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GRANT NUMBER DAMD17-96-1-6237

TITLE: Targeting Mutated Epidermal Growth Factor Receptor

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REPORT DATE: July 1998

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (1 Jul 97 - 30 Jun 98)

4. TITLE AND SUBTITLE

Targeting Mutated Epidermal Growth Factor Receptor

5. FUNDING NUMBERS

DAMD17-96-1-6237

6. AUTHOR(S)

Herlyn, Dorothee M., D.V.M.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Wistar Institute
Philadelphia, Pennsylvania 19104-4268

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This project is aimed at developing specific and effective vaccines for immunotherapy of breast cancer. Mutated epidermal growth factor receptor (mEGF-R) is expressed on a high proportion of breast carcinoma lesions derived from various patients, but not on any normal tissues tested, and is targeted in these studies. The major goal is to develop a rat model of immunotherapy against mammary carcinoma targeting mEGF-R. In this model, vaccines of mEGF-R (peptides, anti-idiotypic antibodies, recombinant antigen) are tested for their effects on the growth of established rat mammary carcinoma cells expressing rat and human mEGF-R. During the first year of funding, rat mammary carcinoma cells transfected with rat mEGF-R cDNA, recombinant baculovirus-derived rat and human mEGF-R protein and five mEGF-R peptides were produced and characterized. While the recombinant proteins and the peptides expressed the mutated epitope, this epitope was absent on the transfectants. During the past (second) year of funding, our efforts focused on the isolation of transfectants expressing the mutated epitope. Furthermore, recombinant adenovirus expressing mEGF-R were produced. Initial immunizations of rats with mEGF-R protein or peptides demonstrated the induction of specific antibodies reactive with the mutated protein.

14. SUBJECT TERMS

Breast Cancer, Vaccines, Mutated Epidermal Growth Factor Receptor

15. NUMBER OF PAGES

14

16. PRICE CODE

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

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Dorothea Herbyn 7-12-98

PI - Signature Date

TARGETING MUTATED EPIDERMAL GROWTH FACTOR RECEPTOR

Table of Contents

Foreword 3

Introduction 5

Body of Work 5

Conclusions 7

References 7

Appendices 10

Introduction (adapted from 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 (mEGF-R) 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). mEGF-R 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 mEGF-R may exert direct anti-proliferative effects (14).

We have chosen a rat model of mEGF-R 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 mEGF-R consists of the minimal sequence, including the mutation, that elicits B- and/or T-cell responses. We have chosen peptides of mEGF-R 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. Anti-idiotypic antibody vaccines will be produced to induce B cell immunity to mEGF-R. Our studies have demonstrated that anti-idiotypes can induce humoral, cellular and protective immunity in colorectal cancer patients (28-30). Molecular cloning of anti-idiotypic antibodies recently developed in our laboratory (31,32) has numerous advantages over traditional approaches, such as high sensitivity, specificity and ease and economy of production.

In conclusion, mEGF-R 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 mEGF-R in the rat mammary carcinoma model may be applicable to other tumor systems, such as lung carcinomas and gliomas which also express mEGF-R (13,33).

Body of Work

During the first year of funding (July 96-June 97), we made the following achievements:

a. Rat mammary carcinoma transfectants were generated which expressed the 145 kDa rat mEGF-R 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 human mEGF-R epitope. They were tumorigenic in syngeneic rats.

b. Rat mEGF-R and human mEGF-R proteins, both specifically reactive with MAb L8 were produced in recombinant baculovirus.

c. Peptides of rat mEGF-R were synthesized.

During the past year of funding (July 1997-June 1998) we focused our efforts on the generation of rat mammary carcinoma transfectants MTLN3 with stable expression of the mEGF-R epitope (defined by MAb L8 to the human mEGF-R epitope) following rat mEGF-R cDNA transfection. One transfectant obtained during the initial funding period expressed the 145 kDa protein characteristic of mEGF-R, but was unreactive with MAb L8 by flow cytometry

analysis with live or fixed (permeabilized) cells. Absence of MAb reactivity with the transfectants was difficult to explain because the cDNA which was used for transfection of the cells did encode the mEGF-R epitope recognized by MAb L8 when transferred to the baculovirus system (see previous report). Furthermore, one of 5 peptides (peptide 4) of rat mEGF-R (described in the previous report), specifically bound MAb L8 (Fig. 1). We therefore sequenced the transfected mEGF-R cDNA. The sequence was only 95% identical with the predicted sequence which may explain absence of reactivity of the transfectants with MAb L8. We therefore tested a total of ~100 drug (G418)-resistant MTLN3 colonies from three independently performed transfections for their cell surface reactivity with MAb L8 by flow cytometry. Two colonies showed low, but significant, binding of MAb L8 (~25% of the cells within a colony bound the MAb). However, antigen expression by the colonies was unstable after about two months in culture. Our previous experience in the colorectal carcinoma (34) and melanoma (unpublished data) systems has indicated stability of the transfected antigen expression in 2-5% of the colonies that all (100%) were antigen-positive initially. Thus, in our current studies, we aim at generating at least 200 transfected colonies that are reactive with MAb L8 initially, in order to obtain 4-10 stable transfectants. This indicates the need for performing transfections of large number of cells (at least 1 x 10^7 cells, assuming a colony forming efficiency in selection media of ~0.2 % and an initial MAb L8 reactivity of ~2% of the colonies).

We have begun immunizations of rats with rat mEGF-R protein and peptides. Since both the mutated protein and one of the 5 peptides (peptide 4) reacted with MAb L8, these two preparations were used in initial immunizations to determine their capacity to induce antibodies.

Rats were immunized with baculovirus-derived rat mEGF-R protein in complete and incomplete Freund's adjuvant (CFA, IFA; see Fig. 2). Although these results are preliminary and include only one rat per immunogen dose, there is a trend of the lowest rat mEGF-R dose (100 μg/injection/rat) yielding higher antibody response than the highest dose (300 μg). The antibodies not only bound to mutated rat EGF-R, but also to normal rat EGR-R protein (Fig. 2A-C). One of the 3 rats (immunized with 200 μg per dose) may have produced antibodies binding not only to normal determinants on the mutated protein, but also to the mutated epitope because sera from this rat specifically bound to rat mEGF-R peptide 4 (Fig. 2E). Thus, rats are not immunologically tolerant to normal EGF-R administered in adjuvant although this protein is widely expressed by their normal organs. Immune responses to normal EGF-R were not accompanied by toxicity as determined macroscopically in those organs which express EGF-R.

Rats were immunized with 5 different peptides of rat mEGF-R (described in the previous report) or with control peptide. Only one peptide (peptide 4) bound to MAb L8 with specificity for the mutated epitope (see Fig. 1). Peptides were incorporated into microspheres and injected with or without Titermax adjuvant. Of the 5 peptides, only peptide 4 (with or without adjuvant; Fig. 3) and peptide 5 (with adjuvant only; not shown) induced antibodies binding specifically to rat mEGF-R protein as compared to BSA. No such antibodies were induced in the control rats.

Additional immunizations of rats with mEGF-R protein or peptides will determine the potential of these vaccines for inducing cellular and tumor protective immunity in the absence of histopathologically evident toxicity. These studies will determine the potential of mEGF-R vaccines for breast cancer patients.

We have produced 4 peptides of human mEGF-R (residues 23-32, 11-38, 30-43, and 30-49) which are described in the Table. The two additional peptides that are also listed in the table (residues 24-32 and 25-33) already had been produced during the first year of funding for immunizations of rats (see above). These two peptides are identical in both rat and human mEGF-R. The six different peptides will be used in future stimulations of breast cancer patients' lymphocytes.

Recently, we have demonstrated inhibition of established colon carcinoma growth in mice vaccinated with recombinant adenovirus expressing a human colon cancer antigen. In contrast, anti-idiotypic antibodies mimicking an epitope of the antigen or the recombinant antigen in various adjuvants were ineffective (34). Therefore, we have begun to produce recombinant adenovirus expressing rat mEGF-R. The details on the production of the construct are illustrated in Fig. 4. The insert in pAd/RmEGF-R vector was sequenced and the sequence showed 100% identity with the predicted sequence of rat mEGF-R (not shown). We are currently producing recombinant adenovirus in A549 cells. The virus will then be purified by CsCl gradient centrifugation, tested for mEGF-R expression, and used as a vaccine in tumor-bearing rats.

Conclusions

During the past funding period, our efforts focused on the establishment of the rat model of active specific immunotherapy against mEGF-R, a breast carcinoma-specific antigen. Rat mammary carcinoma cells transfected during the initial funding period with rat mEGF-R to serve as targets for active specific immunotherapy were further characterized. Although the transfectants expressed the characteristic 145 kDa rat mEGF-R protein, the mutated epitope could not be detected, presumably due to a mutation of the original cDNA after transfection into the cells. Additional transfections were promising, but stable transfectants have yet to be obtained. Initial vaccinations of rats with rat mEGF-R protein or peptides induced antibodies which occasionally bound specifically to the mutated epitope, in addition to binding to the normal EGF-R protein. As a novel, highly promising vaccine, rat mEGF-R was expressed in recombinant adenovirus. Furthermore, peptides of human mEGF-R were produced for future stimulations of breast cancer patients' lymphocytes.

Thus, we have fulfilled the goals originally proposed for the second year of study, with the exception of cellular immune response evaluation in immunized rats. The latter studies could not be performed because we currently lack the appropriate tumor cells expressing rat mEGF-R epitope.

The model system we are establishing will be useful for the evaluation of mEGF-R-specific vaccines against mammary carcinoma in our subsequent studies.

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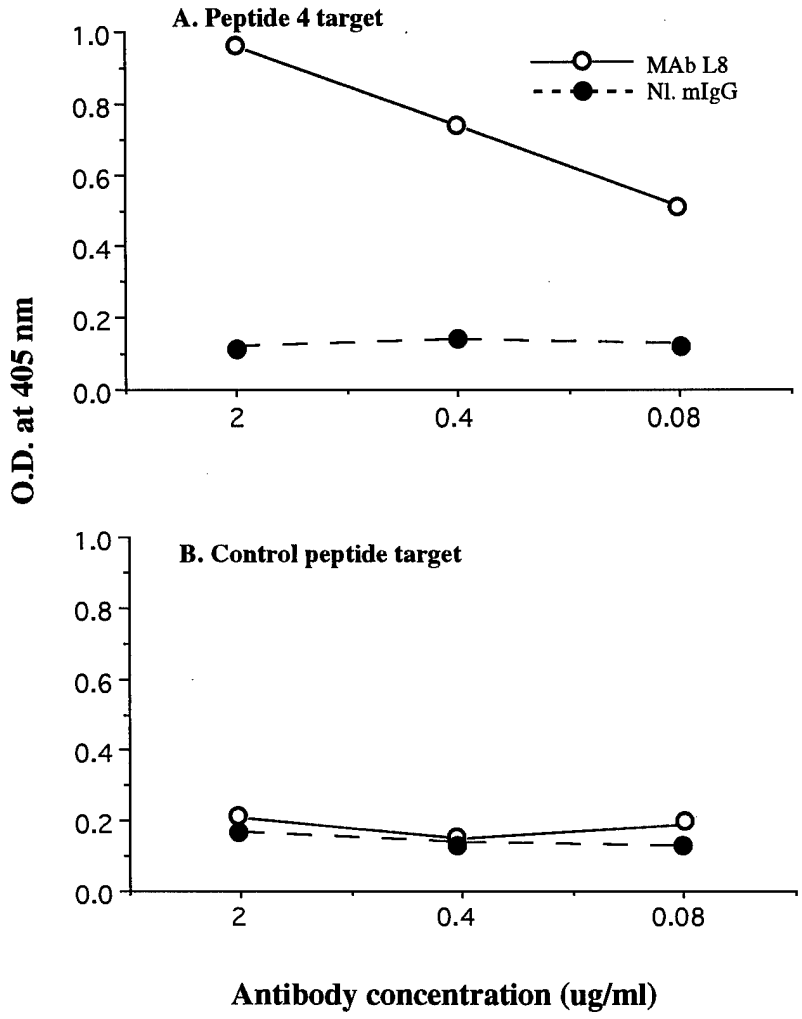

Fig.1. Binding of MAb L8 to rat mEGF-R peptide (peptide 4). Wells of microtiter plates were coated with 25 ng/well of peptide 4 or control peptide. Plates were blocked with 3% BSA in PBS and incubated with various concentration of MAb L8 or normal mouse IgG for 60 min. at R.T. Plates were washed and incubated with POD-labelled goat anti-mouse IgG (1:3,000 dilution) for 60 min. at RT. Plates were washed and O.D. at 405 nm was determined. MAb L8 significantly and specifically bound to peptide 4.

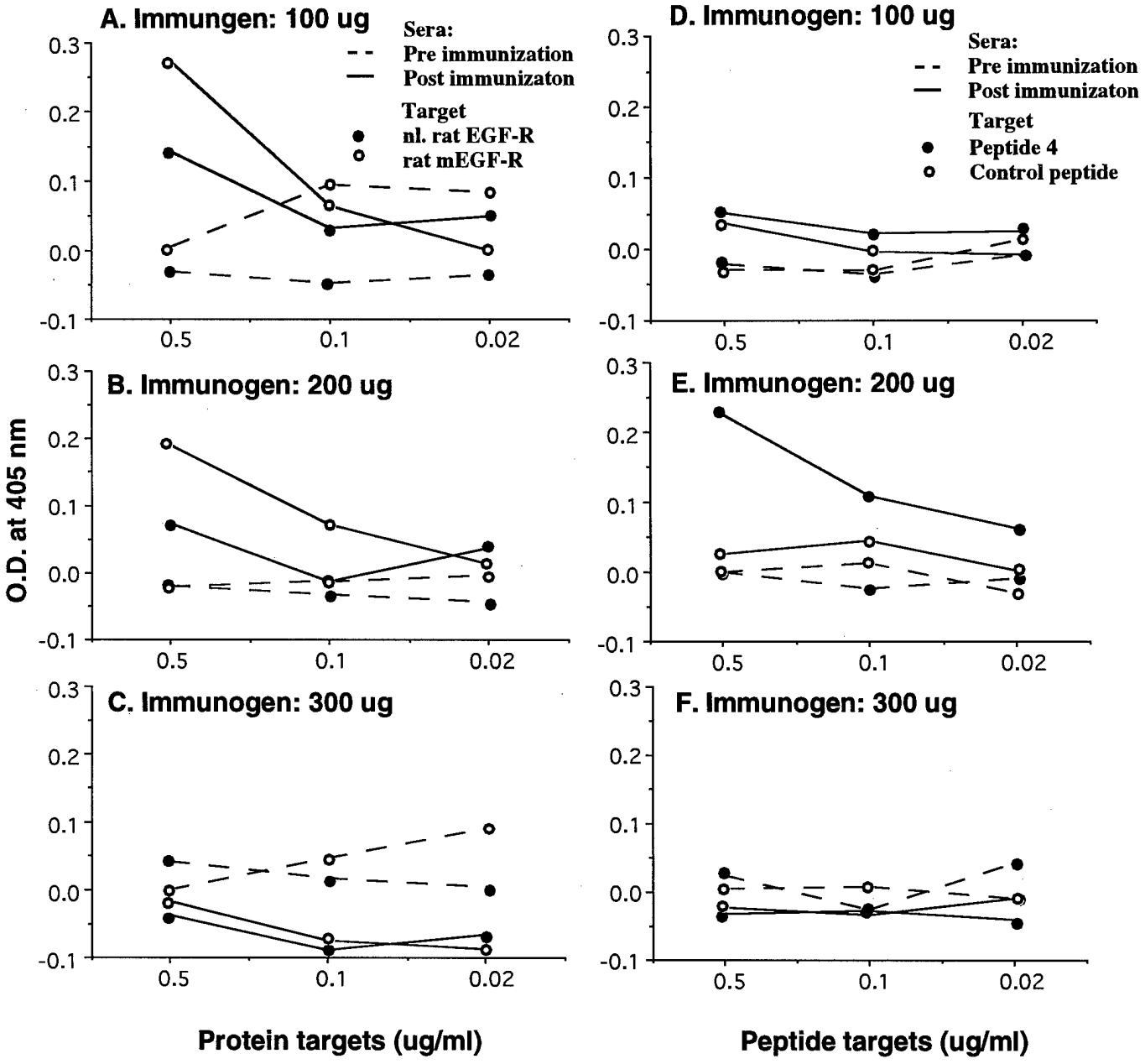

Fig.2. Binding of rat anti-mEGF-R protein sera to mEGF-R protein and peptide. Fisher 344 rats were immunized subcutaneously with 100, 200, or 300 ug of rat mEGFR protein on day 1 in CFA and on day 14 in IFA. Sera were obtained on days 0 and 28 and tested at 1:20 dilution for binding to various concentrations of protein or peptide. Sera were incubated with target protein or peptide for 60 min. at RT. Plates were then blocked with 3% BSA in PBS and incubated with peroxidase labelled-goat anti-rat IgG at 1:3,000 dilution for 60 min. at RT. Plates were washed and OD at 405 nm was determined.

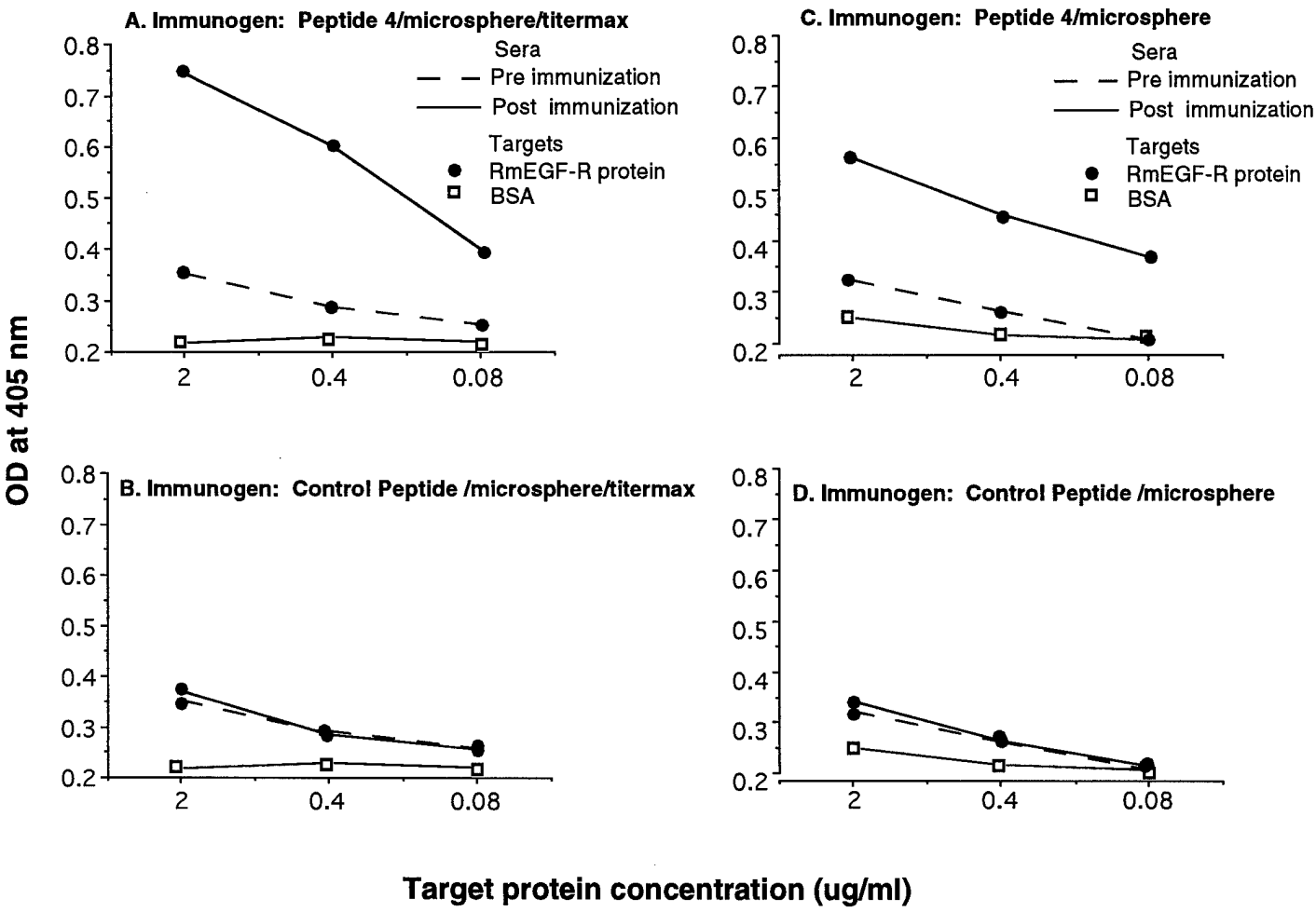

Fig.3. Immunoreactivity of sera from rats immunized with rat mEGFR-R peptide (peptide 4). One rat each was immunized with 50 ug of peptide 4 or control peptide in either microspheres plus titermax adjuvant (A, B) or in microspheres (C, D) on day 1. Sera were obtained on days 0 and 14 were tested at 1:10 dilution for binding to various concentrations of rat mEGF-R protein or BSA control in ELISA. Sera were incubated with target proteins for 60 min. at RT. Plates were then incubated with peroxidase labelled-goat anti-rat IgG at 1:3,000 dilution for 60 min. at RT. Plates were washed and OD at 405 nm was determined.

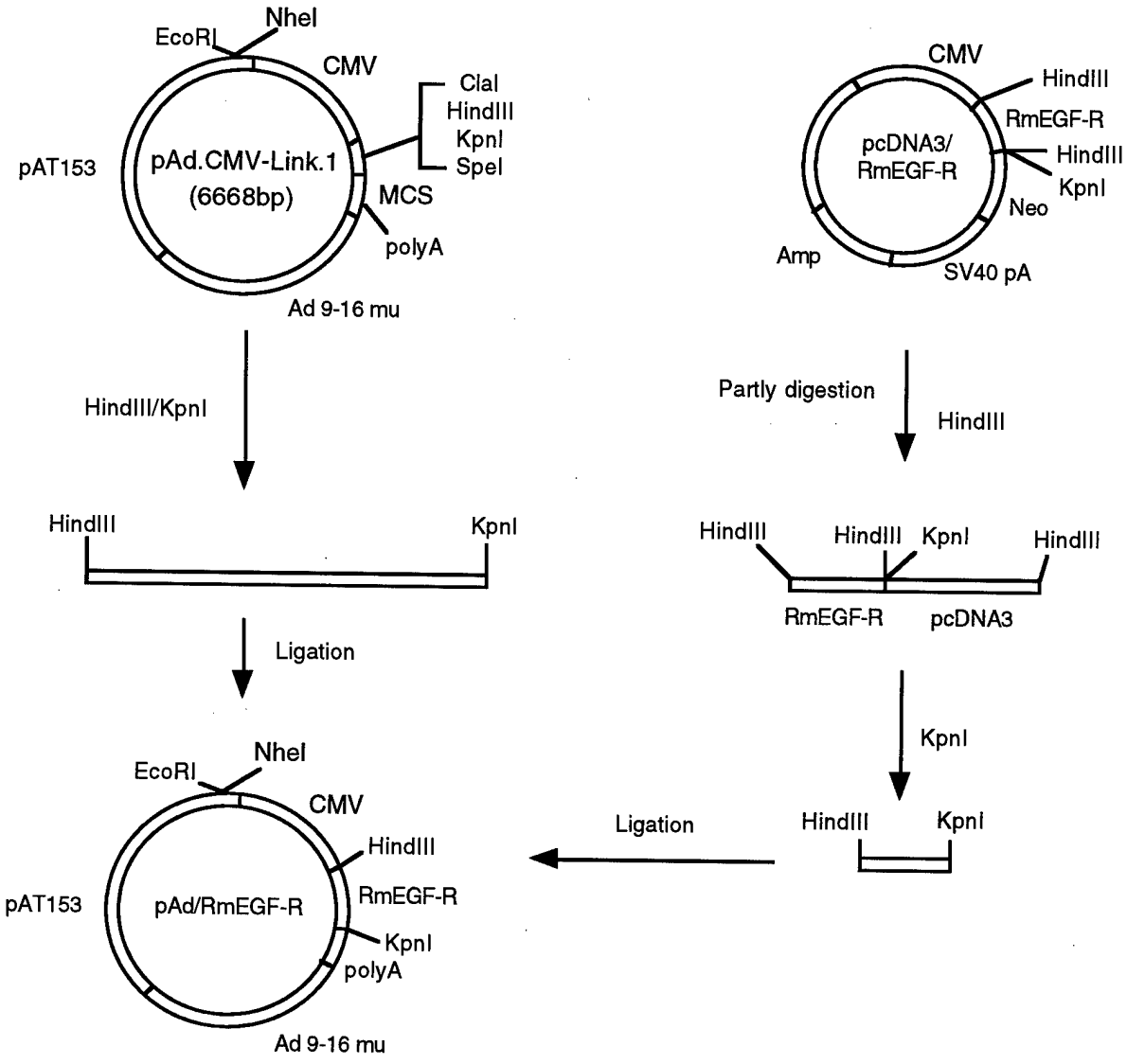

Fig.4. Rat mEGF-R adenovirus vector construction. Full length rat mutated (Rm) EGF-R (3.2kb) in pcDNA3 vector was partially cut with HindIII to linearize the vector. The linearized vector was then cut with KpnI to obtain rat mutated EGF-R with HindIII/KpnI restriction enzyme sites at both ends. The full length RmEGF-R cDNA was then ligated into the HindIII/KpnI site of pAd.CMV-Link.1 (pAd). Sequencing of the insert revealed complete identity of the sequence with the predicted sequence of RmEGF-R.

Peptides of Human mEGF-R

Peptide		Predicted peptide patterns[a]			
Residues	Amino acid sequence[b]	HLA class I binding motif	HLA class II binding motif	Rothbard pattern (residues)	α-amphipathic helix (residues)
24-32	ALEEKKGNY	A1, A3	—	—	—
25-33	LEEKKGNYV	B61 (4006)	—	—	—
23-32	RALEEKKGNY	A1, A3, B61 (4006)	—	23-26	23-25
11-38	LLALLAALCPASRAL→ EEKKGNYVVTDHG	A1, A3, B61 (4006)	DR1(B1*0101)	23-26	12-18, 23-25
30-43	GNYVVTDHGSCVRA	A31, A33, A68.1 B39011	HLA-DR3(b)	—	—
30-49	GNYVVTDHGSCVRA→ CGADSY	B39011, B2702	—	—	—

[a] Based on known human peptide sequences.

[b] Mutated glycine and MHC class I anchors are underlined (solid an dotted lines, respectively). Control peptides will be selected for each human mEGF-R peptide that induces a mEGF-R specific immune response by omitting glycine (position 30) from the specific peptide or by replacing glycine by either proline or glutamic acid.