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Protective Immunity to Vaccinia Virus Induced by Vaccination with Multiple Recombinant Outer Membrane Proteins of Intracellular and Extracellular Virions

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Infectious intracellular and extracellular forms of vaccinia virus have different outer membrane proteins, presenting multiple targets to the immune system. We investigated the immunogenicity of soluble forms of L1, an outer membrane protein of the intracellular mature virus, and of A33 and B5, outer membrane proteins of the extracellular enveloped virus. The recombinant proteins, in 10-μg amounts mixed with a Ribi- or saponin-type adjuvant, were administered subcutaneously to mice. Antibody titers to each protein rose sharply after the first and second boosts, reaching levels that surpassed those induced by percutaneous immunization with live vaccinia virus. Immunoglobulin G1 (IgG1) antibody predominated after the protein immunizations, indicative of a T-helper cell type 2 response, whereas live vaccinia virus induced mainly IgG2a, indicative of a T-helper cell type 1 response. Mice immunized with any one of the recombinant proteins survived an intranasal challenge with 5 times the 50% lethal dose of the pathogenic WR strain of vaccinia virus. Measurements of weight loss indicated that the A33 immunization most effectively prevented disease. The superiority of protein combinations was demonstrated when the challenge virus dose was increased 20-fold. The best protection was obtained with a vaccine made by combining recombinant proteins of the outer membranes of intracellular and extracellular virus. Indeed, mice immunized with A33 plus B5 plus L1 or with A33 plus L1 were better protected than mice immunized with live vaccinia virus. Three immunizations with the three-protein combination were necessary and sufficient for complete protection. These studies suggest the feasibility of a multiprotein smallpox vaccine.

Poxviruses comprise a large family of DNA viruses that infect vertebrates and invertebrates. The *Orthopoxvirus* genus includes about a dozen closely related species, of which variola virus, the causative agent of smallpox, and vaccinia virus, the live vaccine used to prevent smallpox, are best known (26). Interest in orthopoxviruses has increased because of concern that smallpox virus, monkeypox virus, or engineered forms of these viruses could be used as biological weapons (14). Although, the licensed smallpox vaccine provides excellent protection, it routinely causes a pustular skin lesion, frequently induces lymphadenopathy and fever, and occasionally results in life-threatening disease (12). Moreover, vaccination is not recommended for the millions of people and their contacts with immune deficiencies, eczema, atopic dermatitis, or heart disease, who are at increased risk of severe complications. A new vaccine comprised of live vaccinia virus prepared by modern tissue culture methods will probably be protective, but the safety profile may not be improved. Although there is a need for safer vaccines, it will be difficult to evaluate their efficacy in the absence of human smallpox or information regarding the correlates of immunity. Advances in immunology and understanding of poxvirus replication and spread, however, can facilitate the design and testing of new types of smallpox vaccines, such as those based on a highly attenuated vaccinia virus (9), recombinant DNA (17), and recombinant proteins (13).

Infectious intracellular mature virions (IMV), containing a complex core structure and an outer membrane with nonglycosylated viral proteins, are assembled in factory regions within the cytoplasm of vaccinia virus-infected cells. Some IMV migrate out of the factories, become wrapped with an additional double membrane containing viral glycoproteins, and are then transported on microtubules to the periphery of the cell (27, 34). The outer of the two added membranes fuses with the plasma membrane during exocytosis, and the resulting extracellular particles consist of an IMV surrounded by one extra fragile membrane. The majority of extracellular particles, called cell-associated enveloped virions, remain adherent to the cell surface, and some are located at the tips of long microvilli (4, 35). The number of enveloped virions that detach from the cells is virus strain and cell dependent (5, 30). The cell-associated and released extracellular virions (EV) are thought to be largely responsible for direct cell-to-cell and long-range virus spread within a host, respectively (4, 31). Because they have similar or identical outer membranes, we refer to both forms of extracellular virions as EV. After cell lysis, the very stable and abundant IMV may also mediate spread within and between hosts.

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Both IMV and EV are infectious, but they contain different viral outer membrane proteins, bind to cells differently and have different requirements for entry (38). Although the entry process is not well understood, a model consistent with available data is that IMV fuse directly with plasma membrane, whereas EV entry involves endocytosis, low-pH-induced disruption of the outer membrane, and fusion of the exposed IMV with the endosomal membrane. Recent findings that the A28 IMV membrane protein is required for entry of IMV, EV-mediated virus spread, and low-pH-induced fusion provide evidence for a common IMV-mediated fusion step (33). The initial association of IMV with the cell occurs through glycosoaminoglycan binding of the A27, D8, and H3 proteins (7, 18, 24), but no specific cell receptor protein has been identified. The association of EV with the cell surface may be mediated in part through a lectin-binding site of the A34 protein (5).

Several IMV proteins are targets of antibody neutralization in vitro. These include A27 (32), L1 (20, 39), H3 (24), and D8 (18). Mice immunized with recombinant A27 are protected from lethal intraperitoneal infection with vaccinia virus (22). In vitro neutralization of EV has been difficult to demonstrate, with varying results (19, 23, 37). Galmiche et al. (13), however, demonstrated that antibody to B5 neutralizes EV in vitro and that vaccination with recombinant B5 protects mice against intranasal (i.n.) challenge with vaccinia virus. They also found that vaccination with A33 protects in vivo, even though A33 does not appear to be a target of antibody neutralization of EV in vitro (13). Hooper and coworkers (15, 16) showed that DNA immunizations with multiple plasmids encoding L1, A27, B5, and A33 protect mice against intraperitoneal challenge with vaccinia virus better than immunization with any individual plasmid, although the roles of antibody- and cell-mediated immunity were not distinguished.

We engineered soluble forms of several vaccinia virus IMV and EV membrane proteins (unpublished data) to learn more about immunity to poxviruses and to test the proteins as components of a vaccine. In the present study, we immunized mice with recombinant L1, A33, and B5 proteins individually or in combinations and then challenged the mice by the i.n. route with lethal doses of the pathogenic WR strain of vaccinia virus (VV-WR). Under severe challenge conditions, individual IMV and EV proteins protected partially, whereas combinations of IMV and EV proteins protected completely.

(Portions of this work were done to partially fulfill the Ph.D. thesis requirements of C.F. at the University of Maryland.)

MATERIALS AND METHODS

Viruses and cells. HeLa S3 (ATCC CCL-2.2) suspension cells and BS-C-1 (ATCC CCL-26) monolayer cells were maintained in modified Eagle medium for spinner cells (Quality Biologics, Gaithersburg, Md.) supplemented with 5% equine serum (HyClone, Logan, Utah) and in Earle's modified Eagle medium (Quality Biologics) supplemented with 10% fetal bovine serum (FBS) (HyClone), respectively. The vaccinia virus strains used included VV-WR (ATCC VR-1354), IHD-J (from S. Dales, Rockefeller University), recombinant VV-WR-NP-siinfekl-EGFP (1, 29), and Wyeth smallpox vaccine seed (Wyeth Ayerst Laboratories, Marietta, Pa.). Stocks of viruses were grown in suspensions of HeLa S3 cells and purified by sucrose gradient centrifugation (10). Virus titers were determined by plaque assay in BS-C-1 monolayers.

Recombinant proteins. Soluble forms of L1, A33, and B5 were purified by affinity chromatography from the medium of baculovirus-infected insect cells (to be described elsewhere). Protein concentrations were determined by using a bicinchoninic acid assay (Pierce) with bovine serum albumin as a standard. The purity of each protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 12% NuPage gel and 3-(*N*-morpholino)propanesulfonic acid–4-morpholinepropanesulfonic acid running buffer (Invitrogen, Carlsbad, Calif.), followed by staining with GelCode Blue (Pierce, Rockford, Ill.) and by transferring the proteins to NitroPure-supported nitrocellulose (Osmonics, Westborough, Mass.), followed by Western blotting with specific monoclonal antibodies. The same proteins were used for immunization and for antibody binding assays.

Immunization protocols. Female 5- to 6-week-old BALB/c mice were purchased from Taconic (Germantown, N.Y.). Recombinant proteins (10 μg) were diluted in phosphate-buffered saline (PBS) with a Ribi adjuvant system consisting of monophosphoryl-lipid A plus trehalose dicorynomycolate emulsion (MPL+TDM) (Sigma-Aldrich, St. Louis, Mo.) or the saponin adjuvant QS-21 (Antigenics, Inc., New York, N.Y.) for a total injection volume of 0.1 ml. MPL+TDM was prepared immediately before each immunization according to manufacturer's instructions. Aliquots of QS-21 (2 mg/ml in water) were stored at −20°C, and 15 μg was used for each immunization. Proteins were administered subcutaneously at 3-week intervals. Blood was collected from the tail vein 1 day prior to each immunization, and serum was isolated from clotted blood samples by centrifugation. Pools of serum were prepared from each group of mice and heat inactivated for 30 min at 56°C to destroy complement activity.

For live virus immunization, the Wyeth vaccine strain of vaccinia virus (VV-Wyeth) was purified by sucrose gradient centrifugation and diluted with PBS to a titer of 10^9 PFU per ml, and aliquots stored at −80°C. Virus (10 μl) was deposited at the base of the tail, and the skin at the site of the droplet was scarified 25 to 30 times with a 25-gauge needle. After 3 to 4 days, pustules or scabs were observed at the scarification site, indicating a localized vaccinia virus infection.

Vaccinia virus-infected cell lysates. An infected cell lysate for use in antibody binding assays was prepared by inoculating two T-150 flasks of HeLa S3 cell monolayers with VV-WR at a multiplicity of 2. After 3 days, infected cells were harvested and collected by centrifugation at 208 × *g* for 5 min. The cell pellets were resuspended in 10 mM Tris-HCl (pH 8), followed by Dounce homogenization. The homogenates were centrifuged at 208 × *g* for 5 min in order to pellet nuclei, and the supernatants were centrifuged at 112,845 × *g* for 30 min. The pellets were resuspended in 10 mM Tris-HCl (pH 8), and aliquots were stored at −70°C.

ELISA. Polystyrene plates with 96-well round bottoms (Corning, Inc., Acton, Mass.) were coated by incubating them overnight at 4°C with 0.1 ml of a recombinant protein in PBS or at 37°C with a 1:1,000 dilution of infected cell lysate in Universal Plate Coating Buffer (Immunochemistry Technologies, Bloomington, Minn.). The empirically determined optimal amounts of A33, B5, L1, were 90, 100, and 40 ng, respectively. The plates were fixed with 2% paraformaldehyde and washed in a solution of 27 g of NaCl and 3 ml of Tween 20 in 3 liters of deionized water. Plates were incubated for 1 h at 37°C with blocking buffer (5% nonfat dry milk in PBS with 0.2% Tween 20) and washed with additional blocking buffer. Mouse sera were serially diluted in blocking buffer, 0.1 ml was added to each well, and the plates were incubated for 1 h at 37°C and then washed. Anti-mouse immunoglobulin G (IgG) (γ chain) peroxidase-conjugated antibody (Roche Diagnostics GmbH, Mannheim, Germany), diluted 1:5,000 in blocking buffer, was added, and the plates were incubated for 1 h at 37°C. For an isotype-specific enzyme-linked immunosorbent assay (ELISA), horseradish peroxidase-conjugated rat anti-mouse IgG1 or IgG2a (BD PharMingen, San Diego, Calif.) was used at a dilution of 1:1,000. A ready-to-use solution of soluble 3,3′,5,5′-tetramethylbenzidine (BM Blue, POD substrate; Roche Diagnostics) was added to the plates, which were incubated at room temperature for 30 min. The A_{370} and A_{492} were measured with a Spectra MAX Plus plate reader (Molecular Devices, Sunnyvale, Calif.), and the endpoint titer was calculated as the serum dilution resulting in an absorbance greater than 2 standard deviations above the absorbance in wells not incubated with mouse serum.

Neutralization assay and comet reduction test. IMV neutralization titers were determined by incubating serum dilutions in Eagle spinner medium with 2% FBS with a recombinant vaccinia virus (vv-WR-NP-siinfekl-EGFP) which expresses green fluorescent protein and then determining the percentage of infected cells by flow cytometry as described previously (8). The comet reduction test was carried out by infecting confluent BS-C-1 cells in 12-well plates (Costar; Corning, Acton, Mass.) with vaccinia virus IHD-J, which was diluted in Earle's modified Eagle medium with 2% FBS to give approximately 40 plaques per well. After 2 h at 37°C, the inoculum was removed and the cells were washed. Mouse serum diluted in Earle's modified Eagle medium with 2% FBS was added to duplicate wells, and the plates were incubated for 48 h at 37°C and then stained with crystal violet.

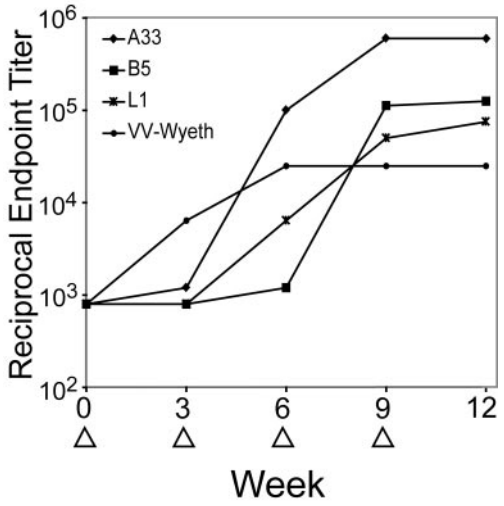

FIG. 1. Temporal induction of binding antibodies. Groups of 12 to 14 mice were inoculated subcutaneously four times with 10 μg of purified recombinant A33, B5, or L1 with MPL+TDM adjuvant or once at zero time with 10^7 PFU of VV-Wyeth by skin scarification. Mice were bled before the first immunization and 3 weeks after each subsequent protein immunization. Mice inoculated with VV-Wyeth were bled before and at 3-week intervals after the single vaccination. Reciprocal end point ELISA titers of pooled sera were determined by using the corresponding recombinant protein as the antigen for the protein immunization groups or a lysate of vaccinia virus-infected cells for the VV-Wyeth group. △, day of immunization.

Vaccinia virus challenge. One day prior to challenge, serum samples were collected and mice were weighed. On the day of challenge, an aliquot of purified VV-WR was thawed, sonicated, and diluted in PBS. Mice were anesthetized by inhalation of isoflurane and inoculated by the i.n. route with a 20-μl suspension of 1×10^6 or 2×10^7 PFU of VV-WR. We determined that the low and high doses of virus correspond to 5 and 100 50% lethal doses (LD_{50}), respectively, for 23-week-old mice. Mice were weighed daily for 2 weeks following challenge and were euthanized when they lost 30% of their initial body weight.

Statistical analysis. Mouse weight data were analyzed with the StatView statistical software package (SAS Institute Inc., Cary, N.C.). Analysis of variance was used to determine the effect of different immunization protocols on weight change resulting from virus challenge. The Fisher protected least-significance-difference test was used to compare pairs of immunization protocols, and significance levels were set at a P value of 0.05.

RESULTS

Antibody responses to recombinant IMV and EV proteins. Secreted, polyhistidine-tagged forms of the A33 and B5 EV proteins and the L1 IMV protein were synthesized in baculovirus-infected insect cells and purified by affinity chromatography (to be described elsewhere). The purity of each protein lot was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie blue staining, as well as by Western blotting with specific antisera (not shown). To assess immunogenicity, mice were inoculated subcutaneously at 3-week intervals with each protein in MPL+TDM adjuvant. Endpoint ELISA titers of the pooled sera were determined by using plates coated with individual recombinant proteins. In each case, antibody was undetectable or minimal after the first inoculation, rose sharply after the second or third inoculation, and increased slightly or not all after the fourth inoculation (Fig. 1). After the fourth inoculation, we also determined the ELISA titers of sera from seven or eight individual mice and found that the majority were within 2- to 4-fold of each other (data not shown). As seen in Table 1, sera from mice immunized with any one of the three proteins exhibited no measurable cross-reactivity with the other proteins, indicating the absence of significant amounts of contaminating baculovirus or insect cell antigens. Additional groups of mice were immunized with combinations of recombinant proteins. Their sera exhibited specific reactivity for each component of the mixture, although there were small decreases in ELISA titers compared to those induced by single-protein vaccines (Table 1). The serum antibodies from mice inoculated with recombinant proteins also reacted with a lysate of vaccinia virus-infected cells (Table 1). However, the ELISA titers obtained on plates coated with infected cell lysate were lower than those on plates coated with individual recombinant proteins, presumably because of differences in the amounts of individual viral proteins or their accessibility to antibody. A33 was the most immunogenic protein, as sera from mice immunized with this recombinant protein had the highest end point ELISA titer whether the plates were coated with recombinant A33 or vaccinia virus-infected cell lysate.

TABLE 1. End point ELISA titers

Immunization	Titer[a] with the following ELISA plate antigen:				IC_{50}[b]
	A33	B5	L1	Lysate	
VV-Wyeth	12,800	3,200	12,800	25,000	6,684
A33	600,000	<200	<200	150,000	<100
B5	<200	125,000	<200	2,400	<100
L1	<200	<200	75,000	6,400	13,890
A33 + B5	400,000	62,500	<200	100,000	<100
A33 + L1	400,000	<200	75,000	100,000	9,765
B5 + L1	<200	75,000	75,000	6,400	6,927
A33 + B5 + L1	400,000	50,000	100,000	87,500	10,992

[a] Results are the averages from two independent experiments, each of which contained 10 to 12 mice per group.
[b] IC_{50}, dilution of serum that reduces plaque number by 50%.

We also determined the ELISA titers of sera from mice that were scarified with 10^7 PFU of live VV-Wyeth. This amount, which was 40-fold higher than the standard human dose, was chosen in order to ensure a reproducibly good skin infection. Antibody to the infected cell lysate was detected at 3 weeks, increased by 6 weeks, and was sustained for at least 12 weeks (Fig. 1). Sera from these mice reacted with A33, B5, and L1, but the ELISA titers were lower than those of sera from mice immunized with the recombinant proteins (Table 1).

IgG isotypes of antibodies. The isotype of IgG can vary with the type of immunogen as well as the adjuvant. In general, IgG2a is associated with a TH1-polarized response induced by virus infection, and IgG1 is associated with a TH2 response induced by immunization with proteins. We used a specific ELISA to compare the IgG isotypes induced by recombinant L1, A33, and B5 with that induced by live vaccinia virus in two independent experiments. IgG1 was the predominant antibody isotype induced by each of the recombinant proteins with MPL+TDM adjuvant (Fig. 2A), indicating a TH2-dominated response. Because studies suggested that QS-21 enhances TH1 responses (25, 28), some animals received recombinant pro-

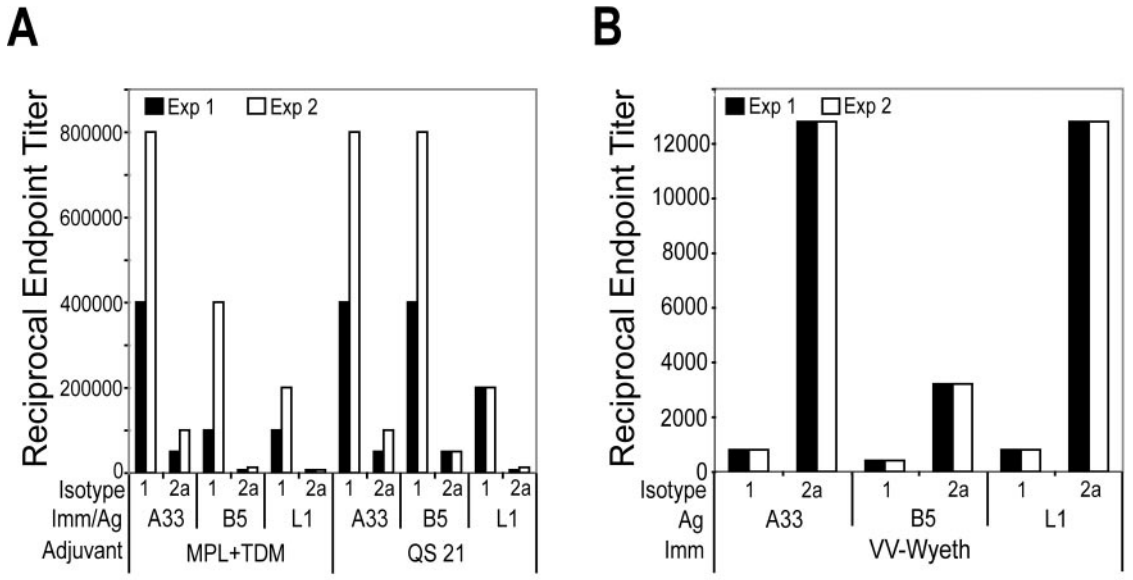

FIG. 2. Isotype-specific IgG responses. (A) Mice were immunized four times as described in the legend to Fig. 1 with recombinant proteins combined with MPL+TDM or QS 21 adjuvant. The sera were collected after the fourth immunization, pooled, and analyzed by using an isotype-specific ELISA. The reciprocal titers shown are from two independent experiments (Exp 1 and Exp 2). In each experiment, sera were pooled from 12 to 14 mice receiving MPL+TDM and from 2 mice receiving QS21. (B) Similar to panel A, except that mice were immunized with vaccinia virus strain VV-Wyeth. Imm, immunization; Ag, antigen.

teins with that adjuvant. Although the ELISA titer to B5 was higher with QS-21 than with MPL+TDM, the antibody to each protein was still predominantly IgG1 (Fig. 2A). In contrast to the IgG1 antibody specificity induced by recombinant protein immunization, live vaccinia virus induced predominantly IgG2a antibodies to A33, B5, and L1 (Fig. 2B), indicating a TH1-dominated immune response.

Neutralization activities of antibodies induced by recombinant proteins. Neutralization of IMV is traditionally measured by a plaque reduction or end point cytopathic effect assay. Our initial experiments indicated that recombinant L1 induced antibodies that neutralized purified IMV, as determined by a reduction in plaque number. These results were confirmed by a quantitative flow cytometric assay, which measures a reduction in the number of cells infected by a recombinant vaccinia virus that expresses green fluorescent protein (8). The IMV neutralization titer induced by L1 increased after the second and third immunizations (Fig. 3A), paralleling that of the ELISA titers (Fig. 1), and was reduced twofold or less by coadministration of the EV proteins A33 and B5 (Fig. 2A; Table 1). The neutralization titer induced by live vaccinia virus immunization, which is presumably caused by antibodies to multiple proteins, was detectable at 3 weeks and remained constant from week 6 to at least week 12 (Fig. 3A). As expected, sera from animals immunized with the EV proteins, A33 and B5, had no neutralizing activity against IMV (Table 1).

Even hyperimmune antiserum has little effect on the direct cell-to-cell spread of vaccinia virus. However, antiserum can prevent the formation of satellite plaques formed following the release of EV into the medium. Because the satellite plaques give the appearance of comets, the antibody inhibition assay is known as the comet reduction test. The cells were infected with the IHD-J strain of vaccinia virus, which releases large amounts of extracellular particles due to a mutation in the gene encoding the A34 EV protein (5), and then antiserum was added to the liquid overlying the cell monolayer. After 48 h, the monolayers were stained in order to visualize the comet-shaped satellite plaques. In the presence of preimmune serum, virus spread by satellite plaques almost obliterated the cell monolayer (Fig. 3B). In contrast, sera from mice immunized with A33 and VV-Wyeth reduced the comet size, though not the parent plaques which form by direct cell-to-cell spread (Fig. 3B). Anti-B5 mouse serum reduced comet size only slightly compared to A33 antiserum (not shown), with the difference perhaps being due to the lower levels of antibody as measured by ELISA (Table 1). As expected, the anti-L1 serum, which reacts with IMV, had no effect on comets formed by EV (data not shown).

Resistance of mice immunized with individual recombinant proteins to an i.n. challenge with 5 LD_{50} of VV-WR. Groups of mice were immunized four times at 3-week intervals with 10 μg of L1, A33, or B5 in MPL+TDM adjuvant. Another group was inoculated once with 10^7 PFU of VV-Wyeth given by skin scratch at the start of the experiment. Three weeks after the last immunization with recombinant protein or 12 weeks after immunization with VV-Wyeth, the mice were challenged by i.n. administration of 10^6 PFU of VV-WR. To evaluate protection, the mice were weighed daily for 2 weeks and individuals were euthanized when their weight loss reached 30% of the starting weight. Similar results were obtained in two independent experiments, each with six mice per group, and the data were averaged and are plotted in Fig. 4. Eight of 12 mice in the unimmunized group died or were sacrificed by day 10,

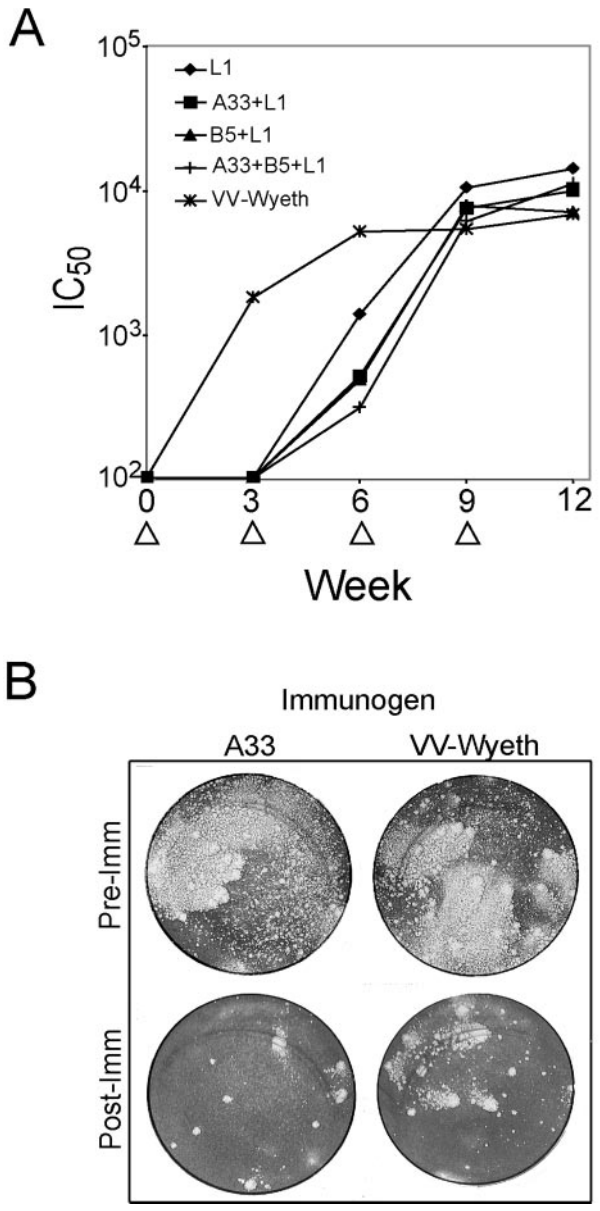

FIG. 3. Induction of neutralizing antibodies. (A) Groups of 8 to 14 mice were inoculated with L1, with L1 plus A33 or B5, or with all three proteins as described in the legend to Fig. 1. In addition, one group received 10^7 PFU of vaccinia virus strain VV-Wyeth by skin scarification. The neutralization of IMV was determined by using a green fluorescent protein reporter assay and is plotted as the serum dilution which reduced the number of fluorescent cells by 50% (IC_{50}). △, day of immunization. (B) Comet inhibition test. BS-C-1 cells were infected with vaccinia virus strain IHD and then overlaid with liquid medium containing a 1:50 dilution of serum pooled from 12 mice prior to immunization (Pre-Imm) or after the fourth immunization with A33 or the single immunization with live VV-Wyeth (Post-Imm). After 2 days, the cell monolayers were stained with crystal violet to visualize the plaques.

whereas all mice immunized with L1, A33, or B5 survived, as did all mice inoculated with VV-Wyeth (Fig. 4). Differences in susceptibility to disease were noted by inspection of the weights of surviving mice. The average weight loss of mice that

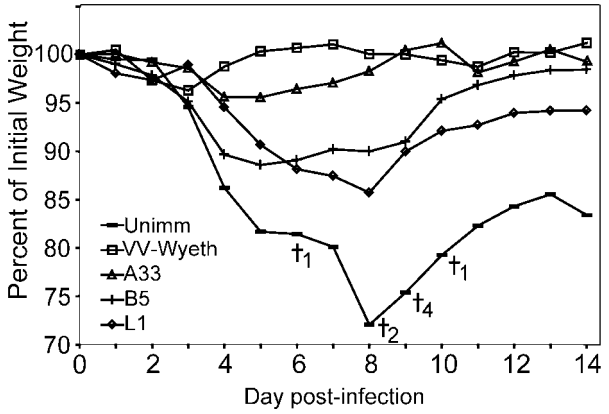

FIG. 4. Resistance of mice immunized with individual recombinant proteins to an i.n. challenge with 10^6 PFU of VV-WR. Mice were immunized four times with 10 μg of A33, B5, or L1 in MPL+TDM adjuvant or once with VV-Wyeth. Mice were challenged i.n. with 10^6 PFU of VV-WR at 3 weeks after the last administration of recombinant protein or 12 weeks after VV-Wyeth inoculation and were weighed daily. The percentage of initial weight was determined for each surviving mouse, and the averages from two experiments with a total of 12 mice were recorded. Unimm, unimmunized; †, number of mice that died on the specified day.

received A33 was less than 5%, providing the best protection of the three recombinant proteins. The difference in weight loss between mice receiving A33 and those receiving B5 or L1 was statistically significant on days 6 to 8 ($P = < 0.02$). There was no statistically significant difference in the protection afforded by A33 versus VV-Wyeth.

Resistance of mice immunized with single recombinant proteins or combinations of recombinant proteins to an i.n. challenge with 100 LD_{50} of VV-WR. We increased the VV-WR challenge dose in order to determine whether a combination of proteins afforded better protection than A33 alone. Groups of mice were immunized with 10 μg of A33, B5, or L1 individually or in all possible double and triple combinations and then were challenged with an i.n. dose of 2×10^7 PFU of VV-WR. Similar results were obtained in two independent experiments, each with 6 to 10 mice per group, and the data were averaged and are plotted in Fig. 5. Under these conditions, all unimmunized mice died within 9 days and 72% or 70% of those immunized with only L1 or B5 succumbed, respectively (Fig. 5A). When mice were immunized with both L1 and B5 or with A33 alone, fewer died (Fig. 5A). The combination of A33 with L1 or B5 further enhanced survival, and complete survival was obtained with the combination of all three proteins (Fig. 5A).

The high level of protection attained with combinations of proteins was also seen by analysis of weight loss of surviving mice (Fig. 5B). Animals immunized with A33 and L1 or with all three proteins had average weight losses of less than 10%, whereas those immunized with VV-Wyeth lost about 20% of their starting weights. The weight differences were statistically significant for days 4 to 6 ($P = < 0.008$ for A33 plus L1 versus VV-Wyeth; $P = < 0.001$ for A33 plus B5 plus L1 versus VV-Wyeth). We analyzed the sera from surviving mice and found that the infection did not increase the already high antibody titers to proteins used for immunization. However, we could now detect antibodies that bound to the recombinant

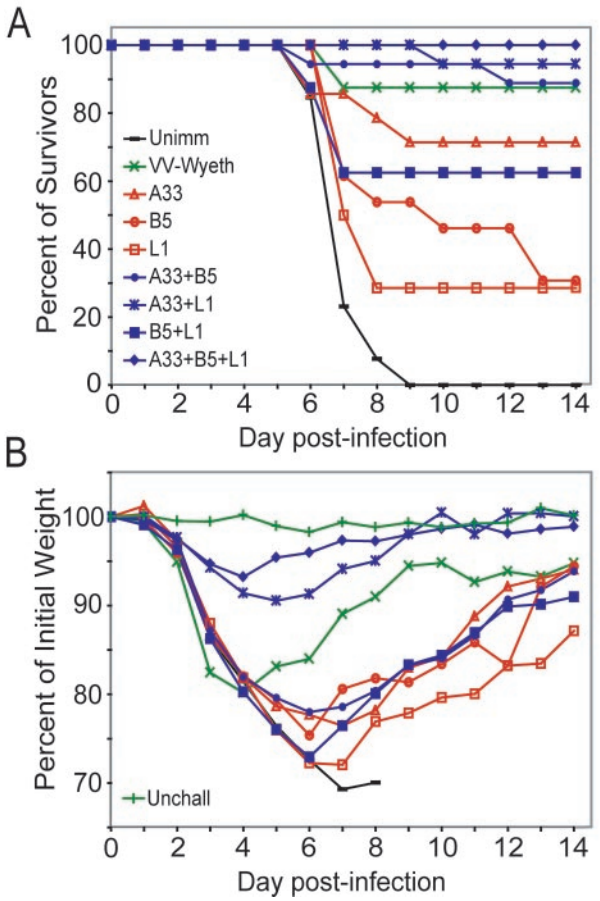

FIG. 5. Resistance of mice immunized with a single recombinant protein or combinations of recombinant proteins to an i.n. challenge with 2×10^7 PFU of VV-WR. Mice were immunized four times with 10 μg of an individual recombinant protein, with mixtures containing 10 μg of each recombinant protein, or with VV-Wyeth as described in the legend to Fig. 4. The i.n. challenge with VV-WR was similar to that described for Fig. 4 except that the dose was increased to 2×10^7 PFU. The percentages of surviving mice (A) and percentages of initial weight (B) are averages from two separate experiments with a total of 13 to 19 mice in each group. Unimm, unimmunized; Unchall, unchallenged.

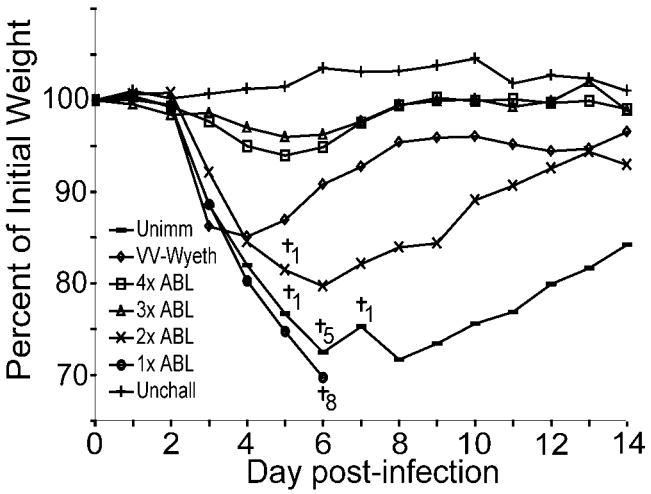

FIG. 6. Effect of number of immunizations on survival and weight loss following an i.n. challenge with 2×10^7 PFU of VV-WR. Groups of eight mice were inoculated with a combination of A33, B5, and L1 from one to four times as described in the legend to Fig. 5. The immunizations were staggered such that all mice received the final one on the same day, and the mice were challenged 3 weeks later. Unimm, unimmunized; Unchall, unchallenged; †, number of mice that died on the specified day; ABL, A33 plus B5 plus L1; 1× one immunization; 2×, two immunizations; 3×, three immunizations; 4×, four immunizations.

proteins not used for immunization. These titers were similar to the ones obtained following vaccination with VV-Wyeth (Table 1).

Number of immunizations needed for protection. In the previous experiments, challenges were carried out after four immunizations. To determine whether this number of immunizations was necessary for protection, groups of mice were inoculated one, two, three, or four times with the combination of L1, A33, and B5. The schedule was arranged so that the final immunizations were on the same day, and the challenge occurred 3 weeks later with 2×10^7 PFU of VV-WR. None of the eight mice immunized only once with recombinant proteins survived, seven of eight mice immunized twice survived, and all eight mice immunized three or four times survived. Analysis of weight loss indicated that mice immunized three or four times with recombinant proteins were equally well protected, whereas mice immunized twice with recombinant proteins or with VV-Wyeth exhibited greater weight loss (Fig. 6). The degree of protection correlated directly with the antibody responses induced by multiple inoculations of proteins (Fig. 1).

DISCUSSION

Previous studies demonstrated that inactivated vaccinia virus, consisting predominantly of IMV, elicited high neutralizing antibody titers but failed to solidly protect animals against vaccinia virus challenge (6, 36). The failure was attributed to the inability of the inactivated vaccine to elicit antibodies that neutralized EV (11). Here we showed that a recombinant multiprotein vaccine elicited antibodies to both IMV and EV forms of vaccinia virus. The recombinant proteins, when given three or more times in an appropriate adjuvant, induced higher titers of antibody than a live virus vaccination. Moreover, the recombinant proteins more completely protected mice against a lethal i.n. inoculation of a pathogenic vaccinia virus than did a live vaccine. IgG1 was the predominant isotype of binding antibody induced by immunization with each of the recombinant proteins with either MPL+TDM or QS21 adjuvant, which is indicative of a predominant TH2 response. In contrast, mice immunized with live vaccinia virus raised predominantly IgG2a antibodies, which are associated with a TH1-polarized response. We did not measure $CD8^+$-T-cell responses in the mice immunized with recombinant proteins, in view of the TH2 response and because such vaccines rarely induce high levels of cell-mediated immunity. While $CD8^+$ T cells are most important in protecting naive animals against orthopoxvirus infections (3, 21), antibodies are sufficient to protect immunized mice against a lethal i.n. challenge (2, 40). In naive mice, the relative importance of $CD8^+$ T cells is probably due to the

time needed to induce a primary antibody response. In contrast, previously immunized mice have preexisting antibodies as well as memory B cells, which can compensate for the absence of $CD8^+$ T cells.

Proper folding of the secreted recombinant proteins may have contributed to their good immunogenicity. The natural viral proteins each have a transmembrane domain, which anchors the protein in the IMV or EV membrane. For production of the recombinant proteins, the open reading frames were modified to remove the natural signal peptide and transmembrane domain and to introduce an insect signal peptide as well as a polyhistidine tag (to be described elsewhere). When insect cells were infected with each recombinant baculovirus, the proteins were secreted into the medium and purified by use of a mild affinity chromatography procedure. L1 normally has intramolecular disulfide bonds, and A33 forms a disulfide-bonded dimer; both of these properties were retained in the purified proteins (our unpublished data). Moreover, all three proteins reacted with monoclonal antibodies that provide passive protection (our unpublished data). A33, B5, and L1 are well conserved among orthopoxviruses, and the amino acid identities of the vaccinia and variola virus orthologs are 94, 93, and 99%, respectively. It seems likely, therefore, that the antibodies to the vaccinia virus proteins will show cross protection against variola virus and other orthopoxviruses. However, the authentic variola virus proteins could be produced by a similar procedure, should that be advantageous for the development of a multiprotein smallpox vaccine.

Our finding that single IMV and EV proteins provided partial protection but that combinations afforded complete protection is in good agreement with studies of Hooper and coworkers (15, 16), who used DNA vaccination and an intraperitoneal route of challenge. We prefer the mouse i.n. challenge model because it mimics the upper respiratory route of transmission of smallpox, has a lower LD_{50} than the intraperitoneal route, and has been widely used to study viral pathogenesis and to evaluate attenuated live vaccines (2, 40). Nevertheless, the differences between our experiments and those of Hooper et al. reinforce the conclusion that protective immunity is best achieved by vaccines that contain or express outer membrane proteins of both forms of the virus. It is interesting to speculate on the relative roles of antibodies to IMV and EV proteins in the mouse i.n. challenge model. The anti-L1 mouse serum neutralized IMV, and previous studies showed that a monoclonal antibody to L1 prevents IMV penetration into cells (20, 39). Since the mice were inoculated with the IMV form of vaccinia virus, as may occur during smallpox transmission, the antibodies to L1 could have a role in neutralizing the inoculum as well as progeny virus. On the other hand, antibodies to EV proteins would have no effect on the inoculum virus but could reduce spread of progeny virus. Galmiche and coworkers (13) previously found that polyclonal antibodies to B5 neutralize isolated EV in vitro, whereas anti-A33 antibodies have little neutralizing activity. Although we also found that anti-A33 polyclonal antibodies had little or no neutralizing activity (S. Lustig, unpublished data), the antisera from mice immunized with A33 prevented virus spread in the comet assay more potently than antisera from mice immunized with B5. Because the A33 antiserum had a higher binding titer than the B5 antiserum, we did not test the latter at a similar concentration, which could account for the difference. Alternatively, antibodies to A33 and B5 might neutralize EV by different mechanisms. In this regard, Vanderplasschen et al. (37) showed that antibodies to vaccinia virus aggregate progeny EV on the cell surface. Such aggregation could inhibit satellite plaque formation in the comet assay but might not play a role in the neutralization of dilute suspensions of EV. Multiple mechanisms of virus inhibition likely contributed to the excellent protection obtained by immunizing mice with the combination of A33, B5, and L1 proteins.

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