

Safety and Immunogenicity of Modified Vaccinia Ankara (ACAM3000): Effect of Dose and Route of Administration

Marissa B. Wilck,^{1,5} Michael S. Seaman,^{2,5} Lindsey R. Baden,^{1,5} Stephen R. Walsh,^{3,5} Lauren E. Grandpre,² Colleen Devoy,² Ayush Giri,² Jane A. Kleinjan,¹ Lizanne C. Noble,¹ Kristen E. Stevenson,⁴ Haesook T. Kim,⁴ and Raphael Dolin^{1,2,5}

¹Division of Infectious Diseases, Brigham and Women's Hospital, ²Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, ³Division of Infectious Diseases, Massachusetts General Hospital, ⁴Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, and ⁵Department of Medicine, Harvard Medical School, Boston, Massachusetts

(See the article by Seaman et al, on pages 1353–1360.)

Background. We conducted a clinical trial of the safety and immunogenicity of modified vaccinia Ankara (MVA) to examine the effects of dose and route of administration.

Methods. Seventy-two healthy, vaccinia virus-naïve subjects received 1 of 6 regimens of MVA (ACAM3000) or placebo consisting of 2 administrations given 1 month apart.

Results. MVA was generally well tolerated at all dose levels and by all routes. More pronounced local reactogenicity was seen with the intradermal and subcutaneous routes than with intramuscular administration. Binding antibodies to whole virus and neutralizing antibodies to the intracellular mature virion and extracellular enveloped virion forms of vaccinia virus were elicited by all routes of MVA administration and were greater for the higher dose by each route. Similar levels of neutralizing antibodies were seen at a 10-fold-lower dose given intradermally (1×10^7 median tissue culture infective doses [TCID₅₀]), compared with responses after 1×10^8 TCID₅₀ given intramuscularly or subcutaneously. T cell immune responses to vaccinia virus were detected by an interferon γ enzyme-linked immunospot assay but had no clear relationship to dose or route.

Conclusions. These data suggest that intradermal immunization with MVA provides a dose-sparing effect by eliciting antibody responses similar in magnitude and kinetics to those elicited by the intramuscular or subcutaneous routes but at a 10-fold-lower dose.

Trial registration. ClinicalTrials.gov identifier: NCT00133575.

An effective vaccination program led by the World Health Organization eradicated smallpox in 1980 [1, 2]. Despite this extraordinary achievement, the immunologic basis for the efficacy of vaccination against smallpox remains incompletely understood, and cor-

relates of protection are not fully defined. Furthermore, despite its effectiveness the use of smallpox immunization with strains of vaccinia virus such as Dryvax can be associated with significant morbidity, particularly in subjects with certain host defense defects and dermatopathologic conditions [3, 4]. Therefore, the development of safer yet efficacious vaccines for future use against smallpox remains of considerable interest.

We studied an attenuated strain of vaccinia virus, modified vaccinia Ankara (MVA) [5–7], which has been reported to be less reactogenic than widely used vaccinia virus strains (such as Dryvax) and yet possibly confers a degree of protection against orthopoxvirus infections (such as those caused by variola virus). MVA is severely host restricted and either is unable to replicate in mammalian cell lines or replicates at a very low level (<2 plaque-forming units [PFUs] per cell) [8–10].

The optimal regimen of immunization with MVA is

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Reprints or correspondence: Dr Raphael Dolin, Beth Israel Deaconess Medical Center, 330 Brookline Ave, E/CLS-1003, Boston, MA 02215 (rdolin@bidmc.harvard.edu).

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not known. Therefore, we conducted a clinical study of immunization with MVA to examine the effects of dose and route of administration on tolerability and immune responses. We were particularly interested in exploring the intradermal route of administration, because it has been associated with levels of immune responses similar to those elicited by higher doses administered subcutaneously or intramuscularly for several vaccines, thus resulting in a potential dose-sparing effect [11–15].

METHODS

Vaccine

The MVA vaccine used in this study was ACAM3000 (lot no. 460304KA; Acambis), formulated with 20 mmol/L Tris, 0.9% NaCl (US Pharmacopeia), and 0.01% neomycin (US Pharmacopeia) at a titer of 2.54×10^8 median tissue culture infective doses (TCID₅₀)/mL. The vaccine was reconstituted with 0.9% NaCl and diluted to the appropriate dose. A sterile saline solution (0.9% NaCl) was used as placebo. The dose of MVA administered was verified by back titration for each dose tier that was studied.

Study Design and Subjects

The study design was a dose escalation of MVA administered intramuscularly at doses of 1×10^7 or 1×10^8 TCID₅₀, subcutaneously at 1×10^7 or 1×10^8 TCID₅₀, or intradermally at 1×10^6 or 1×10^7 TCID₅₀ (or placebo) given in a 2-dose regimen on day 0 and day 28 (1×10^7 TCID₅₀ was the maximum dose that could be given intradermally because of the volume [0.1 mL] that could be administered by that route) (Table 1). Study preparation or placebo was administered under a randomized, double-blind allocation. Subjects were healthy men or women who were at least 18 years of age, were born after 1971, and had no history of smallpox vaccination. Good health was determined on the basis of history, physical examination, and laboratory tests.

Twelve subjects were enrolled sequentially into 6 groups, each of which consisted of 10 vaccine and 2 placebo recipients, for a total of 72 subjects (Table 1). The study was approved by the institutional review board, and written informed consent was obtained from all subjects.

Safety and Reactogenicity Evaluation

To assess reactogenicity after each vaccination, subjects maintained a diary to record daily temperatures and reactions for at least 14 days or until any symptoms resolved if longer. Hematology and chemistry evaluations were then performed on days 4, 7, 14, 28, 32, 35, 42, 56, 84, and 180. Cardiac evaluations using standardized questions were done at each visit. Electrocardiograms were performed and troponin levels were determined on days 14, 28, 42, 56, and 180 after the first vaccination. Nonserious adverse events were recorded through day 28 after

Table 1. Experimental Design of Clinical Trial of Immunization of Subjects with ACAM3000 Modified Vaccinia Ankara (MVA)

Group	Dose, TCID ₅₀	Route
A		
ACAM3000 MVA (<i>n</i> = 10)	1×10^6	ID
Placebo (<i>n</i> = 2)	...	ID
B		
ACAM3000 MVA (<i>n</i> = 10)	1×10^7	IM
Placebo (<i>n</i> = 2)	...	IM
C		
ACAM3000 MVA (<i>n</i> = 10)	1×10^7	SC
Placebo (<i>n</i> = 2)	...	SC
D		
ACAM3000 MVA (<i>n</i> = 10)	1×10^8	SC
Placebo (<i>n</i> = 2)	...	SC
E		
ACAM3000 MVA (<i>n</i> = 10)	1×10^7	ID
Placebo (<i>n</i> = 2)	...	ID
F		
ACAM3000 MVA (<i>n</i> = 10)	1×10^8	IM
Placebo (<i>n</i> = 2)	...	IM

NOTE. Vaccination occurred on days 0 and 28. A total of 72 subjects received either ACAM3000 or placebo. ID, intradermal; IM, intramuscular; SC, subcutaneous; TCID₅₀, median tissue culture infective dose.

the last vaccination, and serious adverse events were recorded throughout the study period. Toxicity was graded on the basis of standard toxicity tables of the Division of Microbiology and Infectious Diseases, National Institute of Allergy and Infectious Diseases.

Cell Lines and Vaccinia Viruses

HeLa, CV-1, and DF-1 cell lines and vaccinia viruses (strain Western Reserve [VV:WR], ACAM3000, recombinants containing a luciferase reporter gene [VV:Luc and MVA:Luc], and strain IHD-J [VV:IHD-J]) are described in the Appendix, which appears only in the online version of the *Journal*.

Immunogenicity Assays

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assays (ELISAs) were performed on serum samples obtained on days 0, 14, 28, 35, 42, 84, and 180 after vaccination, as described elsewhere [16]. See the Appendix for details.

Neutralization assay. Neutralizing antibody responses to vaccinia virus and MVA were measured in serum samples obtained on days 0, 7, 14, 28, 35, 42, 56, 84, and 180 after vaccination by a luciferase-based assay in HeLa or DF-1 cells, as described elsewhere [16]. See the Appendix for details.

Comet-reduction assay. Comet-reduction assays were performed on serum samples obtained on day 42 after vaccination with VV:IHD-J in CV-1 cells and analyzed by densitometry

with Image J software (version 1.40g), as described elsewhere [17]. See the Appendix for details.

T cell interferon γ enzyme-linked immunospot assay. Interferon γ (IFN- γ) enzyme-linked immunospot (ELISPOT) assays were performed on peripheral blood mononuclear cells (PBMCs) obtained on days 0, 14, 28, 35, 42, 56, 84, and 180 after vaccination, as described elsewhere with minor modifications [18–21]. See the Appendix for details.

Statistical Analysis

The Fisher exact test was used to test for associations between categorical variables. The exact Wilcoxon rank-sum test was used to assess group differences in continuous measures. All tests were 2-sided. All 72 subjects received the first vaccination, and 69 (95.8%) received the second. Of the 3 subjects who did not receive the second injection, 2 were in the MVA 1×10^7 subcutaneous group, and 1 was an intradermal placebo recipient. One subject was lost to follow-up, and 2 voluntarily withdrew because of personal reasons unrelated to the study. Thus, the 69 subjects who received both injections were included in an intent-to-treat analysis of immunogenicity. To adjust for multiple comparisons of immunogenicity, *P* values were considered to indicate significance at the .01 level.

RESULTS

Subject characteristics. Seventy-two subjects were enrolled in the study from October 2005 through March 2007. Forty-three participants (59.7%) were female. Fifty-eight subjects (80.5%) were white, 7 (9.7%) were Asian, and 7 (9.7%) were of other racial groups. Subjects ranged in age from 18 to 34 years, with a median age of 25 years.

Safety and reactogenicity. MVA vaccination was well tolerated at all dose levels and by all routes of administration. Local reactogenicity was common in all regimens and consisted of discomfort, erythema, or induration at the inoculation site, which generally resolved within 4–7 days with either no treatment or over-the-counter analgesics (Figure 1A). Severe local reactogenicity (which consisted of erythema and induration of 31–70 mm in size) was more frequent in intradermal (8/20 [40%]) and subcutaneous (5/20 [25%]) vaccine recipients than in intramuscular (0/20 [0%]) vaccine recipients (*P* = .003 and *P* = .047, respectively). Local reactogenicity was correlated with immune responses on days 14, 35, and 42 (for binding antibody responses, *P* < .001; for neutralizing antibody responses, *P* < .001). Thirteen (65%), 17 (85%), and 10 (50%) subjects who received MVA intradermally, intramuscularly, and subcutaneously, respectively, experienced systemic reactogenicity (*P* = .02, *P* < .001, and *P* = .13, respectively, compared with placebo recipients [2/12 {17%}]) (Figure 1B). Systemic reactogenicity, graded as mild or moderate, occurred in 33 subjects and consisted of fever (temperature, 37.8°C–38.3°C) for 1–2 days in 2

subjects and malaise, myalgia, arthralgia, headache, or nausea, which lasted from 1–7 days. Systemic reactogenicity, graded as severe, occurred in 5 subjects and consisted of malaise, headache, or chills, which resolved within 24 h. There were no differences in the frequency or severity of systemic reactions between the higher and lower doses of MVA or among the different routes. There were no differences in reactogenicity noted after the first vaccination compared with that after the second vaccination. All systemic reactogenicities were self-limited and resolved without sequelae. Systemic reactogenicities were not correlated with immune responses.

Adverse events. Two nonserious adverse events were considered to be associated with the vaccine. One was a subcutaneous lump (15 mm in diameter) proximal to the vaccine site; the lump resolved over 7 days without therapy in a subject in the 1×10^7 MVA subcutaneous group. The other adverse event was skin pigmentation at the vaccination site in the form of a reddish brown macule, which also resolved without therapy in a subject in the 1×10^7 MVA intradermal group. No serious adverse events were related to vaccination.

Because of the reports of myopericarditis in recipients of vaccinia virus, subjects were examined closely for possible cardiac effects of immunization. Four subjects experienced mild chest pain or discomfort within 24 h to 3 weeks after the second vaccination. These were found to be related to musculoskeletal or gastrointestinal disorders, resolved, and were deemed not related to the vaccine. No subject had clinical evidence of myopericarditis or electrocardiogram findings or troponin levels suggestive of myopericarditis.

Binding antibody responses detected by ELISA. Binding antibody responses developed to MVA in 93% (54/58) and to vaccinia virus in 88% (51/58) of subjects who received 2 vaccinations. After the first vaccination, elevated anti-MVA titers compared with placebo were first seen on day 14 in the 1×10^8 subcutaneous and 1×10^8 intramuscular groups (*P* ≤ .001 for both) and by day 28 in the 1×10^7 subcutaneous, 1×10^8 subcutaneous, 1×10^7 intradermal, and 1×10^8 intramuscular groups (*P* = .02, *P* < .001, *P* = .01, and *P* < .001, respectively) (Figure 2A). A statistically significant increase in anti-MVA ELISA titers was observed after the second vaccination (day 42) in all MVA groups, compared with those in the placebo group (for the 1×10^6 intradermal group, *P* = .01; for the other 5 groups, *P* < .001). On day 42, the higher-dose groups for the intradermal and subcutaneous routes had greater anti-MVA ELISA titers than did the lower-dose groups (*P* = .005 and *P* = .002, respectively). Importantly, MVA recipients demonstrated serum antibody binding ELISA titers against VV:WR antigen that were similar in time course and magnitude to the responses measured against MVA, suggesting that a high degree of antibody cross-reactivity exists (Figure 2B).

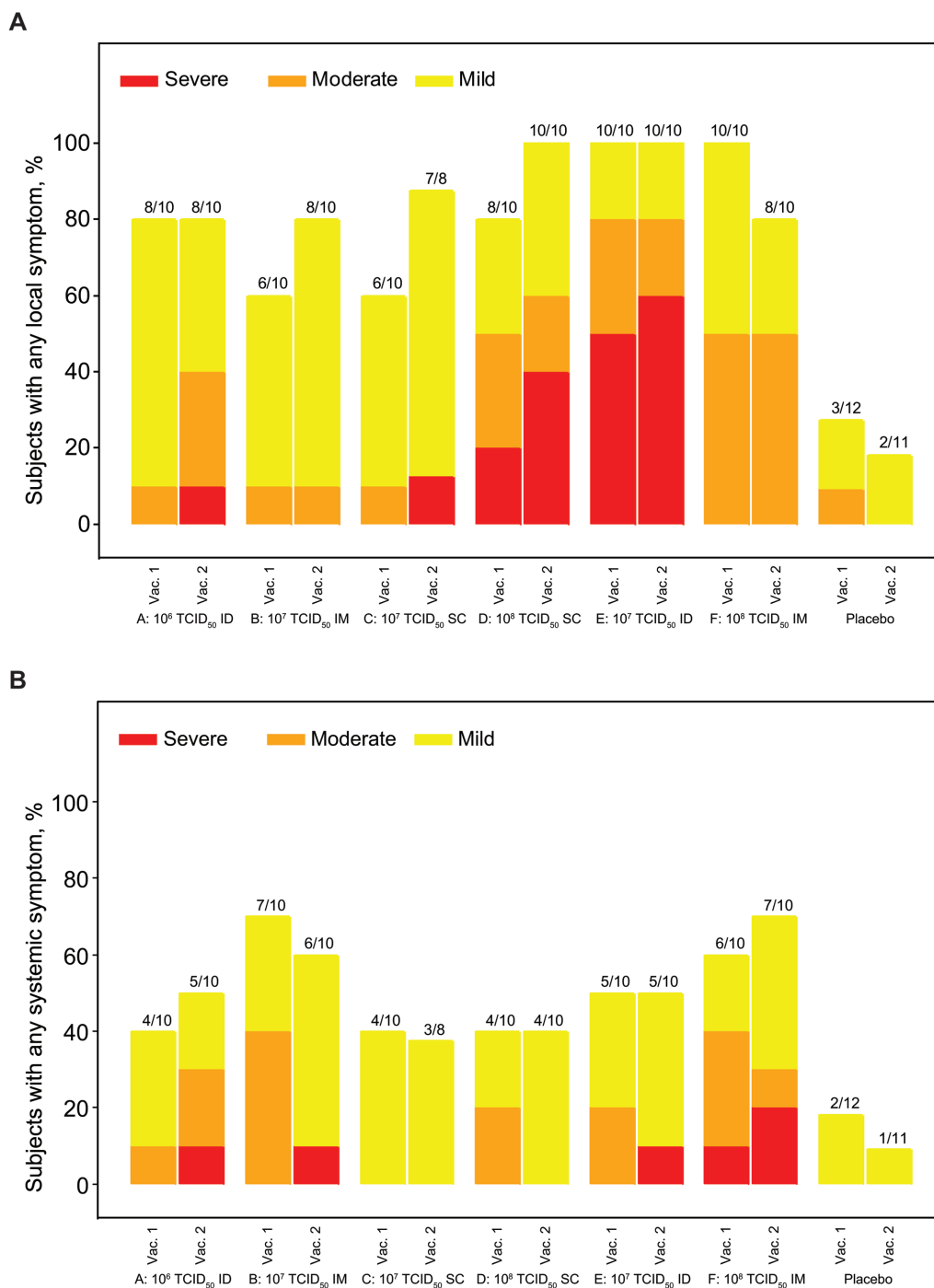


Figure 1. Proportion of vaccine recipients experiencing local (A) or systemic (B) symptoms after the first or second vaccination with modified vaccinia Ankara, by dose and route of administration. Severity of symptoms was graded on the basis of standard toxicity tables of the Division of Microbiology and Infectious Diseases, National Institute of Allergy and Infectious Diseases. ID, intradermal; IM, intramuscular; SC, subcutaneous; TCID₅₀, median tissue culture infective dose.

To further examine MVA-elicited antibodies, we assessed binding antibody responses to intracellular mature virion (IMV)-associated antigens (A27L and L1R) and extracellular enveloped virion (EEV)-associated antigens (A33R and B5R). Data for the higher-dose groups by each route and for the placebo group

are shown in Figure 3. By day 14 after the first vaccination with MVA, 19 (95%) of 20 individuals in the 1×10^8 subcutaneous and 1×10^8 intramuscular groups had ELISA antibody titers against A33R, and 20 (100%) of 20 subjects had titers against B5R and L1R. By day 42, the 1×10^7 subcutaneous (data not

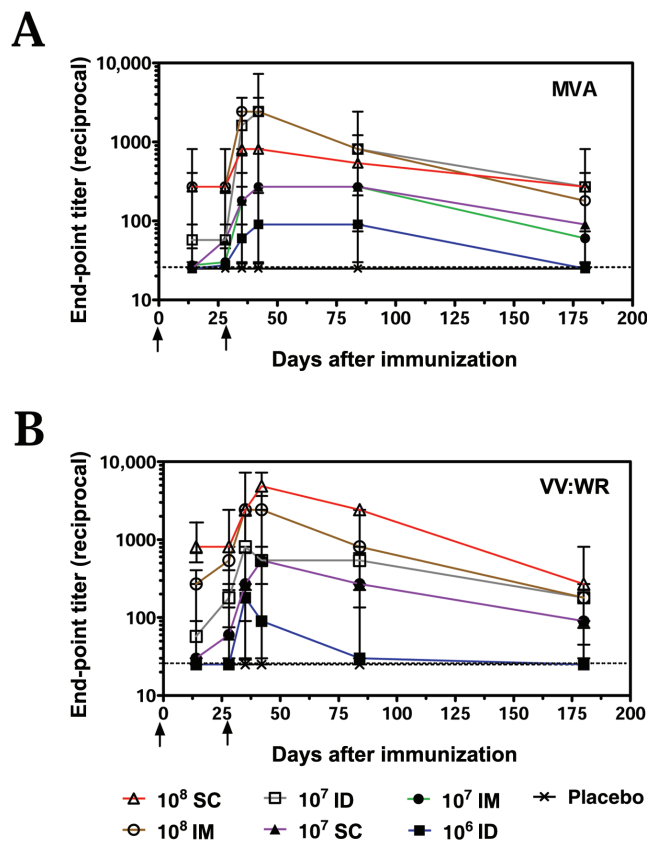


Figure 2. Binding antibody responses elicited by prime-boost vaccinations with modified vaccinia Ankara (MVA). Serum samples were obtained on days 0, 14, 28, 35, 42, 84, and 180 after MVA vaccination. Serial dilutions were tested for binding antibody activity against ACAM3000 MVA (A) or vaccinia virus Western Reserve (VV:WR) (B) by enzyme-linked immunosorbent assay. Data are the median serum end-point titer for each dose and route-of-administration group; error bars indicate interquartile ranges. The dashed line represents the limit of detection (serum end-point titer, 30), and arrows indicate days of vaccination. ID, intradermal; IM, intramuscular; SC, subcutaneous.

shown), 1×10^7 intradermal, 1×10^8 subcutaneous, and 1×10^8 intramuscular groups had titers against all 3 antigens that were significantly greater than those for placebo ($P < .007$), and no statistically significant differences in responses were observed among the higher-dose groups for each route. Responses to A27L were generally quite low and did not consistently increase after the second vaccination, compared with responses to the other 3 antigens.

Neutralizing antibody responses to MVA and VV:WR. Neutralizing antibody responses to MVA were detected in 91% (53/58) of vaccine recipients who received 2 vaccinations (Figure 4A). After primary vaccination, elevated titers were observed on day 14 in the higher-dose group compared with the corresponding lower-dose group (for intradermal, $P = .04$; for intramuscular, $P = .004$; and for subcutaneous, $P = .002$). These responses were increased after the second vaccination, and the

higher-dose groups continued to exhibit higher titers than the lower-dose groups through day 180 ($P < .01$). Peak neutralizing antibody titers typically occurred on days 35–42 (7–14 days after the second vaccination), and median titers were greater than those for placebo for all 6 vaccination groups (for the 1×10^6 intradermal group, $P = .03$; for the other 5 groups, $P < .001$). By day 180, only titers in the higher-dose groups for each route remained significantly increased, compared with those for placebo (for all comparisons, $P < .001$). No differences were observed in the time course or magnitude of the anti-MVA neutralizing antibody responses among the higher-dose groups (1×10^7 intradermal, 1×10^8 subcutaneous, and 1×10^8 intramuscular). These data demonstrate that the 1×10^7 intradermal group, despite receiving a 10-fold lower dose of MVA, elicited neutralizing antibody responses similar to those observed in individuals who received a 1×10^8 dose via the intramuscular or subcutaneous route.

The cross-reactivity of MVA-elicited neutralizing antibody responses to VV:WR was also assessed. Neutralizing antibodies against VV:WR were seen in 81% (47/58) of subjects. Overall, the kinetics of anti-vaccinia virus neutralizing antibody responses for each group were similar to the anti-MVA neutralizing antibody responses, although the magnitude was diminished (Figure 4B). By day 42, the 1×10^7 intradermal, 1×10^7 subcutaneous, 1×10^8 subcutaneous, and 1×10^8 intramuscular groups all had neutralizing antibody titers that were significantly increased compared with those for placebo (for the 1×10^7 subcutaneous group, $P = .007$; for the other 5 groups, $P < .001$), and each higher dose given by a particular route had significantly increased responses, compared with those for the corresponding lower dose (for all comparisons, $P < .001$). No statistically significant differences were observed in the magnitude of anti-vaccinia virus neutralizing antibody titers among the 3 higher-dose groups for each route through day 180. However, differences were found among the 3 1×10^7 groups. The intradermal route had higher anti-vaccinia virus neutralizing antibody titers on day 14 (for 1×10^7 intradermal vs 1×10^7 intramuscular, $P = .05$; for 1×10^7 intradermal vs 1×10^7 subcutaneous, $P = .02$), which peaked by day 42 (for 1×10^7 intradermal vs 1×10^7 intramuscular, $P = .008$; for 1×10^7 intradermal vs 1×10^7 subcutaneous, $P = .009$). No statistically significant differences were observed among these groups by day 180.

Comet-reduction assay. The ability of MVA vaccination regimens to elicit neutralizing antibodies against the EEV form of vaccinia virus was assessed by the comet-reduction assay. Two weeks after the second vaccination (day 42), serum comet-reduction activity in the 1×10^8 intramuscular and 1×10^8 subcutaneous groups was higher than that in the placebo group ($P < .001$ and $P = .005$, respectively) (Figure 5). In contrast, no statistically significant comet reduction was detected in serum samples from each of the lower-dose groups. Importantly,

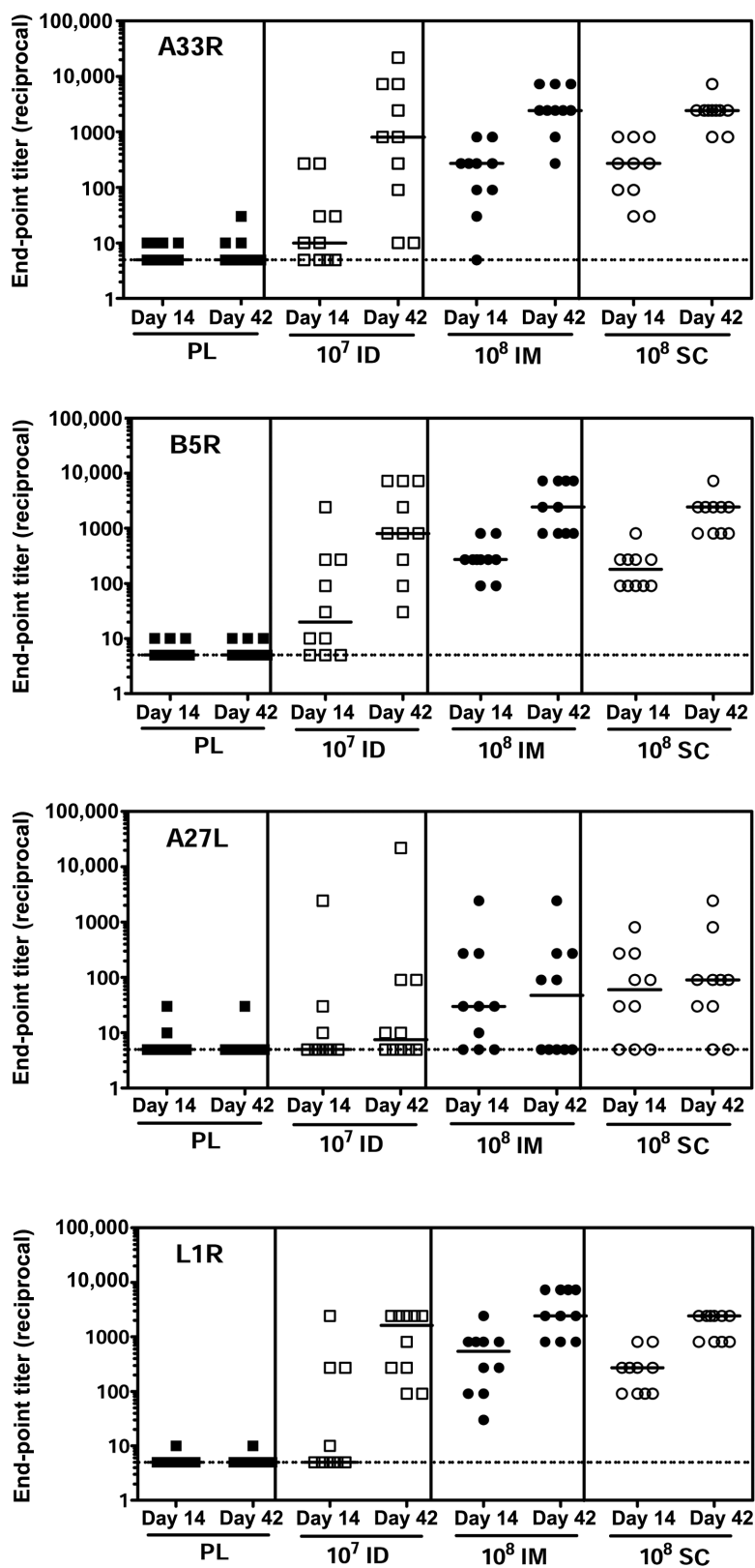


Figure 3. Antibody responses to intracellular mature virion (IMV)-associated and extracellular enveloped virion (EEV)-associated antigens after prime-boost vaccinations with modified vaccinia Ankara. Serum samples were obtained 2 weeks after primary vaccination (day 14) and 2 weeks after booster vaccination (day 42). Serial dilutions were tested for antibody binding activity against 2 IMV-associated protein antigens (A27L and L1R) and 2 EEV-associated protein antigens (A33R and B5R) by enzyme-linked immunosorbent assay. Data are individual end-point titers; horizontal lines indicate the median titer for each dose and route-of-administration group. ID, intradermal; IM, intramuscular; PL, placebo; SC, subcutaneous.

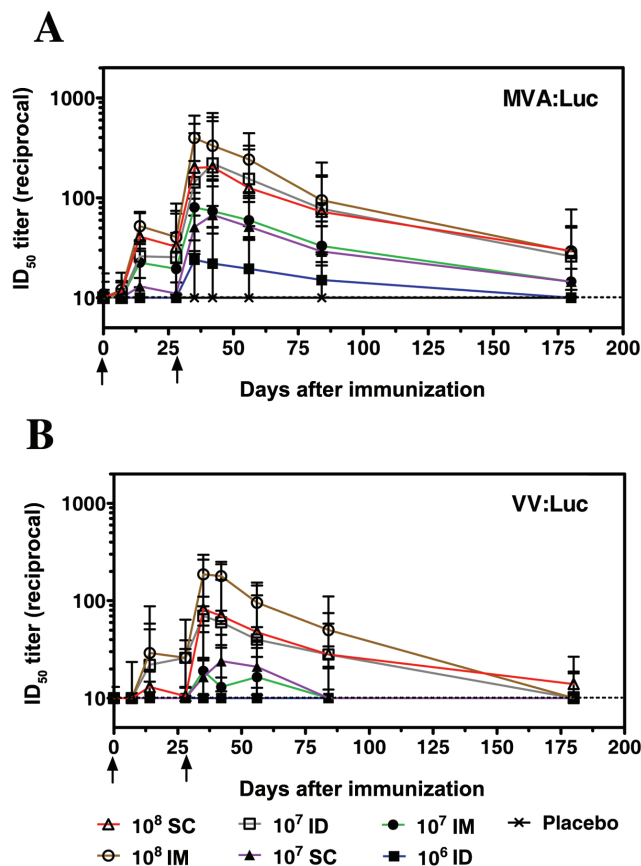


Figure 4. Neutralizing antibody responses elicited by prime-boost vaccinations with modified vaccinia Ankara (MVA). Serum samples were obtained on days 0, 7, 14, 28, 35, 42, 56, 84, and 180 after MVA vaccination. Serial dilutions were tested for neutralizing activity against MVA:Luc (A) or VV:Luc (B) (recombinants containing a luciferase reporter gene). Data are 50% inhibitory dose (ID_{50}) titers for each dose and route-of-administration group; error bars indicate interquartile ranges. The dashed line represents the limit of detection (serum ID_{50} titer, 10), and arrows indicate days of vaccination. ID, intradermal; IM, intramuscular; SC, subcutaneous.

comet-inhibition activity in the 1×10^7 intradermal group was higher than that in the 1×10^7 intramuscular and 1×10^7 subcutaneous groups and was similar to that in the 1×10^8 intramuscular and 1×10^8 subcutaneous groups (Figure 5).

T cell responses by IFN- γ ELISPOT assay. The magnitude and kinetics of anti-vaccinia virus T cell responses were assessed by IFN- γ ELISPOT assay (Figure 6). On day 14 after the first vaccination, only MVA administration by the intramuscular route elicited significantly higher responses than those in the placebo group (for the 1×10^7 group, $P = .005$; for the 1×10^8 group, $P < .001$). Two weeks after the booster vaccination (day 42), both the 1×10^7 and 1×10^8 groups for the intramuscular and subcutaneous routes of administration had statistically significantly higher responses than the placebo group (for all 4 comparisons, $P \leq .007$). T cell responses elicited

by the intradermal route were consistently lower than those measured in the groups receiving MVA via the intramuscular or subcutaneous routes.

DISCUSSION

ACAM3000 was safe and generally well tolerated at all dose levels and by all 3 routes; self-limited local discomfort was the most frequent reactogenicity. More pronounced local reactogenicity was more common in the intradermal and subcutaneous groups than in the intramuscular group. Self-limited systemic reactogenicities were encountered in half of the vaccine recipients and were not statistically significantly different among the various regimens. No serious adverse events were associated with vaccination. Phase 1 studies of other MVA candidate vaccines have shown that they are also well tolerated [22–24].

We extensively characterized antibody responses elicited by MVA vaccination by various routes and doses. Both binding and neutralizing antibody responses to MVA, VV:WR, and 3 individual IMV- and EEV-associated antigens were clearly generated by the 1×10^7 and 1×10^8 doses for the intramuscular and subcutaneous routes and by the 1×10^7 dose for the intradermal route, and the higher-dose groups for each route elicited generally greater responses. Importantly, a single administration of MVA in the higher-dose groups (1×10^7 intradermal, 1×10^8 subcutaneous, and 1×10^8 intramuscular) elicited detectable anti-MVA binding and neutralizing antibody titers in the majority of subjects by day 14. These titers substantially increased by 2 weeks after the second vaccination, and all subjects in the higher-dose groups had detectable responses. Furthermore, antibody responses in the higher-dose groups for each route remained detectable through day 180. Although antibody responses in the lower-dose groups for each route also significantly increased after

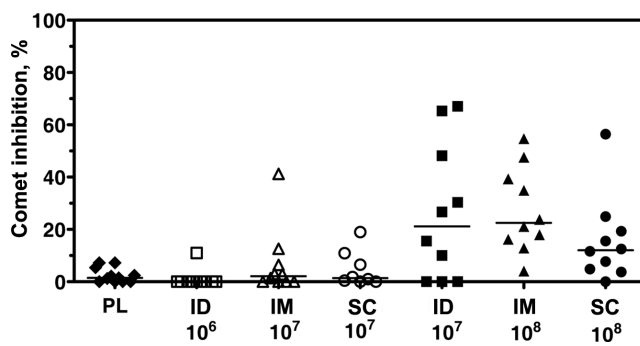


Figure 5. Assessment of anti-extracellular enveloped virion neutralizing antibody responses by comet-reduction assay. Serum samples were obtained 2 weeks after booster vaccination (day 42) and tested by a comet-reduction assay at a 1:50 dilution. Data are the percent comet reduction observed for individual subjects in each dose and route-of-administration group; horizontal lines indicate the median response. ID, intradermal; IM, intramuscular; PL, placebo; SC, subcutaneous.

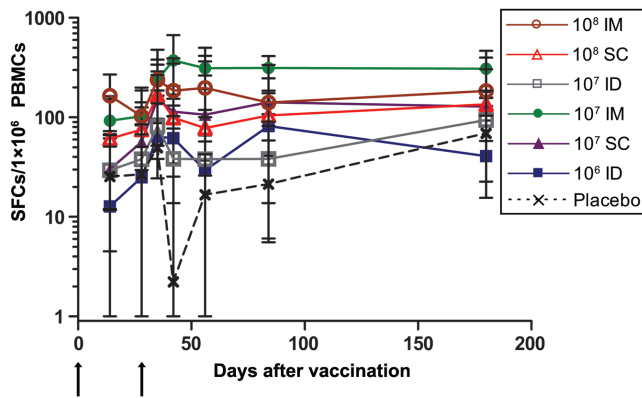


Figure 6. Cellular immune responses elicited by prime-boost vaccinations with modified vaccinia Ankara (MVA). Peripheral blood mononuclear cells (PBMCs) were obtained on days 0, 14, 28, 35, 42, 56, 84, and 180 after MVA vaccination and tested by an interferon γ enzyme-linked immunospot assay against autologous strain Western Reserve-infected target cells. Data are the median number of spot-forming cells (SFCs) per 1×10^6 effector PBMCs for each dose and route-of-administration group after subtraction of responses to medium alone and pre-immune background values; error bars indicate interquartile ranges. ID, intradermal; IM, intramuscular; SC, subcutaneous.

the second vaccination, peak titers were significantly lower than those in the higher-dose groups, and a few individuals in the 1×10^6 intradermal and 1×10^7 intramuscular groups still lacked detectable responses.

Of note, at the maximum doses that were administered there were no statistically significant differences in antibody responses according to route of administration. However, similar responses were obtained with a 10-fold-lower dose of vaccine (1×10^7) administered intradermally, compared with those elicited by 1×10^8 given intramuscularly or subcutaneously. This dose-sparing effect is consistent with that observed with several other vaccines given intradermally [11–15] and suggests that intradermal administration may be a particularly efficient route for administration of certain immunogens. Immune responses after the first intradermal dose were not as robust as those after the first dose given subcutaneously or intramuscularly but were equivalent after the second intradermal dose, reflecting the effect of a prime-boost regimen. The dose-sparing effect of intradermal administration may offer a considerable advantage in terms of availability and cost.

Serum antibody responses to MVA and Dryvax have been reported in studies of immunization with MVA-BN (IMVAMUNE; Bavarian Nordic) and appeared to be highly dose dependent when the vaccine was given subcutaneously [22, 23]. In the study of TBC-MVA (Therion Biologics), a lower dose of vaccine was used (1×10^6 PFUs given intramuscularly), and neutralizing antibody responses were not elicited [24]. This finding is consistent with our observation that MVA given at 1×10^6 TCID₅₀ intradermally generated lower levels of im-

mune responses and suggests that this dose may represent a lower threshold for stimulating immune responses to MVA in humans.

We also assessed the efficiency of antigen cross-recognition by MVA-elicited antibodies by using ELISA and neutralization assays that incorporated VV:WR as target antigens. The magnitude and kinetics of antibody responses recognizing whole vaccinia virus, as determined by ELISA, were similar to the responses observed against MVA, suggesting the presence of a high degree of antibody cross-recognition. We also observed efficient cross-neutralization of VV:WR in serum samples from MVA vaccine recipients, although titers were generally lower than those against MVA. Of interest, a recent study reported that MVA vaccine recipients mounted serum neutralizing antibody responses to variola virus, demonstrating the ability of MVA immunization to elicit cross-reactive immunity against smallpox [25].

Data suggest that optimal protection against orthopoxvirus infection is achieved when antibody responses target 2 structurally and antigenically distinct forms of infectious poxviruses, IMV and EEV [26]. Because the ELISA and neutralization assays described above used the IMV form of virus, we further assessed the ability of MVA immunization to elicit antibody responses to EEV by comet-reduction assay and protein-specific ELISA. Serum samples from all 3 of the higher-dose groups demonstrated neutralizing activity against EEV after the second vaccination. We also assessed the generation of antibodies against 2 IMV and 2 EEV protein antigens that have been implicated in protection against vaccinia virus infection [27–31] and that are expressed by MVA. ELISA responses to the EEV-associated antigens, A33R and B5R, and to the IMV antigen L1R were detected with each route, and higher responses were generated by the higher doses for each route. Of interest, only low-level antibody responses were detected against the IMV antigen A27L. We have previously observed a similar lack of anti-A27L antibody responses in rhesus macaques vaccinated with high doses of MVA, in contrast to vaccination with vaccinia virus [16], and others have also described a lack of anti-A27L antibody responses after NYVAC administration [32]. It may be that responses to this IMV protein are lacking for certain attenuated vaccine strains. Recent reports have described additional antigens against which antibody responses have been reported to convey protection against vaccinia virus infection, including H3L, D8L, and A28L [30, 33, 34]. It will be informative to further characterize the nature of the antibody responses elicited by MVA against a broader panel of vaccinia virus-encoded proteins at the doses and routes described here.

We conducted only limited studies of T cell immune responses, using an IFN- γ ELISPOT assay with VV:WR-infected target cells. T cell responses were observed, but without a clear effect of dose or route of administration. Boosting with the

second dose of MVA appeared to increase the T cell responses for some doses and routes. A previous study of MVA-BN reported T cell responses to Dryvax after MVA immunization but also did not find a dose-response relationship [23]. CD4 and CD8 T cell responses to MVA or Dryvax were reported in subjects vaccinated with TBC-MVA, and responses were augmented after 3 doses of MVA compared with 1 dose [24]. Of interest, we observed that the recall response of T cells after the second MVA vaccination was higher in the 1×10^7 intramuscular group than in the 1×10^8 intramuscular group, despite a higher response in the 1×10^8 group after the first vaccination. Whether this reflects functionally better T cell priming at the lower dose or whether the high level of neutralizing antibodies generated in the higher-dose intramuscular group impaired the ability of the second MVA vaccination to boost cellular immunity remains to be determined. Additional studies of T cell responses to define epitope specificity, breadth, phenotype, and functional characteristics are needed to further characterize the effect of dose and route of administration of MVA on T cell responses.

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