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Award Number: DAMD17-01-1-0384

TITLE: Dendritic Cell-Targeted Phage Vectors for Breast Cancer
Vaccine

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REPORT DATE: June 2004

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGEForm Approved
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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE June 2004	3. REPORT TYPE AND DATES COVERED Annual (1 Jun 2003 - 31 May 2004)	
4. TITLE AND SUBTITLE Dendritic Cell-Targeted Phage Vectors for Breast Cancer Vaccine			5. FUNDING NUMBERS DAMD17-01-1-0384	
6. AUTHOR(S) Stephen Dewhurst, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Rochester Rochester, NY 14627 E-Mail: stephen_dewhurst@urmc.rochester.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			20040930 006	
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) We hypothesize that one can use specific protein or peptide sequences to direct bacteriophage vectors to dendritic cells. We further propose that one can then use such retargeted phage vectors to deliver potentially important antigens to dendritic cells (DC), and that this may allow one to derive vectors capable of eliciting potent immune responses to breast cancer antigens such as her2. These hypotheses are being experimentally tested. During the period covered by this progress report, we have constructed modified lambda phage coat proteins which contain an array of receptor-binding/cell-targeting peptides which are expected to target receptors present on DC. Many of these have high affinities (nanomolar range) for their cognate ligands. We have also constructed modified lambda coat proteins which can be readily coupled to antibody molecules of interest, using engineered IgG binding domains. Finally, we have isolated scFvs that interact with human DC-SIGN on an ELISA assay, and we have generated purified lambda phage particles which contain our modified coat proteins.				
14. SUBJECT TERMS Breast Cancer, dendritic cells, vaccine, immunology, vector			15. NUMBER OF PAGES 17	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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

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



PI - Signature

6/14/04

Date

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INTRODUCTION

The overall purpose of this application is develop a novel method for breast cancer vaccine development. The hypothesis which we propose is as follows: that dendritic cell (DC)-targeted bacteriophage vectors expressing a tumor antigen can be used to elicit specific and potent anti-tumor CD8+ T lymphocyte responses. The experiments being performed under the auspices of this grant award are intended to establish proof-of-concept for the approaches set forth in this application, which are expected to have strong potential for clinical translation and for application to other systems (e.g., retargeting of other virus vectors to DC). If successful, these studies could have important implications since phage vectors are simple and inexpensive to produce, highly stable and not hampered by problems of pre-existing immunity (unlike many mammalian viral vector systems).

BODY

Approved Tasks

The following tasks were outlined in the approved statement of work for this grant:

- *Task 1.* Development of phage vectors that target dendritic cells (months 1 - 15)
- *Task 2.* Generation of DC-targeted phage vectors that express an epitope from human HER-2/neu (DC-HER2 phage) (months 13-29)
- *Task 3.* Analysis of the immunogenicity of DC-HER2 phage (months 30-36)

Research Accomplishments associated with the above tasks

The Progress Summary that follows refers to results for the current reporting period (2003-2004) only

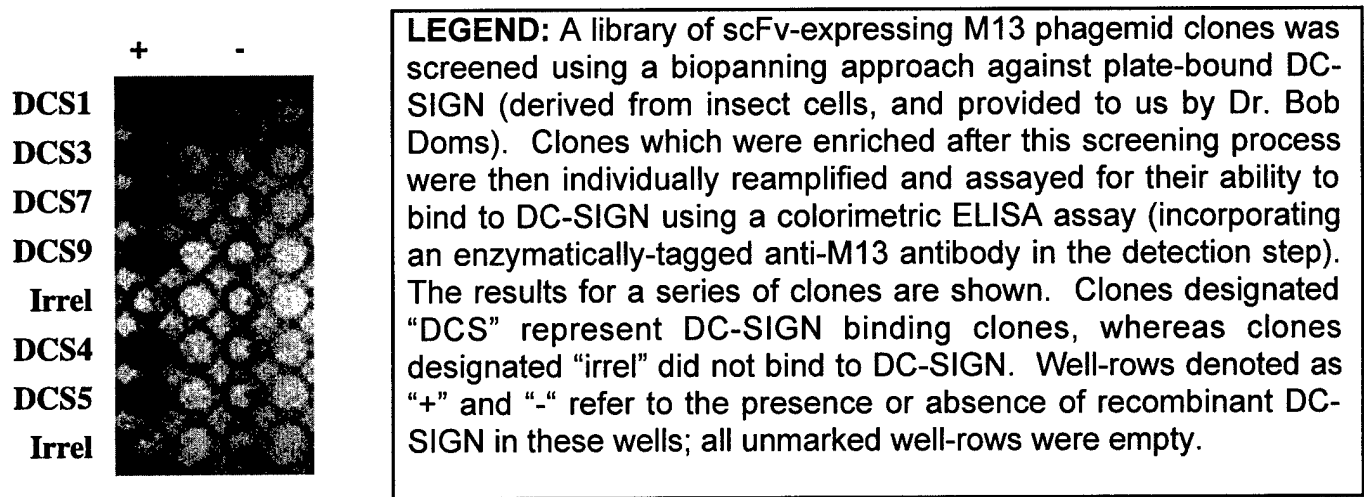
Task 1: Development of phage vectors that target dendritic cells

Current efforts to generate phage vectors that target dendritic cells. In the current project year, we have focussed our efforts on two phage systems – M13 and lambda.

Identification of phage-expressed scFvs that bind to DC-SIGN. In order to facilitate our ability to selectively target phage vectors to dendritic cells (DC), we have performed biopanning experiments against purified receptors known to be present on the surface of DC, using a very large of human single-chain antibody molecules expressed in a M13-phagemid vector (library complexity > 10⁹ unique clones; analyzed in collaboration with Dr. Mark Sullivan, UPMC). These experiments have focussed on a short list of DC surface receptors which we have been able to obtain in highly purified, recombinant form from other investigators – including CD64 (FcγRI) and CD209 (DC-SIGN). Attempts to identify scFv-encoding phage clones that bound to the former protein proved unsuccessful, but we were able to obtain several clones which interacted

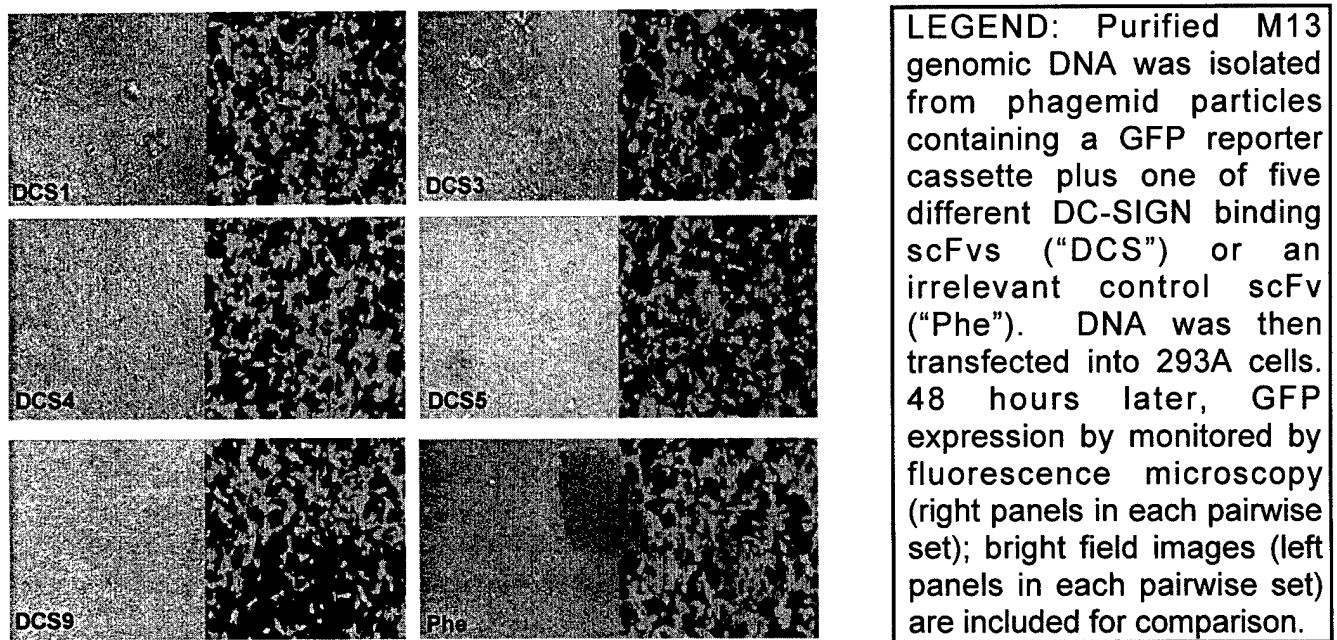
specifically with purified DC-SIGN, when tested by ELISA assay using purified phage clones and immobilized DC-SIGN (Fig. 1).

Fig 1: Identification of scFv-expressing phage clones that bind specifically to DC-SIGN



Construction of modified M13 bacteriophage vectors which contain a mammalian expression cassette encoding GFP. We have inserted a GFP expression cassette (CMV promoter, BGH polyA, and GFP) into the genome of the phagemid clones which contained the DC-SIGN binding scFv clones. We then propagated the phage in *E. coli*, and isolated DNA from CsCl density-gradient purified phage particles. This DNA was then used to transfect 293A cells. The results of one such DNA transfection experiment are shown in Figure 2, and confirm that the M13:GFP phage genomes are indeed capable of expressing GFP in mammalian cells.

Fig 2: Expression of GFP from M13:GFP genomic DNA in 293 cells



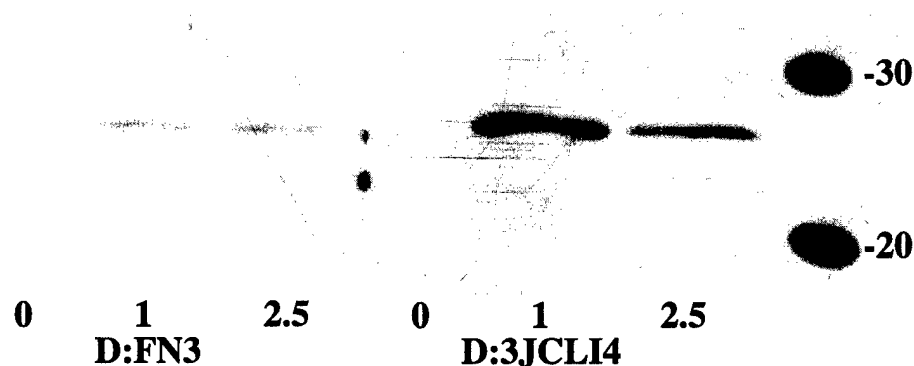
Efforts are presently underway to determine whether the modified phagemid vectors can directly enter DC, and express the encoded GFP indicator gene.

Development of modified lambda phage vectors. We have continued our efforts to derive retargeted lambda phage vectors, by fusing candidate targeting peptides to the terminus of lambda phage gpD. Our ultimate aim is to generate chimeric lambda phage particles which contain a mixture of wild-type and recombinant D-proteins (D-protein being the major lambda capsid protein). This can be achieved by propagation of wild-type lambda phage, or a lambda reporter phage clone (λ -Zap-GFP; *already constructed*), in a host cell that constitutively (or inducibly) expresses the desired recombinant D-protein. Since the normal lambda phage capsid contains 415 copies of D-protein, and because the D-protein is extremely tolerant of even large protein inserts, it is therefore possible to readily generate hybrid phage particles which contain 10-100 or more copies of recombinant D-protein.

We are continuing to move forward with the construction and expression of D-protein fusions containing various cell-binding peptides of interest. In our previous report, we described the generation of D protein fusions containing either the protein transduction domain (PTD) from HIV-1 Tat (previously shown to enhance uptake of lambda phage particles into mammalian cells; (Eguchi *et al.*, 2001)) or a CD40-binding peptide that was previously identified in our laboratory (Richards *et al.*, 2003b).

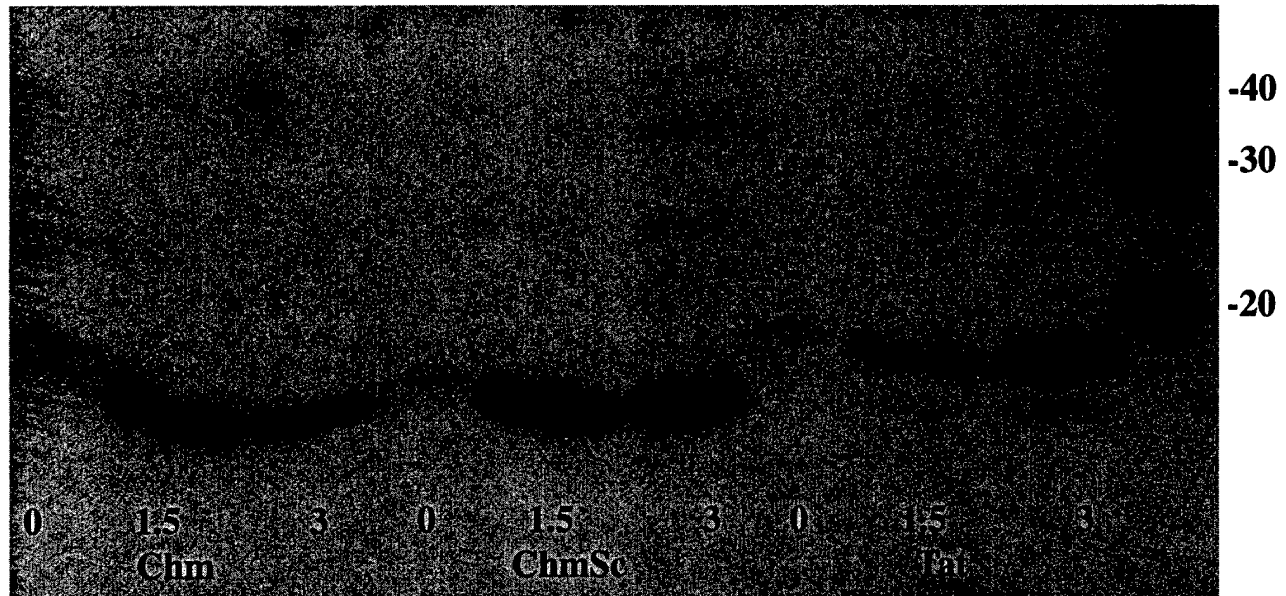
We have now generated additional D protein fusion constructs, including fusions to a high-affinity, $\alpha v \beta 3$ integrin-binding peptide known as 3JCLI4, which was derived from the tenth fibronectin type III domain (FN3), following random mutagenesis and selection for ligand binding using a phage display system (Richards *et al.*, 2003a), as well as fusions to a short peptide derived from chemerin, a cellular chemoattractant that has been shown to possess low nanomolar binding affinity for its cognate receptor (a receptor that is expressed specifically on dendritic cells and macrophages (Wittamer *et al.*, 2003; Wittamer *et al.*, 2004)). Expression results are shown in Figs. 3 and 4.

Figure 3: Expression of D protein fusions to FN3 and its $\alpha v \beta 3$ -binding derivative, 3JCLI4



LEGEND: D-protein fusions were constructed, in which the D protein was fused to the indicated peptides (wild-type FN3 or the $\alpha v \beta 3$ -binding mutant, 3JCLI4). *E. coli* cultures containing these expression plasmids were then grown to log phase, prior to exposure to IPTG (1 mM) for the indicated time periods (hours). Cell lysates were then prepared, and an immunoblot assay was performed using a rabbit antiserum specific for the lambda D protein. The immunoreactive proteins show the anticipated molecular weight (approx 26 kDa). Numbers on the right refer to the size of molecular weight markers (kDa).

Figure 4: Expression of D protein fusions to the chemerin-binding peptide

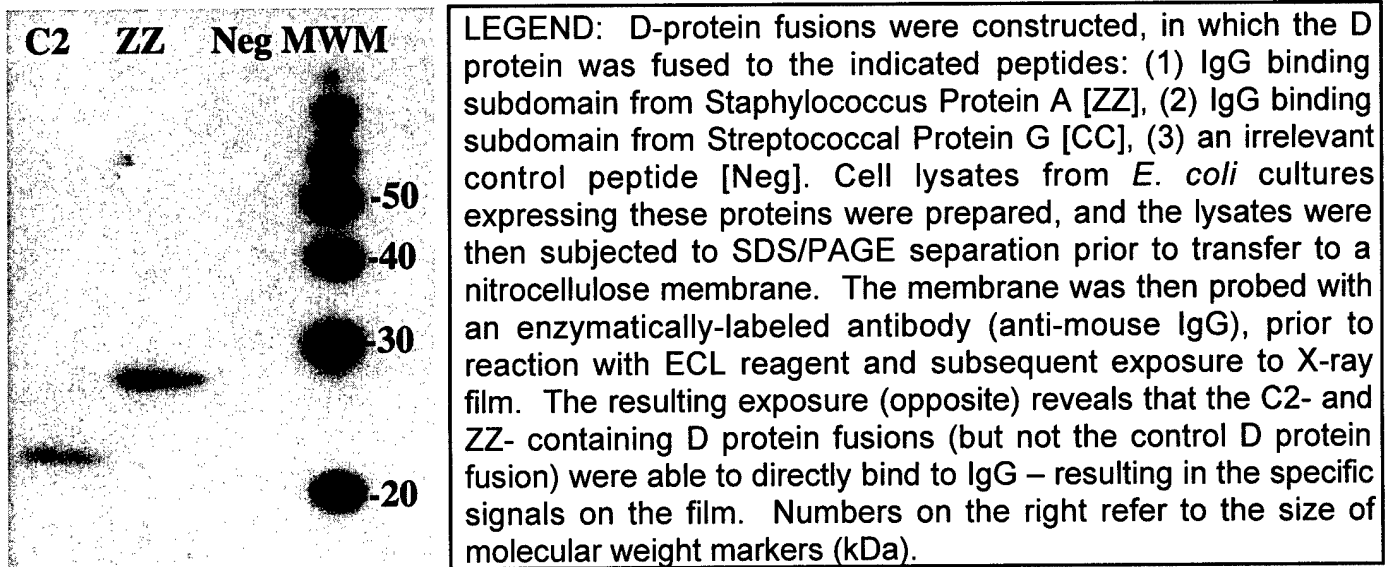


LEGEND: D-protein fusions were constructed, in which the D protein was fused to the following peptides: (1) nonamer peptide from Chemerin [Chm] (Wittamer *et al.*, 2004), (2) scrambled nonamer peptide from Chemerin [ChmSc] and (3) an extended protein transduction domain from HIV-1 Tat (as a control) [Tat] (Eguchi *et al.*, 2001). *E. coli* cultures containing these expression plasmids were then grown to log phase, prior to exposure to IPTG (1 mM) for the indicated time periods (hours). Cell lysates were then prepared, and an immunoblot assay was performed using a rabbit antiserum specific for the lambda D protein. The immunoreactive proteins show the anticipated molecular weight (approx 16 kDa for the Chemerin peptide fusions). Numbers on the right refer to the size of molecular weight markers (kDa).

We have also generated D protein fusions which incorporate two high-affinity IgG-binding domains. In particular, we have inserted the C2 peptide domain, a derivative of streptococcal protein G (SPG) and the ZZ peptide domain, a derivative of staphylococcal protein A (SPA) (Henning *et al.*, 2002). The principal advantage that these IgG domains offer is the ability to conveniently couple modified phage particles to antibodies that target cell surface receptors of interest -- i.e., internalizing receptors present on dendritic cells, including CD64, DEC205, CD207 (Langerin) and CD209 (DC-SIGN). This may provide a high deal of flexibility in the future, and it will also permit us to conduct rapid, proof-of-concept experiments intended to explore the utility of targeting specific cell surface receptors (including those identified above).

The gpD fusion proteins which incorporate the ZZ and C2 domains have been experimentally evaluated for their ability to bind to IgG, and the results are shown in Fig. 5.

Figure 5: Lambda D-protein fusions containing the ZZ and C2 domains bind to IgG



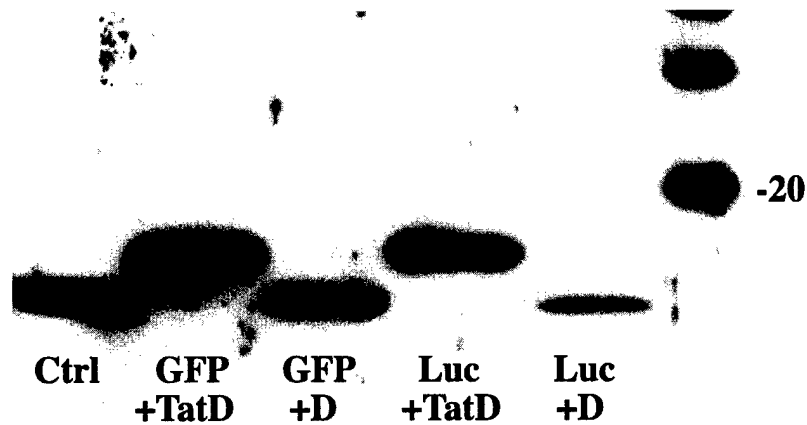
Now that we have a number of modified D protein fusions in hand, we have begun to address the issue of generating lambda phage particles which contain these proteins, and which also contain a reporter gene cassette within the phage genome. To do this, we are working with two recombinant lambda phage lysogens that were kindly provided to us by Dr. Nakanishi of DNAVEC Research Inc. (Eguchi et al., 2001). These phage genomes contain mammalian expression cassettes (promoter – gene – polyA) that encode either the GFP or the luciferase reporter gene (λ D1180-GFP and λ D1180-luc), and are deficient in expression of the lambda D protein.

Briefly, the recombinant phage are being amplified in *E. coli* host cells which supply the various recombinant D protein fusions in trans. This is expected to result in the generation of phage particles, which will contain only the recombinant D protein (since the parental phage genome is defective in the D gene).

For these experiments, we are working with codon-optimized D protein constructs, which offer two important advantages. First, these constructs contain codons that ensure optimal gene expression in *E. coli* (unlike the native D gene, which unexpectedly contains codons that diverge significantly from those which are used by *E. coli* host cells). Second, these constructs have greatly reduced homology (~80%) to the D gene contained within the lambda phage genome. This means there is little or no possibility for recombination between the modified D genes and any D gene (or D gene remnant) that may be present in the D-deficient phage genome.

We have recently completed an immunoblot analysis of CsCl-gradient purified phage particles that were generated from λ D1180-GFP and λ D1180-luc phage, propagated in cells that supplied either wild-type gpD or a gpD:Tat fusion in trans. The results are shown in Fig. 6.

Figure 6: Purified lambda particles contain modified D protein



LEGEND: D-gene deficient λ D1180-GFP (GFP) and λ D1180-luc (Luc) lambda phages were propagated in *E coli* host cells that supplied either wild-type gpD (D) or a gpD:Tat fusion (TatD) in trans. Control denotes wild-type λ (D-intact). Phage particles were purified by CsCl density gradient centrifugation, prior to being solubilized in SDS/PAGE sample buffer. Proteins were then separated by SDS/PAGE, transferred to nitrocellulose, and analyzed by immunoblot assay using a D-protein specific antiserum. The results show that we were able to achieve efficient incorporation of a modified D protein (TatD) into two reporter phage constructs (λ D1180-GFP and λ D1180-luc). Numbers on the right refer to the size of molecular weight markers (kDa); all D-protein fusions were of the correct anticipated size.

Tasks 2, 3: These tasks remain to be initiated. We have made the decision to defer work on the construction of the HER2-expressing phage clones because we believe that the most important phase of the current project relates to the development of modified phage clones which are capable of transducing mammalian cells. Thus, Task 1 has remained the main priority, since it will not be possible to proceed to immunization experiments without a successful gene transfer method. We hope to proceed with insertion of the HER2 expression cassette and the evaluation of the immunogenicity of HER2-expressing phage during the final no-cost extension period.

Modified Statement of Work

In light of the need to extend the unanticipated complexity of Task 1 (noted above), we therefore propose a **modified Statement of Work**, as outlined below (note that the major change here is a revision of the timeline, not the actual work).

1. **Task One (unchanged):** Development of phage vectors that target dendritic cells (*Timeline: months 1-36*).
2. **Task Two (unchanged):** Generation of DC-targeted phage vectors that express an epitope from human HER-2/neu (DC-HER2 phage) (*Timeline: no-cost extension period*)
3. **Task Three (unchanged):** Analysis of the immunogenicity of DC-HER2 phage (*Timeline: no-cost extension period*)

IMPORTANT NOTE: Because of the fact that it will be essential to derive phage vectors that target dendritic cells before we can proceed with immunogenicity experiments, this Task will remain our major focus in the no-cost extension period. This is a scientific necessity. Should progress prove slower than anticipated, it is conceivable that we may not initiate the immunogenicity studies (Task Three). Should that prove to be the case, we will consider a future grant application to undertake this work.

KEY RESEARCH ACCOMPLISHMENTS

Current Project Year (2003-2004)

- Isolation of phagemid clones which encode human scFvs that interact with purified human DC-SIGN in an ELISA assay format.
- Generation of modified phagemid vectors which contain a mammalian expression cassette encoding GFP, and DC-SIGN binding scFvs.
- Generation of recombinant lambda phage coat proteins (modified gD protein) which contain a set of receptor-binding and/or cell-targeting peptides, including: (1) the protein transduction domain (PTD) from HIV-1 Tat; (2) a high-affinity (subnanomolar) $\alpha v\beta 3$ integrin-binding peptide, (3) a high-affinity (low nanomolar) receptor binding peptide derived from the novel chemoattractant, chemerin, (4) a CD40-binding peptide. In addition, we have also generated modified gpD proteins which contain high-affinity IgG-binding domains derived from staphylococcal protein A (ZZ domain) and streptococcal protein G (C2 domain).
- Generation of purified lambda phage particles which contain modified gD protein. *These particles will permit us to move forward with in vitro cell transduction experiments, and with future immunization studies if initial results are promising.*

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations: See Bibliography.

Patents and licenses applied for and/or issued: None

Degrees obtained that are supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc: None

Funding applied for based on work supported by this award: None

Employment or research opportunities applied for and/or received on experiences/training supported by this award:

Research training was provided for the following persons during the present project year:

Ms. Heather Lankes, a Ph.D. trainee in the laboratory. Ms. Lankes is presently conducting her thesis research at the University of Rochester Medical Center, under the supervision of Dr. Dewhurst.

Ms. Christine Gorman-Zanghi, a M.D./Ph.D. trainee in the laboratory. Ms. Gorman-Zanghi is presently conducting her thesis research at the University of Rochester Medical Center, under the supervision of Dr. Dewhurst.

CONCLUSIONS

The conclusions which can be drawn from the third year of our experiments are as follows:

1. We have isolated human scFv clones which may possess the ability to bind to human DC-SIGN.
2. Recombinant T7 bacteriophage clones which contain a mammalian expression cassette encoding a readily-assayable indicator gene (green fluorescent protein; GFP) can be generated, and the inserted indicator gene can be expressed following introduction of phage DNA into human cells.
3. Recombinant lambda phage coat protein (gD protein) can be efficiently produced, bearing a series of receptor-binding/cell-targeting peptides including: (1) the protein transduction domain (PTD) from HIV-1 Tat; (2) a high-affinity (subnanomolar) $\alpha\text{v}\beta\text{3}$ integrin-binding peptide, (3) a high-affinity (low nanomolar) receptor binding peptide derived from the novel chemoattractant, chemerin, (4) a CD40-binding peptide. This will make it possible to derive retargeted lambda phage particles, which may have the ability to directly transduce primary dendritic cells.
4. Recombinant lambda phage coat protein (gD protein) can be efficiently produced, bearing high-affinity, IgG-binding peptides derived from staphylococcal protein A (ZZ domain) and streptococcal protein G (C2 domain). The resulting gD fusion proteins retain the ability to interact with IgG in a simple in vitro assay. This will make it possible to derive retargeted lambda phage particles, which may have the ability to transduce primary dendritic cells after complexation with suitable monoclonal antibodies, directed against DC-surface proteins.
5. We have shown that we can generate purified lambda phage particles which contain modified, recombinant gD. *These particles will permit us to move forward with in vitro cell transduction experiments, and with future immunization studies if initial results are promising.*

“So What Section”

The knowledge gained from these experiments advances the basic goals of this grant application, and brings us closer to being able to test our underlying hypothesis, that genetically modified bacteriophage vectors may represent a useful system for permitting gene (antigen) transfer to dendritic cells, for purposes of breast cancer vaccine development.

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BIBLIOGRAPHY (PUBLICATIONS)

These materials are all also included as Appendices

Abstract:

C. Gorman-Zanghi, S. Dewhurst. Targeted delivery of lambda phage vectors to dendritic cells.
ASM Conference on the New Phage Biology, August 2004 (Key Biscayne, FL).

- 1 page

APPENDIX MATERIALS

Award Number: DAMD17-01-1-0384

TITLE: Dendritic Cell-Targeted Phage Vectors for Breast Cancer Vaccine Development

PRINCIPAL INVESTIGATOR: Stephen Dewhurst, Ph.D.

CONTRACTING ORGANIZATION: University of Rochester Medical Center
Rochester, New York 14642

REPORT DATE: June 2004

TYPE OF REPORT: Annual

List of Materials Appended

New Manuscripts:

None

Abstract:

C. Gorman-Zanghi, S. Dewhurst. Targeted delivery of lambda phage vectors to dendritic cells. ASM Conference on the New Phage Biology, August 2004 (Key Biscayne, FL).

- 1 page

ABSTRACT

C. Gorman-Zanghi, S. Dewhurst. Targeted delivery of lambda phage vectors to dendritic cells. ASM Conference on the New Phage Biology, August 2004 (Key Biscayne, FL).

Abstract: Vaccination through targeted gene delivery by bacteriophage vectors offers a number of advantages over more commonly pursued approaches utilizing mammalian viruses. Unlike mammalian viruses, whose naturally broad tropism must first be abolished before being retargeted to specific cell types, phage lack a native tropism for mammalian cells and may be targeted directly to individual cell types. It is known that mammalian viral vectors selectively targeted to infect dendritic cells produce an enhanced immune response (Banchereau et al. 2001). Therefore, targeting bacteriophage to dendritic cells has the potential to offer a safe and low-cost novel vaccine approach. Previous studies have shown that the protein transduction domain (PTD) of the Tat protein from human immunodeficiency virus type-1 (HIV-1) can be fused to the gpD coat protein of lambda phage and used to deliver reporter genes to mammalian cells (Eguchi et al. 2001). We have constructed individual C-terminal fusions between gpD and the IgG binding domains of Staphylococcal protein A ("Zwt") and Streptococcal protein G ("C2"). Lambda phage with both fusion types were successfully purified by cesium chloride gradient and verified by immunoblot. We subsequently estimated the binding affinity of the modified phage particles for IgG constant regions by ELISA. In the future, the phage will be used in conjunction with antibodies specific for common dendritic cell receptors to target human and murine DCs in vitro. Successful gene transduction will be evaluated by luciferase assay and green fluorescent protein expression.