AD______________

Award Number: DAMD17-98-1-8534

TITLE: Tumor Associated Antigenic Peptides in Prostate Cancer

PRINCIPAL INVESTIGATOR: Raj Tiwari, Ph.D.

CONTRACTING ORGANIZATION: New York Medical College
Valhalla, New York 10595

REPORT DATE: March 2001

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

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.

20010723 092

TIWARI, RAJ K.

# 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 | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | March 2001 | Annual (30 SEP 99 - 28 FEB 01) |

**4. TITLE AND SUBTITLE**
Tumor Associated Antigenic Peptides in Prostate Cancer

**5. FUNDING NUMBERS**
DAMD17-98-1-8534

**6. AUTHOR(S)**
Raj Tiwari, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
New York Medical College
Valhalla, New York 10595

e-mail:
raj_tiwari@nymc.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**
Approved for public release;
distribution unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** ***(Maximum 200 Words)***
We had earlier demonstrated the prophylactic and therapeutic efficacy of tumor derived heat shock protein, gp96-peptide complexes. Since this tumor rejection property was specifically mediated by *tumor derived* and **not** non-tumor derived gp96-peptide complexes, and that gp96 preparations stripped of its peptides are non-immunogenic, we examined the hypothesis whether prostate cancer associated peptides which may act as tumor rejection antigens can be identified in gp96-peptide complexes utilizing a combinatorial single chain phage display antibody library. We have successfully used combinatorial single chain phage display library (scFv) for the detection of tumor rejection antigens and to define the heterogeneity of cancer antigens in prostate cancer. These novel reagents helped us to characterization of the 170 kDa protein specifically expressed in MAT-LyLu cells that could be a target for immunotherapy. These scFvs were used to identify synthetic peptides that mimic the activity of the tumor rejection antigen gp96. We also demonstrated that a tumor protective immune response can be generated using these synthetic peptides. Our results now confirm that T cell defined epitopes can be recognized by antibodies and that there may exist considerable overlap. In this respect, the existing paradigm was challenged.

**14. SUBJECT TERMS**
Prostate Heat shock proteins, gp96, immune response, cancer vaccine

**15. NUMBER OF PAGES**
38

**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 |

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

# FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_____ Where copyrighted material is quoted, permission has been obtained to use such material.

_____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

_✓___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

_____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

_____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

_____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

_____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Raj Tiwari 4/11/01

PI - Signature Date

## TABLE OF CONTENTS

| Items | Page |
|---|---|
| FRONT COVER | 1 |
| SF298 FORM | 2 |
| FOREWORD | 3 |
| TABLE OF CONTENTS | 4 |
| INTRODUCTION | 5 |
| BODY | 5 |
| KEY RESEARCH ACCOMPLISHMENTS | 9 |
| REPORTABLE OUTCOMES | 9 |
| CONCLUSIONS | 10 |
| REFERENCES | 11 |
| APPENDICES | |
| 1. Figures 1 through 23, numbered | 12-34 |
| 2. Tables 1-3 numbered | 35-37 |
| 3. Invention disclosure abstract | 38 |

## INTRODUCTION

The long term goal of our research is to develop active specific immunotherapy for prostate cancer using tumor associated antigens. The identification and isolation of prostate cancer associated antigens was undertaken using an innovative approach that utilized heat shock protein gp96 and the combinatorial antibody phage display library. The choice of gp96 was dictated by published properties of this molecule that it chaperoned peptides in the endoplasmic reticulum (ER) and the gp96-peptide complex had tumor rejection properties (1-4). Since the tumor rejection property was specific and could not be mediated by gp96 preparations from other tissues and that there was no sequence difference or mutation in gp96 genes from different tissues or species and that gp96 stripped of the peptides had lost tumor rejection properties, it was presumed that the imunogenicity resided in the peptide and /or gp96-peptide complexes. It was apparent that the identification of these tumor associated peptides would be useful therapeutic and preventive agents. With this goal in mind we examined the hypothesis if tumor specific immunogenic peptides can be identified, isolated and characterized using single chain phage display antibody library (scFv).

## BODY

### THE FOLLOWING IS THE STATEMENT OF WORK AND TIME SCHEDULE AS PROPOSED IN THE ORIGINAL APPLICATION AND THE PROGRESS.

**Task 1 : 0-12 months**

Identification of prostate cancer associated antigen (PAA) in gp96-peptide complexes isolated from metastatic and non-metastatic prostate cancer cells.

This task has been *completed* as proposed. Differential panning of the combinatorial phage display single chain antibody library over metastatic (MAT-LyLu) and non-metastatic (Dunning G) and non -tumor liver tissue purified gp96-peptide complexes resulted in single chain antibodies (scFvs) that react to PAA specific to metastatic MAT-LyLu cells or non-metastatic Dunning G cells. Distinct set of antibodies react either to cell surface antigens or to intracellular antigens (purified gp96-peptide complexes). The construction of the phage display library is described digramatically in Figure 1 and the panning strategy used for the isolation of tumor specific gp96 reactive phages and soluble antibodies described in Figure 2 and for cell surface antigens in Figure 3.

**A. Isolation of tumor specific single chain antibodies reactive to tumor derived gp96 and their reactivity.**

**(i) The single chain phage display library has a large repertoire of antibody diversity.**
The synthetic phage display library which consist of single chain Fv (scFv) fragments of antibodies displayed on the surface of filamentous bacteriophages was a gift from Dr. G. Winters, MRC Center for Protein Engineering, Cambridge, UK) and is described by Nissim et. al. (5). This library utilizes 50 human germline $V_H$ segments assembled *in vitro* with random synthetic 4-12 residue long CDR3. The $V_H$ segments were cloned into phagemid vector pHEN1 carrying a human $V\lambda3$ light chain to generate a repertoire of $>10^8$ clones

(Figure 1). The scFv fragments can be expressed on phages and in soluble form. In both libraries the scFv fragments have a c-myc tag facilitating the detection of phages carrying the desired antibody, utilizing the anti-c-myc mAb9E10.

**(ii) Panning strategy for the identification of tumor derived gp96-peptide complexes.**
Gp96-peptide complexes purified from MAT-LyLu and liver were the source of the protein that was used to identify phages that specifically reacted to tumor derived gp96-peptide. Several different concentrations of the purified complexes was tested ranging from 10 to 100μg per mL. The panning strategy (Figure 2) was designed such that non-specific phages reacted to liver derived gp96-peptide could be eliminated. Amplification of the phages was undertaken only after sufficient depletion of the phage library with respect to non-tumor derived phages had already taken. The unabsorbed phages were allowed to bind to tumor derived gp96-peptide complexes, eluted, amplified and then tested. For the detection of cell surface antigens the strategy was not of depletion but of affinity selection and bound phages were eluted (Figure 3) which were amplified and passed over Dunning G for reactivity.

**(iii) Isolation of tumor specicfic scFvs**
The reactivity of the eluted phages was screened with MAT-LyLu and liver derived gp96-peptide complexes using the enzyme linked immunosorbent assay (ELISA) and this can be easily done using the 9E10 antimyc antibody as the second antibody and the absorbance read at 490/403 nm. Our results (Figures 4-6) clearly shows that phages of differential reactivity exists in the library and can be separated out. We **successfully** identified phages that react specifically to MAT-LyLu derived gp96-peptide complexes (Figure 7), however, phages that react with both liver and tumor derived gp96-peptide complexes (Figure 5) or phages that react only with liver (Figure 6) were also identified. These experiments were consistent in at least more than three different purified preparations of gp96-peptide complexes. None of these phages reacted with gp96 native protein itself. These results clearly indicate that tumor specific scFvs exist in the library and can be identified from tumor and tissue derived gp96-peptide complexes. To further test the specificity of these scFvs and to characterize the cell surface antigens from metastatic cell line, MAT-LyLu we isolated cell surface antigens reactive phages. We also used gp96-reactive phages to react with intact cells and the results are shown in Figures 8-10. Gp96 reactive scFvs did not react with intact prostate cancer cells (Figure 8) ; cell surface reactive phages panned over MAT-LyLu showed differential reactivity with MAT-LyLu and Dunning G ( Figure 9) [Note that MAT-LyLu is a metastatic variant of Dunning G cell line]; MAT-Ly Lu panned cell surface scFvs did not react to gp96-peptide complexes (Figure 9); Cell surface scFvs panned over MAT-LyLu showed little reactivity to human prostate cancer cells, TSU (Figure 10)

**Conclusion : Tumor specific scFvs reactive to either gp96-peptide complexes or to cell surface can be isolated and their reactivity is specific. Our panning strategy gave adequate number of scFvs for a comprehensive analysis.**

**(iv) Sequence analysis of the scFvs**
Several of these scFvs have been sequenced and $V_H$ -CDR3 sequence of four of them are

presented in Table 1 . Two of the scFvs (E6 and F3 have the same CDR3 sequence and are of DP-32 germline which is different from the germline (DP-38) of cell surface specific scFvs (Table 1).

- specific gp96 protein is associated with peptide and the peptides associated is reflection of the antigenic repertoire of the tissue from which the gp96 preparations are derived. The phage antibodies may be directed against the peptides or recognize a specific conformation of gp96 and a distinct peptide
- the synthetic combinatorial phage display antibody library can be used to differentially separate out the array of antigenic repertoire of a specified tissue.
- several rounds of panning over non-tumor derived gp96-peptide complexes is still not sufficient to completely deplete phages that react specifically only to non-tumor derived gp96-peptide complexes or that react with equivalent avidity to tumor and non-tumor derived gp96-peptide complexes.

**Task 2: 10-24 months**

Identification of the proteins of origin of the peptides isolated from prostate cancer cells.

This task is *complete* using two of the scFvs and one protein identified specifically in MAT-LyLu cells

**(i) Identification of cell specific protein using scFvs**

Identification of a 170 kDa protein specifically expressed in MAT-LyLu cells is presented in Figures 11 and 12. These scFvs panned over the tumor cell line MAT-LyLu and specifically reactive to MAT-LyLu derived gp96-peptide complexes can immuno-precipitate cell specific protein as determined by [$^{35}$-S] methionine metabolic cell labeling [Figures 11 and 12]. A 170 kDa protein is detected in MAT-LyLu cells and **not** in Dunning G [MAT-LyLu cells are a derivative of Dunning G cells]. This was confirmed by immuno-precipitation (IP) of cell lysates by soluble scFv E6, both by metabolic labeling [Figure11] and by staining of electrophoresed IPs by a sensitive Zinc based reagent [Figure 12, Pierce, Rockford, IL]. Both E6 and F3, panned over MAT-LyLu cells, though from distinct phage clones showed similar reactivity and their CDR3 regions are identical which when sequenced corresponded to GKYIRSV of germ line DP-32 origin (Table 1).

**(ii) Structural analysis of the cell specific 170 kDa protein**

The isolated immune complexes are subjected to an acid digestion and the gp96 and peptides separated by Amicon filtration as described in (49,50) and the matric assisted laser desorption ionization mass spectrophotometry (MALDI-MS) peptide mass mapping analysis performed (Figures 13-15). The sequence of the peptides is searched using NCBI and Genpept data bases and match profile suggested that this protein in myosin, heavy

polypeptide 9, non-muscle. The contribution of this protein in imparting specific immunogenicity is under investigation

**(iii) Isolation of synthetic peptide mimotopes of gp96-peptide complexes using tumor specific scFvs**

Since several of these scFvs may react to conformational epitope(s) of tumor derived gp96-peptide complexes it was necessary to examine if these scFvs would react to synthetic peptides in a combinatorial phage display peptide library. These peptide antigens would be conformational mimics of *in vivo* gp96-peptide complexes and thus it would be possible to synthesize standardized immunogens. Two such peptide libraries, LX-8 (consists of conformational epitopes of 12 amino-acids, cysteine each at positions 2 and 11) and X-15 (15 amino-acid linear epitope) were reacted to scFv E6 (tumor specific) and B11(non reactive to tumor gp96-peptide complex). Panning strategy is outlined in Figure 16. The scFv E6, that showed specific reactivity to tumor derived gp96-peptide complexes also reacted to synthetic peptides from *a combinatorial phage display* ***peptide*** *library (6,7). These peptides are presumably mimics of tumor derived gp96-peptide complexes.* Reactivity of scFv E6 to a set of peptide phage clones from the two libraries after four rounds of enrichment is shown in Figures 17 and 18. B11 is an MAT-LyLu derived non-reactive to gp96-peptide phage control. The reactivity of a set of peptide phage clones to the two libraries shown in Figures 19 and 20 and the sequence of some of the synthetic peptides that showed highest reactivity is shown in Table 2. These peptides are synthetic mimotopes to tumor specific gp96-peptide complexes. Several of these phage clones showed similar peptide sequence indicating a selection and enrichment of immuno-dominant peptide epitopes. One such representative peptide from LX-8 having the sequence, YCQEGDSPRLCL (BTE6-LX8b), and one from X-15, GQWQSGDRYWMETST (BTE6-X-15-7) were further analyzed for elicitation of humoral immune response and protective cancer immunity.

**Conclusion : sFvs are useful tools to identify and isolate cell specific proteins and react to synthetic peptides which may be useful immunogens.**

**(iv) Determination of immunogenicity of isolated peptide mimotopes**

Both peptides BTE6-LX-8b and BTE6X-15-7 elicited a distinct high titer humoral immune response with no cross-reactivity (Figure 21). In a preliminary, vaccination study where the animals were vaccinated with 100 μg/ peptide on day 0 and 50 μg peptide/rat as booster on days 14, 21, and challenged with 10,000 MAT-LyLu cells on day 14, a very pronounced delay in tumor incidence, latency and rate of tumor growth (Table 3 and Figure 22 and 23) was noted. Two of four animals vaccinated with the peptides were tumor free even after six weeks. This is a very significant result considering that 10,000 MAT-LyLu cells subcutaneously injected give rise to palpable tumors in ten days. These tumor specific reactivities may all be directed against the 170 kDa protein, since all these studies utilize scFv E6. The differences of gp96-peptide complexes and peptide in their anti-tumor effects may be reflective of antigen differences, dose and large number of antigens, associated with

gp96, superior adjuvant property of gp96, or a cell mediated (for gp96) vs a humoral response (for the peptides), the peptides have a minimal disulfide constraints and thus only partially mimic gp96-peptide complexes. The ability of the synthetic peptides to bind human HLA (A and B)molecules (Web site SYFPEITHI, developed by Hans Georg Rammensee's lab) resulted in a matching score ranging from 12-28, where 30 is the highest HLA-peptide fit score.

**Conclusion : Peptide mimotopes to tumor rejection antigen, gp96-peptide complexes can be identified and are strong candidates as cancer vaccines**

**Task 3: 24-30 months**

Characterization of the distribution of the identified proteins in primary and metastatic prostate cancer lesions and in normal tissues of the proteins of origin of PAA derived peptides.
This task is in progress. Detection of antigens by some of scFvs by immunohistochemistry in frozen and paraffin sections and by western blotting and for cell surface reactivity by immunofluorescence is being examined and is scheduled to be completed in the extension period of this project which is end of March 2002. .

## KEY RESEARCH ACCOMPLISHMENTS

- **Tumor specific single chain antibodies (scFvs) reactive to either gp96-peptide complexes or to cell surface can be isolated and their reactivity is specific. Our panning strategy gave adequate number of scFvs for a comprehensive analysis.**
- **sFvs are useful tools to identify and isolate cell specific proteins and react to synthetic peptides which may be useful immunogens.**
- **Peptide mimotopes to tumor rejection antigen, gp96-peptide complexes can be identified and are strong candidates as cancer vaccines**

## REPORTABLE OUTCOMES

1. Yedavelli SPK, Guo L, Daou ME, Srivastava PK, Mittelman A, Tiwari R.K. Preventive and therapeutic effect of tumor derived heat shock protein, gp96, in an experimental prostate cancer model. Int J Mol Med. 4: 243-248, 1999.

2. Mukhopadhyay S, Yedavelli SPK, Noronha J, Ferrone S, Tiwari R.K. Identification of tumor associated antigens using purified gp96-peptide complexes and the synthetic combinatorial single chain (scFv) phage display library. Proc Amer Assoc for Cancer Res. 40: abstr # 3105, 1999.

3. Chen YG, Mukhopadhyay S, Yedavelli SPK, Chatterjee-Kishore M, Kishore R, Tiwari R.K. The ER-resident heat shock protein gp96 is associated with HLA class I. Proc Amer Assoc for Cancer Res. 40: abstr # 1689, 1999.

4. Yedavelli SPK, Badithe A, Mittelman A, Tiwari RK. Heat shock protein gp96: A novel tumor derived preventive and therapeutic agent. J immunother 22(5) : 469, 1999.

5. Ashok BT, Yedavelli SPK, Chen Y, Mittelman A, Tiwari RK. Identification of a novel tumor rejection antigenic epitopes using synthetic single chain (scFv) combinatorial phage display antibody library. 4th World Congress on Advances in Oncology. October 7-9 1999, held in Athens, Greece.

6. Ashok BT, Chen Y, Yedavelli S, Noronha EJ, Mittelman A, Tiwari RK. Identification of prostate cancer associated immunogens using synthetic combinatorial single chain (scFv) phage display antibody library. Proc. Amer. Assoc. Cancer Res. 41: 698, 2000.

7. Ashok BT, Chen Y, Mittelman A, Tiwari RK. Synthetic peptide mimotopes reacting to single chain antibodies (scFv) directed against tumor rejection antigen gp96 as novel immunogens. Proc. Amer. Assoc. Cancer Res. 42:3664, 2001.

8. Ashok BT, David L, Chander B, Mittelman A, Tiwari RK. Synthetic peptide antigen mimics to HER-2/neu. Proc. Amer. Assoc. Cancer Res. 42: 4150, 2001.

9. Masters thesis of Yedavelli SPK entitled ‘Heat shock protein gp96: a primitive molecule with novel functions’ mentored by Dr. R.K. Tiwari, Spring Semester 1999.

10. Invention disclosure : Cancer vaccine development using novel antigens, tumor rejection antigen.

## CONCLUSIONS

- We have successfully used combinatorial single chain phage display library (scFv) for the detection of tumor rejection antigens and to define the heterogeneity of cancer antigens in prostate cancer. These novel reagents helped us to characterization of the170 kDa protein specifically expressed in MAT-LyLu cells that could be a target for immunotherapy.

- We used the scFvs to identify synthetic peptides that mimic the activity of the tumor rejection antigen gp96. We also demonstrated that a tumor protective immune response can be generated using these synthetic peptides. This is a significant achievement considering that these synthetic peptides are standardized immunogens that can be used as cancer vaccines or prevention of metastases.

- We also challenged an existing paradigm that T cell (cell mediated) and B cell (humoral) defined epitopes are entirely distinct and there are no overlaps. Our results (and results of

several other studies) now confirm that T cell defined epitopes can be recognized by antibodies and that there may exist considerable overlap. In this respect, the existing paradigm was challenged.

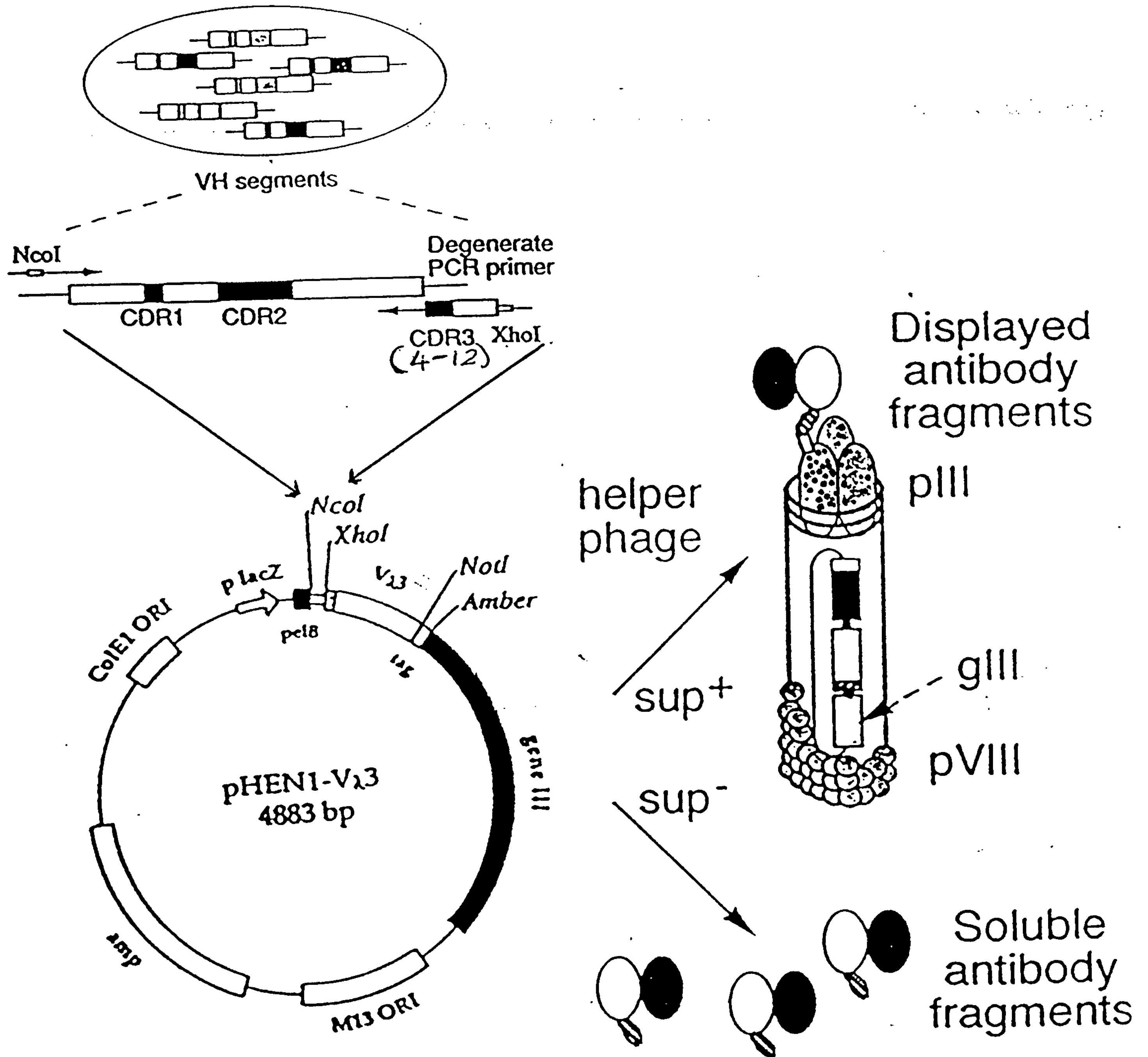
**REFERENCES**

1. Yedavelli SPK, Guo L, Daou ME, Srivastava PK, Mittelman A, Tiwari RK. Preventive and therapeutic effect of tumor derived heat shock protein, gp96, in an experimental prostate cancer model. *Int J Mol. Med. 4:243-248, 1999.*
2. Srivastava PK. Peptide-binding heat shock proteins in the endoplasmic reticulum: role in immune responses to cancer and in antigen presentation. Adv Cancer Res. 62:153-177, 1993.
3. Srivastava PK, Menoret A, Basu S, Binder RJ, McQuade K. Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. Immunity 8:657-665, 1998.
4. Srivastava PK. Immunotherapy of human cancer: lessons from mice. Nature immunology1. 1: (5), 363-366.
5. Nissim, A., Hoogenboom, H.R., Tomlinson, I.M., Flynn, G., Midgley, C., Lane, D., Winter, G. Antibody fragments from `a single pot' phage display library as immunochemical reagent. *EMBO. J.13:692-698, 1994.*
6. Bonnycastle LL, Mehroke JS, Rashed M, Gong X, ScottJK. Probing the basis of antibody reactivity with a panel of constrained peptide libraries displayed by filamentous phage. J. Mol. Biol. 257: 747-762, 1996.
7. Folgari A, Tafi R. Meola A, Felici F. Galfre G, Cortese R, Monaci P, Nicosia A. A general strategy to identify mimotopes of pathological antigen using only random peptide libraries and human sera. EMBO J. 13: 2236-2243, 1994.

Figure 1

# CONSTRUCTION OF PHAGE DISPLAY LIBRARY

Tiwari, Raj K.



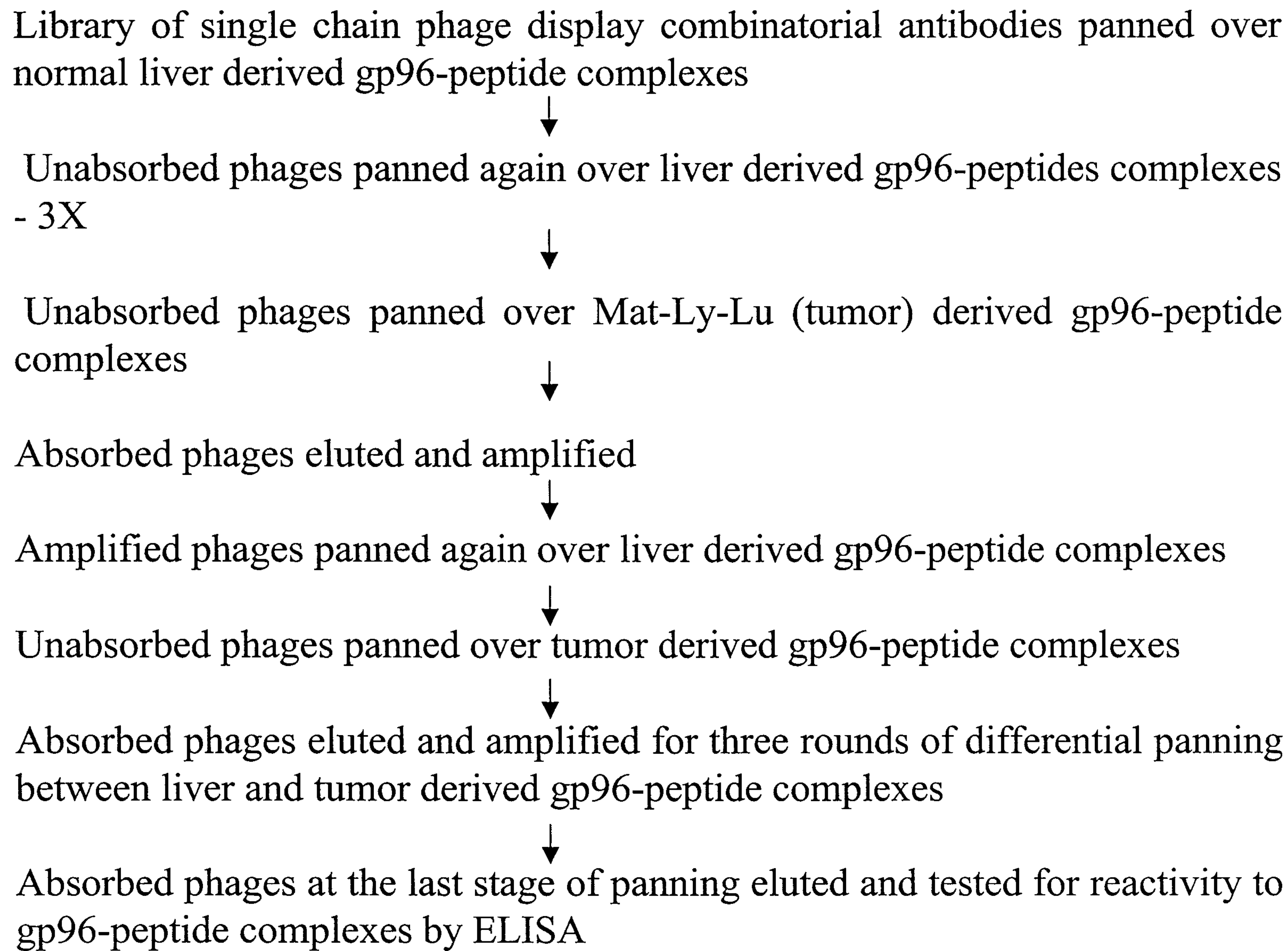


Fig. 2. PANNING STRATEGY FOR gp96-PEPTIDE COMPLEXES
Library of single chain phage display combinatorial antibodies panned over normal liver derived gp96-peptide complexes
↓
Unabsorbed phages panned again over liver derived gp96-peptides complexes - 3X
↓
Unabsorbed phages panned over Mat-Ly-Lu (tumor) derived gp96-peptide complexes
↓
Absorbed phages eluted and amplified
↓
Amplified phages panned again over liver derived gp96-peptide complexes
↓
Unabsorbed phages panned over tumor derived gp96-peptide complexes
↓
Absorbed phages eluted and amplified for three rounds of differential panning between liver and tumor derived gp96-peptide complexes
↓
Absorbed phages at the last stage of panning eluted and tested for reactivity to gp96-peptide complexes by ELISA

## Fig. 3. PANNING STRATEGY FOR CELL SURFACE ANTIGENS

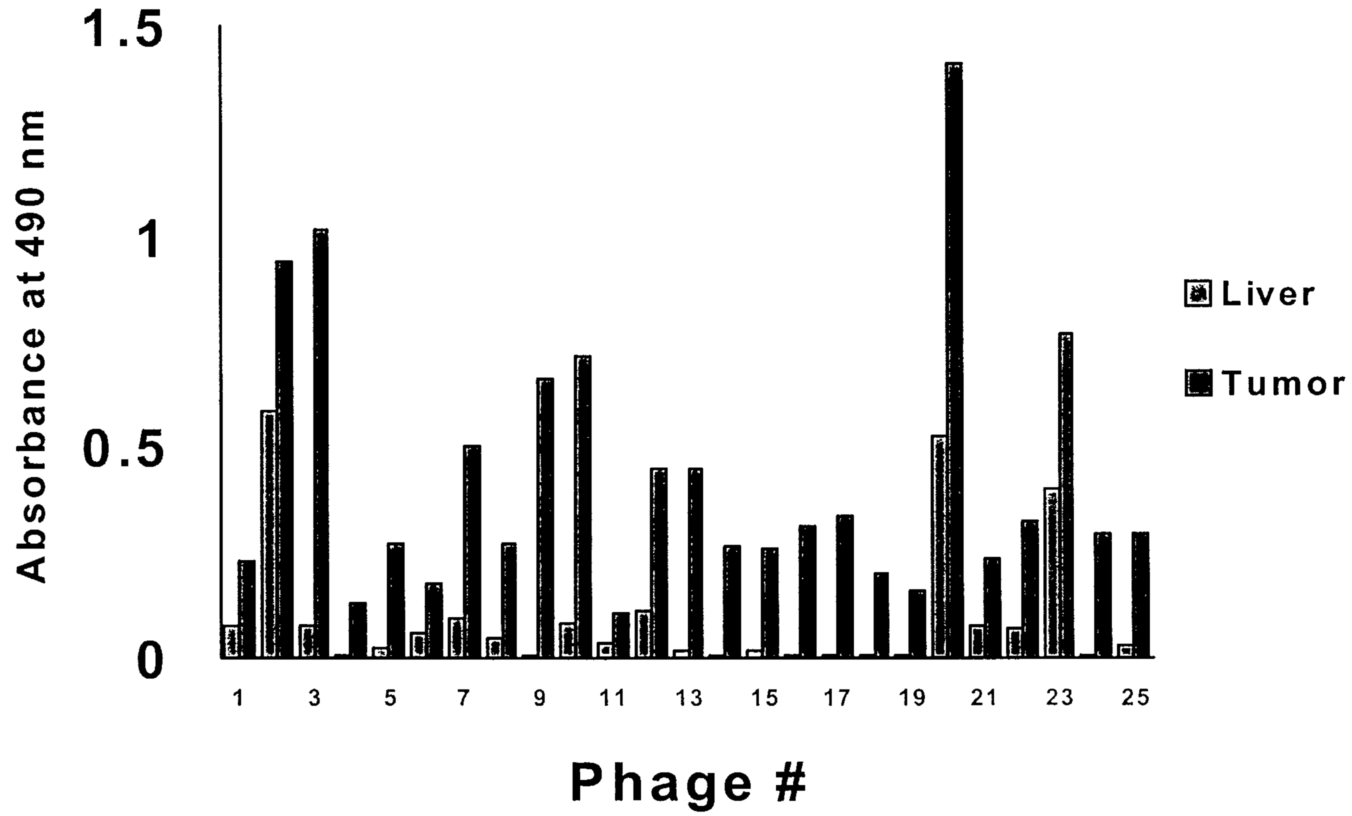
Library of single chain phage display combinatorial antibodies panned over Mat-Ly-Lu (MLL) cells

Bound phages were eluted and amplified

The phages were subjected to two more rounds of panning over MLL cells and bound phages were eluted and amplified each time

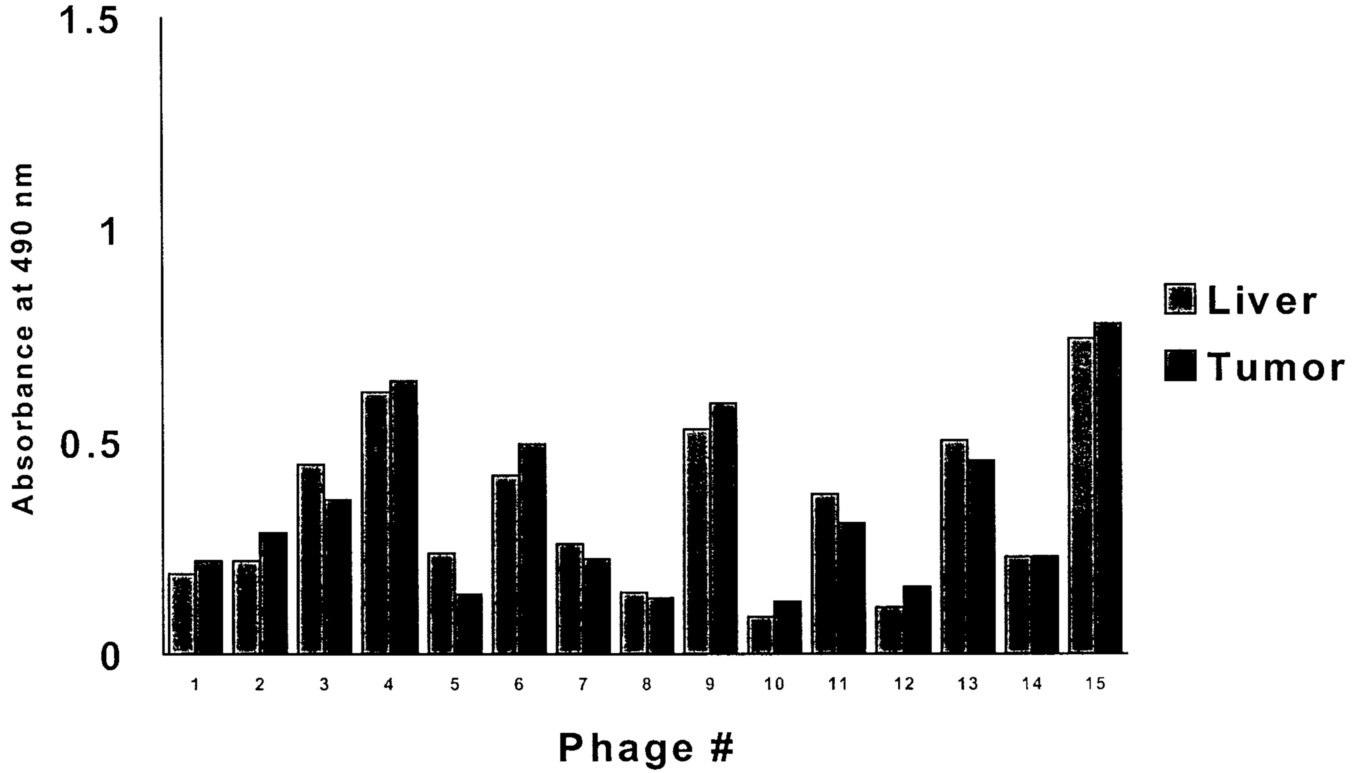
After the last round of panning, single phages were isolated and tested for reactivity to MLL, DG and TSU prostate cancer cell lines by ELISA

**Fig. 4. Phage clones specific for tumor derived gp96-peptide complexes**



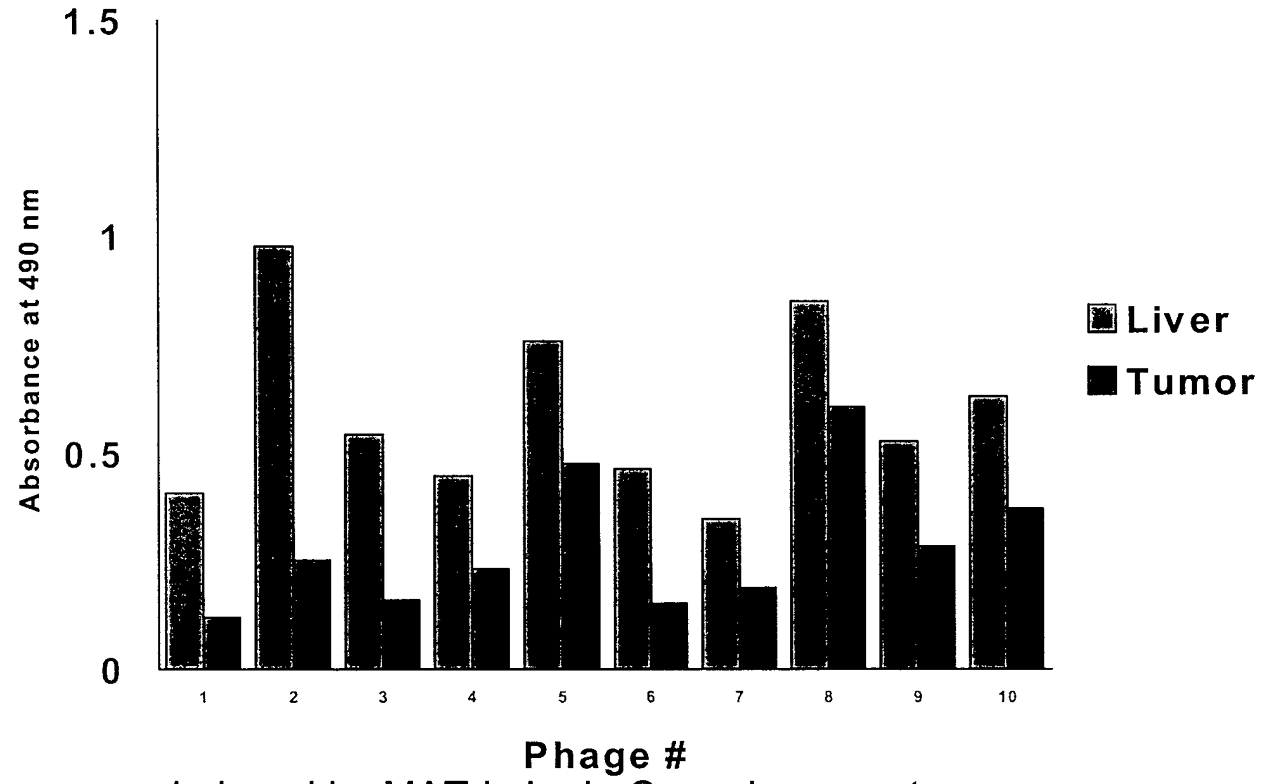

Tumors were induced by MAT-LyLu in Copenhagen rats
Livers for gp96 extraction were tumor free and derived from the same animal

**Fig. 5. Phage clones that react with both liver and tumor derived gp96**




Tumors were induced by MAT-LyLu in Copenhagen rats
Livers for gp96 extraction were tumor free and derived from the same animal

**Fig. 6. Phage clones that have higher reactivity with liver derived gp96**




Tumors were induced by MAT-LyLu in Copenhagen rats
Livers for gp96 extraction were tumor free and derived from the same animal

**Fig. 7. Reactivity of scFv fragments with gp96-peptide complexes (ELISA)**

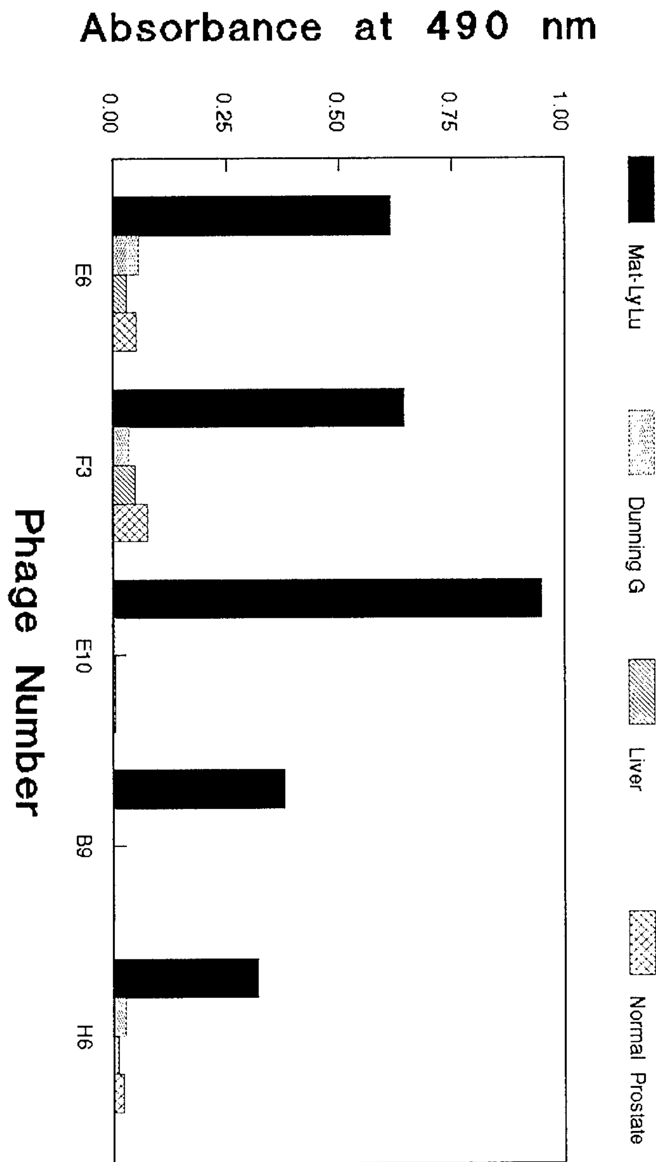


Fig. 8. Reactivity of tumor derived gp96 specific scFv E6 with intact prostate cancer cells

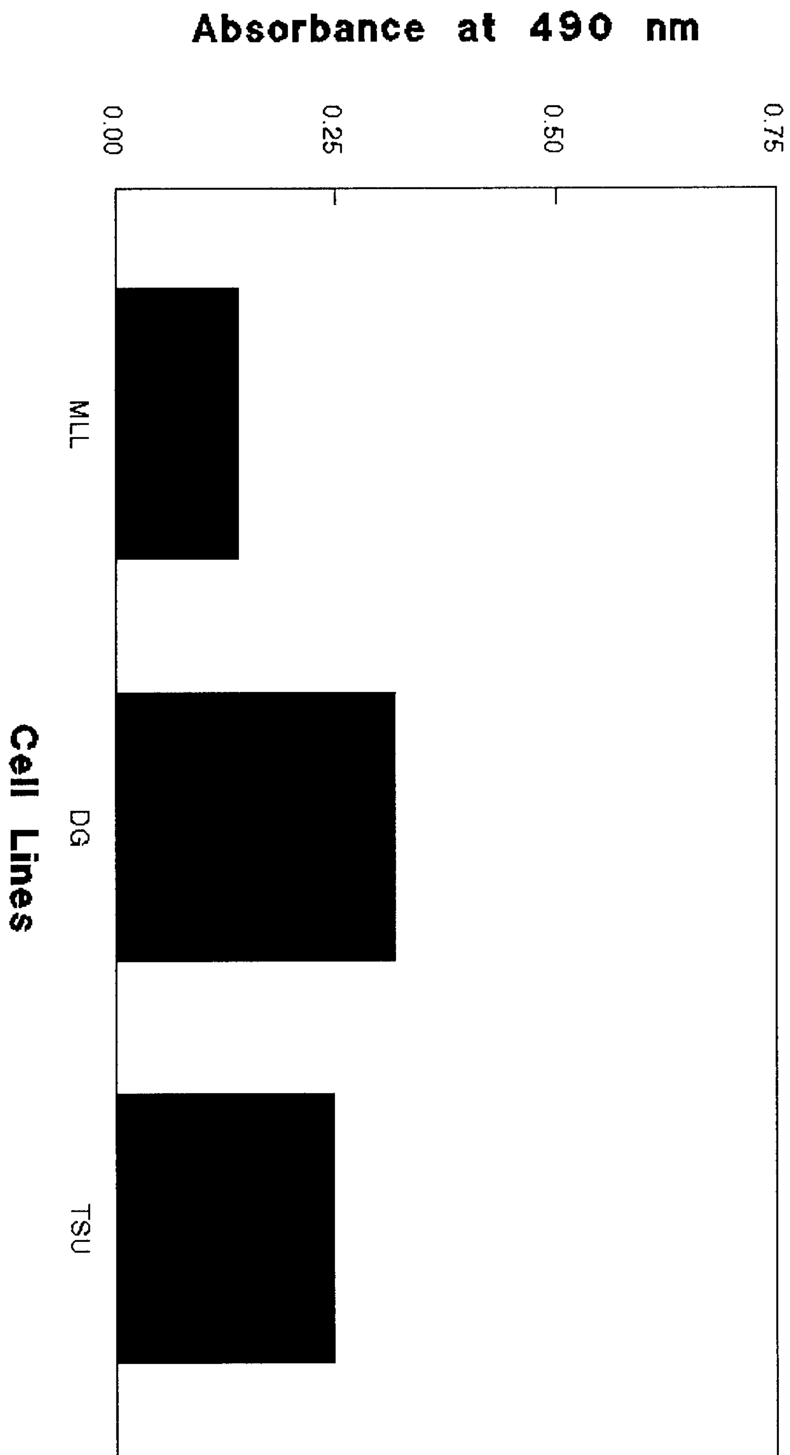
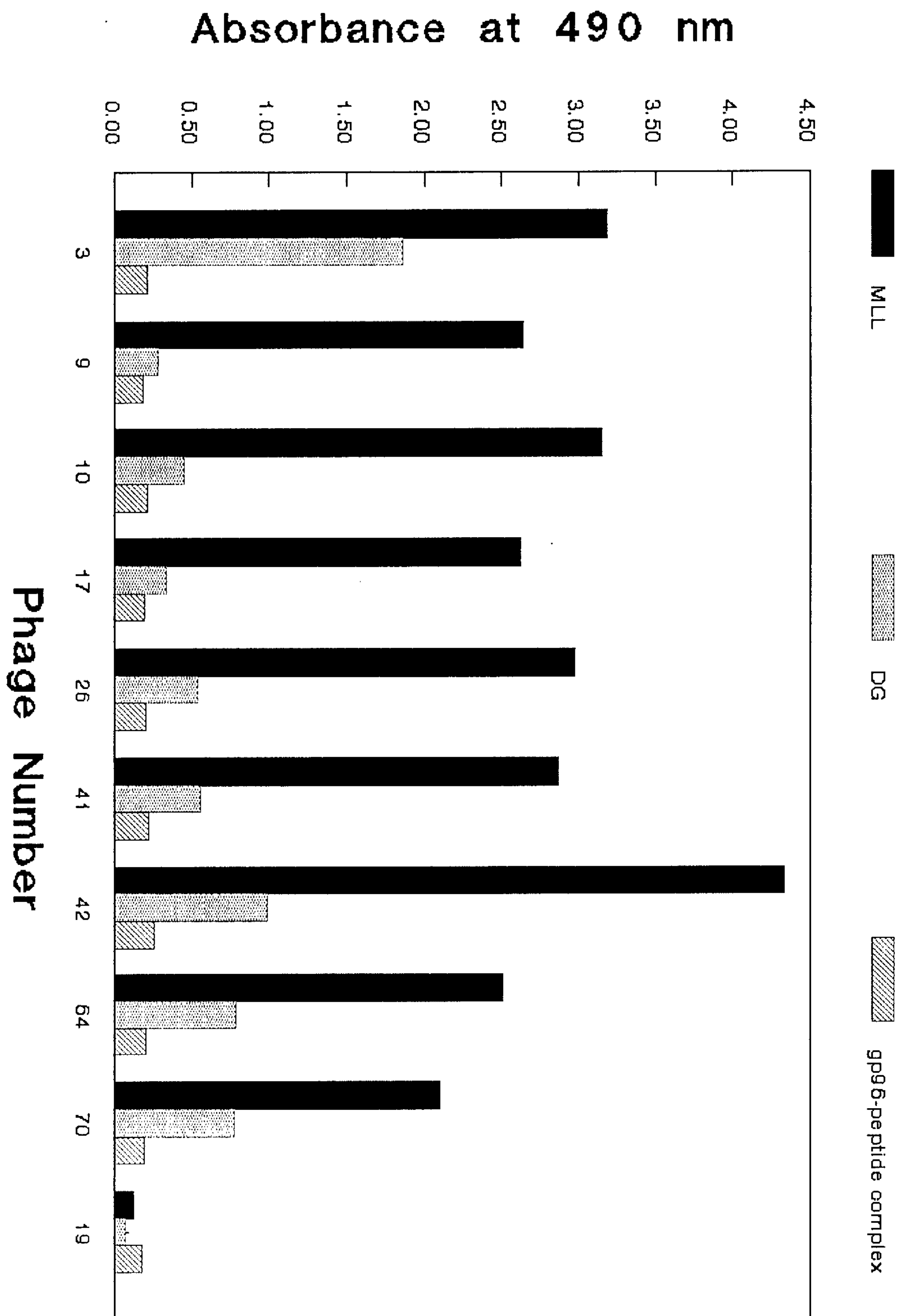


Fig. 9. Differential reactivities of scFvs to cell surface metastatic (MLL) and non-metastatic (DG) proteins and gp96-peptide complex



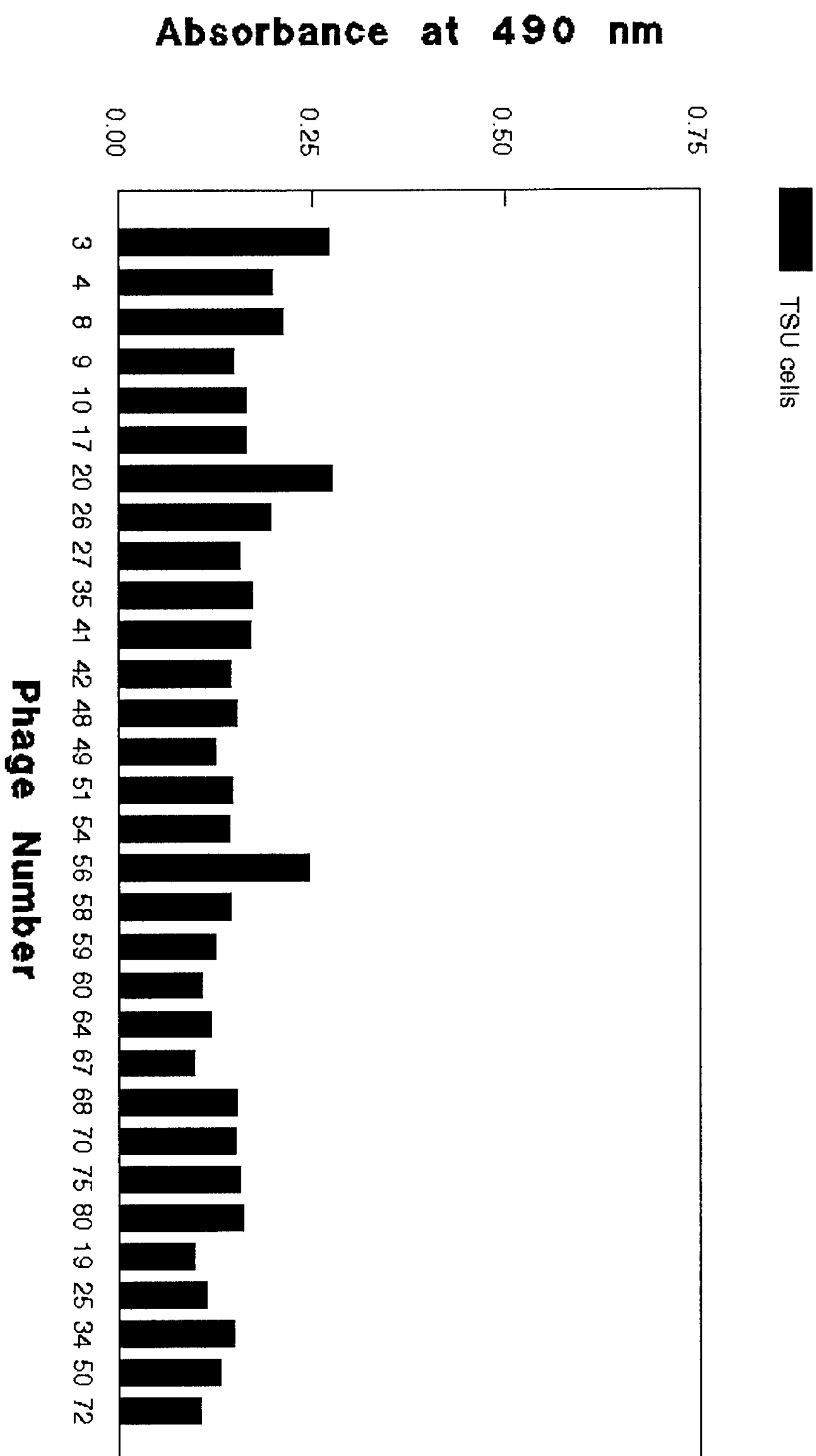



Fig. 10. Reactivity of scFvs with TSU cells

**Fig. 11. scFvs (E6/F3) react with a 170 kDa MAT-LyLu specific protein detected by $^{35}S$-Met metabolic labeling**

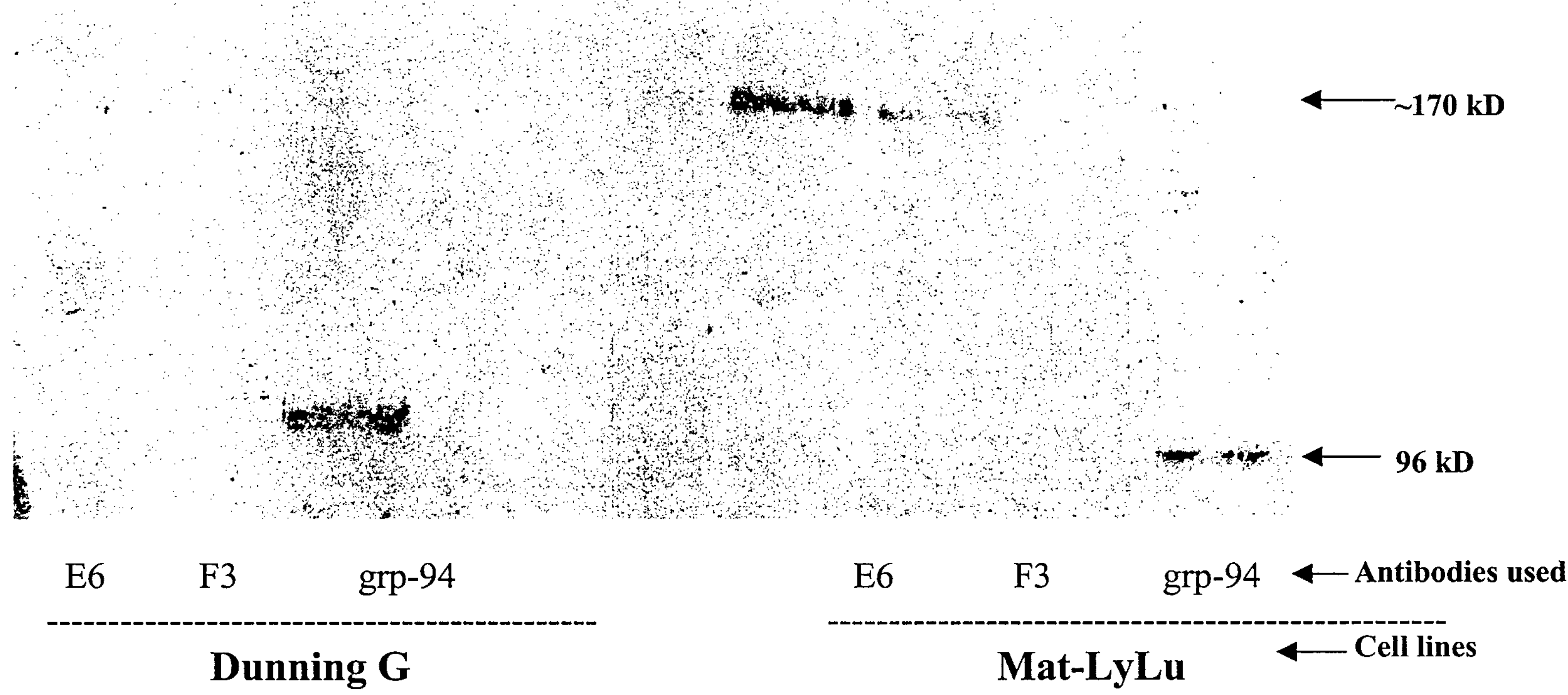


Fig. 12. scFv E6 immunoprecipitates a 170 kD protein in MAT-LyLu cells (detected by reversible E-Zinc stain)

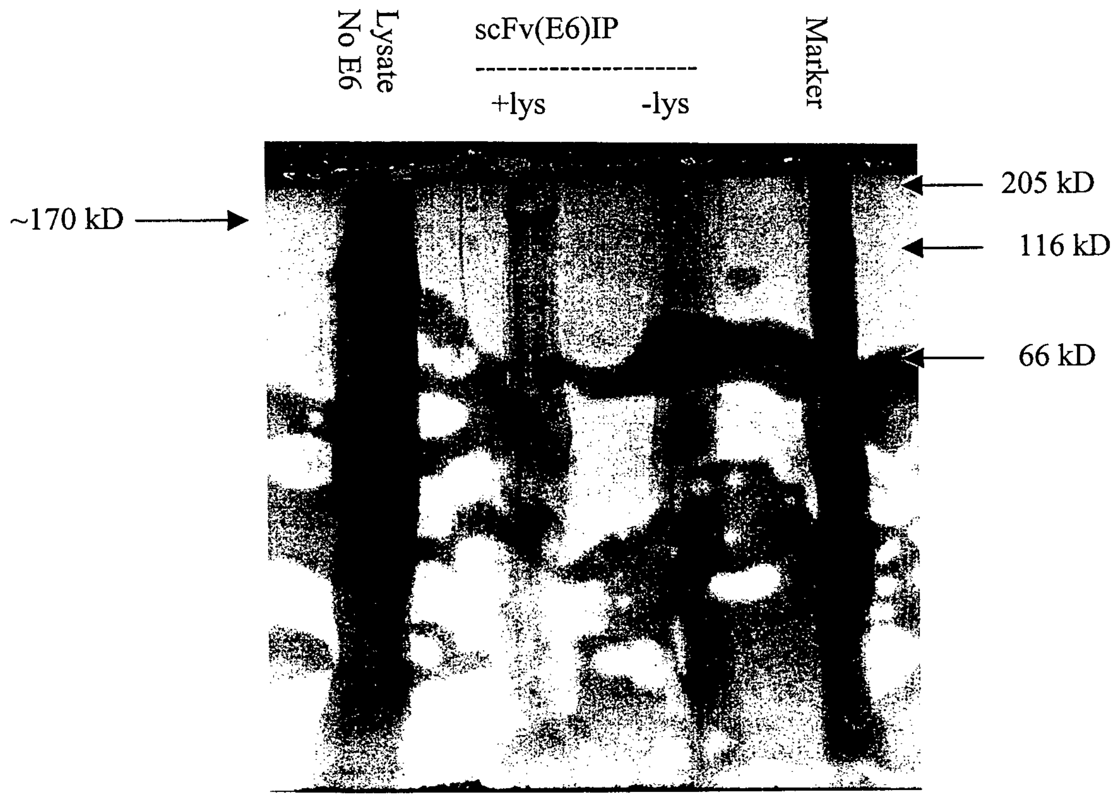


Figure 13

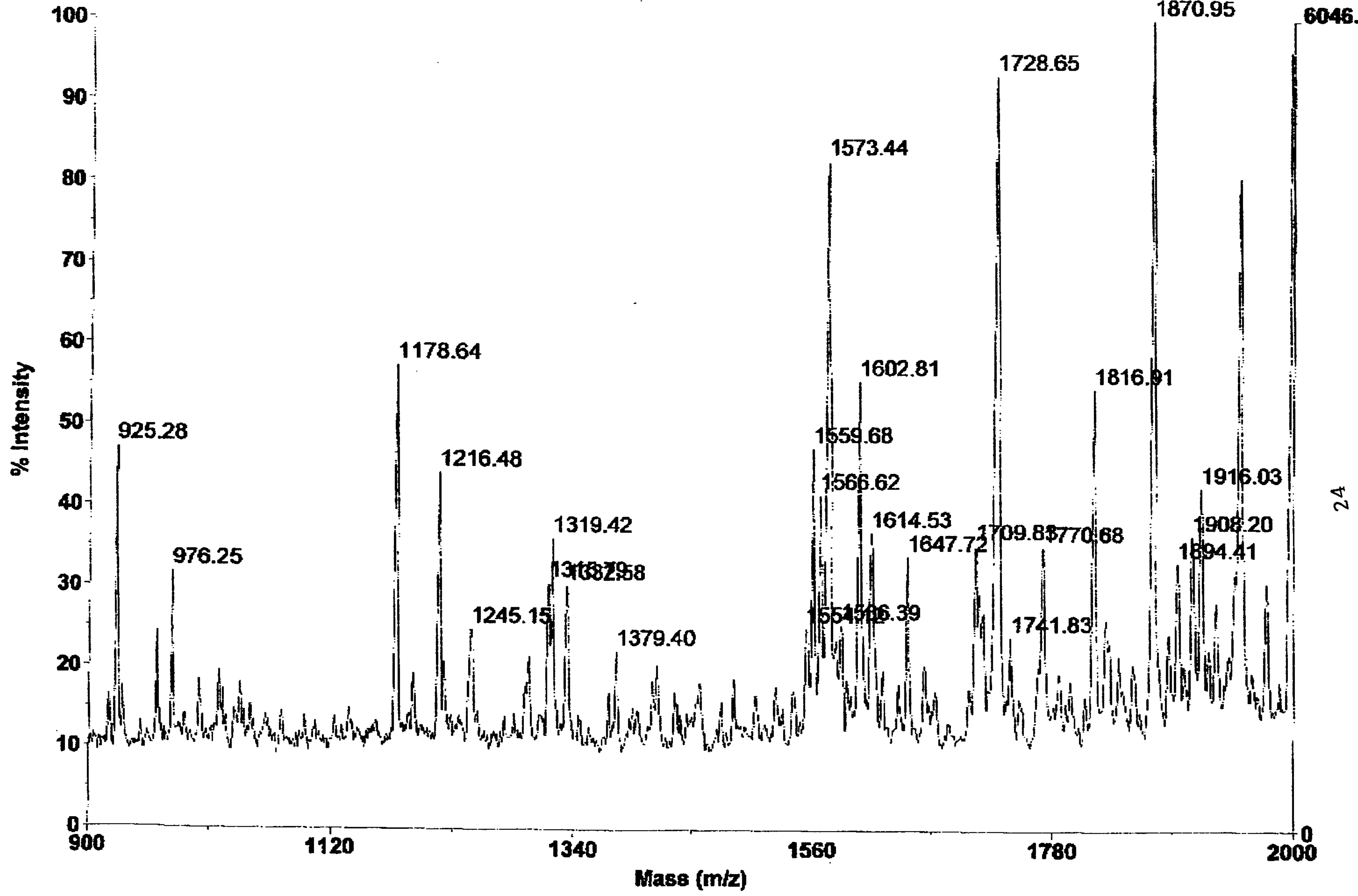


Figure 14

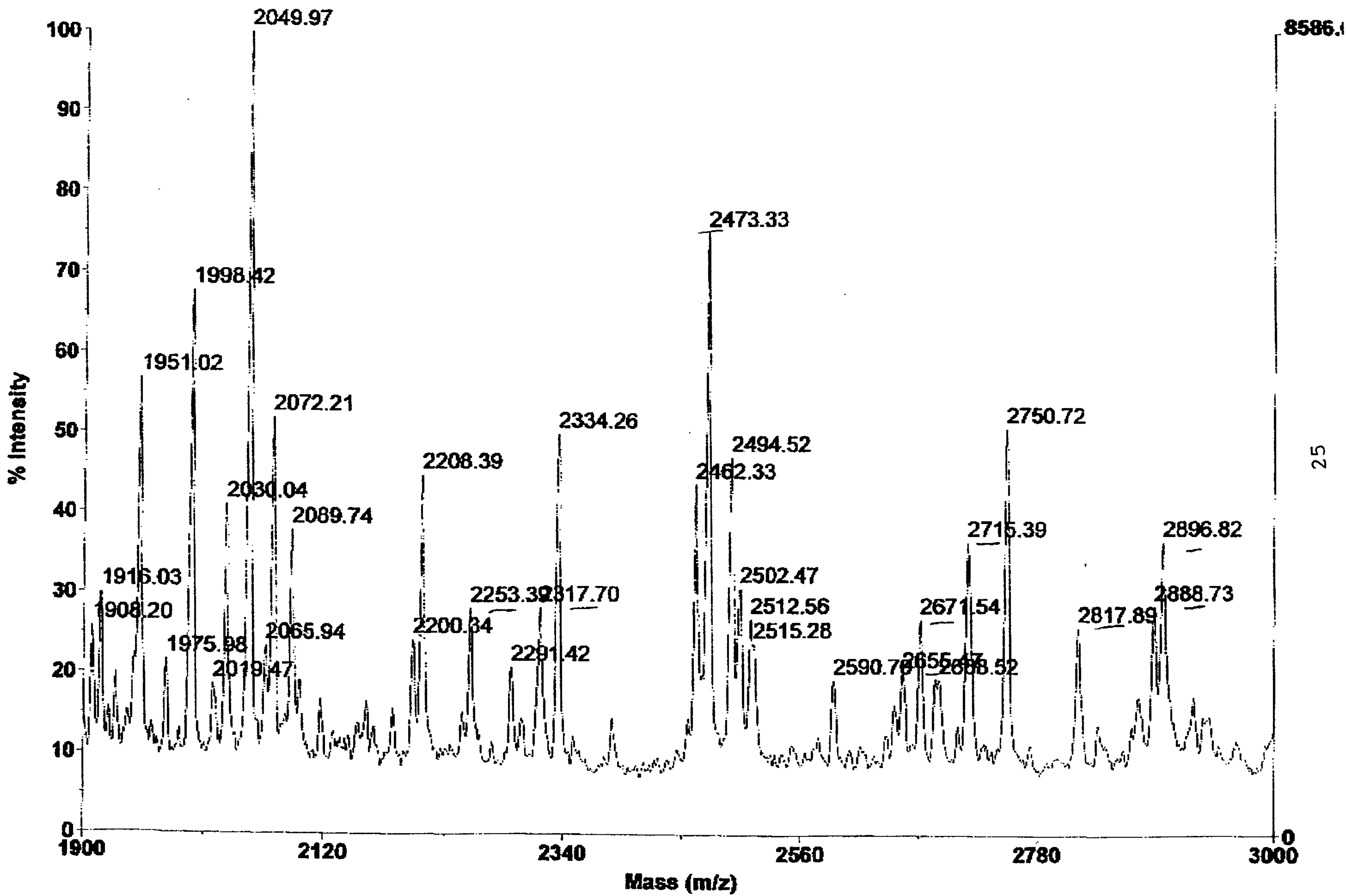
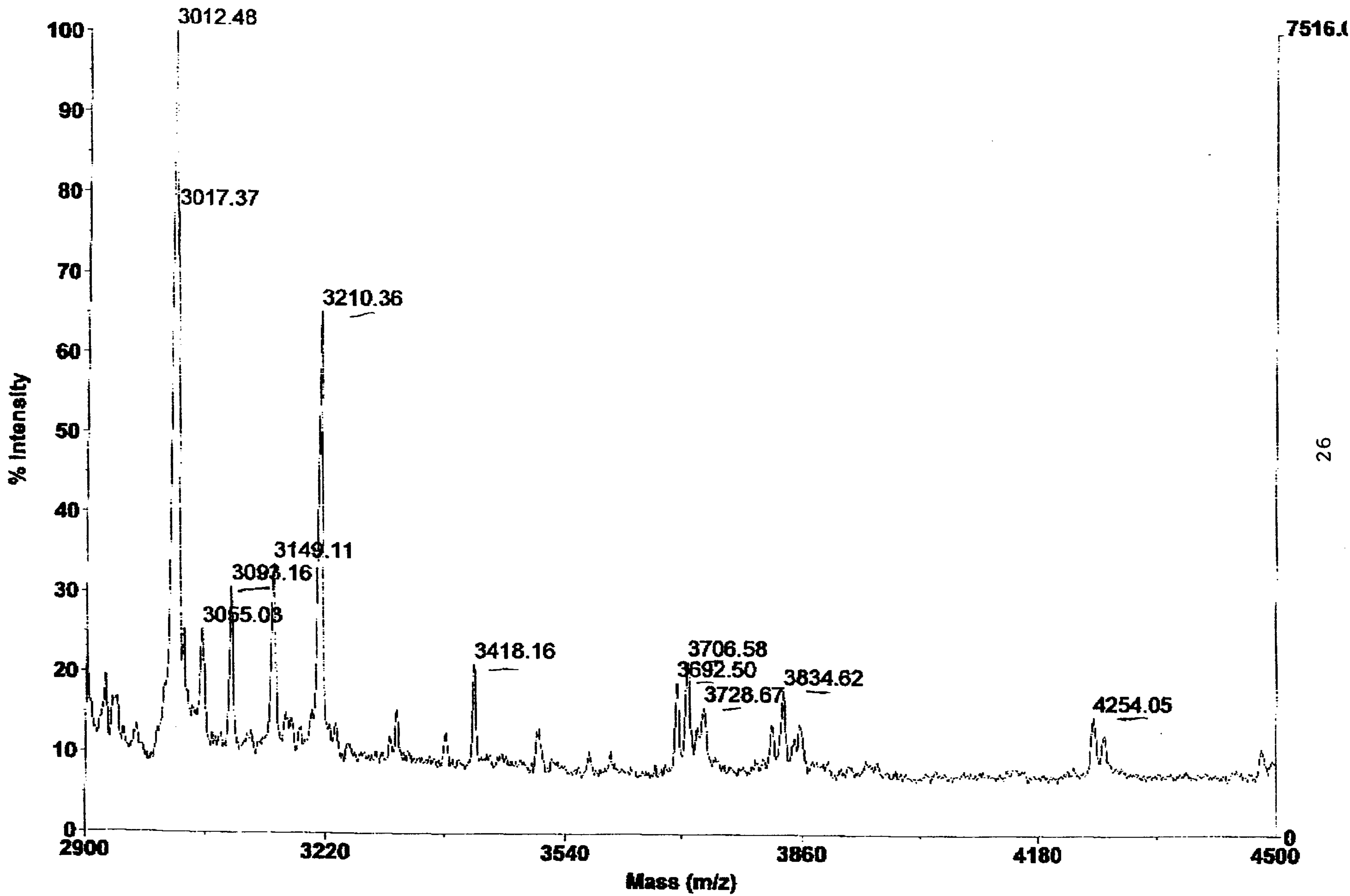


Figure 15



## Fig. 16. PANNING STRATEGY FOR SELECTING PEPTIDE MIMICS

Phage display peptide libraries LX8 (12-mer containing disulfide bridge) and X15 (linear 15-mer) were panned over E6, a MAT-LyLu specific scFv, to affinity select peptides binding to the antibody

↓

Biotinylated E6 (1 μg in 35 μl TBS) was coated to streptavidin coated 96 well microtiter plates for 1 hr at 4°C in a humidified box

↓

Antibody was washed and wells were blocked with 300 μl blotto (5% milk, 10 mM EDTA) containing 0.12 mM biotin at room temperature for 2 hr

↓

Added 50 μl blotto and 100 μl TBS containing $10^{12}$ virions to the well and incubated at 4°C in a humidified box for 4 hr

↓

Wells were washed and bound phages eluted by adding 35 μl of elution buffer (0.1 M HCl, pH 2.2) and incubating at RT for 10 min

↓

Eluted phages were neutralized with 6.6 μl of 1 M Tris, pH 9.1 and then infected into *E coli* K91 strain and amplified for further rounds of panning

↓

Four rounds of panning were performed to select, enrich and amplify specific phages

↓

Specificity of phages tested by immunoscreening and ELISA

↓

Phage clones purified, DNA sequenced and peptide sequences obtained

**Fig. 17. Reactivity of phages of peptide library to soluble single chain Fv antibody E6 by immunoscreening**

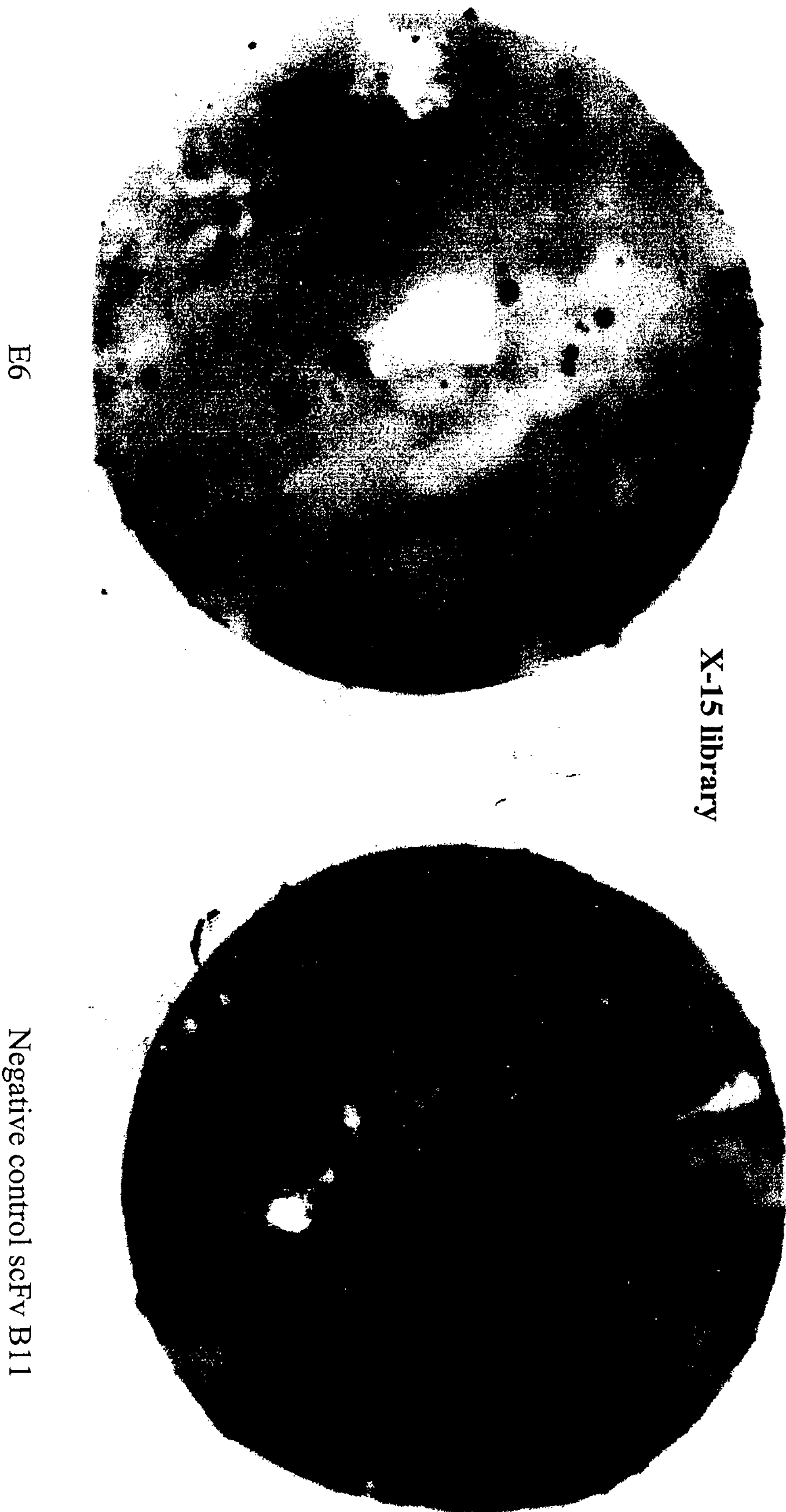
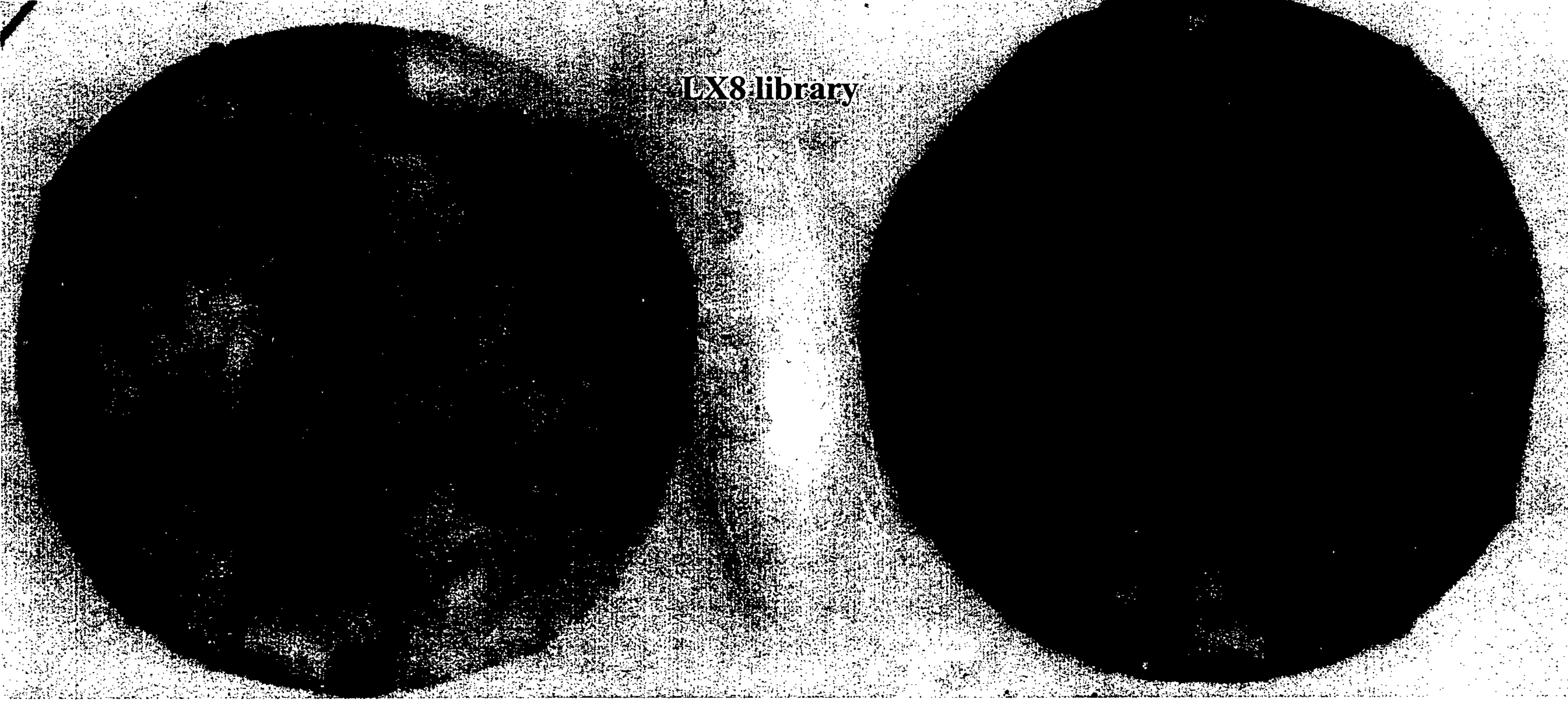


**Fig. 18. Reactivity of phages of peptide library to soluble single chain Fv antibody E6 by immunoscreening**



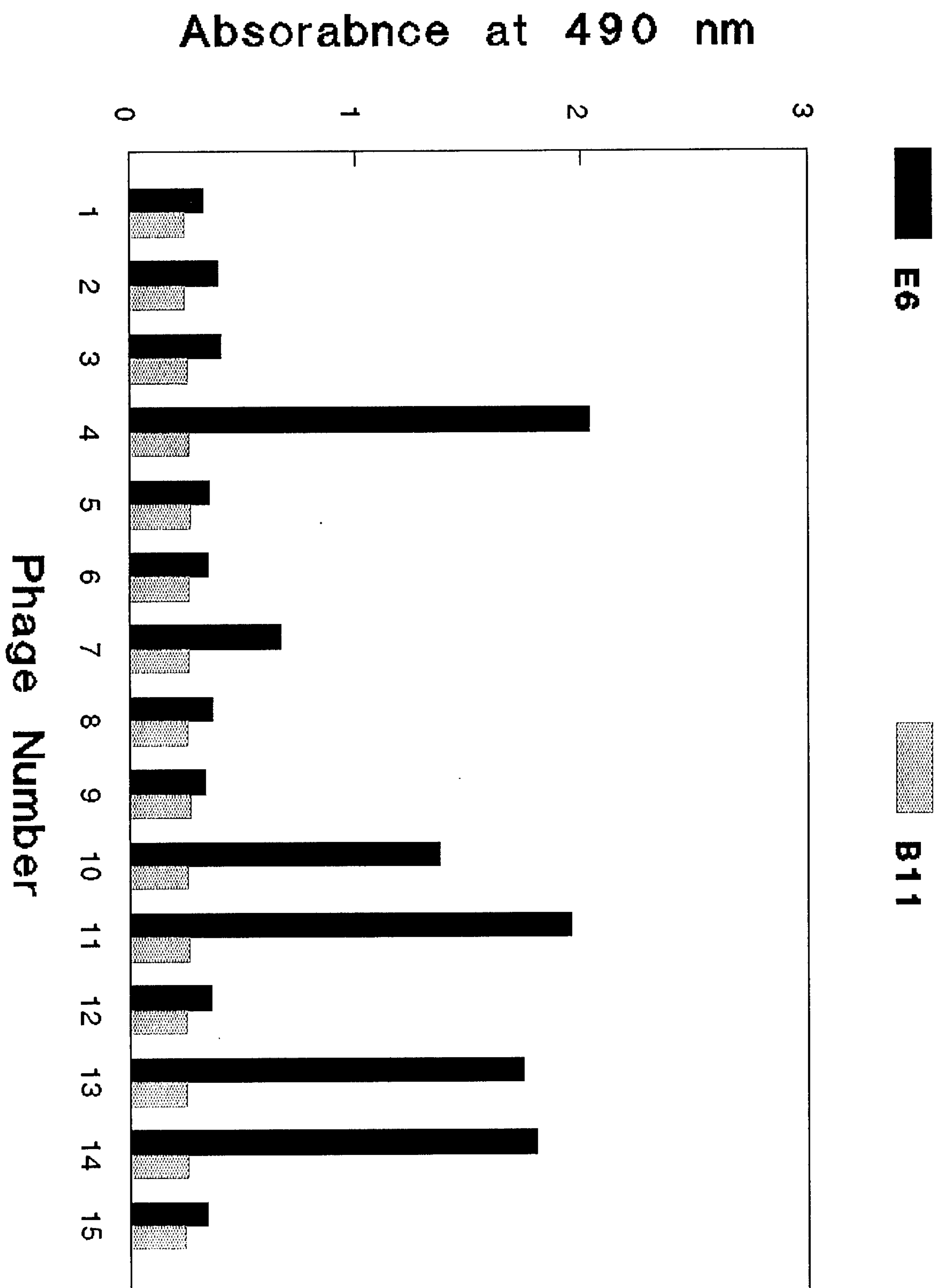



Fig. 19. Reactivity of peptide phages of X-15 library to scFv E6

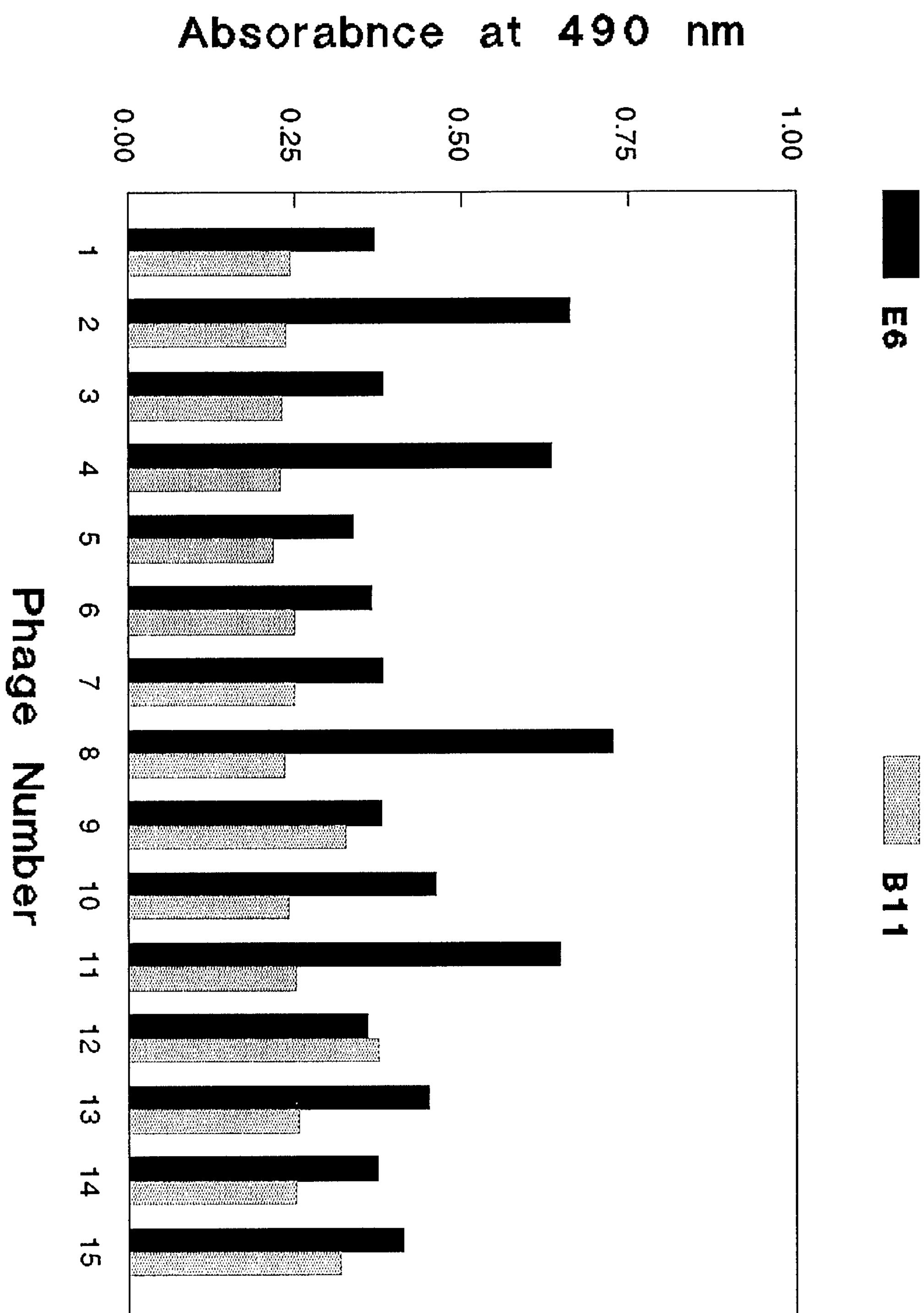



Fig. 20. Reactivity of peptide phages of LX-8 library to scFv E6

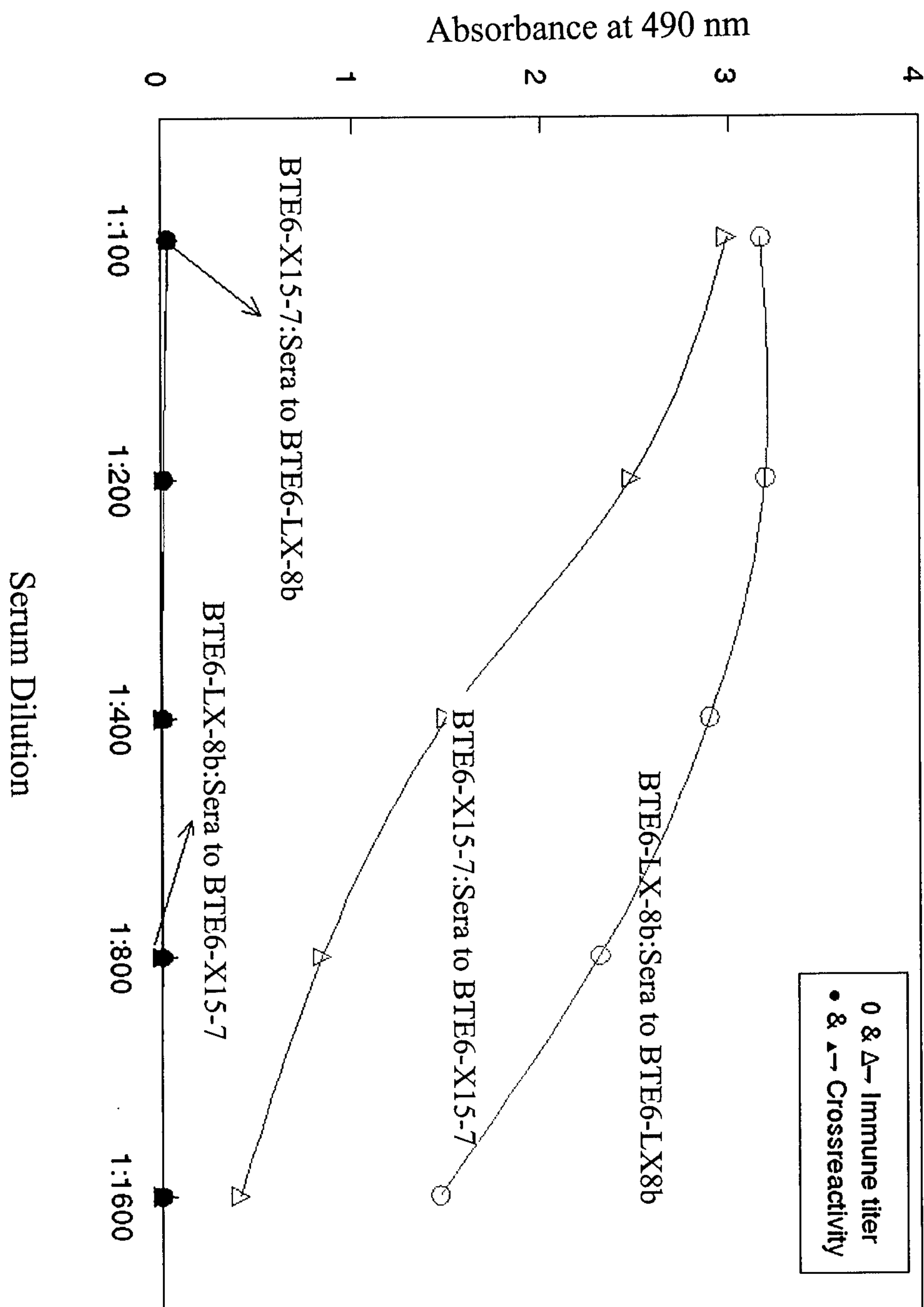



**Fig. 21. Immune reactivity of antisera to peptides**

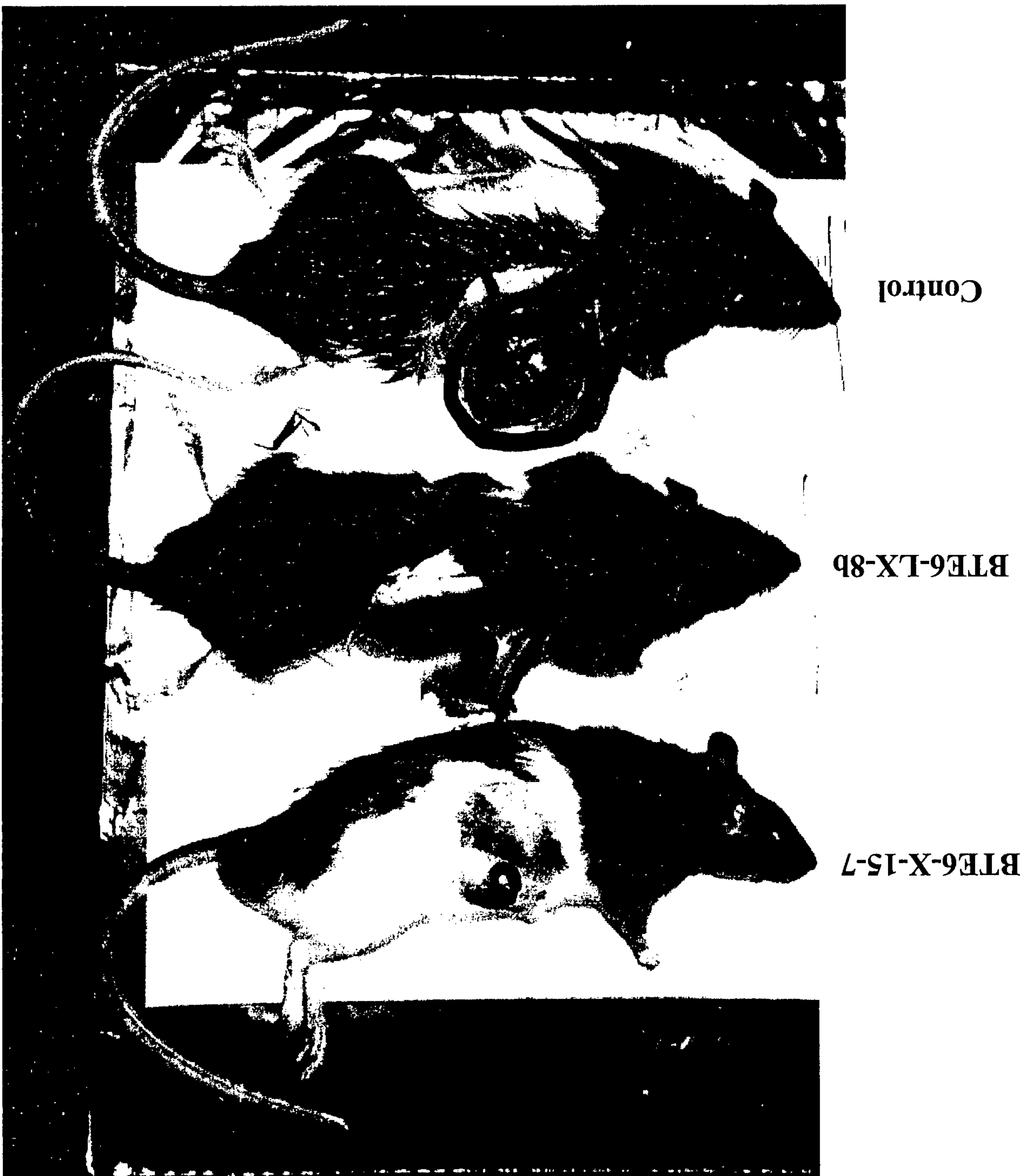



**Fig. 22. Effect of vaccination of synthetic peptides on MAT-LyLu tumor size after 2 weeks**

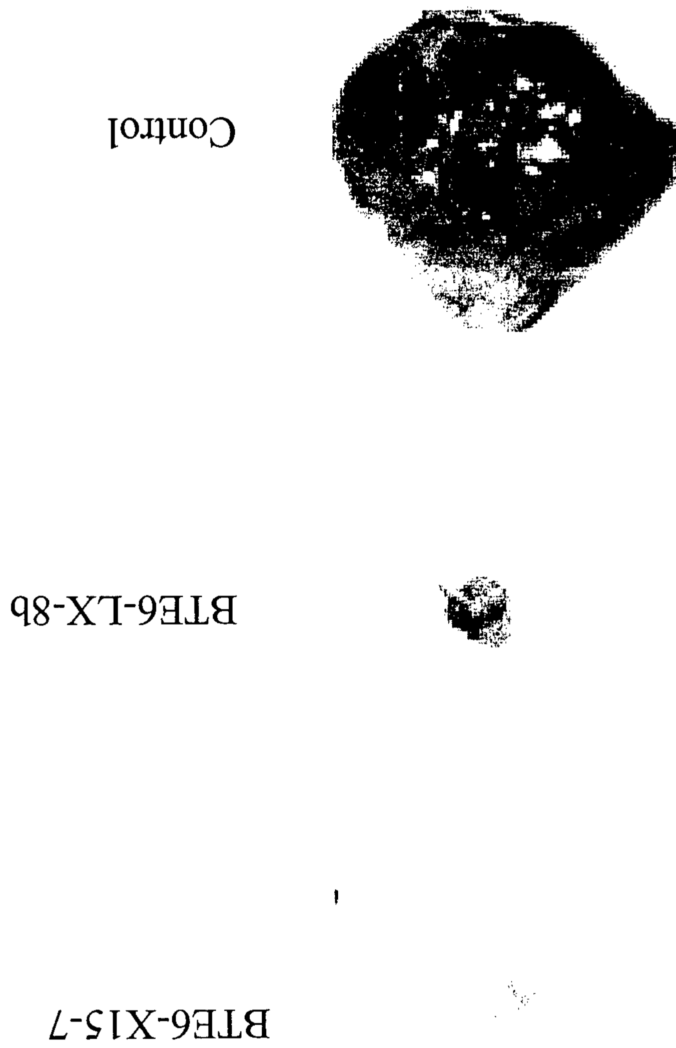



**Fig. 23. Effect of vaccination of synthetic peptides on MAT-LyLu tumor size after 2 weeks**

**Table 1. $V_H$ gene family, germline origin and $V_H$ CDR3 amino acid sequence of the**

| Antigen | scFv# | $V_H$ family | $V_H$-CDR3 | Germline origin |
|---|---|---|---|---|
| MLL-derived | E6 | $V_H3$ | GKYIRSV | DP-38 |
| gp96 | F3 | $V_H3$ | GKYIRSV | DP-38 |
| MLL cells | BTA20 | $V_H3$ | GMRPR | DP-38 |
| | BTA35 | | LSSN | |

**Table 2. Amino acid sequence of synthetic peptides derived by panning with scFv E6**

| Library | Sequence |
|---|---|
| LX-8 | YCQEGDSPRLCL |
| X-15 | YQPPSDALRWILRLQ<br>GQWQSGDRYWMETST |

**Table 3. Effect of vaccination of synthetic peptides on MAT-LyLu tumor incidence**

| Group | Percent tumor bearing animals post tumor cell challenge | | | |
|---|---|---|---|---|
| | 2 weeks | 3 weeks | 4 weeks | 6 weeks |
| Control | 100 (4/4) | 100 (4/4) | 100 (4/4) | 100 (4/4) |
| MLL derived gp96 | 0 (0/4) | 0 (0/4) | 0 (0/4) | 0 (0/4) |
| BTE6-LX-8b | 25 (1/4) | 25 (1/4) | 50 (2/4) | 50 (2/4) |
| BTE6-X15-7 | 25 (1/4) | 25 (1/4) | 50 (2/4) | 50 (2/4) |

Rats were immunized with 40 μg gp96/rat in PBS or with peptide-KLH conjugates at 100 μg/rat in Complete Freund's adjuvant (CFA) (primary immunization). Boosters of 50 μg peptides/rat in incomplete Freund's adjuvant (IFA) or 40 μg gp96/rat were administered on days 14 and 21 after primary immunization. Tumor cells (10,000/rat) were injected sub-cutaneously on day 14.

Tiwari, Raj K.

| **1.Title of the invention:** Cancer vaccine development using novel antigens, tumor rejection antigen | | |
|---|---|---|
| Name<br>Raj K Tiwari | Phone<br>914-594-4870 | Fax<br>914-594-4879 |
| Address<br>Deptt. Microbiology & immunology, BSB Rm 329 | E-mail<br>Raj_tiwari@nymc.edu | |

**INVENTION DISCLOSURE ABSTRACT/WRITE UP**

Novel tumor associated antigens/antigenic peptide mimotopes that can be used for active and passive immunotherapy have been discovered using the synthetic phage display peptide and antibody libraries. Some of these peptides have unique characteristics as they are associated with cell chaperone proteins which have tumor rejection properties. These molecules in addition to their use for cancer therapy can be used for prevention of cancer as cancer vaccines and for prevention of cancer recurrences.

Presently no cancer vaccine exists. The difficulties in the development of a unique vaccine pertains to (a) identification of suitable target such as cancer associated antigen(s) (b) the intrinsic heterogeneity of the expression of different cancer antigens (c) the re-education of the patients immune system that has developed tolerance towards cancer associated antigens (d) lack of appropriate adjuvants and delivery systems to stimulate and maintain a specific immune response. Our discovery collectively addresses all of the present limitations and is a novel application of the phage display synthetic peptide and antibody libraries. These libraries were developed to mimic the immune system in the laboratory and have not been used to identify specific immunogens that can function as tumor rejection antigens for the generation of mimotopes. The use purified tumor derived heat shock proteins (HSPs) as tumor rejection antigens is prior art but these HSPs by themselves cannot stimulate a immune response, since it is a self-protein and its structure does not differ in tumor and non-tumor tissues. The specificity of the immune response is mediated by peptides associated with these proteins which is unique for the tissue from which it is derived, hence HSPs from tumor tissues will be associated with tumor specific antigenic peptides. We have successfully utilized the synthetic antibody library to identify cancer-specific peptide antigens which is one of our unique discovery.

**POTENTIAL USES OF OUR DISCOVERIES**

1. Active and passive immunotherapy for all types of cancer, solid and blood borne.
2. Development of prevention strategies with these antigens as in CANCER VACCINCES and prevention of secondary tumor recurrences
3. Tumor antigens in our methodology include all antigens that have been discovered by us or others to differentially express in specific cancers. This includes onco-proteins, differentiation antigens, embryonic antigens and over-expressed normal proteins
4. Conjugation of synthetic single chain antibodies with any kind of chemically synthesized drugs that have anti-cancer effects
5. Synthetic peptides that are mimotopes may have therapeutic and preventive efficacy against infectious agents, bacteria, viruses , protozoal and parasites. This is on the assumption of shared epitopes between cellular antigens and antigenic epitopes with infectious agents
6. Conjugation of the antibodies and synthetic peptides with macromolecules such as proteins, nucleic acids and chemically synthesized artificial membranes for effective delivery systems
7. The use of heat shock proteins and other cellular chaperone proteins or proteins that have been shown to have affinity for peptide binding as adjuvants for stimulation of an immune response
8. The synergistic biological interaction of the immunogens used for cancer vaccine, active and passive immunotherapy with clinically relevant cytokines.
9. The synergistic biological interaction of the immunogens used for cancer vaccine, active and passive immunotherapy with other clinically relevant mono or poly clonal antibodies and agents that stimulate a antigen specific immune response.

FUNDED BY US ARMY GRANT DAMD 98-1-8534