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13. ABSTRACT *(Maximum 200 Words)*

The objective of this proposal was to identify and isolate novel prostate cancer antigens. We proposed to identify these novel antigens in an experimental rat model using purified preparations of the heat shock protein gp96 and a library of synthetic distinct antibodies that were available in the combinatorial phage display library. We demonstrated the tissue specificity in tumor rejection properties of purified preparations of gp96 by utilizing purified preparations of tumor and non-tumor gp96 preparations. By the use of a library of phage display antibodies we differentially selected out tumor reactive phages. These experiments substantiated our hypothesis that indeed these antibody libraries serve a useful tool to identify tumor specific immunogenic peptides. The specific antibody carrying phages and soluble antibodies produced from these phages were then used to identify novel tumor associated cellular protein. These studies have generated valuable reagents to define the immunological and biochemical properties of novel tumor associated antigens/peptide antigens.

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Ray K Tiwari 10/26/99

PI - Signature Date

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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. 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. It was apparent that the identification of these tumor associated peptides would be useful therapeutic and preventive agents.

We proceeded systemically to analyze the preventive and therapeutic effects of purified preparations of gp96 and to identify the peptides associated with gp96 in a rat experimental model. The R3327 Copenhagen rat model was chosen as it is one of the well studied spontaneous rat model and has several cell lines developed with distinct properties. The two cell lines that we chose to work with in this project were Dunning G which was slow growing, androgen sensitive and non-metastatic, and MAT-LyLu, a derivative of Dunning G that was fast growing, androgen independent and readily metastasizing to the lung and lymph nodes.

We present results that examined the following aims :

1. Preventive and therapeutic effect of tumor derived gp96-peptide complexes on the growth of Dunning G and MAT-LyLu tumors in Copenhagen rats.

2. Identification of phages with synthetic single chain antibodies that recognize specifically tumor associated gp96-peptide complexes.

3. Identification of cellular proteins from which these peptides were generated.

Aims 2 and 3 fulfill Technical Objectives I and II of the specific proposal and Aim 1 establishes the significance of the use of gp96 in a cancer vaccine program for prostate cancer.

BODY

Results

Aim 1. Preventive and therapeutic effect of tumor derived gp96-peptide complexes on the growth of Dunning G tumors in Copenhagen rats.

The details of the experimental design are described (1), some highlights of the results include

(a) Dunning G cells contain gp96

Demonstration of the presence of gp96 in Dunning G cells and that our purification procedures from these cells can identify and purify gp96 . This purified preparation formed the basis of our prophylactic and therapeutic studies. Figure 1 shows the step wise purification of gp96 from Dunning G cells. Although two bands are observed after the

DEAE-Sephacel, this material was not purified further as the antigenic composition of both of these proteins have been found to be similar (2). A typical yield of the purified protein was 400 μg protein per 10 ml packed cell pellet. This protein was recognized by the antibody to grp94 (Neomarkers, Fremont, CA) in a Western blot performed by methods described (3), using anti-rat IgG and iodinated protein G (NEN/Dupont, Boston, MA). Tumor derived gp96-peptide complexes at concentrations of 10 and 40 μg were used as vaccinating agents

(b) Tumor induced protective immunity is detectable in the R3327/ Copenhagen syngeneic model.

To determine if Dunning G induced tumors elicit a protective immune response the following experiment was performed. One million live Dunning G cells was injected subcutaneously and tumors allowed to develop until 5 cm^3 of diameter (twelve weeks after tumor cell injection). The tumors of these was surgically resected and the animals allowed to recuperate for two weeks after which they were injected with one million fresh live Dunning G cells on the flank opposite to the previous tumor. As control, three naive rats of approximately the same age were also injected with one million Dunning G cells. As shown in Figure 2, the naive animals which did not have a prior exposure to tumor cells started developing tumors in about four weeks, whereas, the animals that had prior exposure to Dunning G cells developed tumors by ten weeks, a lag of about six weeks as compared with naive animals. The delay in tumor induction by fresh cells in animals that had prior exposure to these cells is indicative of an active protective tumor response.

(c) Prophylactic effect of tumor derived gp96.

It has been observed that purified preparations of gp96 can protect against subsequent challenges of the tumor cells from which it is derived (4,5). To test if gp96 isolated from Dunning G cells could protect against Dunning G induced tumors in Copenhagen rats, we vaccinated a group of mice at two different concentrations, 10 and 40 μg per rat, and compared this experimental group with rats that were either sham vaccinated with phosphate buffered saline (PBS) or vaccinated with rat albumin. The vaccination schedule was day 0 and day 7 and live cell challenge with one million Dunning G cells at day 7. The effect of Dunning G derived gp96-peptide complexes on tumor incidence and latency is presented in Table 1. By six weeks none of the control animals are tumor free, whereas, 66 % of the rats vaccinated with gp96-peptide complexes are tumor free. All rats vaccinated with gp96 show palpable tumors by nine weeks as opposed to six weeks in the non-immunized group, an increase in the latency of three weeks. The tumor inhibitory effect is also reflected on the rate of tumor growth (Figure 3) and the tumor volume (Table 2). Reduction in tumor volume of fifty percent is observed in the gp96 vaccinated group as compared to the sham vaccinated group. Statistically significant reduction of tumor growth was observed in the rat albumin vaccinated group when compared with control. Rat albumin has peptide binding property and may not be the ideal control. Animals vaccinated with gp96 showed statistically significant reduction in tumor growth when compared with the rat albumin vaccinated group ($P < 0.01$). These results provide strong evidence that gp96-peptide complexes can delay tumor latency and alter tumor growth.

(d) Therapeutic effect of gp96.

Administration of gp96 therapeutically has immediate clinical implications. We tested the therapeutic effect of Dunning G derived gp96 on Dunning G induced tumors in Copenhagen rats (Figure 4). The therapy was started two and a half week after live tumor cell injection. There was no evidence for palpable tumor at initiation of therapy but tumors were predicted to develop in the next two to three weeks. Ten micrograms of Dunning G derived gp96, rat albumin or sham injection of PBS (control animals) was administered to these animals, three times a week for six weeks. Of the four animals that were treated with gp96, three animals showed a reduction or stabilization in tumor growth (Figure 4). Withdrawal of therapy resulted in the rate of tumor growth comparable to the sham or albumin injected animals.

(e) Prophylactic effect of MAT-LyLu derived gp96

To test if gp96 isolated from MAT-LyLu cells could protect against MAT-LyLu induced tumors in Copenhagen rats, we vaccinated a group of rats at 40 μg per rat, and compared this experimental group with rats vaccinated with liver derived gp96. The vaccination schedule was day 0 and day 7 and live cell challenge with ten thousand MAT-LyLu cells at day 7 (Figure 5). Reduction in tumor diameter of fifty percent is observed in the tumor derived gp96 vaccinated group as compared to the liver gp96 group. These results provide strong evidence that specific gp96-peptide complexes can delay tumor latency and alter tumor growth both for Dunning G and MAT-LyLu cells in Copenhagen rats.

AIM 2. Identification of phages with synthetic single chain antibodies that specifically recognize tumor associated gp96-peptide complexes.

It is clear from the results of experiments of Aim 1 that tumor derived gp96 has prophylactic and therapeutic effects and that immunological modulations even in spontaneous cancers such as prostate cancer is possible. It has also been well established in the literature that the immunlogical properties of purified gp96 is due to the peptides that are associated with the purified gp96 preparations and not due to gp96 protein per se (5). Purified preparations of gp96 stripped of the associated peptides lose their immunogenicity and their cancer protective and therapeutic effects. Identification of these cancer specific peptides could lead to well-defined immunogens, some of which can cause tumor regression or inhibition of metastatic propensities. We report the use of combinatorial synthetic phage display antibody library as a source of immunogenic repertoire that could specifically identify tumor derived gp96-peptide complexes.

(a) 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. (6). 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 Vλ3 light chain to generate a repertoire of $>10^8$ clones (Figure 6). 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.

(b) 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 7) 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.

(c) Reactivity of phages eluted after specific binding to tumor derived gp96-peptide complexes

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 8, 9, 10) 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 8), however, phages that react with both liver and tumor derived gp96-peptide complexes (Figure 9) or phages that react only with liver (Figure 10) were also identified. These experiments were consistent in at least more than three different purified preparations of gp96-peptide complexes, though some variations in reactivity was observed through different preparations. None of these phages reacted with gp96 native protein itself. These results clearly indicate that

- 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.

(d) Specificity of reaction of the tumor-specific phages

Some of phages that reacted with MAT-LyLu derived gp96-peptide complexes with high affinity as judged by O.D. readings in ELISA were tested for reactivity with other tissue derived gp96-peptide complexes (Figure 11). The phages were found to be specific and reacted with enhanced affinity to prostate cancer derived gp96-peptide complexes.

The results of these experiments validate TECHNICAL OBJECTIVE 1 in the original proposal which was to test the HYPOTHESIS that prostate cancer associated peptides which may act as tumor rejection antigens can be identified in gp96-peptide complexes utilizing a combinatorial antibody library based strategy.

Aim 3. Identification of cellular proteins from which these peptides were generated.

The identification of specific phages that react with tumor derived gp96-peptide complexes are important reagents to identify and isolate tumor associated antigens. To this end we used two of the phages that specifically reacted with tumor gp96-peptide complexes to immunoprecipitate cellular proteins that were metabolically labeled with [^{35}S] methionine. Metabolic labeling was done as per procedures described in earlier publications (3). Soluble antibodies from these phages was generated by induction with IPTG and these antibodies was used immunoprecipitate cellular proteins. We successfully detected one protein of approximate molecular weight 170 kDa and these antibodies did not immuoprecipitate proteins from Dunning G cell line indicating that this protein was specifically expressed in MAT-LyLu cells.

The results of these experiments fulfil the TECHNICAL OBJECTIVES II of the original proposal that stated that the aim was to identify the proteins of origin of the isolated peptides.

Experiments in progress :

1. Identification of phages that are differentially selected over Dunning G and MAT-LyLu cells to identify cell surface markers

2. Determine the reactivity of cell surface specific phages with cell derived gp96-peptide complexes

3. Identify and isolate specific peptides from a combinatorial phage display peptide library that these phages interact.

4. Examine the tissue distribution of the proteins that these phages identify

5. Examine the biochemical and immunological properties of the tumor associated proteins recognized by these antibodies

KEY RESEARCH ACCOMPLISHMENTS

- Tumor induced protective immunity can be induced in a rat model

- Specific tumor derived gp96-peptide complexes can mitigate tumor delay and enhance latency in a prostate cancer rat experimental model

- The synthetic combinatorial phage display antibody library and purified gp96-peptide complexes can be utilized to differentially separate out prostate cancer associated antigens

- Soluble antibodies produced by specific phages are valuable reagents to identify and isolate cellular proteins that my be differentially expressed in tumor cells

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.

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6. 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.

CONCLUSIONS

- The preventive and therapeutic effect of gp96-peptide complexes in R3327 rat model has been observed
- The use of the combinatorial antibody library can be used as novel reagents to identify cancer associated antigenic epitopes and detect specific cancer related proteins from which these epitopes have been generated.
- Further analyses on the distribution of these antigens needs to be done which is being pursued
- Biochemical and immunological characterization of these antigens needs to done to determine the efficacy of these reagents as cancer preventive and therapeutic agents

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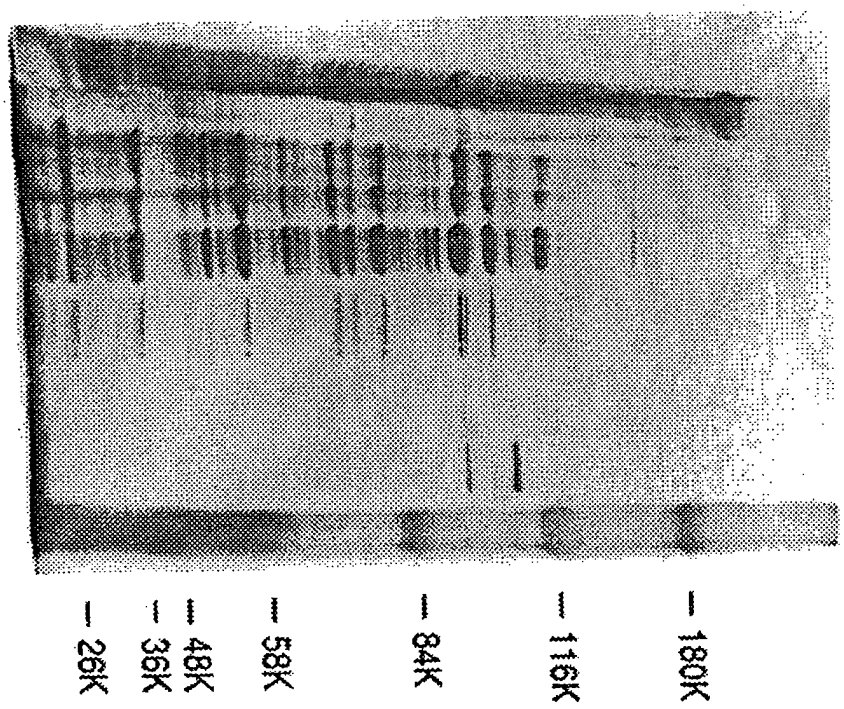
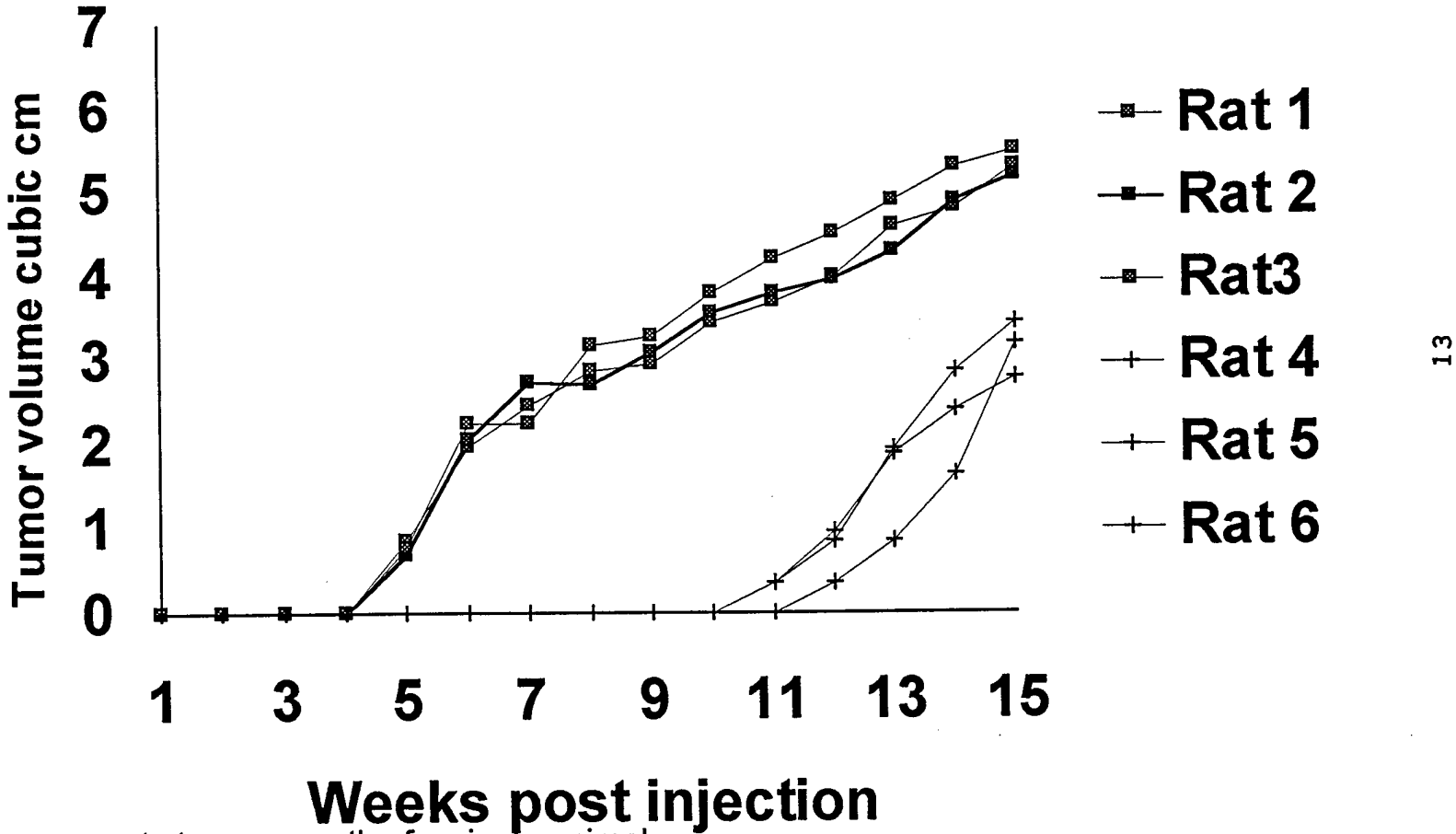

1 50% Amm. Sulfate
2 80% Amm. Sulfate
3 ConA-Sepharose Flow Through
4 PD-10 Column Flow Through
5 DEAE Column Flow Through
6 Gp 96
7 Marker
180K
116K
84K
58K
48K
36K
26K

Figure 1

Figure 2

Protective Immunity of Dunning G induced tumors in Copenhagen

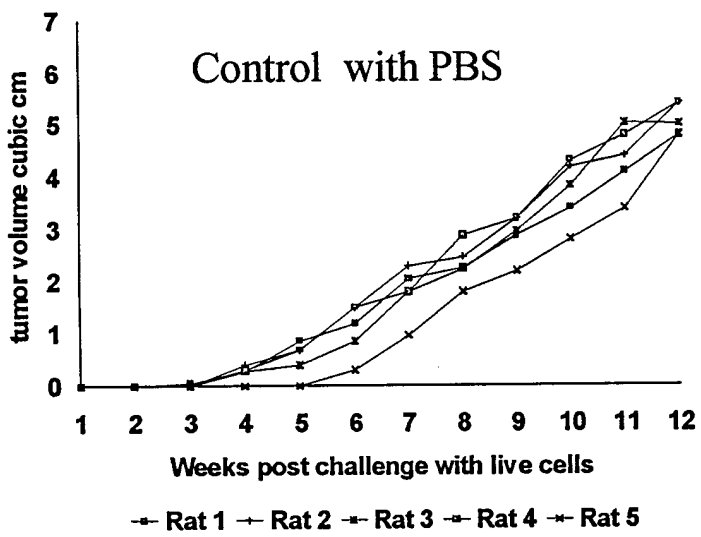
All rats injected with 1 million Dunning G

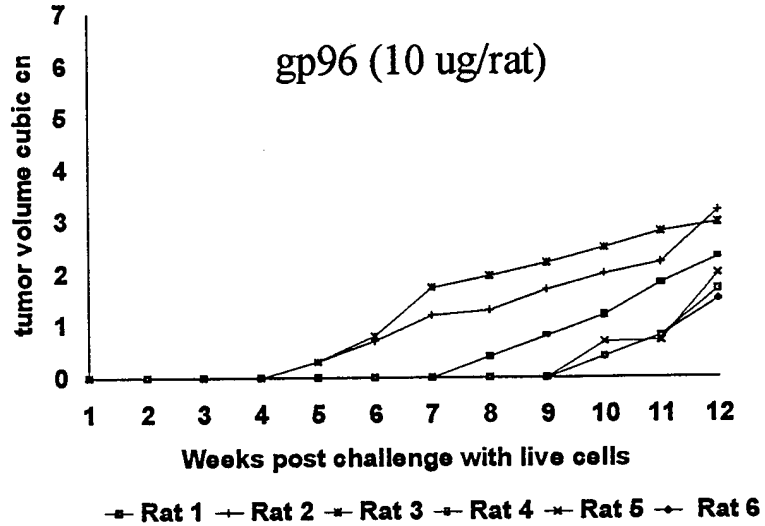


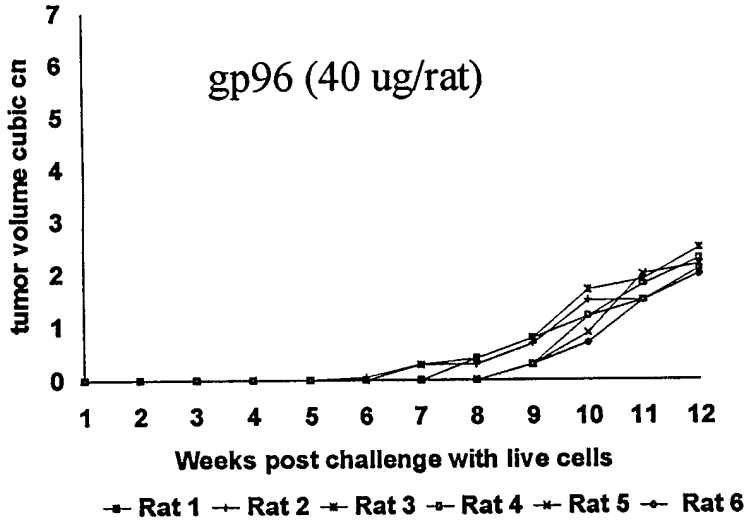
Each line represents tumor growth of a single animal
Rats 1, 2 and 3 are naive rats
Rats 4,5,6 had their tumors (3-5 cms) surgically removed and challenged with fresh live cells

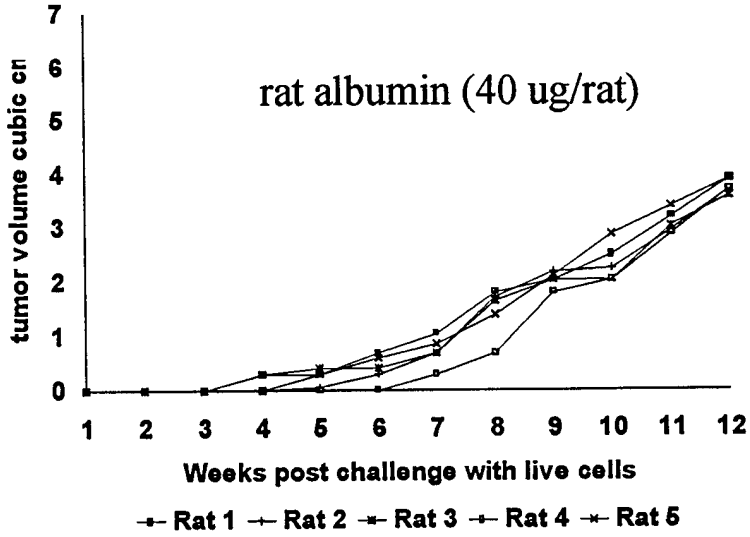
Prophylactic effect of gp96 on the growth of Dunning G in Copenhagen rats

Vaccination on day 0 & day 7, challenge day 7



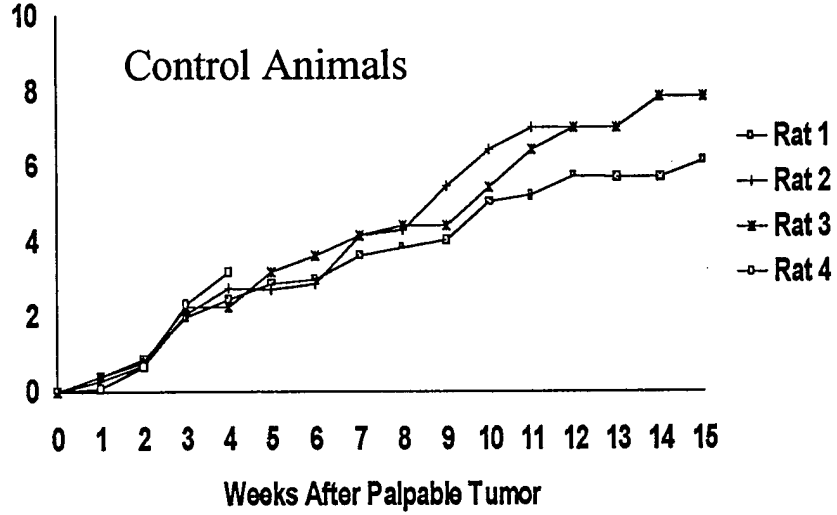


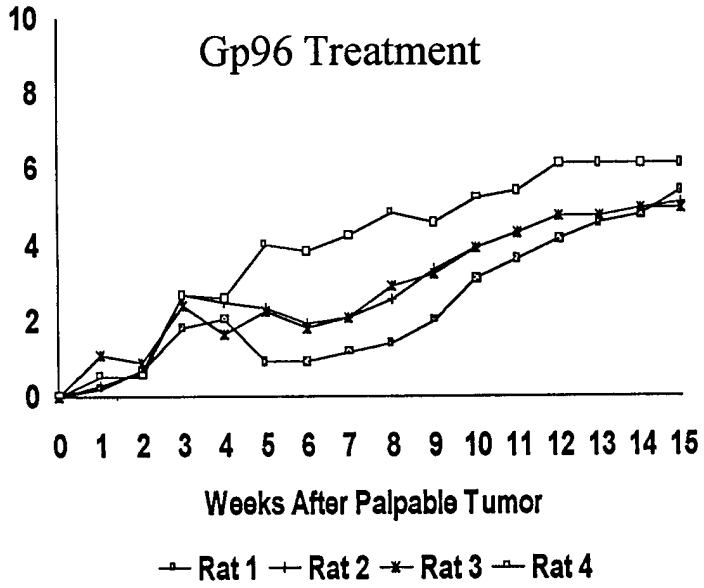




Therapeutic effect of gp96 on the growth of Dunning G in Copenhagen rats

All rats injected with 1 million Dunning G cells eighteen days prior to day 0





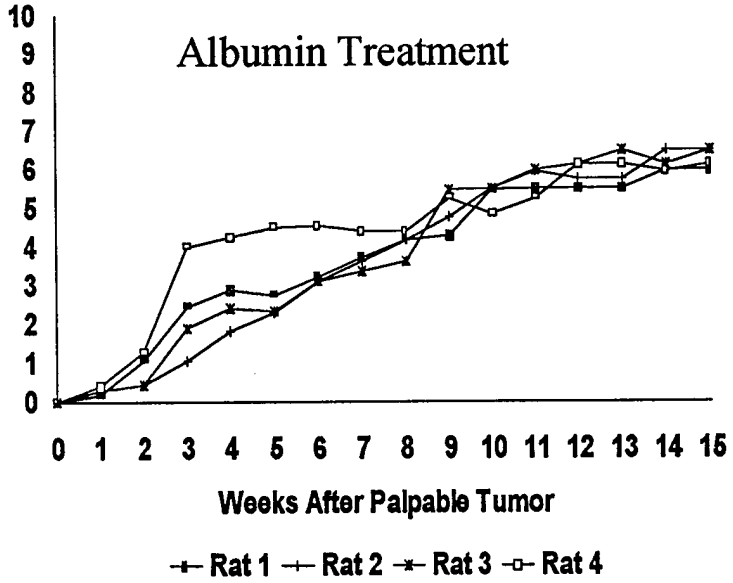
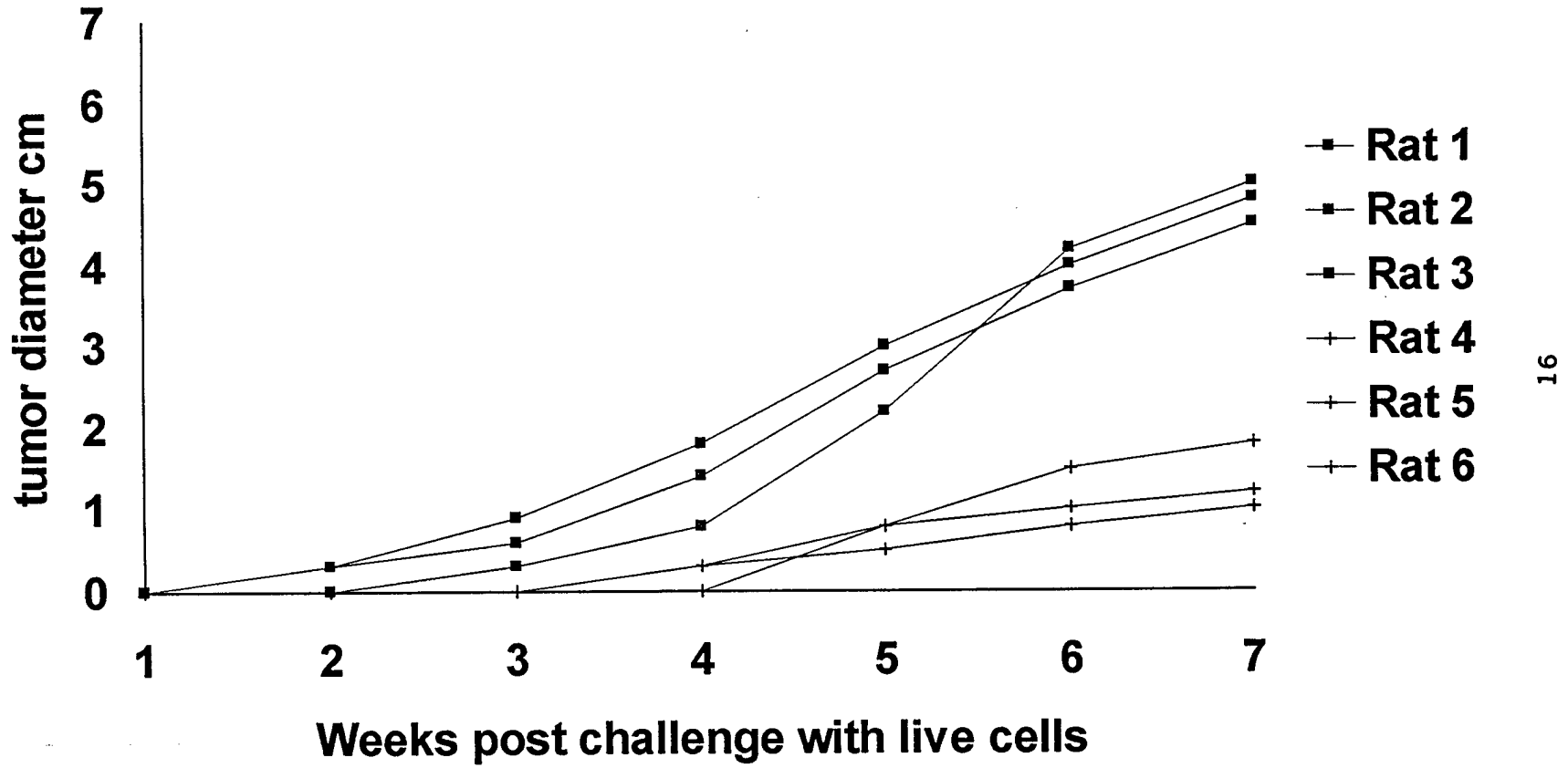


Figure 5

Growth of MAT-LyLu cells in Copenhagen rats

Vaccination with gp96 (liver) or MLL day 0, day 7 challenge day 7



Rats 1,2 3 gp96 liver
Rats 4,5,6 gp96, MLL
tumor cell challenge 10,000

Figure 6

CONSTRUCTION OF PHAGE DISPLAY LIBRARY

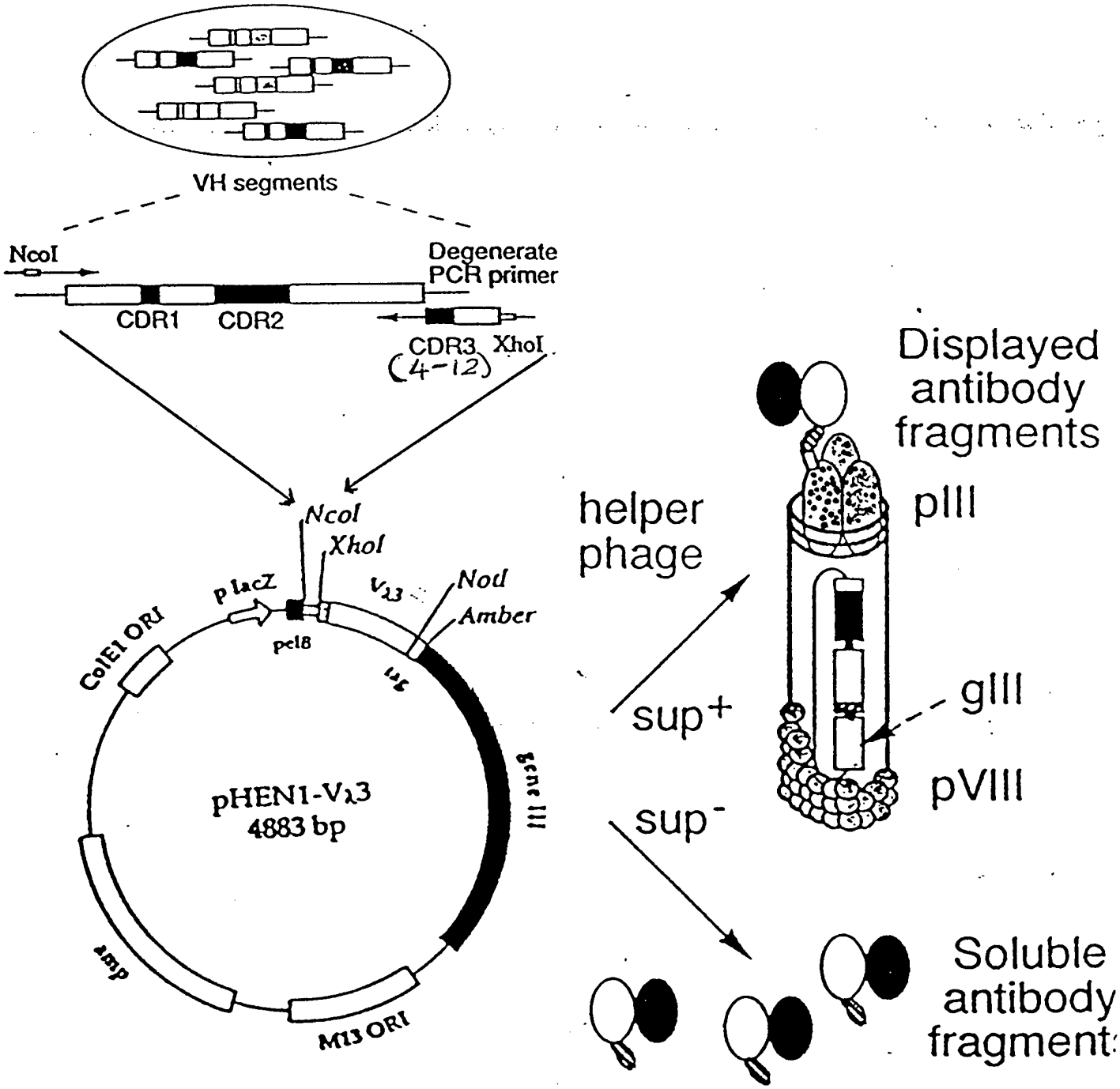


Figure 7

PANNING STRATEGY

Library of single chain phage display combinatorial antibodies panned over normal liver derived gp96-peptide complexes

↓

Unabsorbed phages panned again over liver derived gp96-peptide complexes 3X

↓

Unabsorbed phages panned over MAT-LyLu (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

↓

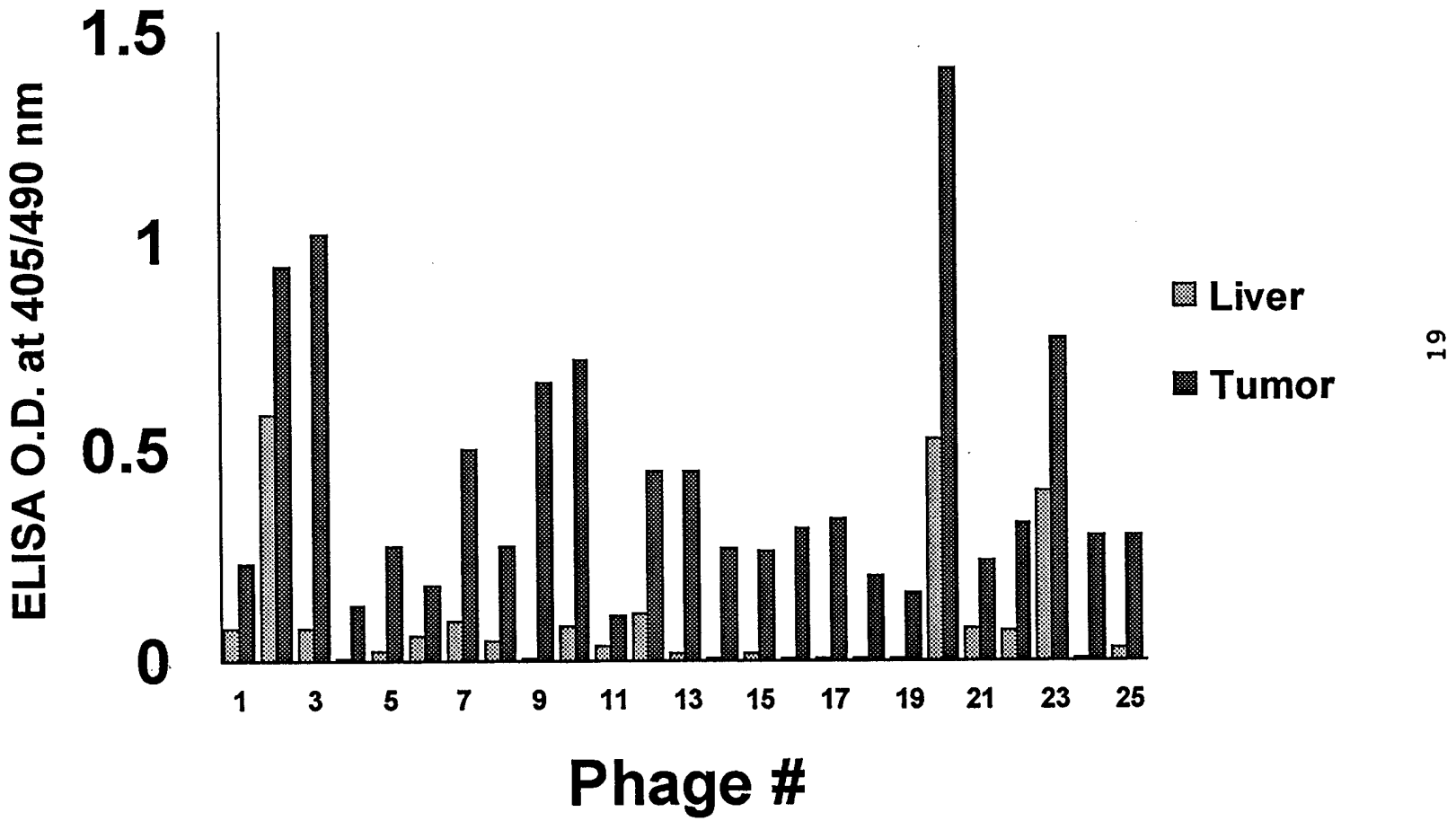
Absorbed phages eluted and amplifed for three rounds of differential panning between liver and tumor derived gp96-peptide

↓

Absorbed phages at the last stage of panning eluted and tested for reactivity to gp96-peptide complexes by ELISA

Figure 8

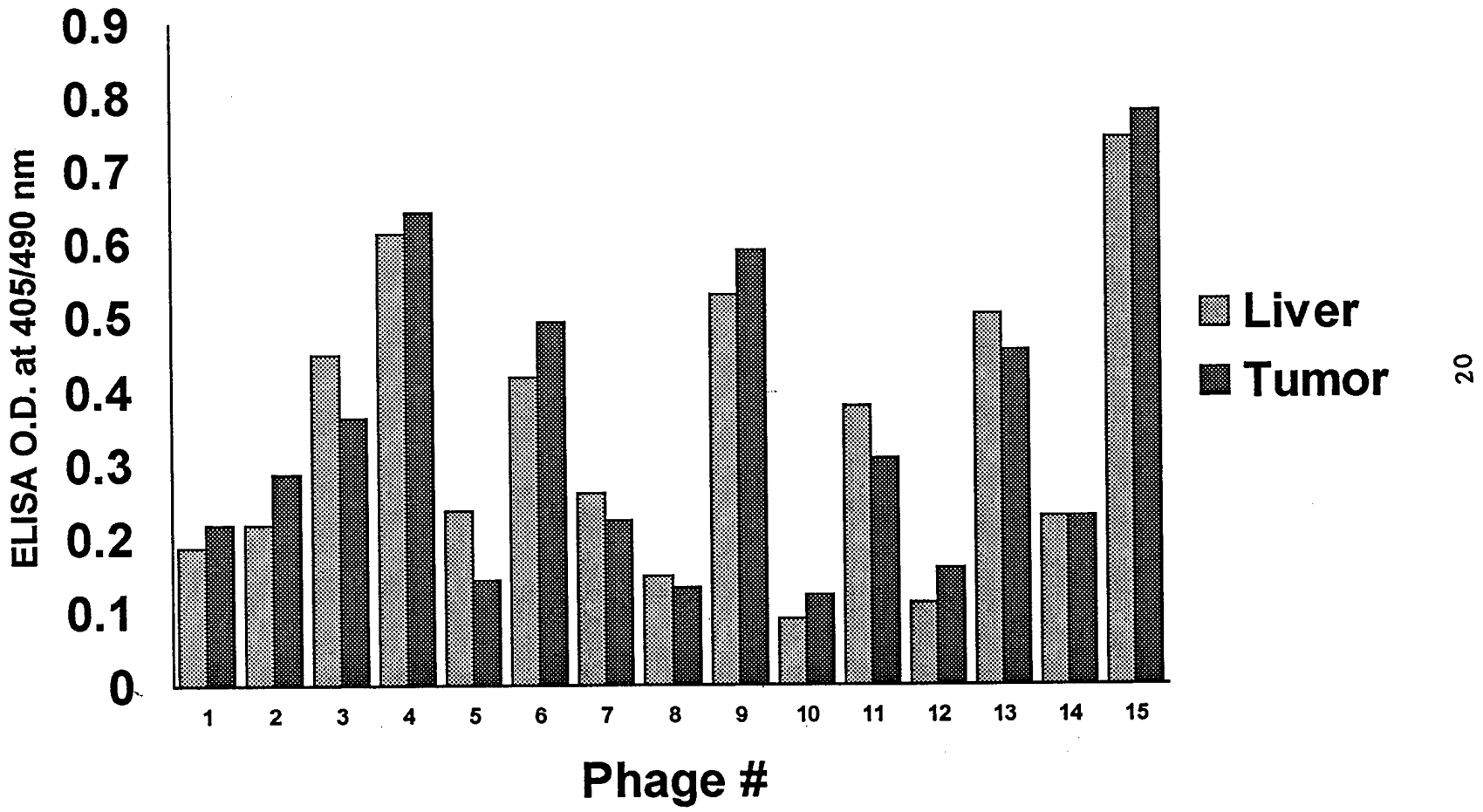
Phage clones specific for tumor derived gp96-peptide complexes



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

Figure 9

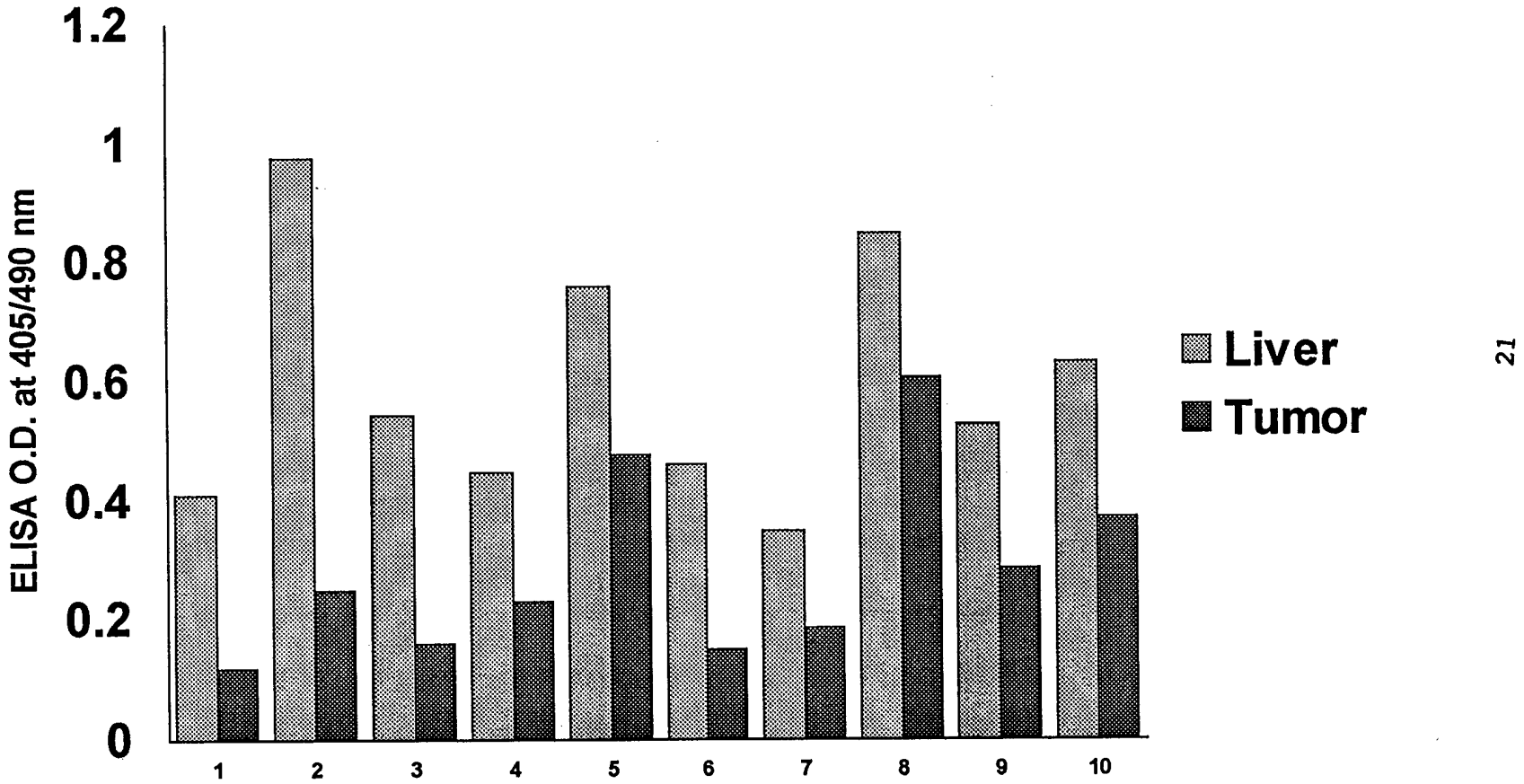
Phage clones that react with both liver and tumor derived gp96



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

Figure 10

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

Figure 11

Reactivity of scFv fragments with gp96-peptide complexes (ELISA)

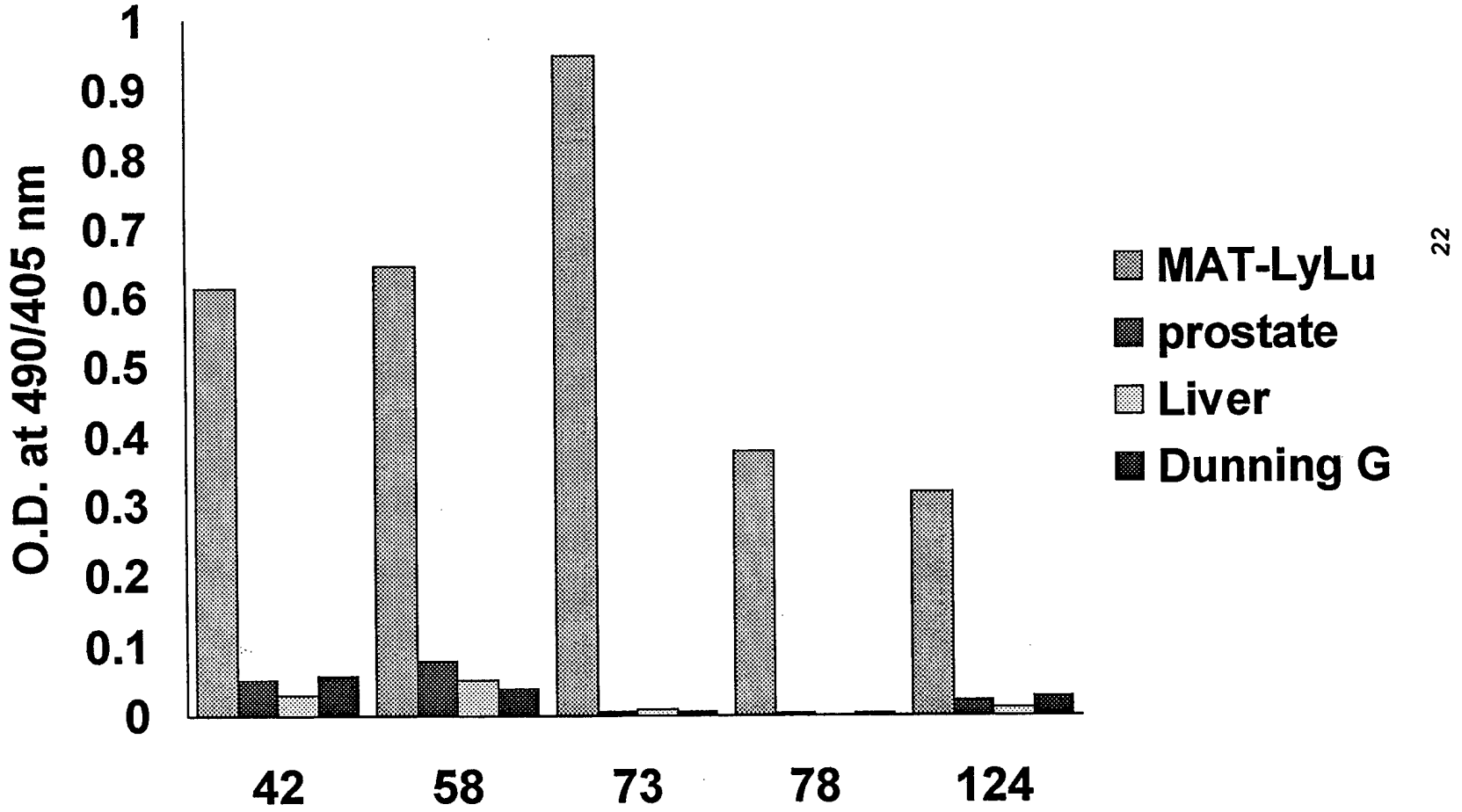


Figure 12

Immunoprecipitation of metabolically labeled cellular lysates of MAT-LyLu and Dunning G with phages E6 and F3 and grp94

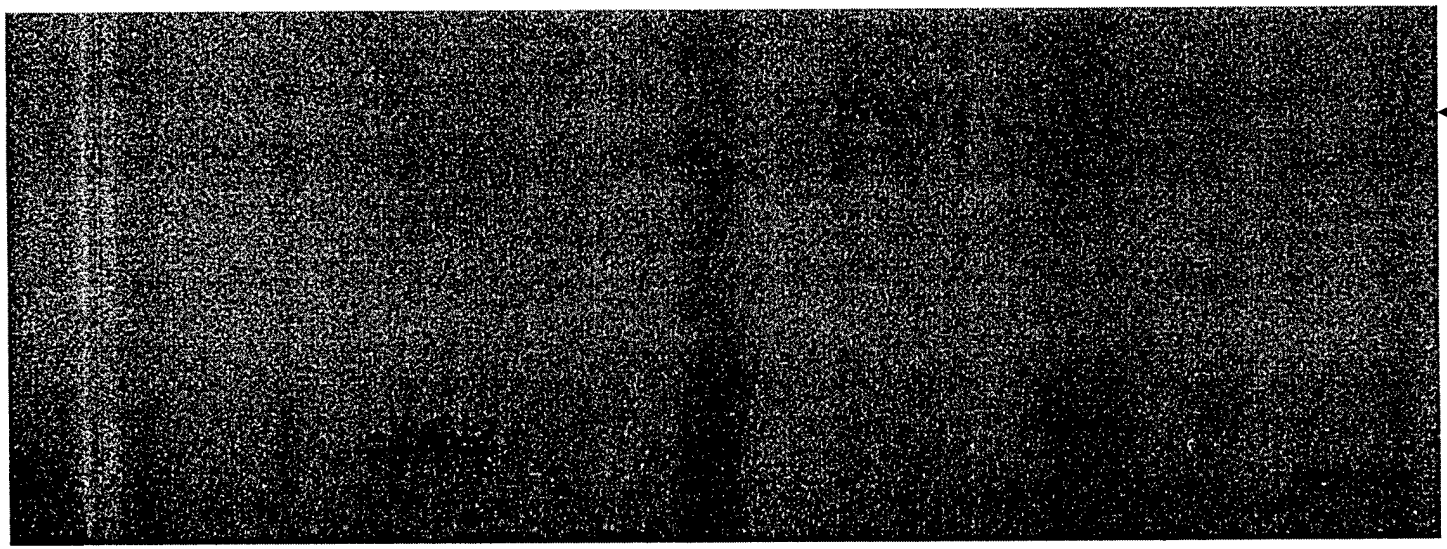


Table 1

Effect of immunization of gp96-peptide complex isolated from Dunning G cells on tumor incidence in syngeneic Copenhagen rats						
	# of weeks post challenge of $1x10^6$ live Dunning G Cells					
	5	6	7	8	9	10
control (non-immunized)	4/5	5/5	5/5	5/5	5/5	5/5
gp96-peptide complex (10μg/rat)	2/6	2/6	2/6	3/6	3/6	6/6
gp96-peptide complex (40μg/rat)	0/6	2/6	3/6	3/6	6/6	6/6
40μg/rat Rat Albumin	5/5	5/5	5/5	5/5	5/5	5/5

Table 2

AVERAGE TUMOR SIZE Mean ± S.D cm^3

Control	5.08 ± 0.30	
gp96 (10µg/rat)	2.27 ± 0.68	P< 0.01
gp96 (40µg/rat)	2.20 ± 0.20	P< 0.01
rat albumin (40µg/rat)	3.80 ± 0.14	P< 0.01

All statistical comparisons were done with control values using a Students `t' test and p< 0.01 was considered statistically significant.

conspicuous infiltrate of CD4 and CD8 lymphocytes in the lung of mice treated with IL-13 + IFN-γ clones. The involvement of T lymphocytes was confirmed by the absence of therapeutic efficacy in athymic nude mice.

#1683 Combination effect of administration of NKT-cell stimulator, α-galactosylceramide and vaccination of IL-12 cDNA transduced tumor cell on subcutaneously inoculated tumor. Kogawa, K., Nishihori, Y., Tanaka, M., Hagiwara, S., Nakamura, K., Kuribayashi, K., Niitsu, Y. *4th Dept of Internal Medicine, Sapporo Medical Univ. Sapporo, 060-0061, Japan.*

α-galactosylceramide (KRN7000) has been reported to exhibit marked anti-metastatic activity through activation of Vα14NKT cells in mice model. However, its anti-tumor activity against subcutaneous tumor is subordinate to the one observed against metastasis. Therefore, we attempted to augment the anti-tumor activity of KRN7000 by combination with immune-gene therapy using IL-12 cDNA transduced tumor cells. Lewis Lung Carcinoma (3LL) cells were transfected with IL-12 bicistronic expression vector (TFG mIL-12, a kind gift from Dr. Tahara, H., Univ. of Pittsburgh) to obtain IL-12 producing 3LL(3LL-mIL12). 3LL cells (8×10^5) were first inoculated subcutaneously into the left back of BDF1 mice. Five days later, 2×10^7 of 3LL-mIL12 were inoculated into right back of the tumor-bearing BDF1 mice, KRN7000 100 μg/kg was intraperitoneally administered and the tumor size was measured at given time intervals. Results indicated that the combination of 3LL-mIL 12 inoculation and KRN7000 administration exhibits enhanced anti-tumor activity against 3LL subcutaneous tumor as compared to either one of 3LL-mIL12 inoculation or KRN7000 administration. Since we found increased expression of MHC class I, II and B7 on 3LL-mIL12 cells, enhancement of anti-tumor effects was considered to be ascribed to the modification of vaccinated tumor cells in addition to the production of IL-12 by tumor cells. These results suggest that combination of KRN7000 and IL-12 immuno-gene therapy is a potent anti-tumor therapy.

#1684 Enhancement of antitumor effect by additional transduction of IL-2 gene into IL-12-gene-transdused Lewis lung carcinoma. Tanaka M., Saijo Y., Tazawa R., Satoh K., and Nukiwa T. *Institute of Development, Aging, and Cancer, Tohoku University, Sendai, Japan.*

IL-2 and IL-12 are key cytokines that exert diverse immunological effects including strong antitumor activity. LLC/IL-12 (9.6 ng IL-12/10^6/24 hr), a mouse Lewis lung carcinoma cell line transduced murine IL-12 gene by retroviral vector, was subcutaneously transplanted into syngeneic C57BL/6 mice. Although reduction of tumor growth was observed by 65% compared to control cells, tumors did develop in all transplanted mice, showing only limited effect of IL-12 on LLC. In order to overcome the limited effect of IL-12 gene transduction, we investigated whether additional IL-2 gene transduction into LLC cells could enhance antitumor responses in mice. IL-2 gene was delivered to LLC/IL-12 using adenoviral vector, Adex-mIL-2 (74.2 ng IL-2/10^6/24 hr/100 M.O.I.). IL-2 and IL-12 co-transduced LLC developed tumors in only 3/12 mice, and reduced tumor growth by 90% compared to LLC/IL-12 or by 83% compared to IL-2 gene transduced LLC (LLC/AdexIL-2). To elucidate the mechanisms of this enhanced antitumor responses, histological and immunological analysis were performed. Splenocytes in mice transplanted either LLC/AdexIL-2 or LLC/IL-12/AdexIL-2 secreted higher IFN-γ compared to LLC or LLC/IL-12. Tumor-specific CTLs were induced only in mice transplanted LLC/IL-12/AdexIL-2. Immunohistrogical staining of tumor sections showed many infiltrations of CD4+ and CD8+ lymphocytes in LLC/IL-12/AdexIL-2 tumor. These findings suggest that local synergistic effect of IL-2 and IL-12 production resulted in the rejection of low immunogenic LLC. Combined transduction of IL-2 and IL-12 gene may provide a potential modality for immunogene therapy against low immunogenic cancers.

#1685 Synergy of SEB superantigen, MHC class II, and CD80 genes in immunotherapy of advanced spontaneous metastatic breast cancer. Pulaski, B.A., Terman, D., Muller, E., & Ostrand-Rosenberg, S. *Dept. of Biological Sciences, University of Maryland, Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250.*

No significant improvements in the treatment of metastatic breast cancer have been developed in the last 20 years and the prognosis for women with this disease remains poor. Progress in understanding the immune response, however, has led to renewed enthusiasm for immune-based anti-cancer therapies. In previous reports, we demonstrated that tumor cell-based vaccines expressing MHC class II and B7.1 (CD80) molecules reduced experimental (i.v.-induced) and established spontaneous metastatic disease, by activating tumor-specific $CD4^+$ T-lymphocytes. We now demonstrate, using the 4T1 mammary carcinoma, that a vaccine combining MHC class II and B7.1 molecules with SEB superantigen in two distinct immunotherapeutic regimens produces an even greater reduction in spontaneous metastatic disease and significant extension of mean survival time. The therapeutic effect is particularly noteworthy because: 1) spontaneous metastatic cancer by 4T1 progresses similarly in comparison to human metastatic mammary cancer, 2) our post-operative model demonstrates that early metastatic lesions are primarily responsible for morbidity, and 3) the disseminated metastatic disease is quite extensive prior to the initiation of immunotherapy in both regimens.

#1686 Irradiation enhances immunogenicity of cells expressing a tumor-specific T-cell epitope. Ciernik I.F., Romero P., Berzofsky J.A., and Carbone D.P. *Centre pluridisciplinaire d' Oncologie, the Ludwig Institute for Cancer Research, CHUV, Lausanne, Switzerland, National Cancer Institute, NIH, Bethesda, MD, and the Vanderbilt Cancer Center, Nashville, TN.*

p53 point mutations may represent potential tumor-specific cytolytic T lymphocyte (CTL) epitopes. We investigated a mutant tumor-specific p53-derived epitope overexpressed in cellular vectors with respect to its ability to induce mutant-specific CTL. P815 mastocytomas expressing the mutant p53 induced mutation-specific CTL in BALB/c mice after iv. injection. Syngeneic fibroblasts or fibrosarcomas endogenously expressing the mutant p53 were able to induce significant mutation-specific CTL only if they were irradiated prior to injection into BALB/c mice. Low dose gamma irradiation of fibroblasts did not alter the expression of cell surface molecules involved in immune induction, nor did it alter the short term viability of the fibroblasts. Radioactively labeled fibroblasts injected into mice after irradiation showed altered trafficking, suggesting that the *in vivo* fate of these cells may play a crucial role in their immunogenicity. These findings indicate that gamma irradiation can alter the immunogenicity of syngeneic normal as well as transformed fibroblasts *in vivo*.

#1687 Treatment of breast cancer with fibroblasts transfected with DNA from breast cancer cells. EP Cohen*, EF de Zoeten*, V Carr-Brendel*, D Markovic* and J Taylor-Papadimitriou#. **Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, Il 60612, USA and #Guy's Hospital London SE1 9RT England.*

A vaccine that prolonged the survival of mice with breast cancer was prepared by transfection of mouse fibroblasts with DNA from breast cancer cells. The underlying rationale was that the immunogenic properties of breast cancer associated antigens (TAAs), the products of mutant or dysregulated genes, would be enhanced if they were expressed by highly immunogenic cells. (Classic studies indicate that transfection of DNA from one cell type into another stably alters both the genotype and the phenotype of cells that take-up the exogenous DNA.) To investigate this question, we transfected LM mouse fibroblasts ($H\text{-}2^k$) modified to secrete IL-2 and to express $H\text{-}2K^b$-determinants with genomic DNA from a breast adenocarcinoma that arose spontaneously in a C3H/He mouse ($H\text{-}2^k$). The cells were then tested in C3H/He mouse for their immunotherapeutic properties.

The results indicated that tumor-bearing mice immunized with the syngeneic / allogeneic transfected cells survived significantly longer than mice in various control groups including mice treated with fibroblasts transfected with DNA from an unrelated tumor (melanoma). Similar beneficial effects were seen in C57BL/6 mice injected with a syngeneic breast carcinoma cell line (E0771) and fibroblasts transfected with DNA from E0771 cells. Supported by DAMD 17-96-1-6178.

#1688 Identification and characterization of mouse prostatic acid phosphatase: Implications for immunotherapy of prostate cancer in animal models. Dirk G. Brockstedt, Michael H. Shapero, Lawrence Fong, Edgar G. Engleman and Reiner Laus. *Department of Pathology, Stanford University School of Medicine, Stanford, CA, USA; Dendreon Corporation, Mountain View, CA, USA.*

In humans, prostatic acid phosphatase (PAP) is selectively expressed in prostatic tissue of normal and malignant origin. Thus far, a murine homologue for PAP has not been described. In order to evaluate its potential utility as a target antigen in animal models for PAP-directed immunotherapy of prostate cancer, we isolated the murine homologue of PAP (mPAP). The full-length cDNA clone encompasses a total of 1455 nucleotides containing the ORF for PAP, which encodes a 353-residue protein with a calculated molecular mass of 41 kDa. A comparison of protein sequences across species reveals 87% sequence identity with rat PAP and 80% sequence identity with human PAP. RT-PCR and Northern Blot analysis of mouse tissues reveals that mPAP expression, unlike its human homologue, is not restricted to the prostate, but is expressed in a wide variety of different tissues including thymus. Interestingly, additional studies in rats reveal that rat PAP exhibits an expression pattern that resembles the human but not mouse tissue distribution. To study the immunological consequences of this differential expression pattern in closely related species we produced recombinant rat and mouse PAP in a baculovirus expression system. We then immunized rats and mice with recombinant protein plus adjuvant and evaluated the induction of antigen-specific immunity. Immunization of mice with mPAP leads only to a weak antibody response in 20% of the mice. In contrast, immunization of rats with rat PAP leads to a significant antibody response in all rats. These findings suggest that the wide tissue expression of PAP in mice may lead to antigen specific tolerance. Thus, rat models are more suitable for evaluation of PAP-specific, prostate-directed immunity in animal models for prostate cancer.

#1689 The ER-resident heat shock protein gp96 is associated with HLA class I. Chen, Y.G., Mukhopadhyay, S., Yedavelli, S.P.K., Chatterjee-Kishore, M., Kishore, R. and Tiwari, R.K. *Department of Microbiology and Immunology, New York Medical College, Valhalla, NY 10595.*

The human leukocyte antigen (HLA) class I serves a key role in the presentation of endogenous peptides to cytotoxic T cells for generation of an immune response. The molecular steps involved in the selection and loading of the peptides on the HLA complex is still unclear. The present study was undertaken to specifically examine the association of the ER-resident, peptide chaperone, gp96, with HLA class I heavy chain in several lymphoma cell lines, including the T2 cell

line that lacks TAP expression. We detected this association using double-determinant immunoadsorbent assay (DDIA), co-immunoprecipitations and western blotting, and cell labeling studies. Protein complexes immunoprecipitated with anti-gp96 antibodies were shown to contain HLA class I heavy chain as detected by western blotting with biotinylated anti-HLA class I monoclonal antibody. These observations were validated using DDIA where the gp96-HLA complex in cell lysates was either captured by anti-gp96 or anti-HLA (w6/32) antibodies and traced with anti-HLA or anti-gp96 antibody. The human erythroleukemic cell line, K562 which lacks the expression of HLA Class I was used for comparison. Metabolic labeling of cells and immunoprecipitation of the labeled lysates provided further evidence of the association of gp96 with HLA class I heavy chain. This association may have implication in the selection of peptides to be loaded on to HLA complex. [Funded by US ARMY grant # 17-98-1-8534].

#1690 DNA as vaccine or therapeutic against cancer and viral infections. Moelling, K., Pavlovic, J., Schultz, J., Schuh, Th., Obraschall, E. and Heinzerling, L. *Institute of Medical Virology, University of Zurich, CH-8028 Zurich, Switzerland.*

Plasmid DNA encoding viral or tumor-associated antigens can induce protective immune response against challenge with virus or tumor cells when the DNA is injected intramuscularly (1-3). We are developing a DNA vaccine against malignant melanoma with a tumor and a metastasis model in mice using a melanoma-associated antigen and a number of cytokine-encoding DNA plasmids such as IL-2, GM-CSF, IL-12, and the costimulator B7.1. Depending on the type of cytokine, some tumor reduction was observed while metastasis in the lung was strongly inhibited. Systemic expression of cytokines was monitored over time and was long-lasting. Non-specific CpG's showed only transient effects. The protective immune response is analyzed using various genetically altered mice such as perforin-, Interferon Receptor (IFN-Rα)-, $CD8^+$-cells- and B-cells- knock-out mice. The mice were also immunized with viral DNA coding for Influenza A hemagglutinin and nucleoprotein, HIV-1 gag-pol, NC, bunya viral antigens and CMV antigens. Combination of viral antigen-encoding DNA with DNA encoding cytokines or peptides are under investigation. (1) Moelling, K., *J. Mol. Med.*, **75**, 242-246 (1997). (2) Moelling, K., *Cytokines Cellular and Molecular Therapy* **3**, 127-136 (1997). (3) Moelling, K., *Gene Therapy 5, 573-574 (1998).*

#1691 Development of a bladder cancer vaccine based on M344, a mucin-associated carbohydrate antigen. Bergeron, A., Champetier, S., LaRue, H. and Fradet, Y. *Cancer Research Center, Centre Hospitalier Universitaire de Québec, Pav. L'Hôtel-Dieu de Québec, Québec, (P.Q.), CANADA, G1R 2J6.*

Bladder cancer offers a unique opportunity to investigate cancer vaccines. Up to 75% of primary bladder tumors are superficial and, although treated effectively by transurethral surgery, recurrences occur in 60% of patients. Moreover, these tumors respond well to non-specific immunotherapy using BCG. We believe that vaccines based on bladder tumor-associated antigens could be used to prevent recurrence of this disease. M344 mAb reacts with an antigen that is expressed on more than 70% of superficial bladder tumors and is also found in premalignant lesions. We previously showed that M344 was a carbohydrate epitope found on a typical mucin. The first objective of this study was to further define the M344 epitope. In competition assays, we showed that M344 totally competed 49H8, a mAb directed against the Thomsen-Friedenreich (T-F) antigen. However, both mAbs react differently with other glycoproteins bearing the T-F antigen thus suggesting that M344 reacts with Galß1-3GalNAc presented in a specific environment. Carbohydrates, as T-cell independent antigens, often elicit incomplete immune response and thus can be advantageously replaced by antigen mimics such as surrogate peptides or anti-idiotype antibodies. A second objective of this study was to produce M344 mimicry antigens and analyze them in order to determine their ability to reproduce the antigenicity of the M344 natural epitope. The potential of these antigen mimics to confer tumor protection will need further study.

#1692 Interleukin 12 and B7.1 costimulatory molecules coexpressed from an adenoviral vector act synergistically to induce antitumor response and suppress tumor formation in Lewis lung carcinoma model. Z.S. Guo, L. Lee, A. Chen, F.L. Graham, and D.S. Schrump. *Surgery Branch, NCI, NIH, Bethesda, MD; and Depts of Biology and Pathology, McMaster University, Hamilton, Ontario, Canada.*

Effective alteration of the host-tumor relationship necessiates presentation of tumor antigen in the context of co-stimulatory molecules and cytokines. The Lewis lung carcinoma is a spontaneous, poorly immunogenic cancer which is highly malignant in syngeneic as well as allogeneic mice. These properties make it an ideal model to study lung cancer immunotherapy utilizing gene transfer techniques. Recently we evaluated the therapeutic efficacy of adenoviral vectors expressing both the IL-12 and B7.1 molecules, or either molecule alone in the Lewis lung carcinoma model in syngeneic C57BL/6 mice. Expression of functional IL-12, B7.1, or both molecules could be detected following transduction of murine and human lung cancer cells. Tumorigenicity was significantly reduced in Lewis lung carcinoma cells transduced with vectors expressing both IL-12 and B7.1 molecules, and to a lesser extent in cells transduced with either molecule alone. Mice with tumors derived from the double transduced LLC1 cells survived much longer than those with tumors derived from cells transduced with a blank vector. Experiments are in progress to evaluate resistance to challenge with parental tumor cells following inoculation of cells transduced with IL-12 and B7.1. These data suggest that combined costimulatory molecules and cytokines expressed from tumor cells may be efficacious for lung cancer immunotherapy.

#1693 Helper virus-free packaged herpes simplex virus type 1 amplicon vectors for GM-CSF-enhanced vaccination therapy of glioma. Herrlinger, U., Jacobs, A.H., Woiclechowski, C., Sena Esteves, M., Fraefel, C., Rainov, N.R., Breakefield, X.O. *Molecular Neurogenetics Unit, Massachusetts General Hospital, Boston, MA 02129; Department of Neurology, University of Tuebingen, Germany.*

Subcutaneous vaccination with glioma cells which are retrovirally transduced to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) has proven effective in the mouse GL261 glioma model. As retroviral vectors only transduce dividing cells, clinical *ex vivo* gene therapy of gliomas with a low growth fraction seems to be difficult. To overcome this obstacle, a herpes simplex virus type 1 (HSV-1) amplicon vector, pHSVGM, expressing the GM-CSF gene from the HSV-1 IE4/5 promoter was constructed. This vector transduces both dividing and non-dividing cells. For mock infection, an amplicon vector encoding the *E. coli* lacZ gene was used. Both HSV-1 amplicon vectors were packaged helper virus-free. Infection of irradiated (35 Gy) GL261 cells with pHSVGM amplicon stocks did not cause any toxicity. The GM-CSF secretion during the first 24 h after infection was 34 ng/10^6 cells/24 h whereas mock-infected cells did not secrete any GM-CSF. In *in vivo* experiments with subcutaneous vaccination of C57BL/6 mice using 5×10^5 irradiated cells seven days prior to intracerebral implantation of 10^6 wildtype GL261 cells, 60% of the animals vaccinated with pHSVGM-infected GL261 cells were long-term survivors. However, in the groups vaccinated with retrovirally transduced GL261 cells or mock-infected GL261 cells 16–20% of the animals were long-term survivors. Vaccination with wildtype GL261 cells did not induce long-term survival. In conclusion, helper virus-free packaged HSV-1 amplicons vectors appear to be promising tools for cytokine-enhanced vaccination therapy of glioma.

#1694 Primary T-cell and activated macrophage response associated with tumor protection using peptide/poly-N-acetyl glucosamine (p-GlcNAc) vaccination. Maitre N, Stack A, Brown JM, Demcheva M, Kelley JR, Vournakis J, Cole DJ. *MUSC Dept of Surgery and CMSB, Hollings Cancer Center, Charleston SC.*

Effective anti-tumor vaccination may depend on the ability to generate an early cell-mediated response. We have previously shown that vaccination with the F2 gel formulation of p-GlcNAc results in cell-mediated tumor protection. The purpose of this study was to evaluate the mechanisms of F2 gel/peptide generation of this response using a murine EG.7-OVA tumor model. C57BL/6 mice were vaccinated with 200 μl in the base of tail/footpad using either F2 gel alone, 200 μg of SIINFEKL minimal peptide (OVA) in F2 gel, or OVA in PBS. Splenocytes at 24 and 48 hours post-vaccination were assayed for cell surface and intracellular markers, and day 10 were tested for a primary CTL response using the LDH cytotoxicity assay. Macrophages were then depleted prior to vaccination (mannan, 10 mg/ml i.p). Vaccination with F2 gel/peptide resulted in a primary T-cell response (25% tumor specific lysis). By 48 hrs, splenic T-cells had increased 4-fold as compared to B cells. Presence of an increased Th1 CD4 helper population was demonstrated by Interferon-γ production. CD4 cells were activated at 24 and 48 hours as shown by IL-2 receptor α chain expression (from 2% to 15% at 48 hrs). Activated splenic macrophages increased from 3 to 8% by 10 hrs with B7-2 expression doubled. Pre-vaccination macrophage depletion abolished any tumor-specific primary CTL response. F2 gel/minimal peptide tumor vaccination primed the immune system in an antigen specific manner with a primary T cell response involving macrophage presence and activation as well as induction of Th1 CD4 cells.

#1695 Induction of specific T cells tolerance to MUC1 antigen in MUC1 transgenic mice. Chen, DS., Koido, S., Li, YQ., Rowse, GJ.,* Gendler, SJ.,* and Gong, JL. *Cancer Pharmacology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115. *Samuel C. Johnson, Medical Research Building, Mayo Clinic Scottsdale, Scottsdale, AZ 85259.*

The C57BL/6 mice transgenic for human MUC1 (MUC1 Tg) have been developed to investigate anti-MUC1 tumor immunity in an animal that expresses MUC1 as a self -antigen. Previous studies showed that MUC1 transgenic mice were tolerant to self-MUC1 tumor associated antigens. In this study, we have investigated the mechanisms of tolerance to human DF3/MUC1 tumor-associated antigen in MUC1 transgenic mice. Immunization with different doses of purified MUC1 antigen or irradiated MUC1-positive tumor cells (MC38/MUC1) was unsuccessful in inducing anti-MUC1 immunity. The MUC1 Tg mice were immunized with MUC1 antigen and MC38/MUC1 shown CD8 T cells at different level reduction, and lack of T cell co-stimulation. By contrast, there were no significant difference in anti-MUC1 antibody secreted in wild-type and MUC1 Tg mice after MC38/MUC1 immunization. Furthermore, compared with irradiated MC38/MUC1 and MC38/β gal immunization, MUC1 Tg induces tolerance to specific MUC1 antigen, not β gal. These findings represent the tolerance induction in adult MUC1 Tg mice, and indicated that specific T cell suppressers play an important role in immune tolerance to MUC1 tumor-associated antigen.

IMMUNOLOGY/PRECLINICAL AND CLINICAL 8: Tumor Antigens II

#3102 Accuracy of TA90 immune complex in the detection of breast carcinoma. Habal, N., Gupta, R., Yee, R., Stern, S., Brennan, M., Brenner, R., Hansen, N., Giuliano, A., Morton, D. *John Wayne Cancer Institute, Santa Monica, CA 90404.*

We have identified a 90-kD glycoprotein serum tumor marker present as a circulating immune complex (TA90-IC) in patients with a variety of solid neoplasms, including breast cancer. This study evaluated the efficacy of TA90-IC in detecting breast cancer, in comparison to the gold standard of mammography. One hundred and forty-seven women (ages 26–82 years) were referred for open breast biopsy because of an abnormal mammogram (n=100) or palpable breast mass (n=47). Fifty-four (37%) patients were younger than 50 years. Preoperative serum samples were tested blindly for TA90-IC using an enzyme-linked immunosorbent assay (ELISA). A positive result was an optical density > 0.410 at an absorbance of 405 nm. Mammogram reports were obtained from the patient's chart and classified as suspicious (indeterminate readings included) or benign. Histopathologic results were classified as positive (infiltrating and intraductal cancer) or negative. Sixty-six (45%) patients had a breast cancer. When compared with mammography, TA90-IC was less sensitive (62% vs. 83%) but more specific (90% vs. 44%) and had a lower false positive rate (10% vs 56%). The difference between TA90-IC values and mammography results was significant ($p<0.0001$ based on McNemar's test.) A combination of tests improved the accuracy of screening (71% for TA90-IC plus mammography vs. 62% for mammography alone) and lowered the rate of false-positives (46% vs. 56%). We conclude that combining TA90-IC and mammography may help reduce the number of unnecessary tests and breast biopsies. The role of TA90 in screening for breast cancer and monitoring progression of disease will be evaluated in a multicenter trial.

#3103 Cell-surface expression of an immunogenic ganglioside (GM2) in pancreatic adenocarcinoma. Chu, K.U., Ravindranath, M.H., Nishmoto, K., Bilchik, A., and Morton, D.L. *John Wayne Cancer Institute, Santa Monica, CA 90404.*

Effective antitumor immunotherapy requires well-exposed immunogenic targets on the surface of tumor cells. Human pancreatic adenocarcinoma cells express gangliosides on their surface. By analyzing antibody levels against different gangliosides, we recently demonstrated that ganglioside GM2 is immunogenic in patients with pancreatic ductal adenocarcinoma. However, these tumor cells have a complex glycocalyx surface component that may mask the expression of GM2. The present study was undertaken to determine whether GM2 antigens are expressed on commonly studied pancreatic adenocarcinoma cell lines and whether the cell-surface expression of GM2 is altered by enzymatic cleavage of the glycocalyx. An anti-GM2 monoclonal antibody in a cell-suspension ELISA was used to examine the expression of GM2 in four pancreatic adenocarcinoma cell lines obtained from ATCC (SU.86.86, CFPAC-1, ASPC-1, and BXPC-3). Cell lines were analyzed before and after enzymatic cleavage of the glycocalyx using O-glycosidase with or without pronase. All four cell lines expressed GM2 in varying amounts that increased when cells were treated with enzymes. Addition of pronase after O-glycosidase did not affect the expression of GM2. These data suggest that GM2 is expressed in pancreatic adenocarcinoma cell lines but is masked by the complex glycocalyx on the cell surface. For effective immunotherapy in patients with pancreatic adenocarcinoma, better exposure of these potential immunogenic antigens on the surface of tumor cells may be needed, and this may be possible by combination chemotherapy that downregulates the expression of the complex glycocalyx.

#3104 Unusual and novel features of peptide binding to MHC Class I molecules. Apostolopoulos, V., McKenzie, I.F.C., Teyton, L., Chelvanayagam, G., and Wilson, I.A. *The Austin Research Institute, Heidelberg, VIC 3084, Australia; The John Curtin School of Medical Research, Canberra, ACT 2601, Australia; The Scripps Research Institute, Department of Molecular Biology, La Jolla, CA 92109.*

MUC1 is a high molecular weight glycoprotein overexpressed by adenocarcinomas. We have been using mannan-MUC1 for the selective delivery of MUC1 peptides to the Class I pathway. MUC1 peptides are presented by a variety of Class I molecules and can generate CTLs. We have described the 9mer epitopes presented by different H-2 Class I molecules and by HLA-A2. For the most part, the 9mer peptides have unusual features in that they lack defined anchors and bind with low affinity. Nonetheless, high avidity CTLs are produced. We also noted that MUC1 9mer peptides were not essential as 5- 8mer peptides could also bind. In addition to VNTR peptides, several mutations and mimics have been generated. This approach leads to the generation of more powerful anti-MUC1 CTLs. The MUC1 peptides have other novel features. They bind in an unusual fashion, in that the mid and C-terminal regions loop out of the groove more than other peptides and are accessible to MUC1 antibody while the N-terminus is buried. Indeed, MUC1 peptides are the only peptides which are accessible to antibody while in the groove of Class I molecules, although other antibodies can react with peptide/MHC molecules or with a MHC dependent configuration. The results clearly indicate that MUC1 is binding in an unusual manner; structural studies in progress confirm this. The studies demonstrate that peptides do not have to be deeply bound within the groove of Class I molecules, indeed low affinity binding peptides can generate CTLs. Thus, a different mode of peptide binding occurs which may have implications in immunity, and in tolerance induction.

#3105 Identification of tumor associated antigens using purified gp96-peptide complexes and the synthetic combinatorial single chain (scFv) phage display antibody library. Mukhopadhyay, S., Yedavelli, S.P.K., Noronha, J., Ferrone, S., Tiwari, R.K. *Department of Microbiology and Immunology, New York Medical College, Valhalla, NY, 10595.*

Tumor derived purified preparation of heat shock protein, gp96, in contrast to non tumor derived gp96 have tumor protective effects. In the absence of any structural difference between gp96 from tumor and non-tumor tissues and the observation that gp96 is associated with a large number of cellular peptides and preparations of gp96 stripped of their associated peptides lose their immunogenicity, it was hypothesized that identification and isolation of peptides associated with gp96 would lead to a standardized treatment. Towards this end, we describe the use of a synthetic combinatorial phage display antibody library in identifying tumor specific gp96 associated peptides. The initial library used for differential panning over purified gp96-peptide complexes from prostate tumor and normal liver contained over 10^8 distinct single chain antibody fragments. After four rounds of differential panning, three hundred phages that specifically adhered to tumor derived gp96-peptide complexes were eluted and individually analyzed. Five phages that specifically reacted to tumor derived gp96-peptide and not to gp96-peptide complexes derived from normal prostate, normal liver were identified. These single chain antibodies provide a useful probe to isolate tumor associated peptides that mitigate tumor rejection. (Funded by the US ARMY Grant # 17-98-1-8534)

#3106 Destruction of human malignant brain tumors by irradiated TALL-104 cells and their movement through normal rat brain: A cellular therapy paradigm. Kruse, C.A., Lamb, C., Gup, C., Hogan, S., Gomez, G., Kleinschmidt-DeMasters, B.K., Visonneau, S. and Santoli, D. *Univ of Colo Health Sci Ctr, Denver, CO 80262 and The Wistar Institute, Philadelphia, PA 19104.*

We are conducting preclinical studies with a human non MHC-restricted killer T cell line, TALL-104, in anticipation of its use in cellular immunotherapy trials for primary malignant brain tumors. The irradiated TALL-104 leukemic cell line was shown to lyse tumor cells across species barriers. In this study we have 1) quantitated in vitro brain tumor cell lysis and cytokine secretion upon TALL-104 cell:brain tumor cell co-incubation, and 2) estimated the damage to normal rat brain and trafficking patterns of the TALL-104 cells when placed intracranially. *In vitro* co-incubation of lethally-irradiated TALL-104 cells with brain tumor cell lines resulted in significant tumor cell lysis. Cytokines TNF-α, TNF-β, γ-IFN, or GM-CSF were variably secreted. Irradiated TALL-104 cells were placed into normal cannulated rat brain multiple times (10^6/injection, days 1, 3, and 7) to mimic the procedure we would follow in humans with a reservoir/catheter system. On days 8, 11 & 15 following the first infusion, histologic analyses of brains showed similar findings. The TALL-104 cells did not cause a widespread allergic encephalitic reaction. Neuronal damage was not evident. Focal sterile abscesses formed at the site of instillation. TALL-104 cells trafficked from the site of instillation through neuropil; a small percentage trafficked into contralateral brain. TALL-104 cell exit was at perivascular and leptomeningeal spaces. Immunostains of rat brain with anti-rat and anti-human CD3 differentiated human TALL-104 cells from endogenous immune rat T cells. Limited immune cell infiltrates (lymphocytes, plasma cells, and small numbers of eosinophils) were present, indicative of an endogenous immune reaction, however, it is unclear if this was a reaction to xenogenic cells, TALL-104 cellular debris, or to viable TALL-104 cell presence. At day 15, cellular debris was being cleared and the instillation cavity was collapsing. (Supported by NIH NS28905 & Univ of Colo Cancer Ctr to CAK & by DAMD17-97-C-7056 to DS).

#3107 The SART-1 antigens as an appropriate vaccine candidate for cancer patients. Sasatomi T., Shichijo S., Niiya F., Yamana H., Ogata Y., Shirouzu K. and Itoh K. *Kurume University School of Medicine, 67-Asahi-machi Kurume 830-0011, Fukuoka, JAPAN.*

We recently reported the SART-1 gene encoding both the SART-1_{259} antigen expressed in the cytosol of squamous cell carcinomas and adenocarcinomas, and the SART-1_{800} antigen expressed in the nucleus of the majority of proliferating cells. (Shichijo et al, J. Exp. Med., 187, 277–288, 1998) The SART-1_{259} antigen was recognized by the HLA-A24-restricted cytotoxic T lymphocytes (CTLs). This study investigated the expression of SART-1_{259} and SART-1_{800} antigens in cancer tissues. The SART-1_{259} antigen was detected in the cytosol fraction of cancer cell lines, and cancer tissues, but not in non-tumorous tissues. The SART-1_{800} antigen was expressed in the nuclear fraction of almost all the cancer cell lines, cancer tissues and few of non-tumorous tissues. The SART-1_{259}^{+} cancer cells were recognized by the HLA-A24 restricted and SART-1 specific CTLs. Therefore, the SART-1_{259} antigens could be an appropriate vaccine candidate for cancer patients.

IDENTIFICATION OF NOVEL TUMOR ASSOCIATED ANTIGENS IN MELANOMA PATIENTS RECEIVING ALVAC-IL-12. B. R. Bellows, R. M. Conry, M. C. Bonnet, A. F. LoBuglio,* T. V. Strong (Department of Human Genetics, Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama, U.S.A; Cancer Program, Pasteur Merieux Connaught, France)

The identification of novel tumor associated antigens may be useful for identifying genes important in the progression of cells from normal to the malignant state, identifying targets for cancer detection/diagnosis, and expanding targets for immunotherapy. In this study, nine patients with surgically incurable melanoma received escalating doses of a canarypox virus (ALVAC) encoding rhIL-12 intra-tumorally in accessible melanoma nodules. Sera was collected from each patient on days 0, 18, and 43 of treatment. A SEREX strategy was used to screen a cDNA library synthesized from the human melanoma cell lines MEL 624 and MEL 888 for the identification of tumor antigens. Sera from patient #1, who experienced a complete remission and has subsequently been disease free for more than 21 months, was used to screen approximately $2x10^5$ plaques. Seven immunologically reactive clones were identified and have been isolated and partially characterized. These proteins have not been previously implicated as melanoma tumor antigens and are not immunologically reactive with a panel of 10 normal sera. All of the proteins are reactive with sera from days 18 and 43, but not with day 0 serum. One of these proteins is reactive with multiple patients, indicating a possible shared antigen. Isotype analysis of the antibody response to these proteins demonstrated predominantly IgG_1 and IgG_3 antibodies. Expression of these genes in the autologous tumor and in normal tissues is under investigation.

Effect Of hGrb10 On Growth And Insulin-like Growth Factor Signaling In Breast Cancer Cells
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Understanding the growth regulation of human breast cancer is critical. Current clinical trials with somastostatin analogues (to decrease IGF-1 levels in serum), anti-Her 2 therapies (to inhibit receptor signal) and farnesylation inhibitors (to disrupt ras function) underscores the importance of cell growth regulatory elements as novel targets for therapeutic intervention. In vivo animal experiments indicate that several growth factors and their receptor tyrosine kinases (RTKS) can affect mammary tumorgenesis. Interestingly, the downstream signaling molecules of RTKS are often co-expressed in breast cancer. hGrb10 is a recently cloned downstream signaling protein of the insulin/insulin-like growth factor (IGF) receptor. In preliminary studies we found that IGF-unresponsive breast cancer cells have increased expression of hGrb10 and IGF-responsive breast cancer cells have decreased levels of hGrb10. We thus hypothesized that hGrb10 may inhibit IGFR action. To test this hypothesis we over-expressed hGrb10, using the tetracycline inducible expression system, in MCF-7 breast cancer cells which are IGF-responsive and hGrb10 negative. We derived several clones of MCF-7 cells that expressed high levels of hGrb10 mRNA and protein after doxycycline (an analogue of tetracycline) exposure. Expression of hGrb10 did not affect serum or IGF-mediated proliferation of MCF-7 cells. Furthermore, hGrb10 expression did not affect the ability of IGF-1 to cause tyrosine phosphorylation of the IGFR downstream signaling molecule insulin receptor substrate-1. We are currently investigating whether hGrb10 interacts with IGFR1 in these cells and whether it can interact with IGF signaling pathways.

HEAT SHOCK PROTEIN, gp96: A NOVEL TUMOR DERIVED PREVENTIVE AND THERAPEUTIC AGENT. S.P.K. Yedavelli, A. Badithe, A. Mittelman, R.K. Tiwari. Department of Microbiology and Immunology and Medicine, New York Medical College, Valhalla, NY.

Tumor derived purified heat shock protein (HSPs), gp96, in contrast, to non-tumor derived, gp96, has tumor protective effect in a number of experimental cancers that include fibrosarcoma, hepatoma, and spindle cell carcinoma. We tested the tumor protective efficacy of heat shock protein, gp96, isolated from a rat tumor cell line, Dunning G, in syngeneic Copenhagen rats. Tumor incidence, latency, and rate of tumor growth were the end points of measurement. Tumor bearing Copenhagen rats, made free of disease by surgical resection of the tumors resisted a fresh challenge of live Dunning G tumor cells. Vaccination with irradiated whole cells failed to elicit any resistance to induction of tumor growth. Vaccination with Dunning G derived purified gp96-peptide complexes delayed both incidence and growth of Dunning G induced tumors. Similar effects were observed when gp96 was used in a therapeutic experimental setting. Our data suggests that tumor derived gp96-peptide complexes can be used as an effective prophylactic and therapeutic agent in prostate cancer. Further investigations will determine specificity of action and define the immunological determinants and experimental conditions for its optimal activity. [Supported by a grant from US Army DAMD 17-98-1-8534, The Zalman A. Arlin Cancer Research Fund and The Zita Spiss Foundation].

DETECTION OF HUMAN ENDOGENOUS RETROVIRUS, HERV-E 4-1, ENVELOPE MRNA EXPRESSION IN PROSTATE ADENOCARCINOMA
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Endogenous retroviruses or retrovirus-like genetic elements are the result of integration of exogenous retroviruses into the human DNA genome. Human endogenous retrovirus sequences are present in thousands of copies in the human genome. We analyzed the mRNA transcriptional activity of HERVs (ERV3, HERV-E 4-1, and HERV-K 22) in normal, benign, and prostate cancer tissues. Using RT-PCR, we found that the env gene of HERV-E 4-1 is detected as mRNA in only prostate cancer tissues. These results were confirmed by RNA in situ hybridization. We detected distinct expression of HERV-E 4-1 mRNA transcripts in prostate tumor epithelial cells using HERV-E 4-1 specific antisense but not sense probes by RNA in situ hybridization. Strong expression of HERV-E 4-1 was detected in prostate adenocarcinoma with increased Gleason Grade. Several RT-PCR fragments of HERV-E 4-1 from prostate adenocarcinoma tissues were cloned and sequenced. The highest alignment scores for the sequenced clones were with previously reported sequences of the HERV-E 4-1 env gene. Additionally, when cDNA fragments were subcloned into a prokaryotic vector, some clones produced full-length fusion proteins. Our results suggest that HERV-E 4-1 env gene products are in fact transcriptionally active and may be translated in prostate cancer cell lines and tissues. The expression of HERV-E 4-1 env gene products in malignant but not in normal and benign prostate tissues suggests that this protein may be a tumor associated antigen in prostate cancer, which can be used in future diagnostic and immunotherapeutic applications.

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Preventive and therapeutic effect of tumor derived heat shock protein, gp96, in an experimental prostate cancer model

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Abstract. Tumor-derived purified heat shock protein (HSP), gp96, has tumor protective effect in a number of experimental cancers that include fibrosarcoma, hepatoma, and spindle cell carcinoma. The rationale for using gp96 as a vaccinating agent stems from the discovery that HSPs, including gp96, chaperone antigenic peptides for eventual recognition and elicitation of an immune response. The immune response generated by the HSP-peptide complex is specific to the tumor from which they are derived. The long-term objective of our studies is to develop a vaccine for primary and metastatic prostate cancer using tumor-derived HSPs. In the present study, we report our results on the tumor protective effect of irradiated Dunning G cells, or purified preparations of g96-peptide complexes as a tumor vaccine. Tumor incidence, latency, and tumor growth were the end points of measurement. Tumor bearing Copenhagen rats, made free of disease by surgical resection of the tumors resisted a fresh challenge of live Dunning G tumor cells. Vaccination with irradiated whole cells failed to elicit any resistance to tumor growth. Vaccination with Dunning G derived purified gp96-peptide complexes delayed both incidence and growth of Dunning G induced tumors. Inhibition of tumor growth was observed when gp96 was administered after tumor induction. Our data suggests that tumor derived gp96-peptide complexes can be used as an effective prophylactic and therapeutic agent even in poorly immunogenic cancer such as prostate cancer. Further investigations will determine specificity of action and define the immunological determinants and experimental conditions for its optimal activity.

Introduction

Prostate cancer is the most common malignancy of American men with approximately 41,000 deaths out of an estimated 180,000 new cases diagnosed every year (1). Clinically, the major concern is metastatic disease, which even if detected early is not amenable to standard surgical or chemotherapeutic treatments (2). Since experimental strategies leading to prevention of primary or metastatic disease has obvious clinical implications, the long term objective of our study is to develop prostate cancer specific vaccines for primary prostate cancer and metastatic disease. Our approach assumes the presence of tumor rejection antigens/antigenic epitopes in tumor, an assumption that is based on seminal observations made in cancer immunology over decades of rigorous research spanning different experimental systems that tumor derived molecules can confer protective tumor immunity (3-5). With recent advances in our knowledge that poorly immunogenic cancers can also be exploited to generate an immune response (6,7), immunopreventive and therapeutic approaches using tumor derived preparations, presumably containing tumor rejection antigenic epitopes, have acquired novel clinical significance. Our experimental approach is to develop a cancer vaccine using tumor derived heat shock protein, gp96-peptide complexes, and test its validity in a preclinical prostate cancer experimental animal model.

HSPs include many proteins which are ubiquitously expressed at a basal level but are specifically induced in response to various stress conditions such as heat, anoxia and metabolic stress. In recent years a fundamental role of HSPs in cell homeostasis has emerged. They bind to a myriad of cellular peptide/protein thus acting as molecular chaperones (8-14). In fact, HSPs isolated from tissues are always associated by tightly bound peptides that presumably represent the antigenic repertoire of the tissue of origin. Some of these HSP bound peptides are eventually processed and presented by the mixed histocompatibility complex (MHC) to evoke an immune response. Immunization of mice with any of the three major HSPs (gp96, HSP90, HSP70)-peptide complexes isolated from malignant cells has been found to induce a tumor specific immunity mediated by $CD8^+$T cells (14-20). It is noteworthy that such an immunity is not induced by HSP-peptide complexes isolated from normal cells. This finding, in conjunction with the lack of sequence variation (s) between HSP isolated from malignant and normal cells has been taken as evidence to predict that tumor specific immunity is not induced by HSPs *per se*, but rather from tumor-specific peptides. This prediction has been corroborated by the loss of tumor-specific immunogenicity of HSP stripped of their peptides. These results, which strongly suggest that HSP-

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Key words: gp96, vaccination, rat model, prostate cancer, protective immunity, irradiated cells

peptide complexes isolated from malignant cells represent a useful source of tumor associated peptides, possibly tumor rejection peptides, have provided the background to investigate the prophylactic and therapeutic effect of tumor derived gp96-peptide complexes in a well characterized rat experimental model that utilizes Dunning G cells grown in syngeneic Copenhagen rats.

The R3327/Copenhagen rat model is a valuable experimental system with several advantages despite the obvious disadvantage that the progression of prostate cancer in an animal model does not resemble the human disease. Besides the predictable time and nature of experimental tumors, the major advantage is the availability of a wide variety of cell lines derived from the original tumor with varied tumorigenic and metastatic properties. Of interest to the present study is the Dunning G cell line which is slow growing, androgen responsive and non-metastatic, whereas, in contrast the MAT-LyLu, a subline derived from the G cells, grows three to four times faster than the G subline, is androgen independent and highly metastatic (21).

Results presented in this study indicate that Copenhagen-Dunning G rat model shows tumor-induced protective immunity and is amenable to immunological experimentation. Further, tumor derived heat shock protein, gp96-peptide complexes has a prophylactic and therapeutic effect as determined by tumor incidence, latency and rate of tumor growth.

Materials and methods

Animal experiments. Four- to five-week old Copenhagen rats purchased from Harlan Sprague Dawley (Indianapolis, MN) were placed in groups of three per cage and were provided with food and drinking water *ad libitum* and were placed in 12-h light and 12-h night cycle. The animals were allowed to acclimate one week prior to experimentation. All injections of either irradiated tumor cells, live tumor cells or purified proteins were done subcutaneously. Live tumor cells were injected on the flank of the animal that were shaved prior to injection. Tumor measurements and weight of the animal were monitored weekly. Vaccination with purified gp96 or with irradiated cells was done at day 0 with a booster at day 7 and live tumor cell challenge at day 7. Tumor measurements were done by a vernier calliper and tumor volume expressed as cubic cm was computed using the formula 0.4 x long diameter x (short diameter)^2.

Cell growth. Dunning G cells were grown in culture in RPMI 1640 containing 10% fetal bovine serum (FBS) and supplemented with 2 mM L-glutamine, 50 IU/ml of penicillin and 50 μg/ml of streptomycin and dexamethasone (0.25 μM). Dunning G cells grown at 80% confluence was the source of purification of gp96 that was used as a vaccinating and therapeutic agent. The rat cell lines were a kind gift of the laboratory of John Isaacs (Johns Hopkins, Baltimore, MD).

Purification of gp96. Dunning G cells grown in T-175 flasks were the source of purification of gp96-peptide complexes essentially following the methods described earlier (14). Briefly packed cell volume of 8-10 ml were used in a single purification experiment. Cells were homogenized in a Dounce homogenizer (30-40 strokes) after allowing to swell in five time cell pellet volume of a hypotonic 30 mM sodium bicarbonate buffer. Cell debris and cellular organelles were removed by ultra-centrifugation at 27,000 rpm and the clear supernatant used for gp96 isolation. After two rounds of ammonium sulfate precipitation, 50% saturation followed by 80% saturation, the solubilized precipitate was subjected to column chromatography using Con-A Sepharose, buffer exchange with PD-10 (Pharmacia Biotech, Piscataway, NJ) and ion-exchange with DEAE-Sephacel. The eluted proteins are separated on an SDS-PAGE and the purified protein visualized by silver staining and quantitated by optical density measurements at 280 and 260 nm.

Results

Dunning G cells contain gp96. Fig. 1 shows the step-wise purification of gp96 from Dunning G cells. Although two bands are observed after the DEAE-Sephacel, this material was not purified further as the antigenic composition of both of these proteins have been found to be similar (22). A typical yield of the purified protein was 400 μg protein per 10 ml packed cell pellet. This protein was recognized by the antibody to grp94 (Neomarkers, Fremont, CA) in a Western blot performed by methods described (23), using anti-rat IgG and iodinated protein G (NEN/Dupont, Boston, MA).

Tumor-induced protective immunity is detectable in the R3327/Copenhagen syngeneic model. To determine if Dunning G induced tumors elicit a protective immune response the following experiment was performed. One million live Dunning G cells was injected subcutaneously and tumors allowed to develop until 5 cm^3 (twelve weeks after tumor cell injection). The tumors were surgically resected and the animals allowed to recuperate for two weeks after which they were injected with one million fresh live Dunning G cells on the flank opposite to the previous tumor. As control, three naive rats of approximately the same age were also injected with one million Dunning G cells. As shown in Fig. 2, the naive animals which did not have a prior exposure to tumor cells started developing tumors in about four weeks, whereas, the animals that had prior exposure to Dunning G cells developed tumors by ten weeks, a lag of about six weeks as compared with naive animals. This observation was repeated twice and was consistent, only one experiment is represented in Fig. 2. The delay in tumor induction by fresh cells in animals that had prior exposure to these cells is indicative of a protective tumor response that may be active, albeit only for a limited duration. The degree of immune response, the determinants of the immune response and the specificity of the immune response needs investigation.

Further, we examined if the tumor protective effect can be elicited by irradiated whole cells. Dunning G cells were irradiated with 3000 rads and used as vaccinating agents. Rats were vaccinated with irradiated Dunning G cells at day 0 and day 7 at doses of five and twenty million per rat and then challenged with live cells on day 14. In a separate experiment live cell challenge was done on day 7. Vaccination with irradiated cells offered no tumor protection and the

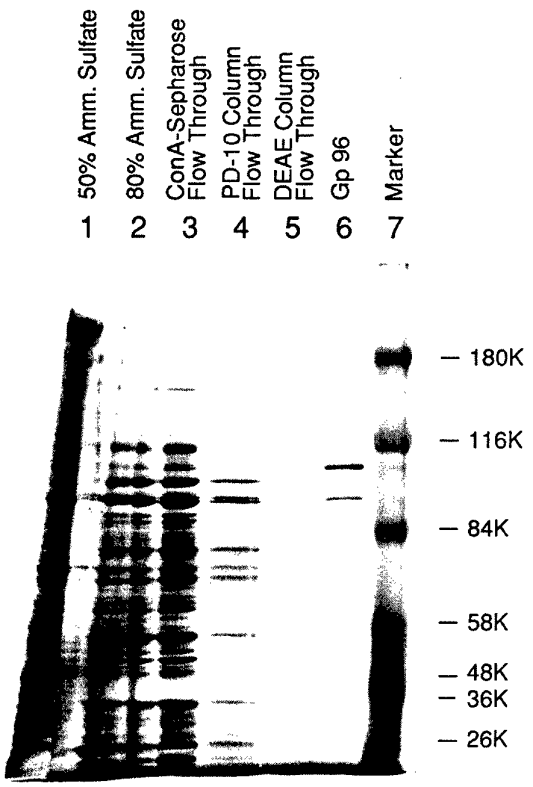

Figure 1. Purification of gp96 from Dunning G cells. SDS-PAGE analysis of cellular proteins followed by silver staining.

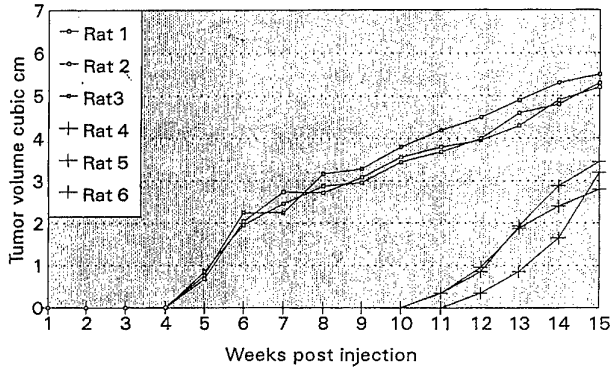

Figure 2. Protective immunity of Dunning G induced tumors in Copenhagen rats. All rats were injected with 1 million Dunning G. Each line represents tumor growth of a single animal; Rats 1, 2 and 3 are naive rats; Rats 4, 5 and 6 had their tumors (3-5 cms) surgically removed and challenged with fresh live cells.

growth rate was comparable in naive and vaccinated animals (data not shown) in any of the experiments. Thus, the agent that mediated the intact-tumor mediated tumor protective effect could not be mimicked by whole cells suggesting the involvement of other intracellular molecules.

Prophylactic effect of tumor-derived gp96. It has been observed in several experimental models that purified preparations of gp96 can protect against subsequent challenges of the tumor cells from which it is derived (19). To test if gp96 isolated from Dunning G cells could protect against Dunning G induced tumors in Copenhagen rats, we vaccinated a group of mice at two different concentrations, 10 and 40 μg per rat, and compared this experimental group with rats that were either sham vaccinated with phosphate buffered saline (PBS) or vaccinated with rat albumin. The vaccination schedule was day 0 and day 7 and live cell challenge with one million Dunning G cells at day 7. The effect of Dunning G derived gp96-peptide complexes on tumor incidence and latency is presented in Table I. By six weeks none of the control animals are tumor free, whereas, 66% of the rats vaccinated with gp96-peptide complexes are tumor free. All rats vaccinated with gp96 show palpable tumors by nine weeks as opposed to six weeks in the non-immunized group, an increase in the latency of three weeks. The tumor inhibitory effect is also reflected on the rate of tumor growth (Fig. 3) and the tumor volume (Table II). Reduction in tumor volume of 50% is observed in the gp96 vaccinated group as compared to the sham vaccinated group. Statistically significant reduction of tumor growth was observed in the rat albumin vaccinated group when compared with control. Rat albumin has peptide binding property and

Table I. Effect of immunization of gp96-peptide complex isolated from Dunning G cells on tumor incidence in syngeneic Copenhagen rats.

	No. of weeks post challenge of $1x10^6$ live Dunning G cells					
	5	6	7	8	9	10
Control (non immunized)	4/5	5/5	5/5	5/5	5/5	5/5
gp96 (10 μg/rat)	2/6	2/6	2/6	3/6	3/6	6/6
gp96 (40 μg/rat)	0/6	2/6	3/6	3/6	6/6	6/6
Rat albumin (40 μg/rat)	5/5	5/5	5/5	5/5	5/5	5/5

Table II. Average tumor size mean ± SD cm^3.

Control	5.08±0.30	
gp96 (10 μg/rat)	2.27±0.68	p<0.01
gp96 (40 μg/rat)	2.20±0.20	p<0.01
Rat albumin (40 μg/rat)	3.80±0.14	p<0.01

All statistical comparisons were done with control values using a Student's t-test and p<0.01 was considered statistically significant.

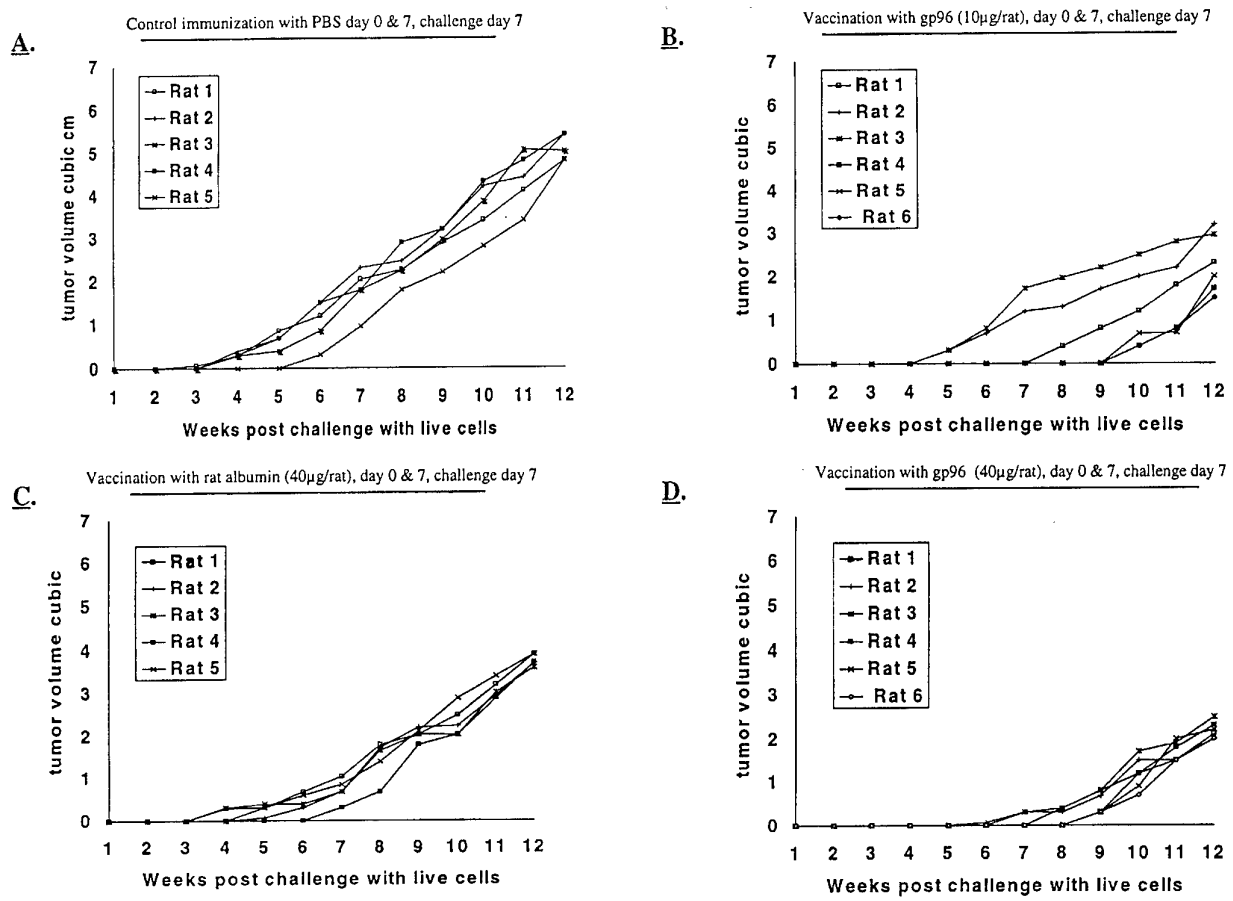

Figure 3. Prophylactic effect of purified gp96 at doses of 10 and 40 µg per rat (panels B and D) as compared with sham vaccination with PBS (panel A) and rat albumin (panel C). Each animal is represented on a single curve.

may not be the ideal control. Animals vaccinated with gp96 from liver tissues showed no protective effect (data not shown). Animals vaccinated with gp96 showed statistically significant reduction in tumor growth when compared with the rat albumin vaccinated group ($p<0.01$). These results provide strong evidence that gp96-peptide complexes can delay tumor latency and alter tumor growth, presumably by alterations of specific immunological response, however, the conditions that can elicit complete tumor regression and render animals vaccinated with gp96 completely tumor free have yet to be determined.

Therapeutic effect of gp96. Administration of gp96 therapeutically has immediate clinical implications. We tested the therapeutic effect of Dunning G-derived gp96 on Dunning G induced tumors in Copenhagen rats (Fig. 4). The therapy was started two and a half weeks after live tumor cell injection. There was no evidence for palpable tumor at initiation of therapy but tumors were predicted to develop in the next two to three weeks. Ten micrograms of Dunning G derived gp96, rat albumin or sham injection of PBS (control animals) was administered to these animals, three times a week for six weeks. Of the four animals that were treated with gp96, three animals showed a reduction or stabilization in tumor growth (Fig. 4). Withdrawal of therapy resulted in the rate of tumor growth comparable to the sham or albumin injected animals. The therapy experiment was also repeated with similar results and only one of the experiments are represented in Fig. 4.

Discussion

Although an experimental animal model that represents the progression of the human prostate cancer does not exist, the R3327 animal model has served a useful purpose in testing the efficacy of various immunological and chemical agents (21,24). The major advantage of this model is the availability of well defined cell lines with predictable tumorigenic and metastatic phenotype, however, the immunological characteristics of the non-metastatic G subline or the metastatic MAT-LyLu has not been examined. We present evidence that tumor induced protective response can be generated in the Dunning G rat model suggesting that preventive and therapeutic strategies using immunological approaches is an option for prostate cancer. A logical avenue for the search of immunological principles that can modulate tumor protective response is the tumor itself, precedence for which exists in defining tumor derived cancer rejection antigens (3-5).

The heat shock protein, gp96, is one such molecule which was discovered as a tumor rejection antigen. After almost

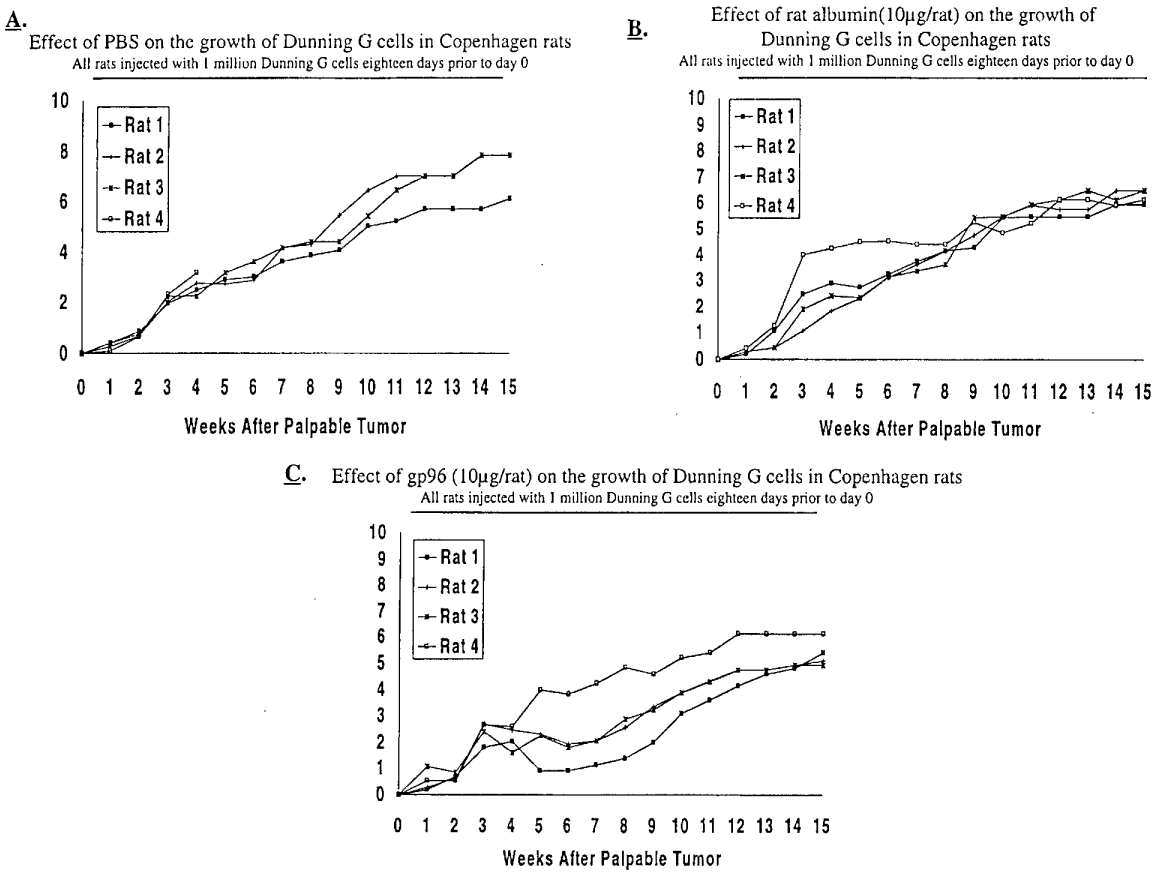

Figure 4. Therapeutic effect of purified gp96 at a dose of 10 µg per rat (panel C) as compared with sham treatment (panel A) and rat albumin at 10 µg per rat (panel B). Each line represents tumor growth of a single animal. Animals were injected with PBS, rat albumin or gp96 subcutaneously three times a week for six weeks.

ten years of intense study, it is now accepted that the immunogenic principle is not the gp96 protein but the complex consisting of gp96 and the myriad of peptides associated with it. It is the peptides that impart specificity of its immunological properties and the enormous diversity of cellular peptides creates the uniqueness of the gp96-peptide complexes that presumably represent a repertoire of cellular antigenic epitopes. The antigenic epitopes represent the entire family of unique, shared and non-specific normal cellular antigens. The presence of unique antigens probably causes tumor regression or suppression and would be most effective clinically, while a gp96 preparation that is only marginally associated with unique antigens and mostly shared antigens would have a reduced level of tumor rejection property. In the case of Dunning G induced tumors, vaccination with gp96 produces a 50% reduction in tumor growth. This may be due to the preponderance of shared antigens over unique antigens in Dunning G derived gp96 preparations. Since similar results were obtained on two separate replications of this experiment it may be assumed as a feature of the tumor system rather than purification artifacts. Alternatively, lack of persistent immune response or a large tumor bolus may be responsible for only a partial regression of these tumors. Careful study with gp96 doses with varying tumor cell challenges will clarify the issue and measurement of the induction of the immune response. Optimization of the vaccination schedule both in terms of number of vaccination, site of injection and the interval between each injections will further determine the extent of gp96-induced tumor regression. Results presented here lay the foundation that preventive immunological approaches can be undertaken with peptide chaperone such as gp96. Identification of the peptides, the immunological principle would also facilitate a standardized vaccination schedule and is being currently being actively examined using a synthetic combinatorial antibody phage display library.

The use of purified gp96-peptide complexes serve as an important repository for the identification of immunologically relevant peptides. Since our data suggests a therapeutic effect as well, the possibility that active specific immunotherapy can be developed with either gp96-peptide complexes or peptides derived from them and natural adjuvants. The selection of specific tumor associated peptides for active immunotherapy for cancer has relied on its tumor cell restricted expression and their ability to function as targets of cytotoxic T lymphocytes (CTL) *in vitro* (6,25). Neither of these criteria identifies tumor rejection antigens/peptides, which may explain the limited success of the use of these immunogens in the clinic. It is our working hypothesis that tumor derived HSP, gp96-peptide complexes that mediate specific protective

tumor immunity are an important source for the identification of tumor rejection antigenic peptides and when combined with gp96 will be a unique preventive and therapeutic agent in prostate cancer.

Acknowledgments

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