Disparity between Levels of In Vitro Neutralization of Vaccinia Virus by Antibody to the A27 Protein and Protection of Mice against Intranasal Challenge[▽]

Christiana N. Fogg,^{1,§†} Jeffrey L. Americo,^{1,§} Patricia L. Earl,¹ Wolfgang Resch,^{1,‡}
Lydia Aldaz-Carroll,² Roselyn J. Eisenberg,² Gary H. Cohen,² and Bernard Moss^{1*}

*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland,¹ and
Schools of Dental and Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania²*

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Immunization with recombinant proteins may provide a safer alternative to live vaccinia virus for prophylaxis of poxvirus infections. Although antibody protects against vaccinia virus infection, the mechanism is not understood and the selection of immunogens is daunting as there are dozens of surface proteins and two infectious forms known as the mature virion (MV) and the enveloped virion (EV). Our previous studies showed that mice immunized with soluble forms of EV membrane proteins A33 and B5 and MV membrane protein L1 or passively immunized with antibodies to these proteins survived an intranasal challenge with vaccinia virus. The present study compared MV protein A27, which has a role in virus attachment to glycosaminoglycans on the cell surface, to L1 with respect to immunogenicity and protection. Although mice developed similar levels of neutralizing antibody after immunizations with A27 or L1, A27-immunized mice exhibited more severe disease upon an intranasal challenge with vaccinia virus. In addition, mice immunized with A27 and A33 were not as well protected as mice receiving L1 and A33. Polyclonal rabbit anti-A27 and anti-L1 IgG had equivalent MV-neutralizing activities when measured by the prevention of infection of human or mouse cells or cells deficient in glycosaminoglycans or by adding antibody prior to or after virus adsorption. Nevertheless, the passive administration of antibody to A27 was poorly protective compared to the antibody to L1. These studies raise questions regarding the basis for antibody protection against poxvirus disease and highlight the importance of animal models for the early evaluation of vaccine candidates.

Smallpox was eradicated following widespread vaccination with live vaccinia virus (VACV) (21). However, most people born within the last three decades have not been vaccinated and consequently are susceptible to smallpox. Because serious side effects are associated with the smallpox vaccine (12), individuals or their close contacts who are immunocompromised or have a history of dermatitis or heart disease might be excluded from future vaccination should the need arise. Attenuated and nonreplicating strains of VACV are being clinically tested as alternative smallpox vaccines (33, 50, 68), but in some cases, high doses are required to achieve good immune responses and the possibility of adverse effects has not been entirely excluded. Additional genetically engineered strains of VACV (14, 62, 67) and recombinant DNA or viral proteins (referenced below) are still in preclinical testing.

Protection against secondary orthopoxvirus infections is largely antibody mediated (48). However, because poxviruses are large and complex, the selection of the best immunogens is a difficult

task. Two major infectious forms of VACV exist: the mature virus (MV) consists of a nucleoprotein core surrounded by a lipoprotein membrane, whereas the enveloped virus (EV) is essentially an MV enclosed by a second viral membrane (45). The intracellular wrapping of MVs facilitates cytoplasmic transport, exocytosis, and cell-to-cell spread (59). Nevertheless, the EV membrane is removed prior to entry, allowing the exposed MV membrane to fuse with the plasma membrane, or following endocytosis (46, 58, 63). Studies of animal models have demonstrated that protection against disease is associated with antibody responses to both viral forms (4, 10, 38, 65). More than 20 proteins are associated with the MV membrane and at least 6 with the EV-specific membrane (45). Mice immunized with recombinant forms of the EV proteins A33 or B5 or the MV protein L1 were partially protected against a lethal VACV challenge (22, 25). We also observed the superior protection of mice given a combination of these EV and MV proteins (22), as did Hooper and coworkers (29, 30) by DNA immunizations. Similarly, greater protection was observed in mice passively immunized with a combination of antibodies specific to A33, B5, and L1 than with individual ones (42). The additional MV proteins A27 (17, 36), H3 (16), and D8 (57) have also been reported to induce neutralizing antibody and provide protection against VACV challenge. The purpose of the present study was to compare the A27 protein to L1 for possible inclusion in a multicomponent recombinant protein vaccine. We found that A27 is immunogenic and that antibodies produced in mice and rabbits neutralize MVs. Nevertheless, active and passive immunization studies indicated that neutral-

* Corresponding author. Mailing address: 33 North Drive MSC3210, Building 33, Room 1E13C.1, NIAID, NIH, Bethesda, MD 20892-3210. Phone: (301) 496-9869. Fax: (301) 480-1535. E-mail: bmoss@nih.gov.

§ Contributed equally to the study.

† Present address: Laboratory of Malaria and Vector Research, NIAID, NIH, 12735 Twinbrook Parkway, Twinbrook 3, Rockville, MD 20852.

‡ Present address: National Center for Biotechnology Information, National Library of Medicine, NIH, 45 Center Drive, Bethesda, MD 20892-6510.

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izing antibody to A27 is less protective than antibody to L1 in the mouse intranasal (i.n.) challenge model.

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MATERIALS AND METHODS

Cells and viruses. BS-C-1 (ATCC CCL-26) monolayer cells were maintained in Earle's modified Eagle medium (EMEM) (Quality Biologicals, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine (Lonza, Walkersville, MD), 10 U/ml penicillin (Invitrogen Gibco, Gaithersburg, MD), and 10 µg/ml streptomycin (Invitrogen Gibco) at 37°C and 5% CO₂. Sog9 (5) and L cells were maintained in Dulbecco's modified Eagle medium (Quality Biologicals) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 U/ml penicillin, and 10 µg/ml streptomycin. HeLa S3 cells (ATCC CCL-2.2) were maintained at 37°C in EMEM for spinner cells containing 586 mg/liter L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Quality Biologicals) supplemented with 5% heat-inactivated equine serum (HyClone). The serum was heat-inactivated by incubation at 56°C for 30 min to destroy complement activity.

The VACV strains used were Western Reserve (WR) (ATCC VR-1354), International Health Department-J (IHD-J) (originally from S. Dales, Rockefeller University), and recombinant VV-WR-NP-siinfekl-EGFP (3, 47). Viral stocks were grown in HeLa S3 suspension cells and purified by sucrose gradient centrifugation (19). Virus titers were determined by a plaque assay in confluent BS-C-1 monolayers grown in six-well cluster plates.

Recombinant proteins. Recombinant proteins were synthesized in insect cells and purified from the medium by affinity chromatography as described previously (1, 22, 71).

ELISAs. Ninety-six-well plates were coated with protein diluted in phosphate-buffered saline (PBS) and incubated at 4°C overnight. The optimal concentration for each protein used to coat the plates, determined by preliminary antibody binding experiments, was 90 ng/well for A33, 150 ng/well for A27, and 40 ng/well for L1. Enzyme-linked immunosorbent assays (ELISAs) were carried out as previously described, and a horseradish peroxidase-conjugated anti-mouse antibody was used to detect mouse immunoglobulin G (IgG) bound to antigen followed by visualization with a chromogenic substrate of a ready-to-use solution of 3,3',5,5'-tetramethylbenzidine (BM Blue, POD substrate; Roche Applied Science, Indianapolis, IN) (22). Spectrophotometric measurements were made at A₃₇₀ and A₄₉₂, and reciprocal endpoint titers were determined as the dilution with an absorbance of 0.1 following subtraction of the background absorbance of serum samples incubated on plates not coated with protein.

MV neutralization and comet reduction assay. MV-neutralizing antibody was measured using a flow cytometric assay with an enhanced green fluorescent protein (EGFP)-expressing recombinant VACV (VV-NP-siinfekl-EGFP) (18). Briefly, purified virus was incubated with twofold serial dilutions of antibody in spinner medium (for HeLa cells) or EMEM (for Sog9 and L cells) with 2% fetal bovine serum at 37°C in a 96-well U-bottom plate. After 1 h, HeLa, L, or Sog9 cells were added, and the mixture was incubated in the presence of cytosine arabinoside for 16 to 18 h at the same temperature. The cells were then fixed with paraformaldehyde, and EGFP-expressing cells were determined by flow cytometry. The serum dilution that reduced the percentage of EGFP-expressing cells by 50% (IC₅₀) was determined by nonlinear regression using PRISM software (GraphPad Software, Inc., San Diego, CA). In a variation of this procedure, virus was added to HeLa cells and incubated at 4°C. After 1 h, the unbound virus was removed by centrifuging and resuspending the cells twice in cold spinner medium containing 2% fetal bovine serum. Serial twofold dilutions of antibody were then added, and the incubation was continued as described above.

Anti-EV antibodies that inhibit secondary satellite plaques in cell culture were observed with the comet reduction assay. BS-C-1 cell monolayers in six-well plates were infected with 80 PFU of VACV strain IHD-J and treated with a 1:50 dilution of serum. The plates were incubated for 40 h at 37°C, and comet-shaped plaques were visualized by the staining of monolayers with crystal violet.

Immunization of mice. Female BALB/c mice, 5 to 6 weeks old, were purchased from Taconic Biotechnology (Germantown, NY) and housed in sterile microisolators. Protein immunizations were carried out as previously described with monophosphoryl lipid A and synthetic trehalose dicorynomycolate adjuvant (MPL+TDM) (22). Three immunizations were given at 3-week intervals, and blood was collected from the tail vein before each immunization and challenge. Serum was separated by centrifugation from clotted blood, and pools were made

from mice immunized with the same proteins. The serum samples were heat-inactivated at 56°C for 30 min to destroy complement activity.

Virus challenge after protein immunizations. Three weeks following the third protein immunization, mice were challenged by the i.n. route with VACV WR. A fresh aliquot of sucrose gradient-purified virus was thawed for each challenge experiment and was diluted in sterile PBS to a final concentration of 1×10^6 or 2×10^7 PFU per 20 µl, corresponding to 50% lethal dose (LD₅₀) values of approximately 10 and 200 determined for 16-week-old female BALB/c mice. Sedated mice were inoculated i.n. with 20 µl of virus (10 µl/nostril) and weighed daily for 2 weeks. Mice were euthanized if their weight was below 70% of the initial value in accordance with a protocol approved by the NIH Animal Care and Use Committee.

Passive immunization and challenge. IgG from the sera of rabbits immunized multiple times with recombinant proteins A27 (R194) or L1 (R180) was purified using protein A Sepharose (Immobilized Protein A Plus; Pierce, Rockford, IL) and dialyzed against PBS. The concentration of IgG was determined from the absorbance at 280 nm, and the neutralizing activity was determined by flow cytometry as described above.

Groups of 7-week-old female BALB/c mice were inoculated intraperitoneally (i.p.) with 4.5 mg of R194 or R180 IgG, 5 mg of nonimmune rabbit IgG (Immunopure rabbit IgG whole molecule; Pierce, Rockford, IL), or 5 mg of vaccinia-immune globulin (VIG) (Cangene, Winnipeg). Nonimmunized controls were injected with the same volume of PBS. At 24 h after immunization, the mice were challenged i.n. with 5×10^4 PFU of VACV WR diluted in PBS containing 0.05% bovine serum albumin. This dose was equivalent to approximately two times the LD₅₀ for these young mice. The mice were weighed daily for 2 weeks and sacrificed if they lost 30% of their starting weights.

Statistical analysis. Weight-loss data were analyzed to determine the significance of differences between the mouse groups. The area under the curve corrected for the follow-up period was calculated for days 2 through 9 as a summary statistic with a trapezoidal rule using all available measurements (32). Area-under-the-curve values were compared between groups using a nonparametric Wilcoxon rank-sum test, adjusting *P* values according to the method described by Holm (28) in order to control the family-wise error rate in multiple tests. For the passive antibody experiments, differences in the weight loss of mice in the different groups were determined by analysis of variance using StatView software (SAS Institute, Inc., Cary, NC).

RESULTS

Characterization of the recombinant A27 protein. VACV A27 is composed of covalently linked trimers and hexamers of a 12.6-kDa protein that is present on the surface of MVs (66). We expressed a secreted form of the A27 protein with a six-histidine tail in insect cells infected with a recombinant baculovirus and purified it from the medium by nickel affinity chromatography (71). The denatured and reduced A27 protein migrated as two major and two minor bands that were detected by silver staining and Western blotting (Fig. 1A and B). Stable higher-molecular-weight oligomeric forms were present when the reducing agent was omitted (Fig. 1A and B). The two major forms may correspond to full-length and cleavage products, as previously detected for A27 made in *Escherichia coli* (35). The two minor bands represent glycosylated forms of the major species as they disappeared after peptide:*N*-glycosidase F digestion (Fig. 1B). Secreted recombinant forms of the VACV L1 and A33 proteins were described previously (1, 22).

Binding and neutralizing antibody responses following protein immunizations. Mice were immunized three times at 3-week intervals with 10 µg of affinity-purified A33, A27, or L1 or with 10 µg each of A33 and L1 or A33 and A27 with adjuvant. One day prior to each immunization or prior to the challenge, the mice were bled. ELISAs were carried out on the pooled serum samples with recombinant A27, A33, or L1 used as binding antigens; the average reciprocal endpoints from two independent experiments are shown in Fig. 2A. In each case,

FIG. 1. Characterization of the recombinant A27 protein. (A) The purified recombinant A27 protein (200 ng/lane) was heated at 100°C for 5 min in denaturing, reducing sample buffer (lane R; 60 mM Tris, 1% sodium dodecyl sulfate [SDS], 20 mM dithiothreitol, 10% glycerol) or for 5 min at room temperature in nonreducing sample buffer (lane N; 60 mM Tris, 0.1% SDS, 10% glycerol). The samples and standards were resolved by electrophoresis through a 10 to 20% Tris-glycine polyacrylamide gel and silver stained. The masses in kDa of the standard proteins are indicated next to their migration positions. (B) Undenatured A27 protein (1 μ g) was mock-digested (–) or digested (+) with 5 U peptide:N-glycosidase F (PNGase F) for 16 h at 25°C. Denaturation and SDS-polyacrylamide gel electrophoresis were as described for panel A, and A27 was detected by Western blotting using a murine MAb (Qiagen) that recognizes the six-histidine tag present at the C terminus of recombinant A27.

antibody titers were low after the first immunization but were boosted after the second and third. A33 induced a higher antibody response than L1 as previously found (22). Similarly, A27 was more immunogenic than L1. When the proteins were combined for immunization, the antibody levels to the individual components were only slightly decreased.

Neutralizing antibodies, measured using a flow cytometric assay with a recombinant VACV-expressing EGFP (18), were detected after two immunizations and further increased after a

FIG. 2. Induction of binding and neutralizing antibodies. (A) Mice ($n = 5$ /group) were subcutaneously immunized at 0, 3, and 6 weeks with 10 μ g of A27, A33, or L1 alone or with A33 plus L1 or A33 plus A27 (10 μ g of each protein) combined with MPL+TDM adjuvant and PBS. Mice were bled 1 day prior to each immunization or to the virus challenge (at 9 weeks), and serum pools were analyzed for binding antibodies to A33, A27, or L1. The reciprocal endpoint titers are the average endpoints measured from two independent mouse experiments; the endpoint titers did not vary more than two dilutions for corresponding groups in the two experiments. (B) Mice were immunized as described for panel A. Neutralizing antibodies in serum pools were measured with an EGFP-based flow cytometry assay, and titers represent the IC_{50} .

FIG. 3. Protection from the low-dose i.n. challenge following immunization with single proteins. Mice ($n = 10$ /group) were immunized three times at 3-week intervals with 10 μ g of A33, A27, or L1 and MPL+TDM adjuvant. An additional group was unimmunized (Unimm), and another was untreated (Untr) as a weight control. Three weeks following the third immunization, mice were challenged i.n. with 1×10^6 PFU of VACV WR except for the untreated group and were weighed daily for 2 weeks. Mice were sacrificed if their weight fell below 70% of their initial value. Only 3 out of 10 unimmunized mice survived after the 7th day postinfection and gained weight, while all immunized mice survived. The data shown is the average percentage of the initial weight for each group from two independent experiments. The bars represent standard errors of the means.

third (Fig. 2B). Similar titers were obtained from mice immunized with A27 or L1 alone and were slightly higher than the titers from mice also given A33. It should be noted that A33 is an EV protein and that antibodies to this protein do not neutralize MVs.

Comet reduction assays (22) were used to determine the anti-EV activity in sera pooled following the third immunization from two separate experiments. Sera from mice immunized with A33 alone or combined with A27 or L1 had reduced comet sizes to similar degrees (data not shown). As expected, no comet reduction was observed in sera from any groups prior to immunization or from mice immunized with either MV protein alone.

Protection of mice following i.n. challenge with VACV. The WR strain of VACV was chosen for animal experiments because of its pathogenicity for mice. The i.n. route was selected over the i.p. route partly because the lethal dose is about 1,000 times lower. Although there are two main infectious forms of VACV, there are several reasons why the MV is used routinely for challenge experiments: MVs can be obtained in much larger amounts than EVs; the outer EV membrane is fragile, causing preparations to be heterogeneous; and owing to the instability of EVs, MVs may be most important in aerosol spread between animals. Previous studies have shown that weight loss, which occurs during the first week, is directly correlated with virus replication and provides an objective, noninvasive way of following disease (38, 41). Three weeks after the third immunization, the mice were challenged with 1×10^6 PFU of VACV WR. The control groups consisted of unimmunized mice that were challenged with virus or remained unchallenged. The mice were weighed daily for 2 weeks and were sacrificed if their weight fell below 70% of the initial value. The average percentage of initial weight for each treatment group combined from two independent experiments is shown in Fig. 3. Three out of 10 unimmunized mice survived beyond the 7th day after the challenge, but all protein-immunized mice survived the challenge. Although the mice immunized with A27 survived, the weight loss was close to that of the few surviving unimmunized mice. In contrast, the mice immu-

FIG. 4. Protection from the high-dose i.n. challenge following immunization with combinations of proteins. Mice ($n = 10$ mice/group) were immunized three times at 3-week intervals with $10 \mu\text{g}$ of A33, A27, or L1 or $10 \mu\text{g}$ of each component of the combinations A33 plus A27 or A33 plus L1 and MPL+TDM adjuvant. An additional group was unimmunized (Unim), and another was untreated (Untr). Three weeks after the third immunization, the mice were challenged i.n. with 2×10^7 PFU of VACV WR except for the untreated group and were weighed daily for 2 weeks. Mice were sacrificed if their weight fell below 70% of their initial value. (A) Percentage of survivors each day after the challenge; (B) average percentage of initial weight following challenge. The data shown above was calculated from the averages of two independent experiments. Bars represent standard errors of the means.

nized with L1 lost less weight than the A27-immunized mice ($P = 0.015$), similarly to the mice immunized with A33.

Previous studies showed that the combination of L1 and A33 provided more robust protection than either protein alone (22). We therefore wanted to see whether A27 could enhance the protection achieved with A33. Mice were immunized with A27 and A33 or L1 and A33, as well as with the individual proteins. Three weeks after the third immunization, the mice were challenged i.n. with 2×10^7 PFU rather than the 1×10^6 PFU dose used in the above experiment. This challenge dose, which is highly lethal for animals immunized with a single protein, is suited to discriminate between the efficacies of protein combinations. All of the mice immunized with A27 alone and all but one of the nonimmunized mice died or were sacrificed due to more than 30% weight loss (Fig. 4A). In addition, 80% of those immunized with L1 alone and 50% of those immunized with A33 alone died. In contrast, all of the mice receiving L1 plus A33 survived, as did most receiving A27 plus A33. Nevertheless, the weight-loss curves revealed more severe disease in mice immunized with A27 plus A33 than those immunized with L1 plus A33 (Fig. 4B).

Protection of mice passively immunized with antibodies to A27 or L1. The protective effects of protein immunizations are usually ascribed to antibody rather than to cellular effectors. Passive immunization experiments were carried out to confirm that the superiority of L1 over A27 was due to antibodies and

FIG. 5. Neutralizing activity of anti-L1 and anti-A27 IgG. Neutralization titers of IgG from rabbits R180 and R194, immunized with recombinant L1 and A27, respectively, were determined by the flow cytometric EGFP assay with HeLa cells.

not to effector T cells. Because of the difficulty in obtaining sufficient amounts of the mouse antibodies for the passive transfer experiments, IgG was purified from sera collected from rabbits that had been repeatedly immunized with A27 (R194 serum) or L1 (R180 serum). A previous study had shown that the injection of 4 to 5 mg of rabbit IgG to mice gives serum levels of 0.87 to 1.2 mg/ml with a half-life of 5 to 7 days with no evidence of immune elimination over a 2-week period (15). Using the EGFP reporter gene VACV neutralization assay, we found that the potencies of the rabbit anti-A27 IgG, rabbit anti-L1 IgG, and human VIG were similar with IC_{50} values of 834, 1,590, and 1,052 ng/ml, respectively, with no evidence of a neutralization-resistant virus fraction (Fig. 5). A virus dilution assay indicated that the relative IC_{50} values did not reflect artifacts associated with virus aggregation rather than neutralization (2).

Seven-week-old mice were injected i.p. with IgG from non-immune rabbits or rabbits immunized with A27 or L1. VIG was used as a positive control. After 24 h, the mice were challenged i.n. with 5×10^4 PFU of VACV WR, determined to be twice the LD_{50} for this young age group. All mice given nonimmune or anti-A27 IgG died between days 8 and 10 after the challenge, whereas 40% of the mice given anti-L1 IgG and 80% of those given VIG survived (Fig. 6A). This pattern was reflected in the steeper rates of weight loss of mice that had received anti-A27 IgG compared to those that received anti-L1 IgG (Fig. 6B). The difference in weight loss between the anti-A27 and anti-L1 groups was statistically significant on days 4 to 7 ($P \leq 0.01$). Therefore, while the anti-A27 IgG was slightly more potent than the anti-L1 IgG for in vitro neutralization of VACV, it did not protect against VACV challenge in vivo. This outcome is consistent with the result obtained when mice were immunized with A27 or L1 and made their own antibodies. The superiority of VIG in this experiment can be attributed to the presence of additional antibodies to EV proteins. Our previous studies show that a combination of antibodies to L1, A33, and B5 provides better protection than VIG (42). Importantly, the disparity between the neutralization titers of rabbit anti-A27 and -L1 and their protective activity was consistent with the results obtained by the active immunization of mice with A27 and L1 proteins.

Further characterization of the IgG-neutralizing activities. Additional neutralization assays were carried out in an effort to

FIG. 6. Passive immunization of mice. Seven-week-old mice ($n = 5$ /group) were inoculated i.p. with 4.5 mg of anti-A27 (R194) or anti-L1 (R180) IgG, 5 mg of VIG, or 5 mg of nonimmune rabbit IgG (Unimm IgG). At 24 h after immunization, the mice were challenged i.n. with 5×10^4 PFU of VACV WR except for the untreated (Untr) group. Mice were weighed daily for 2 weeks and sacrificed if their weight diminished to 70% of their starting weight. (A) Percentages of survivors; (B) average weight loss.

explore the striking difference between in vitro neutralization and protection. The neutralization assay, for which the results are depicted in Fig. 5, was performed with human HeLa cells. Although there was no precedent, we considered that the A27 antibodies might not prevent the infection of mouse cells as well as human cells. However, the neutralizing activity of the anti-A27 IgG appeared to be greater than that of the anti-L1 IgG in mouse L cells (Fig. 7A). A27 is known to contribute to the binding of VACV to heparin glycosaminoglycans (GAGs) on the surfaces of cells (13). Therefore, we contemplated the possibility that the anti-A27 antibodies might only prevent the infection of cells with GAGs and that cells lacking GAGs might remain susceptible and enable the spread of VACV in animals. Therefore, we compared the neutralizing activities of the antibodies in mouse Sog9 cells, which are deficient in GAGs (5). However, even in Sog9 cells, the anti-A27 neutralizing activity was equivalent to or greater than the anti-L1 activity (Fig. 7B). Anti-L1 antibody was previously shown to neutralize VACV even after attachment to cells (69). Since A27 is thought to play a role in cell attachment, we also considered that the anti-A27 antibody might be effective only if it is incubated with virus prior to attachment. This was not the case; the anti-A27 IgG had slightly more neutralizing activity than the anti-L1 IgG even when added after virus adsorption to HeLa cells (Fig. 7C). Thus, anti-A27 IgG was neutralizing under all in vitro conditions tested.

DISCUSSION

The purpose of the present study was to guide the selection of appropriate recombinant proteins for a third generation

FIG. 7. Further characterization of the neutralizing activities of anti-L1 and anti-A27 IgG. Neutralization was carried out as described in the legend to Fig. 5 except that L cells (A) or Sog9 cells (B) were used instead of HeLa cells. (C) Purified MVs were adsorbed to HeLa cells in suspension for 1 h at 4°C, and the cells were washed by centrifugation and then incubated with the indicated concentrations of antibody.

smallpox vaccine. Our previous studies indicated that the immunization of mice and monkeys with a recombinant L1 MV protein combined with recombinant EV proteins A33 or B5 induces a protective immune response against a lethal chal-

lenge with VACV and monkeypox virus (22, 23). A27, another MV protein, appeared to be a possible alternative or supplement to L1, since antibodies to A27 are raised after smallpox vaccination (39, 53) and the active immunization with recombinant A27 protein and the passive transfer of a monoclonal antibody (MAb) to A27 were reported to protect mice against a lethal VACV challenge (17, 36, 54). Additionally, the gene encoding A27 is a component of candidate DNA and protein vaccines (8, 30, 31, 52, 57), though in most cases the contribution of A27 toward protection was not established. We therefore compared A27 and L1 individually as well as in combination with the A33 EV protein. Both the A27 and L1 proteins were secreted from insect cells, purified from the medium by affinity chromatography, and induced similar levels of neutralizing antibody when administered with MPL+TDM in mice. Nevertheless, mice immunized with L1 were better protected against an i.n. challenge with VACV than mice immunized with A27. Furthermore, A27 combined with A33 was not as protective as L1 with A33.

The disparity between the *in vitro* neutralization by antibody to A27 and protection was surprising since numerous studies point to the importance of the induction of antibody by a vaccine for protection against orthopoxvirus infections (7, 20, 48, 49, 70, 72). Nevertheless, it was possible that L1 elicited a more protective T-cell response than A27. Therefore, we used passive transfer experiments to analyze the effects of antibody specifically. We prepared IgG from rabbits that were immunized with recombinant A27 or L1. Although the anti-A27 IgG had equivalent or greater VACV neutralizing activity than the anti-L1 IgG, it did not protect mice as well against the VACV challenge. Thus, neither A27 antibody made in mice following protein immunization nor exogenously added antibody provided potent protection.

There are some apparent inconsistencies in the literature regarding the protective effects of A27 immunizations that may be related in part to the route of challenge with VACV. Using the same recombinant proteins as in the present study, but with a different adjuvant (alum and CpG), Xiao et al. (71) compared the effectiveness of trivalent vaccines consisting of A33 and B5 plus either L1 or A27 and a tetravalent vaccine containing all four proteins. After two immunizations, the L1-containing trivalent vaccine was more protective than the A27-containing trivalent vaccine, consistent with our data. However, the difference appeared to be due to the absence of MV-neutralizing antibody in sera from mice receiving the latter vaccine. Other investigators have reported protection with A27 DNA or antibodies to A27 using an i.p. challenge model. The LD₅₀ for the i.p. challenge is about 1,000-fold higher than that for the i.n. route, the kinetics of disease and pathogenesis are different, and vaccine formulations appear to be more protective when mice are challenged i.p. than i.n. (57). Using DNA immunization and the i.p. route of challenge, Hooper et al. (30) found that neither the A27 gene nor the B5 gene alone was protective but that the combination was protective. He et al. (26) reported that the passive administration of neutralizing antibodies to A27 protected mice; however, in those experiments, the antibody was mixed with challenge virus prior to i.p. inoculation. Esteban and coworkers (17, 36, 54) also reported that the A27 protein and antibody were protective. Their success may have been related to the synthesis of the protein in

E. coli, the use of Freund's adjuvant, and passive vaccination with a mouse MAb. In addition, the challenge virus was administered i.p., which was also the site of the active and passive immunizations. Thus, to some extent, neutralization may have occurred locally rather than systemically. In the present studies, we avoided the local effects by administering the challenge virus i.n., while giving the active protein immunization subcutaneously and the passive antibody immunization i.p.

Although the *in vitro* neutralization assay can provide important information, it is a simplified surrogate for the complex situation that occurs *in vivo*. This is particularly true for VACV since two infectious forms exist and the relative roles of each are not well understood. *In vivo* imaging and fluorescence microscopy show that after i.n. inoculation, VACV WR replicates extensively in the nose and produces a focal infection around the bronchioles in the lungs, probably due to the aspiration of liquid (41). In addition, infection occurs in the anterior and inferior aspects of the frontal lobes of the brain, which are adjacent to the olfactory bulbs, suggesting that the mechanism of neuroinvasion is the direct spread of virus from the nasal mucosa, as also suggested earlier (64). However, the protection of mice from VACV WR correlates with a reduction of virus replication in the lungs but not in the brain (38). The spread of VACV WR in tissue cultures is largely due to cell-associated EVs (9), and a similar spread by EVs is also likely in the nasal epithelium (51). Although the cell-to-cell spread of VACV in tissue cultures is antibody resistant (37), antibodies to EV proteins can prevent the formation of satellite plaques (4) and antibodies to MV proteins can enhance this effect in the presence of complement (43). Since the mouse challenges were carried out with MVs, circulating antibodies to MV proteins might effectively prevent the primary infection. In addition, MVs released by the lysis of infected cells would be susceptible to antibodies to MV proteins. Furthermore, complement or other effectors that disrupt the EV membrane might enhance the efficacy of antibodies to some MV proteins on the surface of virus progeny. Similarly exposed MV proteins on the cell surface could mark the cells for destruction by humoral and cellular mechanisms.

We considered that the discrepancy between the anti-L1 and -A27 antibodies might be related to the different functions of these proteins. L1 is a myristylated transmembrane component of the MV (24, 69). Considerable information is available regarding its structure and one of its neutralization epitopes (60, 61). Although L1 was reported to be essential for virus assembly (55), recent data demonstrate that the primary role of L1 is in virus entry (H. Bisht and B. Moss, unpublished data). A27 has an oligomeric structure with a rigid hydrophobic coiled-coil region at the C terminus (27, 40). A27 lacks a transmembrane domain and binds to the MV surface through an interaction with the A17 transmembrane protein (66). The epitope targets of some A27-neutralizing antibodies have been found (44). A27 has been reported to have a number of different roles, including wrapping of the MV, membrane fusion, and binding to heparin proteoglycans on the cell surface (13, 34, 56). Because cells may vary in their content of GAGs, its role in attachment may be cell-type specific (11). However, we found that the anti-A27 IgG had equivalent or higher virus-neutralizing activity than the anti-L1 IgG when tested with human HeLa cells and mouse L cells, which display GAGs on their cell

surface, and with Sog9 cells, which do not (5). Furthermore, both the anti-A27 IgG and the anti-L1 IgG neutralized VACV when added following virus adsorption to cells. Thus, anti-A27 was neutralizing under each of the in vitro conditions tested. Although our findings may have more generality because they were carried out with polyclonal antibodies, studies with well-characterized neutralizing MAbs might help to better understand differences in the mechanism of virus neutralization.

The disparity between the in vitro neutralization of VACV and the protection of animals may not be unique to A27 and may be true of other components of VIG. Furthermore, non-neutralizing antibody may be protective. For example, passive immunization with antibodies to the EV proteins A33 and B5 is protective (42). Of the two, however, only the anti-B5 antibody neutralizes EV (6). Thus, the present and previous findings raise questions regarding the basis of protection by antibody and whether it occurs by direct virus neutralization or involves complement and Fc-receptor interactions. The use of mice with genetic alterations, such as complement and Fc-receptor deficiencies, may help to resolve this issue.

Although caution is needed to extrapolate from mouse to human, our data support the use of recombinant L1 in preference to A27 as a component of a poxvirus vaccine. This is of practical importance because cost necessitates that there be a selection of the most protective immunogens for the formulation of a multicomponent protein vaccine. More broadly, our studies highlight the importance of animal models for the early evaluation of recombinant vaccines and antibodies.

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