A Recombinant Flagellin-Poxvirus Fusion Protein Vaccine Elicits Complement-Dependent Protection Against Respiratory Challenge with Vaccinia Virus in Mice

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Abstract

Bacterial flagellin is a potent adjuvant that enhances adaptive immune responses to a variety of protein antigens. The vaccinia virus antigens L1R and B5R are highly immunogenic in the context of the parent virus, but recombinant forms of the proteins are only weakly immunogenic. Therefore we evaluated the humoral response to these antigens in mice when flagellin was used as an adjuvant. Flagellin-L1R and flagellin-B5R fusion proteins were more potent than flagellin, L1R, and B5R as separate proteins. At least three immunizations with flagellin-L1R and flagellin-B5R fusion proteins were required to confer protection in mice against challenge with vaccinia virus. Immune mice exhibited only limited signs of disease following challenge. Additionally, virus neutralization titers correlated with protection. Depletion of complement using cobra venom factor resulted in a marked decrease in the survival of immunized mice after challenge with vaccinia virus. Our results are consistent with the conclusion that flagellin-L1R and flagellin-B5R fusion proteins are effective in eliciting protective immunity against vaccinia virus that is dependent, in large part, on complement.

Introduction

VACCINIA VIRUS IS HIGHLY IMMUNOGENIC, promoting protective humoral and cell-mediated immune responses in humans and animals. However, when outer membrane protein antigens (e.g., L1R, B5R, A33, and A27L) were used as recombinant proteins in experimental vaccines, they exhibited markedly reduced immunogenicity (10,16,18,22,25–27). For example, Fogg *et al.* (18) demonstrated that four immunizations with relatively large amounts of these proteins were required to promote protection against respiratory challenge with vaccinia virus in mice. In this study, the authors used trehalose dicorynomycolate emulsion or saponin as adjuvants to enhance the response to L1R, B5R, and A33R.

Work from our laboratory (3,4,24,39,42,43) as well as that of other investigators (5–8,11,28,29,32,33,37,38) has established that flagellin, the major structural protein of gram-negative bacteria, is a potent adjuvant when administered with an antigen by any of several different routes. Flagellin binds to a leucine-rich repeat of toll-like receptor 5 (TLR5) (40), and induces downstream signaling in a MyD88-dependent manner (21). The adjuvant effect of flagellin on the humoral immune response is due to at least three important actions: (1) induction of cytokine production by non-lymphoid cells (1,2,11,12,23); (2) increased T and B

lymphocyte accumulation in draining lymph nodes (3); and (3) activation of *tlr5*^{+/+} CD11c⁺ cells (4). The ability of flagellin to bind with high affinity to TLR5 on CD11c⁺ antigen-presenting cells provides an explanation for the enhanced potency of flagellin-antigen fusion proteins over flagellin + antigen in the induction of CD4⁺ T-cell dependent humoral immunity (39).

The adjuvant effect of flagellin has been demonstrated in a variety of pathogen models. By using flagellin as an adjuvant with one or more recombinant antigens, antibody-dependent protective responses have been obtained against a variety of pathogens, including influenza (1,7,29,30,33,38), *Yersinia pestis* (24,39), West Nile virus (37), and *Pseudomonas aeruginosa* (42,43). These protective responses characteristically exhibit remarkably high titers of antigen-specific IgG when the vaccine is administered intranasally or intramuscularly, and IgG and secretory IgA when the vaccine is administered intranasally. For example, a vaccine containing a flagellin fusion protein that incorporates two protective antigens of *Y. pestis* provides complete protection against respiratory challenge with large numbers of virulent *Y. pestis* in mice (39).

To date, the recombinant antigens used with flagellin have been highly immunogenic, and thus the question remains as to whether or not flagellin can promote robust responses to weakly immunogenic proteins. To address this important

question, we asked if flagellin can promote a protective adaptive immune response in mice when combined with the weakly immunogenic recombinant vaccinia virus proteins, L1R and B5R.

Materials and Methods

Mice

Female BALB/c mice (6–8 wk of age) were purchased from Charles River Laboratories (Wilmington, MA) and were housed in specific pathogen-free facilities. All animal work was done in accordance with protocols approved by the Wake Forest University School of Medicine Animal Care and Use Committee.

Cell lines

RAW264.7 and HeLa cell lines were purchased from ATCC. RAW424 cells were generated by stably transfecting RAW264.7 cells with an expression plasmid containing TLR5 linked to eYFP (45). Plasmid expression is maintained by the addition of 400 µg/mL of G-418 to culture medium.

Virus production and purification

Vaccinia Virus Western Reserve (VV-WR) was produced by infecting HeLa cells at an MOI of 5 for 48 h. The cells were pelleted by centrifugation and lysed by at least four freeze-thaw cycles. Further lysis was performed by three cycles of sonication at full power for 30 sec. Virus was centrifuged 80 min at 32,900×g and 4°C on a 36% sucrose cushion in 10 mM Tris-Cl (pH 9.0). The pellet was resuspended in 1 mM Tris-Cl (pH 9.0) and stored at –80°C until use. Virus titers were determined by a plaque assay using COS-1 cells.

Baculovirus production of L1R and B5R

Baculoviruses encoding ectodomains of L1R and B5R were obtained from J.C. Whitbeck, the Schools of Dental and Veterinary Medicine, University of Pennsylvania, Philadelphia, PA. Viral proteins were prepared by infecting SF9 cells (Invitrogen Corp., Carlsbad, CA) grown in SF-900II Serum Free Medium (Gibco, Carlsbad, CA) at an MOI of 3–10. Supernatants were harvested on day 3 and concentrated using a filter with a 10-kDa cutoff (Millipore, Billerica, MA). The his-tagged proteins were purified using a Ni-NTA Superflow column (Qiagen Inc., Valencia, CA). Proteins were then dialyzed into PBS pH 7.0 (L1R) or PBS pH 5.9 (B5R). To remove endotoxin and DNA contaminants the proteins were run through Acrodisc units with Mustang E (B5R) or Q (L1R) filters (Pall Corporation, Port Washington, NY). Endotoxin levels were <20 pg/µg of protein (Limulus Amoebocyte Assay; Cape Cod Inc., East Falmouth, MA).

Generation of flagellin and flagellin-poxvirus fusion proteins

FliC (hereafter referred to as flagellin) from *Salmonella enterica* serovar enteritidis and the 229-truncation mutant were prepared as previously described (23,36). The 229-truncation mutant of flagellin contains only amino acids 297–471 of the hypervariable region, and therefore does not signal via TLR5 (23). Two approaches were used to generate fusion proteins containing flagellin and L1R or B5R or flagellin,

L1R, and B5R. Plasmids containing L1R and B5R cDNA were kindly provided by J.W. Hooper (U.S. Army Medical Research Institute of Infectious Diseases [USAMRIID], Ft. Detrick, MD). To generate a flagellin-B5R (FB) fusion, a cDNA encoding the ectodomain of B5R (defined as nucleotides 57–837) was cloned into the *fliC* gene lacking the majority of the hypervariable region (lacking nucleotides 586–1134). The L1R-flagellin (LF) fusion protein was generated by inserting the cDNA encoding the ectodomain of L1R (nucleotides 1–543) at the N-terminus of full-length *fliC*. These constructs are illustrated in Fig. 1. Each construct was cloned into the pET29a expression vector (Novagen, Madison, WI).

Purification of proteins

Plasmids were transformed into *E. coli* BL-21 (DE3) for protein production. All proteins used in this study contained a 6-His tag to facilitate rapid purification using Talon metal affinity resin (Clontech, Mountain View, CA) as previously described (23). It is important to emphasize that we have never detected the generation of antibodies against the 6-His tag itself when mice were immunized with any of the recombinant proteins used in this study. To remove endotoxin and nucleic acid contaminants, the purified proteins were passed through Acrodisc Mustang Q filters (Pall Corporation). LPS levels (measured by Limulus Amoebocyte Assay, Cape Cod Inc.) were <20 pg/µg of protein.

TLR5-specific signaling activity of flagellin and flagellin fusion proteins

In-vitro TNF-α production was used as a measure of flagellin signaling activity (36,45). To ensure that the signaling was TLR5 specific, we assessed activity in cultures of RAW 264.7 cells, a mouse macrophage cell line that does not express TLR5, and in cultures of RAW 264.7 cells that stably express TLR5 (RAW 424 cells) (45). These cell lines were stimulated for 4 h with flagellin-containing proteins and supernatants were harvested for analysis of TNF-α by ELISA using a commercial kit (BD Biosciences, San Jose, CA) per the manufacturer's instructions. Units are calculated as the inverse of the concentration giving a 50% maximal response, which are then standardized to units per milligram.

Immunizations

For intramuscular (IM) immunizations, groups of at least 7 mice were anesthetized with tribromoethanol, and then 20 µL of vaccine mixture was injected into the right rear flank muscle. Groups of 7 mice received two immunizations on days 0 and 28, three immunizations on days 0, 28, and 42, or four immunizations on days 0, 28, 42, and 56. Blood samples were collected via tail vein bleeding 10–14 d after each immunization and the plasma prepared for analysis of antibody titers.

Determination of plasma anti-L1R and anti-B5R IgG titers by ELISA

ELISA plate wells (Immunosorb 96-well plates; Nalge Nunc, Rochester, NY) were coated with 100 µL of 10 µg/mL recombinant antigen (L1R or B5R) in PBS overnight at 4°C. The plates were washed and then blocked with PBS + 10%

FIG. 1. Recombinant proteins used in this study. Chimeric proteins were produced containing the ectodomains of L1R and B5R linked to flagellin. Diagrams illustrate antigen placement in the fusion proteins. L1R is present at the N-terminus of full length flagellin. B5R was substituted for the hypervariable domain of flagellin. TLR5-stimulating activity is calculated as units per milligram. A unit is the inverse of the concentration yielding a half maximal response. This value is then standardized to units per milligram.

newborn calf serum. Plasma samples were then added to triplicate wells and the plates were incubated overnight at 4°C. The plates were then washed, and rabbit anti-mouse IgG conjugated to horseradish peroxidase was added and the plates were incubated for 2 h at room temperature, followed by a 30-min incubation with 3,3',5,5'-tetramethylbenzidine liquid substrate. The reaction was stopped by the addition of 2N H₂SO₄, and absorbances were read at 450 nm. Titers were defined as the inverse dilution of plasma that yields an absorbance of 0.1 over background (established using naïve plasma).

Respiratory challenge with vaccinia virus

The 50% maximally tolerated dose (MTD₅₀; equivalent to LD₅₀) for BALB/c mice was determined for each vaccinia virus preparation. Groups of 7 mice were anesthetized with tribromoethanol, and 10 µL of virus was administered dropwise to alternating nares. The mice were observed daily for signs of disease. Symptoms were scored according to the following disease index: hunched posture (no = 0, yes = 1), respiratory stress (no = 0, mild-moderate = 1, severe = 2). Conjunctivitis and lethargy were evaluated on the same scale as respiratory stress. A mouse that received a disease score of 2 for any of the latter three symptoms or lost 30% of its initial weight was sacrificed.

Vaccinia virus neutralization

A flow cytometry-based assay was used to assess vaccinia virus neutralization (14). Vaccinia virus expressing eGFP was diluted in DMEM with various dilutions of heat-inactivated mouse plasma and incubated for 1 h. HeLa cells (seeded in 96-well plates at 1×10^5 cells/well) were infected at an MOI of 0.25 with the plasma-treated virus and incubated for 6 h. The cells were then treated with trypsin and fixed with PFA before FACS analysis. Virus incubated in medium only was used as a control (0% neutralization).

Complement depletion

Mice were treated with 5 µg (1 unit) of cobra venom factor (CVF) (Calbiochem, San Diego, CA) IV 18 h before challenge with vaccinia virus. A second treatment of 5 µg of CVF was administered 4 d after the initial injection (3 d post-infection). Complement activity in the mouse sera collected at various time points was measured based on a previously described method with minor modifications (41).

Measurement of C-reactive protein

Plasma samples were collected from mice 1 d after the mice had been immunized two or three times. Plasma was prepared as described above and stored at -80°C until

analysis. C-reactive protein was measured by an ELISA kit (KT-095; Kamiya Biomedical, Seattle, WA) per the manufacturer's instructions. Plasma was diluted 1:5 instead of the recommended 1:10.

Statistical analysis

Statistical analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA). Mouse titer data were assumed to be non-parametric, thus the Mann-Whitney *U* test was utilized to evaluate differences in titers. Survival data was evaluated using the integrated survival analysis tool.

Results

Immunization with flagellin and poxvirus antigens results in a robust humoral response and protection against respiratory challenge with vaccinia virus

A number of studies using recombinant L1R and B5R proteins or DNA encoding these proteins have demonstrated that recombinant forms of these antigens are only weakly immunogenic (17,18,22,25–27). As previously noted, several recent studies have demonstrated that flagellin fusion proteins enhance responses to strong antigens such as influenza virus epitopes, the F1 and V antigens of *Y. pestis*, and the EIII antigen of West Nile virus. Since these fusion proteins promoted a more robust response than separate antigens plus flagellin, we tested the immunogenicity of recombinant L1R and B5R when presented in the context of flagellin fusion proteins. Schematics for the flagellin/poxvirus antigen fusion proteins are shown in Fig. 1. To determine if the fusion proteins retained the ability to signal via TLR5, we assessed their ability to stimulate TNF- α production in cultures of TLR5⁺ RAW 424 cells. To control for TLR5-independent signaling, we also assayed TNF- α production in cultures of TLR5-negative RAW 264.7 cells. As shown in Fig. 1, the LF and the FB retained full TLR5 signaling activity relative to flagellin, as evidenced by the high specific activity of each fusion protein. None of the proteins induced TNF- α production in cultures of TLR5-negative RAW 264.7 cells (data not shown).

Having established that the fusion proteins retained TLR5 signaling activity, we next compared the ability of the fusion

proteins with that of the separate proteins (flagellin + L1R + B5R) to induce IgG responses against L1R and B5R. Groups of 7 mice were immunized IM with equimolar doses of L1R (1.3 μ g) + B5R (2.4 μ g) + flagellin (3.7 μ g), or LF (5 μ g) + FB (5 μ g). As shown in Fig. 2A and B, the fusion proteins elicited a dramatically more robust humoral response to L1R and B5R than the separate proteins + flagellin when administered IM. This difference was most evident with the B5R response. Furthermore, the variability in response within the fusion protein groups was far less than that observed when separate proteins were used for immunization.

Immunization with LF and FB promotes the production of antigen-specific IgG₁ and IgG_{2a}

To determine the extent to which fusion proteins can promote anti-L1R and anti-B5R IgG, we immunized mice two, three, or four times IM with 5 μ g each of LF and FB (Fig. 3). Total anti-L1R and B5R IgG titers as well as IgG₁ and IgG_{2a} increased with increasing numbers of immunizations. The total anti-B5R and anti-L1R IgG (Fig. 3A and B) and IgG₁ (Fig. 3C and D) titers appear to plateau after three immunizations. Although not all of the mice produced anti-B5R IgG_{2a} after two immunizations, the responding mice exhibited titers that were not dramatically different from the titers obtained after three or four immunizations (Fig. 3E and F). More than two immunizations resulted in a marked increase in the number of mice producing high titers of anti-B5R IgG_{2a}. To determine if a higher dose of LF and FB would alter the balance of antigen-specific IgG₁ and IgG_{2a}, BALB/c mice were given 3 immunizations with 20 μ g of LF and FB. Although the variability in response was markedly reduced, the IgG₁ and IgG_{2a} titers were not significantly different in mice given 20 μ g of the fusions compared to 5 μ g (data not shown).

Immunization with LF and FB confers protection against respiratory challenge with vaccinia virus

To determine if fusion proteins decrease the number of immunizations required for protection against vaccinia virus, mice were immunized two, three, or four times IM with 5 μ g each of LF and FB, and then challenged intranasally with

FIG. 2. Vaccine efficacy can be enhanced using flagellin-poxvirus antigen fusion proteins. (A and B) Groups of 7 BALB/c mice were immunized two times IM with 5 μ g of the flagellin-antigen fusion proteins LF and FB or equimolar doses of the separate proteins. Asterisks indicate significant differences in titer ($p < 0.05$).

FIG. 3. IgG, IgG₁, and IgG_{2a} responses following immunization with LF and FB. Mice were immunized IM with 5 μ g of LF and 5 μ g of FB. (A and B) Anti-B5R and L1R total IgG. (C and D) IgG₁. (E and F) IgG_{2a} titers after each immunization were determined by ELISA. Each symbol represents the results from an individual mouse, and the dashed lines at 10^3 are the limit of detection for the assay. Numbers below the dashed lines indicate the number of mice whose titers were below the limit of detection (non-responders). In A–D all groups are significantly different unless noted by n.s. In E and F significant differences are indicated by asterisks.

20 MTD₅₀ vaccinia virus. As shown in Fig. 4, despite high B5R- and L1R-specific antibody titers (Fig. 3), at least three immunizations were required to achieve 100% protection (Fig. 4A). Although mice given three or four immunizations survived challenge, they still lost a significant amount of weight (Fig. 4B). To evaluate disease symptoms other than weight loss, we used a disease index score that evaluated hunched posture, respiratory distress, conjunctivitis, and leth-

argy. As shown in Fig. 4C, mice that received three or four immunizations exhibited only minimal disease symptoms. In contrast, mice that were immunized only twice had disease symptom scores that approached those of animals that were not immunized. Since 40% of the mice immunized twice with the fusion proteins did not produce anti-B5R IgG_{2a}, it was possible that the lower level of survival might be associated with this subgroup of animals. However,

FIG. 4. Three or more immunizations with flagellin/poxvirus fusion proteins confers protection against vaccinia virus challenge. Groups of 10 BALB/c mice who received two to four IM immunizations of LF and FB at the indicated doses were challenged with 20 MTD₅₀ vaccinia virus. (A) Survival, (B) weight loss, and (C) disease index were measured following infection.

analysis of individual animals did not reveal a correlation between anti-B5R IgG_{2a} titer and survival (data not shown). It seems likely that the very high titers of anti-B5R IgG₁ obtained following immunization with the fusion proteins may compensate for the absence of IgG_{2a} in some of the mice.

Virus neutralization titers of plasma from immunized mice

To evaluate the virus neutralization titer of antibodies generated by immunization with LF + FB, *in-vitro* vaccinia

virus neutralization was performed using pooled heat-inactivated plasma collected from mice prior to challenge. Plasma from immunized mice was incubated with eGFP-expressing vaccinia virus for 1 h prior to incubation with HeLa cells for 6 h. Virus neutralization was defined as a 50% reduction in eGFP expression compared to virus that was not incubated with plasma. All samples from immunized mice neutralized significantly more virus than plasma from mock-immunized animals (Fig. 5). There was a significant difference in neutralization between two and three immunizations with 5 μ g of the fusion proteins LF and FB (titers of 321

FIG. 5. *In-vitro* neutralization of vaccinia virus by plasma from mice immunized with LF + BF. Pooled plasma from mice receiving two to four immunizations with 5 μ g of LF and FB was incubated at three dilutions with eGFP-expressing vaccinia virus *in vitro* at 37°C for 1 h. The plasma-treated virus was added to HeLa cells and incubated for 6 h at 37°C. Infected cells were detected by flow cytometry and the percentage of neutralization was calculated by standardizing to mock-infected cells or cells infected with virus that was not treated with plasma. Neutralizing titer is defined as the dilution at which 50% of virus is neutralized, and is calculated by non-linear regression. The dashed line shows 50% neutralization.

versus 902). Mice immunized by a single sublethal dose of vaccinia virus (convalescent) had titers that were almost threefold higher than any of the LF + FB immunized mice. It is interesting to note that the neutralizing titers correlated with the severity of disease. Naïve mice all succumbed to infection and had neutralizing titers under 100. Mice immunized twice were partially protected, lost weight, and had elevated disease scores and had an average neutralization titer of approximately 300. The mice that received three or four immunizations were protected and had low disease scores, but lost significant weight. Their average neutralizing titers were approximately 900. The mice that received a sublethal dose of vaccinia virus exhibited complete immunity and did not exhibit any disease symptoms or weight loss. These mice had titers ≥ 2000 .

Analysis of C-reactive protein levels following immunization

Although the changes in neutralizing titer may account for the difference in protection observed between two and three immunizations with LF + FB, one or more additional factors may be involved. We evaluated the possibility that three immunizations with LF + BF might trigger the production of liver-derived C-reactive protein, an acute phase reactant with complement-activating and opsonic activities, and thus possibly compensate for insufficient levels of IgG_{2a}. To evaluate this possibility, mice were immunized IM two or three times with 5 μ g of LF and FB or PBS, and plasma samples were collected 24 h after the final immunization. Plasma C-reactive protein levels were determined by ELISA. There was no change in circulating C-reactive protein levels of mice

immunized with the flagellin fusion proteins compared to PBS-injected mice (data not shown).

Complement plays a role in the protective effect of LF + FB

Since IgG_{2a} is known to be an efficient activator of complement in mice and monoclonal anti-B5R IgG_{2a} protects against vaccinia virus challenge (9), we evaluated whether complement-mediated immunity was critical to the protective response generated in response to LF and BF immunization (Fig. 6). BALB/c mice were immunized three times IM with 5 μ g each of LF and FB. One group of immunized mice was depleted of complement by IV administration of CVF 18 h before challenge with 20 MTD₅₀ of vaccinia virus. A second treatment of CVF occurred on day 3 post-infection. In preliminary experiments, we established that the first treatment with CVF resulted in an almost complete depletion of circulating C3 within 18–24 h. As expected, all of the mice that were immunized with LF and BF, but not treated with CVF, survived infection (Fig. 6A). However, 50% of the immunized mice that were treated with CVF succumbed to infection. Although the degree of weight loss between the two groups was similar (Fig. 6B), the complement-depleted mice had significantly higher disease scores (Fig. 6C). In view of these results, we hypothesized that about 50% of the CVF-treated mice may have had a much slower rate for restoration of circulating C3 levels, and were therefore more likely to succumb to vaccinia virus infection. In a subsequent experiment we evaluated this possibility by measuring the *in-vitro* complement-dependent red blood cell lytic activity in serum samples from mice treated twice with CVF. A group

FIG. 6. Complement depletion reduces the protective effect of flagellin/poxvirus fusion protein immunization. BALB/c mice were immunized three times IM with LF and FB. Six mice were treated with cobra venom factor (CVF) 1 d before challenge and 3 d after challenge with 20 MTD₅₀ vaccinia virus. (A) Survival, (B) weight loss, and (C) disease index were measured following infection.

of 5 mice received two injections of PBS and a second group of 10 mice received two injections with CVF (days 0 and 3). Blood was obtained from the mice in each group on days 0, 1, 3, 5, 7, and 10, and assayed for complement activity *in vitro*. As shown in Fig. 7, the CVF-treated mice differed dramatically in the time course for the return of complement activity. For example, the day 7 (a time at which morbidity and mortality are quite high) samples from 50% of the CVF-treated mice exhibited <10% hemolytic activity. Taken together, these results are consistent with the conclusion that complement component C3 plays a crucial role in LF+BF-induced protection against vaccinia virus challenge in mice.

Discussion

A major goal of these studies was to evaluate the efficacy of flagellin as an adjuvant with viral proteins that are poorly immunogenic in their recombinant forms. Our studies clearly demonstrate that flagellin is an effective adjuvant with weakly immunogenic poxvirus antigens. Although administration of fusion proteins resulted in a robust humoral response and protection against challenge with vaccinia virus, there was still a requirement for at least three immunizations with LF and FB for protection against respiratory challenge with vaccinia virus. Fogg *et al.* (18) found that four immunizations with recombinant poxvirus antigens were required to achieve high antibody titers and protection against challenge. In contrast to the more limited response to the recombinant poxvirus antigens, a single exposure to vaccinia virus elicits high titers of anti-L1R and anti-B5R antibodies. At the most simple level, a sublethal dose of vaccinia virus may be more effective than immunization with a small number of recombinant antigens due to the availability of a dramatically larger number of potentially protective antigens during virus infection. However, it is also possible that there is some feature of antigens such as L1R and B5R in the context of the viral infection that cannot be replaced or overcome by flagellin or other adjuvants (15,18,22,25–27).

In addition to the variety of viral antigens present during a viral infection, the duration of antigen availability repre-

sents another major difference between live virus vaccination and recombinant protein immunization. The persistence of antigen in the viral infection, as opposed to the limited availability of the flagellin fusion proteins following immunization, provides a reasonable explanation for the difference in the level of the induced antibody response. Dryvax immunization results in the shedding of live vaccinia virus for 3–4 wk (19,31). In contrast, flagellin administered IM is detectable in draining lymph nodes for up to 10 h, but is absent after 24 h (J.T. Bates and S.B. Mizel, unpublished observations). In addition to antigen availability, the poxvirus-induced innate immune response may promote a more robust activation of dendritic cells, and thus enhanced helper T-cell activation and associated B-cell responsiveness. Finally, it may be that the conformations of the recombinant L1R and B5R do not precisely mirror those of the same proteins in the context of a vaccinia virus-infected cell. In contrast to immunization with a sublethal dose of vaccinia virus, significant weight loss is observed in mice immunized with recombinant L1R and B5R. It is interesting to note that this weight loss in the LF+FB-immunized mice occurred in the absence of other disease symptoms.

Complement is known to be a part of the host response to vaccinia virus, especially in promoting lysis of the extracellular enveloped virus (EEV) (34,35). Clearance of EEV is very important, as this form of the virus enters cells more rapidly than the intracellular mature virion (IMV) form (13), and it is released earlier in infection (before lysis of the host cell). In a recent study, Benhnia *et al.* (9) generated a series of monoclonal anti-B5R antibodies and screened them for their ability to protect mice from vaccinia virus. The only monoclonal antibody that conferred full protection was of the IgG_{2a} isotype—an isotype in mice that is particularly efficient at activation of the classical complement pathway. Furthermore, Benhnia *et al.* (9) demonstrated that depletion of complement reduced the protective effect of passive immunization with anti-B5R IgG_{2a}. We found that 50% of immune mice given the CVF treatment survived vaccinia virus challenge. This finding is not surprising given the slow rate at which about 50% of the mice are able to restore complement

FIG. 7. Time course for the restoration of complement activity in mice treated with CVF. Mice were treated with PBS (A) or CVF (B) as described in Fig. 6. At the indicated time points following treatment, the mice were bled and samples were assayed for the ability to promote red blood cell lysis.

activity 7 d after CVF depletion (Fig. 7). LF + BF-immunized mice generate multiple antigen-specific IgG isotypes. Although IgG₁ is a less efficient activator of the classical complement pathway than IgG_{2a} in mice (20,44), it is quite likely that sufficiently high titers of antigen-specific IgG₁ may compensate for reduced inherent complement activating activity, especially if there is high epitope density on the target (20). Additionally, the LF + BF-induced IgG may promote clearance of virus by opsonization or direct neutralization. Although a mechanism for neutralization of vaccinia virus is not known, it has been hypothesized to be caused by steric hindrance (35).

Despite the fact that immunization with fusion proteins generated greater antigen-specific IgG responses to poxvirus antigens than the separate proteins, three immunizations were still required for all of the mice to survive vaccinia virus challenge. The finding that three immunizations results in a higher level of vaccinia virus neutralizing activity than two immunizations raises the possibility that elevation of the neutralizing titer following a third immunization is a key event. Fogg *et al.* (18) also observed an increase in neutralizing titer between two and three immunizations. Our neutralization data may provide a guide to the minimal levels of neutralizing IgG required for survival. With average neutralization titers of ~300, some mice survive infection while others succumb. Mice that had mean neutralization titers ranging from 900–1300 all survived and showed few to no disease symptoms. However, mice in this range did lose weight at the peak of the infection, but this weight loss was reversible. When mice were immunized with sublethal doses of vaccinia virus the average neutralization titer was ~2700. These mice did not lose weight when challenged with high doses of vaccinia virus, thus exhibiting maximal protection. Thus it appears that a neutralizing titer between approximately 300 and 900 is required for survival, whereas a titer above 1300 is required for protection against any significant weight loss.

Taken together, our results establish that flagellin can promote robust responses against weakly immunogenic poxvirus antigens. Although weight loss is observed, the immunized mice do not exhibit other signs of disease and survival challenge. It may be that the overall effectiveness of a flagellin/poxvirus fusion protein vaccine may be enhanced by inclusion of additional target antigen/flagellin fusion proteins.

Acknowledgments

This study was supported by National Institutes of Health grant P01 AI060642 (S.B.M.). We would like to acknowledge J.W. Hooper of USAMRIID, Ft. Detrick, MD, for plasmids containing the A33R, B5R, and L1R genes, J.C. Whitbeck of the Schools of Dental and Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, for baculoviruses expressing the ectodomains of L1R and B5R, and Drs. John Bates and Eric Weimer, as well as Aaron Graff for technical assistance.

Author Disclosure Statement

No competing financial interests exist.

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Received December 9, 2009; accepted January 7, 2010.