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ORIGINAL ARTICLE

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Phage-displayed random peptide libraries in mice: toxicity after serial panning

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Abstract *Purpose:* In vivo screening of phage-displayed random peptide libraries (RPLs) has been used to identify peptide ligands to targets found on endothelial cells of blood vessels supplying specific tissues such as brain, kidney, and tumor tissue. Peptides that bind specifically to blood vessels supplying tumor tissue have been conjugated to cytotoxic agents and used to successfully eradicate tumors in a mouse model. With the ultimate goal of developing similar methods for treating human cancer, we describe an in vivo RPL screening process that, unlike previous in vivo experiments, does not harm the animal being screened. *Methods:* RPLs were administered to FVB, BalbC, and tumor-bearing MRL/MpJ-fas$_{LPR}$ mice in a variety of dosing formats. Tumor nodules were excised 10 min following infusion and phage were amplified from the specimens. Phage were reinjected into the same animal within 48 h. This process was repeated twice for a total of three in vivo screens of mouse tumor tissue within the same animal. Mice were observed for systemic side effects, histopathologic damage, and presence of phage in organs. Peptide sequences were determined from several third-pan phage clones. *Results:* Overall there was minimal toxicity from administration of single or repeat doses of RPLs. Amino acid consensus sequences were identified and some of the sequences were similar to those of peptide ligands that bind matrix metalloproteinases. *Conclusions:* Serial administration of an RPL is well tolerated and serial panning in individual mice leading to consensus sequence motifs is possible. Based on these preclinical data the Food and Drug Administration has approved the implementation of human clinical trials with this technique.

Keywords Phage-displayed random peptides · Toxicity · Ligands · Cancer · Mouse

Abbreviations *IHC:* immunohistochemistry · *PBS-EPI:* phosphate-buffered saline and eukaryotic protease inhibitors · *PBS-PPI:* phosphate-buffered saline and prokaryotic protease inhibitors · *RPL:* phage-displayed random peptide library · *TU:* transducing unit

Introduction

Approval of trastuzumab (Herceptin; Genentech, South San Francisco, Calif.) in September 1998 by the Food and Drug Administration for the treatment of breast cancer was a major clinical milestone in the field of targeted therapeutics [4, 51]. Despite the recent and exciting success of Herceptin and other notable exceptions [23, 28, 49], the field of antibody-based therapy and diagnosis of solid tumors is extremely limited. Antibody-based therapy has a number of important limitations that have prevented its rapid development and translation to the clinic. Failure of antibodies in the clinic is likely due to unfavorable pharmacokinetics, lack of tumor penetration, immunogenicity, and undesirable uptake by the reticuloendothelial system [9, 12, 15, 17, 18, 19, 22, 30, 42]. Should the antibody be conjugated to a toxic molecule, destruction of normal cells in the reticuloendothelial system may limit the deliverable dose [40]. In addition, antibodies are complex biologic molecules that are not readily prepared for human use. Efforts to diminish the size of the antibody molecule, for example single-chain antibodies of about 25 kDa, have resulted in improved pharmacokinetics. There are clear

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indications that, for ligands, smaller is better [24, 27, 38, 45, 58, 62, 63].

Peptide ligands (1 to 2 kDa) that are much smaller than antibodies (150 kDa) but still retain selective binding affinity to target molecules may overcome several of the limitations of antibody therapy. An attractive source of peptide ligands is phage display technology [32, 44, 48]. Phage-displayed random peptide libraries (RPLs), which contain 10^6 to 10^8 different peptide sequences, are particularly powerful in that the peptide ligands are physically linked to their encoding DNA. DNA is easily amplified for sequencing and the sequence of one binding peptide out of millions of non-binding peptides can be determined. Although a variety of formats have been developed, the most common phage-displayed RPL involves insertion of random synthetic DNA into the gene coding for the minor coat protein pIII [47] or the major coat protein pVIII [20, 29]. The foreign DNA is expressed as random peptides in limited copies at the free N-terminus of pIII or pVIII. Each phage particle displays a different peptide.

Phage-displayed RPLs have been used to isolate small ligands, some with nanomolar and even picomolar affinity, to a large variety of clinically important targets including cell membrane receptors [3, 14, 46, 54, 59, 60], tumor-associated antigens [5, 11, 53], hormones and cellular messengers [6, 26], matrix-related elements [21, 31, 57], immunoglobulins [10], DNA [7], intracellular signal transduction molecules [13, 35], and nuclear receptors [33].

Most of these ligands have been identified using in vitro screening techniques which involve binding the target (purified protein or cells) to a matrix, incubating immobilized target with the peptide-phage library, washing away nonspecific binders, eluting specifically bound phage, amplifying eluted phage, and sequencing the DNA to determine the identity of the peptide responsible for the binding activity. In vivo RPL screening in animal models has resulted in ligands to organ-specific vasculature and to implanted tumor xenografts [2, 36, 37, 41, 43]. Administration to tumor-bearing mice of peptide-doxorubicin conjugates with affinity to tumor xenografts has been shown to result in a marked decrease in doxorubicin toxicity, selective tumor destruction, and improved animal survival [2]. These results are rather dramatic and represent a promising and unique approach to clinically relevant targeted therapeutics.

We are interested in adapting and applying in vivo ligand selection to human cancer patients. In preparation for human clinical studies, we evaluated in a murine model the toxicity of repeated administration of naive (full primary library) and enriched RPLs in conjunction with tumor harvesting and collection of phage. The results of this preclinical study are presented here and are the basis for approval by the United States Food and Drug Administration (Investigational New Drug BB-IND#9145) to begin human cancer patient studies.

Materials and methods

Phage-displayed RPL

The RPL used in these studies was constructed in the fUSE5 gene III phage-display system [10, 47]. The fUSE5 vector and *E. coli* host strains were a generous gift from Dr. George Smith at the University of Missouri. The half-site cloning method used by Cwirla et al. was employed in RPL construction. The following oligonucleotides were synthesized, annealed and ligated into 10 μg of SfiI-digested fUSE5 phage vector: 5′ pGGGCTTGC$(NNK)_9$ TGCGGGGCCGCTG 3′, 5′ GCAAGCCCCGT 3′, and 5′ CGG-CCCCGCA 3′, where N represents a position in the oligonucleotide with an equal chance of being occupied by G, A, C or T, and K represents a position with an equal chance of being occupied by either G or T. The ligation was transformed into electrocompetent *E. coli* (MC1061F') cells using electroporation (BIO-RAD E.coli Pulser;BIO-RAD, Hercules, Calif.). Ligation of an insert into the fUSE5 tetracycline-resistant vector causes a gene III frame-shift. A vector without an insert is non-infective and therefore will not grow on a tetracycline-containing agar plate. Therefore, only clones having a frame-restoring insert can contribute infectious particles to a library and the library complexity can be determined by counting the original number of transformants. After electroporation, the library was amplified overnight to produce thousands of copies of each peptide-phage particle library member. DNA sequencing of the N-terminal region of gene III of randomly chosen phage clones confirmed the presence of correct inserts. The peptides were nine amino acids long and flanked by cysteine residues believed to form disulfide-linked cyclic peptides. While each phage displays only one specific peptide, the complexity of this library contains phage displaying approximately 2×10^6 different peptides.

Preparation of the RPL for intravenous injection

Peptide-phage were prepared from *E. coli* cultures grown at 37°C overnight on 2×YT medium agar plates (Tryptone 16 g/l and yeast extract 10 g/l; Mikrobiologie). The plates were supplemented with kanamycin (Sigma, St Louis, Mo.) and tetracycline (Sigma). fUSE 5 contains a tetracycline-resistance gene that allows phage-infected *E. coli* to grow as colonies in the presence of tetracycline. The phage particles were resuspended in phosphate-buffered saline containing prokaryotic protease inhibitors (PBS-PPI; Sigma P8465). The phage suspension was centrifuged twice to remove bacterial cells and filtered with a 0.22-μm polyethersulfone membrane (Corning, Corning, N.Y.) to completely remove any remaining *E. coli* cells. The phage were concentrated by precipitation with 0.15 ml cold polyethylene glycol per milliliter of filtrate and the precipitate centrifuged. The resulting pellet was resuspended in fresh PBS-PPI and filtered through a pyrogen-free 0.2-μm cellulose acetate filter (Schleicher & Schuell, Keene, N.H.).

Endotoxin removal and testing

Endotoxins were removed from the preparation by performing three 1% (v/v) Triton X-114 (Sigma) extractions [1]. The phage were concentrated with polyethylene glycol again and the resulting pellet resuspended in PBS-PPI by shaking for 10 min at 200 rpm on ice followed by centrifugation. The resulting suspension containing the peptide-phage was passed through a 0.45-μm cellulose acetate filter, followed by passage through a pyrogen-free 0.2-μm cellulose acetate filter to sterilize the preparation. The Limulus Amebocyte Lysate gel clot assay (Endosafe, Charles River Laboratories, Charleston, S.C.) was used to determine the level of endotoxins remaining in the preparation and to check for potentially interfering substances in the preparation that might inhibit the gel clot reaction.

Sterility testing

Sterility of the phage preparations was tested according to the guidelines of the US FDA Code of Federal Regulations (21CFR610.12), by inoculation of the preparation into fluid thioglycolate medium and tryptic soy broth (Difco Laboratories, Detroit, Mich.). These tests confirmed the sterility of the preparations, as expected after 0.2-μm filtration.

Description of mice

Three strains of mice were used for the toxicity studies: FVB, BalbC, and MRL/MpJ-fas$_{LPR}$(MRL) (Jackson Laboratories, Bar Harbor, Me.). FVB and BalbC mice are normal strains. MRL mice, beginning around 8 weeks of age, develop lymphoproliferative disease resulting in massive lymph node enlargement. MRL mice were chosen for in vivo screening because they formed multiple, large, superficial tumors that were surgically accessible. Tumors chosen for excision varied in size from approximately 5 to 10 mm. MRL mice have the disadvantage of dying rather early and somewhat unpredictably compared to other strains of mice.

Survival surgery protocol

The University of Vermont Institutional Animal Care and Use Committee approved all animal procedures. Mice were weighed, positioned on a warming pad to maintain body temperature, and anesthetized with halothane. Ophthalmic ointment was applied to the eyes which were protected from bright light. Breathing pattern and toe pinch were used to monitor the level of anesthesia. A warm compress was applied to dilate the tail vein. Through a 29-gauge needle, 250 μl or less of sterile peptide-phage preparation was injected into the tail vein. Electric clippers were used to shave the area immediately surrounding the tumor to be excised. Isopropyl alcohol was used to cleanse the operative field and sterile drapes and instruments were used. The subcutaneous tumor of interest was excised through a small skin incision 10 min following injection. The incision was closed using interrupted 5-0 nylon sutures. Mice were injected subcutaneously with buprenorphine (0.05 mg/kg) for pain and again 12 h after surgery.

Collection of phage from harvested tumors

The tumor was rinsed with phosphate-buffered saline-eukaryotic protease inhibitors (PBS-EPI), weighed, minced and homogenized in homogenization buffer (RPMI supplemented with 1.8 μg/ml insulin, 2 m*M* L-glutamine, and 10% calf bovine serum). The homogenate was centrifuged and rinsed several times with homogenization buffer to eliminate unbound phage, and the final pellet was resuspended in homogenization buffer. An excess of Kan *E. coli* cells were added to rescue the remaining tissue-bound phage. The suspension was incubated while gently shaking for 1 h at 37°C, followed by the addition of tetracycline (0.2 μg/ml) and a 25-min incubation with vigorous shaking at 37°C. The suspension was centrifuged and the supernatant containing peptide-phage removed for quantitation and amplification. An aliquot was saved for titering. The remainder of the rescued phage were plated on 2×YT agar plates supplemented with kanamycin/tetracycline and amplified overnight. Amplified peptide-phage (φAmp1×) was subsequently harvested and purified for injection as described above in Methods.

Quantitation of phage in harvested tissues

Tissues for titering were weighed, homogenized with disposable pestles in a small amount of PBS-EPI, and incubated with an equal volume of *E. coli* cells for 1 h at ambient temperature. Tetracycline (0.2 μg/ml) was added to the suspension followed by vigorous shaking for 25 min at 37°C. The suspension was concentrated by centrifugation (6600 *g*, 5 min at 4°C), resuspended in approximately 50 μl PBS-EPI and plated on LB Kan/Tet plates (Luria-Bertani). Plates were incubated overnight at 37°C. Each colony represented one transducing unit (TU). In some cases, heparinized blood (20 μl) was also titered essentially as described above, but without homogenization.

DNA and amino acid sequence determination of harvested phage

Several isolated *E. coli* colonies from the final peptide-phage amplification titer plates were grown further in 5 ml LB broth in order to amplify and isolate the DNA (Qiagen mini-prep kit; Qiagen, Valencia, Calif.). Using this DNA as a template, DNA sequence analysis was performed using the fUSE5 sequencing primer CCCTCATAGTTAGCGTAACG. The amino acid sequences of peptides displayed by peptide-phage eluted from tumor tissue were deduced from the DNA sequence of the corresponding phage clones. Consensus sequence identification was performed by visual inspection and with the PILEUP program in the GCG DNA analysis software package (Wisconsin package, Genetics Computer Group, Madison Wis.).

Analysis of organs

Mice were killed and samples from ten organs (brain, diaphragm, heart, kidney, lung, bone marrow, lymph node, spleen, gonads, and liver) were immediately harvested from each mouse for analysis by (1) hematoxylin & eosin staining to assess pathology, (2) immunohistochemistry (IHC) to look for the presence of phage particles, and (3) phage titering to determine the number of infective phage. Organ pieces were fixed in buffered formalin, embedded in paraffin, sectioned and mounted onto slides. Rabbit α-M13 (Sigma) 7.3 and anti-rabbit polymer HRP (Dako) were used for IHC evaluation of phage. Positive control tissues for phage IHC were prepared from the organs of a mouse that had phage injected 10 min prior to organ harvest. The project pathologist (D.L.W.) evaluated all slides.

Systemic toxicity endpoints

Animals were observed for signs of toxicity by daily monitoring of behavior (posture, activity level, and grooming), gross appearance (coat), and body weight.

Protocols for intravenous administration of RPLs

Group-1 mice: single injection of naive phage: Eight mice (four FVB, four BalbC) were injected with a single dose of naive library phage. Two control mice (FVB) were injected with saline. Two mice of each strain and one control were killed for organ harvest at 3 days (to assess acute toxicity). The remaining mice were killed for organ harvest at 3 weeks (to assess chronic toxicity).
Group-2 mice: single injection of phage amplified from a tumor: Seven mice (three FVB, four MRL) were treated with 6.4×10^8 TU of peptide-phage that had been passaged through tumor tissue once (φAmp1×). A second set of eight mice (four FVB, four MRL) were injected with 8.2×10^{10} TU of peptide-phage that had been passaged through tumor tissue twice (φAmp2×). Two mice of each strain were killed for organ harvest at 3 days (to assess acute toxicity) or 3 weeks (to assess chronic toxicity).
Group-3 mice: serial injection phage: Six mice (MRL) were injected with 3.8×10^9 TU of naive peptide-phage. At 48 h intervals the mice were additionally injected with 3.6×10^9 TU of φAmp1× and then 2.8×10^9 TU of φAmp2×. Blood was drawn 4 days following injection, and then twice a week until it was shown to be clear of infective phage by titering. The mice were killed at 3 weeks and organs harvested for analysis.

Group-4 mice: serial injection of phage and tumor harvesting: Three MRL mice each bearing at least three palpable tumors were evaluated. Naive RPL was injected and 10 min later a tumor nodule was excised. The animals were allowed to recover. Phage were recovered from the tumor, amplified and labeled as φAmp1×. The mice were injected 1 to 2 days later with φAmp1× recovered from their own tumor. After 10 min a second tumor was excised and the mice were again allowed to recover. Phage recovered from tumor 2 was labeled φAmp2×. The mice were injected 1 to 2 days later with φAmp2× recovered from their own tumor. A third tumor was excised 10 min after intravenous injection of φAmp2×, the incision sutured and the animal allowed to recover. Peptide-phage were eluted from the third tumor and amplified for DNA sequence analysis. Mice were killed 3 weeks following the third phage injection/surgery and organs were harvested for analysis.

Immune response by ELISA

Over 3–5 days, BalbC mice were injected two or three times with phage library (9.4×10^8 TU/dose). Blood was drawn from the saphenous vein for serum IgG measurements prior to phage administration and at 1, 2, and 3 weeks following phage injections. Phage (1×10^7 TU/well) was coated on microtiter wells (Nunc MaxiSorp; Nunc, Naperville, Ill.) overnight at 4°C. Wells were washed five times with 10 m*M* Tris, 0.15 *M* NaCl, pH 7.5, containing 0.1% (v/v) Tween 20 (TTBS). Wells were then blocked with 1% (w/v) casein in TBS, pH 7.4, (Pierce, Rockford, Ill.) at ambient temperature for 2 h. After washing with TTBS again, mouse serum (diluted 1:1000), 73 ng rabbit IgG (Sigma I5006), or 73 ng rabbit α-M13 IgG (Sigma B7786) was added followed by incubation for 2 h at ambient temperature. Wells were washed again and goat α-mouse IgG HRP (Sigma 4416) diluted 1:4000 or donkey α-rabbit IgG HRP (Santa Cruz Biotechnology, Santa Cruz, Calif.) diluted 1:2000 were added to detect the binding of mouse α-M13 IgG or rabbit α-M13 IgG, respectively. After 2 h at ambient temperature the wells were washed again and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)diammonium salt (ABTS) was added according to the manufacturer's instructions (Sigma). Color reactions were read at 405 nm using a plate reader (Bio-Tek, Santa Barbara, Calif.; model EL310).

Results

Survival

All mice in this study, except two, lived to the end of the study period. The first mouse died prior to receiving any peptide-phage injections while in the restraint used to hold the mouse for injections. The second mouse died under anesthesia during the second tumor surgery. This appeared to be due to excess administration of halothane anesthesia. The mouse up to that time showed normal behavior and appearance.

Weight

Mice in group 2 which had been injected with φAmp1× showed an average decrease in body weight of 9.1% on day 1 following injection but their weights had returned to baseline by day 2. Mice in group 4 showed a minor weight loss the day after surgery but this had returned to normal the following day. The weights of all other mice remained stable relative to the control mice.

Activity, behavior and appearance

During the first day after surgery, group-4 mice were less active but had returned to normal by the next day. All other mice had normal activity, behavior and appearance throughout the study.

Histopathologic evaluation

Histologic analyses of 320 organs from 32 mice injected with phage were performed. In group 2, three FVB mice had hepatic inflammation and one FVB mouse had lymphoid aggregates in the hepatic lobules. Sections of liver from mice with hepatic inflammation were subsequently evaluated using a silver stain (Steiner) to rule out *Helicobacter* or *Clostridium* infection. Although these tests were negative, they do not test for all causes of infectious hepatitis in mice. All other organs in all other mice appeared normal for that strain.

IHC detection of phage particles

In group-1 mice, 3 days after injection most tissues were negative. In this group, the spleens from all phage-injected mice ($n=4$) showed trace to 1+ staining in the germinal centers of the lymphocytes. At 3 weeks after phage injection all organs were negative. In group-2 mice, all tissues were negative for phage staining 3 days after injection of φAmp1×. Most tissues from mice injected with φAmp2× were negative at 3 days with the exception of three livers, two spleens, one lymph node, and one kidney. In group-2 mice all tissues 3 weeks after phage injection were negative except for the liver of one mouse and a lymph node of another mouse, both from the φAmp2×-injected group. In group-3 mice, all tissues were negative for phage 3 weeks after phage injection. Among the group-4 mice, the organs of one mouse were available for end-experiment IHC detection of phage and all tissues were negative.

Titering of phage from organs and blood

In group-1 mice, all organs were negative at 3 weeks in the phage-titering assay. In group-2 mice, 3 days after φAmp1× phage injection, there were infective phage present in all the tissues except the blood and liver of mouse no. 3 and the spleen of mouse no. 2. In this group, no phage were detected in any of the tissues collected 3 weeks after injection of either φAmp1× or φAmp2×. In group-3 mice, blood was free of infective phage 11 days after the third and final injection of peptide-phage. No infective phage were detected in any of the tissues collected 3 weeks after the third injection of phage. Among the group-4 mice, one mouse was available for 3-week titering and most tissues were positive.

Endotoxin test results

Initial RPL preparations contained roughly 10^5 times more endotoxin than is permissible for intravenous administration to humans. Using the Triton X-114 extractions (see Methods section), the amount of endotoxins decreased by several orders of magnitude to levels permissible by the FDA for administration to humans.

Immune response

In response to phage injection, serum IgG levels increased. Serum levels had increased by the end of the 1st week over baseline and continued to increase following serial injections (Fig. 1).

Consensus amino acid sequences of phage recovered from tumors of MRL mice

Among the group-4 mice, 20–70 clones per mouse were sequenced and several amino acid sequence consensus patterns emerged (Fig. 2). Of particular interest, one consensus pattern had strong homology with a peptide previously shown to bind to and inhibit matrix metalloproteinases (MMPs) 2 and 9 (consensus C in Fig. 2). These molecules are strongly associated with the metastatic phenotype and are promising tumor targets [21].

Discussion

Bacteriophages have been injected intravenously into thousands of humans and even neonates for the diagnosis and evaluation of immune function and disorders. Although they are very uncommon, non-fatal adverse reactions in patients with unusual genetic immune deficiencies have been reported over the past 30 years [8, 16, 34, 39, 55]. These injections have been performed singly or serially, similar to the process of in vivo screening performed in our mouse study. There has also been extensive use of over 250 strains of bacteriophage, including 39 that infect *Escherichia* bacteria, which have been administered orally, externally, or intraperitoneally for treatment of infection [25, 52, 56]. Not only have side effects been reported as extremely rare, but the phage treatments have been reported to be effective in eliminating the bacterial infection.

Consistent with the reported literature on administration of phages to humans, our study in mice demonstrated that injection of naive and amplified phage using a variety of dose schedules resulted in minimal toxicity. One mouse died prior to receiving any phage injections and one mouse died intraoperatively during the second surgical procedure. The second mouse was well and acting normally until the second surgery. It appeared that the mouse had an excessive exposure to halothane that was irreversible. No other cause of death was discernible.

Out of 32 mice, 4 had identifiable hepatic abnormalities. Only the FVB mice had these findings. Histologic findings included focal acute lobular hepatitis with no evidence of fibrosis. Focal processes were diffusely distributed in small foci. Each nidus consisted of apoptotic and degenerating hepatocytes primarily associated with neutrophils in the larger foci. These histologic findings were present in two of the four mice. A third had mild changes with no necrotic hepatocytes and the fourth mouse had lymphoid aggregates only. No other mice, including those that underwent multiple phage injections had any hepatic changes. While it is possible that this strain had underlying hepatitis or are particularly sensitive to phage, future in vivo studies will be designed to take into account the possibility of hepatic reactions to phage injection.

Acute anaphylactic allergic reactions were a main concern and none was observed despite repeated injections of phage. Another concern was exposure to toxic peptides following injection of enriched phage populations. Following enrichment of phage there are higher copy numbers of enriched peptide sequences. However, the final number of enriched peptide molecules would be extremely small (≤ 20 pg) and the lack of expected toxicity was supported by the study results.

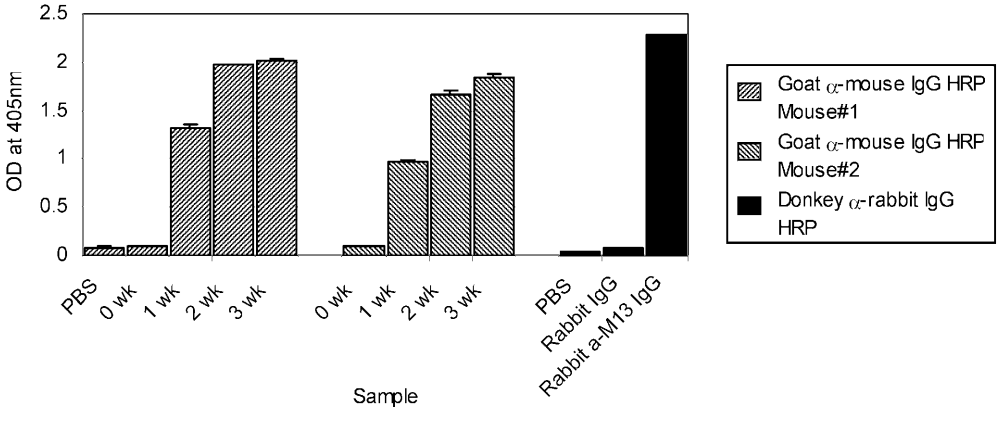
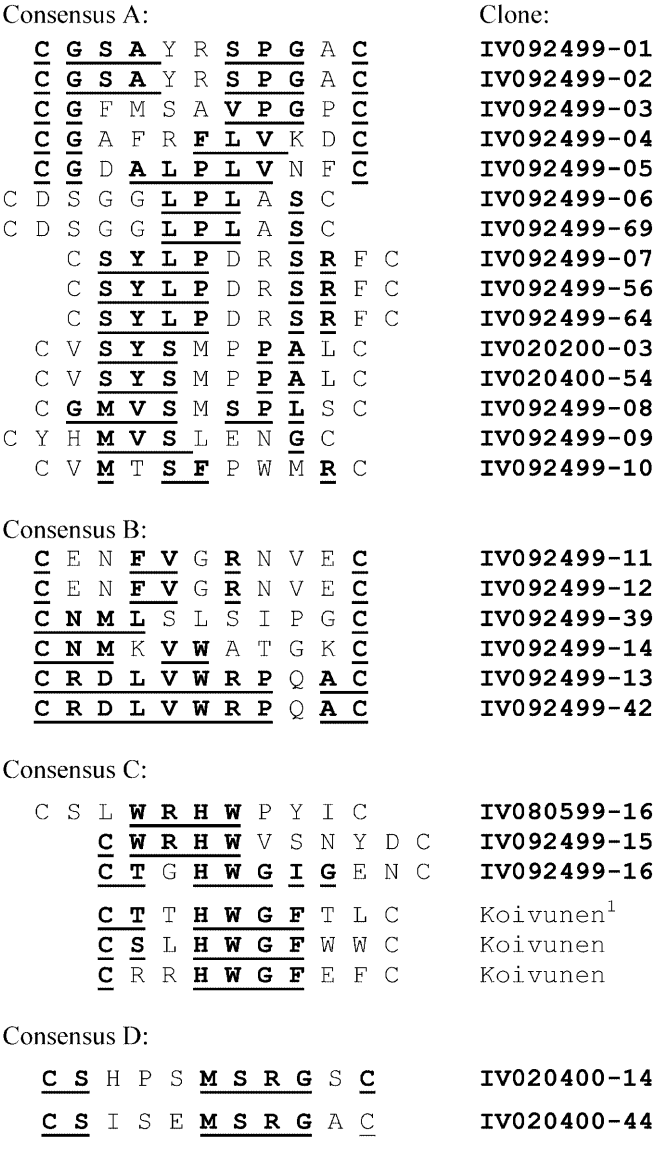


Fig. 1. Mouse serum IgG following i.v. administration of library phage, analyzed by ELISA. Mouse no. 1 was given two doses of phage over 3 days. Mouse no. 2 was given three doses over 6 days



[1] Sequences previously reported by panning RPL to purified MMPs.[21]

Fig. 2. Consensus amino acid sequences of peptide-phage isolated from tumor tissue. Amino acids that appear at least two times in vertical alignments are *underlined and in bold type*. Amino acids that are similar but not identical are *underlined and not in bold type*. Although the end cysteines were constant in all peptides and homology may not be as significant as the amino acids within the loop, they are still underlined when they line up in the consensus sequence to emphasize which peptides fall into an identical register with respect to the disulfide loop

Previous studies on panning in a murine model have utilized single injections per mouse [2, 36, 41]. Mice were killed and immersed in liquid nitrogen, and the phage collected and amplified, and injected into a next mouse. Such a panning scheme is not possible in humans with cancers. Our mouse study was designed to mimic planned human studies so as to allow serial panning in cancer patients. The fact that minimal toxicity was noted with single and repeated injections suggests that repeat panning in humans is feasible.

Clearance of phage was evaluated by IHC and titering. IHC appeared to be less sensitive than titering for detecting phage in organs. Most organs were IHC-negative for phage within 3 days. Virtually all were negative by 3 weeks. Titering demonstrated phage at 3 days in most tissues in most mice. Blood was negative by 11 days and all tissues in most mice by 3 weeks. An exception was one group-4 mouse that had positive titers in all organs at 3 weeks. These findings are similar to biodistribution of a phage library displaying a Fab fragment evaluated in nude mice [61].

Elevated IgG levels were found 1 week following the last serial injection of peptide-phage library. The levels increased during the 2nd and 3rd week. This response is normal and documents the immune competency of the mice used in this study. This is important since side effects related to immunologic reactions are potentially the most serious in humans. These findings support the validity of these experiments as a relevant preclinical model for subsequent human studies.

It was very encouraging that consensus amino acid sequence patterns were identified from peptide-phage eluted from tumor tissue, and especially, that a peptide was identified that has strong homology to an inhibitor of MMPs associated with the metastatic phenotype [21]. Interestingly, the lymph node tumors in these mice, while multiple and large, do not appear to metastasize or invade tissue outside the capsule of the lymph node. The tumor tissue histologically appears to be similar to normal lymph node tissue. However, for tumors to grow this large (more than a centimeter), a substantial amount of tissue remodeling must take place, and MMPs are likely to play a prominent role in this process.

There are several potential benefits of panning human cancers in vivo rather than on purified material ex vivo or on whole cells. Panning in a human exposes the peptide-phage library to exclusively human targets as opposed to a human xenograft model. A tumor is composed of a complex of tumor cells and host stroma such as fibroblasts and blood vessels. A human xenograft is not purely human but contains host murine blood vessels, stroma, and blood components. Vascular targets in a xenograft model are not human and may be sufficiently different to not be clinically applicable. Human tumors are spontaneous as opposed to the artificial setting of a xenograft model in which host tumor relationships may be quite different. An additional potential advantage of in vivo panning is that all tumor targets will be in their native conformation with all their human post-translational modifications.

An additional benefit of in vivo panning is that phage that have cross-reactive affinity to nonmalignant targets are negatively selected against tumor tissue. This makes cross-reactive phage less available to the tumor. This may be a powerful feature of this method since it should minimize cross-reactivity to nonmalignant targets. The importance of maximizing specificity through subtraction is highlighted by the unexpected cardiac

cytotoxicity of Herceptin which appears to be related to cross-reactive binding to the heart [50].

Unlike in vitro panning in which a known purified target is panned against, target identification is not necessary for initial in vivo panning events. For targeted therapy with ligand/toxin conjugates, it may be only necessary to know that the target is unique to the cancer. In addition, in vivo panning also allows selection of stable peptides since only peptides that are durable in blood will be able to bind.

We conclude that serial administration of a naive and enriched phage-displayed RPL is minimally toxic in a murine model. The majority of phage appeared to be cleared by 3 days and with few exceptions were nondetectable at 3 weeks by IHC and phage titering. An immune response occurred but, at least under the study conditions, anaphylactic reactions were not observed. In vivo RPL screenings performed serially in the same animal resulted in enrichment of peptide-phage. Several amino acid sequence motifs were identified and one was highly homologous to a known MMP ligand. The results from this study have led to approval by the FDA to begin human cancer patient studies at the University of Vermont similar to the preclinical mouse study presented here.

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