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Construction and analysis of variants of a polyvalent Lyme disease vaccine: approaches for improving the immune response to chimeric vaccinogens

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Abstract

There is currently no Lyme disease vaccine commercially available for use in humans. Outer surface protein C (OspC) of the *Borrelia* has been widely investigated as a potential vaccinogen. At least 38 OspC types have been defined. While the antibody response to OspC is protective, the range of protection is narrow due to the localization of protective epitopes within OspC type-specific domains. To develop a broadly protective vaccine, we previously constructed a tetravalent chimeric vaccinogen containing epitopes from OspC types A, B, K, and D. While this construct elicited bactericidal antibody against strains bearing each of the four OspC types, its solubility was low, and decreasing IgG titer to epitopes near the C-terminus of the construct was observed. In this report, construct solubility and immunogenicity were increased by dialysis against an Arg/Glu buffer. We also demonstrate the immunogenicity of the construct in alum. To further optimize epitope-specific immune responses, several constructs were generated with differing epitope organization or with putative C-terminal protective motifs. Analyses of murine antibody titers and isotype profiles induced by these constructs revealed that while the C-terminal tags did not enhance antibody titer, specific epitope reorganization and reiteration did. These analyses provide important information that can be exploited in the development of chimeric vaccinogens in general.

Keywords

Lyme disease vaccine; *Borrelia burgdorferi*; Outer surface protein C

1. Introduction

Lyme disease is caused by the spirochetes *Borrelia burgdorferi*, *B. garinii* and *B. afzelii* and is transmitted to humans by infected *Ixodes* ticks [1,2]. It is the most common vector-borne disease in the North America and Europe, and remains an emerging disease of considerable importance due to its potentially serious cardiac, neurological, and arthritic sequelae [3-10]. The need for a Lyme disease vaccine has been clearly established [11,12]. The outer surface

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protein A (OspA) based LYMERix vaccine was available for several years but was removed from the market over concerns of potential adverse effects [13]. OspC, a ~22 kDa, surface-exposed, highly antigenic lipoprotein is an attractive vaccine candidate [14-17]. OspC is upregulated within the tick during transmission and expressed during early infection in humans [18]. In contrast, OspA is rapidly downregulated upon spirochete entry into the mammal [19, 20]. Immunization with OspC is protective but the protection range is narrow [14-16, 21-29]. In an analysis of *B. burgdorferi* sensu stricto, 21 different OspC phylogenetic types were described and designated by letter (A-U) [30, 31]. In a recent study we demonstrate the existence of additional OspC types and hypothesize that approximately 30 OspC types represent the majority of human infectious isolates (unpublished data).

Since the OspC sequence varies by an average of 35% between types, a broadly protective vaccine will have to be designed to incorporate multiple, type-specific epitopes [32, 33]. Towards this goal, we have begun identifying the immunodominant linear epitopes of diverse OspC types. To date, these epitopes have been identified for OspC types A, B, K, and D, and the epitopes have been incorporated into a tetravalent chimeric vaccinogen [17, 33, 34]. This construct, designated as the ABKD vaccinogen, elicited an antibody response against each of the component epitopes, and those antibodies were bactericidal in a complement-dependent manner [17]. However, construct solubility was not optimal and the relative antibody titer to each epitope progressively decreased from the N- to the C-terminus of the construct. In this study, we sought to improve construct solubility and assess the impact of epitope placement, epitope reiteration, and the inclusion of putative C-terminal protective motifs on the immune response. These analyses provide new insight into design strategies for a broadly protective OspC vaccine, and for construction of chimeric vaccines in general.

2. Materials and Methods

2.1 Expression and purification of recombinant OspC

Recombinant full length OspC proteins of types A, B, K and D (minus the leader peptides) were generated as previously described, using a PCR based approach and ligase-independent cloning (LIC) methodologies with the pET-32 Ek/LIC vector (Novagen) [17, 33]. The DNA sequences of these and all other constructs described in this report were confirmed (MWG Biotech). The r-proteins were purified by nickel affinity chromatography using standard methods (Novagen). The r-proteins were dialyzed extensively against phosphate buffered saline (PBS; pH 7.4) across a 10kDa molecular weight cut-off membrane (Slide-a-lyzer, Pierce), the protein concentration was quantified by the BCA assay (Pierce), and the purity of the preparation was assessed by SDS-PAGE.

2.2 Construction, expression, and purification of ABKD vaccine variants

Variants of the ABKD construct with reordered epitopes, reiterated epitopes or specific C-terminal protective motifs were constructed. The original ABKD vaccinogen contains the loop 5 region of type A (amino acids (aa) 131 to 149) and the alpha helix 5 regions of types B (aa 160-201), K (aa 161-201), and D (aa 161-201) (Fig. 1 inset) [17]. Variants in which a Pro-Pro-Ala (ABKDppa construct) or Gly-Gly (ABKDgg construct) were added were generated by amplification of the original ABKD construct using reverse primers (Table 1) that carry the appropriate 5' overhang (Fig. 1A). Other vaccine variants were made by overlap annealing and PCR using techniques similar to those used in construction of the original ABKD vaccinogen as detailed in Fig. 1B, C, D [17]. In all cases, PCR was performed with GoTaq Green (Promega) using an initial 2 min 94°C denaturation step, followed by 35 cycles of denaturation at 94°C for 15 sec, primer annealing at 50°C for 30 sec, and extension at 72°C for 60 sec, with a final 72°C extension for 7 min. All PCR products were gel purified (Qiagen) prior to use as templates in subsequent PCR reactions. Final amplicons were annealed to the pET-46 Ek/LIC vector

using ligase independent cloning methodologies and r-proteins were generated and purified as described above. After purification the r-proteins were dialyzed across a 10kDa molecular weight cutoff membrane (Slide-a-Lyzer, Pierce) against three changes of either PBS (pH 7.4) or a pH 8 buffer containing 100 mM phosphate, 100 mM NaCl, 50 mM arginine, 50 mM glutamic acid (Arg/Glu buffer) [35]. The soluble protein concentration was quantified by the BCA assay (Pierce), and the purity of the constructs was assessed by SDS-PAGE.

2.3 Mouse immunizations

Six week old male C3H/HeJ mice were immunized with the vaccinogen variants (3 mice per construct). The r-proteins were administered on a molar basis to compensate for differences in the number of epitopes per unit of vaccinogen mass. Each mouse received ~ 2.8 nmols of protein per vaccination which is equivalent to 50 µg of constructs ABKD, ABKDppa, ABKDGg, and ADBK or 62.5 µg of constructs ABKDD and ADBKD. Initial doses were delivered with complete and boosts with incomplete Freund's adjuvant (weeks 2 and 4). In a second set of mice, the ABKD vaccinogen was delivered using Alum (Imject Alum, Pierce). Sera was collected by tail nick prior to the first immunization and at week 6.

2.4 Assessment of epitope-specific IgG titer induced by vaccine variants

The immunogenicity of each vaccinogen was assessed by immunoblot and ELISA with full length r-OspC of types A, B, K, and D serving as the test antigens. All immunoblot and ELISA methods were as previously described [17]. For immunoblotting, a 1:2500 dilution of each antiserum was used (1% BSA in PBS with 0.2% Tween-20 (PBS-T); 1 hr; 3 washes). Equal protein loading was verified by screening with anti-His tag mAb (1:2000; Novagen). Secondary detection was by peroxidase-conjugated goat-α-mouse IgG (1:40,000; Pierce) and chemiluminescence (Super Signal Pico, Pierce). For ELISA analyses, r- OspC proteins were coated (100 ng well⁻¹; 16h; 4°C; carbonate buffer, pH 9.6) onto 96 well plates (Costar 3590). The plates were blocked (1% BSA/PBS-T; 2 hr), and serially diluted anti-vaccinogen antiserum (100 µL) was added to the wells of duplicate plates (1 hr). HRP-conjugated goat-α-mouse IgG (1:20000) served as the secondary antibody and ABTS as the chromogenic substrate. The absorbance was read at 405 nm in an ELISA plate reader (ELx 808; Biotek) while the reaction rate was linear. Titers are reported as the inverse dilution corresponding to 50% of the maximum absorbance plateau.

2.5 Determination of epitope-specific immunoglobulin isotype profiles

The isotype profiles of the antibody response to the ABKD, ABKDD, and ADBKD vaccine constructs were assessed by ELISA, as described above. Antisera were analyzed in duplicate (100 µL; 1:10000; 1 hr) and epitope-specific Ig was detected with isotype specific, biotinylated secondary antibodies (1 hr; Mouse isotyping kit; Zymed). The secondary antibodies were detected by peroxidase-conjugated streptavidin (30 min) and the ABTS chromogen. All incubations were completed at room temperature.

3. Results

3.1 Construction, expression and purification of variant vaccine constructs

The properties of the ABKD vaccinogen and the variants generated as part of this report are presented in Fig. 1 and Table 2 [36]. Upon purification of the r-vaccinogens by nickel chromatography, a significant proportion of the protein precipitated during dialysis against PBS. To increase solubility, a modified phosphate dialysis buffer (100mM NaCl, 50 mM Arg, 50 mM Glu; pH 8.0) was employed [35]. Buffer pH was increased from 7.4 (PBS) to 8.0 to increase the difference between buffer pH and the pI of the r-proteins (Table 2). In addition, the NaCl concentration was reduced from 150 to 100 mM, and 50 mM arginine and 50 mM

glutamic acid was added [35]. Using this buffer, no precipitation of the r-proteins was observed and there was a marked increase in protein solubility (Table 3). As determined by SDS-PAGE, the r-proteins were essentially homogenous (Fig. 2).

3.2 Immunogenicity of vaccine variants

The relative immunogenicity of the r-proteins was assessed by immunization of mice with the vaccinogens in Freund's adjuvants. Epitope-specific reactivity of the sera was assessed by immunoblotting using r-OspC of each of the four types as test antigens. Vaccinogen-dependent differences were observed (Fig. 3A). These differences were quantified by ELISA using full length, r-OspC proteins as the immobilized antigens. The differences in the determined titers were reflective of that seen with the immunoblots (Fig. 3B). The most marked differences were seen in reactivity with the type D epitope, with particularly low titers seen for the ABKDppa, ABKDgg and ADBK variants. These data demonstrate that the organization of the constructs influences the immune response to individual epitopes.

3.3 Isotype profiles of vaccine variant-specific immunoglobulins

Epitope-specific immunoglobulin isotype profiles were determined for the vaccinogen variants deemed to have the greatest vaccine potential (ABKD, ABKDD, and ADBKD). In general, IgG1 dominated with smaller amounts of IgG2b and IgG2a and minimal IgG3 or IgM (Fig. 4). This pattern has been previously noted with the ABKD construct [17]. For all epitopes and all vaccine variants, the pattern of Ig isotype was similar, with one exception. There were higher levels of type K and D epitope-specific IgG2a and IgG2b in mice immunized with the ABKDD than with the ABKD or the ADBKD variants.

3.4 Effect of adjuvant type on antibody titer and isotype profile

To compare the effects of adjuvant on the response to the vaccinogen, mice were immunized with the ABKD protein emulsified in Freund's adjuvants or adsorbed to alum. There was a marginal decrease in IgG titers against the whole vaccinogen and individual constituent epitopes (Fig 5A). While the level of vaccinogen-specific IgG1 was similar with both adjuvants, the levels of IgG3, IgG2a, and IgG2b (Fig 5B) were slightly lower in mice immunized with alum (Fig 5A). Epitope-specific isotype profiles were similar to that seen using the whole vaccinogen (data not shown).

4. Discussion

The use of chimeric proteins containing multiple B-cell epitopes has potential advantages over whole-protein polyvalent vaccinogens and peptide conjugates in vaccine development. The exclusive use of protective epitope sequences reduces the potential for misdirection of the response to irrelevant epitopes. In the context of OspC, this is particularly important as vaccination with full length r-OspC elicits immune responses to conserved domains that are not presented by the bacteria during infection [33,37,38]. In this study, we extend our efforts to develop and optimize a recombinant, polyvalent, chimeric OspC vaccinogen. We have previously demonstrated that a chimeric construct consisting of epitopes from OspC types A, B, K, and D was immunogenic in mice, induced IgG that bound native OspC at the cell surface and elicited complement-dependent killing [17]. However, the ABKD construct was of low solubility in PBS, and C-terminal located epitopes were less efficient in eliciting IgG than N-terminally located epitopes. The goals of this study were to improve these particular properties of the vaccinogen through the use of modified vaccinogens that differed in epitope placement, epitope reiteration, and the inclusion of protective motifs to improve the total and the epitope-specific immune response.

In an earlier study, it was noted that there was significant precipitation of recombinant ABKD vaccinogen following dialysis against PBS [17], with a maximum achieved soluble concentration of 0.5 mg/mL. This poor solubility may have impacted vaccine immunogenicity. The Arg/Glu dialysis buffer significantly increased the solubility of the r-proteins (Table 3). Enhanced solubility may result from the interaction of Arg and Glu with exposed residues of opposite charge [35]. Dialysis against the Arg/Glu buffer resulted in dramatically higher titers compared with that seen with vaccinogen dialyzed against PBS [17] (Fig. 3B). Dialysis against buffers containing Arg and Glu may be a useful tool to improve the solubility of other novel chimeric proteins.

Decreased titer to C-terminally located epitopes in chimeric proteins has been previously reported for the chimeric streptococcal M-protein vaccinogen. In that case, it was speculated that structural issues or proteolytic degradation by carboxypeptidases was responsible [39-41]. Numerous methods have been proposed for protection of peptides and recombinant proteins from protease activity, including amidation or PEGylation of the C-terminus, acetylation of the amino (N)-terminus, and addition of protective amino acid motifs [42-47]. Two protective motifs, Gly-Gly and Pro-Pro-Ala, were assessed for their ability to enhance antibody responses to the ABKD vaccinogen. The addition of neutral, hydrophilic Gly residues may reduce the activity of carboxypeptidases C and D, which are specific for hydrophobic and basic C-terminal amino acids, respectively [45,46,48]. The Pro-Pro-Ala motif may sterically hinder carboxypeptidase progression through the juxtaposed, bulky proline residues [47]. To assess the impact of addition of these motifs, mice were immunized with the ABKDgg or ABKDppa constructs (Fig. 1A). In both cases, the sera had lower mean IgG titers against one of more epitopes, compared with those immunized with the unmodified ABKD construct (Fig. 4) indicating that the addition of the tags actually had a negative impact on immunogenicity. In addition, the data suggest that the basis for the lower titer of antibody to the C-terminal epitopes is not due to the activity of carboxypeptidases.

To investigate whether epitope placement and organization were factors that contributed to the lower antibody titer elicited to the C-terminal epitope, additional constructs were created. In one, the C-terminal type D epitope was reiterated to yield ABKDD. Epitope repetition has been previously demonstrated to increase antibody titer by an unknown mechanism [41,49-51]. To determine if decreased titer could be simply due to C-terminal epitope location, the type D epitope was relocated in the construct (ADBK). Epitope reiteration in the ABKDD vaccinogen doubled the type D-specific IgG titer, but simultaneously caused a decrease in the titer against the adjacent type K epitope (Fig. 3B). In the ADBK construct, relocation of the D epitope led to decreased type-D specific IgG titer while at the same time the antibody titer to the K epitope was improved over that observed with the ABKD construct. Lastly, adding a C-terminal type D epitope (ADBKD) to ADBK did not improve the type K-specific titer compared to ADBK; however, it yielded a significantly improved titer against the type D epitope. These results indicate that C-terminal placement of an epitope in and of itself does not lead to a general decrease in titer. The data presented here highlight the need for the individual optimization of epitope placement in chimeric constructs.

Determination of the IgG isotype profile induced upon vaccination is an important consideration in vaccine development since it can influence protective efficacy. The epitope-specific isotype profiles were determined for the ABKD, ABKDD, and ADBKD constructs using r-OspC of types A, B, K or D as the immobilized antigens. Bound antibody was detected with isotype-specific antisera. As previously reported for the ABKD vaccinogen [17], the predominant isotype was IgG1 with less IgG2a and IgG2b. Only low levels of IgM and IgG3 were detected (Fig. 4). The epitope specific Ig isotype profiles were similar between the ABKD and ADBKD antisera, with a decrease in the levels of IgG2a and IgG2b from the N- to the C-terminal epitope, mimicking the total IgG titer. The profile for ABKDD had a consistent level

of IgG2a and IgG2b across all of the epitopes, despite the lower type K-specific total IgG titer. While the above data indicate the dominance of IgG1, which in the mouse does not activate complement [52,53], we have previously demonstrated antibodies elicited by the ABKD vaccinogen are in fact bactericidal by a complement-dependent mechanism [17]. Hence, while IgG1 may dominate, the levels of the complement-fixing isotypes are sufficient to allow for protection.

Alum is currently the only adjuvant approved for use in humans, so a comparative assessment of the response elicited by alum versus Freund's is an essential aspect of human vaccine development. These adjuvants have been demonstrated in some cases to elicit differences in immune responses due to their influence on Th cytokine profiles [54-58]. As part of this report, we compared the total and epitope-specific IgG titers and Ig isotype profiles associated with these two adjuvants. Vaccination in alum elicited a high, but slightly lower total IgG titer than that observed with Freund's adjuvant, a finding that was not unexpected. Regarding the isotype profile, the overall profile was not significantly influenced by adjuvant choice. In summary, it can be concluded that sufficiently high titers of antibody capable of complement activation are elicited upon delivery of the vaccinogen in either Freund's or alum adjuvant.

In this study, we have generated several variants of an OspC-based chimeric polyvalent Lyme disease vaccinogen in an effort to optimize the induced humoral immune response. The solubility and immunogenicity of the construct was improved by dialysis against an Arg/Glu buffer. In addition, variations in vaccinogen organization revealed that the immune response against component epitopes appears to be dependent on epitope position and copy number. This provides critical information that can be used in the design of even more broadly protective vaccinogens. Lastly, comparative analyses of alum and Freund's adjuvants revealed that delivery of the vaccinogen in alum is likely to induce appropriate immune responses for protection in humans. The information obtained from these analyses also provides important information that can be used to improve the immune responses to chimeric vaccinogens in general.

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Figure 1.

Schematic representation of the construction of the ABKD vaccine variants. ABKDppa (Panel A) was constructed by amplification of the original construct using a reverse primer with a 5' overhang to add the C-terminal amino acids. ABKDg (not shown) was made in an identical manner, but using the OCA5LICg primer. ABKD (Panel B), ADBK (Panel C), and ADBKD (Panel D) were all made by PCR amplification of constituent sequences using primers that added tails encoding linker sequences. The resultant PCR products were gel purified, joined by overlap annealing and extension, and amplified by PCR. The final products were cloned into the pET-46 Ek/LIC vector. OspC type-specific epitope containing regions are denoted by letter, and linker sequences by number (see inset for encoded amino acid sequences). Arrows denote primers, and 5' overhanging LIC tails or linker sequences are noted on the tail of each primer arrow.

Figure 2.

Coomassie stained SDS-PAGE gel of the chimeric vaccinogen test constructs. Vaccinogen r-proteins were expressed in *E. coli*, affinity purified by nickel chromatography, and quantified by the BCA method. Two μ g of the purified proteins were electrophoresed on a 15% SDS-PAGE gel (Criterion; Bio-Rad) and stained with Coomassie G-250. No contaminating proteins were noted, and there was minimal or no degradation of the recombinant proteins.

Figure 3.

Assessment of mouse vaccine serum recognition of full length r-OspC. In panel A, r-OspC of types A, B, K, and D were electrophoresed and blotted to PVDF (type indicated at top; 500 ng/lane), and probed with a 1:2500 dilution of representative sera from mice vaccinated with each of the variant constructs (indicated at left). Secondary detection was by peroxidase-conjugated goat-α-mouse IgG (1:40000) and chemiluminescence. Molecular masses are indicated at the right. In panel B are the results of a quantitative ELISA titration of mouse vaccine serum reactivity with full length r-OspC. Sera generated against each vaccine construct (noted at bottom) were titrated against immobilized, full length, r-OspC of types A, B, K, and D. Also included are the titers from the ABKD construct dialyzed against PBS (ABKD*) [17]. Bars denote the mean titer against OspC types A (black), B (grey), K (open), and D (hatched). Titters from individual mice are denoted by open triangles. Listed below are the mean numerical titers, as well as the titers indexed either to the corresponding titer of the ABKD construct dialyzed against PBS (ABKD*) or against Arg/Glu buffer (ABKD).

Figure 4.

Epitope-specific isotype responses to three vaccine constructs. OspC types A, B, K, and D were immobilized on ELISA plates and probed with immune sera from mice vaccinated with the ABKD, ABKDD, or ADBKD constructs in duplicate. Bound Ig isotypes were detected with biotinylated isotype-specific secondary antibodies and peroxidase-conjugated streptavidin.

Figure 5.

Assessment of the antibody response to the ABKD vaccinogen administered in Freund's adjuvants or alum. In panel A are the results of a quantitative ELISA titration of IgG in mouse sera generated against the ABKD vaccine emulsified in Freund's adjuvants (grey) or adsorbed to alum (hatched). The sera were titrated against immobilized ABKD vaccinogen and full length, r-OspC of types A, B, K, and D. In panel B is shown the isotype profile of the sera bound to immobilized ABKD vaccinogen. The bound Ig isotypes were detected with biotinylated isotype-specific secondary antibodies and peroxidase-conjugated streptavidin.

Table 1

Primers used in construction of the chimeric vaccinogens. LIC tails are in bold, and linker sequences are underlined.

Primer	Sequence	Description
OCAL5LIC(+)	GACGACGACAAGATT TCTGAAACAT TACTAATAAATTTAAAGAAAAAC	Amplifies type A from aa 131 and adds LIC tail
OCAL5L5(-)	<u>TAAAGCTGACATAGCACCTTCTTTAC</u> CAAGATCTGTGTG	Amplifies type A up to aa 149 and adds linker 5 (GAMSAL)
OCBH5L1(+)	<u>GGTAGCATGGGTATGTTAAAAGCAA</u> ATGCAGCGGG	Amplifies type B from aa 160 and adds linker 1 (GSMGML)
OCKH5L3(-)	<u>TAAAACGCTCATGCTACTTGTAAGCT</u> CTTTAACTGAATTAGC	Amplifies type K up to aa 201 and adds linker 3 (SSMSVL)
OCKH5LIC(-)	GAGGAGAAGCCCGGTTTAACTTGTA AGCTCTTTAACTGAATTAGC	Amplifies type K up to aa 201 and adds an LIC tail
OCDH5LIC(-)	GAGGAGAAGCCCGGTTTAACTTGTA AGCTCTTTAACTGAATTAG	Amplifies type D up to aa 201 and adds an LIC tail
OCDH5ppaLIC(-)	GAGGAGAAGCCCGGTTTATGCAGG AGGACTTGTAAGCTCTTTAACTGAAT TAG	Amplifies type D up to aa 201 and adds 'PPA' and an LIC tail
OCDH5ggLIC(-)	GAGGAGAAGCCCGGTTTATCCTCC ACTTGTAAGCTCTTTAACTGAATTAG	Amplifies type D up to aa 201 and adds 'GG' and an LIC tail
OCDH5L1(-)	<u>TAACATACCCATGCTACCACTTGTA</u> GCTCTTTAACTGAATTAG	Amplifies type D up to aa 201 and adds linker 1 (GSMGML)
OCDH5L4(+)	<u>AGTTCAAGCCAAGGCTTAAAAACAC</u> ATAATGCTAAAGACAAG	Amplifies type D from aa 161 and adds linker 4 (SSSQGL)
OCDH5L4(-)	<u>TAAGCCTTGGCTTGA</u> ACTTGTAAGC TCTTTAACTGAATTAGC	Amplifies type D up to aa 201 and adds linker 4 (SSSQGL)
OCDH5L5(+)	<u>GGTGCTATGTCAGCTTTAAAAACAC</u> ATAATGCTAAAGACAAG	Amplifies type D from aa 161 and adds linker 5 (GAMSAL)
3'REVLIC(-)	GAGGAGAAGCCCGGT	Amplifies up to the 3' LIC tail
pET46 T7(+)	CGAAATTAATACGACTCACTATAGG GG	Amplifies from pET46, 123 bases upstream of cloning site
pET46 T7(-)	GCTAGTTATTGCTCAGCGG	Amplifies up to pET46, 117 bases downstream of the cloning site

Table 2

Physicochemical properties of the vaccinogens.

Construct	Amino acids	Molecular mass (Da)	Isoelectric point	Instability index
ABKD	169	18014.4	6.49	10.14
ABKDppa	172	18279.7	6.49	14.93
ABKDgg	171	18128.5	6.49	10.86
ABKDD	214	22632.7	6.85	15.49
ADBK	170	18027.4	6.49	8.51
ADBKD	215	22645.7	6.85	14.18

Table 3

Concentration of soluble protein for vaccinogens dialyzed against PBS or Arg/Glu buffer.

Construct	Protein concentration (mg mL ⁻¹)	
	PBS	Arg/Glu buffer
ABKD	0.15	3.04
ABKDppa	0.20	3.78
ABKDgg	0.22	5.99
ABKDD	0.80	4.66
ADBK	0.04	4.37
ADBKD	1.12	4.66