Subunit Recombinant Vaccine Protects against Monkeypox

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The smallpox vaccine Dryvax, a live vaccinia virus (VACV), protects against smallpox and monkeypox, but is contraindicated in immunocompromised individuals. Because Abs to VACV mediate protection, a live virus vaccine could be substituted by a safe subunit protein-based vaccine able to induce a protective Ab response. We immunized rhesus macaques with plasmid DNA encoding the monkeypox orthologs of the VACV L1R, A27L, A33R, and B5R proteins by the intradermal and i.m. routes, either alone or in combination with the equivalent recombinant proteins produced in Escherichia coli. Animals that received only DNA failed to produce high-titer Abs, developed innumerable skin lesions after challenge, and died in a manner similar to placebo controls. By contrast, the animals vaccinated with proteins developed moderate to severe disease (20–155 skin lesions) but survived. Importantly, those immunized with DNA and boosted with proteins had mild disease with 15 or fewer lesions that resolved within days. DNA/protein immunization elicited Th responses and binding Ab titers to all four proteins that correlated negatively with the total lesion number. The sera of the immunized macaques recognized a limited number of linear B cell epitopes that are conserved among orthopoxviruses. Their identification may guide future efforts to develop simpler, safer, and more effective vaccines for monkeypox and smallpox. The Journal of Immunology, 2006, 177: 2552–2564.

Smallpox and monkeypox are closely related orthopoxviruses that differ in their pathogenicity for humans. Although smallpox is highly transmissible among humans and causes death in approximately one-third of infected individuals, monkeypox is transmitted less efficiently and has a lower mortality rate (1, 2). Smallpox has been eradicated worldwide and it is not a public health concern except for its possible use as a bioterrorism threat. In contrast, outbreaks of monkeypox have often been reported in Africa since 1970 (3, 4) and, unexpectedly, in the United States (5, 6). The U.S. outbreak (37 confirmed cases within a few weeks) was due to exposure to monkeypox-infected prairie dogs that had contracted the disease from imported African rodents (5). Although an embargo on the importation and sale of African rodents would prevent exposure, this episode nevertheless indicates the unpredictable nature of zoonotic infection of humans.

Thus, the development of safer vaccines or drugs to prevent or treat such infection will serve the public.

The existing smallpox vaccine, Dryvax, protects humans from both smallpox and monkeypox (7). However, there are numerous adverse events that can affect both the vaccinee and persons in contact with the vaccinee (e.g., contact vaccinia) (8, 9). Monkeypox virus infection of healthy rhesus macaques appears to be a suitable model to study protective immune responses against monkeypox (10). Indeed, macaques vaccinated with Dryvax are protected from monkeypox (10–14). Recent data have shown that the major mode of protection from monkeypox afforded by the current nonattenuated smallpox vaccine is mediated by Abs (14). Depletion of either CD4+ T cells or CD8+ T cells in vaccinated animals before monkeypox virus challenge does not affect survival, whereas B cell depletion before and during immunization abrogates vaccine-induced protection. Accordingly, passive administration of vaccinia virus (VACV) Abs confers protection from subsequent lethal monkeypox (14). Thus, next-generation smallpox vaccines may not need to be based on replicating vectors, provided that they elicit appropriate Ab responses. The definition of VACV protective Ags has been limited by the complexity of the VACV proteome that encodes some 200 proteins. Nevertheless, proteins L1R and A27L, specific to the intracellular mature virus (IMV), and proteins A33R and B5R, specific to the extracellular enveloped virus (EEV), have been shown to be immunogenic and protected mice from VACV (15–17). In addition, vaccination with a single protein, A33R, was shown to protect against a lethal challenge with ectromelia virus, which is a highly virulent natural pathogen in mice (18). EEV are produced when IMV wrap in

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3 Abbreviations used in this paper: VACV, vaccinia virus; IMV, intracellular mature virus; EEV, extracellular enveloped virus; Ct, threshold cycle; β-Gal, β-galactosidase; mOD, milli-OD; MVA, modified vaccinia virus Ankara.
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additional cellular membranes, move to the cell surface, and release from the cell (19). Both the IMV and EEV forms of poxviruses are infectious. Recently, protection from monkeypox-in-duced severe disease was also observed following gene gun immunizations with only four VACV DNA plasmids encoding these four proteins (10). Thus, smallpox vaccines based on recombinant proteins or peptides should be able to confer protection from monkeypox/smallpox. In this study, we demonstrate that this hypothesis is plausible. A combination of recombinant vaccine modalities (DNA plus proteins) was superior to either DNA or proteins alone and conferred protection against severe monkeypox infection of rhesus macaques. Protection from disease correlated with the titers of binding Abs to all proteins and to the extent of virus-specific CD4+ T cell responses elicited by vaccination.

Materials and Methods

Animals, immunization, and monkeypox virus challenge

Fourteen colony-bred rhesus macaques (Macaca mulatta), obtained from Covance Research Products, were housed and handled in accordance with the standards of the American Association for the Accreditation of Laboratory Animal Care. The study was reviewed and approved by the animal care and use committees at Advanced BioScience Laboratories and Southern Research Institute. Each DNA immunization consisted of four plasmids (4 mg each) given i.m. (3 mg) and intradermally (1 mg) at different locations. Proteins (100 μg each) were either formulated in alum (Rehydragen) or preincubated with vSC56 virus for 60 min at 37°C and then dispensed into port Vaccine and validated at the Center for Biologics Evaluation and Administration (FDA) Standard Reference Vaccinia Ig obtained from Dyno-Cell Vaccines and described previously (23).

Real-time PCR to detect monkeypox virus genomes

DNA was extracted from frozen blood samples using the QIAamp DNA mini kit (Qiagen) (20). The primers OPHA-F89 (5'-GATGATGACGAC TATCTATGTA-3') and OPHA-R219 (5'-GTTATTATTAACAAACAC GAGTTCT-3') and the probe OPHA-p1435-MGB (5'-FAMAGTGCGTTG GATGACGACGATCTATGTA-3') were either formulated in alum (Rehydragen) daily for activity, the appearance of skin lesions, and development of lesions through the stages of papule, vesicle, pustule, and scab. The 5' nucleic acid PCR and amplification conditions were performed using Platinum TaqDNA polymerase (Invitrogen Life Technologies). All reactions included at least one positive control containing target DNA and one no-template control. The positive control for each run established the threshold cycle (Ct) value for positive samples. Samples yielding Ct values that marginally exceeded the threshold value were retested. If the Ct value was confirmed as exceeding the threshold after retesting, the sample was considered negative (contained <5000 copies).

Measurement of VACV-neutralizing Abs

Sera from monkeys were collected 3 wk after the last protein immunization (1 wk before challenge), heat-inactivated (56°C for 30 min), and evaluated for the presence of VACV-neutralizing Abs using a novel assay based on expression of a reporter gene, β-galactosidase (β-Gal) (21).

Briefly, a rVACV vSC56, expressing β-Gal under the control of a synthetic early/late promoter (22), was used to develop a neutralization assay based on a single-round infection of HeLa cells (CCL-2; American Type Culture Collection). This is a rapid (24 h), high throughput assay that was shown to have similar sensitivity to the classical plaque reduction neutralization tests. Each assay includes as a positive control Food and Drug Administration (FDA) Standard Reference Vaccinia Ig obtained from Dyno-Port Vaccine and validated at the Center for Biologics Evaluation and Research (FDA). Negative controls included plasma from unvaccinated children and albumin. Four serial dilutions of each monkey plasma were preincubated with vSC56 virus for 60 min at 37°C and then dispensed into 96-well round-bottom plates containing 1 × 106 HeLa cells/well (five replicates per Ab dilution). Plates were incubated for an additional 16 h at 37°C in a humidified CO2 incubator. Cells were then lysed with the de-tergent Igepal CA630 (Sigma-Aldrich). In the second stage of the assay, β-Gal enzymatic activity in each well was measured using 96-well Immu-nol 2 plates (Thermo Labsystems). Each plate included a β-Gal standard curve using a β-Gal enzyme (Roche Diagnostics). Chlorophenol red β-D-galactopyranoside monosodium salt substrate (Roche Diagnostics) was added to all wells for 30 min at room temperature in the dark, and the enzymatic reaction was stopped with 1 M Na2CO3 solution. OD was determined at 575 nm by an ELISA reader. OD readings were transferred to Microsoft Excel for further analysis. The β-Gal standard curves were used to convert OD values into β-Gal activity per experimental or control group (in milliunits per milliliter). The β-Gal activity of each experimental group (virus mixed with a given dilution of test plasma) was expressed as per-centage β-Gal activity in the virus-only control wells. Microsoft Excel was used to plot the percentage of control values for the serial dilutions of each protein. The logarithm of the reciprocal of the OD50 was calculated as the neutralization titer. Titers represent the reciprocal of the highest dilution resulting in a 50% reduction in the number of plaques.

EEV spread inhibition assay

This assay is similar to a comet inhibition assay; however, a semisolid overlay is added after monkeypox virus EEV are released from initially infected cells; the resulting satellite plaques are given a few days to enlarge and are then counted. Specifically, 200 μl of complete medium containing 20–30 PFU of monkeypox virus was adsorbed to monolayers of BSC-1 cells in 6-well plates for 1 h at 37°C 5% CO2 incubator. Unbound virus was removed by rinsing once with 2 ml of complete medium. Mon-KEYY was added to each serially diluted serum sample and the monolayers were incubated for 1 h at 37°C 5% CO2 incubator for 4 days for VACV or 6 days for monkeypox virus. Cells monolayers were stained with 1 ml of crystal violet staining solution (2% crystal violet, 70% ethanol). Plaques were counted and the percent neutralization was calculated relative to the number of plaques in the absence of Ab. Titers represent the reciprocal of the highest dilution resulting in a 50% reduction in the number of plaques.

Plasmid production and bacterial expression

The monkeypox orthologs of the VACV A27L, A35R, B5R, and LJR genes are A29L, A35R, B6R, and M1R, respectively. In this manuscript, we will refer to the protein products of the monkeypox A29L, A35R, B6R, and M1R open reading frames as A27Lo, A33R0, B5R0, and L1R0, respectively, where the “0” is included to indicate that the protein is an ortholog of the VACV Copenhagen protein. The VACV designations are retained to simplify referrals to earlier work involving the VACV orthologous proteins.

DNA vaccine plasmids containing the monkeypox A27L, A35R, B5R, and M1R genes were produced by PCR amplification from DNA plasmid pMPOX/A27Lo (where the o refers to ortholog), pMPOX/ A33R0, pMPOX/B5R0, and pMPOX/L1R0, respectively, by the same methods previously (23).

The monkeypox genes were cloned from the aforementioned DNA vaccine plasmids into prokaryotic expression vectors to produce recombi-nant proteins. The recombinant proteins were expressed in the yeast expression system (Invitrogen Life Technologies). The recombinant plasmids were PCR-amplified from DNA plasmid pMPOX/A27Lo using Hot Start Taq Polymerase (Qiagen) and PCR primers made by Invitrogen Life Technologies (5'-GATATACATATGGACGCGAAAACCTTCT
subsection recombinant vaccine for monkeypox/smallpox

**Detection of Ab response**

ELISA was conducted with E. coli-purified monkeypox proteins as a solid-phase antigen. Ninety-six-well microtiter plates were coated with either purified monkeypox A27Lo, A33Ro, B5Ro, or L1Ro at a concentration of 50 ng/well in coating buffer (50 mM NaHCO3, pH 9.6). The plates were blocked with postcoating buffer (5% sucrose, 1.25% nonfat dry milk, and 0.05 M NaCl) at 37°C followed by washing. The plates were coated overnight at 4°C. Nonspecific adsorption was prevented with 200 μl/well of blocking buffer (2% nonfat dry milk) for 1 h at 37°C. After washing (BioTek) with wash buffer (0.02% Tween 20 in PBS), 100 μl of diluted test sera (1/100 in blocking buffer) were added to each well in triplicate (two coated wells and one uncoated well). Plates were incubated for 1 h at 37°C, washed four times, and incubated for 1 h at 37°C with a 1/10,000 dilution of HRP-conjugated goat anti-human IgG Ab (Jackson ImmunoResearch Laboratories). Plates were washed with buffer four times followed by distilled water. One hundred microliters of Super AquaBlue ELISA substrate (eBioscience) was added to each well and plates were read immediately using a Dynex Technologies microplate reader. The rate of color change in milli-OD (mOD) per minute was read at a wavelength of 405 nm every 9 s for 5 min with the plates shaken before each measurement. The mean mOD per minute reading of duplicate wells was calculated, and the background mOD per minute was subtracted from the corresponding well.

**Intracellular cytokine staining**

Frozen PBMC were thawed and incubated overnight in a 37°C/5% CO2 incubator in RPMI 1640/20% FCS. In a 96-well plate, 104 cells were incubated in 0.2 ml of RPMI 1640/10% FCS for 1 h at 37°C in the absence or presence of a pool of overlapping peptides (207 total peptides) of monkeypox proteins A33Ro, A27Lo, L1Ro, and B5Ro (1 μg/ml each) supplemented with costimulators CD28 and CD49d (1 μg/ml each). After addition of 10 μg/ml brefeldin A (Sigma-Aldrich), cells were incubated for 5 h at 37°C and processed for surface and intracellular cytokine staining. Briefly, cells were washed with 1% FCS in PBS, surface stained for 30 min with CD4-PerCP and CD8-PE (2 μl each; BD Biosciences), washed again, and permeabilized with FACSperm (BD Pharmingen) for 10 min at room temperature in the dark. Following two further washes, cells were intracellularly stained with IFN-α, anti-IFN-γ (both allophycocyanin conjugated; 2 μl/well each; BD Pharmingen), and FITC-conjugated anti-IL-2 (4 μl/well each; BD Pharmingen). Cells were incubated for 30 min at 37°C, fixed with 200 μl 1% paraformaldehyde (Sigma-Aldrich) in PBS, and analyzed by four-color flow cytometry (FACSCalibur-Multiwell Plate Manager; BD Biosciences).

**Results**

**Generation of monkeypox immunogens and study design**

To assess whether a subunit vaccine could confer protection from monkeypox, we expressed four monkeypox virus proteins in E. coli. The cDNAs encoding the monkeypox protein orthologs to the VACV L1R, A33R, and B5R gene products were first modified by deleting their transmembrane region (Fig. 1A) to optimize expression and purification in E. coli. In contrast, the A27Lo protein was expressed from the unmodified cDNA. All purified proteins were used either alone or in conjunction with the corresponding native cDNA plasmids to immunize healthy rhesus macaques. Two groups of macaques (groups 1 and 4) were immunized by the i.m. and intradermal routes with the unmodified DNA plasmids encoding the EEV monkeypox proteins A33Ro and B5Ro and the IMV proteins A27Lo and L1Ro (Fig. 1A). Group 1 was boosted by the i.m. route with all the recombinant proteins, which for simplicity are designated with the same nomenclature as the VACV proteins A27L, A33R, B5R, and L1R with the addition of an α that stands for ortholog, adjuvanted with CpG. Two additional groups were immunized with the recombinant protein alone either in Cpg
Neutralizing Abs to IMV and EEV forms induced by vaccination

Neutralizing Ab titers to the IMV form of VACV were measured using either a β-Gal-based assay or a plaque reduction assay. The plaque reduction assay was also used to measure neutralizing Abs to the IMV form of monkeypox virus. In the case of EEV, neutralizing activity was measured using a novel monkeypox virus EEV spread inhibition assay. Macaques immunized with DNA only had no serum neutralizing Abs in any of these assays (Table I), making the differences across the groups that were immunized with proteins significant \((p = 0.017\) by the exact Kruskal-Wallis test corrected for multiple testing), but no other significant differences in the titers of binding Abs among these three groups were observed \((p > 0.50\)). Each of these groups had substantially higher Ab titers than animals immunized with DNA only (Table I), making the differences over all four groups significant with one minor exception \((p < 0.06\) for each protein). Using kinetic ELISA, the Ab responses to monkeypox proteins were compared with the responses of the corresponding proteins from vaccinia. There was a strong correlation \((R = 0.95; p < 0.001)\) between the homologous proteins (Fig. 2B) indicating that inducing monkeypox-specific responses will provide cross-reactive Abs to vaccinia.

Vaccination-induced T cell response

The overall T cell responses were measured by intracellular cytokine staining following specific stimulation with a pool of overlapping peptides encompassing the four proteins used as immunogens. The ability of CD8\(^+\) and CD8\(^-\) T cells to produce cytokines was assessed by flow cytometry. Staphylococcal enterotoxin B stimulation was used as a positive control to assess the ability of the cells to respond to Ags. Representative raw data of virus-specific CD8\(^+\) and CD8\(^-\) T cell response (expression of IFN-γ, TNF-α, and IL-2) are provided for animals 482, 498, and 481 at 2 wk after the last immunization (Fig. 3, A and B). Thereafter, the mean percentage of CD8\(^+\) and CD8\(^-\) T cells producing IL-2 or dilution or kinetic readout. DNA immunization alone elicited low levels of binding Abs to all four immunogens (Fig. 2A). In contrast, two boosts of DNA-primed animals with the four recombinant proteins elicited high levels of Abs whose titers were higher than those elicited by two immunizations with proteins alone, regardless of the adjuvant used except in the case of L1Ro (Fig. 2A). Indeed, 2 wk after the last immunization, the macaques immunized with DNA prime/protein boost had the highest Ab titers to three of the four monkeypox proteins. A similar pattern was observed in the kinetic ELISA (data not shown). At 1 wk before challenge exposure to monkeypox, the wide range of the Ab titers to A33Ro across the groups that were immunized with proteins was significant \((p < 0.001)\) between the homologous proteins (Fig. 2B) indicating that inducing monkeypox-specific responses will provide cross-reactive Abs to vaccinia.

Binding Abs specific for A33Ro, B5Ro, A27Lo, and L1Ro

The Ab response to the four monkeypox recombinant proteins in the immunized animals was studied using ELISA with endpoint
IFN-γ and TNF-α in the various groups was measured at weeks 0 and 3 before challenge exposure (Fig. 3B). Overall, CD4⁺ T cell responses tended to be higher than CD8⁺ T cell responses, consistent with the ability of DNA and proteins to induce helper response. The variation over the groups in the CD8⁺ (CD4⁺) T cell response measured as the ability of T cells to produce IFN-γ and TNF-α was significant (p = 0.042 by the exact Kruskal-Wallis test corrected for multiple tests), due primarily to the higher responses in the macaques that received DNA plus proteins than those in all other groups. Only animals from groups 1 and 2 showed a significant increase of IL-2 in both CD4⁺ (p = 0.0021 and p = 0.0019, respectively) and CD8⁺ (p = 0.037 and p = 0.029, respectively) T cell responses compared with other groups before or after immunization. Group 1 also showed a significant increase of
TNF-α/IFN-γ in CD4<sup>+</sup> T cells but not in CD8<sup>+</sup> T cells. In some animals, the background level of cytokine production in samples obtained before immunization precluded proper assessment of CD4<sup>+</sup> and CD8<sup>+</sup> T cell response.

Subunit vaccine confers protection against lethal monkeypox virus challenge

Macaques in groups 1, 4, and 5 were challenged 5 wk after the last protein boost with 5 × 10<sup>7</sup> PFU of monkeypox virus (Zaire 79 strain) i.v. Groups 2 and 3 were challenged identically but 4 wk after the last protein boost. The virological and clinical outcomes postchallenge were assessed by monitoring the number of skin lesions, the health status (10, 11, 25), and by measuring DNA viral genomes in blood by quantitative real-time PCR DNA assay (20). Macaques immunized with the DNA prime/protein boost regimen had a mild disease according to the World Health Organization scoring system with a lesion number of <25 (Table III). Importantly, in most of these animals, papules did not evolve to vesicles and disappeared within a few days. In the two groups immunized with proteins together with either CpG or alum, two animals had mild disease, three moderate (25–99), two severe (100–200), and none grave (>200). All animals from these groups completely resolved their lesions (Table III). In contrast, all macaques immunized with DNA developed innumerable lesions that progressed

### Table I. Humoral immune response in macaques before monkeypox virus challenge<sup>a</sup>

<table>
<thead>
<tr>
<th>Macaque</th>
<th>Treatment</th>
<th>L1Ro</th>
<th>B5Ro</th>
<th>A33Ro</th>
<th>A27Lo</th>
</tr>
</thead>
<tbody>
<tr>
<td>482</td>
<td>DNA + protein-CpG</td>
<td>50/17,820</td>
<td>50/74,300</td>
<td>50/60,420</td>
<td>50/59,340</td>
</tr>
<tr>
<td>489</td>
<td>DNA + protein-CpG</td>
<td>50/15,120</td>
<td>50/72,190</td>
<td>50/30,000</td>
<td>50/70,570</td>
</tr>
<tr>
<td>496</td>
<td>DNA + protein-CpG</td>
<td>50/12,790</td>
<td>50/26,090</td>
<td>50/22,940</td>
<td>50/12,930</td>
</tr>
<tr>
<td>498</td>
<td>Proteins-CpG</td>
<td>50/7,820</td>
<td>50/27,090</td>
<td>50/11,630</td>
<td>50/7,900</td>
</tr>
<tr>
<td>499</td>
<td>Proteins-CpG</td>
<td>50/12,260</td>
<td>103/62,980</td>
<td>50/14,050</td>
<td>50/13,100</td>
</tr>
<tr>
<td>500</td>
<td>Proteins-CpG</td>
<td>50/14,090</td>
<td>50/21,970</td>
<td>50/9,190</td>
<td>50/7,290</td>
</tr>
<tr>
<td>501</td>
<td>Proteins-CpG</td>
<td>50/20,760</td>
<td>50/56,660</td>
<td>50/26,410</td>
<td>50/21,230</td>
</tr>
<tr>
<td>490</td>
<td>Proteins + Alum</td>
<td>50/14,060</td>
<td>50/25,300</td>
<td>50/3,630</td>
<td>50/14,240</td>
</tr>
<tr>
<td>481</td>
<td>Proteins + Alum</td>
<td>50/18,620</td>
<td>50/69,050</td>
<td>50/5,810</td>
<td>12/36,840</td>
</tr>
<tr>
<td>497</td>
<td>DNA</td>
<td>50/10,660</td>
<td>50/7,880</td>
<td>50/2,380</td>
<td>50/6,160</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sera from all animals were collected before immunization and 1 wk before challenge exposure. Values were calculated from duplicate assays.

### Table II. Humoral immune response elicited by vaccination<sup>a</sup>

<table>
<thead>
<tr>
<th>Macaque</th>
<th>Treatment</th>
<th>Binding Ab Titers to Monkeypox Proteins</th>
<th>Prevaccination/Prechallenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L1Ro</td>
<td>B5Ro</td>
</tr>
<tr>
<td>479</td>
<td>DNA</td>
<td>50/50</td>
<td>50/50</td>
</tr>
<tr>
<td>488</td>
<td>DNA</td>
<td>50/50</td>
<td>50/50</td>
</tr>
<tr>
<td>491</td>
<td>DNA</td>
<td>50/50</td>
<td>127/180</td>
</tr>
</tbody>
</table>

<sup>a</sup> Titers of ELISA-binding Abs in monkey sera before vaccination and 1 wk before challenge exposure. Values were calculated from duplicate assays.
FIGURE 3. CD4⁺ and CD8⁺ T cell responses. A and B, Representative data from blood of animals 482, 498, and 481 at 2 wk after the last immunization. C, Percentage of CD4⁺ and CD8⁺ T cells that express IL-2 or IFN-γ and TNF-α after stimulation with the peptide pools derived from the A27Lo, A33Ro, B5Ro, and L1Ro proteins at weeks 0 and 3 (postimmunization) before challenge exposure to monkeypox virus. The columns represent the mean percentage of positive cells after subtraction of the background, with error bars extending 1 SD. Values of p were obtained using the Student paired t test, with a two-tailed distribution.
from papules to vesicles to pustules. The animals were euthanized at days 11, 17, and 21 postinfection (Table III). Thus, macaques immunized with subunit vaccines based on four monkeypox proteins with or without DNA priming were protected from a lethal injection of monkeypox virus. Among these groups, however, animals immunized with a combination of DNA and proteins fared much better. Quantitative analysis of the monkeypox DNA genomes in blood demonstrated equivalent exposure to monkeypox in all animals (Table IV). Following exposure, monkeypox genomes were detectable mainly in macaques immunized with DNA only (Table IV), consistent with the overall clinical findings (Table III).

**Correlates of protection**

As we had observed a significant difference between CD4+ T cell responses in the animals that fared better following monkeypox virus exposure, we performed a regression analysis of the percentage of virus-specific CD4+ T cells at the time of monkeypox virus challenge and the number of lesions that developed following exposure. A significant negative correlation was found between the percentage of IL-2-producing and IFN-γ/TNF-α-producing CD4+ T cells and lesion number ($R = -0.71, p = 0.0083; R = -0.68, p = 0.013$, respectively), suggesting the importance of the induction of a Th1 helper response (Fig. 4).

To establish the role of Abs in protection from disease, titers of neutralizing Abs to VACV (measured with the β-Gal assay) and monkeypox as well as binding Ab titers to A27Lo, A33Ro, B5Ro, and L1Ro (Table I) were analyzed with respect to the time of lesion development and the maximum number of lesions. Neutralizing Ab titers to the IMV form of VACV did not correlate with neutralizing Abs to VACV (measured with the β-Gal assay) and $R = 0.68, p = 0.013$, respectively), suggesting the importance of the induction of a Th1 helper response (Fig. 4).

Table IV.  **Viral genomes in blood following monkeypox challenge exposure**

<table>
<thead>
<tr>
<th>Macaque</th>
<th>Treatment</th>
<th>3 min</th>
<th>Monkeypox Virus Genomes/ml Blood (log 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>Group 1</td>
<td>DNA + proteins-CpG</td>
<td>482</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>489</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>496</td>
<td>6.2</td>
</tr>
<tr>
<td>Group 2</td>
<td>Proteins-CpG</td>
<td>498</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>499</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>501</td>
<td>6.1</td>
</tr>
<tr>
<td>Group 3</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>481</td>
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</tr>
<tr>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>491</td>
<td>5.8</td>
</tr>
<tr>
<td>Group 5</td>
<td>CpG + Alum</td>
<td>485</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*The limit of detection is 5000 copies/ml of blood. All prechallenge samples were below detection.*
suggesting that the extent of neutralizing Abs to IMV may contribute to the delay in the appearance of skin lesions. In contrast, the titer of binding Ab induced by vaccination to all four monkeypox proteins inversely correlated with the maximum lesion number (p < 0.01 for all groups, Spearman correlation coefficients ranging from -0.80 to -0.88), suggesting that binding Abs with this specificity participate in the containment of pocks (Fig. 6). Because each animal’s binding Ab titers tended to be high or low across all four proteins (correlation coefficients between pairs of titers ranging from 0.72 to 0.95), each trend in Fig. 6 was adjusted for these correlations in a rank-based partial correlation analysis. The results suggested that the A33Ro titer was the most strongly related to the number of pocks and that the correlation of the B5Ro titer was the most weakly related to the number of pocks, but the limited number of animals may render these findings statistically insignificant.

As we observed a correlation between the extent of binding Abs and severity of disease, we postulated that we should find significant differences in Ab titers in the animals that had mild (<25 pocks) or moderate/severe disease (>25 lesions but <200). Therefore, the animals were divided into two groups according to their lesion number (Table V). A significant difference in mean binding titers to L1Ro, B5Ro, and A27Lo was found between the groups that had mild or moderate/severe disease (Fig. 7). In the case of A33Ro, the difference between the two groups only approached statistical significance. A significant difference was found between the two groups when the titers to all proteins were averaged for each animal and analyzed (p < 0.013), providing further support to the importance of Abs in protecting from lethal monkeypox (Fig. 7). A similar correlation with disease severity was seen when the protein-specific Ab responses were measured in the kinetic ELISA; L1Ro: p < 0.22, B5Ro: p = 0.047, A27Lo: p = 0.028, and A33Ro: p = 0.006.

B cell epitopes recognized by the immunized macaques

To identify B cell epitopes recognized by the immunized macaques, we performed ELISA on the macaque sera using overlapping peptides derived from the amino acid sequence of the monkeypox virus B5Ro, A33Ro, L1Ro, and A27Lo proteins. An example of the raw data for these assays is given for B5Ro, A33Ro, and L1Ro on sera of animals from group 1 (Fig. 8). Two regions were recognized within the B5Ro protein by the sera of protected animals: peptides 12–13 (aa 49–64) located in the short consensus repeat 1 and peptides 60–63 (aa 237–263) that are part of the stalk region (Lyd) adjacent to the transmembrane region Lyd (26). Interestingly, several mAbs able to neutralize the EEV form of VACV have been mapped to these discontinuous linear epitopes (16, 26–28). The amino acid sequence of these peptides is well conserved among several orthopoxviruses, including VACV and variola virus; however, there are 1–2 aa changes in the peptides for every virus evaluated (Fig. 8A).

In the case of A33Ro, a single region spanning peptides 25–29 (aa 97–127) is recognized by the sera of protected animals (Fig. 8B). The N terminus but not the C terminus region of this peptide is highly conserved among orthopoxviruses, including variola. In the case of L1Ro, the sera mainly recognized the regions spanning peptides 18–20 (aa 69–91) and peptides 35–36 (aa 137–155), which are identical with the VACV and variola virus orthologs (Fig. 8C).
Discussion

The live vaccine (Dryvax) that has eradicated smallpox worldwide poses serious side effects in a subset of people with acquired or congenital defects in the immune system. Moreover, the live virus vaccine is infectious and can be transmitted from the vaccinee to close contacts, including children and persons with weakened immune systems. Thus, vaccination was halted in the late 1970s as it was perceived that the risks of vaccination outweighed its benefits, in the absence of a known smallpox threat. Recent sociopolitical changes worldwide, however, have raised concerns about the possibility of a deliberate introduction of smallpox in humans. As Dryvax is a replicating vaccine strain and a mass vaccination with it could result in serious adverse effects for high-risk individuals (29), efforts were devoted to the development of safer attenuated smallpox vaccines. Modified vaccinia virus Ankara (MVA) and NYVAC, both VACV derivatives, have been shown to be safe in immune-compromised macaques (14, 30) and both can protect immune-competent macaques from lethal monkeypox virus challenge (11) (our unpublished results with NYVAC).

There is evidence that the humoral response to vaccination is a necessary and sufficient component of smallpox vaccine-mediated protective immunity. Abs play a pivotal role in protection from monkeypox (14); therefore, live poxvirus vectors may not be needed if a subunit vaccine can elicit Abs that protect macaques against monkeypox. Here, we obtained the proof of principle that indeed it is possible to induce protective Ab responses using rDNA and proteins. Interestingly, DNA alone or proteins alone did not confer acceptable protection, whereas the combination of DNA and proteins did.

The aim of this study was not to model a real vaccine for smallpox or monkeypox, as multiple immunizations are likely impractical, but rather to assess the feasibility of protecting from severe disease using subunit monkeypox vaccines. It has been demonstrated that a DNA vaccine comprised of the VACV A27L, A33R, B5R, and L1R genes administered by gene gun can confer protection from severe monkeypox (10); however, the vaccine-elicited immunity did not fully block viral replication in two of three animals and some pox lesions were still observed. Here, we tested the

Table V. Binding Ab titers to monkeypox correlated with the maximum number of lesions

<table>
<thead>
<tr>
<th>Disease</th>
<th>Macaque</th>
<th>Treatment</th>
<th>Maximum Number of Pocks</th>
<th>Binding Ab Titers to Monkeypox Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>496M</td>
<td>DNA + Proteins-CpG</td>
<td>4</td>
<td>L1Ro 12,790 26,090 A33Ro 12,930 A27Lo</td>
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<td></td>
<td>482M</td>
<td>DNA + Proteins-CpG</td>
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<td>L1Ro 18,200 24,300 A33Ro 19,700 A27Lo</td>
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<td></td>
<td>489M</td>
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<td>L1Ro 15,120 12,790 A33Ro 12,930 A27Lo</td>
</tr>
<tr>
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<td>501M</td>
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<td>L1Ro 20,760 56,660 A33Ro 21,230 A27Lo</td>
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<tr>
<td></td>
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<td>22</td>
<td>L1Ro 18,620 79,050 A33Ro 36,840 A27Lo</td>
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<tr>
<td>Moderate/severe</td>
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<td>31</td>
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</tr>
<tr>
<td></td>
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<td>L1Ro 7,820 27,090 A33Ro 7,900 A27Lo</td>
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<td></td>
<td>499M</td>
<td>Proteins-CpG</td>
<td>37</td>
<td>L1Ro 12,260 62,980 A33Ro 13,100 A27Lo</td>
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<tr>
<td></td>
<td>500M</td>
<td>Proteins-CpG</td>
<td>120</td>
<td>L1Ro 14,000 21,970 A33Ro 7,290 A27Lo</td>
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<tr>
<td></td>
<td>497M</td>
<td>Proteins + Alum</td>
<td>155</td>
<td>L1Ro 10,660 7,880 A33Ro 6,160 A27Lo</td>
</tr>
</tbody>
</table>

* According to the World Health Organization scoring system, monkeys were divided into two groups: animals with mild disease (<25 pocks) and animals with moderate to severe disease (25–200 pocks).
i.m. route of DNA administration using the monkeypox ortholog genes. This route of DNA delivery was poorly immunogenic on its own; however, when the DNA prime was followed by a protein boost, we observed a higher Ab titer against monkeypox protein orthologs (A27Lo, A33Ro, B5Ro, and L1Ro), few lesions (<15), and better protection from disease. Although the viral load in the plasma was under our limit of detection in group I, the appearance of pox lesions indicated that viral dissemination was not completely halted by this immunization regimen. This may be explained either by the relatively high limits of detection or by the fact that the virus reservoirs were in internal organs rather than in the plasma. Thus, strategies to further increase the immunogenicity of these vaccine platforms are needed to achieve full protection from monkeypox infection. In our study, we focused on protection elicited by multiple Ags. We do not know whether one Ag provided most of the protective immunity because of the correlation between titers. Testing separately the protective efficacy of each Ag will be necessary to address the relative contribution of each protein. Several studies in mice have shown the importance of the combination of the four proteins for full protection from vaccinia (10, 15, 23).

Simple vaccines constituted of well-characterized DNA and proteins are amenable to manipulation with specific immune modulators to increase their immunogenicity and efficacy. In contrast, because live poxvirus vectors already express a plethora of cytokines and chemokines, the effects of immunomodulatory approaches may be difficult to dissect. Of note, neither the nonattenuated Dryvax nor the MVA or NYVAC protect immune-deficient animals (CD4⁺ T cells <300) infected with SIV from lethal monkeypox, likely because of a defect in the maturation of high-affinity protective Abs in conditions of CD4⁺ T cell depletion (13). We believe that our demonstration that simple subunit-based vaccines can be protective provides the platform to manipulate the immune response of simple immunogens and generate vaccines for smallpox that are safe and may also confer protective immunity to people with congenital or acquired immune deficiency.

In this study, we demonstrate that a monkeypox-based subunit vaccine elicited sufficient Ab titers to protect against severe monkeypox and that monkeypox proteins can elicit Abs that are cross-reactive with homologous vaccinia proteins. In addition, the data suggest that priming with DNA may provide qualitative differences in the immune response by inducing a better CD4 helper response. Indeed, we observed that the group primed with DNA and immunized with protein (group I) had a higher CD4 helper type I response (Fig. 3C) and, importantly, this response significantly correlated with a fewer number of lesions (Fig. 4). CD4 helpers are important but not sufficient, as we observed that groups that received only DNA showed the same response 2 wk after the last DNA immunization (data not shown). These data are consistent with findings that CD4⁺ T cell depletion following Dryvax vaccination was associated with a small number of pocks, whereas depletion of CD8⁺ T cells was associated with complete protection (14). In mice, both CD4⁺ T cells and Abs have been shown to be important in protection from a VACV challenge (31), and, along the same line, CD4 or MHC class II knockout mice were poorly protected by MVA (32).

Our study further highlights the importance of Abs, as high neutralizing Ab titers of IMV correlated with a delay in the time of appearance of pox lesions and binding Ab titers inversely correlated with the lesion number. Our finding that macaque sera recognized epitopes within the BSR regions short consensus repeat 1 (20–72) and the stalk region (238–275), which are also recognized by mAbs that neutralize EEV and inhibit “comet” formation (16, 26), underscores the importance of these epitopes in halting virus spread in the host. Indeed, the sera of these animals had inhibitory activity in a novel EEV spread inhibition assay (Table II). Recent studies using passive immunization of mice with chimpanzee/human anti-BSR mAb showed a protection of mice intranasally challenged with virulent VACV. The protective mAbs bound to an epitope that maps within the same amino acid stretch of BSR recognized by the sera from our protected animals (33).

Additional experiments will be needed to explore the specificity of Ab response and the full functional spectrum of these Abs. Complementary and/or Ab-dependent cell cytotoxicity may also be involved in protection. Indeed, in one study, individuals vaccinated 15–18 years before the time of testing showed residual immunity only in an Ab-dependent cell cytotoxicity assay (34). Further mapping the epitopes that can mediate Ab-dependent cell cytotoxicity in immunized/protected animals may be instrumental in the identification of specific peptides able to induce a strong, long-lasting protective response.

Monkeypox transmits poorly from person to person and has a lower rate of mortality (4–15%) (1, 3) compared with smallpox (~30%). However, in contrast to smallpox, monkeypox cannot be eradicated. The virus has an unknown animal reservoir and the existence of more virulent strains is plausible. The 2003 U.S. human monkeypox outbreak (5) was the first to be seen outside Africa; however, cases have continued to occur in central Africa in the decades following the cessation of smallpox vaccination. A safe, noninfectious vaccine that confers protective immunity could be used in endemic areas to prevent the suffering caused by this disease.

Acknowledgments
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FIGURE 8. Identification of B cell epitopes. Peptide scans were performed by ELISA for the B5Ro protein (A), A33Ro (B), and L1Ro (C). In the middle of each figure, the amino acid sequence in a single letter amino acid code is given for related orthopoxviruses. On the bottom, B cell-predicted epitopes obtained from the BcePred software (35).
Disclosures
The authors have no financial conflict of interest.

References