

UNCLASSIFIED

AD NUMBER
ADB275102
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Jul 2001. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Ft. Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 28 Aug 2002

THIS PAGE IS UNCLASSIFIED

AD _____

Award Number: DAMD17-96-1-6237

TITLE: Targeting Mutated Epidermal Growth Factor Receptor

PRINCIPAL INVESTIGATOR: Dorothee M. Herlyn, D.V.M.

CONTRACTING ORGANIZATION: The Wistar Institute
Philadelphia, Pennsylvania 19104

REPORT DATE: July 2001

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Jul 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020215 032

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-96-1-6237
Organization: The Wistar Institute

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Alvinna Swan
01/15/2002

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2001	3. REPORT TYPE AND DATES COVERED Final (01 Jul 96 - 30 Jun 01)	
4. TITLE AND SUBTITLE TITLE: Targeting Mutated Epidermal Growth Factor Receptor			5. FUNDING NUMBERS DAMD17-96-1-6237	
6. AUTHOR(S) Dorothee M. Herlyn, D.V.M.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Wistar Institute Philadelphia, Pennsylvania 19104 E-Mail: DHerlyn@wistar.upenn.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only (proprietary information, Jul 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) During this four-year funding and one-year no cost extension periods we have established a rat model of active specific immunotherapy against mutated epidermal growth factor receptor (EGF-RvIII) specifically expressed by breast carcinomas. The mutation is not expressed by normal breast tissues. The model system consists of rat mammary carcinomas expressing EGF-RvIII after cDNA transfer and EGF-RvIII vaccines, including recombinant EGF-RvIII protein, EGF-RvIII expressed in adenovirus vector and peptides mimicking EGF-RvIII. Expression of EGF-RvIII by the transfected rat mammary carcinoma cells was instable due to vector mutations. The vector was corrected but rat mammary carcinoma cells transfected with the vector did not stably express EGF-RvIII. Future studies will be aimed at obtaining stable transfectants. These will allow us to test the available vaccines for their tumor growth inhibiting properties. We have also evaluated breast cancer patients' immune responses to EGF-RvIII. Most patients with EGF-RvIII-positive lesions had circulating antibodies, and a few patients also EGF-RvIII specific lymphoproliferative responses. These responses may be beneficial to the patients, and this issue will be addressed in future studies in a larger population of patients.				
14. SUBJECT TERMS Breast cancer			15. NUMBER OF PAGES 25	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover	
SF 298	2
Table of Contents	3
Introduction	5
Body	5
Key Research Accomplishments	10
Reportable Outcomes	10
Conclusions	11
References	12
Appendices	15-25

INTRODUCTION

(adapted from the previous report)

Clinical trials of active immunotherapy in breast carcinoma patients have suffered from the use of vaccines that induce only humoral, but not cellular, immunity (1,2) or lack specificity (3). Preclinical and clinical studies with cancer vaccines have demonstrated a correlation between the induction of humoral or cellular immunity and tumor growth inhibition (4-12). Thus, tumor vaccines ideally should induce both humoral and cellular immunity and induced immunity should be specific for the tumor cells. The major goal of this study is to test tumor-specific vaccines against breast carcinoma in a relevant rat model. Mutated epidermal growth factor receptor (EGF-RvIII) is expressed by a high proportion of breast carcinoma tissues derived from various patients, but not several normal tissues tested (13; and our unpublished data described in the original proposal). EGF-RvIII is expressed both on the surface and in the cytoplasm of tumor cells (13), rendering it a target for both B and T cells. Furthermore, targeting of EGF-RvIII may exert direct anti-proliferative effects (14).

We have chosen a rat model of EGF-RvIII vaccines for the proposed studies because of the availability of cloned normal rat EGF-R (15) and MHC class I and II positive rat mammary carcinoma cells with either high or low metastatic capability (16,17). The most specific vaccine of EGF-RvIII consists of the minimal sequence, including the mutation, that elicits B- and/or T-cell responses. We have chosen peptides of EGF-RvIII for induction of T cells, analogous to studies performed successfully with peptide vaccines by other groups (18-24) and our collaborators (25-27) in various antigen systems. In addition, recombinant, extracellular EGF-RvIII protein and full-length EGF-RvIII expressed in adenovirus vector have been chosen for induction of protective humoral and cellular immunity, respectively.

In conclusion, EGF-RvIII is a unique target for active specific immunotherapy of breast carcinoma, based on its specificity, frequency of expression, potential for activating both B and T cells, and availability of an ideal animal model of active immunotherapy. The studies will provide the rationale for specific active immunotherapy of breast carcinoma patients. The results we will obtain with EGF-RvIII in the rat mammary carcinoma model may be applicable to other tumor systems, such as lung carcinomas and gliomas which also express EGF-RvIII (13,28).

BODY

This final report includes a 4-year funding period and a 1-year no-cost extension period. The final report has been adapted from the previous report and the progress made during the past year (no cost extension period) for tasks 1 and 10 is indicated in italics.

Task 1: Transfection of two rat mammary carcinoma cell lines with available EGF-RvIII cDNA. Characterization of the transfectants.

Rat mammary carcinoma transfectants MTLN3 were generated which expressed the 145 kDa rat EGF-RvIII protein by Western blot analysis of whole cell extract, but did not express the protein in membrane extract. The transfectants did not react with murine monoclonal antibody

(MAb) L8 directed to the mutated epitope on EGF-RvIII. Thus, the transfectants did express EGF-RvIII protein, but lacked expression of the mutated epitope. They were tumorigenic in syngeneic rats. We had attributed our failure to obtain rat mammary carcinoma cells stably expressing rat EGF-RvIII protein to mutational events that possibly had occurred in the transfectants in vivo. Therefore, in order to obtain stable transfectants, we performed four independent transfections of 115 million cells total using various methods of transfection. Forty-eight colonies were isolated that showed 40% or more of the cells within a colony expressing EGF-RvIII defined by MAb L8A4. However, all colonies lost EGF-RvIII expression after 3-4 months in culture. Furthermore, attempts to isolate EGF-RvIII cells by fluorescence-activated cell sorting were unsuccessful. We therefore sequenced the EGF-R PcDNA3 vector used for cell transfection. There were two mutations each in the extracellular and intracellular domain of the EGF-RvIII vector. These mutations must have occurred at some point during EGF-RvIII vector propagation in vitro as they were not found in the vector when it was originally constructed and sequenced.

The data summarized above have been described in detail in Fig. 2 of the July 1997 report, and in the 1998 and 1999 reports.

During year 4, we sequenced the normal (non-mutated) EGF-R vector and found the sequence to be identical with the published sequence in Gene Bank. This vector was then used to correct the EGF-RvIII vector. We combined the correct 5' piece of EGF-RvIII with the 3' piece of the wild-type (wt) EGF-R (pLXSN vector supplied by Dr. Shelton Earp). A 1.4 kb *HindIII/BamHI* fragment of the EGF-RvIII clone was ligated into pcDNA3.1 Zeo. One of several positive clones (clone 5) was used for all further constructions. DNA of clone 5 and also pLXSN wtEGF-R were digested with *PpuMI/PstI*. *PpuMI/PstI* cuts 212 bp downstream of the desired deletion mutation and before the first undesired mutation. The 3.3 kb *PpuMI/PstI* fragment derived from pLXSN/wtEGF-R was ligated into the cut clone 5/EGF-RvIII/pcDNA3. The corrected EGF-RvIII vector was sequenced and the sequence was found to be 100% identical with the published sequence in gene bank.

During the past year (no cost extension period) the corrected EGF-RvIII vector was used to transfect rat mammary carcinoma cells MTLN3. Six independent transfections were performed using lipofectamine and FuGene methods. Transfectants were selected by FACS analyses using anti-EGF-RvIII MAb L8. However, there was no enrichment for antigen-positive cells (<5% cells positive after sorting). In contrast, we have successfully transfected a rat glioma cell line. These transfectants are >80% positive for EGF-RvIII. Thus, the vector is functional, but the rat mammary cells MTLN3 do not stably integrate the vector. We will now try to transfect another rat mammary carcinoma cell line (MTCT10).

Task 2: Production and characterization of peptides of rat EGF-RvIII and corresponding control peptides.

Five different rat EGF-RvIII peptides were selected for expression of MHC class I and/or class II binding motives. Only one peptide (peptide 4) bound specifically to MAb L8 (see Table 1 of the July 1997 report and Fig. 1 of the July 1998 report for details).

Task 3: Production and characterization of rat EGF-RvIII derived from recombinant baculovirus.

A baculovirus expression vector for rat EGF-RvIII was constructed and the presence of the correct insert was confirmed by sequencing. Recombinant baculovirus was produced and used to infect insect cells. The supernatant of the infected insect cells was tested by Western blot analysis for the presence of the mutated protein (extracellular domain), using MAb NCL-EGF-R to the extracellular domain of human wt EGF-R as a probe. MAb NCL-EGF-R reacted with the 41 kDa band characteristic of the extracellular domain of rat EGF-RvIII in 24 hr supernatants. The protein was purified from the supernatant by HPLC and tested by Western blot analysis and ELISA using MAb L8. The characteristic 41 kDa band of EGF-RvIII was detected by Western blot analysis and MAb L8 specifically reacted with EGF-RvIII protein in ELISA.

Rats were immunized with rat EGF-RvIII protein in complete and incomplete Freund's adjuvant. There was a trend for the lower antigen dose yielding higher antibody response than the higher dose. The antibodies not only bound to rat EGF-RvIII, but also to normal rat EGF-R protein. Thus, rats are not immunologically tolerant to normal EGF-R administered in adjuvant although this protein is widely expressed by normal rat tissues. Immune responses to normal EGF-R were not accompanied by toxicity as determined macroscopically in those organs which express EGF-R. These data have been described in detail in Figs. 4, 5, and 6 of the July 1997 report and Figs. 2 and 3 of the July 1998 report.

Task 4: In vitro responses of rat lymphocytes to stimulation with peptides.

These studies could not be performed because we lack syngeneic rat mammary carcinoma cells expressing the EGF-RvIII epitope. These cells are needed to test proliferating lymphocytes from EGF-RvIII-immunized mice for cytolytic activity.

Task 5: Production and characterization of six peptides of human EGF-RvIII and corresponding control peptides.

We have produced six peptides of human EGF-RvIII which were described in detail in the previous reports. The peptides were selected for expression of anchors for HLA class I and/or II (for details see Table in the 1998 report).

Task 6: Immunization of rats with rat EGF-RvIII peptides and characterization of immune responses.

Peptides of rat EGF-RvIII were incorporated into microspheres and injected with or without Titermax adjuvant. Of the five peptides, only peptide 4 (with or without adjuvant) and peptide 5 (with adjuvant only) induced antibodies binding specifically to EGF-RvIII as compared to bovine serum albumin. No such antibodies were induced in the control-immunized rats. These data have been described in detail in Fig. 3 of the 1998 report.

Task 7: Tumor protection studies with peptides in rats.

These studies could not be performed because we lack syngeneic rat mammary carcinoma cells expressing the EGF-RvIII epitope.

Task 8: Production and in vitro characterization of up to 20 anti-idiotypes.

We will receive from Dr. Darell Bigner two anti-idiotypic antibodies directed against monoclonal anti-EGF-RvIII antibodies Y10. These anti-idiotypes mimic EGF-RvIII in vitro and induce tumor protective immune responses in a mouse tumor model (D. Bigner, personal communication). The anti-idiotypes will be tested in our rat model of EGF-RvIII when the target cells become available. The murine anti-idiotypes most likely will be more immunogenic in xenogeneic rats than in syngeneic mice (our unpublished observations) and, therefore, they may have superior anti-tumor activity in our rat model as compared to Dr. Bigner's mouse model.

Task 9: In vivo characterization of 2-3 selected anti-idiotypes.

These studies will be performed when syngeneic EGF-RvIII expressing mammary carcinoma cells will be available.

Task 10: In vitro stimulation of patients' lymphocytes with peptides and anti-idiotypes.

We have produced peptides of human EGF-RvIII (see task 5) and recombinant human EGF-RvIII protein for the stimulation of cancer patients' lymphocytes in vitro. Positive lymphocyte stimulation would indicate that the peptides and/or the protein may serve as vaccines for breast cancer patients. Human EGF-RvIII extracellular domain was produced in recombinant baculovirus. Western blot analysis of purified human EGF-RvIII protein derived from baculovirus-infected insect cells revealed the 30 kDa protein characteristic of the extracellular domain of human EGF-RvIII. Analysis of the purified protein in ELISA shows its reactivity with both MAb NCL-EGF-R to human wtEGF-R and MAb L8 to human EGF-RvIII. These data have been described in detail, supported by Figs. 5 and 6, in the July 1997 report.

Preliminary studies shown in the previous report (July 1999) have suggested that breast cancer patients with EGF-RvIII-positive lesions produce specific antibodies against EGF-RvIII and show lymphoproliferative responses specific for EGF-RvIII. There seemed to be a correlation between the expression of EGF-RvIII by tumor tissue and humoral immune response development. However, these studies included only five patients. During the fourth year of funding an additional eleven patients were included in these studies. Figure 1 (A-G) shows

EGF-RvIII specific antibody responses determined in the sera of 7 breast cancer patients. The details of the assay used are described in Fig. 1 legend. The antibodies which bound to EGF-RvIII did not bind to wt EGF-R or carcinoembryonic antigen (CEA). In positive control experiments, antibodies to wtEGF-R or CEA bound to their respective antigen (Fig. 1H). The sera of nine additional breast carcinoma patients did not bind to EGF-RvIII (Fig. 2 A-I), neither did the sera of three healthy donors (Fig. 3). *During the past year we have tested serum reactivity of the patients against human EGF-RvIII positive transfectants NR6M and found that in 5 of the 7 patients who had developed EGF-RvIII protein-reactive antibodies these antibodies also reacted with EGF-RvIII positive cells (Table).*

We also tested lymphoproliferative responses of the above described breast cancer patients after stimulation of the lymphocytes with EGF-RvIII protein, EGF-RvIII peptides or control preparations. The details of the assay used are described in Fig. 4 legend. As shown in Fig. 4, three patients had significant lymphoproliferative responses to stimulation with EGF-RvIII protein compared to stimulation with wtEGF-R or no stimulation. Two of the three patients (DM and TF; Fig. 4 B and C) also had specific and significant lymphoproliferative responses to stimulation with EGF-RvIII peptide in PLG microspheres. In contrast, 13 other breast cancer patients did not show EGF-RvIII-specific lymphoproliferative responses, although their lymphocytes responded to phytohemagglutinin (PHA) stimulation (Fig. 5). Although we had originally planned to stimulate patients' lymphocytes with anti-idiotypes mimicking EGF-RvIII, these studies were not performed because F(ab')₂ fragments of the anti-idiotypic are needed for lymphocyte stimulation. These fragments were not available in sufficient quantities. Instead, we used recombinant baculovirus-derived EGF-RvIII protein for lymphocyte stimulation (see Figs. 4 and 5).

To determine whether there was a correlation between immune response to EGF-RvIII and EGF-RvIII expression by the patients' tumors, tumors were evaluated for EGF-RvIII expression by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry with MAb L8 (Table). *RT-PCR determinations were completed in all patients' tissues (except for patient EJ from whom no more tissue sample was available) during the past year. Nine of 15 patients' tissues were positive for EGF-RvIII by RT-PCR. Four of these patients (DI, KJ, MC, HrJ) had developed antibodies binding specifically to the mutated protein.*

All five patients with lesions positive for EGF-RvIII by immunohistochemistry (KJ, MC, BD, EJ, KrJ) had developed specific antibodies to EGF-RvIII baculovirus-derived protein (Table). One patient (TF) had humoral and cellular immune responses, but EGF-RvIII-negative lesions as determined both by RT-PCR (see above) and immunohistochemistry. The specimen obtained from this patient's lesion may not be representative of the tumor as a whole.

Only three patients (KJ, TF, DM) had EGF-RvIII-specific lymphoproliferative responses. These responses correlated with tumor EGF-RvIII expression in only one patient (KJ).

There was a significant correlation ($p=0.03$; Fisher's exact test) between patients' humoral immune responses to EGF-RvIII protein and EGF-RvIII tumor expression (determined

by immunohistochemistry. There was no significant correlation between cellular immune responses and EGF-RvIII expression by immunohistochemistry or either cellular or humoral immune responses and EGF-RvIII detection by RT-PCR. Absence of a correlation between immune response and EGF-RvIII presence by RT-PCR is not surprising in the absence of protein expression (patients PL, RS, BR, DM). Immunohistochemistry and RT-PCR data also did not significantly correlate in the group of patients, presumably because of sensitivity differences between the two assays or different parts of the specimens used in the two assays which are not representative of the tumor as a whole. Immune responses to EGF-RvIII protein also did not correlate with the HER2-neu or estrogen receptor status of the tumors. A larger study is needed to establish such correlation.

KEY RESEARCH ACCOMPLISHMENTS (4-year funding period and 1 year no cost extension)

- Rat mammary carcinoma cells were transfected with rat EGF-RvIII, but expression of the mutated epitope was instable, probably due to additional mutations in the rat EGF-RvIII vector. The vector has been corrected, *but was unable to transfer EGF-RvIII expression to rat mammary carcinoma cells.*
- Various human and rat-derived EGF-RvIII peptides, recombinant proteins, and EGF-RvIII expressed in adenovirus have been produced. These reagents serve as targets or stimulants for the determination of humoral or cellular immune responses in rats and humans.
- Rats produced antibodies to EGF-RvIII peptides and baculovirus-derived protein. Immune responses were directed both to wt and mutated sequences of EGF-RvIII. Thus rats are not immunologically tolerant to wtEGF-R despite the expression of this protein by many normal rat tissues.
- Breast cancer patients develop antibodies and proliferative lymphocyte responses to EGF-RvIII expressed by their tumors. These responses are specific for the mutated epitope on EGF-RvIII. *Antibody responses to EGF-RvIII significantly correlated with EGF-RvIII protein expression by the patients' tumors.*

REPORTABLE OUTCOMES

Abstracts (see appendix)

Immune responses of breast cancer patients to mutated epidermal growth factor receptor (mEGF-R); Purev, E., Cai, D.W., Miller, E., Birebent, B., Somasundaram, R., Mayer, T., and Herlyn, D.; Albert Einstein Med. Ctr., Philadelphia, PA, Memorial Hosp. of Burlington, Mt. Holly, NJ, and The Wistar Institute, Philadelphia, PA. Scientific Proceedings, 91st Annual Meeting of the American Association for Cancer Research, April 1-5, 2000, San Francisco, CA.

Immune responses of breast cancer patients to mutated epidermal growth factor receptor (mEGF-R); Purev, E., Cai, D.W., Miller, E., Birebent, B., Somasundaram, R., Mayer, T., and Herlyn, D.; The Wistar Institute, Philadelphia, PA 19104, Memorial Hospital of Burlington, Mt. Holly, NJ 08060, and Albert Einstein Medical Center, Philadelphia, PA 19104. Era of Hope Meeting, June 8-11, 2000, Atlanta, GA.

Development of tissue and serum repositories.

Tumor tissue, lymphocyte, and serum repositories have been developed from sixteen breast cancer patients, most of them with primary tumors without lymph node involvement.

Funding applied for.

We have applied to the National Cancer Institute for funding of a study on immune responses of breast cancer patients to EGF-RvIII and the prognostic value of EGF-RvIII for clinical outcome. The revised application will be submitted 11-1-01.

CONCLUSIONS

We have developed a rat model of active specific immunotherapy against mammary carcinoma targeting EGF-RvIII. EGF-RvIII is a tumor-specific antigen which is not expressed by any normal tissues tested. During the funding period, various vaccines of rat and human EGF-RvIII have been developed. These include peptides, recombinant proteins and the protein expressed in adenovirus which will be tested for their tumor growth inhibiting properties in vivo when rat mammary carcinoma cells expressing the EGF-RvIII epitope become available. Although we performed numerous transfections of rat mammary carcinoma cells with rat EGF-RvIII cDNA, the transfectants expressed the mutated protein but not the mutated epitope. Our future studies are aimed at generating transfectants which express the mutated epitope and use them in studies of cellular and protective immunity against EGF-RvIII.

We have shown that breast cancer patients raise specific immune responses to EGF-RvIII. These responses are expected to be induced by EGF-RvIII expressed by the patients' tumors, and there was a statistically significant correlation between the humoral immune response to EGF-RvIII protein and EGF-RvIII expression by the patient's tumor. Future studies will determine whether EGF-RvIII expression by the patients' tumors is a prognostic factor which determines clinical outcome of the disease in breast cancer patients.

REFERENCES

1. MacLean, G.D., Reddish, M., Koganty, R.R., Wong, T., Gandhi, S., Smolenski, M., Samuel, J., Nabholz, J.M., and Longenecker, B.M. 1993. Immunization of breast cancer patients using a synthetic sialyl-Tn glycoconjugate plus Detox adjuvant. *Cancer Immunol. Immunother.* 36:215-222.
2. MacLean, G.D., Reddish, M.A., Bowen-Yacyshyn, M.B., Poppema, S., and Longenecker, B.M. 1994. Active specific immunotherapy against adenocarcinomas. *Cancer Invest.* 12:46-56.
3. Lytle, G.H., McGee, J.M., Yamanashi, W.S., Malnar, K., and Bellefeuille, C. 1994. Five-year survival in breast cancer treated with adjuvant immunotherapy. *Am. J. Surg.* 168:19-21.
4. Livingston, P.O., Natoli, E.J., Calves, M.J., Stockert, E., Oettgen, H., and Old, L.J. 1987. Vaccines containing purified GM2 ganglioside elicit GM2 antibodies in melanoma patients. *Proc. Natl. Acad. Sci. USA* 84:2911-2915.
5. Wallack, M.K., Bash, J.A., Leftheriotis, E., Seigler, H., Bland, K., Wanebo, H., Balch, C., and Bartolucci, A.A. 1987. Positive relationship of clinical and serologic responses to vaccinia melanoma oncolystate. *Arch. Surg.* 122:1460-1463.
6. Mittelman, A., Chen, Z.J., Yang, H., Wong, G.Y., and Ferrone, S. 1992. Human high molecular weight melanoma-associated antigen (HMW-MAA) mimicry by mouse anti-idiotypic monoclonal antibody MK2-23: induction of humoral anti-HMW-MAA immunity and prolongation of survival in patients with stage IV melanoma. *Proc. Natl. Acad. Sci. USA* 89:466-470.
7. Portoukalian, J., Carrel, S., Dore, J.-F., and Rumke, P. 1991. Humoral immune response in disease-free advanced melanoma patients after vaccination with melanoma-associated gangliosides. *Int. J. Cancer* 49:893-899.
8. Riethmüller, G., Schneider-Gädicke, E., Schlimok, G., Schmiegel, W., Raab, R., Höffken, K., Gruber, R., Pichlmaier, H., Hirche, H., Pichlmayr, R., Buggisch, P., Witte, J., and the German Cancer Aid 17-1A Study Group. 1994. Randomised trial of monoclonal antibody for adjuvant therapy of resected Dukes' C colorectal carcinoma. *Lancet* 343:1177-1183.
9. Sahasrabudhe, D.M., deKernion, J.B., Pontes, J.E., Ryan, D.M., O'Donnell, R.W., Marquis, D.M., Mudholkar, G.S., and McCune, C.S. 1986. Specific immunotherapy with suppressor function inhibition for metastatic renal cell carcinoma. *J. Biol. Resp. Mod.* 5:581-594.
10. Mitchell, M.S., Kan-Mitchell, J., Kempf, R.A., Harel, W., Shau, H., and Lind, S. 1988. Active specific immunotherapy for melanoma: phase I trial of allogeneic lysates and a novel adjuvant. *Cancer Res.* 48:5883-5893.
11. Berd, D., Maguire, H.C., Jr., McCue, P., and Mastrangelo, M.M. 1990. Treatment of metastatic melanoma with an autologous tumor-cell vaccine: clinical and immunologic results in 64 patients. *J. Clin. Oncol.* 8:1858-1867.
12. Liebrich, W., Schlag, P., Manasterski, M., Lehner, B., Stohr, M., Moller, P., and Schirmacher, V. 1991. In vitro and clinical characterization of a Newcastle disease virus-modified autologous tumor cell vaccine for treatment of colorectal cancer patients. *Eur. J. Cancer* 27:703-710.

13. Wikstrand, C.J., Hale, L.P., Batra, S.K., Hill, M.L., Humphrey, P.A., Kurpad, S.N., McLendon, R.E., Moscatello, D., Pegram, C.N., Reist, C.J., Traweck, S.T., Wong, A.J., Zalutsky, M.R., and Bigner, D.D. 1995. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res.* 55:3140-3148.
14. Nishikawa, R., Ji, X.-D., Harmon, R.C., Lazar, C.S., Gill, G.N., Cavenee, W.K., and SuHuang, H.-J. 1994. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc. Natl. Acad. Sci. USA* 91:7727-7731.
15. Petch, L.A., Harris, J., Raymond, V.W., Blasband, A., Lee, D.C., and Earp, H.S. 1990. A truncated, secreted form of the epidermal growth factor receptor is encoded by an alternatively spliced transcript in normal rat tissue. *Mol. Cell. Biol.* 10:2973-2982.
16. Neri, A., Welch, D., Kawaguchi, T., and Nicolson, G.L. 1982. Development and biologic properties of malignant cell sublines and clones of a spontaneously metastasizing rat mammary adenocarcinoma. *J. Natl. Cancer Inst.* 68:507-517.
17. Lichtner, R.B., Kaufmann, A.M., Kittmann, A., Rohde-Schulz, B., Walter, J., Williams, L., Ullrich, A., Schirmacher, V., and Khazaie, K. 1995. Ligand mediated activation of ectopic EGF receptor promotes matrix protein adhesion and lung colonization of rat mammary adenocarcinoma cells. *Oncogene* 10:1823-1832.
18. Peace, D.J., Smith, J.W., Chen, W., You, S.-G., Cosand, W.L., Blake, J., and Cheever, M.A. 1994. Lysis of *ras* oncogene-transformed cells by specific cytotoxic T lymphocytes elicited by primary in vitro immunization with mutated *ras* peptide. *J. Exp. Med.* 179:473-479.
19. Yanuck, M., Carbone, D.P., Pendleton, C.D., Tsukui, T., Winter, S.F., Minna, J.D., and Berzofsky, J.A. 1993. A mutant *p53* tumor suppressor protein is a target for peptide-induced CD8+ cytotoxic T-cells. *Cancer Res.* 53:3257-3261.
20. Peace, D.J., Chen, W., Nelson, H., and Cheever, M.A. 1991. T cell recognition of transforming proteins encoded by mutated *ras* proto-oncogenes. *J. Immunol.* 146:2059-2065.
21. Fenton, R.G., Taub, D.D., Kwak, L.W., Smith, M.R., and Longo, D.L. 1993. Cytotoxic T-cell response and in vivo protection against tumor cells harboring activated *ras* proto-oncogenes. *J. Natl. Cancer Inst.* 85:1294-1302.
22. Fossum, B., Olsen, A.C., Thorsby, E., and Gaudernack, G. 1995. CD8+ T cells from a patient with colon carcinoma, specific for a mutant p21-ras-derived peptide (GLY¹³ØASP), are cytotoxic towards a carcinoma cell line harbouring the same mutation. *Cancer Immunol. Immunother.* 40:165-172.
23. Feltkamp, M.C.W., Smits, H.L., Vierboom, M.P.M., Minnaar, R.P., de Jongh, B.M., Drifjhout, J.W., ter Schegget, J., Melief, C.J.M., and Kast, M.W. 1993. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur. J. Immunol.* 23:2242-2249.
24. Apostolopoulos, V., Xing, P.-X., and McKenzie, I.F.C. 1994. Murine immune response to cells transfected with human MUC1: immunization with cellular and synthetic antigens. *Cancer Res.* 54:5186-5193.
25. Dietzschold, B., Wang, H., Rupprecht, C.E., Celis, E., Tollis, M., Ertl, H., Heber-Katz, E., and Koprowski, H. 1988. Induction of protective immunity against rabies by

- immunization with rabies virus ribonucleoprotein. *Proc. Natl. Acad. Sci. USA* 84:9165-9169.
26. Ertl, H.C.J., Dietzschold, B., Gore, M., Otvos, L., Jr., Wunner, W.H., and Koprowski, H. 1989. Induction of rabies virus-specific T-helper cells by synthetic peptides that carry dominant T-helper cell epitopes of the viral ribonucleoprotein. *J. Virol.* 63:2885-2892.
 27. Ertl, H.C.J., Dietzschold, B., and Otvos, L., Jr. 1991. T helper cell epitope of rabies virus nucleoprotein defined by tri- and tetrapeptides. *Eur. J. Immunol.* 21:1-10.
 28. Garcia de Palazzo, I.E., Adams, G.P., Sundareshan, P., Wong, A.J., Testa, J.R., Bigner, D.D., and Weiner, L.M. 1993. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinoma. *Cancer Res.* 53:3217-3220.

APPENDICES

Abstracts, Table, Figures 1-5.

SCIENTIFIC PROCEEDINGS

91st Annual Meeting of the American Association for Cancer Research

San Francisco, CA
April 1-5, 2000

#4443 IMMUNE RESPONSES OF BREAST CANCER PATIENTS TO MUTATED EPIDERMAL GROWTH FACTOR RECEPTOR (MEGF-R). Enkhtsetseg Purev, D. W. Cai, E. Miller, B. Birebent, R. Somasundaram, T. Mayer, and D. Herlyn, *Albert Einstein Med Ctr, Philadelphia, PA, Memorial Hosp of Burlington, Mt. Holly, NJ, and The Wistar Institute, Philadelphia, PA*

mEGF-R is expressed by carcinomas of the breast, ovary, lung and colon, and also by gliomas, but not by normal tissues. The mEGF-R is the result of an 801 bp deletion within the extracellular domain of normal EGF-R and is expressed both on the surface and in the cytoplasm of the tumor cells. Thus, mEGF-R is a potential tumor-specific target for B and/or T cells in active and passive immunotherapy against tumors. We have evaluated humoral and cellular immune responses to mEGF-R in eight breast cancer patients and three healthy donors. Four patients with tumors expressing mEGF-R developed mEGF-R-specific humoral immune responses and two of these patients also developed mEGF-R-specific cellular immune responses. None of the patients with mEGF-R negative tumors developed cellular immune responses, and only one patient developed antibodies to mEGF-R. None of the three healthy donors demonstrated mEGF-R-specific humoral or cellular immune responses. These studies demonstrate that breast cancer patients can immunologically recognize mEGF-R and suggest that enhancement of the immune responses may be possible by vaccination of the patients against mEGF-R. (Supported by DAMD17-96-1-62 37 from the U.S. Department of Defense.)

Era of Hope Meeting
June 8-11, 2000
Atlanta, GA

**IMMUNE RESPONSES OF BREAST CANCER PATIENTS TO
MUTATED EPIDERMAL GROWTH FACTOR RECEPTOR (MEGF-R)**

**E. Purev, D.W. Cai, E. Miller, B. Birebent,
R. Somasundaram, T. Mayer, and D. Herlyn**

The Wistar Institute, Philadelphia, PA 19104; Memorial
Hospital of Burlington, Mt Holly, NJ 08060; and Albert
Einstein Medical Center, Philadelphia, PA 19104

Epidermal growth factor binds to epidermal growth factor receptor (EGF-R) located on normal cells. The binding initiates cell division and growth. When cells become cancerous, EGF-R is mutated (mEGF-R) due to the deletion of 261 amino acids in the extracellular domain. MEGF-R expression is directly correlated with the degree of cancer malignancy. Several cancers including carcinomas of breast, ovary, lung and gliomas express mEGF-R. Normal cells do not express mEGF-R. Thus, mEGF-R is a potential target for active specific immunotherapy against cancer. We have evaluated the cellular and humoral immune responses to mEGF-R in eight breast cancer patients and three healthy donors. Four patients with tumors expressing mEGF-R developed antibodies binding specifically to mEGF-R, but not to normal EGF-R. Only one of the four other patients with tumors that lacked mEGF-R expression developed antibodies to mEGF-R. None of the three healthy donors demonstrated mEGF-R specific antibodies. Two of eight patients developed lymphoproliferative responses to stimulation with mEGF-R protein, but not with control proteins. Both patients' tumors expressed mEGF-R. None of the three healthy donors demonstrated such lymphoproliferative responses. This study demonstrates that breast cancer patients can immunologically recognize mEGF-R and suggests that enhancement of the immune responses may be possible by vaccination of cancer patients against mEGF-R. (Supported by DAMD17-96-1-62 37 from the U.S. Department of Defense).

Table. Summary of patients' clinical status and immune responses

Initials (Age in years)	Patients	HLA-type	Lymph node involvement	Tumor Diameter (cm)	Estrogen Receptor	HER2-neu	Time interval between surgery and blood sampling (months)	Tumor ^a expression of EGF-RvIII		Immune responses to EGF-RvIII		
								RT-PCR	Immunohistochemistry	Humoral	Protein	Cells
DI (74)	A*02: B*07:1401; Cw*07:1505; DRb1*0101,11; DQb1*0301,0501; DRb3*02		+	5.0	+	NT	2.5	+	-	+	+	-
KJ (51)	A*01: B*57; Cw*06; DRb1*0701,11; DQb1*0301,0303; DRb3*02; DRb4*01		-	2.0	+	NT	37	+	+	+	+	+
MC (71)	A*01,03; B*15,57; Cw*03,06; DRb1*1501,11; DQb1*0301,06; DRb3*02; DRb5*01		-	1.4	+	NT	2.5	+	+	+	+	-
TF (62)	A*01,03; B*07; Cw*07; DRb1*0101,1501; DQb1*0501,06; DRb5*01		-	4.5	+	NT	44	-	-	+	+	+
CR (45)	A*02,03; B*07,44; Cw*0501,0702; DRb1*1501,14; DQb1*0503,06; DRb3*02; DRb5*01		-	1.5	NT	NT	38	-	-	-	-	-
FM (36)	A*0201; B*27,40; Cw*0102,0304; DRb1*1501,0801; DQb1*04,06; DRb5*01		+ ^b	2.5	-	NT	44	-	-	-	-	-
GI (70)	A*01,24; B*07,08; Cw*07,0702; DRb1*03,04; DQb1*02,03; DRb3*01; DRb4*01		+	5.0	NT	NT	45	-	-	-	-	-
BD (48)	A*01,03; B*07,08; Cw*07; DRb1*03,0402; DQb1*02,03; DRb3*01,4*01		+	2.0	-	-	before surgery	+	+/-	+	-	-
PL (42)	A*01,02; B*08; Cw*0701; DRb1*03,15; DQb1*02,06; DRb3*01,5*01		-	2.0	+	-	before surgery	+	-	-	-	-
RS (66)	A*02,09; B*44; Cw*05,1601; DRb1*04,04; DQb1*03,03; DRb4*01		-	2.1	NT	+	before surgery	+	-	-	-	-

Table. Summary of patients' clinical status and immune responses (continued)

Initials (Age in Years)	Patients HLA-type	Lymph node involvement	Tumor			Time interval between surgery and blood sampling (months)	Immune responses to EGF-RvIII				
			Diameter (cm)	Estrogen Receptor	HER2-neu		Tumor ^a expression of EGF-RvIII	Humoral	Cellular		
							RT-PCR	Immunohistochemistry	Protein	Cells	Cellular
BR (61)	A*01,6601; B*5802; Cw*0602,1801; DRb1*13,1602; DQb1*0303,05;DRB 3*02, 5*02	-	1.2	+	+	before surgery	+	-	-	-	-
DM (66)	A*02,3402; B*35,53; Cw*0401,0602; DRb1*03,1503; DQb1*02,0506;DRB 3*02, 5*00	-	1.1	NT	+	before surgery	+	-	-	-	+
DP (38)	A*01; B*08; Cw*0701; DRb1*03,15; DQb1*02,06;DRb 3*01, 5*01	+	1.4	-	+	before surgery	-	-	-	-	-
GB (52)	A*0201,02,32; B*07,35; Cw*04,0702; DRb1*08,11; DQb1*03,04;DRb 3*02	-	1.5	NT	+	before surgery	-	-	-	-	-
EJ (69)	A*0202,02,03; B*15,41	-	1.9	+	-	before surgery	NT	+/-	+	+	-
KJ (71)	A*01,2501; B*18,44; DRb1*14,15; DQb1*0503,06;DRb 3*02, 5*01	-	4	-	NT	before surgery	-	+	+	-	-

^a Primary tumor.

^b Expression of EGF-RvIII was tested on the primary breast cancer lesion.

NT not tested

FIGURE LEGENDS

Fig. 1. Antibodies to EGF-RvIII in sera of breast cancer patients. Sera from breast cancer patients (panel A-G; for EGF-RvIII expression by the lesions, see table) were tested for binding to EGF-RvIII by measuring their capacity to inhibit binding of ^{125}I -labeled anti-EGF-RvIII MAb L8A4 to EGF-RvIII in radioimmunoassay. Wells coated with 10 $\mu\text{g}/\text{ml}$ of anti-wtEGF-R MAb 425 were incubated first with 10 $\mu\text{g}/\text{ml}$ of purified EGF-RvIII protein and then with serum dilutions (or 1-20 $\mu\text{g}/\text{ml}$ of positive control MAb L8A4; panel H). ^{125}I -labeled MAb L8A4 (20 K cpm/well) was added and % inhibition of binding of ^{125}I -MAb L8A4 to EGF-RvIII by sera or MAb L8A4 was calculated relative to buffer control.

To measure binding of the sera to wtEGF-R, wells coated with MAb 425 were incubated with 10 $\mu\text{g}/\text{ml}$ of purified wtEGF-R protein followed by the addition of serum dilutions (panels A-G) (or 1-20 $\mu\text{g}/\text{ml}$ positive control MAb NCL directed to wtEGF-R, panel H) and 20K cpm per well of ^{125}I -labeled MAb NCL.

To measure binding of the sera to CEA, wells were coated with 10 $\mu\text{g}/\text{ml}$ of anti-CEA MAb followed by incubation with 10 $\mu\text{g}/\text{ml}$ of CEA, serum dilutions (panels A-G) (or 1-20 $\mu\text{g}/\text{ml}$ of polyclonal antibody [PAb] to CEA, panel H) and 20K cpm per well of ^{125}I -labeled anti-CEA PAb. *, values obtained in the EGF-RvIII system are significantly (at $p < 0.05$ level; two-sided student's t-test) different from the control values obtained in the wtEGF-R or CEA systems.

Fig. 2. Absence of binding of breast cancer patients' sera to EGF-RvIII. Sera from breast cancer patients (panels A-I) with EGF-RvIII lesions were tested for binding to EGF-RvIII, wtEGF-R or CEA as described in Fig. 1 legend. As positive control inhibitors MAb L8A4, MAb NCL and polyclonal antibody (PAb) to CEA were used as described in Fig. 1 legend. *, values obtained with MAb and PAb are significantly (at $p < 0.05$ level; two-sided student's t-test) different from the buffer control values.

Fig. 3. Absence of healthy donors' sera binding to EGF-RvIII. Sera from healthy donors (panels A-C) were tested for binding to EGF-RvIII, wtEGF-R or CEA as described in Fig. 1 legend. As positive control inhibitors MAb L8A4, MAb NCL and polyclonal antibody (PAb) to CEA were used as described in Fig. 1 legend. *, values obtained with MAb and PAb are significantly (at $p < 0.05$ level; two-sided student's t-test) different from the buffer control values.

Fig. 4. Proliferation of breast cancer patients' PBMC to stimulation with EGF-RvIII protein. For expression of EGF-RvIII by patients' lesions see Table. PBMC were stimulated for 10 days (TF) or 5 days (KJ, DM) with 10 $\mu\text{g}/\text{ml}$ of EGF-RvIII or wtEGF-R protein, 25 $\mu\text{g}/\text{ml}$ of EGF-RvIII peptide (patient DM: peptide GAALLALLAALCPASR ALEEKKGNY; patients KJ, TF: peptide ALEEKKGNY), in the presence of 1 $\mu\text{g}/\text{ml}$ of $\beta 2$ microglobulin, the EGF-RvIII peptides in PLG microspheres, or control (co) peptide GEXXLAEKLN. PHA was used as a positive control. PBMC were then pulsed with ^3H -thymidine and ^3H -thymidine incorporation was measured. Values with identical letters differ significantly from each other in two-sided student's t-test.

Fig. 5. Absence of lymphoproliferative responses to EGF-RvIII stimulation in breast cancer patients. For EGF-RvIII expression by patients' lesions see Table. PBMC were stimulated for 10 days (GI) or 5 days (DI, MC, CR, FM, KrJ, BD, PL, RS, BR, DP, GB, EJ) with EGF-RvIII or wtEGF-R protein, EGF-RvIII peptide (patients DI, FM, RS, GB: peptide GAALLALLAALCPASR ALEEKKGNY; patients MC, CR, KrJ, GI, BD, PL, BR, DP, EJ: peptide ALEEKKGNY), control (co) peptide GEXXLAEKLN or PHA as described in Fig. 4 legend. PBMC were then pulsed with ^3H -thymidine and ^3H -thymidine incorporation was measured. Values with identical letters differ significantly from each other in two-sided student's t-test.

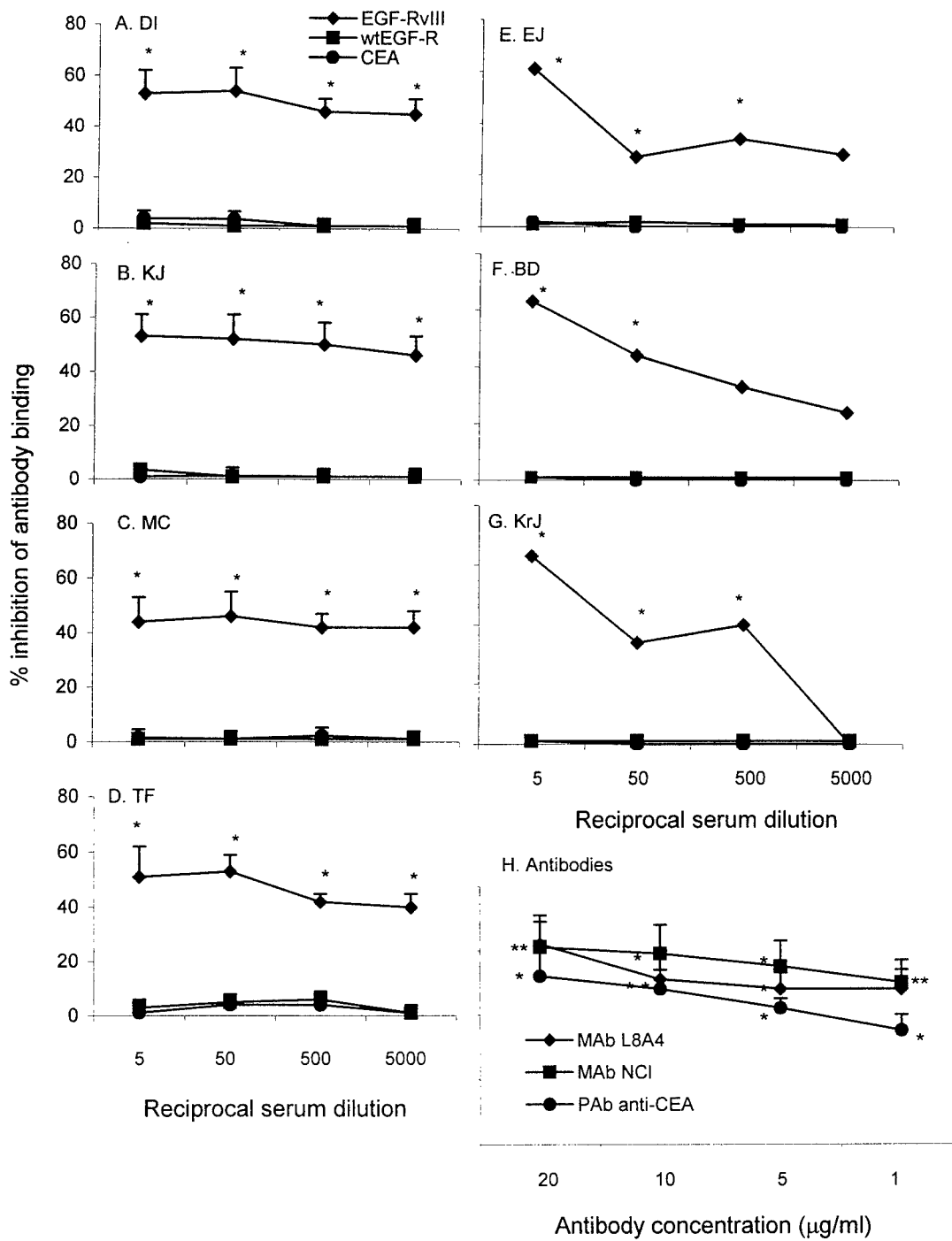
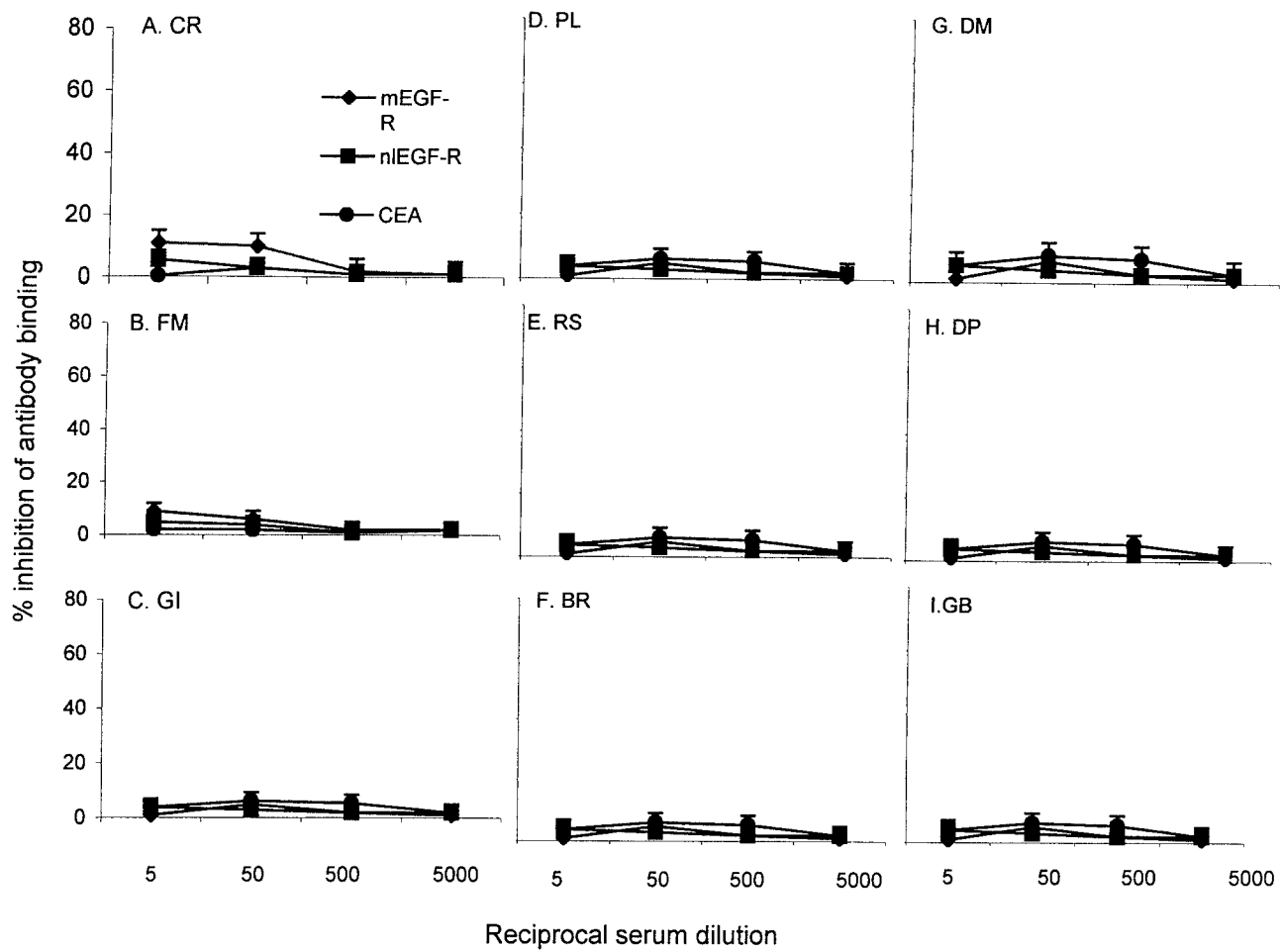


Figure 1



Reciprocal serum dilution

J. Antibodies

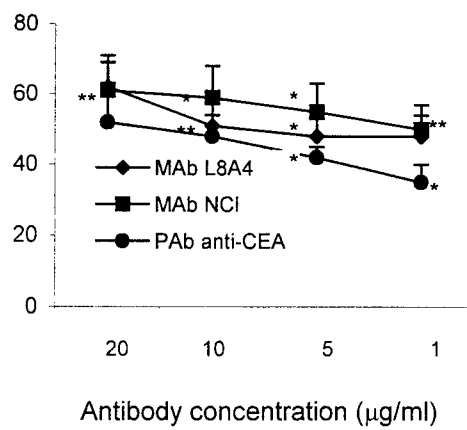


Figure 2

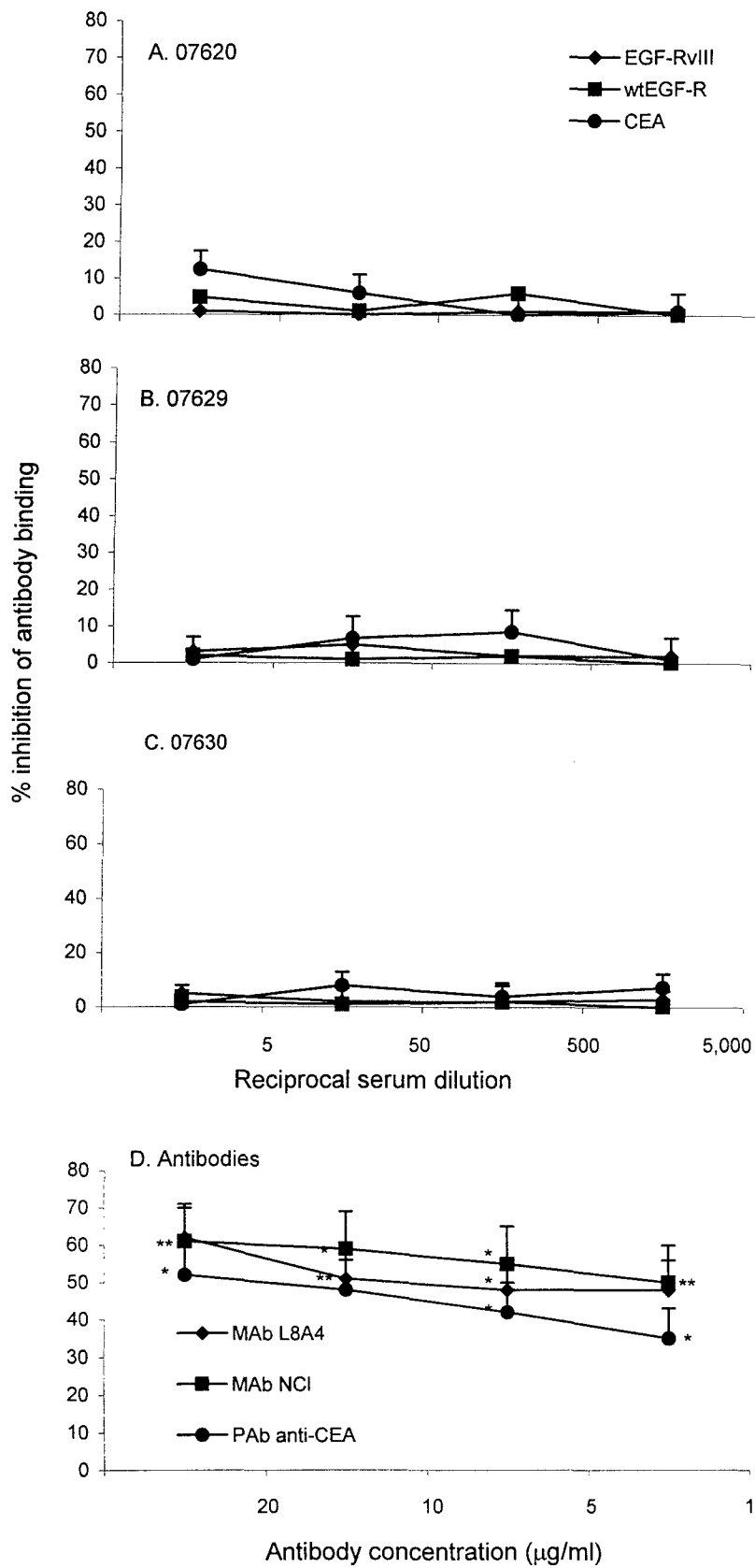


Figure 3

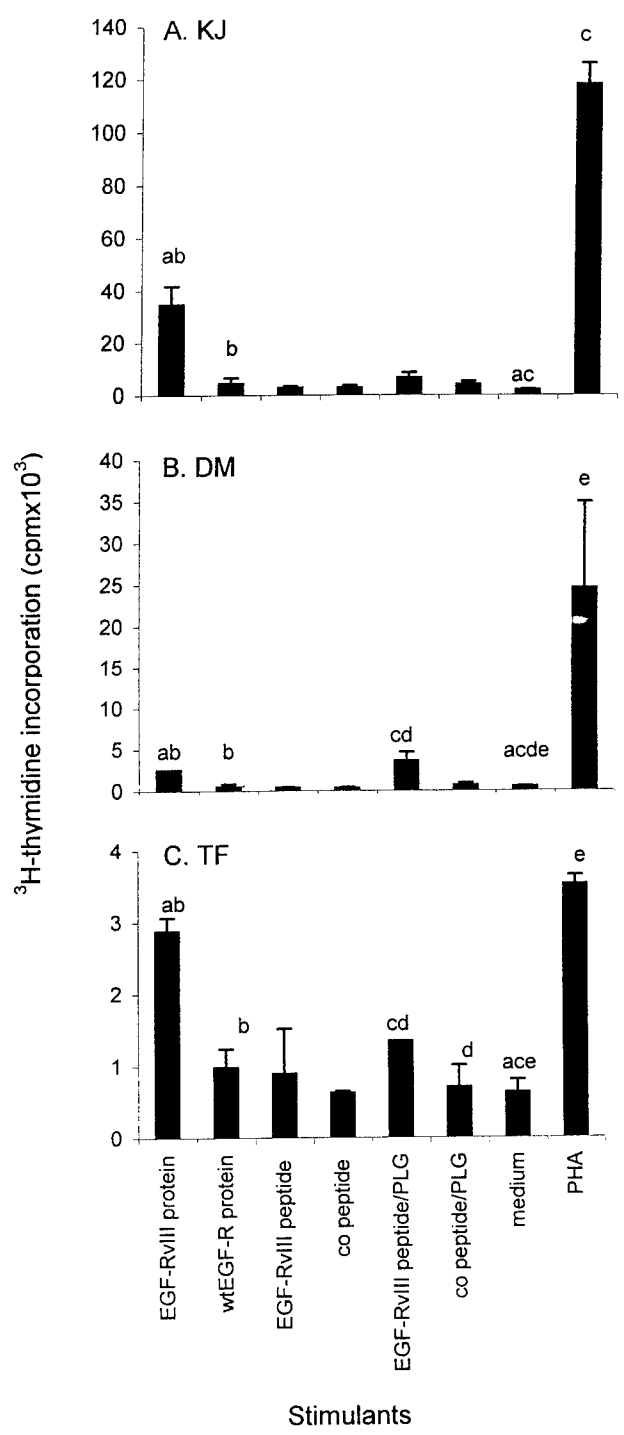


Figure 4

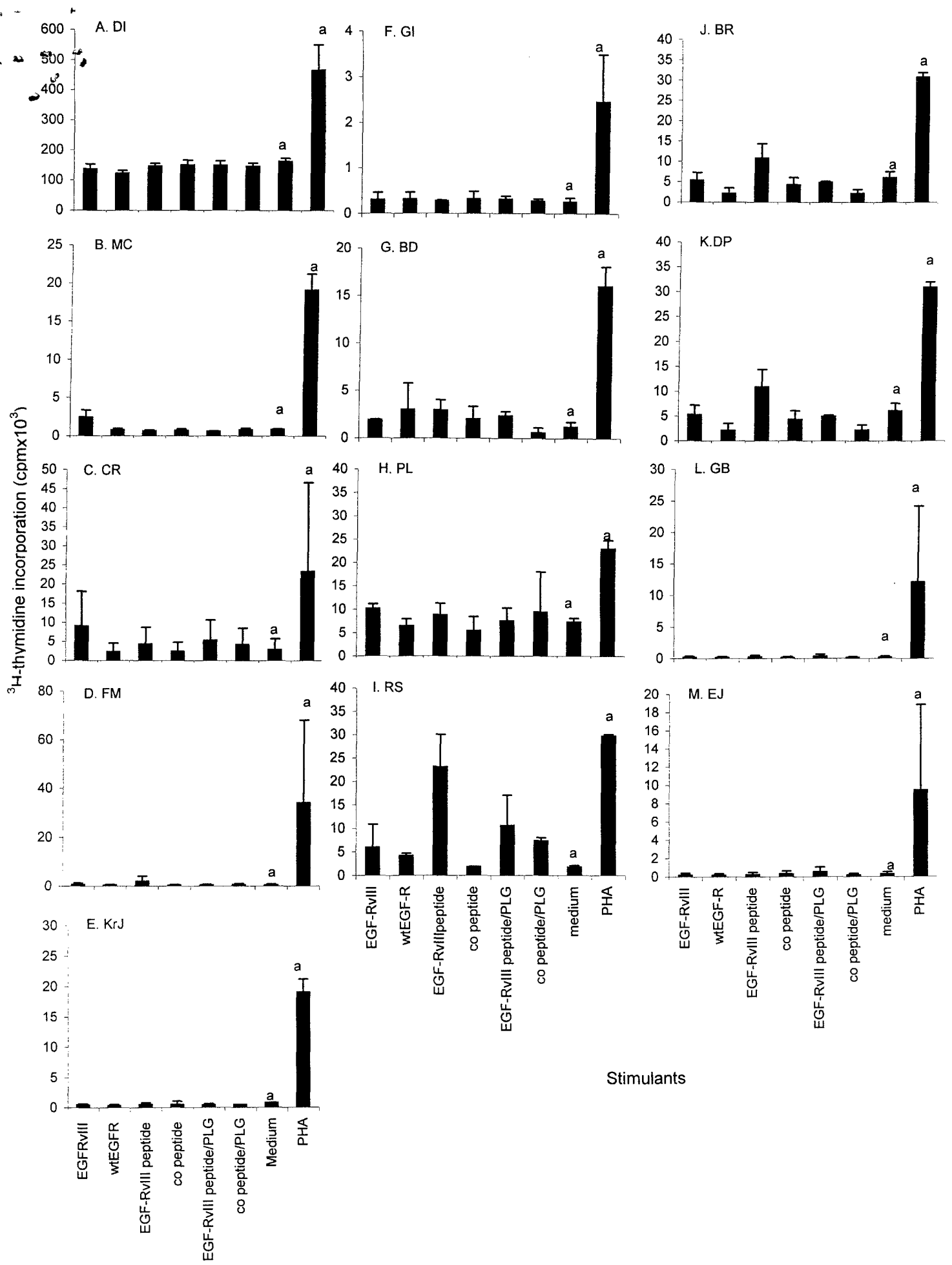


Figure 5



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 S. ...
FORT DETRICK ...

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

28 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218


SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Enc.


PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB231838
ADB240253
ADB251610
ADB275099
ADB253637
ADB261538
ADB275186
ADB264648
ADB275102
ADB241899
ADB259033
ADB266113
ADB275663
ADB254489
ADB262700
ADB276708
ADB274345
ADB274844
ADB275154
ADB275535
ADB275101
ADB275451
ADB274597
ADB273871
ADB275145
ADB274505
ADB275851
ADB274459
ADB277942
ADB277404
ADB277494
ADB277536