Antibodies to the A27 Protein of Vaccinia Virus Neutralize and Protect against Infection but Represent a Minor Component of Dryvax Vaccine–Induced Immunity

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The smallpox vaccine Dryvax, which consists of replication-competent vaccinia virus, elicits antibodies that play a major role in protection. Several vaccinia proteins generate neutralizing antibodies, but their importance for protection is unknown. We investigated the potency of antibodies to the A27 protein of the mature virion in neutralization and protection experiments and the contributions of A27 antibodies to Dryvax-induced immunity. Using a recombinant A27 protein (rA27), we confirmed that A27 contains neutralizing determinants and that vaccinia immune globulin (VIG) derived from Dryvax recipients contains reactivity to A27. However, VIG neutralization was not significantly reduced when A27 antibodies were removed, and antibodies elicited by an rA27 enhanced the protection conferred by VIG in passive transfer experiments. These findings demonstrate that A27 antibodies do not represent the major fraction of neutralizing activity in VIG and suggest that immunity may be augmented by vaccines and immune globulins that include strong antibody responses to A27.

The threat of bioterrorism has stimulated new smallpox vaccine initiatives. Although the US-licensed smallpox vaccine Dryvax (Wyeth Laboratories) composed of replication-competent vaccinia virus (VACV) is highly effective, it poses safety risks, especially for immunocompromised individuals. Vaccines with improved safety profiles are being developed [1, 2], but, in the absence of an orthopox virus outbreak, evaluations of efficacy and protection experiments in animal models [3].

Information on protective immune responses induced by Dryvax would aid the development of new vaccines. Traditional VACV vaccines establish local cutaneous infections that trigger humoral and cellular responses. Both arms of immunity likely contribute to protection [2, 4], but experience with passive antibody transfer [5, 6] and depletions of B or T cells in monkeypox protection models [7] have shown the importance of antibodies for protection. Indeed, the vaccinia immune globulin (VIG), derived from persons recently immunized with Dryvax, is recommended for treating certain vaccine complications, including progressive vaccinia, eczema vaccinatum, severe generalized VACV infection, and infections of the eyes and mouth [8].

Current knowledge about protective and neutralizing determinants in VACV is limited. Among the >200 VACV proteins, at least 5 encoded by the genes H3L [9], A27L [10], BSR [11], D8L [12], and L1R [13] are known to contain neutralizing determinants, whereas the protein encoded by the A33R gene may induce different protective antibody responses [14]. One of the
best characterized of these is the 14-kDa protein (A27) encoded by A27L gene. A27 is a trimer on the surface of the mature virus (MV) [15–17], an infectious form that is believed to be important for spread between hosts. A27 facilitates virus entry and is critical for virus maturation [15, 18–21]. Monoclonal antibodies specific for A27, isolated from VACV-immunized mice, neutralize virus and protect immune-competent mice in passive transfer studies [10, 22, 23]. Immunization with a recombinant form of A27 (rA27) also protected immune-competent mice from a lethal intranasal challenge [24]. These studies have suggested that antibodies to the A27 protein may be an important component of protective immunity. However, other studies involving DNA or recombinant proteins for A27 immunizations have reported protection to varying degrees [1, 25, 26], suggesting that source, dose, or formulation of antigen may affect outcome.

Here we evaluate A27 antibodies elicited by Dryvax and further investigate the potential of an rA27 to induce protective antibodies. We confirm that rA27 elicits robust neutralizing antibodies and further show that anti-rA27 antibodies confer partial protection and augment VIG in immunocompromised mice. Additionally, we show that Dryvax induces anti-A27 neutralizing antibodies but that they represent only a small fraction of the total neutralizing activity in VIG. These findings demonstrate the potential for enhancing immunity to poxviruses by approaches that elicit strong humoral responses to A27.

**MATERIALS AND METHODS**

**Viruses and cells.** VACV strains Western Reserve (VACV-WR; ATCC VR-1354) and vSC56 [27] expressing β-galactosidase and lacking functional TK genes (provided by B. Moss, National Institutes of Health, Bethesda, MD) were propagated and titered in BS-C-1 cells and purified by sucrose gradient centrifugation [28].

**Recombinant proteins and peptides.** rA27 was made as described elsewhere [29], using the VACV-WR clone of the New York City Board of Health strain of vaccinia virus (NYCBOH-VACV). Dryvax is also derived from NYCBOH-VACV. Briefly, the A27L gene was amplified by polymerase chain reaction, cloned into pAED4 [30], and mutated to replace cysteine residues 71 and 72 with alanines, creating pAED4-A27-AA. The alanine substitutions aided purification and refolding and have been previously shown not to impair A27 function [15]. The residues glycine-serine-serine-glycine-glycine-cysteine were added to the C terminus of rA27 (rA27-GGC). Plasmids were expressed in bacteria (BL21[DE3]/pLysS; Novagen), and recombinant proteins were isolated from soluble fraction, purified to homogeneity by high-performance liquid chromatography (HPLC), and refolded by dialyzing in PBS at 4°C. Purity was assessed by gel electrophoresis (10% NuPage gel; Roche) and staining with GelCode Blue (Pierce). Oligomerization was analyzed by gel filtration (Waters Protein PAK 300 SW column) and cross-linking with bis(sulfosuccinimidyl)suberate (BS3; Pierce). Briefly, 50 μg of rA27 was mixed with 20 molar excess of BS3 and incubated for 1 h at room temperature before quenching with Tris-HCl. Synthetic A27 peptides N24 (LKQRLLNEKITTNYTTFKKEQEK), C31 (NDEVFLRLEHATLRAAMISLAKIDVQTG), N20 (MDGTLPFGDIDLAPTEFF), N12 (STKAAKPKDPRK), and N26 (STKAKKEPKQVKADEDDNNEET) were made by standard 9-fluorenylmethoxy carbonyl chemistry and purified by HPLC (Center for Biologics Evaluation and Research [CBER] Facility for Biotechnology Resources, US Food and Drug Administration, Rockville, MD). Peptides and recombinant proteins were confirmed to have the expected molecular weight by matrix-assisted laser desorption ionization–time-of-flight mass spectroscopy.

**Antibodies and immunizations.** VIG, a commercial immune-globulin preparation from Dryvax-immunized persons, was provided by Dr. Dorothy Scott (US Food and Drug Administration, Rockville, MD). Two to six New Zealand white rabbits were primed subcutaneously with 200 μg of rA27 or unconjugated, purified peptide mixed with Freund’s complete adjuvant, followed by 2–4 boosts of 100 μg of antigen mixed with Freund’s incomplete adjuvant, at 4-week intervals. Serum samples were heated to 56°C for 30 min to inactivate complement. Control immune globulins using an irrelevant HIV antigen were prepared similarly.

**ELISAs.** For ELISAs, 96-well polystyrene plates (Corning) were coated with rA27 or peptides (75 pmol) in PBS overnight, washed with Tris-buffered saline with 0.5% Tween 20 (TBS-T), and blocked with 5% nonfat dry milk in TBS-T. Serial dilutions of serum or immune globulins (IgG) were incubated for 1 h at 37°C and washed 4 times in TBS-T before 1 h incubation with peroxidase-conjugated anti-rabbit or anti-human antibody (Roche Diagnostics) diluted 1:5000. After the application of soluble 3,3′,5,5′-tetramethylbenzidine (BM Blue, POD substrate; Roche Diagnostics) for 30 min, samples were read for absorbance (optical density at 450 nm). The end-point serum titer (or milligrams per binding unit for IgG) was determined to be the highest serum dilution (or least amount of antibody) resulting in an absorbance >3 SDs above that of the negative control antibody at the lowest dilution.

**Neutralization assay.** Neutralization titers were determined by incubating serum or IgG dilutions in Dubelco’s modified Eagle medium with 10% fetal calf serum with VACV-WR in plaque reduction neutralization (PRNT) assays [28]. Briefly, 0.5 mL of VACV, containing 100–300 pfu, was incubated with serial dilutions of antibodies for 30 min at 37°C before inoculating BS-C-1 monolayers in 12-well plates for 1 h at 37°C, followed by 2 washes with PBS and culturing for 2 days at 37°C under liquid overlay. Plaques were counted after staining with 0.1% crystal violet. The neutralizing titer was defined as the
Figure 1. A, Map of A27 domains and peptides. B, Analyses of recombinant A27 protein (rA27) oligomerization. The chromatograph shows a gel-filtration profile of rA27. Inset, SDS-PAGE of rA27 with or without a bis(sulfosuccinimidyl)suberate (BS3) cross-linker. Monomers, trimers, and molecular-weight markers are indicated.

**Affinity chromatography.** rA27-GGC was biotinylated at the C terminus with 1-biotinamido-4-(4′-[maleimidomethyl]cyclohexanecarboxamido) butane (Pierce) to create rA27-GGC–biotin, which was purified to homogeneity by C-18 HPLC and verified by mass-spectroscopic analysis and SDS-PAGE. rA27-GGC–biotin (7 mg) was immobilized on 2 mL of streptavidin resin (Pierce). VIG (550 mg) or A27 IgG (100 mg) was incubated with rA27-AA-GGC–biotin streptavidin resin at 4°C overnight. After extensive washing with PBS, the A27-specific antibody was eluted with 0.1 mol/L glycine (pH 2.5) and neutralized immediately with 1 mol/L Tris-HCl (pH 8.0). Each fraction was analyzed for rA27 binding activity by ELISA and neutralization activity by PRNT assay. Total rA27 binding units (activity) in each fraction were calculated by dividing the total amount of IgG measured in that fraction (in milligrams) by the least amount of antibody from that fraction giving an absorbance >3 SDs above that of the negative control (in milligrams per binding unit). Total neutralizing units (activity) in each fraction were calculated by dividing the total amount of IgG measured in that fraction (in milligrams) by the least amount of IgG from that fraction needed to result in 50% plaque reduction (in milligrams per neutralizing unit).

**Abrogation of neutralization.** Neutralizing antibodies in a quantity close to the IC_{50} were mixed in the presence or absence of the indicated concentrations of rA27 for 30 min before incubating with virus for PRNT assays.

**Vaccinia virus challenge.** Six 9-week-old female BALB/c AnNcr-nu/nu mice were given intraperitoneal inoculations with 1 × 10^7 pfu of purified strain vSC56 that was premixed with VIG or rabbit IgG–purified antibodies at room temperature for 30 min. Prior experiments showed that this procedure conserved the amount of antibodies needed for protection, compared with procedures dosing mice with antibodies before inoculation. Mice were weighed daily after challenge and were euthanized when they lost 25% of their initial body weight. All mice were handled according to CBER Institutional Animal Care and Use Committee standards.

**Statistical analyses.** In the abrogation assays, comparable samples with and without rA27 were analyzed with Student’s 2-tailed, paired t test. In the protection studies, the log-rank test was used to determine significance of differences in survival between the groups. Statistical analyses used JMP software (version 5.1; JMP).

**RESULTS**

**Immunogenicity of rA27 and A27 peptides.** Full-length rA27 and peptides corresponding to domains in A27 described elsewhere [20] (figure 1A), including peptides corresponding to a potential α-helical region near the N terminus (N24), a C-terminal leucine zipper region (C-31), a putative heparin-binding region (N12 and N26), and an N-terminal region (N20), were used to immunize rabbits and to evaluate neutralizing determinants in A27. Gel chromatography (figure 1B) and chemical cross-linking (inset) showed that rA27 predominantly formed trimers. All immunogens except N12, a 12-residue lysine/arginine-rich peptide, elicited end-point titers against cognate antigens in the range of 10^{5}–10^{7} (table 1, top). The peptide antisera also demonstrated significant reactivity to rA27 but much less, compared with the cognate antigen (table 1, top). Conversely, rA27 antisera end-point titers were 2–3 logs lower to individual or the combination of peptides than they were to rA27 (not shown), which suggests that responses are predominantly against conformational determinants. Analysis of A27-specific antibodies induced by Dryvax showed that VIG contained significant reactivity to rA27 (table 1, bottom), but it was 3.5 logs lower than reactivity from immune globulins isolated from rabbits immunized with rA27 (A27 IgG). VIG was 20-fold less reactive to N20 and 100-fold less reactive to the other peptides, compared with rA27 (data not shown).
Neutralizing activity of anti-A27 antibodies. Functional antibody responses were investigated in PRNT assays. Among the A27 immunogens, only rA27 demonstrated potent neutralization activity (table 1, top). The combination of 5 antigen sera at 1:20 dilution for each serum also lacked neutralization activity (not shown), which suggests that neutralizing antibodies are not efficiently elicited by peptides. Although several anti-A27 neutralizing monoclonal antibodies bind linear epitopes [31, 32], they were elicited by vaccination with VACV recombinant A27 protein; VIG, vaccinia immune globulin.

<table>
<thead>
<tr>
<th>Antigen, sample</th>
<th>ELISA titera (log10 serum dilution)</th>
<th>Neutralizing titerb (log10 serum dilution)</th>
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</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Cognate peptide rA27</td>
<td></td>
</tr>
<tr>
<td>N12</td>
<td>2.77 ± 0.30</td>
<td>3.08 ± 0.31 &lt;1.3</td>
</tr>
<tr>
<td>N20</td>
<td>6.05 ± 0.55</td>
<td>5.32 ± 0.14 &lt;1.3</td>
</tr>
<tr>
<td>N26</td>
<td>5.80 ± 0.25</td>
<td>5.19 ± 0.23 &lt;1.3</td>
</tr>
<tr>
<td>N24</td>
<td>5.91 ± 0.25</td>
<td>4.75 ± 0.27 &lt;1.3</td>
</tr>
<tr>
<td>C31</td>
<td>6.41 ± 0.16</td>
<td>5.04 ± 0.25 &lt;1.3</td>
</tr>
<tr>
<td>rA27</td>
<td>NA</td>
<td>6.31 ± 0.15 3.54 ± 0.53</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SD values. ELISA plates were coated with indicated antigens. Antibodies were diluted to determine end point for reactivity. Results shown are from 5 independent experiments. NA, not applicable; rA27, recombinant A27 protein; VIG, vaccinia immune globulin.

A27 antibodies induced by Dryvax. We assessed the relative amount of A27-specific antibodies in VIG by measuring the neutralization activity of VIG before and after the depletion of A27 antibodies using 2 independent approaches. In the first approach, we fractionated VIG using an affinity column created with rA27 and tested loading (total VIG), flow-through, and eluate fractions for rA27 reactivity and neutralization activity in ELISA and PRNT assays, respectively (table 2 and figure 2). We first characterized the binding capacity of the column by measuring its ability to remove A27-specific antibodies. After 100 mg of purified anti-A27 IgG containing ~7 × 10⁸ binding units was loaded onto the column, all but 1 × 10⁸ binding units (the flow-through fraction) were removed, and almost all (6.7 × 10⁸ binding units) were recovered in the eluate fraction. Similarly, of the 2 × 10⁷ Nt₅₀ loaded onto the column, only an extremely small fraction remained in the flow through (1 × 10⁵ Nt₅₀), and ~9 × 10⁷ Nt₅₀ were recovered in the eluate. The incomplete recovery of the neutralizing activity in the eluate likely reflects combined losses of neutralizing activity due to acid elution buffers and an inability to completely recover high-affinity, adsorbed antibodies. Importantly, these data show the binding capacity of the column (6 × 10⁸ binding units) and its ability to remove A27-specific antibodies with neutralizing activity.

To fractionate VIG, 550 mg of purified IgG containing 6.3 × 10⁴ binding units and 4.7 × 10⁴ Nt₅₀ units was applied to the column (table 2). Similar to the rA27 IgG, the column removed nearly all of the A27 binding activity in VIG, which was recovered in the eluate fraction. By contrast, the vast majority of the neutralization activity (3.6 × 10⁴ Nt₅₀) remained in the flow-through fraction, and <1% was recovered in the eluate (2 × 10⁴ Nt₅₀). This result was not due to column overload, because the column was capable of removing a much greater amount of A27-specific antibodies (table 2). Thus, the A27-specific antibodies elicited by Dryvax vaccine are neutralizing, but they represent only a small fraction of the neutralizing antibodies present in VIG. Similarly, when we measured specific

Table 2. Fractionation of A27-specific antibodies.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>A27 IgG</th>
<th>VIG</th>
</tr>
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<tbody>
<tr>
<td>Binding unitsa</td>
<td>Loading 6.99 × 10⁴</td>
<td>6.26 × 10⁴</td>
</tr>
<tr>
<td>Flow-through</td>
<td>7.62 × 10⁴</td>
<td>9.61 × 10⁴</td>
</tr>
<tr>
<td>Eluate</td>
<td>6.71 × 10⁴</td>
<td>6.12 × 10⁴</td>
</tr>
<tr>
<td>Nt₅₀ unitsb</td>
<td>Loading 2.10 × 10⁵</td>
<td>4.74 × 10⁴</td>
</tr>
<tr>
<td>Flow-through</td>
<td>1.02 × 10⁵</td>
<td>3.63 × 10⁴</td>
</tr>
<tr>
<td>Eluate</td>
<td>9.00 × 10⁴</td>
<td>2.06 × 10⁵</td>
</tr>
</tbody>
</table>

NOTE. Vaccinia immune globulin (VIG) or rabbit anti–recombinant A27 protein (rA27) antibodies (A27 IgG) were applied to an rA27 column (“loading”) and separated according to whether the material passed through the column (“flow-through”) or was retained and eluted (“eluate”). All fractions were assessed for ELISA binding and neutralization activity. Data represent a single fractionation. Activity measurements are averages of 3 independent assays.

a Calculated by dividing total amount of IgG measured in each fraction (in micrograms, determined by the optical density at 280 nm) by the least amount of IgG (micrograms at the end-point titer) that gave an OD₅₄₀ nm >3 SDs above background in rA27 ELISA.

b Calculated by dividing the total amount of IgG measured in each fraction (in micrograms, determined by the optical density at 280 nm) by the amount of IgG (in micrograms) needed to reduce plaques by 50% in a neutralization assay.
the host, we undertook in vivo experiments to further assess the potency of our polyclonal A27 antibodies and whether they could augment VIG. These studies involved the transfer of antibodies into immunodeficient nude mice and lethal challenge with $1 \times 10^7$ pfu of vSC56, a thymidine-kinase–deficient VACV. Suboptimal doses of immune globulins, determined in preliminary experiments (data not shown), were used to evaluate whether benefit could be obtained from combining A27 IgG and VIG. Low or high doses of either VIG or rA27 antibodies were compared using a combination of low-dose antibodies that equaled the total amount of high-dose VIG (figure 4). Antibodies were normalized for total amount of IgG rather than PRNT activity, because neutralizing activity against both MVs and EVs would be relevant in vivo.

Figure 4 shows partial protection at both doses in the groups treated with A27 IgG (8 and 24 mg) and VIG (4 and 12 mg), compared with control groups receiving only VACV and VACV treated with 24 mg of normal rabbit immune globulin (NR IgG) ($P < .005$ for both comparisons). Both control groups lost weight progressively and were euthanized when their weight dropped by 25%, starting at day 20. Mice in the untreated and NR IgG groups all died by day 45 (mean time to death, 31 days for both groups). By contrast, both groups treated with high or low doses of A27 IgG were partially protected from infection in a dose-dependent manner. Three of six mice in the high-dose (24 mg) A27 IgG group survived until the experiment was terminated on day 140 after challenge. In the low-dose (8 mg) A27 IgG group, mice showed a significant extension of survival, to an average of 60 days ($P < .005$). Previously, a monoclonal antibody to A27 (C3) was shown to protect immunocompetent mice from a lethal intranasal challenge [23], but, to our knowledge, our results are the first

**Protection studies with anti-A27 antibodies and VIG.**

Because PRNT assays measure only neutralizing activity against MVs, and EVs are believed to be important for spread within...
Figure 4. A27 antibodies conferring passive protection. Nude mice were challenged intraperitoneally with 1 × 10^7 pfu of vaccinia virus (vSC56) alone or mixed (+) with the indicated immune globulins. NR, normal rabbit; VIG, vaccinia immune globulin.

demonstration that polyclonal antibodies to recombinant A27 protein can passively protect immunodeficient animals from lethal challenge.

As expected, VIG also protected mice by significantly extending survival, compared with the control groups \((P < .005)\), without a significant difference between the high-dose (12 mg) and low-dose (4 mg) VIG groups \((P = .47)\). The low-dose combination of (8 mg) A27 IgG plus (4 mg) VIG extended survival to an average of 87 days, compared with 60 days for 8 mg A27 IgG \((P < .005)\) and 52 days for 4 mg VIG \((P < .001)\). Importantly, survival was better in the group that received a low-dose combination of A27 IgG (8 mg) and VIG (4 mg) than in the group that received an equivalent amount (12 mg) of VIG (56 days) \((P < .0001)\). We measured IgG concentrations in the mice at several intervals to check for potential differences in antibody clearance and found that the average half-life for rabbit antibody and VIG did not differ significantly among the groups (data not shown), consistent with other reports of similar clearance rates for rabbit and human antibodies [5]. These protection studies demonstrate that antibodies to A27 can augment protection by VIG and suggest that current vaccines could be enhanced by improving the antibody response to A27.

DISCUSSION

A27 antibody responses are frequently detected after immunization with VACV-based vaccines [29, 33], but their significance in protection is unknown. To inform the design and evaluation of new smallpox vaccines and therapeutic immune globulins, we investigated the contribution of A27-specific antibodies elicited by Dryvax to virus neutralization and protection and further explored the potential of A27 antibodies to augment protection.

Full-length rA27 induced high-titered antibodies with potent neutralizing activity (table 1) that enhanced passive protection by VIG in immunodeficient mice (figure 4). These results are consistent with our findings that Dryvax does not induce large amounts of A27 antibodies (figures 2 and 3). A recent report examining antibody responses in persons immunized with the Lister strain of VACV also suggested that MV neutralization is not dominated by A27 responses, unlike EV neutralization, which appears to be dominated by a single protein (BS) [34]. Further research is needed to determine whether antibodies to other proteins potentially involved in a multiprotein fusion-entry complex are important components of vaccine-induced immunity [35, 36].

Vaccine studies involving various MV and EV proteins, delivered as DNA, recombinant proteins, or combinations, have shown that protection in some animal models can be achieved by immunizations with single proteins, although combinations are often better against more stringent challenges, especially if both EV and MV antigens are included [1, 5, 14, 25, 37, 38]. It has also been suggested that the addition of A27 antigen in a combination vaccine may confer little advantage [25]. However, many factors—including the source and dose of immunogen, its mode of delivery, adjuvant formulation, the number of immunizations, and challenge model—differ among the studies, making comparisons difficult to interpret. Perhaps the many different combinations of antigens that can confer protection in some animal models mirror the redundancy in protective immune responses that are achieved after VACV infection, which in turn may also explain the effective, long-lasting immunity seen after vaccination with VACV or recovery from natural infection [39]. Because the outcome of natural infection is determined by the race between host immunity and virally encoded immune modulators that interfere with the immune response, smallpox vaccines may only have to reduce the initial viral inoculum below a threshold to allow the immune response to gain an upper hand in controlling infection. If so, there may be many ways for vaccines to sufficiently blunt the start of an infection. Our studies highlight opportunities for developing new smallpox vaccines through approaches that induce strong antibody responses to A27. Similarly, our results indicate that immune globulin cocktails that are enriched in protective an-
tibodies, such as anti-A27, could offer more potent supplements or alternatives to VIG.

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References