Vaccine 30 (2012) 998–1008

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Peptide vaccination is superior to genetic vaccination using a recombineered bacteriophage λ subunit vaccine

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ARTICLE INFO

Article history:
Received 29 April 2011
Received in revised form
12 December 2011
Accepted 14 December 2011
Available online 28 December 2011

Keywords:
Bacteriophage λ
Phage display
Recombineering
Subunit vaccine
Th1 response
Peptide vaccination
Peptide immunization

ABSTRACT

Genetic immunization holds promise as a vaccination method, but has so far proven ineffective in large primate and human trials. Herein, we examined the relative merits of genetic immunization and peptide immunization using bacteriophage λ. Bacteriophage λ has proven effective in immune challenge models using both immunization methods, but there has never been a direct comparison of efficacy and of the quality of immune response. In the current study, this vector was produced using a combination of *cis* and *trans* phage display. When antibody titers were measured from immunized animals together with IL-2, IL-4 and IFNγ production from splenocytes *in vitro*, we found that proteins displayed on λ were superior at eliciting an immune response in comparison to genetic immunization with λ. We also found that the antibodies produced in response to immunization with λ displayed proteins bound more epitopes than those produced in response to genetic immunization. Finally, the general immune response to λ inoculation, whether peptide or genetic, was dominated by a Th1 response, as determined by IFNγ and IL-4 concentration, or by a higher concentration of IgG2a antibodies.

1. Introduction

With the advent of recombinant DNA technology, the speed at which vaccines can be produced has improved, but still lags the speed at which viruses may adapt. It therefore remains a challenge to rapidly produce new vaccines in response to epidemics. To this end, we have been interested in bacteriophage lambda (λ) as a subunit vaccine platform, as it has several advantageous characteristics. Bacteriophage have been used for therapeutics in humans since their discovery nearly a century ago [1]. They are inexpensive, are stable at room temperature, can be produced quickly in large quantities, and can also be genetically engineered with ease, which is a boon where vaccines must be quickly generated against evolving or emerging pathogens.

Phage display was developed as a technique to clone proteins recognized by antibodies, and it remains a technique widely used in vaccine studies [2,3]. Filamentous bacteriophage (M13/fd) was first used for phage display, [3] and it is still the predominant phage strain, as no simple methods are available to display proteins on λ. However, filamentous phage display is not efficient at displaying large fusion proteins or libraries of complex cDNAs [4]. λ is superior to other bacteriophage for protein display, as it has been proven to stably display fusion proteins larger than a few amino acids on its capsid, with copies per virion that are two to three orders of magnitude higher than other phage display vectors [4–10]. For example, with λ, it is possible to display large functional proteins off all 420 copies of the gpD capsid. Functional proteins such as β-lactamase [11–13], luciferase (a 61 kDa protein) [14], or even β-galactosidase (a 465 kDa protein) [11,12] have all been displayed on λ with negligible effects on morphology and viability. In comparison, large proteins can be displayed on M13 bacteriophage, but only off the pIII capsid protein, which

Abbreviations: λ, bacteriophage lambda; (λ-wt) or (wtλ), wildtype bacteriophage lambda; (TLR), toll-like receptor; (IFNγ), interferon gamma; (PRR), pattern-recognition receptor; (PAMP), pathogen associated molecular pattern; (λgfp10), bacteriophage lambda gt10 with EGFP cloned into the EcoRI site; (λgfp10-TAT), bacteriophage lambda with EGFP cloned into the EcoRI site displaying gpD-TAT; (λgfp10-GFP-TAT), bacteriophage lambda with EGFP cloned into the EcoRI site displaying gpD-TAT and gpD-GFP; (pDE), pBluescript containing gpD to gpE in the MCS; (IP), intra peritoneal; (Th), T helper cell; (MHC), major histocompatibility complex.

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doi:10.1016/j.vaccine.2011.12.070

has a valency of 3–5 [5,15]. The difference in valency is pertinent to vaccine development, for the degree of antibody response appears to be proportional to the valency of the displayed peptides [16].

One vaccination approach is genetic vaccination, which involves the direct administration of DNA or the *ex vivo* transfection of dendritic cells [17,18]. A genetic vaccine induces both humoral and cellular immunity [18,19]. Production of genetic vaccines is facilitated by the inherent properties of DNA: (1) unlike recombinant proteins, there is no risk of misfolded products; (2) no expensive purification techniques are necessary; (3) there is no risk of vaccines becoming pathogenic; (4) DNA can be produced on a large scale; (5) refrigeration is unnecessary for DNA; and (6) vaccines made from DNA can be stored for long periods. Unfortunately, although immune protection is achieved in mice, genetic immunization produces insufficient immune protection in higher primates and in human clinical trials [19]. It is possible that genetic immunization could be improved through the use of adjuvants.

Adjuvants are critically important for vaccines, as without proper innate immune activation protective immune responses are not possible [20]. Alum, an adjuvant in use since the 1920s, is known to produce a Th2 polarized immune response [22], and this has recently been shown to occur from the activation of the pattern recognition receptor (PRR), Nlrp3 [21]. Typically PRR's bind a pathogen associated molecular pattern (PAMP). The classic paradigm being lipopolysaccharide (LPS), an outer cell wall component of gram-negative bacteria, is the PAMP responsible for binding its cognate PRR, toll-like receptor 4 (TLR4) [60]. Although the adjuvant activity of alum was found to be TLR independent, [23] the best described family of PRRs are TLRs and the intrinsic adjuvants in most vaccines activate TLRs [61]. The telltale result of TLR signaling is a Th1 polarized immune response [24,25]. DNA immunization is believed to act through TLR9, but *in vivo* TLR9s effect is negligible and TBK1 is instead responsible for the innate immune responses to naked DNA [26]. In fact, TBK1 defines immune responses from antigen specific B and $CD4^+$ T cells by signaling through cells of the haematopoietic lineage. In contrast, antigen specific $CD8^+$ T cells arise from TBK1 signaling through cells of non-haematopoietic lineage [26]. It remains controversial what new PRR(s) cause TBK1 signaling.

In the current study, we evaluate the relative merits of genetic vaccination and protein/peptide subunit vaccination, which has not been previously evaluated. With λ, it is possible to delineate the effects of each vaccination method using the same vector, controlling for the confounding effects of different PAMPs being present in different vaccines. A number of genetic vaccines based on λ have been described [27–35], as have λ-based peptide/protein vaccines [36,37]. Here we characterize the quality and magnitude of an immune response to a λ-based protein vaccine. We also find that all λ based vaccines are Th1 biased. Our systematic comparison demonstrates that protein/peptide immunization with λ is superior to genetic immunization. Specifically, genetic immunization resulted in no antibodies that bind proteins adsorbed at physiological pH, whereas protein/peptide immunization with λ produced significant antibody titers.

2. Materials and methods

2.1. Construction of pGEX-DLT-GFP

The plasmid pGEX-DLT, is a general purpose vector for IPTG-inducible expression of gpD fusion proteins. In pGEX-DLT, a fragment containing the glutathione S-transferase (GST) gene, a thrombin recognition sequence and the Sma I-cloning site of pGEX-2T (Amersham-Pharmacia) has been replaced with sequences encoding the λ capsid protein, gpD, a linker peptide (GKYTSSGQAGAAASES), a thrombin recognition sequence (LVPRGS), and a multiple cloning site: Not I - Pac I - EcoR I. The three nucleotides, TTC, immediately preceding the translation initiation codon, ATG, of GST was mutated to CAT in order to introduce an Nde I-site, CATATG, and the linker was flanked by Hpa I and Pvu II sites. The first three nucleotides of the Hpa I-site, GTTAAC, encode the last amino acid the gpD protein, and the latter 3 nucleotides, add an additional amino acid, Asn, to the N-terminal of the linker. Likewise, first half of the Pvu II-site, CAGCTG, adds an additional amino acid, Gln, to the C-terminal of the linker and latter half encodes the N-terminal amino acid, Leu, of a thrombin recognition site. The sequence encoding the gpD protein was isolated by PCR from wild type λ DNA using the forward primer: 5′-CCA GTG TAA GGG ATG CAT ATG A-3′ and the reverse primer: 5′-AAC GAT GCT GAT TGC CGT TCC-3′. The sequence encoding EGFP was isolated from pEGFP-F (Clontech) by PCR using the forward primer; 5′-AGA AAA AAG CGG CCG CCA TGG TGA GCA AGG GCG AG-3′ and the reverse primer; 5′-TCG AAT TCC TTG TAC AGC TCG TCC ATG CC-3′. The result is EGFP without the farnesylation site. Following this, a Not I/EcoR I digestion of the PCR product, was inserted between the Not I and EcoR I sites of the pGEX-DLT vector. The resulting plasmid, pGEX-DLT-GFP, expresses a "gpD-linker-thrombin recognition site-EGFP" fusion protein upon addition of IPTG.

2.2. Construction of λgfp10 and λgfp10-TAT

pEGFP-F (Clontech) was cut with EcoR I and cloned into the compatible site of λgt10 (Promega), producing λgfp10. Prior to cloning, the COS ends of λgt10 were melted at 65 °C for 10 min. and annealed in TE (10 mM Tris, 1 mM EDTA pH 8) at 42 °C for 1 h. The COS ends were then ligated, and λgt10 was digested with EcoR I and dephosphoryled. EcoR I digested pEGFP-F was then ligated into λgt10. DNA was packaged into λ capsids using the gigapackIII packaging extract (Stratagene). 200 ng of DNA was added to 500 μL of packaging extract and was left at 4 °C for 2 h and 100 μL of SM buffer was added. Where SM is 0.1 M NaCl, 5 mM $MgSO_4$, 50 mM Tris pH 7.5, and 0.01% gelatin. λ was then amplified by growth on a top agar plate containing LE392 (Promega). Screening for λgfp10 clones was performed using PCR and sequencing (ABI).

Recombineering was used to insert the synthetic oligonucleotide 3′ of the gpD sequence of λgfp10 [38–41]. The oligonucleotide was designed with a linker sequence, a c-Myc tag, and the protein transduction domain of the TAT protein from HIV1, in order from 5′ to 3′ off of the 3′ end of the gpD directly preceding the TAG stop codon. After recombineering this construct into λ the last codon of gpD became the GTT codon. The stop codon became an amber stop codon, TAG, that can be suppressed in a $SupF^+$ host. The linker was inserted 3′ of gpD with Hpa I and Not I into pDE (see below), a plasmid we built by cloning gpD to gpE into the MCS of pBluescript SK+(Stratagene). The linker was a 16 amino acid sequence designed using LINKER [42]. The linker oligonucleotide sequence for the sense strand in 5′–3′ order was: TAG GGC AAG TAC ACC AGC TCT GGC CAA GCA GGC GCC GCT GCG AGC GAA TCT GCG GCC GC. The antisense strand was the compliment of this. The oligonucleotide with the c-Myc tag and TAT was: GGC CGC CGA GCA GAA ACT GAT CTC TGA AGA GGA TCT GTA TGG CCG TAA AAA ACG TCG TCA GCG TCG TCG TTA ATG. The antisense strand, 5′–3′, was: AAT TCA TTA ACG ACG ACG CTG ACG ACG TTT TTT ACG GCC ATA CAG ATC CTC TTC AGA GAT CAG TTT CTG CTC GGC. Everything was codon optimized for expression in *E. coli*. The c-Myc tag was EQKLISEEDL, which was based on the pCMV-Tag5 vector (Stratagene). The TAT PTD sequence was modeled on Dr. Dowdy's work where it is: YGRKKRRQRRR [43]. All oligonucleotides were synthesized and gel purified by Operon (Eurofins MWG Operon).