Subunit Recombinant Vaccine Protects against Monkeypox[1]

Jean-Michel Heraud,* Yvette Edghill-Smith,*† Victor Ayala,‡ Irene Kalisz,‡ Janie Parrino,§ Vaniambadi S. Kalyanaraman,‡ Jody Manischewitz,¶ Lisa R. King,¶ Anna Hryniewicz,*‖ Christopher J. Trindade,* Meredith Hassett,* Wen-Po Tsai,* David Venzon,# Aysegul Nalca, Monica Vaccari,* Peter Silvera,† Mike Bray,†† Barney S. Graham,§ Hana Golding,¶ Jay W. Hooper,** and Genoveffa Franchini[2]***

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 highly 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)[3] 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

*Animal Models and Retroviral Vaccines Section, National Cancer Institute, Bethesda, MD 20892; †Southern Research Institute, Frederick, MD 21701; ‡Advanced BioScience Laboratories, Kensington, MD 20895; §Vaccine Research Center, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892; ¶Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892; ‖Department of General and Experimental Pathology, Medical University of Bialystok, Bialystok, Poland; #Biostatistics and Data Management Section, National Cancer Institute, Bethesda, MD 20892; **U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702; and ††Biodefense Clinical Research Branch, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892

Received for publication March 13, 2006. Accepted for publication May 27, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research; the National Institute of Allergy and Infectious Diseases Intramural Biodefense Research Award program; and National Institutes of Health Contract N01-AI-15451.

[2] Address correspondence and reprint requests to Dr. Genoveffa Franchini, National Cancer Institute, Building 41, Room D-804, Bethesda, MD 20892-5065. E-mail address: franchig@mail.nih.gov

[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.

0022-1767/06/$02.00

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-induced 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 (Rehydragel HPA; Reheis) or mixed with 2 mg of CpG-B ODN 2006 (TCGTCGTTT TGTCGTTTTGTCGTT) (Coley Pharmaceutical Group) given by the i.m. route. Monkeypox virus (Zaire 79 strain) was administered by the i.v. route 5 wk after the last protein boost (week 35 from the beginning of immunization) at a dose of 5×10^7 PFU to animals from groups 1, 4, and 5. Macaques in groups 2 and 3 were challenged at week 41 with the same dose of the same viral stock. Following challenge, animals were monitored daily for activity, the appearance of skin lesions, and development of lesions through the stages of papule, vesicle, pustule, and scab.

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′-GATGATGCAACTC TATCATGTA-3′) and OPHA-R219 (5′-GTATAATTATCAAAATACAA GACGTC-3′) and the probe OPHA-p143S-MGB (5′-FAMAGTGCTTGG TATAAGGAGMGBNFQ-3′) were selected from the hemagglutinin gene (GenBank no. L22579; ORF J7R). Although the primers were synthesized by Invitrogen Life Technologies, the TaqMan probe was synthesized by Applied Biosystems and contained FAM in the 5′ end and a nonfluorescent quencher and a minor groove binder in the 3′ end.

The 5′ nuclease PCR and amplification conditions were performed using Platinum *Taq*DNA polymerase (Invitrogen Life Technologies). All reactions included at least one positive control that had 100 copies of cloned target DNA and one no-template control. The positive control for each run established the threshold cycle (Ct) value for positivity. 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 Dynport 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 1×10^5 HeLa cells/well (five replicates per Ab dilution). Plates were incubated for an additional 16 h at 37°C in a humidified CO_2 incubator. Cells were then lysed with the detergent Igepal CA630 (Sigma-Aldrich). In the second stage of the assay, β-Gal enzymatic activity in each well was measured using 96-well Immunlon 2 plates (Thermo Labsystems). Each plate included a β-Gal standard curve using a rβ-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 Na_2CO_3 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 percentage 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 plasma vs log dilutions. The equation of each curve was used to calculate the ID_{50}.

The plaque reduction assays were performed essentially as described previously (17); however, BSC-1 cells and a semisolid overlay were used. Briefly, VACV strain IHD-J or monkeypox virus Zaire 79 was diluted in complete medium (Earle's MEM containing 5% heat-inactivated FBS, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin), and 10 mM HEPES) to give ~500 PFU/ml. Aliquots of this viral suspension (100 μl) were incubated with an equal volume of serum diluted in complete medium (serum samples were heat activated, 56°C for 30 min, before dilution) for 1 h at 37°C and then 180 μl of sample was adsorbed to confluent BSC-1 cell monolayers in 6-well plates for 1 h in a 37°C 5% CO_2 incubator. A 2-ml semisolid overlay (Earle's basal MEM, 1.5% methyl cellulose, 5% heat-inactivated FBS, antibiotics) was added to each well. Plates were incubated in a 37°C 5% CO_2 incubator for 4 days for VACV or 6 days for monkeypox virus. Cell 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.

EEV spread inhibition assay

This assay is similar to a comet inhibition assay; however, a semisolid overlay is added after monkeypox virus EEV have been 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 in a 37°C 5% CO_2 incubator. Unbound virus was removed by rinsing once with 2 ml of complete medium. Monkey serum serial-diluted in 1.5 ml of complete medium was then added to duplicate wells. A no-inhibition control plate was overlaid with complete medium containing no serum, and a no-spread control plate was overlaid with semisolid overlay. The plates were incubated in a 37°C 5% CO_2 incubator for 48 h to allow EEV to release from infected cells. The liquid overlay was then removed and 2 ml of semisolid overlay was added to each well to prevent additional EEV spread. After an additional 2 days (5 days after virus adsorption), the monolayers were stained with 1 ml of crystal violet staining solution. Plaques were then counted. EEV spread was determined by subtracting the average number of plaques in the no-spread control wells from the number of plaques in the no-inhibition control wells. EEV spread inhibition titers represent the reciprocal of the highest serum dilution that inhibited EEV spread by 60%.

Plasmid production and bacterial expression

The monkeypox orthologs of the *VACV A27L*, *A33R*, *B5R*, and *L1R* 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, A33Ro, B5Ro, and L1Ro, respectively, where the "o" 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 *A29L*, *A35R*, *B6R*, and *M1R* genes pMPOX/A27Lo (where the o refers to ortholog), pMPOX/A33Ro, pMPOX/B5Ro, and pMPOX/L1Ro, respectively, have been described previously (23).

The monkeypox genes were subcloned from the aforementioned DNA vaccine plasmids into prokaryotic expression vectors to produce recombinant proteins from *Escherichia coli*. The monkeypox gene *A29L* (GenBank no. AY160186) was PCR-amplified from DNA plasmid pMPOX/A27Lo using Hot Star *Taq* Polymerase (Qiagen) and PCR primers made by Invitrogen Life Technologies (5′GATATACATATGGACGGAAACTCTTT

TCCCCGGAGAT-3′, 5′CTCGAGTGCGGCCGCCTCATAGGGACGCC GTCCAGTCTGTACAT-3′). The gene was digested with the restriction enzymes *Nde*I and *Not*I and directionally cloned into the expression vector pET26b (Novagen), which contains a 6-HIS tag on the C terminus. The monkeypox genes *A33Ro* (GenBank no. AY160188), *B5Ro* (GenBank no. AY160189), and *L1Ro* (GenBank no. AY160187) were constructed in a similar method with the exception that these proteins were cloned to express only their extracellular domains to increase protein expression and facilitate purification without compromising the immunogenic determinants of the extracellular domain. The monkeypox A33RoΔTM gene was amplified from N (60)-T (181) using the primers 5′-GATATA CATATGAATCAATGCATGTCTGCTAACG-3′ and 5′-CTCGAGT GCGGCCGCTGTACAAAAATACTTTCTAACTTCTTGTGATACAT-3′. The monkeypox *B5RoΔTM* gene was amplified from M (1)-H (279) using the primers 5′-GAGATA TATACATATGAAAACGATTTCCGT TGTTACGTTGTTATG-3′ and 5′-GCTCGAGTGCGGCCGCATGATA AGTTGCTTCTAACGATTCT-3′. The monkeypox *L1RoΔTM* gene was amplified from A (3)-Q (185) using the primers 5′-GAGATATACATAT GGCAGCAAGCATACAGACGACTGTGAA-3′ and 5′-GTCGAGTGC GGCCGCAAACTGAACTCCTGTACCAGCAACTT-3′. The extracellular domains were determined using the software TM Predict (⟨www.ch.embnet.org⟩), which was also used for comparison of structural similarities to VACV proteins. Plasmid constructs were confirmed by enzyme restriction digestion and by PCR.

The bacterial expression plasmids pETMPOX/A27Lo, pETMPOX/A33RoΔTM, pETMPOX/B5RoΔTM, and pETMPOX/L1RoΔTM were transformed into the expression host cells BL21(DE3) (Novagen). Cells were grown in LB-Kan (50 μg/ml) at 37°C to a cell density of OD_{600} = 0.6–1. Cells were induced with 1 mM isopropyl β-D-thiogalactoside for 2 h at 37°C. The recombinant monkeypox proteins were named using the nomenclature for the equivalent VACV proteins. The monkeypox A27o protein was expressed as a soluble protein. The monkeypox A33Ro, B5Ro, and L1Ro proteins were expressed in inclusion bodies. The cells were harvested by centrifugation at 5000 × *g* for 15 min and stored at −70°C until purification.

Protein purification

The bacterial cell pellet for monkeypox A27Lo was chemically lysed with buffer A (50 mM Tris-HCL (pH 7.5), 300 mM NaCl, 10% sucrose, 10 mM imidazole, 0.1% Triton X-100, 1× protease inhibitors, and 50 μg/ml lysozyme). The cells were resuspended in buffer A and incubated at 21°C for 15 min with gentle rocking. Viscosity of the lysate was reduced by the addition of benzonase (Novagen) at 1 U/μl with further incubation at 21°C for 10 min. The cell lysate was centrifuged at 16,000 × *g* for 45 min to remove the insoluble material. The protein was then bound on the immobilized metal affinity chromatography HIS Select (Sigma-Aldrich). The protein was eluted with 50 and 250 mM imidazole in buffer B (50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol). Imidazole was removed from the protein using a PD10 desalting column (Amersham) and collected with buffer B. Proteins A33Ro, B5Ro, and LIRo were expressed and purified from inclusion bodies using the Protein Refolding kit (Novagen). The cell pellets were chemically lysed similar to monkeypox A27Lo without the addition of imidazole. The inclusion bodies were homogenized, washed three times with wash buffer (20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1% Triton X-100), and resuspended in solubilization buffer (50 mM CAPS, 0.3%N-Lauroylsarcosine, and 0.1 mM DTT (pH 11)) for 15 min at 21°C. The supernatant was clarified by centrifugation at 10,000 × *g* for 10 min. Protein refolding was accomplished through dialysis against 20 mM Tris (pH 8.5) and 0.1 M DTT. Residual detergent was removed from the proteins by anionic exchange on a Dowex column (Bio-Rad). Subsequently, the proteins were bound to the immobilized metal affinity chromatography, HIS Select (Sigma-Aldrich) and were eluted with 50 mM imidazole in buffer B. As before, imidazole was removed, as described earlier. Proteins were confirmed by SDS PAGE and Western blot using NYVAC-positive sera.

Detection of Ab response

ELISA was conducted with *E. coli*-purified monkeypox proteins as a solid-phase bound Ag. 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 $NaHCO_3$ (pH 9.6)). The plates were blocked with postcoating buffer (5% sucrose, 1.25% nonfat dry milk, and 2.5% BSA) for 1 h at 21°C. Serial dilutions of the monkey sera in Dilsim II (BioMerieux) were applied and incubated for 1 h at 37°C. After washing, 1/60,000 peroxidase goat anti-human IgG (Kirkegaard & Perry Laboratories) was applied and the plates were incubated for an additional hour at 37°C followed by washing. The plates were developed by the addition of Enhanced K-Blue (Neogen) and incubated at 21°C for 15 min. The colorimetric assay was stopped by adding 2 N H_2SO_4. Absorbance values were measured at 450 nm on a microtiter plate reader (BioTek). All tested samples were assayed in duplicate. Results of the ELISA were expressed as endpoint titers, calculated as the reciprocal values of the lowest dilution with an OD of 0.3, which is the equivalent of twice the OD mean preimmune serum samples at a dilution of 1/50 dilution.

In a procedure modified from Boyce et al. (24) kinetic ELISA was performed coating plates at 50 ng/well (0.05 M Na carbonate buffer (pH 9.6)) with either one of the purified monkeypox proteins listed above or one of four homologous vaccinia proteins (A27L, A33R, B5R, and L1R) produced from baculovirus and obtained from Drs. G. H. Cohen and R. J. Eisenberg at the University of Pennsylvania (Philadelphia, Pennsylvania). Duplicate wells were coated with 100 μl of the diluted Ag, and 100 μl of carbonate buffer was added to an additional well for each sample to be tested. 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 wash 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% CO_2 incubator in RPMI 1640/20% FCS. In a 96-well plate, 10^6 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 CD8β-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 anti-TNF-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. 1*A*) 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. 1*A*). 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 o that stands for ortholog, adjuvanted with CpG. Two additional groups were immunized with the recombinant protein alone either in CpG

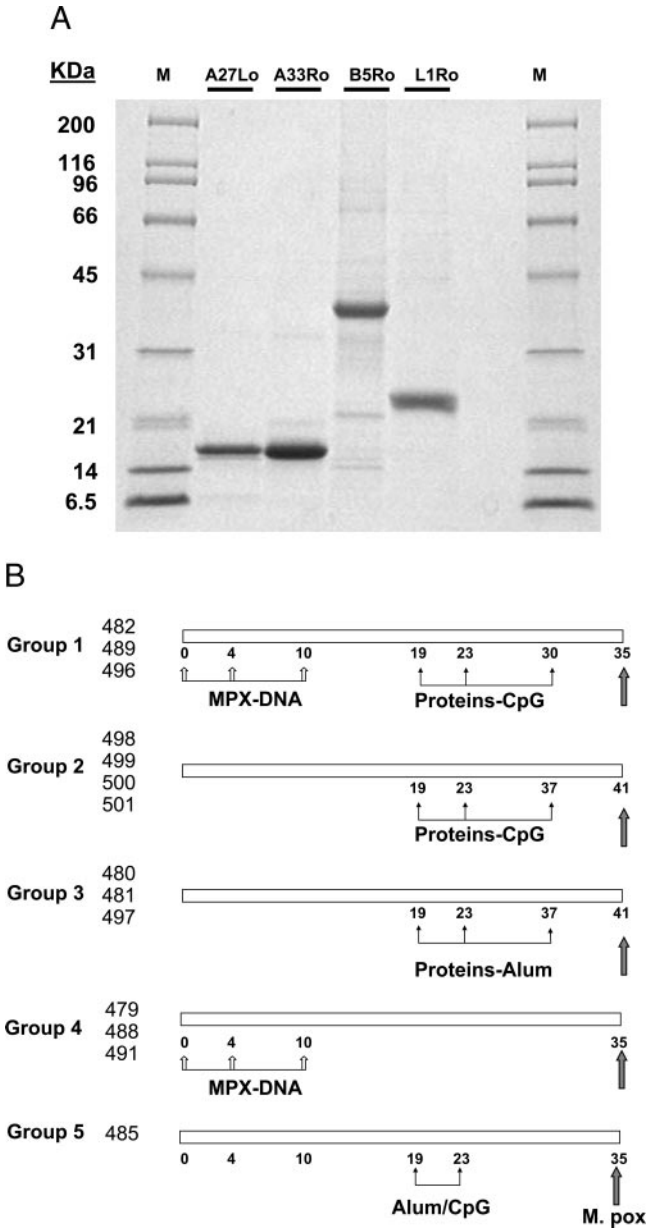

FIGURE 1. *A*, Bacterial expression of the VACV ortholog monkeypox proteins A27Lo, B5Ro, L1Ro, and A33Ro. Coomassie blue staining of recombinant proteins. The bacterial expression plasmids pETMPOX/A27Lo, pETMPOX/B5RoΔTM, pETMPOX/L1RoΔTM, and pETMPOX/A33RoΔTM were transformed into the expression host cells BL21(DE3). The monkeypox virus A27Lo protein was expressed as a soluble protein. Monkeypox B5Ro, L1Ro, and A33Ro proteins were expressed in inclusion bodies. After purification, protein expressions were confirmed by Western blot using VACV-immune sera. *B*, Study design. Each DNA immunization consisted of four plasmids (4 mg each) given i.m. (3 mg) and intradermally (1 mg). Proteins (100 μg each) were either formulated in alum or mixed with 2 mg of CpG class B (TCGTCGTTTTGTCGTTTTCTCGTT) given by the i.m. route. Challenge with monkeypox virus Zaire 79 (5×10^7 PFU) was performed i.v. at the time (in weeks) indicated for each group.

(group 2) or alum (group 3) to assess the relative ability of proteins alone with the two different adjuvants to elicit protective Ab responses. One control animal (in group 5) was mock immunized with a combination of alum and CpG (Fig. 1*B*).

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 dilution or kinetic readout. DNA immunization alone elicited low levels of binding Abs to all four immunogens (Fig. 2*A*). 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. 2*A*). 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 ($p = 0.017$ 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. 2*B*) indicating that inducing monkeypox-specific responses will provide cross-reactive Abs to vaccinia.

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 II) whereas all animals in other groups developed neutralizing Ab titers to VACV and monkeypox virus IMV. Overall, reasonable consistency was found in VACV-neutralizing assays using the β-Gal and the plaque reduction assay (Table II). Importantly, sera positive in the VACV assays also had neutralizing activity to the IMV form of monkeypox ($p = 0.0054$ by the exact Spearman rank correlation method in groups 1–3). Differences in neutralizing Ab titers among groups 1–3 were not significant.

To assess whether immunization with B5Ro and A33Ro, the proteins specific for the EEV form, elicited neutralizing Abs to EEV, we performed a monkeypox EEV spread inhibition assay. With this assay, no inhibition was found in sera from animals immunized with DNA alone, whereas all the sera of animals immunized with the DNA prime/protein boost regimen and some of those immunized with proteins alone inhibited monkeypox virus EEV spread (Table II). The variation across groups 1–3 is significant ($p = 0.021$ by the exact Wilcoxon-Gehan test corrected for multiple comparisons).

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

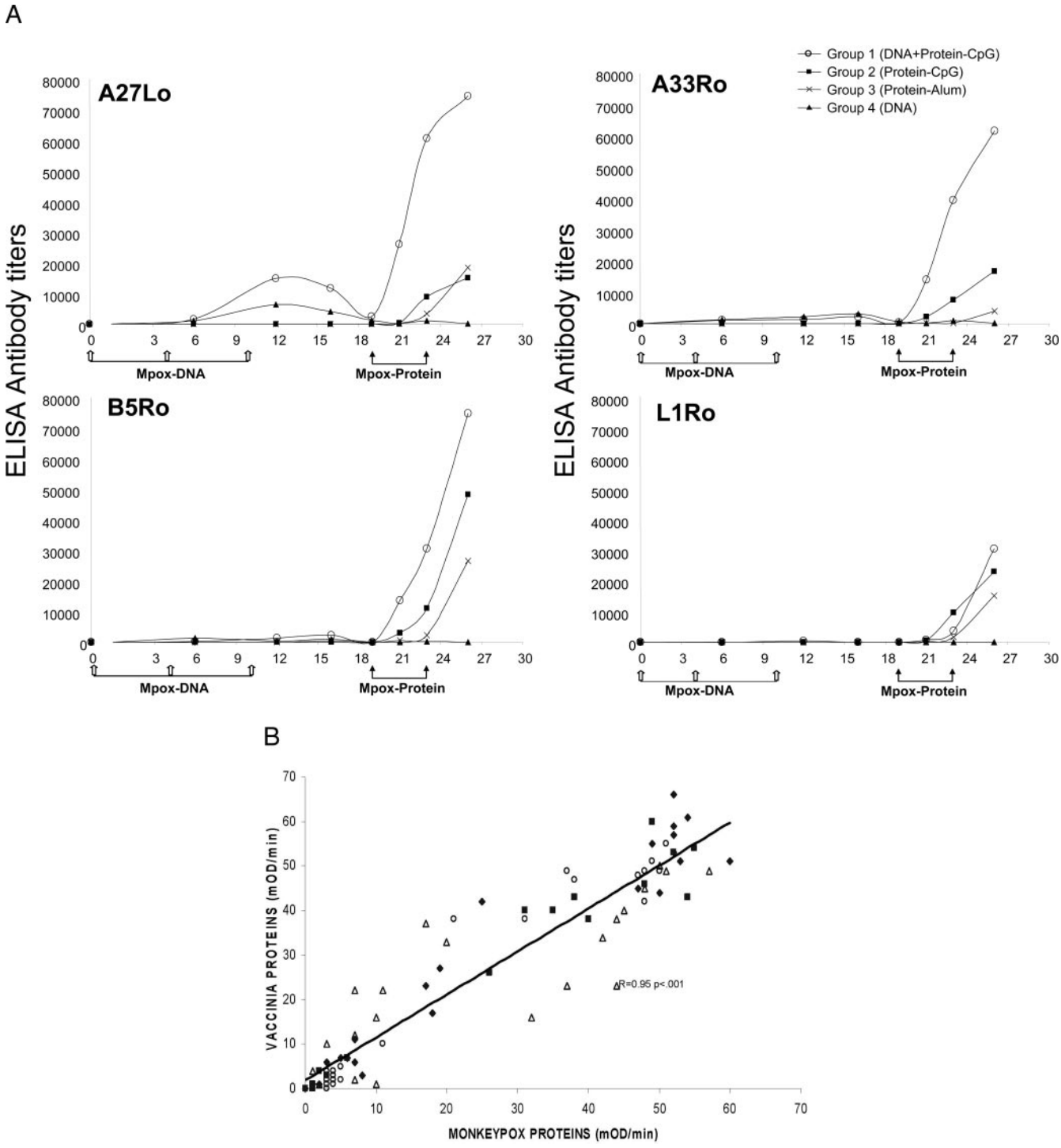

FIGURE 2. *A*, ELISA Ab titers to monkeypox virus-purified proteins. Sequential sera during the immunization regimen were tested to detect the presence of Abs directed against the different monkeypox proteins (A27Lo, A33Ro, B5Ro, and L1Ro). Each data point on the *y*-axis represents the average of the response of all animals in each group. On the *x*-axis, the time of immunization is presented in weeks. *B*, Ab response to monkeypox proteins correlates cross-reactive response to homologous vaccinia proteins. The kinetic ELISA data in mOD per minute for each protein and its ortholog are plotted against each other to demonstrate the significant antigenic cross-reactivity between the homologous proteins from the two distinct orthopoxviruses. ♦, B5R/B5Ro; △, A35R/A35Ro; ○, L1R/L1Ro; ■, A27L/A27Lo.

IFN-γ and TNF-α in the various groups was measured at weeks 0 and 3 before challenge exposure (Fig. 3*B*). 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

Table I. *Humoral immune response in macaques before monkeypox virus challenge*[a]

Macaque	Treatment	Binding Ab Titers to Monkeypox Proteins Prevaccination/Prechallenge			
		L1Ro	B5Ro	A33Ro	A27Lo
Group 1	DNA + protein-CpG				
482		50/17,820	50/74,300	50/60,420	50/59,340
489		50/15,120	50/72,190	50/30,000	50/70,570
496		50/12,790	50/26,090	50/22,940	50/12,930
Group 2	Proteins-CpG				
498		50/7,820	50/27,090	50/11,630	50/7,900
499		50/12,260	103/62,980	50/14,050	50/13,100
500		50/14,000	50/21,970	50/9,190	50/7,290
501		50/20,760	50/56,660	50/26,410	50/21,230
Group 3	Proteins + Alum				
480		50/14,060	50/25,300	50/3,630	50/14,240
481		50/18,620	50/69,050	50/5,810	12/36,840
497		50/10,660	50/7,880	50/2,380	50/6,160
Group 4	DNA				
479		50/50	50/50	50/190	50/80
488		50/50	50/30	50/270	50/60
491		50/50	127/180	50/50	50/240

[a] Titers of ELISA-binding Abs in monkey sera before vaccination and 1 wk before challenge exposure. Values were calculated from duplicate assays.

TNF-α/IFN-γ in $CD4^+$ T cells but not in $CD8^+$ T cells. In some animals, the background level of cytokine production in samples obtained before immunization precluded proper assessment of $CD4^+$ and $CD8^+$ 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 5×10^7 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 II. *Humoral immune response elicited by vaccination*[a]

Macaque	Treatment	Neutralizing Ab Titer to Vaccinia (IMV)			Neutralizing Ab Titer to Monkeypox (IMV)	Neutralizing Ab Titer to Monkeypox (EEV)	
		β-Gal assay		Plaque reduction assay	Plaque reduction assay	Spread reduction assay	
		Prevaccine	Prechallenge	Prechallenge	Prechallenge	Prevaccine	Prechallenge
Group 1	DNA + proteins-CpG						
482		<20	1:214	80	202	<25	100
489		<20	1:237	≥640	640	<25	100
496		<20	1:304	160	202	<25	50
Group 2	Proteins-CpG						
498		<20	1:294	160	80	<25	<25
499		<20	1:325	80	80	<25	<25
500		<20	1:121	80	160	nd	25
501		<20	1:506	320	1280	nd	<25
Group 3	Proteins + Alum						
480		<20	1:1780	160	320	<25	25
481		<20	1:744	320	453	nd	50
497		<20	1:421	≥640	1280	nd	100
Group 4	DNA						
479		<20	1:76	<20	<20	nd	<25
488		<20	1:97	<20	28	nd	<25
491		<20	1:93	<20	<20	nd	<25

[a] Sera from all animals were collected before immunization and 1 wk before challenge exposure to monkeypox virus. In the neutralization assay for VACV IMV with the β-Gal assay, each sample was tested in four replicates at four consecutive dilutions in two independent assays. In the monkeypox IMV plaque reduction assay, the values represent the geometric mean titers (50% neutralization) of two independent experiments. The monkeypox EEV spread inhibition assay titers represent the reciprocal of the highest serum dilution inhibiting the formation of satellite plaque numbers by at least 60% (titers represent results obtained from one assay). Prebleed sera for 482, 489, 496, 498, 499, and 480 were tested, and all exhibited titers <25 (data not shown). nd=not done.

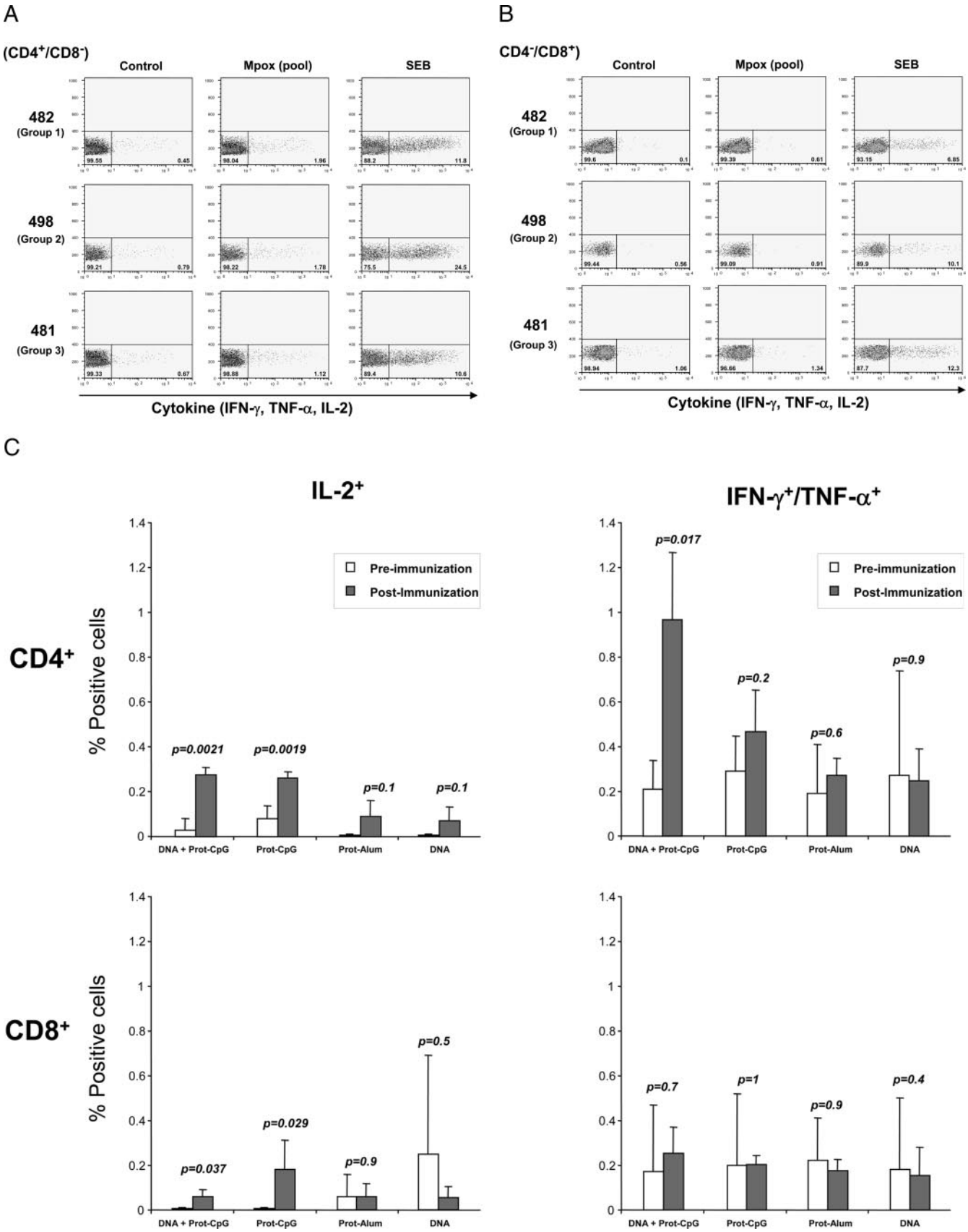

FIGURE 3. $CD4^+$ and $CD8^+$ T cell responses. *A* and *B*, Representative data from blood of animals 482, 498, and 481 at 2 wk after the last immunization. *C*, Percentage of $CD4^+$ and $CD8^+$ T cells that express IL-2 or IFN-γ and TNF-α after stimulation with the peptide pools derived from the A27Lo, 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.

Table III. *Clinical outcome of monkeypox challenge exposure*[a]

Macaque	Treatment	Pox Lesion Count								
		Day 0	Day 2	Day 4	Day 7	Day 9	Day 11	Day 14	Day 17	Day 21
Group 1	DNA + proteins-CpG									
482		0	0	0	6	4	3	0	0	0
489		0	0	0	15	0[a]	0	0	0	0
496		0	0	4	3	3	0	0	0	0
Group 2	Proteins-CpG									
498		0	0	5	37	37	36	31	0[a]	0
499		0	0	2	22	37	37	4	0[a]	0
500		0	0	6	108	120	120	24	0[a]	0
501		0	0	0	14	20	20	1	0[a]	0
Group 3	Proteins + Alum									
480		0	0	0	2	31	31	5	0[a]	0
481		0	0	1	5	13	22	0[a]	0	0
497		0	0	11	148	148	155	5	0[a]	0
Group 4	DNA									
479		0	0	TNTC	TNTC	TNTC	[b]			
488		0	0	6	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
491		0	0	TNTC	TNTC	TNTC	TNTC	[b]		
Group 5	CpG + Alum									
485		0	0	TNTC	TNTC	TNTC	[b]			

[a] Macaques were challenged with 5×10^7 PFU of monkeypox Zaire 79 by the i.v. route. Characteristic pox lesions (whether papular, vesicular, or scabs[a]) were counted on the whole body of each animal. When the number of lesions were too numerous to count (TNTC), and when vital signs were critical, animals were euthanized ([b]).

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 the maximum number of pocks but correlated significantly ($R = 0.72$, $p = 0.034$) with the time of appearance of pocks (Fig. 5),

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

Macaque	Treatment	Monkeypox Virus Genomes/ml Blood (log 10)				
		3 min	Day 2	Day 4	Day 7	Day 9
Group 1	DNA + proteins-CpG					
482		6.4	<3.6	<3.6	<3.6	<3.6
489		5.6	<3.6	<3.6	<3.6	<3.6
496		6.2	<3.6	3.7	<3.6	<3.6
Group 2	Proteins-CpG					
498		5.8	<3.6	<3.6	<3.6	<3.6
499		5.8	<3.6	<3.6	<3.6	<3.6
500		5.5	<3.6	<3.6	<3.6	<3.6
501		6.1	<3.6	<3.6	<3.6	<3.6
Group 3	Proteins + Alum					
480		5.5	<3.6	<3.6	<3.6	<3.6
481		6.1	<3.6	<3.6	<3.6	<3.6
497		5.6	<3.6	<3.6	<3.6	<3.6
Group 4	DNA					
479		6.0	<3.6	4.3	6.3	7.4
488		6.9	<3.6	<3.6	<3.6	7.4
491		5.8	<3.6	3.7	7.4	7.4
Group 5	CpG + Alum					
485		6.5	4.5 (day 3)	5.6 (day 6)	5.6	7.2

[a] The limit of detection is 5000 copies/ml of blood. All prechallenge samples were below detection.

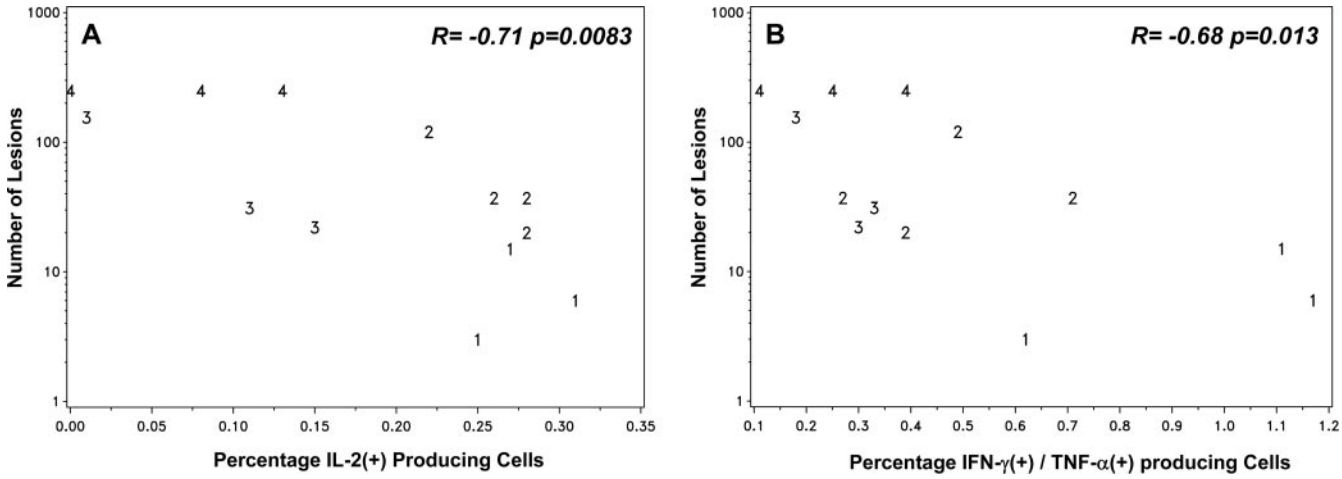

FIGURE 4. Correlates of protection. Inverse correlation between maximum lesion number and monkeypox-specific $CD4^+$ T cells. Regression analysis of the percentage of $CD4^+$ T cell responses and the maximum number of lesions measured as $CD4^+$ T cells producing IL-2 (*A*) or TNF-α and IFN-γ (*B*). Each number refers to data from animals of groups 1–4. Lesions too numerous to count were assigned the value 250 for plotting.

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: $p = 0.006$.

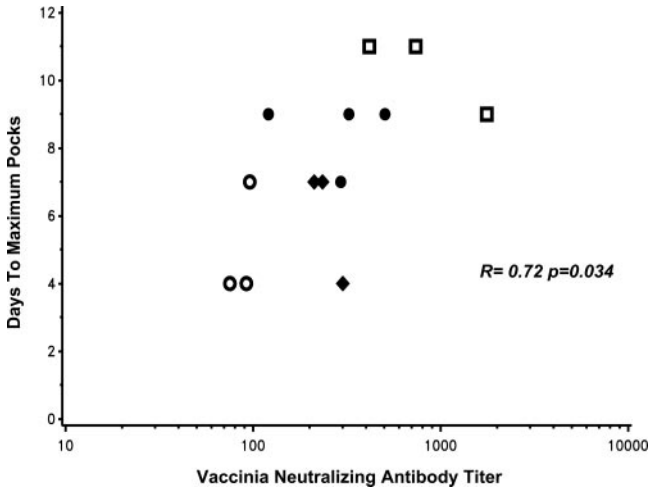

FIGURE 5. Regression analysis of IMV VACV neutralizing Ab titers and time to maximum number of pox lesions. ○, Animal from the group immunized with DNA. ●, Animal from the group immunized with protein plus CpG. □, Animal from the group immunized with protein plus alum. ♦, Animal from the group immunized with DNA plus proteins.

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 immunized and 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. 8*A*).

In the case of A33Ro, a single region spanning peptides 25–29 (aa 97–127) is recognized by the sera of protected animals (Fig. 8*B*). 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. 8*C*).

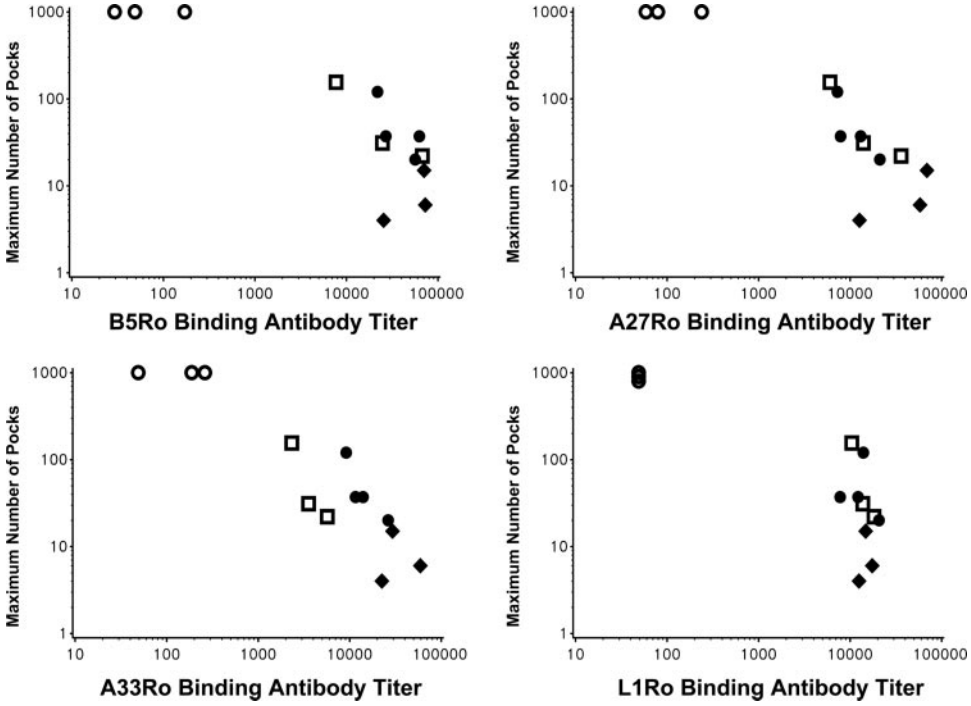

FIGURE 6. Regression analysis of maximum number of pocks and Ab titers to L1Ro, B5Ro, A33Ro, and A27Lo. ○, Animal from the group immunized with DNA. ●, Animal from the group immunized with protein plus CpG. □, Animal from the group immunized with protein plus alum. ◆, Animal from the group immunized with DNA plus proteins. Lesions too numerous to count were assigned the value 1000 for plotting.

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*[a]

Disease	Macaque	Treatment	Maximum Number of Pocks	Binding Ab Titers to Monkeypox Proteins			
Mild				L1Ro	B5Ro	A33Ro	A27Lo
	496M	DNA + Proteins-CpG	4	12,790	26,090	22,940	12,930
	482M	DNA + Proteins-CpG	6	17,820	74,300	60,420	59,340
	489M	DNA + Proteins-CpG	15	15,120	72,190	30,000	70,570
	501M	Proteins-CpG	20	20,760	56,660	26,410	21,230
	481M	Proteins + Alum	22	18,620	69,050	5,810	36,840
Moderate/severe							
	480M	Proteins + Alum	31	14,060	25,300	3,630	14,240
	498M	Proteins-CpG	37	7,820	27,090	11,630	7,900
	499M	Proteins-CpG	37	12,260	62,980	14,050	13,100
	500M	Proteins-CpG	120	14,000	21,970	9,190	7,290
	497M	Proteins + Alum	155	10,660	7,880	2,380	6,160

[a] 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).

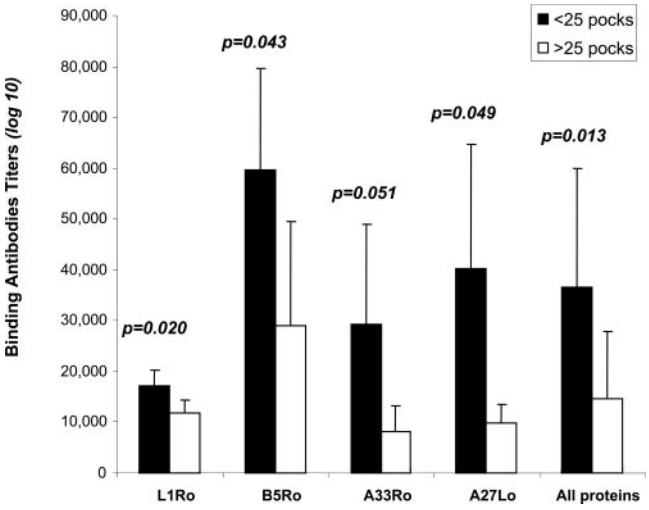

FIGURE 7. Sera from animals 1 wk before challenge were tested for the presence of binding Abs to different proteins (L1Ro, B5Ro, A33Ro, and A27Lo). According to the World Health Organization scoring system, monkeys were divided into two groups: animals with mild disease (<25 pocks) and animals with moderate to severe disease (25–200 pocks). Each column represents the average of the response of the animals in each group to each protein or to the average by animal over all proteins, with error bars extending one SD. Values of p were obtained using the t test with Satterthwaite's approximation for unequal variances.

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 1, 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 1) had a higher CD4 helper type 1 response (Fig. 3*C*) 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 B5R 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-B5R 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 B5R 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

We thank Steven Snodgrass (National Cancer Institute (NCI)) for editorial assistance and David Liewehr (NCI) for help with statistics. Thanks to Man Chen and Martha Nason (National Institute of Allergy and Infectious Diseases).

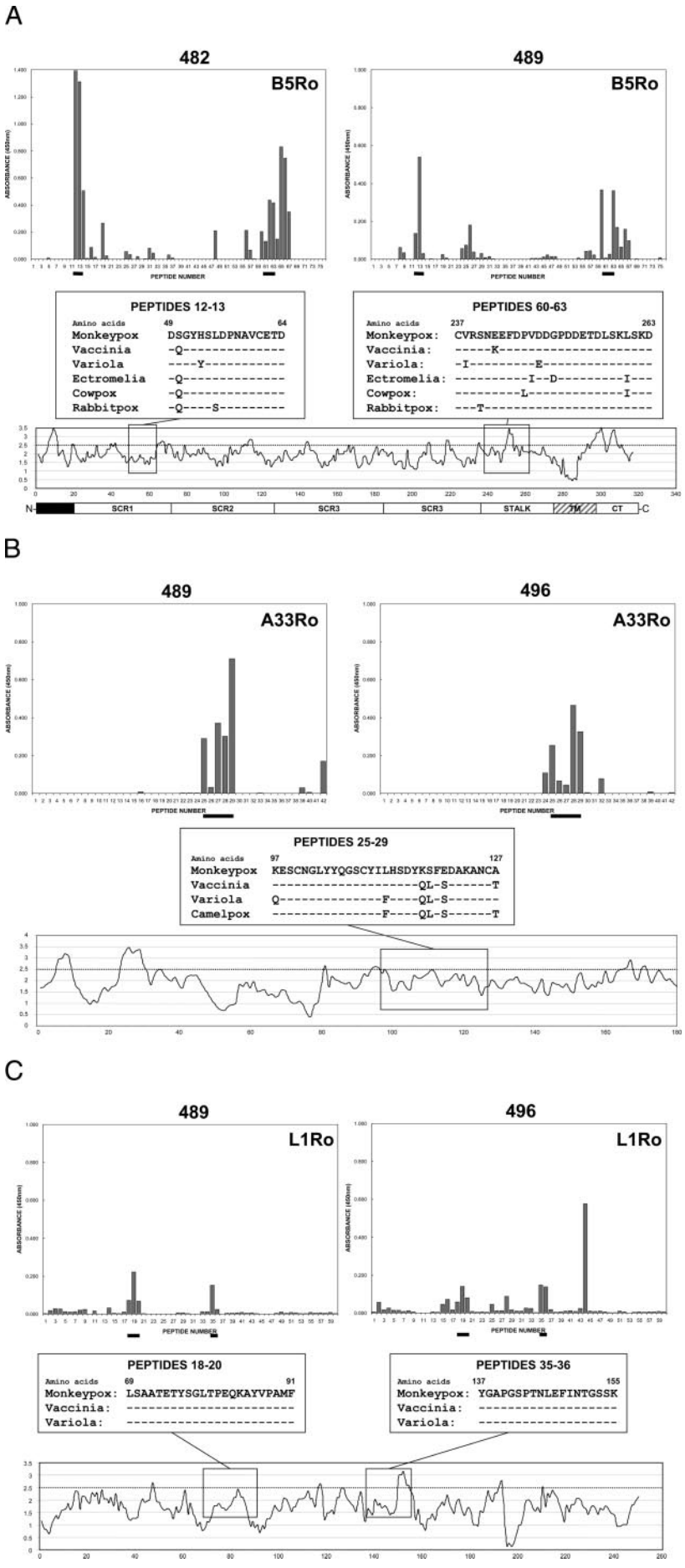

FIGURE 8. Identification of B cell epitopes. Peptide scans were performed by ELISA for the B5Ro protein (*A*), A33Ro (*B*), and L1Ro (*C*). In the middle of each figure, the amino acid sequence in a single letter amino acid code is given for related orthopoxviruses. On the bottom, B cell-predicted epitopes obtained from the BcePred software (35).

Disclosures

The authors have no financial conflict of interest.

References

1. Jezek, Z., S. S. Marennikova, M. Mutumbo, J. H. Nakano, K. M. Paluku, and M. Szczeniowski. 1986. Human monkeypox: a study of 2,510 contacts of 214 patients. *J. Infect. Dis.* 154: 551–554.
2. Jezek, Z., M. Szczeniowski, K. M. Paluku, and M. Mutombo. 1987. Human monkeypox: clinical features of 282 patients. *J. Infect. Dis.* 156: 293–294.
3. Hutin, Y. J., R. J. Williams, P. Malfait, R. Pebody, V. N. Loparev, S. L. Ropp, M. Rodriguez, J. C. Knight, F. K. Tshioko, A. S. Khan, et al. 2001. Outbreak of human monkeypox: Democratic Republic of Congo, 1996 to 1997. *Emerg. Infect. Dis.* 7: 434–438.
4. Meyer, H., M. Perrichot, M. Stemmler, P. Emmerich, H. Schmitz, F. Varaine, R. Shungu, F. Tshioko, and P. Formenty. 2002. Outbreaks of disease suspected of being due to human monkeypox virus infection in the Democratic Republic of Congo in 2001. *J. Clin. Microbiol.* 40: 2919–2921.
5. Centers for Disease Control. 2003. Multistate outbreak of monkeypox–Illinois, Indiana, and Wisconsin, 2003. *Morb. Mortal. Wkly. Rep.* 52: 537–540.
6. Nalca, A., A. W. Rimoin, S. Bavari, and C. A. Whitehouse. 2005. Reemergence of monkeypox: prevalence, diagnostics, and countermeasures. *Clin. Infect. Dis.* 41: 1765–1771.
7. Hammarlund, E., M. W. Lewis, S. V. Carter, I. Amanna, S. G. Hansen, L. I. Strelow, S. W. Wong, P. Yoshihara, J. M. Hanifin, and M. K. Slifka. 2005. Multiple diagnostic techniques identify previously vaccinated individuals with protective immunity against monkeypox. *Nat. Med.* 11: 1005–1011.
8. Centers for Disease Control. 2001. Vaccinia (smallpox) vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2001. *Morb. Mortal. Wkly. Rep.* 50: 1–26.
9. Centers for Disease Control. 2003. Supplemental recommendations on adverse events following smallpox vaccine in the pre-event vaccination program: recommendations of the Advisory Committee on Immunization Practices. *Morb. Mortal. Wkly. Rep.* 52: 282–284.
10. Hooper, J. W., E. Thompson, C. Wilhelmsen, M. Zimmerman, M. A. Ichou, S. E. Steffen, C. S. Schmaljohn, A. L. Schmaljohn, and P. B. Jahrling. 2004. Smallpox DNA vaccine protects nonhuman primates against lethal monkeypox. *J. Virol.* 78: 4433–4443.
11. Earl, P. L., J. L. Americo, L. S. Wyatt, L. A. Eller, J. C. Whitbeck, G. H. Cohen, R. J. Eisenberg, C. J. Hartmann, D. L. Jackson, D. A. Kulesh, et al. 2004. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. *Nature* 428: 182–185.
12. Edghill-Smith, Y., D. Venzon, T. Karpova, J. McNally, J. Nacsa, W. P. Tsai, E. Tryniszewska, M. Moniuszko, J. Manischewitz, L. R. King, et al. 2003. Modeling a safer smallpox vaccination regimen, for human immunodeficiency virus type 1-infected patients, in immunocompromised macaques. *J. Infect. Dis.* 188: 1181–1191.
13. Edghill-Smith, Y., M. Bray, C. A. Whitehouse, D. Miller, E. Mucker, J. Manischewitz, L. R. King, M. Robert-Guroff, A. Hryniewicz, D. Venzon, et al. 2005. Smallpox vaccine does not protect macaques with AIDS from a lethal monkeypox virus challenge. *J. Infect. Dis.* 191: 372–381.
14. Edghill-Smith, Y., H. Golding, J. Manischewitz, L. R. King, D. Scott, M. Bray, A. Nalca, J. W. Hooper, C. A. Whitehouse, J. E. Schmitz, et al. 2005. Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. *Nat. Med.* 11: 740–747.
15. Fogg, C., S. Lustig, J. C. Whitbeck, R. J. Eisenberg, G. H. Cohen, and B. Moss. 2004. Protective immunity to vaccinia virus induced by vaccination with multiple recombinant outer membrane proteins of intracellular and extracellular virions. *J. Virol.* 78: 10230–10237.
16. Galmiche, M. C., J. Goenaga, R. Wittek, and L. Rindisbacher. 1999. Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. *Virology.* 254: 71–80.
17. Hooper, J. W., D. M. Custer, C. S. Schmaljohn, and A. L. Schmaljohn. 2000. DNA vaccination with vaccinia virus L1R and A33R genes protects mice against a lethal poxvirus challenge. *Virology* 266: 329–339.
18. Fang, M., H. Cheng, Z. Dai, Z. Bu, and L. J. Sigal. 2006. Immunization with a single extracellular enveloped virus protein produced in bacteria provides partial protection from a lethal orthopoxvirus infection in a natural host. *Virology* 345: 231–243.
19. Smith, G. L., and M. Law. 2004. The exit of vaccinia virus from infected cells. *Virus Res.* 106: 189–184.
20. Kulesh, D. A., R. O. Baker, B. M. Loveless, D. Norwood, S. H. Zwiers, E. Mucker, C. Hartmann, R. Herrera, D. Miller, D. Christensen, et al. 2004. Smallpox and pan-orthopox virus detection by real-time 3′-minor groove binder TaqMan assays on the Roche LightCycler and the Cepheid Smart Cycler platforms. *J. Clin. Microbiol.* 42: 601–609.
21. Manischewitz, J., L. R. King, N. A. Bleckwenn, J. Shiloach, R. Taffs, M. Merchlinsky, N. Eller, M. G. Mikolajczyk, D. J. Clanton, T. Monath, et al. 2003. Development of a novel vaccinia-neutralization assay based on reporter-gene expression. *J. Infect. Dis.* 188: 440–448.
22. Chakrabarti, S., J. R. Sisler, and B. Moss. 1997. Compact, synthetic, vaccinia virus early/late promoter for protein expression. *BioTechniques* 23: 1094–1097.
23. Hooper, J. W., D. M. Custer, and E. Thompson. 2003. Four-gene-combination DNA vaccine protects mice against a lethal vaccinia virus challenge and elicits appropriate antibody responses in nonhuman primates. *Virology* 306: 181–195.
24. Boyce, T. G., W. C. Gruber, S. D. Coleman-Dockery, E. C. Sannella, G. W. Reed, M. Wolff, and P. F. Wright. 1999. Mucosal immune response to trivalent live attenuated intranasal influenza vaccine in children. *Vaccine* 18: 82–88.
25. Zaucha, G. M., P. B. Jahrling, T. W. Geisbert, J. R. Swearengen, and L. Hensley. 2001. The pathology of experimental aerosolized monkeypox virus infection in cynomolgus monkeys (*Macaca fascicularis*). *Lab Invest.* 81: 1581–1600.
26. Aldaz-Carroll, L., J. C. Whitbeck, M. Ponce de Leon, H. Lou, L. Hirao, S. N. Isaacs, B. Moss, R. J. Eisenberg, and G. H. Cohen. 2005. Epitope-mapping studies define two major neutralization sites on the vaccinia virus extracellular enveloped virus glycoprotein B5R. *J. Virol.* 79: 6260–6271.
27. Engelstad, M., S. T. Howard, and G. L. Smith. 1992. A constitutively expressed vaccinia gene encodes a 42-kDa glycoprotein related to complement control factors that forms part of the extracellular virus envelope. *Virology* 188: 801–810.
28. Law, M., and G. L. Smith. 2001. Antibody neutralization of the extracellular enveloped form of vaccinia virus. *Virology* 280: 132–142.
29. Kemper, A. R., M. M. Davis, and G. L. Freed. 2002. Expected adverse events in a mass smallpox vaccination campaign. *Eff. Clin. Pract.* 5: 84–90.
30. Osterhaus, A. D., C. A. van Baalen, R. A. Gruters, M. Schutten, C. H. Siebelink, E. G. Hulskotte, E. J. Tijhaar, R. E. Randall, G. van Amerongen, A. Fleuchaus, et al. 1999. Vaccination with Rev and Tat against AIDS. *Vaccine* 17: 2713–2714.
31. Snyder, J. T., I. M. Belyakov, A. Dzutsev, F. Lemonnier, and J. A. Berzofsky. 2004. Protection against lethal vaccinia virus challenge in HLA-A2 transgenic mice by immunization with a single $CD8^+$ T-cell peptide epitope of vaccinia and variola viruses. *J. Virol.* 78: 7052–7060.
32. Wyatt, L. S., P. L. Earl, L. A. Eller, and B. Moss. 2004. Highly attenuated smallpox vaccine protects mice with and without immune deficiencies against pathogenic vaccinia virus challenge. *Proc. Natl. Acad. Sci. USA* 101: 4590–4595.
33. Chen, Z., P. Earl, J. Americo, I. Damon, S. K. Smith, Y. H. Zhou, F. Yu, A. Sebrell, S. Emerson, G. Cohen, et al. 2006. Chimpanzee/human mAbs to vaccinia virus B5 protein neutralize vaccinia and smallpox viruses and protect mice against vaccinia virus. *Proc. Natl. Acad. Sci. USA* 103: 1882–1887.
34. Moller-Larsen, A., and S. Haahr. 1978. Humoral and cell-mediated immune responses in humans before and after revaccination with vaccinia virus. *Infect. Immun.* 19: 34–39.
35. Saha, S., and G. P. S. Raghava. 2004. BcePred: Prediction of continuous B-cell epitopes in antigenic sequences using physico-chemical properties. In *ICARIS 2004, LNCS 3239.* G. Nicosia, V. Cutello, P. J. Bentley, and J. Timis, eds. Springer, Berlin, pp. 197–204.