predominantly found in peptides with HSA, while 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 & P4) with low HLA-A2 stabilizing ability was observed. Similar results were obtained from the analysis of the percent expression of hydrophilic residues at each position in the peptides. (**Figure 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 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 and compared with W6/32 epitope expression. C85 was chosen because, when presented by T2 cells, forms an 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.

Since the low stabilizing ability 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 first group of substituted peptides was synthesized containing at P1 either Thr (designated as 103T), Gly (103G), Lys (103K), or Phe (103F). The reasons for selecting these amino acids were as follows: **(1)** aromatic amino acids at P1 were preferentially associated with good binding (21); **(2)** the presence of Lys at P1 is expected to facilitate the stabilization of the complex through charge interactions with the negatively charged pocket A (26); **(3)** the small volume and the lack of side chains of Gly will not only avoid steric interactions with mAb MA2.1 but also may provide additional conformational flexibility for the peptide chain (27-29); and **(4)** the group Ser, Thr, Cys was found with higher frequency at P1 in our LSA, than in HSA HER-2 peptides (**Table II**). However the presence of this group at P1 was associated with 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. Since 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 III**. 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 compared with wild-type peptide C85, while for W6/32 and MA2.1 the change in MCF indicate 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 show 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  $\rightarrow$  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. For the double substituted peptides (designated 104, T, G, K, F) different results were obtained. 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 staining 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), while for 104F increase in staining was observed over C84 for both BB7.2 and MA2.1, suggesting synergy in inducing conformational changes in HLA-A2 by F (P1) and V (P9) in the C85 peptide.

These results show that for the LSA peptide C85, substitution Met  $\rightarrow$  Val at P9 in C85 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 Glu  $\rightarrow$  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 affect

significantly less the BB7.2 epitope. Substitutions Glu → Lys (P1) while stabilizing the peptide HLA-A2 complex apparently induced strong conformational changes in the peptide-HLA-A2 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 structure, 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 **Figure 2**, staining by W6/32 and BB7.2 of T2 cells incubated with E51 significantly decreased. Interestingly, staining by the same mAb of T2 cells incubated with E54 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 at 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 **Figure 1**, that the presence of hydrophobic aliphatic residues at P4 is associated with LSA peptide. The substitution Phe → Val (P6) in C83 peptide (81) increased the binding of all mAb tested over the levels detected for peptide C83. In this sequence context, when the MCF for all mAb tested for the peptide pairs C83-E53 and C81-C82 are compared, the substitution Ser → Lys (P4) affected both the stability of the HLA-A2 peptide complex and possibly its conformation. Substitution Glu → Gly (P5) in C82 lead to further reduction of mAb 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 not only dependent of 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 CTL.*

To define the effects of sequence changes on the recognition by peptide specific CTL we conducted cytotoxicity experiments. CTL-41 induced by peptide C84 (13) were used as indicators, because they were developed against conservatively substituted C85 peptide. Thus, they offer 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 tumor infiltrating lymphocytes. 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. Since this may result in a small number of peptide HLA-A2 complexes, we investigated their recognition over a range of concentration. The results are presented in the **Figure 3A**. These results show that even at high concentration (20 $\mu$ M), the P3-P5 substituted peptides were either 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 140nM was needed for induction of the half-maximal cytolytic effect of the CTL-41. This value is significantly higher than the amount of C84 (40 pM) giving SD50.

Recognition of P1 and P1P9 substituted peptide by CTL-41 is shown in Figure 3B. Recognition of all peptides was tested at the same concentration in the assay. The results indicate that G1 and F1 substituted peptides are recognized at similar extent with C84, while 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 efficiency with C84. These results show that recognition of peptides substituted at P1 is sequence specific. G1M9 and F1M9 substituted peptides were recognized by CTL with similar efficiency with C84 (E1V9) although F1M9 showed significantly higher stabilizing ability than G1M9. There was no direct correlation between the ability of these peptides to affect either the conformational epitope BB.7.2, or MA2.1 or the magnitude of change in HLA-A2 expression and CTL recognition. All double substituted peptides showed significantly higher stabilizing ability than C84. The half-maximal stabilization by P1 substituted peptides was observed 2-6  $\mu$ M, while for C84 was observed with M (Savary, Fisk, Hudson, manuscript in preparation). This suggests that a significantly higher number of P1 and/or P9 substituted peptide - HLA-A2 complexes are needed for recognition by TCR than for recognition of C84 or C85, and these analogs are recognized with lower affinity than C84.

## Discussion

In this paper we have investigated the ability of 26 HER-2 and 5 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 peptide are of low binding affinity (15/26 and 4/5 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) while 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 selected from the same protein, allow us to identify the role of the protein sequence in selection of HLA-A2 binding peptides from a tumor protein which is the target of a CTL response. We found that for residue at position 9 replacement of Met with Val enhance the stability of the HLA-A2 peptide complex. Comparison of the recognition of the conformational epitopes BB7.2 and MA2.1 in comparison with the monomorphic epitope W6/32 indicates very little change in the recognition of these antibodies over W6/32, suggesting that conformational changes induced by the M -> V substitution are minimal.

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 utilization 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 tumor specific CTL induction.

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 CTL. The non-permissivity 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 on these positions bulge away from the  $\alpha 1$  - and  $\alpha 2$  helices and are likely to be TCR contacts.

Since 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  $\mu\text{g/ml}$  none of these peptides reached SD50 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 CTL.

The implications of these findings are two fold: **(1)** 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; **(2)** The selective use of substituted peptides from self-proteins as immunogens leads to CTL which 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 gp100 peptides may be expressed at lower amounts on normal tissues than on tumor cells (31). Thus the epitope density may be a limiting factor in epitope recognition (32). However, when CTL 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 anti-tumor response.

Induction of CTL by substituted peptides will lead to effectors that recognize the natural peptides with lower affinity than the peptide used as immunogen (33). Since the low affinity CTL need significantly higher concentrations of antigen to engage TCR than high affinity CTL effective lysis of targets may be expected only in

the case of tumors overexpressing the HER-2 or gp100 protein, but not of normal tissues. Furthermore, the induced "neo-antigenicity" of the peptides may be beneficial for induction of tumor reactive CTL. Although the rules defining CTL cross-reactivity are still unclear, for humoral responses, high affinity antibodies are more cross-reactive with various antigens than low-affinity antibodies (35,36).

To date there is little information on induction of CTL specific for the naturally presented epitope on human tumors using peptides as immunogens. The difficulties in inducing CTL 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 (37), and **(c)** peptide presentation is determined by corresponding HLA molecule co-expression (38). Thus not only induction of CTL specific for these peptides would require breaking of tolerance in the non-responder population of patients which express the Ag e.g. as demonstrated for tyrosinase (39,3), but induction of CTL using various approaches recognizing self-peptides with high affinity also raise concerns about recognition of normal tissues. 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, raise the possibility of using P1P9 substituted peptides for tumor specific/reactive CTL induction. These approaches are currently under investigation in our laboratory (Hudson, Fisk, et.al. manuscript in preparation) using dendritic cells isolated from bone-marrow precursors and T cells from PBMC from healthy donors and ovarian and breast cancer patients.



**Legend to Table 1**

HER-2 and FBP peptides were selected from the corresponding protein sequences as we have previously described (13). The code for each nonapeptide is the code assigned for the particular synthetic peptide 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 above. 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).

## Legends to the Figures

**Figure 1.** Frequency of hydrophilic residues at each position in the HSA and LSA nonapeptides. Each amino acid was designated as either hydrophilic or hydrophobic according to the hydropathy scale of Fauchere-Plitska ( ). (-■-) HSA peptides (□) LSA peptides.

**Figure 2.** Stabilization of HLA-A2 specific 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 the Materials and Methods. (-) Indicates residues identical with the unsubstituted peptide. Cells were stained by indicated mAb (W6/32, B.B.7.2 and MA2.1) The antibody specifications are listed in the Results Section.

**Figure 3. A.** Concentration dependent recognition of HER-2:971-979 peptide C85 and its substituted analogs in the central area (E51 ( $\Delta$ ), E52 ( $\blacktriangle$ ), E53 ( $\square$ ), E54 ( $\circ$ )). by CTL-41. The sequences of these peptides are presented in **Figure 2**. CTL-41 were induced by stimulation with peptide C84 ( $\blacksquare$ ). T2 cells were incubated with various concentration 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%. **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  $\mu$ g/ml before being used as targets in CTL assay. 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 e.g. M9, V9. E:T ratio was 10:1.

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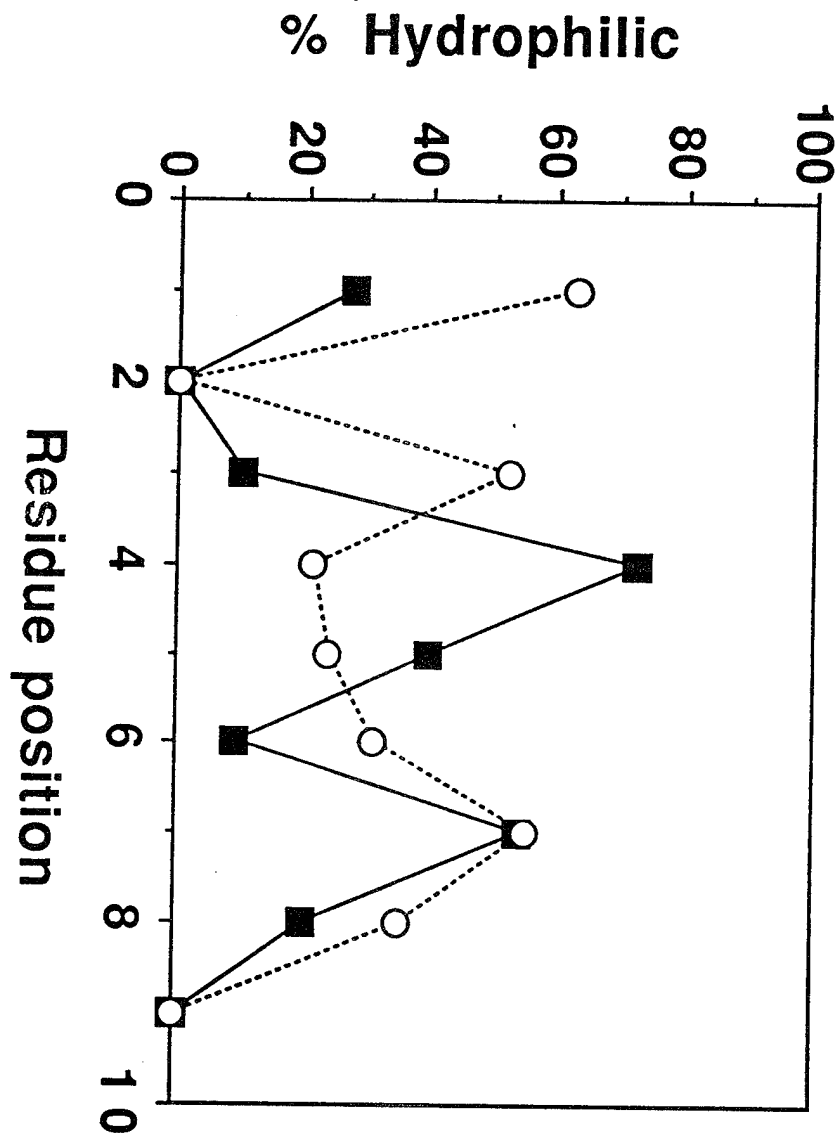
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Figure 1



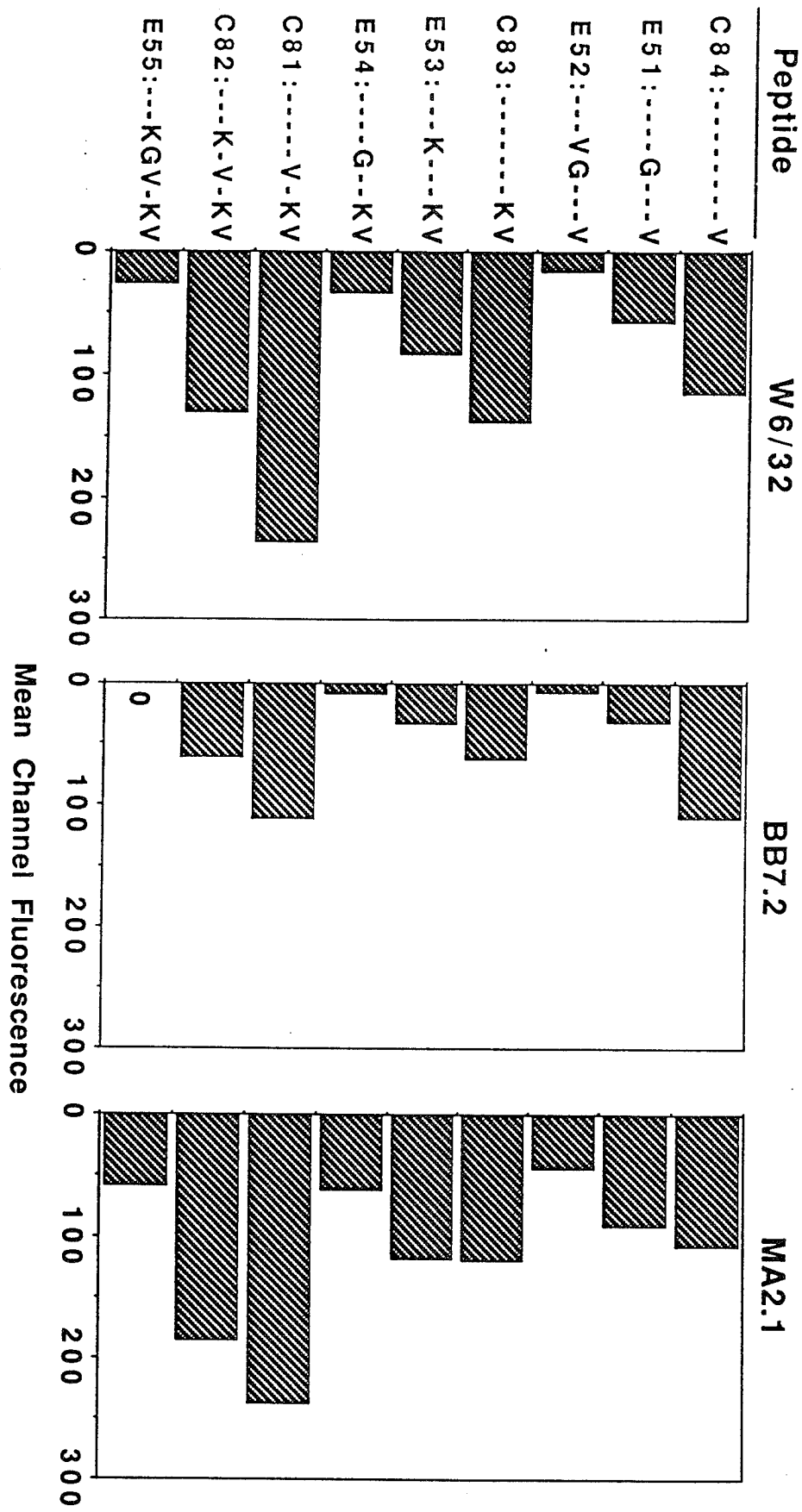


Figure 2



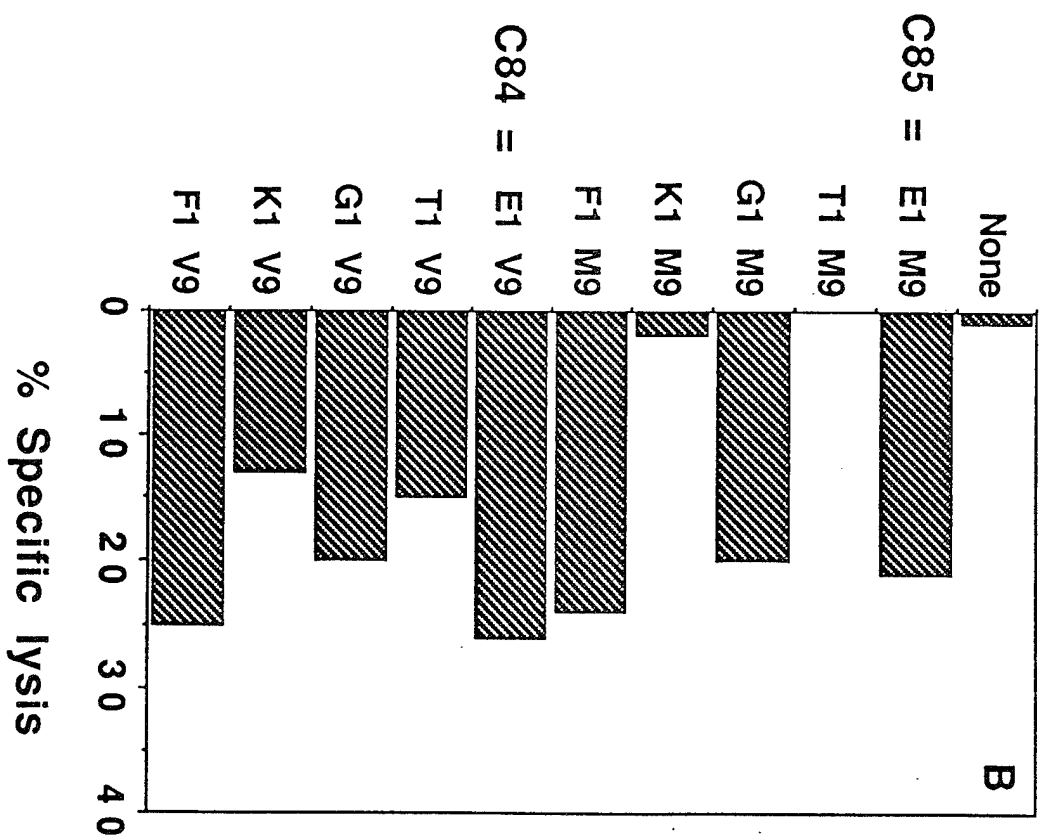
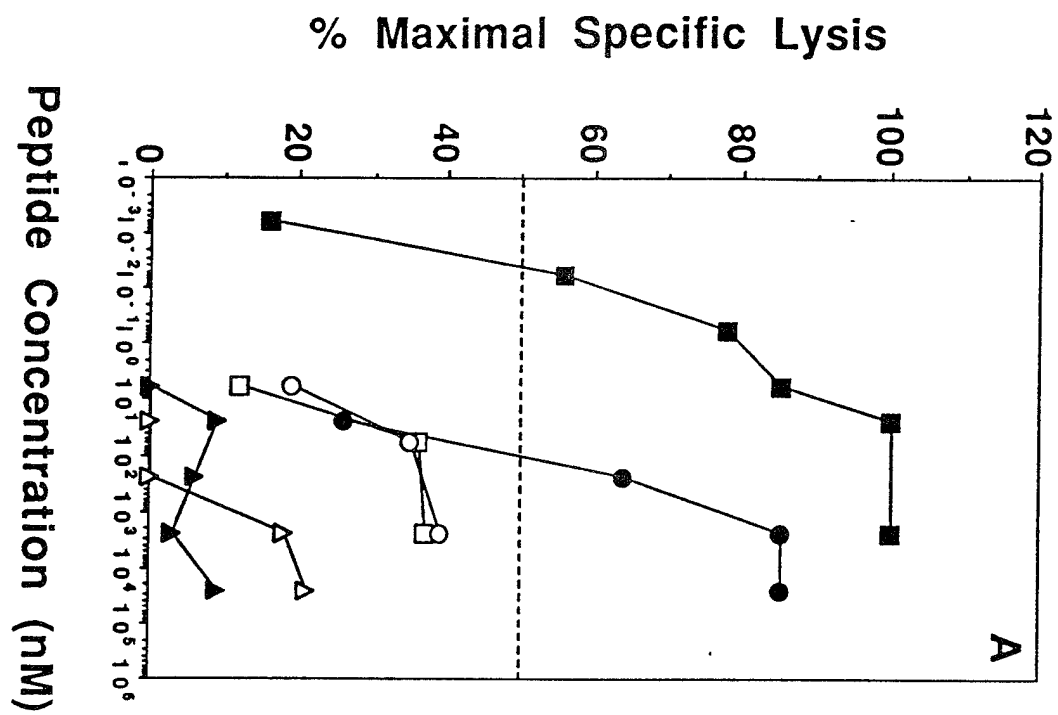


Figure 3

**Table I. Stabilization of BB7.2 epitope by HER-2 and FBP peptides.**

Code	Position	Sequence	MCF-R	
			BB7.2	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

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

	RESIDUES AT POSITIONS/FREQUENCIES(%)*				
	1	2	3	4	5
	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
S,T,C	16.7 : <b>26.3</b>	0	25.0 : 31.6	16.7 : 21.0	<b>33.3</b> : 05.2
A	<b>16.7</b> : 00.0	0	00.0 : <b>15.8</b>	00.0 : 10.6	00.0 : <b>15.8</b>
G	08.3 : 00.0	0	<b>16.7</b> : 00.0	08.3 : 00.0	16.7 : 10.6
P	08.3 : 10.5	0	00.0 : 05.3	08.3 : 15.8	00.0 : 00.0
L,V,I,M	16.7 : 10.5	100 : 100	<b>25.0</b> : 10.6	08.3 : <b>31.6</b>	16.7 : <b>31.6</b>
Q,N	08.3 : 21.0	0	00.0 : <b>15.8</b>	<b>25.0</b> : 00.0	00.0 : 10.6
R,H,K	<b>25.0</b> : 10.5	0	08.3 : 15.8	<b>25.0</b> : 05.3	08.2 : 08.2
D,E	00.0 : <b>15.8</b>	0	08.3 : 00.0	08.3 : 05.3	01.0 : 10.6

\*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 HSA and 19 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.

TABLE II (cont.)

RESIDUES AT POSITIONS/FREQUENCIES					
	6	7	8	9	
	HSA LSA	HSA LSA	HSA LSA	HSA LSA	
Y,P,W	08.3 : 05.2	08.3 : 05.2	16.7 : 16.7	0	
S,T,C	25.0 : 15.8	16.8 : <b>42.0</b>	08.3 : 10.2	0	
A	08.3 : 00.0	08.3 : 00.0	08.3 : 00.0	0	
G	08.3 : 15.8	08.3 : 05.2	08.3 : 15.8	0	
P	08.3 : 05.2	00.0 : 00.0	00.0 : 00.0	0	
L,V,I,M	33.0 : 26.0	16.7 : 21.0	33.0 : 37.0	100 : 100	
Q,N	08.3 : 05.2	<b>33.0</b> : 10.0	08.3 : 05.2	0	
R,H,K	00.0 : <b>21.0</b>	00.0 : 10.0	16.0 : 10.0	0	
D,E	00.0 : 05.2	08.3 : 10.0	00.0 : 10.0	0	

The frequency of appearance of a certain group of residues at a position in the sequence was considered significant if exceeded by at least 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 aminoacids 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.

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

		MCF <sup>a</sup> (channel no.)		
Peptide	Amino Acid	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

<sup>a</sup>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 mAb. 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).

## **Generation of Tumor-Binding Dendritic Cells from CD34+ Progenitors**

### **Cultured with IL-2 and Stem Cell Factor (SCF)<sup>1</sup>**

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Key Words: dendritic cells, CD34, cytotoxic T lymphocytes, HER-2/*neu*, interleukin-2

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**Abbreviations used:** APC, antigen-presenting cell; DC, dendritic cells; DNase, deoxyribonuclease; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HEPES, 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PE, phycoerythrin; S-RPMI, supplemented-RPMI 1640 medium; SCF, stem cell factor; TAA, tumor-associated antigens; TBC, tumor-binding cells.

## ABSTRACT

Because dendritic cells (DCs) constitutively express HLA-Class II and CD80/86 costimulatory molecules and a high density of adhesion structures, they are considered to be the most efficient of antigen-presenting cells (APC). DCs, though, are present in very low frequency in all tissues so that efforts have been made to propagate these cells *in vitro*. Although GM-CSF has been shown to promote DC growth, little is known concerning the role of other cytokines in DC-genesis. We show that cells with the morphology and phenotype of DCs are generated in relatively large numbers from CD34+ bone marrow progenitors of normal donors and cancer patients after 7-10 days culture with IL-2 and stem cell factor (SCF). Interestingly, the DCs showed a striking propensity to bind to K-562 tumor cells as detected by 2-color fluorescence-activated cell sorting. The marrow-derived DCs were also found to be efficient in inducing cytolytic CD8+ T lymphocytes (CTL) specifically reactive against a synthetic HER-2/*neu* nonapeptide E75 (369-377) which has been previously reported to reconstitute recognition of ovarian tumor-reactive CTL. Our results suggest that marrow-derived DCs can efficiently present MHC Class I binding tumor- antigens to autologous CTL...



## INTRODUCTION

Studies of *in vitro* cytokine-expanded peripheral blood and tumor-infiltrating lymphocytes of cancer patients have provided evidence that T cells recognize antigenic determinants on autologous malignant cells [1-3]. The targets of tumor-reactive lymphocytes include the products of genes that are overexpressed in malignant cells (e.g. HER-2/*neu*), or silent genes that become activated during malignant transformation (e.g. the MAGE gene family) [4-8]. Additionally, novel proteins resulting from gene mutation are also immunogenic[6].

With the molecular characterization of some tumor-antigens (TA), and our new understanding of peptide-major histocompatibility complex (MHC) interaction, *in vitro* immunization of lymphocytes against TA has become a technical reality [7, 9]. The effectiveness of lymphocyte immunization, however, relies on optimum presentation of the antigen by autologous antigen-presenting cells (APC). According to the 2-signal model of lymphocyte activation, a positive response requires that the APC: (a) present peptide bound to autologous MHC Class I or Class II molecules, and (b) provide a costimulatory signal directed through CD80/86, CD54, or other yet to be defined structures [10, 11]. Attention has focused recently on the use of dendritic cells (DC) for *in vitro* immunization, since they are considered to be the most efficient APC [12].

All DCs are derived from bone marrow and migrate via blood to virtually all tissues where they are poised to capture antigen for transport to lymph nodes and presentation to lymphocytes [12]. Under appropriate cytokine stimulation, less mature DC are capable of protein ingestion and processing [13-15]. By comparison, more mature DC are less efficient at antigen processing, but are more effective immunostimulatory cells due to expression of a high density of MHC, adhesion, and costimulatory molecules. In fact, it is the property of constitutive expression of HLA Class I and Class II molecules, and CD80/86 that distinguish Dcs from other APC, and that allow them to activate both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes. Thus, use of DCs as APC would be expected to improve the quantity, activity and diversity of tumor-reactive clones generated in *in vitro* immunization systems using proteins and peptides.

The precise identification of cytokines involved in DC maturation, function and growth have not yet been fully delineated. In both murine and human models, GM-CSF stimulates the activity and growth of DCs isolated from peripheral blood [15-17], and induces DC differentiation from CD34<sup>+</sup> bone marrow progenitors [18, 19]; other cytokines, including IL-4 and SCF synergize with GM-CSF in this activity [15, 18, 20]. We present evidence that cells with DC-like morphology and phenotype are generated from CD34<sup>+</sup> marrow progenitors in short-term cultures supplemented with IL-2 and SCF. We show that these cells have tumor-binding capacity, and function as APC in *in vitro* induction of CD8<sup>+</sup> lymphocytes specific for a known CTL epitope, HER-2/*neu*: 369-377.

## MATERIALS AND METHODS

### *Target Cells.*

K-562 and 174CEM.T2 (T2) tumor cells were maintained as continuous cultures in RPMI-1640 medium supplemented with HEPES buffer, 10% fetal bovine serum (FBS), antibiotics, and glutamine. K-562 was originally established from a patient with chronic myelogenous leukemia and was obtained from ATCC. The T2 cell line was a kind gift of Dr. Peter Cresswell (Yale University, New haven, CT); these cells have a defect in the TAP transport system and express HLA-A2.1 occupied only by signal peptides [21].

### *Generation of Dendritic Cells.*

Cryopreserved bone marrow cells of normal donors and breast cancer patients were thawed and washed in  $\text{Ca}^{++}/\text{Mg}^{++}$ -free phosphate buffered saline (PBS) containing 100 U/ml DNase, and incubated overnight in RPMI 1640 medium supplemented with HEPES buffer, 10% fetal bovine serum (FBS), glutamine, antibiotics, nonessential amino acids, sodium pyruvate, and 2-mercaptoethanol (S-RPMI). The cells were then separated on a gradient of 35% Percoll to remove dead cells and debris.  $\text{CD34}^{+}$  cells were positively selected by paramagnetic bead separation technology using a CD34 isolation kit purchased from Miltenyi Biotec, Inc. (Sunnyvale, CA). Briefly, the marrow cells were incubated for 15

min at 4°C with human IgG blocking solution (reagent A1) and 15 min with modified QBEND/10 anti-human CD34 IgG<sub>1</sub> antibodies (reagent A2), washed and then incubated an additional 15 min with superparamagnetic microbeads recognizing reagent A2 (reagent B). The cells were then passed through an iron bead column placed within a strong magnetic field and the adherent cells eluted from the column after removal from the magnet. The adherent cells were passed through a second column to further enrich for CD34<sup>+</sup> cells. The efficiency of the separation was evaluated by staining an aliquot of the sorted cells with phycoerythrin (PE)-conjugated HPCA-2 anti-CD34 (Becton Dickinson Monoclonal Center, Mountain View, CA) which reacts with an epitope distinct from that of the QBEND antibody; the purity of CD34<sup>+</sup> populations was 95-99% as evaluated by flow cytometry.

The CD34<sup>+</sup> cells were seeded into the wells of tissue culture plates at an initial concentration of 10<sup>5</sup> cells/ml in S-RPMI medium containing 10<sup>3</sup> U/ml of highly purified human recombinant IL-2 (18 x 10<sup>6</sup> IU/mg, kindly provided by Cetus), and/or 2-5 U/ml SCF (kindly provided by Immunex, Seattle, WA ). The cultures were maintained for 7-10 days at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

#### *Phenotypic Analysis of Cells.*

Cells were labelled with fluorescein isothiocyanate (FITC)- or PE-conjugated monoclonal antibodies recognizing the following cell surface determinants: CD1a, CD2, CD3, CD4, CD8, CD14, CD16, CD19, CD25, CD33, CD34, CD38, CD45RA, CD45RO, CD56,

CD80, HLA-DQ (Becton Dickinson); HLA-DR (DAKO Corp., Carpinteria, CA); CD122 (Endogen, Boston, MA). Briefly,  $1-5 \times 10^5$  cells were incubated for 20 min with mouse IgG antibodies to block nonspecific binding. Test antibody was then added and the cells placed at 4°C for 30 min and washed. Isotype control antibodies unreactive to human cells were used to control for background fluorescence. Samples were analyzed using a FACScan flow cytometer equipped with a single 488 nm argon laser and Consort 30 or FACScan Research Software (Becton Dickinson).

#### *Analysis and Sorting of Tumor-Binding Cells (TBC).*

The ability of CD34<sup>+</sup> cells from 7-10 day cultures to form conjugates with K-562 cells was analyzed by 2-color flow cytometry. K-562 target cells were labeled with FITC and effector cells from IL-2+SCF cultures were labeled with PE-conjugated antibodies as described in the Results. The two populations were mixed in 1:1 ratio in 0.2 ml of S-RPMI, centrifuged for 5 min, and gently resuspended. The cells were analyzed for conjugate formation immediately, and after 10 min of incubation at 37°C in a water bath. Forward and side scatter gates were set to include all targets, effectors, and conjugates, but excluding debris.

For fluorescence-activated cell sorting (FACS) of tumor-effector conjugates, FITC-labelled K-562 cells were irradiated with 5000 cGy gamma irradiation delivered from a

cesium source, and mixed together with effector cells which had been pre-labelled with PE-conjugated anti-CD38 antibodies. The dual-stained conjugates were then sorted at a rate of 1500 events/second using a VANTAGE cell sorter (Becton Dickinson), and returned to culture for 20 hrs prior to analysis by light microscopy.

*Immunization of Lymphocytes Against HER-2/neu Peptides.*

Cryopreserved bone marrow cells from patients with breast cancer were pre-screened for reactivity to the BB7.2 antibody recognizing the  $\alpha$ -2 domain of HLA-A2 Class I, and the CD34<sup>+</sup> progenitors from an HLA-A2<sup>+</sup> patient were placed into culture in IL-2+SCF supplemented medium. Highly purified T lymphocytes were isolated by sheep erythrocyte-rosetting from the CD34-depleted fraction of the same marrow sample, and cryopreserved at -85°C in 90% human AB serum + 10% dimethylsulfoxide until use. After 10 days in culture, when DCs were observed, the cells were collected from the IL-2+SCF cultures and depleted of any mature cells using superparamagnetic bead separation. This was done by incubating the cells with a cocktail of purified mouse antibodies recognizing human CD3, CD16, CD19 and CD14, followed by incubation with paramagnetic bead-conjugated anti-mouse antibodies (Miltenyi Biotec, Inc.) and passage through a magnetic field. The cells in the effluent were returned to culture in serum-free medium and pulsed for 1 hr with 1 mM of a synthetic nonapeptide of HER-2/neu, E75 (HER-2, 369-

377:KIFGSLAFL), shown in previous experiments to bind with high affinity to HLA-A2 molecules [22]. The autologous T cells, which had been thawed and cultured overnight in complete RPMI medium without peptide, were then added in serum-supplemented medium containing IL-2 (50 U/ml final concentration), and the cultures continued for an additional 3 weeks with weekly restimulation and IL-2 supplementation.

## RESULTS

The experiments described below were designed to characterize the populations expanded from CD34<sup>+</sup> progenitors in 7-10 day IL-2+SCF culture. We found that the cells in these cultures bound to K-562 cells and phenotypically resembled DC. The results of these experiments and subsequent studies to further characterize the DC-like cells generated in IL-2+SCF marrow cultures are described below.

### *Phenotype of cells in short-term cultures established from bone marrow CD34<sup>+</sup> progenitors*

When CD34<sup>+</sup> bone marrow progenitors were cultured in the presence of IL-2 and SCF for 7 to 10 days, a 5- to 10-fold expansion of cells was observed as determined by direct enumeration of cells using a trypan blue dye exclusion technique. Flow cytometric analysis

of the cells in these cultures indicated that only negligible numbers (<2%) displayed a phenotype consistent with that of mature T cells (CD3<sup>+</sup>), B cells (CD19<sup>+</sup>), monocyte/macrophages (CD14<sup>+</sup>), granulocytes (CD16<sup>+</sup>), or NK cells (CD56<sup>+</sup>, 16<sup>+</sup>). Most cells lacked CD2, CD5, CD7, CD8, or CD25 (IL-2R $\alpha$ ) cell surface determinants (Fig. 1). Instead, the majority of cells expressed CD33 suggestive of differentiation along the myeloid pathway [28]. An average of 58%, 50% and 22% of cells displayed CD4, HLA-DR and HLA-DQ, respectively, while a small subset expressed CD80 (B7) or CD1a; CD122 (IL-2R $\beta$ ) was detected on 20% of cells.

#### *Tumor-binding capacity of cells from IL-2+SCF cultures*

When irradiated FITC-labelled K-562 target cells were mixed in 1:1 ratio with PE-anti-CD38 labelled cells from 7-10 day cultures and analyzed by 2-color flow cytometry, we observed that 12-16% of cells formed conjugates with the tumor (Fig. 2 and Table 1); these cells are designated as tumor-binding cells (TBC). CD38 expression was chosen for these studies, since virtually all of the bone marrow cells from these cultures expressed this surface marker (Fig. 2). It was necessary to differentially label K-562 and TBC, since some overlap in the forward- and side-light-scatter characteristics of these cells was observed (data not shown). Table 1 indicates that a similar proportion of TBC was observed in



cultures established from CD34<sup>+</sup> progenitors of normal donors and breast cancer patients, as well as from fresh and cryopreserved marrow samples.

Next, we sorted the dual-staining conjugates by FACS and returned them to culture for subsequent analysis of their morphology and phenotype. When examined 20 hr later by light microscopy, many of the sorted TBC were found to have a morphology akin to DCs, i.e. displaying numerous and long dendritic processes (Fig. 3). Time-lapse videomicroscopic analysis of these cells showed them to be highly active and motile (data not shown). As depicted in Fig. 3, the DCs were usually seen beneath clusters of K-562 cells. Closer examination of unseparated cultures revealed that the DC-like cells were often not readily visible because of their tendency to reside beneath clusters of round cells (Fig. 4). However, they were more easily seen if the cultures were very gently resuspended. Although the dendritic-like cells were observed to be in contact with the plastic surface of the culture vessel, they were only loosely adherent and could be detached by routine pipetting. Similar, but fewer, dendritic-like cells were detected in cultures supplemented with IL-2 or SCF alone (data not shown).

#### *Phenotype of Tumor-Binding Dendritic Cells.*

We next examined the phenotype of the cells binding to K-562. Fig. 5 shows the results of an analysis of TBC from cultures established from marrow of a normal donor and

a breast cancer patient. Virtually all of the TBC from either donor expressed HLA-DR and 20-40% displayed HLA-DQ. The majority of TBC from the normal donor were CD4<sup>+</sup>, while approximately half of the TBC from the breast cancer patient were CD1a<sup>+</sup>. A small proportion of TBC from both donors expressed CD80. Further studies will be required to determine if the differences in the phenotypic profile of TBC is disease-related.

*Immunization of Autologous Lymphocytes to HER-2/neu Peptides Using Marrow-Derived Dendritic Cells.*

We next tested whether the DCs generated in IL-2+SCF cultures would be effective APC in immunization of autologous lymphocytes against TAA *in vitro*. DCs were generated from CD34<sup>+</sup> marrow progenitors of an HLA-A2<sup>+</sup> breast cancer patient and pulsed with a synthetic HER-2/*neu* nanopeptide (E75) shown previously to bind to the HLA-A2 antigen and to be recognized by ovarian tumor-reactive CTL [22]. These APC were then incubated together with autologous T lymphocytes that had been collected from the marrow during CD34 purification and cryopreserved. The lymphocytes were expanded in culture with 50 U/ml of IL-2 and weekly restimulation with E75-pulsed APC, and then tested for their ability to lyse peptide-pulsed T2 target cells in a <sup>51</sup>Cr release assay [22]. Fig. 6 shows that the immunized T cells lysed T2 targets which had been pulsed with E75 peptide, but not those pulsed with medium alone or a different peptide (E90; HER-2/*neu*

789-797). Phenotypic analysis of the lymphocytes showed that they were >95% CD3<sup>+</sup>,CD8<sup>+</sup> T cells and <1% CD56<sup>+</sup>,3- NK cells, suggesting that the APC from IL-2+SCF cultures were effective in immunizing CD8<sup>+</sup> T lymphocytes against MHC Class I binding HER-2/*neu* peptide.

## DISCUSSION

We have shown that cells with DC morphology (stellate appearance with multiple long cytoplasmic processes) and phenotype (CD4<sup>+</sup>, HLA-DR<sup>+</sup>, HLA-DQ<sup>+</sup>, CD1a<sup>+</sup>, and CD80<sup>+</sup>) are generated from CD34<sup>+</sup> bone marrow progenitors in short-term cultures supplemented with IL-2 and SCF. Cells with similar phenotype were also generated in cultures supplemented with IL-2 alone or SCF alone, but these conditions did not promote as high a degree of proliferation as supported by the combination of cytokines. While SCF has been shown to enhance the effect of GM-CSF on DC growth and function [20], ours is the first observation that IL-2 may contribute to DC-genesis from bone marrow progenitors. Although the cultures established in our studies were not deliberately supplemented with GM-CSF, it is possible that this cytokine was produced endogenously - a question currently being addressed. However, the presence of DCs in GM-CSF knockout mice suggests that GM-CSF is contributory, but not essential, for DC development [29].

In studies of hematopoiesis, IL-2 has been shown to promote the expansion of both myeloid- and lymphoid-committed cells [23-27]. SCF generally has little differentiative

activity, but acts synergistically with most colony stimulating factors to increase progenitor frequency. As DC and monocytes/macrophages appear to arise from a common precursor [18] and a large number of cells in early IL-2+SCF cultures expressed the CD33 determinant associated with myeloid-commitment [28], it is possible that these cytokines promote expansion of the common DC/monocyte precursor. While a small percentage of monocytes have been shown to express CD4 [30], the presence of this determinant on a large subset of the cultured cells which lack CD14, could indicate either that other stimuli are required for further differentiation of monocytes or that the cells generated in IL-2+SCF cultures have been directed along the DC-differentiation pathway.

We and others have reported previously that long-term (4-5 week) IL-2-supplemented cultures established from human CD34<sup>+</sup> bone marrow progenitors support the generation of CD56<sup>+</sup>,3<sup>-</sup> NK cells [23-26]; subsequently it was shown that SCF increases the frequency of NK progenitors within these IL-2-dependent cultures [27; our unpublished observations]. These data suggest that IL-2+SCF may support progenitor populations committed to different lineages. However, it cannot be excluded that NK and DC, which share a primary role as sentinel cells, may be derived from a common precursor and/or that the earlier appearing DCs may contribute to NK cell differentiation. These questions may be answered by further analysis of the differentiation pathways of phenotypically defined cell subsets within early IL-2+SCF cultures.

The tumor-binding capacity of DCs is not surprising considering the high density of adhesion molecules displayed by these cells [12]. This property, along with the ability of

DCs to cluster with lymphocytes, could provide a means of enhancing the antitumor response, i.e. by facilitating the interaction between tumor and oncolytic lymphocytes. However, tumor-binding by DCs could be a two-edged sword as far as metastatic tumor cells are concerned: while capture (or transport) of micrometastasis by DCs to the lymphocyte-laden lymph node might be advantageous, capture within the marrow might contribute to the formation of metastatic lesions.

Previously, it was found that CD4<sup>+</sup> T lymphocytes from some breast cancer patients could be sensitized to a HER-2/*neu* peptide presented by CD34 progenitor-derived DCs [19]. Our results show the first time, that such DCs from a breast cancer patient can also induce CD8<sup>+</sup> cytolytic T lymphocytes recognizing an immunodominant nonapeptide product of the HER-2/*neu* proto-oncogene [22]. In mice, antigen-specific cytolytic CD8<sup>+</sup> T cells have also been induced by marrow-derived DCs pulsed with an MHC Class I-restricted peptide of ovalbumin [31]. Together, these data support our hypothesis that DCs are effective for *in vitro* immunization of both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes.

Our studies are also unique in that we used T lymphocytes derived from the bone marrow as responders. While it is possible that some of these cells may be derived from peripheral blood contaminating the marrow sample, it is also possible, that the marrow may provide a source of naive T cells more amenable to immunization. Future studies will examine this possibility.

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## FIGURE LEGENDS

**FIGURE 1.** Phenotype of cells in 7-10 day IL-2+SCF cultures. CD34<sup>+</sup> bone marrow progenitors from normal donors and breast cancer patients were cultured for 7-10 days with  $10^3$  U/ml IL-2 and 2-5 U/ml SCF; the phenotype of cells in culture was analyzed by flow cytometry. Bars represent the mean  $\pm$ S.E. of 4 donors. Since no differences between normal individuals and cancer patients was observed, the data were pooled.

**FIGURE 2.** Detection of tumor-binding cells by flow cytometry. Cells from 7-10 day IL-2+SCF cultures established from CD34<sup>+</sup> progenitors were labeled with PE-anti-CD38 and mixed together with FITC-labeled K-562 in 1:1 ratio. The cells were analyzed immediately (0 min) and after 10 min. incubation at 37°C. Quadrant 1: nonbinding CD38<sup>+</sup> cells; Quadrant 2: tumor-binding CD38<sup>+</sup> cells; Quadrant 3: nonbinding CD38<sup>+</sup> cells; Quadrant 4: unbound K-562..

**FIGURE 3.** Morphology of tumor-binding cells in IL-2+SCF cultures. Dual-labeled tumor-binding cells (detected as described in Fig. 2) from a 7 day IL-2+SCF culture were sorted, returned to culture, and analyzed microscopically 20 hrs later. The digital image shows a DC with long processes binding to K-562 tumor cells. Magnification 200x.

**FIGURE 4.** Generation of dendritic cells from CD34<sup>+</sup> progenitors. CD34 progenitors were isolated from fresh bone marrow of a normal donor and cryopreserved (3 yr) marrow from a breast cancer patient (31), and cultured as described in the legend of Fig. 1. The DCs are identified by their long processes. The differences in concentration of DCs in these images is due to dilution rather than disease-related factors.

**FIGURE 5.** Phenotype of TBC from IL-2+SCF cultures. Cells from 7-10 day cultures established from CD34<sup>+</sup> progenitors were labeled with PE-conjugated antibodies and allowed to bind to FITC-labeled K-562. The bars represent the percent of total TBC (i.e. %CD38<sup>+</sup> TBC) expressing the particular surface determinant.

**FIGURE 6.** Immunization of T cells against HER-2/*neu* peptide. *Left panel:* Immunization scheme. DCs were generated from CD34 marrow progenitors of a breast cancer patient (HLA-A2<sup>+</sup>) and pulsed with a nonapeptide of HER-2/*neu* binding with high affinity to HLA-A2 [22]. Autologous T cells and IL-2 were then added and the cultures continued for 3 more weeks with weekly restimulation. *Right panel:* The lymphocytes were then tested for lytic activity against T2 cells pulsed with the same HER-2/*neu* peptide used for immunization (E75), an unrelated peptide (E90), or no peptide.

**Table 1.**

*Tumor-binding cells in IL-2+SCF cultures*

Expt. <sup>1</sup>	Donor	%TBC
1	NORMAL	13.5
2	NORMAL <sup>2</sup>	14.5
3	PATIENT	16.1
4	PATIENT <sup>2</sup>	13.7
5	PATIENT <sup>2</sup>	12.1

<sup>1</sup>CD34+ progenitors of normal donors and breast cancer patients were cultured in LTBM for 7-10 days, and the percentage of cells binding (TBC) to K-562 determined by flow cytometry.

<sup>2</sup>Cryopreserved/thawed marrow.

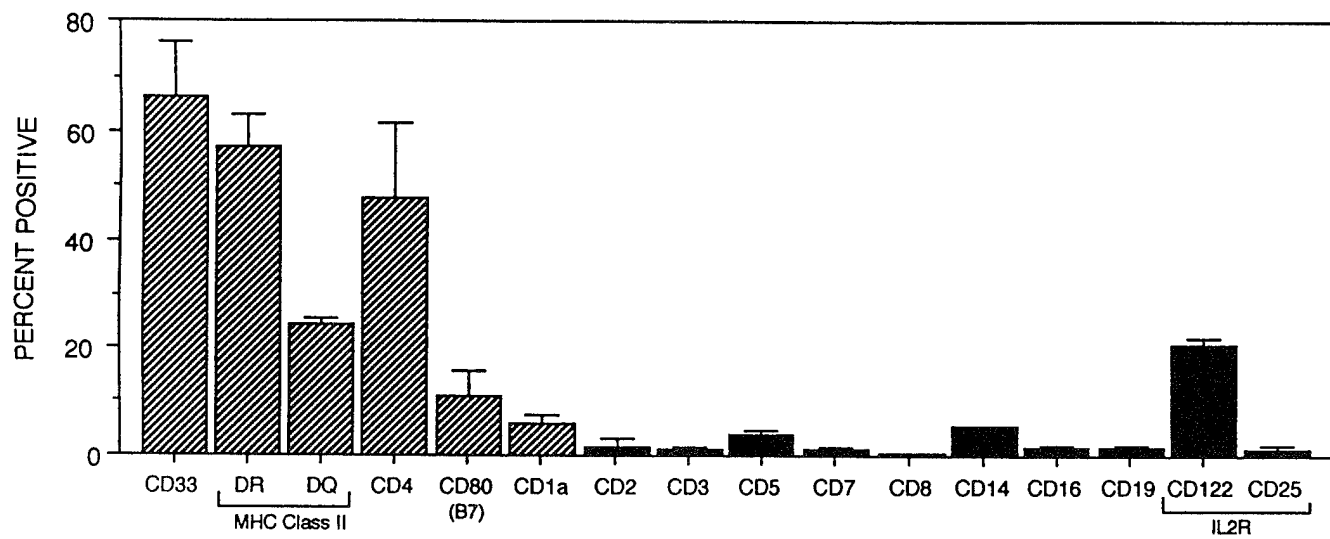


Figure 1

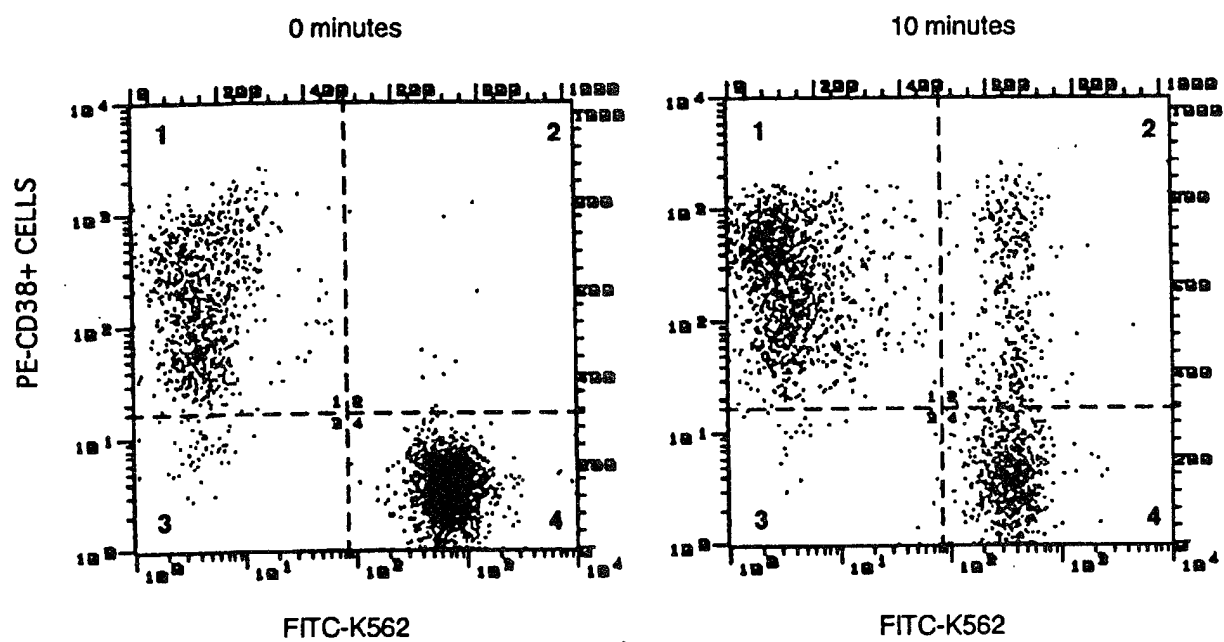


Figure 2



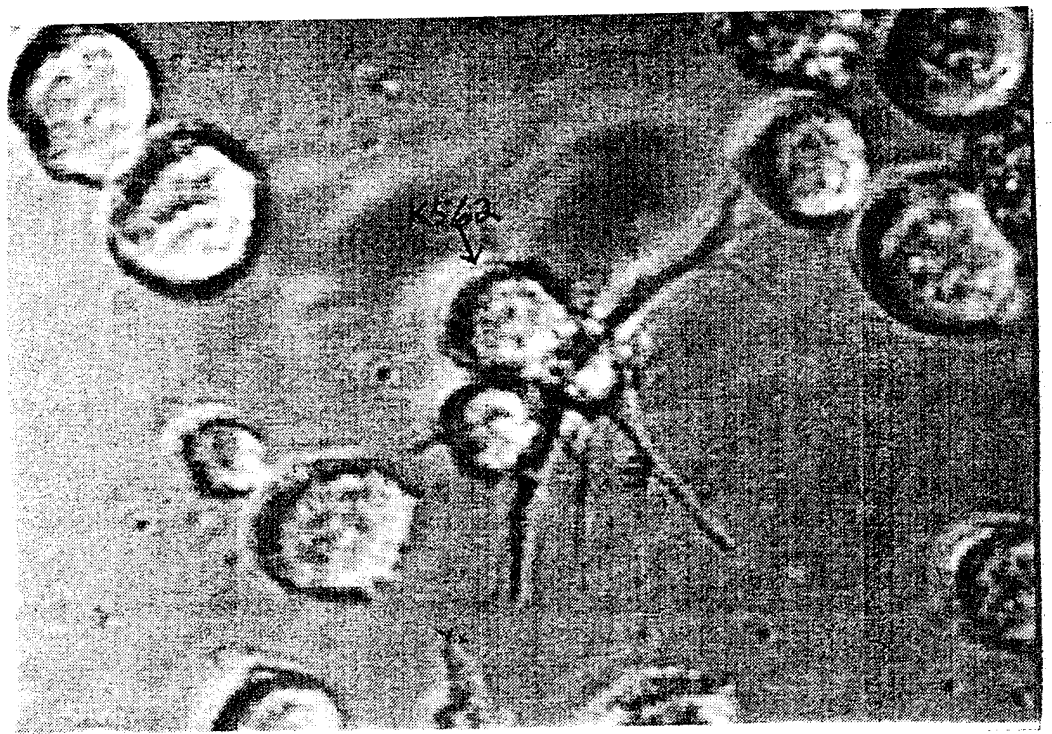


Figure 3

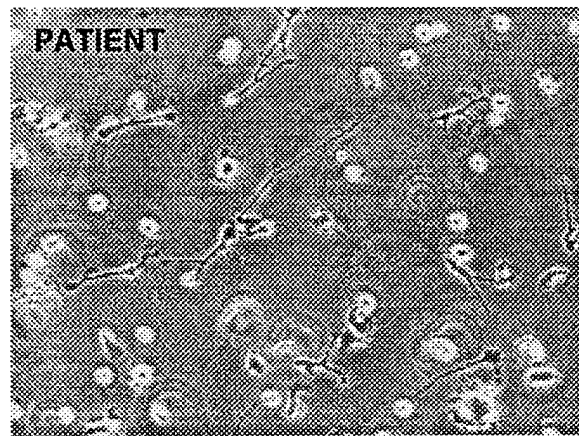
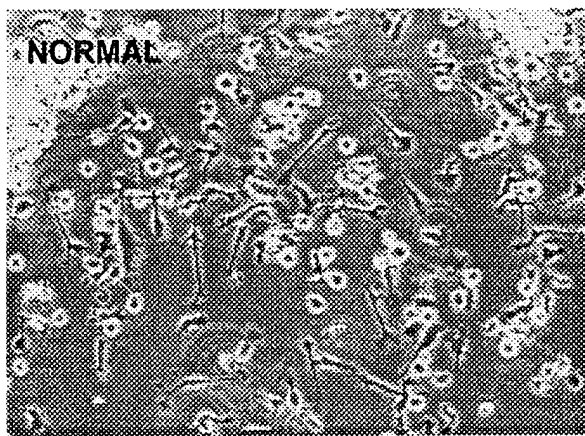


Figure 4

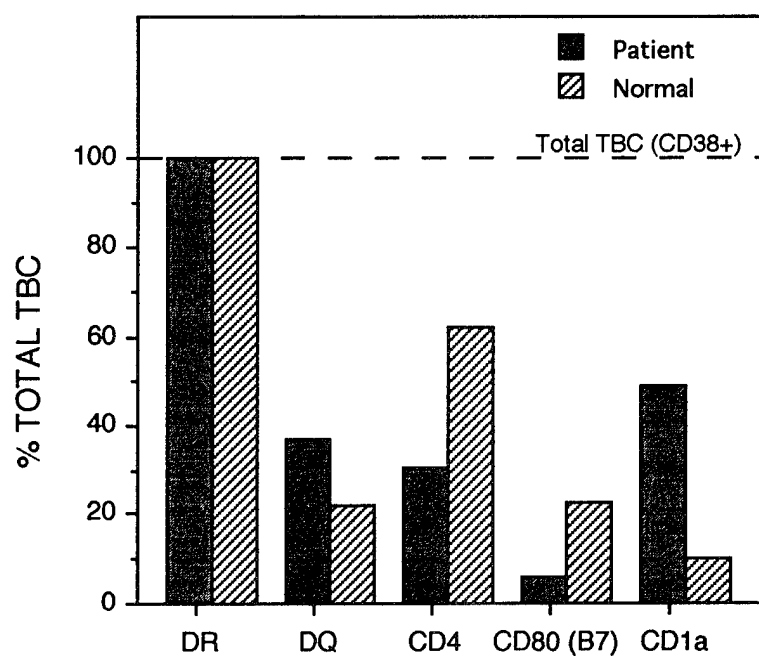


Figure 5

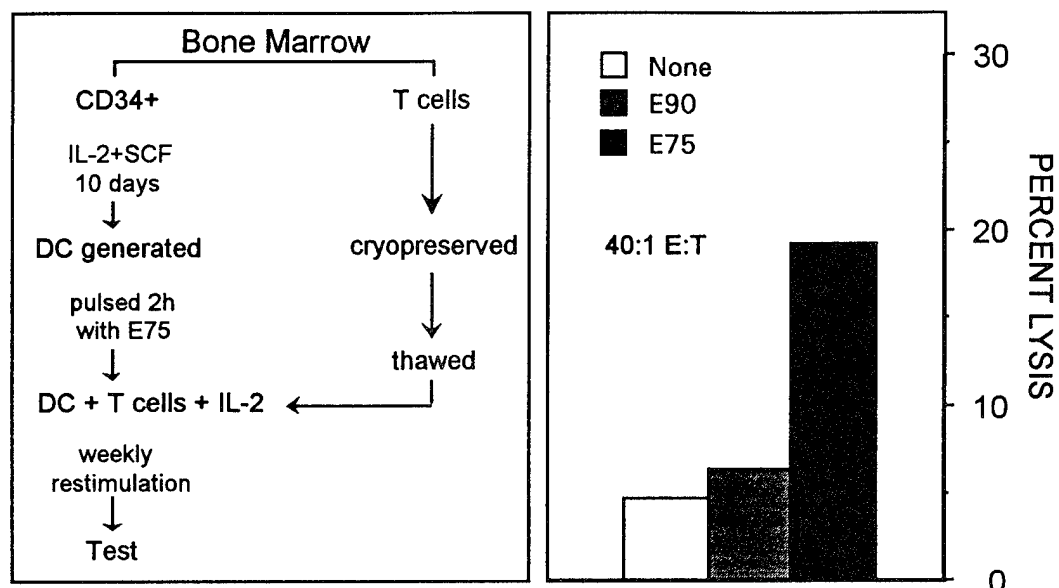


Figure 6

## Chapter 22

# TUMOR IMMUNITY

Constantin G. Ioannides and Elizabeth A. Grimm

In vitro cellular immune responses to tumors have been demonstrated by activated macrophages, natural killer (NK) cells, lymphokine-activated killer cells (LAK), and T cells. Manipulations of the human immune system in cancer clinical trials have been attempted by the use of anti-idiotypic antibodies, NK/LAK cells, tumor vaccines, adjuvant immunotherapy, and adoptive immunotherapy with tumor infiltrative lymphocytes (TIL) comprising mainly T cells, and has shown that a certain level of clinical responses can be sustained by the use of these immunological agents<sup>1-15</sup> (Chapters 123 and 124). No causal relationship has yet been demonstrated between either the nature of effectors and treatment outcome, or the association of the levels of clinical responses to tumors with particular effectors; also, no quantitative (dose-response) correlations can yet be drawn. The fact that such responses exist provides a rationale for investigation of the mechanisms that regulate the immunity to human tumors.

An underlying assumption is that responsive patients have some level of preexisting tumor immunity, defined as tumor-specific antibodies or cytotoxic T lymphocytes, however, the same responses have been found in patients with progressive cancers.<sup>16-18</sup> Thus, this preexisting immunity is unable to control tumor progression. Treatments with bacteria (the Coley toxin, bacillus (BCG), Calmette-Guérin (*C. parvum*) cytokines (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-2 (IL-2), and more recently granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-12 (IL-12), etc.), and nonspecific immune modulators, in general, have shown tumor regression (although in most cases incomplete), suggesting that either they can amplify a preexisting immune response, to as yet unknown antigens expressed on tumors, or induce a potent antigen nonspecific one, or both. The central questions of tumor immunity are: (1) What are the target antigens of an antitumor response, (2) why are these responses inefficient in controlling tumor growth, (3) how can these responses be targeted and amplified to be effective and, (4) if these responses are targeted to self-antigens, do they reflect an an-

titumor response (because of overexpression/posttranslational modification of the target antigen) or a response to tissue injury by tumor expansion. If the latter is true, a physiologic response to tumor would be expected to "suppress" the local inflammatory and consequently the antitumor response.

This chapter focuses first on the specific immunity to human tumors illustrated by T cell responses to autologous human tumor cells. It discusses the relevance of what is known for understanding the mechanisms of human tumor recognition by T cells, and the control mechanisms that may regulate this recognition. Second, it addresses the nonspecific arm of tumor immunity, describing the antitumor functions of NK, LAK, and macrophages.

### T CELLS IN TUMOR SURVEILLANCE

Of the fundamental questions on the function of the immune system for surveillance against emergence of tumors and control of tumor progression and metastasis, the question of how T cells recognize autologous human tumors is farthest from an answer. The concept of tumor surveillance by T cells implies that circulating T cells are able to detect antigenic (Ag) changes on the surface of malignant cells. This recognition leads to elimination of the tumor cells and generation of memory T cells capable of recognizing the same antigen (Ag) at reencounter.

It is considered a favorable prognostic indicator when solid tumors are infiltrated by T cells,<sup>18-20</sup> since this is suggestive of the ability of the patient's immune system to develop a local antitumor response. However, freshly isolated lymphocytes from infiltrations of solid tumors (tumor infiltrative leukocytes) (TIL) show poor cytolytic function against all targets, whereas the same cells after in vitro culture in the initial presence of autologous tumor and IL-2, mediate tumor lysis.<sup>21-23</sup> Moreover, cultured TIL when adaptively transferred to cancer patients were reported to induce partial or complete clinical responses.<sup>11,14</sup> Studies initiated in the last 5 years to address hypotheses explaining this apparent paradox have contributed a large body of in-

**KEY CONCEPTS****Tumor Immunity**

1. T cell immunity to tumors
  - a. Tumors are infiltrated by T cells known as TILS
  - b. TILS exhibit both antigen-specific and antigen-non-specific reactivity to tumor cells
  - c. Failure of TILS to eliminate tumors due to suppression of T cell activity and induction of T cell anergy
2. Cell surface "tumor" antigens on human tumors
  - a. Differentiation antigens
  - b. Oncofetal antigens
  - c. Activation antigens
3. Nonspecific mechanisms of tumor immunity
  - a. Natural killer cells
  - b. Lymphokine-activated killing activity
  - c. Macrophages
  - d. IL-2 and other cytokines

formation which improves our understanding of the way T cells interact with autologous tumors. We have analyzed this information in the context of the basic mechanisms that govern T cell responses to Ag, whose specificity and functionality can be defined in terms of Ag recognition, lymphokine secretion, and T cell receptor usage.

The immune system is controlled by the regulation of differentiation and expansion of T and B lymphocyte clones, each bearing unique clonally distributed receptors, T cell antigen receptors (TCR), and membrane immunoglobulins (Igs), respectively. It is still unclear whether antigens recognized by T and B cells on human tumors are proteins expressed *de novo* on malignant cells and absent from their normal counterparts, or normally occurring overexpressed cellular proteins which, by virtue of increased intracellular concentration, may provide more self-products (peptides) than normal cells or provide mutated self-products to be presented to T cells. T and B lymphocytes can potentially develop a response to any foreign molecule and can distinguish changes in the primary structure at the level of one nonconservative amino acid substitution. Therefore, they are able to distinguish mutated self-products.

The concept of self-tolerance implies that autoreactive clones are silenced or eliminated before they acquire functional competence.<sup>24,25</sup> If we assume that human cancers are only another expression of the self, then it would be expected that no specific T and B cell response at all will be mounted against malignant cells. If, on the other hand, we assume that the malignant transformation, as a consequence of either DNA mutations or activation of normally expressed genes, results in the expression on tumor cells of novel cellular proteins, theoretically T cell responses detecting structural changes and increased levels of immunogens will be expected. This will be true unless: (1) tumor cells are defective in Ag expression, due to either decreased expression of major histocompatibility complex (MHC) molecules or defective tumor Ag presentation (transporter systems); (2) suppressor factors or cytokines for differentiation and functional maturation of T cells are released by the tumor or host tissue; (3) immune suppressor cells or circuits block

an immune response by T cells against the tumor; or (4) lymphokines essential for functional maturation of tumor reactive T cells and B cells and antibody secretion are not released in the tumor environment.

Another factor that needs to be considered is whether T cells bearing receptors for self-proteins are eliminated after responding to a particular tumor Ag. It has recently been demonstrated that clonal deletion appears not to be restricted to immature T cells and can be the endpoint of a powerful immune response, e.g., to superantigens.<sup>26</sup> Selective elimination of V $\beta$ 2<sup>+</sup> cells has been reported in mice bearing preneoplastic lesions.<sup>27</sup> In contrast with experimental tumors induced by, e.g., mutagens that express immunologically definable unique tumor Ags, most serologically defined human tumor Ags are structurally similar to normal tissue proteins. The fact that certain of these proteins may be expressed at fetal stages strengthens the hypothesis that recognition of such antigens during development can lead to elimination of self-reactive clones and therefore to the lack of observed responses to tumor cells. However, from studies in experimental animals it is clear that immunization with self-antigens can unmask the presence of autoreactive T cells at a clonal level and induce autoimmune disease.<sup>28-30</sup>

In cancer patients, humoral and cellular responses to a number of differentiation antigens are not entirely suppressed by tolerance. For example, glycoprotein (gp75) is a differentiation antigen since it is specific for cells of the melanocyte lineage.<sup>16</sup> gp75 was recognized by antibodies in the serum of a melanoma patient,<sup>31</sup> while epitopes in tyrosinase (a member of the gp75 family) were found to be targets of CTL in melanoma patients.<sup>32</sup> The extent to which *in vitro* stimulation (IVS) with tumor of the TIL, demonstrated to be a critical step in generation of *in vitro* functional tumor reactive T cells, reflects induction of autoreactive tissue-specific T cells, needs to be studied in the future. Since tumors may express cellular proteins not found in the thymus, it is now clear that more complex mechanisms regulate the apparent unresponsiveness of T and B cells to tumors.

**THE CONCEPT OF TUMOR REACTIVE T CELLS**

In classic studies, freshly isolated T lymphocytes from human tumors, in most instances, show minimal cytolytic function against autologous and allogeneic targets and poor proliferative responses to Ag or mitogens compared with peripheral blood mononuclear cells (PBMC) from the same donor.<sup>19,21-23</sup> Since the proliferative responses to mitogens and antigens to cancer patients' PBMC are in general lower than those of healthy individuals, this raised the question of whether lymphocytes infiltrating tumors (TIL) or associated with tumors (TAL) in solid cancers and malignant effusions are functional.

Studies by Rosenberg and collaborators, Whiteside, Herberman and collaborators, and other groups have shown that tumor-reactive T cells isolated from TIL can be expanded *in vitro* in large numbers, and for long time intervals. Results from studies with TIL/TAL and PBMC from patients with tumor vaccines became the main source of information on the nature and specificity of T cells reacting with autol-

ogenous human tumors. Much of the information analyzed here was obtained from these systems. When TIL from different sources are cultured in the presence of autologous tumor and low (5 to 20 U/ml) or moderate (50 to 100 U/ml) amounts of recombinant IL-2, they expand, often as CD3<sup>+</sup> T cell lines of either CD4<sup>+</sup> CD8<sup>-</sup> or CD4<sup>-</sup> CD8<sup>+</sup> phenotype.<sup>33-36</sup> In many instances these cells have shown autologous tumor cytotoxicity, proliferation, and specific lymphokine production. On the other hand, TIL derived from tumors and cultured in high concentrations of IL-2 (1000 U/ml) can in some cases (e.g., TIL from ovarian carcinoma) lead to expansion of effectors with NK-like characteristics<sup>37</sup> and in other cases (TIL from melanoma) lead to expansion of autologous tumor-specific T cells.<sup>33</sup> To some extent this variability may reflect differences in the amount of IL-2 used in the cultures since IL-2 concentrations expressed in units may, in reality, reflect very different concentrations of IL-2.<sup>34-36</sup> Furthermore, regardless of the apparent poor proliferative responses initially observed when T cells isolated from tumor infiltrations are cultured in the presence of lymphokines, higher frequencies of tumor-reactive T cells have been found in cloned tumor-infiltrating lymphocytes (TIL) than in peripheral blood, suggesting the presence of a local Ag priming event.<sup>34,36</sup> The implications of these observations are twofold: (1) either human tumors express molecules (tumor antigen) not present in the normal tissues capable of Ag priming T cells; or (2) clonal abortion of self-reactive clones is incomplete. The ease with which it is now possible to recover reactive T cells from human tumors suggests that, at the least, clonal abortion of cells expressing self-antigens or autologous tumors is incomplete. Therefore, other mechanisms may be present that abolish the capacity of tumor-reactive T cells to eliminate human tumors through recognition of novel tumor Ags, or via potential autoreactive responses to normal self-antigens, or both.

Other important questions that remain to be answered are whether such activated lymphocytes that appear to possess receptors for human tumor Ag or self-antigens exit the tumor environment and circulate to other tissues. Such circulation could lead to autoimmune reactions in the recipient tissues and there would have to be mechanisms to control (suppress) such reactions. Animal studies have shown that in experimental tumor models adoptively transferred antigen-specific T cells as well as TIL, can proliferate *in vivo* and persist long term as memory T cells.<sup>38</sup> Although survival of neo<sup>+</sup> marked human TIL was found to be comparable with that of murine TIL, there are major differences between murine and human TIL (e.g., HLA-DR expression, production of interleukins) and possibly differences in the nature of murine and human tumor Ag.<sup>39</sup> Thus, the persistence of TIL in animals cannot be presumed for humans.

#### TARGET SPECIFICITY OF AUTOLOGOUS TUMOR-REACTIVE T CELLS

It is very well known that T cells recognize antigens only when presented on the surface of other cells (target cells, antigen presenting cells (APCs), as short peptides bound to MHC molecules (Chapter 7). Due to the high MHC polymorphism, individual T cells recognize the peptide Ag in

the context of a particular allelic product, a phenomenon that is called MHC restriction.<sup>40</sup> With the notable exception of mucin-reactive T cells,<sup>41-43</sup> most T cells apparently follow this rule. The key characteristics of a T cell response to Ag are specificity and memory, i.e., the particular TCR of a T cell clone recognizes an unique epitope, and reencounter of the same but not of a different epitope triggers responses that are defined as proliferation, lymphokine secretion, and, in the case of cytotoxic T lymphocytes (CTL), lysis of the targets bearing this epitope.<sup>44</sup>

In describing the nature of T cell response to human tumors, a large body of evidence indicates that T cells isolated mainly from TIL after culture *in vitro* with IL-2 can express "specific" lysis, proliferation, and lymphokine secretion upon encountering autologous tumor cells.<sup>36</sup> With respect to the "specificity" of the response, this is generally defined as a higher level of lysis or proliferation in response to autologous than to allogeneic tumor cells, and significantly lower level of lysis of NK-sensitive targets (K562 cells) and LAK-sensitive targets (Daudi cells).<sup>36</sup> However, it was recently demonstrated that cloned T cells specific for autologous tumors can recognize a number of allogeneic tumors and lyse K562 cells employing probably distinct mechanisms to accomplish each function.<sup>45</sup> Since allogeneic tumors differ not only in putative tumor Ag expression, but also in the MHC class I and class II phenotype, the response is considered to be MHC class I restricted if it is also inhibited by anti-MHC class I monoclonal antibody (mAb). These features are necessary conditions in defining specificity, however, they are not sufficient because in most instances the normal counterparts of the tumor targets are not available. Therefore, it is unclear whether this reflects specific tumor Ag recognition or autoreactivity (recognition of a self-allelic form of a particular MHC molecule plus a self-peptide).

Recognition of human tumor Ag by T cells is inferred from studies that used cloned T cells to address the recognition of cloned tumor targets. Recognition of some but not of all autologous tumor clones with the same human leukocyte antigen (HLA)-phenotype indicates that Ags expressed on these tumor clones are different.<sup>46</sup> However, formal proof from parallel studies with normal tissue cells and isolation of tumor peptides is needed to address this question. The ability of CD8<sup>+</sup> cytotoxic T lymphocyte (CTL)-TIL to lyse allogeneic tumors or tumors with different histology to a similar extent as autologous tumors has been initially interpreted as an expression of "nonspecific" lytic function. However, since many of the initial studies on TILs' target specificity were performed with targets that were not HLA-typed, the results of these studies should be interpreted with caution because they may reflect recognition of a shared Ag. It was initially believed that the putative tumor Ags are individual restricted. Recently, it became apparent from studies with tumor targets sharing MHC class I molecules that there is also MHC-restricted recognition of allogeneic tumors sharing HLA-A2, -A1, etc. with the effectors. These findings are suggestive of a novel class of T cell recognized Ag on tumor cells: common (shared) tumor Ag.<sup>47-49</sup> Their presence is inferred from evidence that the same effectors that lyse a group of

allogeneic tumors in an MHC restricted, and usually HLA-A2 associated, fashion fails to lyse other targets that share the presenting element. The fact that the resistant targets are sensitive to lysis by autologous CTL-TIL serves as evidence for the hypothesis that both unique and shared Ag are expressed on tumor cells.

The specificity of tumor-reactive T cells as described previously has been defined with CD8<sup>+</sup> cells. The specificity of recognition of tumor-reactive CD4<sup>+</sup> T cells it is still unclear. Since both MHC class I and class II epitopes can be presented by the same APC, presence of epitopes of both CD8<sup>+</sup> and CD4<sup>+</sup> cells on the same protein is expected to produce more efficient interactions between CD8<sup>+</sup> and CD4<sup>+</sup> cells. Cultured CD4<sup>+</sup> TIL were reported in many instances to mediate target and APC lysis,<sup>50</sup> and tumor lysis,<sup>51</sup> although it is still unclear whether acquisition of this function is a consequence of prolonged culture of CD4<sup>+</sup> cells with IL-2.<sup>52</sup> The tumor lytic function of CD4<sup>+</sup> TIL has been reported to be mainly non-MHC restricted. However, in certain instances T cell clones of CD3<sup>+</sup> CD4<sup>+</sup> CD8<sup>-</sup> phenotype express autologous tumor-restricted lytic function that is MHC class I restricted.<sup>53</sup> These findings can be understood in the light of the recent demonstration that murine CD4<sup>+</sup> clones have the ability to recognize MHC class I determinants in the context for MHC class I or class II determinants.<sup>54</sup>

Another criterion used to define functional specificity of tumor-reactive T cells is their ability to release lymphokines upon encounter with autologous tumor. It was initially believed that this role is reserved for tumor-specific or perhaps autoreactive CD4<sup>+</sup> cells. Lymphokines released by CD4<sup>+</sup> cells, IL-2, TNF- $\alpha$ , IFN- $\gamma$ , and even IL-4, may be essential for functional activation of CD8<sup>+</sup> cytolytic effector T cells. It remains unclear whether this function reflects tumor Ag recognition or autoreactivity with tumors or activated CD8<sup>+</sup> cells (which in humans express MHC class II) since MHC class II-restricted CD4<sup>+</sup> TIL cannot recognize tumor Ag on MHC class II negative tumors (e.g., ovarian tumor cells), but probably they can still recognize tumor Ag presented by infiltrating macrophages. The fact that human CD8<sup>+</sup> T cells secrete lymphokines, although at somewhat lower levels than CD4<sup>+</sup> cells,<sup>55,56</sup> and that CD8<sup>+</sup> TIL can specifically secrete lymphokines in response to autologous tumors, may serve as an indicator of functionality.<sup>57</sup> This possibility has received increased attention recently with the demonstration in experimental tumor models that the therapeutic potential of T cells of murine TIL correlates better with specific release of TNF- $\alpha$  and IFN- $\gamma$ , than with tumor lytic function *in vitro*.<sup>58</sup> The cellular basis for this observation is still unclear. It should be noted, however, that recently murine studies have demonstrated a functional dissociation between target lysis and specific lymphokine production. This phenomenon, defined *in vitro* as "split anergy," defines CD8<sup>+</sup> cells that maintain lytic function but fail to release lymphokines upon specific stimulation.<sup>59</sup>

#### ACCESSORY SIGNALS IN TUMOR RECOGNITION AS PARAMETERS OF T CELLS FUNCTIONALITY

Expression of Ag on tumor or APC provides a ligand for the TCR. Following Ag recognition, additional signals

are required to trigger an effector T cell function. In addition to accessory cell surface molecules that through their participation enhance Ag recognition, inducible signals provide accessory function for differentiation to effector T cell function. Such signals involve the release of lymphokines, and/or interactions with T cell surface receptors such as that between CD28 or CTLA4 and B7.<sup>60-62</sup> Since the outcome of the interaction between T cell and Ag depends on the function of APC, this raises the question of whether the autologous tumor can provide adequate accessory signals for activation of CTL which, in fact, are expected to recognize Ag on its surface. Therefore, if Ag presentation by the tumor fails to provide the accessory signals needed by T cells, this may lead not only to an incomplete activation process but also to a state of unresponsiveness to other stimuli.<sup>63,64</sup> The fact that in many instances lymphocytes infiltrating human tumors are outnumbered by the tumor cells may provide an additional mechanism for tumor-reactive T cell inactivation. High concentrations of surface-bound tumor Ag and/or soluble tumor Ag (resulting from tumor necrosis) by T cells in the absence of APC may lead to Ag-specific T cell inactivation, in parallel with findings in other experimental systems.<sup>65</sup> Similarly, when anergic T cells are stimulated with low concentrations of Ag they fail to proliferate.<sup>64</sup> Studies by Schwartz and colleagues tentatively defined the state of anergy (unresponsiveness) of mature T cells as the inability of Ag-stimulated T cells to up-regulate IL-2R and to release IL-2 at restimulation with a particular Ag. Since the state of anergy can be reversed by using high concentrations of IL-2 in culture,<sup>64</sup> these observations show a striking similarity with the functional characteristics of the freshly isolated TIL described in earlier studies.<sup>19,37</sup>

The possibility that tumor-induced T cell tolerance is a form of T cell anergy has only recently begun to be investigated. Thus, it is still unclear whether any or all of the TIL recovered from solid tumors are in the anergic state. In addition, the reversibility of the anergic state supports the concept that a down-regulatory network of T cells and cytokines function to maintain the T cells' unresponsiveness over time. Of particular relevance for studies on tumor recognition is the fact that anergic T cells maintain a somewhat diminished ability to release TNF- $\alpha$ , GM-CSF, and IFN- $\gamma$ , which may be associated with antitumor effects, and release only very low levels of IL-2 at restimulation with high concentrations of Ag.<sup>64,66</sup>

Although definition of T cell anergy as a functional state depends on the assays used to evaluate functional competence, it is clear that the lytic or proliferative function of tumor-reactive T cells *in vitro* needs to be correlated with specific lymphokine secretion,<sup>67</sup> and even more, the levels of lymphokines secreted need to be quantitatively evaluated for differences between individual cytokines—e.g., IL-2 and TNF- $\alpha$ . Because of *in vitro* secretion of certain lymphokines, IL-2 and IFN- $\gamma$ , and IL-2 and GM-CSF, may not always reflect fully functional T cells. It has been shown in other systems of cloned human T cells that frequent exposure (restimulation) with Ag lead to tolerization of Ag-reactive clones which nevertheless retain the ability to secrete TNF- $\alpha$  and GM-CSF.<sup>66</sup> The potential induction of an anergic state may provide a mechanism for the anti-CD3-induced



activation<sup>67-69</sup> of proliferative and effector function of human TIL (either fresh isolated or cultured) that became refractory to IL-2-induced expansion. It should be noted that studies in other systems have shown that while anti-CD3 mAb can induce unresponsiveness on unprimed T cells, it does not affect previously primed T cells.<sup>70</sup>

Lymphokines and cytokines are, in general, short-lived substances and both in vivo and in vitro lymphokine consumption may lead to misleading conclusions on the ability of human TIL to secrete lymphokines. Examination of freshly isolated TIL for IL-2 and TNF- $\alpha$  messages (mRNA), may help distinguish between functional and anergic tumor-reactive T cells.<sup>71</sup>

In addition to the balance between Ag recognition and essential lymphokine production required for maturation of tumor-reactive T cells and up-regulation of a specific antitumor immune response in vivo, this system is under continuous influence of immunosuppressive factors. These factors are produced either by the tumors, by the tissue hosting the tumor, or by the lymphocytes themselves. Activation of suppressor circuits<sup>72</sup> is likely to occur in the case of immunogenic tumors that, being able to induce an antitumor response, may consequently activate the down-regulatory (suppressor) cells. Mukherji and collaborators have extensively analyzed the phenomenon of cell mediated suppression in regulating T cell responses to autologous tumors, and a comprehensive analysis of this cell function can be found elsewhere.<sup>73</sup>

Functional definition of the suppression may vary according to the condition of the experimental assay and particular tumor system. For example, TGF- $\beta$  by itself is well known as a potent inhibitor of lymphocyte proliferation and cytotoxicity.<sup>74</sup> However, in the presence of immobilized anti-CD3 mAb, TGF- $\beta$  exerts a costimulatory effect on T cell proliferation, which is independent of the IL-2 and IL-2R pathways.<sup>75</sup> Another example of the complexity of suppressor circuits in tumor recognition by T cells is illustrated by the reported effects of TNF- $\alpha$  in the ovarian systems. For example, addition of TNF- $\alpha$  to TIL cultured in IL-2 leads to preferential outgrowth of CD8<sup>+</sup> cells, more restricted target specificity, and up-regulation IL-2R of TIL.<sup>76</sup> These effects may be interpreted as reversal of TGF- $\beta$ -induced suppression. However, a number of ovarian tumors secrete TNF- $\alpha$ <sup>77</sup> which may function as a paracrine growth factor. Therefore in certain instances TNF- $\alpha$  may increase the tumor mass, leading eventually to Ag specific inactivation as discussed above.

There is a large body of literature on soluble mediators secreted by tumors,<sup>78</sup> several of which are known to induce impaired lymphocyte responses to mitogens and Ags. The inhibitory function of these factors is usually tested by evaluating the ability to tumor cell culture supernatants to inhibit lymphocyte activation. Identification of the function of a particular factor in the supernatants is demonstrated by neutralizing the suppressor activity with mAb specific for, e.g., TGF- $\beta$  or TGF- $\alpha$ .<sup>79</sup> There is, however, little knowledge of how these immunosuppressive influences actually influence lymphoid cell reactions to the tumor. Another confounding influence is that tumor progression and metastasis at distant sites is facilitated by organ-derived growth factors.<sup>78,80</sup> T lymphocytes synthesize mitogenic growth factors for vas-

cular cells and fibroblasts.<sup>81</sup> Such factors may in fact control the process of organ repair and may affect the proliferation of tumor cells. For example, TGF- $\alpha$  may function as a physiologic regulator of liver regeneration, and TGF- $\beta$  may control hepatocyte replication.<sup>82</sup> Such factors, and others, released by host organs following injury, are known to inhibit lymphocyte activation and function.

In summary, the evidence available suggests that active suppressive processes (mediated by soluble factors released by tissues, tumors, or lymphocytes themselves) lead to impaired immune function. These findings raise the question of whether it is possible to design therapeutic approaches leading to inhibition of secretion of suppressor factors, or to improve the responses to immunologic therapies.

### T CELL RECEPTOR USAGE BY TUMOR-REACTIVE T CELLS AS AN INDICATOR OF CLONALITY OF THE ANTITUMOR RESPONSE

It has been reported that preferential usage of specific TCR V $\beta$  products occurs in the T cells involved in pathologic processes.<sup>83,84</sup> TCR analysis of TIL from uveal melanoma has shown a predominant usage of the V $\alpha$  7 family.<sup>85</sup> Studies with the cultured murine and human TIL have shown preferential increase in cells bearing TCRs of certain V $\beta$  families. The significance of the selective TCR V $\beta$  usage in these cultures is still unclear; it may reflect differential T cell proliferation in the presence of IL-2 of: (1) T cells primed in vivo by tumor; (2) T cells stimulated in vitro by autologous tumor T cells; (3) preferential in vitro proliferation of T cells of certain V $\beta$  subsets and lack of proliferation of others that may be functionally suppressed. The fact that TILs only require exogenous/endogenous lymphokine (IL-2) for proliferation and for the observed TCR V $\beta$  selectivity is in opposition to the notion of selective/preferential expansion of certain TCR V $\beta$  families since, if all T cells are activated in vivo and in vitro, then all T cells are expected to proliferate at the same rate. This is also true if we hypothesize the opposite, i.e., if all lymphocytes infiltrating and interacting with the tumor are immunosuppressed or anergic, since in this case T cells expanding in vitro consist exclusively of lymphocytes from "passenger" blood contaminations. Thus, in certain of these cases skewed or preferential usage of certain TCR V $\beta$  will not be detected.

Since the T cell specificity for an MHC-tumor peptide complex is defined only by the TCR, a somewhat limited diversity of TCR V $\beta$  usage, together with preferential increase in particular TCR V $\beta$  families in TILs with different HLA-phenotypes, raises the question of whether a more restricted TCR V $\beta$  repertoire reflects recognition of a common or tumor Ag.

The restricted TCR V $\beta$  usage may have important practical applications for immunotherapy in that it may allow preselection of T cells by sorting with appropriate mAb with V $\beta$  specificity. Demonstration of in situ amplification of melanoma TIL expressing an unique TCR V $\beta$  gene segment supports the immunosurveillance concept in regressive melanoma.<sup>86,87</sup> It should be noted that recent models of TCR recognition suggest that V $\alpha$  and V $\beta$  domains recognize mainly MHC chains whereas diversity in Ag recognition results from combination of V $\alpha$  with J $\alpha$  and V $\beta$  with D $\beta$

and J $\beta$  respectively, i.e., the CDR3 regions.<sup>88</sup> Recent studies on the primary structure of TCRs of clones nad hybridomas specific for numerous protein Ag have demonstrated that T cells recognizing MHC class II-peptide complexes exhibit a limited TCR diversity but significant sequence diversity in the CDR3 regions of both V $\alpha$  J $\alpha$  and V $\beta$  J $\beta$ .<sup>89-91</sup> If these observations are further confirmed for tumor-reactive T cells, it would be possible to characterize the appropriate V $\alpha$ /V $\beta$  combination for recognition of well-defined tumor Ag presented by an allelic form of the MHC.

In contrast to the situation in inbred mice strains, the situation in humans is more complex. It was recently suggested that HLA is the major genetic component influencing TCR V $\alpha$ /V $\beta$  segment frequency, and T cells expressing particular V $\beta$  segments may be positively selected by either MHC class I or MHC class II proteins.<sup>92</sup> Therefore, CD4<sup>+</sup> cells may preferentially use certain TCR V segments whereas others are used by CD8<sup>+</sup> cells.<sup>93</sup> The V $\beta$ , and sometimes V $\alpha$ , dominance may not only reflect selection of particular TCR domains interactive into MHC (self-reactive?) but also peptide Ag (tumor Ag) recognition.

There is limited information on the CDR3 sequences of TCR recognizing human tumor antigens; therefore, it is still unclear whether TCRs recognizing self-antigens show a limited diversity, or reactivity with self-antigens reflects molecular mimicry (cross-reactivity) in recognition of self-antigens by TCR with different specificities.<sup>94,95</sup> This possibility should be seriously considered since a number of tumor CTL epitopes, e.g., from MART-1,<sup>96</sup> are derived from hydrophobic (leucine, isoleucine, and valine-rich) areas, and a number of viral CTL epitopes, e.g., from matrix protein, show the same alternation of nonpolar and polar residues. Taking into consideration that the peptide epitope is expected to interact with the TCR/CDR3 region by establishing either a salt bridge (by charged residues) or hydrogen bonds (by hydroxy groups in, e.g., serine, treonine, or tyrosine), the hydrophobic nonpolar aliphatic residues in these tumor peptide are expected to establish weak interactions with TCR (low TCR affinity) such as by van der Waals forces.<sup>97,98</sup>

If we also take into consideration that there is significant cross-reactivity between proteins at tri- and tetrapeptide level, only TCR sequencing and comparison of the sequences of TCRs reacting with antigens with similar structure will address whether these CTL recognize bona fide tumor antigens.

The picture is even less clear with respect to antibodies recognizing tumor antigens. Although it is expected that the CDR regions of the Ig molecules recognize Ag, there is a limited number of human monoclonal antibodies and even more limited information on the sequence of human antibodies recognizing tumor antigens, and conclusions cannot be inferred from sequence information of murine Ag.

#### TUMOR ANTIGENS RECOGNIZED BY T CELLS AS POSSIBLE TARGETS FOR ACTIVE OR ADOPTIVE SPECIFIC IMMUNOTHERAPY

As noted previously, tumor-reactive T cells recognize Ag bound to and presented by MHC molecules. The failure of earlier studies with mAb to serologically defined tumor an-

tigens to inhibit cell-mediated cytotoxicity by TIL demonstrate, that, even if we assume that T cells recognize peptides derived from these proteins, the epitopes recognized by mAb and T cells are different. Therefore, the notion of tumor Ag recognition in reality reflects recognition of tumor-derived peptides that are approximately nine amino acids in length for CD8<sup>+</sup> cells<sup>99</sup> and somewhat longer (16-18 residues) for CD4<sup>+</sup> cells.<sup>100</sup> Although these peptides lack serological definition, they originate as products of intracellular degradation in the cytosol or extracellular degradation by activated M $\phi$  of cellular proteins. It is still unknown what tumor-reactive CD4<sup>+</sup> T cells recognize but recent reports show that melanoma TIL can recognize epitopes on the same proteins as the CD8<sup>+</sup> cells, e.g., tyrosinase<sup>101</sup> or heat shock proteins.<sup>102</sup>

#### MOLECULAR CHARACTERIZATION OF TUMOR ANTIGENS RECOGNIZED BY T LYMPHOCYTES

Studies in animal models using tumors demonstrated that CTL recognize tumor-specific antigens. Since T cells can distinguish nonconservative substitutions in a protein sequence, the initial expectations were that human tumor specific Ag would be derived from activated oncogenes, tumor suppressor genes, or as yet unknown proteins uniquely expressed on tumor cells. Recent studies have shown that CD4<sup>+</sup> cells from peripheral blood of healthy individuals, or that CD8<sup>+</sup> CTL from cancer patients can recognize mutated ras peptide analogs, but the tumor cells harboring this mutation were absent from the cancer patient.<sup>103,104</sup> Although these findings exist, most of the recently identified human tumor Ag are either: (1) developmental antigens reexpressed during tumorigenesis,<sup>16,105</sup> seldomly expressed on normal tissues: e.g., in melanoma the MAGE family<sup>106,107</sup> or (2) lineage-specific differentiation antigens,<sup>16,105</sup> e.g., tyrosinase, gp100, or MART-1<sup>96,108-111</sup> and they are common (shared) antigens. Based on the recognition by CTL from HLA-A1<sup>+</sup> and HLA-A2<sup>+</sup> melanoma patients, gp100 is a membrane glycoprotein on melanomas and adult melanocyte on normal skin and is recognized by a series of mAbs.<sup>108</sup> This antigen has been recognized by TIL associated with tumor regression in a melanoma patient. MART-1 expression is similar to that of gp100. MART-1- and gp100-specific CTL are not cross-reactive.<sup>96-109</sup>

Other groups of candidate tumor antigens include: (1) cellular proteins that are either overexpressed (e.g., HER-2), likely a target of CTL on ovarian and, perhaps breast tumors<sup>112-115</sup> and (2) polymorphic epithelial mucin (Muc-1) core peptides, exposed on epithelial tumors, because of incomplete glycosylation.<sup>42,43,116</sup> Recognition of Muc-1 is non-MHC restricted because of the ability of the repetitive unit of the peptide core to engage (cross-link?) a large number of TCRs, thus bypassing the need for MHC presentation.<sup>42,43</sup>

It should be mentioned that at the time of this writing—with only one exception (gp100 peptide 946: YLEPGPVTA)<sup>111</sup>—none of the antigens listed above has been identified using strictly biochemical approaches, i.e., elution of peptide from the HLA, purification, sequence characterization, and reconstitution of cytolytic activity. The bioactive fractions eluted from HLA contain a large number of peptides at very low concentrations, thus making Ag

Table 22-1. Candidate tumor Ag recognized by T cells in human cancers\*

Antigen	Tumor
MAGE 1,3	Melanoma
Tyrosinase	Melanoma
MART-1/Melan A	Melanoma
gp100/pMel 17**	Melanoma
HER-2/NEU	Ovary, breast, lung
p21RAS	Colorectal cancer
Muc-1	Pancreas, breast, ovary

\*Peptide analogs of defined sequence from these proteins were found to reconstitute the activity of tumor-reactive CTL.

\*\*A peptide isolated from gp100 was the first Ag to be confirmed by strictly biochemical approaches.

identification difficult. The molecular strategies for identification of tumor antigens used either cloning from a tumor the gene that transfers sensitivity to CTL to a resistant tumor and narrowing down the region of interest, followed by assessment of recognition of synthetic peptide analogs of this area, or mapping CTL activity with synthetic peptides encompassing the potential HLA-A2 binding sites (Table 22-1). Ongoing studies should address the chemical nature of naturally processed peptides and confirm the identity of epitopes mapped using different methods.

It should be mentioned that both peptide 946<sup>111</sup> and a number of other model peptide CTL epitopes derived from either wild-type p53 or HER-2/neu proto-oncogene bind HLA-A2 with low affinity.<sup>115,117</sup> This finding suggests that affinity of the peptide-HLA complex for TCR and not the affinity of peptide for HLA may be the critical determinant for tumor recognition.

Assuming that abundantly expressed tumor proteins can provide peptides at concentrations above a certain threshold that can compete with other self-peptides for presentation to the T cells, recognition of a tumor Ag by the T cell will be a function of: (1) peptide-MHC class I assembly and stabilization of the complex; (2) the efficiency of processing and intracellular trafficking of tumor Ag; and (3) equally important, the presence in the primary structure of MHC binding motifs (allele specific consensus sequences) and of TCR binding motifs (T cell epitopes).

For class I molecules, stable assembly of peptide-HLA heavy chain complexes appears to be the most important step for peptide binding, because the presence of  $\beta$ -2-microglobulin and a tightly bound peptide confer on these molecules their conformational integrity.<sup>118,119</sup> One of the most critical elements in ensuring normal MHC class I expression on tumors rests in the normal function of the pair of peptide transporters (TAP1 and TAP2) located on the endoplasmic reticulum membrane of the tumor cells. The human peptide transporter *TAP* gene maps to the HLA class II region, and appears to be a member of the ABC (ATP binding) family of transporters that comprise the MDR1 gene encoding P-glycoprotein.<sup>120</sup>

These recent findings prove a very important point. Normally, the expression of a functional peptide transporter gene is a necessary condition for MHC class I expression on the cell surface. Tumor invasion and metastasis most often correlate with decreased levels of MHC class I expres-

sion on tumor cells.<sup>121</sup> Since MDR-like transporters have only recently been demonstrated to be critical for MHC class I expression, this finding may raise the question of whether the impaired expression of MHC class I chains reflect in part altered signal transduction where the signal is the putative tumor peptide.<sup>122</sup>

Both human and experimental tumors resistant to chemotherapy have shown that in certain instances drug resistance may be associated with elevated levels of P-glycoprotein. It is not known whether enhanced MDR1 expression correlates with enhanced peptide transporter gene expression. However, if up-regulation of peptide transporters (and of MHC class I expression) is observed following chemotherapy, there may be a reason for immunotherapy with T cells after chemotherapy. It should be noted that Mokyr and Dray have reported that low doses of chemotherapeutic drugs potentiate antitumor immunity in experimental tumor models.<sup>123</sup> Although different hypotheses can be advanced to explain this phenomenon, these findings show that chemotherapy can interfere with specific cellular immune responses to autologous tumors.

On the basis of recent findings, the difference between "normal" and tumor antigen (Ag) may be predicated on differences between the primary structures of nonapeptides. The question arises as to how extensive these differences are and where in the peptide sequence they locate. Sequencing of peptides eluted from both murine and human MHC class I chains reveals allele specific sequence motifs (consensus) that are distinct for the various MHC allotypes. These consensus motifs are characterized by particular residues occupying certain positions in the peptide sequence.<sup>124</sup> Based on the frequency of appearance in these positions, they are defined as primary (dominant) and secondary (auxiliary) anchor.<sup>125,126</sup> Therefore, the consensus motifs appear to be a minimal requirement of peptide MHC class I binding, and impose a strong reduction in the number of sequences from a tumor protein that can be presented to a T cell. This applies also to CD4<sup>+</sup> T cells. A number of binding motifs have been identified for the major HLA-DR antigens expressed in human population. When we searched the sequence of HER-2/neu proto-oncogene for the presence of such sequences, we found a large number of epitopes containing predicted anchor motifs for HLA-DR1, HLA-DR3, but very few for HLA-DR4 and HLA-DQ7 (Blevins and Ioannides, unpublished data).

From the data presented above it is clear that tumor Ags, in addition to their ability to bind MHC, should be able to interact with TCR. Initially, Berzofsky and DeLisi reported that T cell epitopes were found preferentially in segments of the Ag that have the potential to form amphiphilic  $\alpha$ -helices.<sup>127</sup> Although this feature is not universally applicable for the definition of T cell sites, it has led to studies focused on the identification and characterization of T cell epitopes.<sup>128</sup> The advantage of the amphiphilic  $\alpha$ -helical structures over  $\beta$ -propensive areas may be due to increased resistance to proteolytic digestion during processing,<sup>129</sup> increased ability to interact with membranes,<sup>130</sup> and, most importantly, the specific and stable positioning of critical residues by amphiphilic  $\alpha$ -helical structures. The ability to form  $\alpha$ -helices and the proper hydrophilic-hydrophobic balance are essential for high-affinity binding of model peptides

at the cell surface.<sup>130</sup> It is also thought that peptides resulting from protein digestion that lack signal peptide would be more amenable to being transported across membranes when their overall structure is amphiphilic, either through passive or active (transporter-mediated) mechanisms.<sup>131</sup> A number of tumor cells show diminished expression of HLA-molecules, which in certain instances correlated with lack of transporter proteins, or impaired transporter function. Studies with mutant cell lines deficient in TAP function have identified peptides bound in HLA-A2. These peptides appear to be derived from highly hydrophobic areas of proteins, such as signal and maybe transmembrane domains.<sup>132</sup> Although signal peptides have not yet been identified as tumor Ag, the epitope of MART-1 recognized by CTL is derived from the transmembrane area, raising the question of whether a number of tumor Ag can bind HLA through passive transporter mechanisms.

An additional structural feature of T cell epitopes used for focusing the search for potential helper T cell sites is provided by Rothbard's epitope motifs—charged-hydrophobic-hydrophobic-polar, and charged-hydrophobic-hydrophobic-hydrophobic/proline-polar—that define tetra- and pentapeptide epitopes.<sup>133</sup> Selection by potential amphiphilic  $\alpha$ -helical sequences and by Rothbard's motifs are rather complementary methods, because a succession of Rothbard's epitope motifs can sometimes assume a helical conformation due to the periodical alternation of hydrophilic and hydrophobic residues. In any case, algorithms initially described by Margalit et al.<sup>128</sup> can be applied to the analysis of primary and secondary structures of tumor protein to identify potential T cell epitopes.

Although neither amphiphilic peptides nor the presence of Rothbard's epitope's motifs are restrictive features in selection of tumor Ag (and in other systems sequences with  $\beta$ -propensive structure have been reported to include T cell epitopes), the search for such sequences may allow a focused search for T cell epitopes. Recent crystallographic and computer modeling studies show that peptides bound within the two antiparallel helices of the  $\alpha 1$  and  $\alpha 2$  domains of the MHC class I adopt an extended conformation.<sup>134</sup> However, the conformation of peptide in the trimolecular complex MHC-peptide-TCR in solution should be determined to address this point.

## CONCLUSIONS AND PERSPECTIVES CONCERNING T CELL RESPONSES TO TUMORS

Attempts to develop novel therapeutic approaches to human cancer, focusing in the last 5 years on TIL, have been instrumental in improving our understanding of the nature of interactions between T cells and human tumors. It is clear that the infusion into patients of cultured TIL, either alone or combined with interleukins, has certain limitations, which are probably the result of either tumor- or immune cell-induced suppression, or tolerance to self. Breaking the suppression or tolerance would require novel approaches for intratumor delivery of essential lymphokines and inhibition of the suppressor networks. Chemical characterization of tumor peptides shared by human tumors and cultured tumor cell lines, and recognition by CTL, may offer an alternative approach for in vitro stimulation and expansion of tumor-

reactive T cells isolated from both peripheral blood or tumor infiltrations and development of tumor vaccines. If such approaches are found to be more effective than the TIL used now in clinical trials, engineering of adequate vehicles for tumor peptide (tumor Ag) gene delivery after modification to optimize their binding to HLA and stabilize both the HLA-peptide complex and TCR engagement may provide an alternative approach to tumor vaccination in generating in vivo tumor fighting cells.

## ANTIBODY RESPONSES TO HUMAN TUMORS

Antibody therapy of cancer is one of the oldest aims of cancer therapy. The expectations from an antibody response against tumor cells were and still are that: (1) the antibodies recognize novel antigens/epitopes expressed only on human tumors; (2) these tumors and antigen are accessible; (3) these antigens internalize but do not shed after antibody induced receptor aggregation; and (4) internalization of the receptor-Ab complex disrupts the cell cycle of the tumor target. Most of the currently available antibodies do not meet one or both of these following characteristics: (1) the Ag are also expressed in the normal tissues, and (2) the antibody (Ab) alone is inefficient to deliver a lethal hit to a target cell, consequently "armed" antibodies (coupled to toxins, or radionuclides) are currently used for therapy. In the last decade, analysis of the surface antigens of human tumor cells has focused on: (1) identification and definition of differences between normal and cancer cells, and (2) use of these newly identified differences for development of passive and active immunotherapy approaches to cancer.

## CELL SURFACE ANTIGENS OF HUMAN CANCERS RECOGNIZED BY ANTIBODIES

Initial studies on cell surface antigen expression by human tumors suggested that tumors express: (1) differentiation antigens corresponding to the lineage and stage of their closest normal counterpoint; (2) oncofetal antigens, shared with fetal or embryonic cells; and (3) activation antigens, shared with activated adult tissues. This initial attempt of classification has been recently replaced by the concept of "modular differentiation antigens." These antigens include leukocyte surface protein antigens (e.g., CD56) lipids (e.g., gangliosides), and complex carbohydrates (e.g., blood group antigens).<sup>135</sup>

Modular differentiation antigens can be divided in several categories using immunogenetic principals. The first group is clonogenetic epitopes, resulting from genetic changes in unique tumor genes. Relevant examples are: (1) T and B cell expressing unique TCR or immunoglobulin idiotypes, that comprise the majority of leukemias and lymphomas; (2) mutated oncogenes (e.g., *ras*, *myc*); and (3) mutated tumor suppressor genes (e.g., *p53*). These clonogenetic changes either define the malignant phenotype (1), or may result from mutation that alone or in association with other factors are essential to pathogenesis (2,3). For example, *p53* is overexpressed in transformed cells, but is expressed at very low levels in normal cells, *p53* accumulation is a consequence of its stabilization due to a point mutation that modifies its conformation and, apparently, its rate of turnover.<sup>117</sup>

The second group of epitopes consists of aberrantly expressed alloantigens (allogenetic epitopes) usually detected among blood group-related carbohydrate determinants (e.g., Thomsen-Friedenreich, (T) and Tn antigens).<sup>18</sup>

The third group of antigens (xenogenetic epitopes) includes epitopes of viral proteins associated with human cancers. This group includes proteins, derived from Epstein-Barr virus (EBV) associated with Burkitt lymphoma, immunoblastic B cell lymphoma, nasopharyngeal carcinoma, and possibly Hodgkin disease. Human papillomavirus (HPV) associated with a number of skin and anogenital cancer, and HTLV (human T cell leukemia/lymphoma virus) associated with adult T cell leukemia.

The fourth group of tumor epitopes consists of epigenetic epitopes (differentiation antigens) that are encoded by the same cellular genes in normal and cancer cells, but may be expressed at different levels in normal and tumor cells. This category includes the majority of antigens detected using patients' sera or human and murine monoclonal antibodies.<sup>16,135</sup>

### ANTIBODY RESPONSES TO HUMAN TUMOR-ASSOCIATED ANTIGENS

The main approach to the examination of the immunogenicity of human tumors is to analyze the ability of antibodies from human sera to react with autologous or allogeneic tumors. Studies characterized by this approach used either sera from cancer patients before or after therapy, to identify prognostic and follow-up parameters or immunoglobulins isolated from surgically removed tumor cells. Thus, these studies characterized tumor-specific Ab component, or antibodies from patients treated with tumor vaccines, to identify target antigens of the antitumor immune response. With few exceptions, only a few antibodies were found of restricted specificity for tumor cells. However, this approach has led to identification of novel antigens expressed on melanoma cell surface, such as p97.<sup>136</sup> The main groups of antigenic specificities recognized by natural antibodies from the sera of cancer patients are summarized below.

#### Antiheterophile antibodies

Sera from healthy individuals contain antibodies to blood groups, or antigens expressed in red blood cells, or heterophile antigens (which react with tissues from species other than the immunizing species). A number of such antigens such as Forsman (FS) and Hanganutziu-Deicher (HD) antigens are absent from human cells, but serum antibodies to these specificities are present. Biochemical analysis of target antigens has identified that the targets of anti-T and anti-Tn antibodies are carbohydrate epitopes in glycoproteins (mucins), while for the anti-FS and anti-HD antibodies the targets are glycosphingolipids or glycoproteins and the novel antigenic specificity reflects the presence of a novel sugar *N*-glycolyl neuraminic acid (NeuGc).

Studies of anti-T and anti-Tn and Fs antibodies in the sera of patients with breast, lung, pancreatic, and gastric cancer have found significantly lower levels of such antibodies compared with normal individuals and those with benign disease. In contrast, anti-HD antibody levels were

found increased in a variety of human cancers. However, other studies failed to confirm these findings. A possible explanation for these discrepancies may rest in the low or variable levels of these antigens on human tumors. For these reasons, the use of tests in which Fs and HD antigen levels are used to follow therapy is questionable.<sup>18</sup>

#### Antiganglioside antibodies

Pioneering work by Livingston, Old, and their colleagues contributed much to identification of serological specificities recognized by sera from melanoma patients. A number of previously described melanoma antigens were found to be gangliosides (glycosphingolipids containing neuraminic acid). From these studies, as well as from others of antibodies from patients treated with melanoma vaccines,<sup>137-138</sup> it was concluded that gangliosides GM2 (melanoma antigen, OFA-I-1), GM- and GD2 (oncofetal antigen I-2), but not GM3 and GD3 are immunogenic in melanoma patients.<sup>18</sup> Further studies will be needed to address whether this strong humoral response to gangliosides reflects an antitumor response, or is a consequence of an autoimmune response to high concentration of antigen.

#### Antiprotein antibodies

B cell responses to tumor protein antigens are of major interest for understanding tumor immunity in humans because Ab responses to protein antigens are T cell dependent, which points not only to the involvement of T cell for induction to this type of tumor immunity (autoimmunity) but also to the existence of a network of B cells, T cells, and lymphokines capable of modulating and/or stimulating tumor growth.

Although the evidence for such responses is still limited, several such antigens have been recently identified.

1. gp75 (melanosomal protein) expressed on melanomas and normal melanocytes. gp75 is a member of the tyrosinase family—peptides that are recognized by melanoma-specific CTL.<sup>16</sup> This protein is a target for both humoral and cellular responses and is now a target for active immunotherapy studies.

2. p97 (melanoferrin)<sup>136</sup> is abundantly expressed in melanoma cells, but at lower levels in normal cells. A mutated variant of p97 (FD antigen) can be recognized by serum antibodies from a melanoma patient on its autologous tumor, suggestive of the case where antibody responses against a mutated self-protein can be induced by tumor vaccination.<sup>138</sup> Antibodies to the wild-type p97 have not yet been reported.

3. 58kd melanoma chondroitin sulfate proteoglycan.<sup>134</sup> Natural antibodies to this glycoprotein have not been yet detected in cancer patients. However, antibodies to this antigen were induced in patients with melanoma by immunization with a murine anti-idiotypic antibody,<sup>137,139</sup> suggesting cross-reactivity (molecular mimicry) of the melanoma antigen epitope with a murine epitope on the immunizing antibody.<sup>140</sup>

4. A number of other melanoma antigens have been detected by the reactivity of antibodies from patients' sera with melanoma cell lines, spent culture medium, and patient's urine, and await sequencing.<sup>138</sup>

5. Melanotransferrin identified by antibodies from a pa-

tient treated with melanoma viral oncolysates. The antibody specificity of the patients serum appears to be directed against a unique determinant.<sup>140</sup>

6. Wild-type p53. The antibody response was detected in 15% of primary breast cancer patients, directed against two immunodominant regions located at the amino- and carboxy-terminal domain of the protein. Importantly, these responses were outside the area expected to be the mutational hot spot, indicating again the preferential induction of tumor immunity to self-determinants.<sup>141</sup>

7. HER-2/neu. Both antibody and cellular proliferative (likely T cell) responses to this protein have been recently reported using serum and antibody from a breast cancer patient.<sup>43</sup>

We have also found cellular proliferative responses to HER-2 peptides by peripheral blood mononuclear cells (PBMC) from ovarian cancer patients and some normal donors (Ioannides, et al, manuscript in preparation). Since antibodies can induce receptor internalization, which may disrupt tumor cell growth, this may be suggestive of an antitumor humoral response.<sup>113</sup> However, it is still unknown whether antibodies and cytokines released by the tumor may lead to tumor stimulation thorough receptor activation.

#### TUMOR ANTIGENS DETECTED BY MONOCLONAL ANTIBODIES

Studies of antitumor humoral responses in patients' sera failed to reveal the complex antigenic phenotype of human tumors, because of differences in responses from individual to individual, the low affinity for the antigen of the serum immunoglobulins, and ultimately the difficulties in defining specificities in a mixture of antibodies.<sup>18</sup>

Murine monoclonal antibodies developed against a large number of human tumors have proved useful in defining the antigenic phenotype of cancer cells and because of their restricted specificity provide an important tool for diagnosis and follow-up of cancer patients (e.g., OC125 for ovarian carcinoma). Detailed presentation of the mAb application for immunodiagnostic is made in a number of books (e.g., Herberman RB, Mercer DW: *Immunodiagnosis*, 1990, New York, Marcel Dekker.).

Development of these mAb antibodies has been instrumental in biochemical characterization and functional analysis of molecules on the tumor cell surface, particularly of leukemias and lymphomas. The epitope specificity of these mAb has raised the possibility of using them for treatment of various malignancies, mainly hematologic cancer, or treatment of residual disease of micrometastases. Both unmodified antibodies and conjugates with toxins or radio-nuclides are currently being evaluated (Chapter 135). We will mention only briefly the limitations of use of murine mAb for cancer therapy, and the current directions of research for overcoming these limitations.

Use of mAb for therapy attempts to overcome a number of limitations: (1) the low efficiency of unmodified antibodies to treat malignant diseases, (2) the ability of the immune system to destroy xenogeneic antibody-coated cells, and (3) the development of human antimouse antibodies with concerns of potential anaphylactic and serum sickness reactions. To overcome these limitations a number of ongoing

studies are focused on: (1) development of human monoclonal antibodies, or subsequent "humanization" or murine antibodies by replacing the murine Fc regions with a human Fc region; and (2) engineering of single-chain antibodies made of VH and VL regions (sFv) fused with effector molecules such as cytotoxic drugs, toxins, growth factors, and receptor domains. Because of their small size, single-chain antibodies can penetrate tissues more efficiently.<sup>142</sup>

#### TUMOR DETERMINANTS THAT ARE POTENTIAL TARGETS OF AN ANTITUMOR HUMAN RESPONSE

A number of cancers express particular determinants that can be targets of humoral and cellular response. Although immune responses to such antigens have not been detected in patients, xenogeneic sera or mAb have been raised to allow utilization of these antigens as promising diagnostic markers.<sup>135,143</sup> These tumor proteins presented briefly below are products of: (1) fusion gene (*BCR-ABL*); (2) abnormal genes with in frame deletion mutations (*EGFR*); (3) stem cells on oncofetal cells antigens of highly restricted expression in normal and tumor cells (e.g., HBA71 antigen expressed Ewing sarcoma in normal thymocytes, and folate binding protein from human trophoblast expressed only in ovarian carcinoma); and (4) growth-controlled or growth controlling cell surface molecules (e.g., lymphokine receptors, HER-2/neu).

1. A translocation in chronic myelogenous leukemia cells joins two genes: the *c-ABL* proto-oncogene with breakpoint cluster region (BCR) on chromosome 22, resulting in a fusion gene *BCR-ABL*. The resulting cytoplasmic protein p210<sup>BCR-ABL</sup> has unique epitopes that may be targets for a diagnostic test for cellular responses to CML.

2. Epidermal growth factor receptor (EGF-R) is expressed both in normal and malignant cells, and is a specific receptor for transforming growth factor (TGF) related peptides. In a significant number of gliomas (40%), an abnormal form of EGF-R is amplified. In-frame deletion mutation in the *EGF-R* gene extracellular domain results in cell surface expression of truncated receptors or fused proteins containing new sequences.

3. HGA71 antigen was found by its reactivity with mAb to be expressed in more than 95% of patients with Ewing sarcomas. The corresponding protein is absent from most normal tissues and tumor cells with the exception of normal thymocytes.

4. The human trophoblast antigen is identical to the antigen recognized by MOv18/MOv19 mAb, and appears to be restricted to ovarian cancer. Sequencing of the gene shows that both antigens are in fact the folate binding protein.<sup>144</sup>

#### NONSPECIFIC IMMUNITY

As described in the introductory section, the modern era of tumor immunology began with observations of tumor regression after administration of bacterial extracts and products. These antitumor responses were considered "non-specific" since no tumor specific immunogen was administered. Today most investigators believe that the tumor regression in those studies was a by-product of either local



Table 22-2. Nonspecific immune system effectors

Effector	Stimulus	Target range	Killing characteristics
NK Cells	Constitutively expressed, IL-12 up-regulates	K562 and selected targets	4-hr assay
LAK Cells	IL-2, possibly IL-7, IL-12, and IL-15	Most fresh and cultured tumors	4-hr assay
Macrophages	IFN $\gamma$ + LPS, IL-2, liposomal MTP	Most fresh and cultured tumors (same as LAK)	18-hr assay

or systemic inflammatory reactions. While the molecular basis for any specific cross-reactivity of bacteria with tumors is not yet understood, cross-reactive responses to superantigens or heat shock proteins are considered likely candidates. It is also possible that all tumors express common determinants that identify them as abnormal and may be exploited for recognition. In this context, the distinction of "self" vs. "nonself" for immune recognition becomes controversial. Some immunologists suggest that "lack of normal" is defined by fundamental recognition by the more conserved and primitive components of the immune system such as NK cells, LAK cells, and macrophages (Table 22-2). This section reviews the background and clinically relevant highlights of these three immune system components as well as some of the cytokines that are now considered the primary inducers of "nonspecific" immune responses.

#### Natural killer cells

NK cells are peripheral blood lymphocytes with distinct functional and morphological characteristics (Chapter 19). They are described as large granular lymphocytes (LGL) with the cell surface expression of CD6 (FcRIII+), CD56 (N-Cam), and the IL-2R $\beta\gamma$  complex, but lack the expression of CD3 (see refs. 145-147 for excellent reviews). These cells express an apparent constitutive killing activity for a very limited subset of tumor targets *in vitro*, for which the erythroleukemia, K562, is the prototype. Direct contact of NK with the target is required for this lysis to occur. The presence of the receptor for the Fc portion of antibody also confers the ability to mediate killing of antibody-coated targets, by antibody-dependent cellular cytotoxicity (ADCC). NK cells do not require immunization, but their killing activity can be boosted by exposure to cytokines such as IL-2, interferons, and TNF.

Most fresh human tumors are NK resistant, but after long-term culture some acquire NK sensitivity. Culture-induced loss of certain molecules was one hypothesis forwarded for the induction of NK sensitivity. Consistent with the loss of a masking epitope are data indicating that the absence of MHC class I expression often (but not always) increases to tumor sensitivity to NK, suggesting that MHC class I molecules may mask the NK recognition determinant.<sup>148</sup> Much research is currently addressing the molecular nature of the NK recognition system. If sensitivity to NK cell cytotoxicity could be induced on tumors *in vivo*, then increased therapeutic potential would be likely.

NK activity is often considered important in regulation of blood-borne metastases. In this regard, correlation of higher NK activity with fewer metastases in certain animal

models. In the human, it has been noted that patients with a deficiency of functional NK cells (Chédiak-Higashi syndrome) manifest a dramatic increase in malignant disease,<sup>149</sup> providing strong circumstantial evidence of their role in surveillance.

Unfortunately, human tumors arise and metastasize in the presence of NK activity, which often does not appear significantly decreased in patients. Clinically, NK cell activity should be considered from several perspectives. First, tumor immunotherapy trials have attempted to exploit the capacity of NK cells to mediate ADCC, especially after boosting of the cells with IFN or low-dose IL-2. Second, the NK cell activity found in the blood has been considered reflective of the generalized cellular immune status of an individual, since NK cells are sensitive to various immunosuppressive agents such as prostaglandins, corticosteroids, TGF- $\beta$ , and IL-10. If NK activity is low or absent, then the potential of the cellular arm of the immune system is likely to be similarly suppressed. Third, NK cells can be stimulated to produce inflammatory cytokines themselves, so that these may contribute to immune amplification. Finally, NK cells are the major precursors for the generation of lymphokine-activated killing activity (LAK) in response to IL-2.

#### Lymphokine activated killing activity

LAK cells can be generated from almost any source of lymphoid cells, including blood, bone marrow, lymph node, thoracic duct, etc. In response to intermediate levels of IL-2, NK cells and others expressing the IL-2R $\beta\gamma$  differentiate to express a pattern of tumor recognition that is very broad<sup>150</sup> (Chapter 124). Noncultured normal cells are LAK cell resistant, although reports of cultured normal cells acquiring sensitivity do exist. IL-2 alone drives the 2 to 3 day differentiation cascade that leads to the development of LAK cells.<sup>151</sup> Therefore, IL-2 drives not only proliferation of antigen-stimulated cells, but it also initiates differentiation of a tumoricidal activity as well as a tumor recognition capacity that was not expressed on NK or other precursor cells.<sup>152</sup> Direct contact of LAK cells with the tumor target cells is required for lysis, as is the case with NK cells and with macrophages. Tumor lysis proceeds quickly, within 3 to 4 hours. As with NK cells, no prior antigenic exposure is required, and lymphoid cells of normal individuals as well as cancer patients can be induced to express this activity.

From the clinical perspective, LAK cells are the most potent and specific antitumor effector cells known to date. The poorly understood LAK cell recognition of tumor is extremely interesting and suggests that common tumor de-

terminants exist, since various allogeneic tumors will inhibit each other in cold-target inhibition assays. NK cell sensitive targets also inhibit LAK killing, suggesting that NK recognition is preserved as a subset of the LAK system. NK-resistant and LAK-sensitive tumor targets do not inhibit NK killing of K562, clearly distinguishing the NK from the LAK repertoire.<sup>150,151</sup>

Data presented in this chapter indicate that tumor-specific cytotoxic T lymphocytes, including those generated from TIL can be generated and used in therapy experiments. It is our hope that such systems will reconstitute the tumor specific responses and provide the memory necessary to maintain a systemic surveillance against micrometastatic disease. There is no doubt that from today's perspective LAK are much more accessible and can destroy very large numbers of tumor cells rapidly. The activation of LAK may also be preferred, if particular tumors do not express any antigenic epitopes or if they modulate to become resistant to TIL or CTL. The clinical application of LAK has proceeded from two perspectives: The first approach is the direct adoptive therapy of LAK cells using intravenous injection for systemic therapy, as well as localized injections, while the second approach has been the localized generation of LAK cells at the tumor site, by localized and slow release of IL-2. In both types of clinical trials, a constant supply of IL-2 is needed to maintain the LAK, and variable severe toxicity was observed, particularly that of hypotension and vascular leak resulting in dose-limited toxicity. Adoptively transferred LAK were documented to lodge mostly in the lungs and liver after intravenous injection, and in some cases where massive tumor burdens existed in these sites tumor regression did occur.<sup>153-155</sup> Unfortunately, the highly publicized dramatic responses were erratic and not reproducible, clearly indicating that factors other than the LAK were influencing the patient response. Two other cytokines that activate LAK are IL-12<sup>156-158</sup> and the recently identified IL-15.<sup>159</sup> Common functional qualities of these cytokines make them attractive candidates for future study.

### Macrophages

In a manner similar to the NK cells, macrophages must be "activated" by bacterial products or cytokines in order to kill tumor cells (Chapter 20). Activation of murine macrophages requires two types of signals, resulting in an increased motility, phagocytic capability, and tumor killing ability. The combination of IFN $\gamma$  and lipopolysaccharide (LPS) makes up the prototype signals for murine splenocytes; inability to consistently activate human blood monocytes has hampered widespread clinical studies, but preliminary data indicate that IFN $\gamma$  and LPS are responsive to either IFN $\gamma$  alone or IL-2 alone (Grimm, submitted for publication). After activation, the tumor target spectrum is diverse, and may be identical to that of LAK, although the mechanism of killing is apparently different since macrophages require longer in vitro incubation times (usually 12 to 15 hours) in order to lyse the same tumors that are rapidly killed by LAK cells in 3 to 4 hours. The homeostatic role of macrophages appears to be that of tissue turnover and elimination of damaged or senescent cells.<sup>160,161</sup> The blood-borne monocytes and tissue macrophages are functional in

both the afferent and efferent limbs of the immune system, and subclasses of these cells are yet to be delineated.

Attempts to use macrophages clinically for destruction of tumors have been best achieved to date by taking advantage of the liposomes for which biologic selectivity exists for uptake by macrophages.<sup>162</sup> The encapsulation of macrophage activation such as muramyl dipeptide<sup>163</sup> provides a unique and relatively nontoxic means to target this family of immune activators. Recently, liposomal encapsulated tripeptide phosphatidyl-ethanolamine (MTP-PE) has shown successes in reducing the appearance of lung metastases in children with osteosarcoma (Kleinerman, personal communication). Macrophage activation may be important in combination with certain chemotherapeutic regimens (discussed later in this chapter) however, the data of Kleinerman indicate that addition of cisplatin,<sup>164</sup> methotrexate,<sup>165</sup> or adriamycin<sup>166</sup> did not enhance the effect of the liposomal encapsulated MTP-PE. Liposomal IL-2 is also a very interesting consideration, since both macrophages and local lymphoid cells may be activated; clinical trials are also, currently under way examining this agent in a variety of malignancies in Phase I settings.<sup>167</sup>

### IL-2 and other cytokines

In 1992, the Food and Drug Administration (FDA) approved IL-2 for treatment of renal cell carcinoma (RCC) in the United States. IL-2 was the second immunomodulator approved for use in cancer therapy (after IFN $\alpha$ ). The systemic use of IL-2 has resulted in response rates of 15% to 30% for RCC and slightly lower rates for malignant melanoma<sup>166-169</sup> (Chapter 133). Unfortunately, in the human IL-2 manifests serious dose-limiting toxicity, mainly vascular leak and hypotension; this has precluded IL-2 usage at concentrations found efficacious in mouse model systems.

As information accumulated indicating the sometimes powerful effects of IL-2 for cancer therapy, researchers questioned why such responses were not consistent when assessed in similar patients with similar disease burdens and performance status. It is not clear why some patients respond to IL-2 and others do not. More recently, it has become apparent that some tumors make immunologically active cytokines. It is possible that endogenous local cytokine elaboration potentiates exogenously provided ones, resulting in generation of local immune effector function from activated macrophages and/or LAK cells. Current research is directed to the identification of individual cytokine profile of each patient's tumor, in order to tailor the treatment to take advantage of the local environment.

There is no doubt that IL-2 induces an immune amplification cascade that in some individuals causes a potent antitumor response; however, in most patients the toxicity induced is limiting and no significant or lasting tumor regression occurs. Attempts to reduce the toxicity have led to several strategies, including lowering the doses of IL-2 and combining it with other cytokines,<sup>170</sup> or providing the IL-2 directly to the tumor site<sup>171</sup> including sites in the central nervous system,<sup>172,173</sup> where documentation of local secondary cytokine release as well as generation of cellular infiltrates in response to the IL-2 alone suggest possible



mechanisms. In animal models, expression of an IL-2 transfect gene in tumor cells results in abrogation of the tumorigenicity and long-term immunity in selected cases.<sup>174</sup> IL-2 has also been injected directly into accessible tumor sites in slow release form<sup>175</sup> and this approach is now being attempted in humans providing liposomal IL-2<sup>167</sup> (personal communication). Another attractive approach is the use of mutant IL-2 molecules, which have various amino acid substitutions in order to limit the toxicity-inducing interactions. Studies by Heaton et al.<sup>176,177</sup> using IL-2 analogs that have had their capacity for interaction with the IL-2Ra eliminated, have been found in comparison to the wild-type IL-2 to produce significantly less secondary cytokines but only minimally reduced LAK cell activity.

The approach of reducing IL-2 toxicity by combining lower doses with other cytokines such as the IFNs, TNF, IL-1, etc. has gained popularity as the possibility of more efficacious approaches are continuously developed in response to the development of clinical-grade quantities of other cytokines and interleukins. However, to date no consistent combination has proved better than IL-2 alone.<sup>170</sup>

The newest approach for immunotherapy with cytokines has been the delivery of these molecules after recombinant DNA transfection into tumor cells. Although this is often called "gene therapy," this term is a misnomer since no therapy to a gene is attempted; in the case of cytokine gene transfer and cytokine elaboration, this type of immunotherapy is merely another approach at localized cytokine delivery. Although there are numerous reports of successful tumor elimination after gene transfer of various cytokines in mouse models, IL-2 appears to be the most effective way in generating long-term CTL-like activity<sup>174</sup> as well as the most effective method of overcoming the more serious immunosuppressive factors such as TGF- $\beta$  known to be produced by tumors.<sup>178</sup>

## BIOCHEMOTHERAPY

Because the various antiproliferative and immunosuppressive properties of most chemotherapy drugs are well known, combining these with biologics for treatment of cancer was not especially attractive except in the hope of immunorestitution. Even when trials of combinations were initiated such as MTP-PE mentioned above, poor results were found. Fortunately, some other combinations have been tried based on empirical considerations, and surprisingly some very good responses have been reported. Response rates up to 60% have been obtained by combining cisplatin (CDDP), DTIC, IL-2, and  $\alpha$ IFN for treatment of malignant melanoma in over 200 patients.<sup>179-181</sup> Many refinements to this approach are in progress and include research addressing the possible biological mechanisms. One attractive concept involves indications that cytokines elaborated from the response to the biologics, which then enhance the DNA strand breaks. It is known that IL-2 induces IL-1 production from lymphocytes and monocytes; Braunschweiger et al.<sup>182</sup> reported that IL-1 enhances CDDP-induced cytotoxicity, possibly by release of reactive oxygen or nitrogen species from adjacent endothelia and/or from macrophages resident in the tumor.

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Protein Kinase C Activation and the Intrinsic Drug Resistance of Human Colon Cancer

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## I. Intrinsic drug resistance in human colon cancer - a clinical perspective.

Colorectal cancer is a major cause of cancer-related death in the United States and other industrialized countries (1,2). This unfortunate fact is due in part to the marked resistance of colon cancer cells to available therapeutic agents (Table I) (2,3). In fact, the sole, truly effective therapy available for colorectal cancer at present is surgical intervention. Thus, there is no truly effective therapy available to manage metastatic colorectal cancer. Neither chemotherapeutic drugs nor biological response modifiers, *e.g.* interferons, have achieved major improvements in the survival of colon cancer patients (2). The inherent resistance of colon cancer to chemotherapeutic agents is termed intrinsic drug resistance. In contrast, acquired drug resistance is commonly observed in cancers that initially respond to chemotherapy, *e.g.*, breast cancer. Acquired drug resistance is defined as resistance that develops as a result of exposure of cancer cells to chemotherapy (4,5).

Because intrinsic drug resistance to cancer chemotherapy is generally observed in cancers that arise from tissues which have detoxifying functions *e.g.*, the colon, stomach and kidney, it is thought that tissue-specific detoxification mechanisms are operative in the cancer cells and protect them from chemotherapy (6,7). Evidence has been presented in support of this hypothesis. The drug-efflux pump P-glycoprotein is a contributing factor in drug resistance in cancer, and it is thought to provide a detoxification mechanism in normal cells (6,7). Human colon cancer and other intrinsically drug resistant cancers have been shown to express the message that encodes P-glycoprotein more abundantly than human cancers that are initially responsive to therapy (5). It should be noted that, although this suggests the potential value of P-glycoprotein-targeted drugs in colon cancer therapy, attempts to reverse clinical



drug resistance in solid cancers with P-glycoprotein-binding drugs have resulted in unacceptable levels of toxicity, and enthusiasm for this approach in the treatment of solid cancers has declined (8).

5-fluorouracil is the most effective chemotherapeutic agent available for colon cancer therapy (2). This drug achieves a partial response rate of about 15-20% of advanced colorectal cancers (1,2). Modest improvements in the efficacy of 5-fluorouracil against human colon cancer have been achieved by combination therapy (2,9,10). Colon cancer therapy has been attempted by combining 5-fluorouracil with other cytotoxic chemotherapeutic agents, with agents that serve as biochemical modulators of 5-fluorouracil, and with biological response modifiers (2). Although the relative merits of these therapies remain controversial, there is a consensus that a combination of 5-fluorouracil and levamisole is indicated as surgical adjuvant chemotherapy in the treatment of advanced colon cancer (10). Levamisole is an antihelminthic agent with immunomodulatory activity (1). The mechanism of action of levamisole in colon cancer therapy remains unclear, and it has yet to be determined whether the combination of 5-fluorouracil and levamisole is actually more effective than 5-fluorouracil alone (9,10).

The lack of a significant breakthrough in the management of advanced colon cancer during the past three decades supports the view that a completely new approach to the treatment of this disease is needed. In this chapter, we will focus on protein kinase C (11) as a potential target for therapeutic intervention in human colorectal cancer. We will review studies on the expression of the protein kinase C isozyme family in the colonic epithelium and in colon carcinomas, and we will also review the evidence that protein kinase C plays a major role in the intrinsic drug resistance of human colon cancer. Finally, we

will consider the potential value of inhibiting protein kinase C as a strategy for sensitizing human colon cancer to chemotherapeutic drugs.

## II. An overview of the protein kinase C isozyme family.

The identification of protein kinase C as the phorbol-ester tumor promoter receptor in 1982 revealed the importance of this enzyme in cell growth and differentiation (12). At approximately the same time, the central importance of protein kinase C in mammalian signal transduction was revealed by the identification of the second messenger diacylglycerol as the endogenous activator of protein kinase C (13). These discoveries set into motion more than a decade of intensive research dedicated to this pivotal signal-transducing enzyme (14,15).

Over the past several years, more than ten protein kinase C isozymes have been identified (11,16,17). These isozymes can be categorized into three groups, based on structural homology and cofactor requirements (Table II) (11,14-16). The common protein kinase C isozymes (cPKC's) are activated by diacylglycerol in a  $\text{Ca}^{2+}$ - and phosphatidylserine (PS)-dependent manner. Four cPKC's have been identified: cPKC- $\alpha$ , cPKC- $\beta_1$ , cPKC- $\beta_2$ , and cPKC- $\gamma$ . The novel protein kinase C isozymes (nPKC's) are distinguished from the cPKC's by their independence of  $\text{Ca}^{2+}$ . nPKC's are activated by diacylglycerol in a PS-dependent manner. These isozymes are nPKC- $\delta$ , nPKC- $\epsilon$ , nPKC- $\eta$ , nPKC- $\theta$ , and nPKC- $\mu$ . The atypical protein kinase C isozymes (aPKC's) are independent of diacylglycerol and phorbol esters (11,16). The messenger molecule(s) responsible for their activation *in vivo* has not been definitively identified, although evidence has been presented that atypical protein kinase C isozymes can be activated by specific

polyphosphoinositides (18). aPKC activity is  $\text{Ca}^{2+}$ -independent and PS-dependent. Two aPKC's have been identified, aPKC- $\zeta$  and aPKC- $\lambda$  (11,16).

In the cPKC isozymes, the conserved region C2, which is present in the regulatory domain, is responsible for the  $\text{Ca}^{2+}$ -dependence of the kinase activity. The C2 region is not present in  $\text{Ca}^{2+}$ -independent protein kinase C isozymes (11). The shared phorbol ester/diacylglycerol binding site is present in the conserved region C1, which is also within the regulatory domain of protein kinase C. Although the C1 region is present in all protein kinase C isozymes, it is truncated in the diacylglycerol-independent isozymes, i.e., the aPKC's (11).

In addition to differences in cofactor dependence among protein kinase C isozymes, the isozymes have distinct substrate specificities (16,19,20) and distinct patterns of subcellular localization (21) and tissue expression (11,22). Proteins that anchor particular protein kinase C isozymes appear to play an important role in the subcellular localization of the isozymes (23,24). The isozymes cPKC- $\alpha$ , nPKC- $\delta$ , and aPKC- $\zeta$  are expressed universally in mammalian tissues; more restricted patterns of expression have been reported for the other isozymes (11). Expression of protein kinase C isozymes is regulated by transcriptional controls and also by protease-mediated downregulation of activated isozymes (25). Based upon distinctions among the catalytic and regulatory properties of the isozymes and their patterns of expression, it has been inferred that the isozymes also have distinct functions in mammalian cells (16). In fact, there is substantial evidence that the contribution of protein kinase C to multidrug resistance in cancer cells, which is the focus of this chapter, is primarily due to the action of the isozyme cPKC- $\alpha$  (26).

### III. Protein kinase C activity and isozyme expression in human colon carcinogenesis.

Protein kinase C activity levels have been determined in normal, premalignant, and transformed human intestinal mucosal tissue specimens. In the normal human intestinal mucosa, the level of protein kinase C activity is highest in the distal ileum, lowest in the rectum, and intermediate in intervening segments (27). Protein kinase C activation is often associated with translocation of cytosolic protein kinase C to the particulate fraction of mammalian cells (25), and approximately the same percentage of protein kinase C activity is membrane-associated in each segment (27). Because cancer is rare in the small intestine, these results indicate that reduced protein kinase C activity levels are associated with increased cancer risk in the intestinal mucosa of healthy individuals (27).

A positive correlation between reduced protein kinase C activity and increased cancer risk is also observed when protein kinase C activity levels are compared in the colonic mucosa of healthy individuals and the uninvolved colonic mucosa of colon cancer patients (28). Both cytosolic and particulate fractions of the uninvolved colonic mucosa have significantly less protein kinase C activity than normal controls, and the uninvolved mucosa has an increased percentage of membrane-associated protein kinase C activity (28). Furthermore, colon adenomas are premalignant lesions, and the level of protein kinase C activity is significantly lower in human colon adenomas (27) and in their particulate fractions (29) than in the adjacent normal-appearing mucosa. Based on these observations, it can be hypothesized that the reduction in the level of colonic mucosal protein kinase C activity may be an early event in human colon carcinogenesis. Furthermore, in human colon carcinomas, the level of protein

kinase C activity is significantly reduced with respect to adjacent normal-appearing mucosa in both cytosolic and particulate fractions (27,29,30). This suggests that a progressive downregulation of protein kinase C may occur during human colon carcinogenesis.

Similarities between 1,2-dimethylhydrazine (DMH)-induced rat colon carcinogenesis and human colon carcinogenesis have been noted in studies of the level of protein kinase C activity and its intracellular distribution in normal, premalignant, and transformed colonic mucosal tissues (31,32). For example, protein kinase C activity in the uninvolved colonic mucosa of tumor-bearing DMH-treated rats is reduced compared with control rats, and an even sharper decline in protein kinase C activity is observed in the colon tumors (32). The parallel alterations in colonic mucosal protein kinase C activity in human colon carcinogenesis and DMH-induced rat colon carcinogenesis (27-32) strengthen the evidence that progressive loss of protein kinase C activity is a critical event in human colon carcinogenesis.

Prolonged exposure to phorbol-ester tumor promoters results in downregulation of protein kinase C in mammalian cells (25). Various oncogene-transformed cultured fibroblasts, including ras transformants, express elevated levels of sn-1,2-diacylglycerol and reduced levels of protein kinase C (33-35). Because phorbol-ester tumor promoters and sn-1,2-diacylglycerol activate protein kinase C by the same mechanism (25), it is thought that sn-1,2-diacylglycerol may mediate a partial downregulation of protein kinase C in the transformants (33,35). Since human colon cancers often express activated ras (36), it would seem likely that chronically elevated sn-1,2-diacylglycerol levels in the cancer cells could account for the reduced level of protein kinase C activity. However, this is not the case. In fact, human colon adenomas and carcinomas have less sn-

1,2-diacylglycerol than the adjacent normal mucosa (37,38). Thus, cellular sn-1,2-diacylglycerol levels do not offer an explanation for the relative protein kinase C activity levels in these tissues.

Members of the common, novel, and atypical protein kinase C subfamilies have been detected in tissue specimens of normal human colonic mucosa. The isozyms that have been detected in this tissue by Western analysis are cPKC- $\alpha$ , cPKC- $\beta$ , nPKC- $\delta$ , nPKC- $\epsilon$ , nPKC $\eta$ , and aPKC- $\zeta$  (39-42). There is a lack of agreement among reports from different groups concerning the relative levels of the individual isozyms detected by Western analysis in normal and transformed human colonic mucosal specimens. Three groups report similar levels of cPKC- $\alpha$  in specimens of normal human colonic mucosa and colon carcinoma (39-41), but one group observed decreased cPKC- $\alpha$  expression in human colon carcinomas (42). One report observed decreased cPKC- $\beta$  and nPKC- $\epsilon$  expression and increased nPKC- $\delta$  expression in human colon carcinomas (40), another report noted increased expression of cPKC- $\beta$ , nPKC- $\delta$ , nPKC- $\epsilon$ , nPKC $\eta$ , and aPKC- $\zeta$  in the carcinomas (41), while a third report observed decreased expression of nPKC- $\delta$ , nPKC- $\epsilon$ , nPKC- $\eta$ , and aPKC- $\zeta$  in human colon carcinomas (42). Further studies will be necessary to address the contradictory conclusions reached by these reports.

In a report that focused on the metastatic potential of human colon carcinomas in nude mice, increased metastatic potential was associated with a loss of the cPKC- $\beta$  message and increased expression of messages encoding nPKC- $\delta$ , nPKC $\eta$ , nPKC- $\theta$ , and cPKC- $\alpha$  (43). Other investigators have shown that overexpression of rat brain PKC- $\beta$ , in human colon cancer HT29 cells markedly reduces their tumorigenicity in nude mice (44). These findings suggest that cPKC- $\beta$  may be a tumor suppressor in the colonic epithelium (44).

#### IV. The contribution of protein kinase C- $\alpha$ to the intrinsic drug resistance of human colon cancer cells *in vitro*.

Multidrug resistant (MDR) cancer cells are characterized by broad spectrum resistance to chemotherapeutic drugs and sharply reduced intracellular accumulation of the drugs (4,6). Typically, MDR tumor cell lines are selected *in vitro* by exposure to a cytotoxic chemotherapeutic agent, and they express high levels of the drug-efflux pump P-glycoprotein (4,6). The cells are generally resistant to chemotherapeutic drugs that are efficiently transported by P-glycoprotein, and the reduction in the intracellular concentration of the drugs largely accounts for the resistance of the cells to their cytotoxic effects. This type of drug resistance in cancer cells is termed P-glycoprotein-mediated MDR or classical MDR (Table III) (4,6). A hallmark of classical MDR is potent reversal of the drug resistance by verapamil, which inhibits P-glycoprotein function by directly binding to the drug-efflux pump (4,6,45). The high level of expression of the P-glycoprotein-encoding message observed in surgical specimens of human colon cancer provides evidence that classical MDR may be a component of the intrinsic drug resistance of clinical colon cancer (5).

A prominent role for protein kinase C in P-glycoprotein-mediated MDR (classical MDR) has been demonstrated (26,46,47). Convincing evidence has been presented that protein kinase C directly stimulates the drug-efflux activity of P-glycoprotein by phosphorylating the pump. Protein kinase C-catalyzed phosphorylation of the linker region of P-glycoprotein in MDR cancer cells is tightly coupled to a sharp reduction in the intracellular accumulation of cytotoxic drugs and a significant enhancement of the drug resistance phenotype (48-52). Although multiple protein kinases have been shown to phosphorylate P-glycoprotein, only protein kinase C has been shown to modulate its function (26).

In human breast cancer drug-sensitive MCF7 cells and Adriamycin-selected MCF7-MDR cells, the enhancement of drug resistance and the reduction in intracellular drug accumulation achieved by phorbol ester-mediated activation of protein kinase C is reversed by verapamil. This provides evidence that the phorbol ester effects on MDR are largely due to protein kinase C-catalyzed P-glycoprotein phosphorylation in the MCF7 cell lines (53,54). Furthermore, transfection of drug-sensitive MCF7 cells with PKC- $\alpha$  does not alter the chemosensitivity of the cells. However, if MCF7 cells are first transfected with P-glycoprotein, transfection with PKC- $\alpha$  significantly enhances the MDR phenotype of the cells, and this is associated with substantial P-glycoprotein phosphorylation (55). Recent studies in a baculovirus expression system have shown that PKC- $\alpha$  directly phosphorylates isolated P-glycoprotein and thereby stimulates the ATPase activity of the pump (56). Thus, PKC- $\alpha$ -catalyzed P-glycoprotein phosphorylation may be a contributing factor in the intrinsic drug resistance of human colon cancer.

It is now evident that P-glycoprotein-independent drug resistance mechanisms are of particular importance in the clinical drug resistance of colon and other solid cancers (8). We have developed an *in vitro* model of the intrinsic drug resistance of human colon cancer (39, 57) which shows that protein kinase C activation can play a major role in non-P-glycoprotein-mediated (nonclassical) MDR in colon cancer. In the model, a metastatic human colon cancer cell line that had never been exposed to cytotoxic drugs is rendered transiently multidrug resistant by protein kinase C activation (57). Both phorbol ester and diacylglycerol protein kinase C activators induce resistance to multiple cytotoxic drugs in the cultured human colon cancer KM12L4a cells, and the induction of resistance is antagonized by protein kinase C inhibitors (57).



The  $IC_{50}$ 's of the cytotoxic drugs (drug concentrations that cause 50% cell growth inhibition) are increased significantly by 2- to 3-fold as a consequence of phorbol ester exposure, and the phorbol ester-induced resistance correlates with protein kinase C activation (rather than with its downregulation) (57).

The phorbol ester-induced resistance in human colon cancer KM12L4a cells resembles P-glycoprotein-mediated MDR in three important ways. First, the same spectrum of drugs appears to be affected in the phorbol ester-induced resistance in KM12L4 cells and in the P-glycoprotein-mediated MDR phenotype. Adriamycin, vincristine, and vinblastine are affected by phorbol esters in KM12L4a cells, and 5-fluorouracil is not (57). Second, the phorbol ester-induced drug resistance in KM12L4a cells and P-glycoprotein-mediated MDR are both associated with a pronounced defect in the intracellular accumulation of affected drugs (39,57). Third, activation of the isozyme PKC- $\alpha$  enhances P-glycoprotein-mediated MDR and triggers the induction of MDR in KM12L4a cells in response to phorbol esters (39).

The phorbol ester-induced MDR phenotype in KM12L4a cells is, however, distinguished from P-glycoprotein-mediated MDR in important ways. While P-glycoprotein-mediated MDR is associated with an increased rate of cellular drug efflux (4), phorbol ester-induced MDR in KM12L4a cells is associated with a decreased rate of drug uptake by the cells and with no apparent alteration in the rate of drug efflux (57). Similar phorbol ester effects on cellular drug uptake and efflux rates have been noted in murine leukemia P388 cells (58). Verapamil and cyclosporin A are potent reversal agents of P-glycoprotein-mediated MDR (4), but they have no effect on either phorbol ester-induced resistance or basal chemosensitivity in KM12L4a cells (Gravitt and O'Brian, manuscript in preparation). The apparent P-glycoprotein-independence of phorbol ester-induced

MDR and basal chemosensitivity in KM12L4a cells is consistent with the lack of detectable P-glycoprotein expression in the cells, as measured by Western analysis (Gravitt and O'Brian, manuscript in preparation). Thus, phorbol ester-induced MDR in KM12L4a cells and P-glycoprotein-mediated MDR are, in fact, distinct phenomena. The mechanism of the phorbol ester-induced MDR phenotype of KM12L4a cells is still unknown.

**V. The colonic lumen as a repository of endogenous protein kinase C-stimulatory agents--implications in the progression and intrinsic drug resistance of human colon cancer.**

Stimulators of protein kinase C activity are included among the major components of the contents of the colonic lumen. These protein kinase C-stimulatory agents include bile acids and dietary fat-derived diacylglycerols and free fatty acids (Table IV) (59-61). Protein kinase C-stimulatory activity appears to be a general feature of bile acids, as it has been observed with primary and secondary bile acids in both conjugated and unconjugated forms (62). Bile acids stimulate protein kinase C by direct effects on the enzyme and by stimulating the production of sn-1,2-diacylglycerol by phospholipase C (62-64). Among diet-derived diglycerides in the colonic lumen, only those with the sn-1,2 configuration have been shown to stimulate protein kinase C activity (65,66). Human fecal bacteria produce sn-1,2-diacylglycerols from dietary lipids in the colon (66). Unsaturated free fatty acids have direct stimulatory effects on protein kinase C and also induce the production of sn-1,2-diacylglycerol in colonic epithelial cells (67).

The ability of bile acids such as deoxycholate and free fatty acids to serve as tumor promoters in the colonic lumen has been demonstrated, although the

role of protein kinase C modulation in the tumor promotion of the colonic epithelium by these agents has not been defined (61). It has been inferred from the potent tumor-promoting activity of specific protein kinase C activators, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), that chronic activation of colonic epithelial protein kinase C by bile acids and dietary fat metabolites plays an important role in the mediation of tumor promotion by these agents in the colonic epithelium (59-61). Thus, chronic protein kinase C activation has been implicated in the progression of human colorectal cancer. Limiting the activation of colonic epithelial protein kinase C is therefore seen as a potentially valuable strategy for chemoprevention of the malignant disease (60).

Our model of phorbol ester-induced intrinsic drug resistance in cultured human colon cancer cells directly implicates luminal sn-1,2-diacylglycerols in the intrinsic drug resistance of clinical colon cancer (57), because phorbol esters and sn-1,2-diacylglycerols activate protein kinase C by closely related mechanisms (68). It follows logically that other protein kinase C-stimulatory agents in the colonic lumen, e.g., bile acids and free fatty acids, may also induce multidrug resistance in human colon cancer cells. In fact, we have shown that deoxycholate transiently induces a multidrug resistance phenotype in murine fibrosarcoma cell lines (63). The induction of multidrug resistance in fibrosarcoma cells by deoxycholate appears to be a result of protein kinase C activation, since phorbol-ester protein kinase C activators induce a similar response (63). Thus, protein kinase C-stimulatory luminal contents may induce multidrug resistance in colon cancer cells *in vivo* and thereby contribute to the intrinsic drug resistance of clinical colon cancer.

## VI. Targeting protein kinase C- $\alpha$ as a novel strategy for reversal of drug resistance in colon cancer therapy.

The intrinsic drug resistance of human colon cancer accounts for the limited value of chemotherapy in the management of the disease (1-3). Rational design of strategies to reverse intrinsic drug resistance in human colon cancer will require an understanding of the molecular events underlying the major drug resistance mechanisms operative in the disease. Evidence has been presented that at least three distinct mechanisms of drug resistance may be operative in human colon cancer. These mechanisms are P-glycoprotein-mediated MDR (5), DNA topoisomerase I-mediated MDR (69), and P-glycoprotein-independent, protein kinase C- $\alpha$  mediated MDR (39,57). The heterogeneous nature of drug resistance mechanisms in human colon cancer suggests that a combination of agents that antagonize drug resistance by distinct mechanisms may be required to reverse clinical drug resistance in colon cancer and improve the ultimate therapeutic outcome for the patient. Conceptually, such an approach to the reversal of drug resistance is analogous to the standard use of combination chemotherapy in cancer treatment to address the heterogeneous nature of tumor cell populations (26).

It is anticipated that P-glycoprotein-binding drugs will be of limited value in colon cancer therapy. This is based on problems of toxicity that have already been encountered in attempts to target P-glycoprotein directly for reversal of clinical drug resistance in solid tumors (8). This underscores the importance of defining the magnitude of the contribution of protein kinase C- $\alpha$ -mediated MDR to the intrinsic drug resistance of clinical colon cancer. Furthermore, definition of the importance of DNA topoisomerase I-mediated resistance in the clinical disease needs to be accomplished. Identification of additional mechanisms of drug resistance in clinical colon cancer is also a

worthwhile goal that may lead to effective strategies of chemosensitization of the disease to therapy.

Strategies for the reversal of protein kinase C- $\alpha$ -mediated MDR in colon cancer could involve targeting of either protein kinase C- $\alpha$  or molecules that function upstream or downstream of protein kinase C- $\alpha$  in the MDR phenotype. Protein kinase C- $\alpha$  is expressed ubiquitously in mammalian tissues (22,25). If it should turn out that protein kinase C- $\alpha$  inhibition is not well-tolerated in cancer patients, effective reversal of intrinsic drug resistance might still be accomplished by blocking downstream events in protein kinase C- $\alpha$ -mediated resistance. Elucidation of the mechanism of protein kinase C- $\alpha$ -mediated MDR in human colon cancer will be required to identify potential downstream targets for therapeutic intervention.

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Table I

Profile of Intrinsic Drug Resistance in Human Colon Cancer: Agents that are  
Inactive Against the Disease

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Cisplatin	Vinblastine
Carboplatin	Methotrexate
Cyclophosphamide	Etoposide
Melphalan	Tamoxifen
Vincristine	Interferons
Mitoxantrone	Interleukin-2
Doxorubicin	Tumor Necrosis Factor

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Table II  
The Protein Kinase C Isozyme Family

<u>Subfamily</u>	<u>Cofactor Dependence</u>	<u>Members</u>
Common Protein Kinase C	Ca <sup>2+</sup> , phosphatidylserine, diacylglycerol	cPKC- $\alpha$ , cPKC- $\beta_1$ , cPKC- $\beta_2$ , cPKC- $\gamma$
Novel Protein Kinase C	Phosphatidylserine, diacylglycerol	nPKC- $\delta$ , nPKC- $\epsilon$ , nPKC- $\eta$ , nPKC- $\theta$ , nPKC- $\mu$
Atypical Protein Kinase C	Phosphatidylserine, ?	aPKC- $\zeta$ , aPKC- $\lambda$



Table III

Anticancer Drugs Affected by P-glycoprotein-Mediated Multidrug Resistance

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Doxorubicin (Adriamycin)	Daunorubicin
Vincristine	Vinblastine
Etoposide	Taxol
Mitoxantrone	Actinomycin D

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Table IV

Protein Kinase C-Stimulatory Agents Present in the Colonic Lumen

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unsaturated free fatty acids

sn-1,2-diacylglycerols

unconjugated primary bile acids

tauro-and glyco-conjugated primary bile acids

unconjugated secondary bile acids

tauro- and glyco-conjugated secondary bile acids

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