Elsevier has created a Monkeypox Information Center in response to the declared public health emergency of international concern, with free information in English on the monkeypox virus. The Monkeypox Information Center is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its monkeypox related research that is available on the Monkeypox Information Center - including this research content - immediately available in publicly funded repositories, with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the Monkeypox Information Center remains active.

Vaccine (2008) **26**, 933–946

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/vaccine

Immune responses to the smallpox vaccine given in combination with ST-246, a small-molecule inhibitor of poxvirus dissemination

Douglas W. Grosenbach, Robert Jordan, David S. King, Aklile Berhanu, Travis K. Warren, Dana L. Kirkwood-Watts, Shanthakumar Tyavanagimatt, Ying Tan, Rebecca L. Wilson, Kevin F. Jones, Dennis E. Hruby*

SIGA Technologies, 4575 SW Research Way, Suite 230, Corvallis, OR 97333, United States

Received 7 August 2007; received in revised form 26 October 2007; accepted 29 November 2007
Available online 26 December 2007

KEYWORDS
Smallpox;
Vaccine;
ST-246

Summary The re-emerging threat of smallpox and the emerging threat of monkeypox highlight the need for effective poxvirus countermeasures. Currently approved smallpox vaccines have unacceptable safety profiles and, consequently, the general populace is no longer vaccinated, leading to an increasingly susceptible population. ST-246, a small-molecule inhibitor of poxvirus dissemination, has been demonstrated in various animal models to be safe and effective in preventing poxviral disease. This suggests that it may also be used to improve the safety of the traditional smallpox vaccine provided that it does not inhibit vaccine-induced protective immunity. In this study, we compared the immune responses elicited by the smallpox vaccine alone or in combination with ST-246 in mice. Normal lesion formation following dermal scarification with the attenuated New York City Board of Health strain (Dryvax), commonly referred to as a vaccine ''take'', was not inhibited although severe lesions and systemic disease due to vaccination with the virulent Western Reserve (VV-WR) strain were prevented. The vaccine given with ST-246 did not affect cellular immune responses or neutralizing antibody titers although anti-vaccinia ELISA titers were slightly reduced. Vaccination in combination with ST-246 provided equivalent short- and long-term protection against lethal intranasal challenge with VV-WR when compared to vaccine alone. These results suggest that ST-246 does not compromise protective immunity elicited by the vaccine and provide the basis for future studies examining the efficacy of ST-246 in preventing or treating adverse events due to vaccination.

Introduction

Smallpox has been a scourge of humanity throughout recorded history. It is estimated to have killed, crippled or disfigured 1/10 of all humankind—300 million people in the

* Corresponding author. Tel.: +1 541 753 2000; fax: +1 541 753 9999.
E-mail address: dhruby@sgph.com (D.E. Hruby).

0264-410X/$ – see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2007.11.095

20th century alone. Smallpox is caused by variola virus, an extremely infectious orthopoxvirus. Virtually all susceptible individuals will contract disease if exposed and, historically, up to 30% of unvaccinated individuals will succumb to disease. Smallpox is a renewed threat due to its potential use as a biowarfare agent. In addition to the threat of smallpox, it is possible to genetically engineer lethality into laboratory strains of poxviruses via the introduction of genes for toxins, altered tropism or immunomodulatory factors [1,2]. There is also the threat of zoonotic spread of orthopoxviruses found in nature, such as monkeypox [3]. Fortunately, all of these orthopoxviruses exhibit significant homology at the nucleotide and amino acid levels [4]. A vaccine effective against one appears to provide protection against another. The vaccine used to eradicate smallpox is a live vaccinia virus, a close relative of variola. In the U.S., the most widely used version of the vaccine is an attenuated New York City Board of Health strain of vaccinia manufactured by Wyeth and licensed under the trade name Dryvax®. Although extremely effective, a number of severe adverse events are associated with the vaccine [5]. Following the eradication of smallpox, vaccination of the general public has been discontinued due to safety concerns, resulting in an ever-increasing number of naïve individuals susceptible to smallpox, monkeypox or engineered orthopoxvirus variants.

Vaccinia is widely studied, and is considered the prototypic orthopoxvirus—the genus to which variola, monkeypox, ectromelia, cowpox and vaccinia virus (among others) belong. The virus life cycle is extremely complex, yet has been characterized in detail and serves as a model for other orthopoxvirus infections [6]. Productive viral replication leads to the formation of a number of phenotypically distinct, infectious virion forms. Mature virus (MV) is formed first and is fully infectious, but is retained intracellularly until cell lysis. A fraction of the MV particles acquire additional membranes within the cell and are released in a non-lytic fashion and are referred to as EV (for enveloped virus). The formation of EV is of particular importance in the virulence of orthopoxviruses. EV is the form of virus involved in the *in vivo* dissemination of the virus from the site of original inoculation [7,8]. Mutant viruses defective for EV production show greatly reduced virulence *in vivo* and display a small-plaque phenotype in tissue culture [9—12]. While other viral and perhaps cellular proteins are involved, the virus F13L gene product, also called p37, plays a central role in the formation of EV [9]. As such, F13L represents a viable target for drugs that would be capable of inhibiting smallpox and also for preventing or treating adverse events following vaccination.

ST-246 is a novel, orally bioavailable compound that is a potent, non-toxic and specific inhibitor of EV formation and thus reduces virus dissemination both *in vitro* and *in vivo* [13,14]. *In vitro*, ST-246 treatment inhibits plaque formation and reduces the formation of EV without affecting the production of MV particles or EV-associated proteins. In tissue culture, ST-246 was demonstrated to be effective against a number of orthopoxvirus strains including variola, vaccinia, cowpox, ectromelia, monkeypox and camelpox viruses [13]. The target of ST-246 was discovered to be p37, the product of the F13L gene in vaccinia. This protein is highly conserved amongst orthopoxviruses, particularly in the region of the protein targeted by ST-246. *In vivo*, oral administration of ST-246 protected 100% of mice from challenge with a 40,000 LD_{50} dose of ectromelia virus and reduced viral titers in the lungs, livers and spleens of infected mice by at least five orders of magnitude relative to control vehicle-treated mice [13]. Maximal protection required treatment for 14 consecutive days following challenge while treatment for 5 days protected 80% of the mice. In a separate experiment, mice were challenged with IHD-J, a strain of vaccinia whose virulence is due in part to enhanced EV release and dissemination [7], and then treated with ST-246 for 14 days. All ST-246-treated mice survived [13]. Furthermore, in the IHD-J challenge model, surviving mice were resistant to rechallenge suggesting that protective immunity was elicited even though viral dissemination was inhibited by the drug [13]. The models in which ST-246 has been tested so far are designed to mimic lethal exposure of naïve individuals to smallpox virus and subsequent therapeutic treatment with ST-246. These experiments may also be considered a model for adverse events following prophylactic vaccination with the smallpox vaccine and suggest that ST-246 may be used in combination with smallpox vaccination to reduce the occurrence of adverse events associated with the vaccine. Prior to testing in more stringent models for adverse events, it must first be demonstrated that vaccine efficacy is not compromised by ST-246 in immunocompetent mice. In this study, we compared immune responses of immunocompetent mice to the smallpox vaccine given in combination with ST-246 to standard smallpox vaccination.

Materials and methods

Viruses and cells

All tissue culture media and reagents were purchased from Invitrogen (Carlsbad, CA) except where otherwise indicated. Cell lines and viruses (with the exception of the Wyeth Dryvax vaccine) were purchased from the American Type Culture Collection (Manassas, VA). BSC40 (CRL-2761) cell monolayers were maintained in a humidified atmosphere at 37 °C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 10 μg/ml gentamicin sulfate. A-20 (TIB-208) cells are a B-cell lymphoma expressing both class I and class II major histocompatibility complex proteins of the *H-2*^{d} haplotype and are syngeneic to BALB/c mice. A-20 cells were maintained at 37 °C and 5% CO_2 in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (penicillin-streptomycin solution, Mediatech Inc, Herndon, VA), 50 μM 2-mercaptoethanol, and 10 mM HEPES (complete media). Stocks of vaccinia virus Western Reserve (VV-WR, VR-1354) and IHD-J (cultured from a stock originally obtained from S. Dales) were cultured in BSC40 cells and purified by sucrose gradient centrifugation [15]. The New York City Board of Health strain of vaccinia manufactured by Wyeth Laboratories (Wyeth Ayerst Laboratories, Marietta, PA) under the trade name Dryvax® (Dryvax) was obtained from the Centers for Disease Control (Atlanta, GA). The lyophilized stock was resuspended in phosphate-buffered saline (PBS) at a concentration of 5×10^7 plaque-forming units/ml (pfu/ml). In some experiments, animals were

vaccinated with the unpassaged virus stock. In other experiments, the stock virus was cultured for a single passage in BSC40 cells as described above for VV-WR and IHD-J. Viral titers were determined by plaque-assay in monolayers of BSC40 cells.

ST-246

The identification of ST-246 as an inhibitor of poxvirus dissemination as well as its chemical structure have been previously reported [13]. ST-246 was synthesized by Pharmacore (Highpoint, NC) and delivered as a lyophilized powder. The drug was prepared for oral dosing of animals by suspension in an aqueous solution containing 0.75% methylcellulose (Sigma—Aldrich, St. Louis, MO) and 1% Tween-80 (Sigma—Aldrich) at a concentration of 10 or 20 mg/ml. Two mg/mouse (approximately 100 mg/kg) of ST-246 was delivered in a volume of 100 or 200 μl by oral gavage using a curved gavage needle. Animals were dosed daily at 24-h intervals for 7 or 14 consecutive days beginning on the day of vaccination with Dryvax or VV-WR.

Vaccination protocols

All animal protocols were designed and carried out according to the *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by the Oregon State University Institutional Animal Care and Use Committee. Female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). All mice were 7 weeks of age on the first day of each experiment. In all experiments in which mice were vaccinated with Dryvax, a ''standard'' dose of 5×10^5 pfu/mouse was used in order to closely approximate the dose given when vaccinating humans. On the day of vaccination, Dryvax was diluted to 5×10^7 pfu/ml in PBS and deposited at the base of the tail in a volume of 10 μl followed by scarification with a 25-gauge needle approximately 30 times in a cross-hatch pattern through the droplet. In a separate experiment, VV-WR was used to vaccinate/challenge mice by dermal scarification. VV-WR was diluted to 1×10^8 pfu/ml ml in PBS and deposited at the base of the tail in a volume of 10 μl (1×10^6 pfu/mouse) followed by scarification, as described above. ST-246 treatment was initiated immediately following vaccination and continued for 7 or 14 days as described above. Naïve mice and some groups of vaccinated mice were treated with vehicle (aqueous 0.75% methylcellulose, 1% Tween-80) similar to ST-246 treatment. In one set of experiments mice were sacrificed at 7, 14, 33, or 180 days post-vaccination. Serum was isolated from clotted blood obtained by cardiac puncture and spleens were removed for use in immune response assays. In other experiments, vaccinated mice were challenged with lethal doses of VV-WR at 21 or 180 days post-vaccination.

Lethal virus challenge

The mean lethal dose (LD_{50}) of our preparation of VV-WR delivered intranasally was determined to be 1.26×10^5 pfu/mouse in 10-week-old mice and in 6-month-old mice, according to the method of Reed and Muench [16] (data not shown). The LD_{50} titrations were performed immediately prior to the challenge experiments using identical preparations of virus and identically aged mice. In all challenge experiments, mice were anesthetized by inhalation of 3% isoflurane (IsoSol, Vedco Inc., St. Joseph, MO). While anesthetized, the challenge virus was delivered in a total of 10 μl using a pipet to administer approximately half of the dose to each naris. In all challenge experiments, naïve mice and Dryvax-vaccinated mice were used as controls. In one set of experiments, 7-week-old mice were vaccinated with Dryvax on day 0 and treated with ST-246 or vehicle for 14 days, as described above. On day 21 post-vaccination, mice (now 10 weeks old) were challenged by intranasal inoculation with VV-WR at 10, 100 or 1000 times the LD_{50}. Mice were then monitored for survival, signs of disease, weight and temperature for 14 days. Mice were preemptively euthanized when exhibiting severe disease symptoms (such as >30% weight loss, labored breathing, hunched posture, lack of mobility and socialization) and/or were moribund. In a second set of experiments, 7-week-old mice were vaccinated with Dryvax on day 0 and treated with ST-246 or vehicle for 14 days as described above. On day 180 post-vaccination, mice were intranasally challenged with a 10 LD_{50} dose of VV-WR. Mice were then monitored for survival, as above, for 14 days. Mice were euthanized when appropriate as described above.

Bacterial expression and purification of VV proteins for ELISA

The cloning, bacterial expression and purification of vaccinia antigens D8L, L1R, A27L, B5R, and A33R are to be described in detail elsewhere. In brief, the open-reading frames for each gene were amplified by polymerase chain reaction (PCR) from vaccinia DNA (Copenhagen strain). Restriction sites engineered into the PCR primers were utilized in cloning the DNA in the pET41B plasmid (Novagen/EMD Biosciences, San Diego, CA) placing the genes adjacent to the T7 promoter and creating gene fusions with glutathione-*S*-transferase (GST). The DNAs were transformed into *Escherichia coli* strains Rosetta or BL21 (DE3) (EMD Biosciences) that express an inducible T7 polymerase. Upon induction, the bacteria produced amino-terminal GST fusion proteins that were then purified from cell lysates using a glutathione column. Protein purity was confirmed by gel electrophoresis. The proteins were dialyzed against PBS and diluted to 5 μg/ml in PBS for coating ELISA plates.

Immunoglobulin G ELISA

ELISAs were performed to measure the humoral response to whole vaccinia and to the individual vaccinia proteins, D8L, L1R, A27L, B5R, and A33R. The whole vaccinia ELISA was performed as follows: VV-WR was cultured in BSC40 cells as described above. The purified virus was UV-inactivated, diluted in PBS to 1×10^7 pfu/ml and 50 μl/well was added to Nunc maxisorp (eBioscience, San Diego, CA) 96-well ELISA plates and allowed to adsorb overnight at 4 °C. The coating solution was removed and the plates were blocked for 1 h

at 37 °C using 200 μl well of blocking solution (1× PBS, 5% Casein, 1% Brij and 0.06% Kathon microbicide). Each mouse was analyzed individually: the sera were diluted in blocking solution starting with a 1:5 dilution of neat sera and making 1:5 dilutions thereafter. After blocking the plates, the diluted sera were added in duplicate in a volume of 50 μl/well. The plates were then incubated at 37 °C for 3 h. The plates were washed and a goat-anti-mouse IgG (heavy and light chain) antibody conjugated to horse-radish peroxidase (Bio-Rad Laboratories, Hercules, CA), diluted 1:10,000 in blocking buffer was added to each well in a volume of 50 μl. The secondary antibody incubation was at 37 °C for 1 h. The plates were washed and 100 μl of (3,3′,5,5′)-tetramethylbenzidine (TMB) colorimetric substrate (Pierce, Rockford, IL) added to each well. The reaction was stopped after approximately 20 min by addition of 100 μl/well of 2 M sulfuric acid. The OD_{450} was measured with a Spectrafluor Plus plate reader (Tecan, San Jose, CA). Background absorbance was measured in wells that were only treated with PBS and blocking solution. Background absorbance was subtracted from all test absorbance values. The ELISA for individual vaccinia proteins was performed in a similar fashion as the whole vaccinia ELISA with the following exceptions: Individual vaccinia proteins were diluted to 5 μg/ml in PBS and 50 μl/well was added to the ELISA plates. Coating of the plates was performed at room temperature overnight. Each mouse was analyzed individually. The sera were diluted 1:50 in blocking buffer as a starting dilution and 1:5 dilutions were made thereafter. All other aspects of the assay were identical to the whole vaccinia ELISA. Endpoint titers were calculated as the reciprocal of the serum dilution resulting in an absorbance greater than 2 standard deviations above the absorbance in wells incubated with the same dilution of naïve mouse serum. Calculations were performed using XLFit curve-fitting software (IDBS, Alameda, CA).

Splenocyte proliferation assay

Freshly harvested spleens from vaccinated (±ST-246 treatment) mice and naïve mice as controls were dispersed into single-cell suspensions by grinding through a cell strainer. Splenocytes, from 5 mice/group, were pooled. The red blood cells were lysed by treatment with ACK lysis buffer (0.15 mM NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA). The lymphocytes were washed and resuspended in complete medium at 1×10^7 cells/ml. Heat-killed VV-WR was used for antigenic stimulation. Heat-killed virus was prepared as follows: VV-WR, at 5×10^{10} pfu/ml (in PBS), was boiled for 30 min. The virus was diluted in complete medium to 5×10^8 pfu equivalents/ml and was further diluted in 1:2 increments for a total of seven dilutions, all in complete medium. The virus dilutions were added in 100 μl volumes to triplicate wells on 96-well flat-bottom plates. Splenocytes were added to the diluted virus in a volume of 100 μl/well such that each well contained 1×10^6 splenocytes and heat-killed virus at concentrations ranging from 5×10^7 to 1.5×10^5 pfu equivalents in a total volume of 200 μl complete medium. The assay was cultured in a humidified atmosphere at 37 °C and 5% CO_2 for 4 days. Cell proliferation was measured using the colorimetric development reagent CellTiter 96® AQ_{ueous} One Solution (Promega, Madison, WI) according to the manufacturer's instructions with the following exceptions: Splenocytes were added at a concentration of 1×10^6 cells/well to ensure adequate cell-to-cell contact and antigen presentation to T cells. The manufacturer recommends concentrations not exceeding 1×10^5 cells/well as high cell concentrations can interfere with the linear relationship between cell number and absorbance at OD_{490}. To compensate for this, after 4 days incubation, the cells were resuspended in the culture medium and 20 μl of the culture contents (1/10 of the culture) were diluted into 80 μl of PBS on a new 96-well plate. The development reagent was added in a volume of 20 μl and the color development proceeded for 4 h at 37 °C. Color development was measured at OD_{490} in a Spectrafluor Plus plate reader. Background absorbance values were measured in wells containing media, PBS and development reagent. Control absorbance values were measured in wells that contained splenocytes that had not been stimulated with antigen. The stimulation index was calculated using the formula: (test absorbance − background absorbance)/(control absorbance − background absorbance).

Intracellular cytokine stain

Intracellular cytokine staining was performed essentially as previously described [17]. Initially, vaccinia-infected naïve splenocytes were used as stimulator cells as A-20 cells were unavailable. In later experiments that were exact repeats of earlier experiments, A-20 cells were used and yielded very similar results in terms of cytokine production. A-20 cells were used in subsequent intracellular cytokine staining experiments and cytokine release assays (see below) for convenience as they are easily maintained in tissue culture. Naïve splenocytes or A-20 cells were infected with VV-WR at a multiplicity of infection of five, 6 h prior to setting up the assay. Mock-infected cells were maintained as controls. Just prior to setting up the assay, the cells were washed and resuspended in complete medium at a concentration of 3×10^6 cells/ml. Splenocytes were prepared in an identical manner as described above for the proliferation assay. The lymphocytes were washed and resuspended in complete medium at a concentration of 1×10^7 cells/ml. In a 96-well plate, 100 μl of VV-WR infected splenocytes or A-20 cells (or mock-infected controls) were incubated with 100 μl of lymphocytes to stimulate VV-specific interferon-γ (IFN-γ) production. Human IL-2 (BD Biosciences, San Jose, CA) was added to a final concentration of 20 U/ml and brefeldin A (BD Biosciences) was added to a final concentration of 3 μg/ml. The assay was set up in duplicate. The stimulation was performed in a humidified atmosphere at 37 °C and 5% CO_2 for 12−16 h prior to staining. The duplicate cultures were pooled, and then the cell surface was stained with anti-CD8α-FITC and anti-CD4-PE-CY5. The cells were then fixed and permeabilized using reagents from eBioscience (San Diego, CA), followed by intracellular staining for IFN-γ using anti-IFN-γ-PE. All antibodies were from BD BioSciences. Samples were acquired using a Cytomics FC500 flow cytometer (Beckman Coulter Inc., Miami, FL) and CXP software. Data was analyzed using WinMDI software version 2.8 (J. Trotter, Scripps Institute, San Diego, CA).

Cytometric bead array analysis of cytokine release

The culture setup for the cytometric bead array (CBA, BD BioSciences) was identical to the ICCS setup with the exceptions that IL-2 and brefeldin A were omitted from the culture, and the culture period was extended to 48 h. Culture supernatants were collected at 48 h post-setup and diluted 1:5 in PBS. The concentrations of TNF-α, IFN-γ, IL-5, IL-4 and IL-2 were determined using the Mouse Th1/Th2 CBA kit according to the manufacturer's instructions. Samples were acquired using a Cytomics FC500 and the data were analyzed using the CBA software. The concentration of each cytokine in the supernatants was calculated based on comparison to the corresponding standard curve generated using purified cytokines from the kit.

Virus neutralization and comet inhibition assay

Virus neutralization

BSC40 cells were seeded at a density of 2.5×10^5 cells per well on 12-well plates 24 h prior to the assay. Test sera were heat inactivated by incubation at 56 °C for 30 min. For the neutralization assay, the sera from individual mice were diluted 1:50 in 100 μl of PBS for a starting dilution and 12 twofold serial dilutions in PBS were made thereafter. The diluted sera were mixed 1:1 with approximately 50 pfu of VV-WR in DMEM supplemented with 2.5% FCS, 2 mM L-glutamine and 10 μg/ml gentamicin sulfate (DMEM-2.5) and incubated overnight at 37 °C, as previously described [18]. The growth medium was aspirated from the BSC40 cells and the virus + serum dilution was added to each well in a volume of 200 μl followed by addition of 1 ml of DMEM-2.5. The infected cells were cultured for 24 h after which the monolayer was fixed and stained with 0.1% crystal violet in 30% ethanol to visualize plaques. The reciprocal of the serum dilution resulting in a 50% reduction in the initial number of plaque-forming units (using naïve mouse serum as a negative control), termed IC_{50}, was calculated using XLFit curve-fitting software (IDBS, Alameda, CA).

Comet-inhibition assay

BSC40 cell monolayers in 12-well plates, set up as described for the virus neutralization assay, were infected with the IHD-J strain of vaccinia at approximately 20 pfu/well in a volume of 200 μl of PBS, for 1.5 h at 37 °C. The inoculum was aspirated and 1 ml of DMEM-2.5 containing a 1:50 dilution of sera (pooled from five mice per experimental group) was added to each well. Sera from naïve mice and infections in the absence of sera were used as controls. The infected cells were fixed and stained with crystal violet at 36 h post-infection.

HPLC analysis of serum ST-246 concentrations

ST-246 was diluted in MeOH from a (1 mg/ml stock) and 10 μl of each dilution was added to 100 μl normal mouse serum (Lot# 23417, Biomeda) to generate a standard curve. One hundred microlitres pooled mouse serum samples (from day 7 post-vaccination $\pm$ST-246 and day 14 post-vaccination $\pm$ST-246) were analyzed for comparison to the standard curve. Acetonitrile was added into the standard curve and experimental samples to a final volume of 500 μl followed by vigorous vortexing for 15 s. The resulting mixtures were centrifuged at 13,000 rpm for 10 min at 4 °C. The upper supernatant of 450 μl was transferred to a microcentrifuge tube and dried in a centrifugal vacuum evaporator. One-hundred microlitres MeOH was added to the dried sample and vortexed briefly, followed by centrifugation at 13,000 rpm for 10 min at 4 °C. Seventy microlitres supernatant was transferred to a sample vial with a glass insert in preparation for injection. The analysis was performed on a HPLC system consisting of the Agilent 1100 series with Quaternary Pump and Diode Array Detector. Chromatographic separation was performed on a Zorbax Eclipse XDB C18 solvent saver plus column (3.0 mm $\times$ 100 mm, 3.5 μm; Agilent). The mobile phase was delivered at 0.5 ml/min consisting of 55% 2.5 mM NH_4OH (pH 3.5, adjusted with formic acid) and 45% acetonitrile. The column temperature was controlled at 28 °C. The detection wavelength for ST-246 was 226 nm. The injection volume was 10 μl.

Statistical analysis

Student's *t*-test were performed to compare one test group to another at a specific experimental point such as dilution of sera in ELISAs or dilution of antigen in the proliferation assays. The *t*-test were performed using Microsoft Excel software and all tests were two-tailed, unpaired with equal variance. For comparison of all experimental groups within an experiment, analyzing outcomes such as change in weight or temperature, ANOVA, using a Pairwise Multiple Comparison Procedures (Tukey test) was performed using SigmaPlot software (Systat Software Inc., San Jose, CA).

Results

ST-246 is currently being developed as a post-exposure therapeutic for smallpox. Toward that end, ST-246 has been demonstrated to be efficacious in animal models for lethal poxvirus challenge [14,19]. This suggests the possibility that ST-246 may also be used as an adjunct to the smallpox vaccine to protect against or treat adverse events due to vaccination. Importantly, though, it must first be demonstrated that ST-246 does not adversely affect vaccine efficacy. In this study, we report on the clinical and immunological responses to the smallpox vaccine in immunocompetent BALB/c mice. Furthermore, vaccination $\pm$ST-246 is evaluated for protection from lethal challenge in short- and long-term experiments.

Clinical responses to dermal scarification with VV-WR or Dryvax

VV-WR is a commonly used laboratory strain of vaccinia virus. It is often used as a surrogate virus for the authentic smallpox vaccine (although the routes of administration and doses used vary considerably) and also for lethal poxvirus challenge in mice. VV-WR is more virulent in mice than approved vaccine strains such as the New York City Board of Health strain of vaccinia that was manufactured by Wyeth and licensed under the trade name Dryvax®. In our ini-

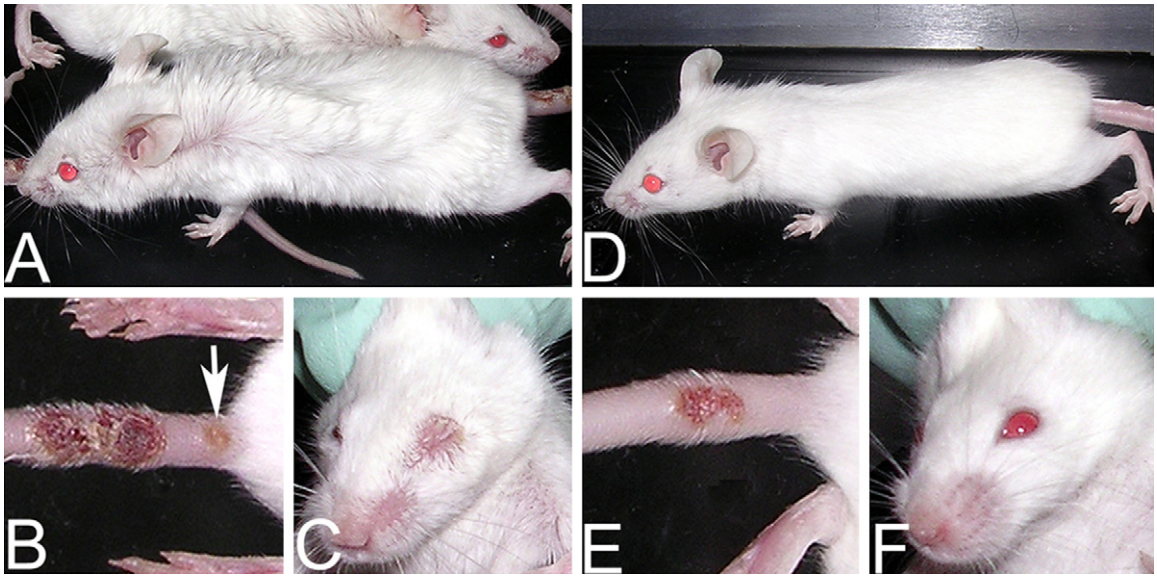

Figure 1 Lesion formation and systemic disease following dermal scarification with VV-WR ±ST-246. Seven-week-old BALB/c mice were challenged with 1×10^6 pfu of VV-WR by the dermal scarification route. One group of mice was treated with vehicle (A, B and C) while the other group of mice was treated with ST-246 (D, E and F). The mice were monitored for general disease symptoms such as wasting, ruffling of the fur (A and D), lesion formation and severity (B and E), and evidence of dissemination such as viral conjunctivitis (C and F) or satellite lesion formation (arrow in B). Mice were photographed on day 9 post-challenge. Images are representative of three mice per group.

tial studies we used VV-WR to vaccinate 7-week-old female BALB/c mice. We administered a dose of 1×10^6 pfu/mouse by dermal scarification of the tail in order to approximate the human vaccine dose and route of administration. This dose also corresponds to approximately 10 times the mean lethal dose (10 LD_{50}) of virus when mice are challenged intranasally. One group of vaccinated animals was treated with vehicle while the other was treated with ST-246 as detailed in Materials and Methods. Vaccinated mice that were treated with vehicle formed tail lesions characterized by redness and induration on days 3 through 5, ulcerated blistering on days 6 through 8, crusting and scab formation on days 9 through 14 with eventual release of the scab within 3—4 weeks post-vaccination and residual scarring. Lesions appeared most severe on or about day 9 (Fig. 1B) and often involved tissue layers beneath the dermis. Although none of the mice died as a result of VV-WR vaccination, all exhibited apparent systemic disease characterized by ruffling of the fur and wasting, with some animals experiencing conjunctivitis (Fig. 1A and C) and satellite lesion formation (Fig. 1B arrow). Mice that were treated with ST-246 formed lesions with the same characteristic stages of progression and resolution but they were much less severe at each stage (Fig. 1E) and did not extend beyond the dermis. Furthermore, ST-246-treated mice exhibited no symptoms of systemic disease such as fur-ruffling, wasting and conjunctivitis seen with the vehicle-treated mice (Fig. 1D and F). This demonstrates that after oral delivery, ST-246 is present in the skin at concentration sufficient to arrest viral dissemination and prevent severe lesion formation.

For the remainder of the study we used Dryvax for vaccinations. The vaccine dose was reduced to 5×10^5 pfu/mouse in order to remain consistent with precedent in the literature where doses ranging from 1×10^5 to 5×10^5 pfu/mouse are typically described [20,21]. Seven-week-old female BALB/c mice were vaccinated with 5×10^5 pfu of Dryvax but otherwise in an identical manner as the VV-WR-vaccinated mice. To date we have treated 95 Dryvax vaccinated mice with ST-246. We have observed lesion formation in 93 of 95 mice (this study and unpublished observations D. Grosenbach). In no case have the animals exhibited disease symptoms and all have remained healthy in appearance throughout the experiments. Progression through the characteristic stages and timing of lesion formation and resolution was similar to VV-WR vaccination although less severe at every stage (Fig. 2). Lesions were superficial and restricted to the dermis. While ST-246 dramatically reduced lesion severity in all VV-WR-vaccinated mice, there was no consistent evidence of a reduction in lesion severity when Dryvax vaccinated mice were treated with ST-246. This is likely due to the reduced virulence of Dryvax relative to VV-WR.

Short- and long-term protective immunity elicited by Dryvax vaccination in combination with ST-246 treatment

Intranasal challenge of BALB/c mice with VV-WR is a well-accepted model for lethal poxvirus challenge and is commonly used to evaluate vaccine efficacy. In order to compare the protective efficacy of standard vaccination with vaccine given in combination with ST-246, mice were vaccinated with 5×10^5 pfu Dryvax by dermal scarification. One group of 15 vaccinated mice was treated with vehicle while another group of 15 vaccinated mice was treated with ST-246. Naïve mice were maintained as controls. Lesions formed as a result of the scarification in all mice regardless of treatment with vehicle or ST-246 (data

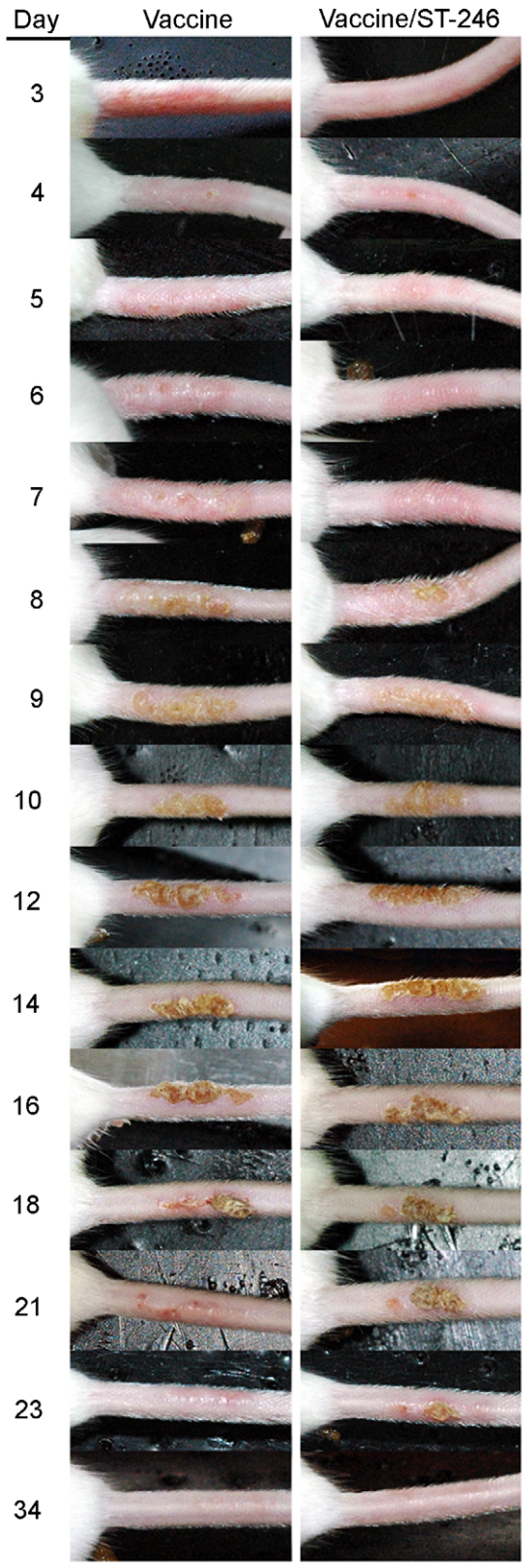

Figure 2 Lesion formation following dermal scarification with Dryvax ±ST-246. Seven-week-old BALB/c mice were vaccinated with 5×10^5 pfu of Dryvax by dermal scarification. One group of mice was treated with vehicle while the other group was treated with ST-246. All mice in each group were photographed daily. Individual mice shown here were chosen as representative of their respective groups by independent evaluation of all images and selection by the majority of seven laboratory personnel (see acknowledgements).

not shown; see Fig. 2). All lesions were fully resolved prior to challenge. At 21 days post-vaccination, the mice were challenged with 10, 100 or 1000 LD_{50} doses of VV-WR by the intranasal route. One group of naïve mice was challenged with a $10 \times LD_{50}$ dose while another group of naïve mice was not challenged. The mice were then monitored 14 days for evidence of disease, weight loss, and failure to maintain body temperature. The naïve mice, receiving a 10 LD_{50} challenge showed progressive evidence of disease, loss of weight and failure to maintain body temperature until day 8, when they were euthanized for humane reasons. All vaccinated mice survived the challenge regardless of treatment (±ST-246) or the challenge dose (Fig. 3A). The most pronounced effect of the challenge was evident as weight loss on days 4 and 6 post-challenge in vaccine/ST-246-treated mice receiving the 1000 LD_{50} dose (Fig. 3B). The weight of these mice was significantly reduced on day 4 post-challenge ($86 \pm 9.85\%$ of starting weight) relative to all other groups (all P values <0.05) with the exception of the naive mice receiving a 10 LD_{50} dose ($81 \pm 3.12\%$ of starting weight) ($P = 0.727$). All other challenged mice maintained weights that were not significantly different than unchallenged mice. On Day 6, the weights of the vaccine/ST-246-treated mice receiving the 1000 LD_{50} dose increased and were significantly better than the naïve mice receiving a 10 LD_{50} dose ($P = 0.034$) but remained significantly lower than all other groups (all P values <0.05). By day 8, the weights of the vaccine/ST-246-treated mice receiving the 1000 LD_{50} dose were near normal and not significantly different than any of the vaccine-protected groups. It is notable that the naive mice, challenged with 10 LD_{50} showed a significant drop in temperature beginning on day 4, while all groups of vaccinated mice (±ST-246) maintained temperatures that were never significantly different than unchallenged naïve mice regardless of the challenge dose (Fig. 3C). Furthermore, vaccinated mice (±ST-246) showed no other signs of disease during the course of the study regardless of challenge dose. These results suggest that vaccine efficacy is not compromised by adjunct treatment with ST-246.

We also evaluated long-term immunity elicited by vaccination in combination with ST-246. Seven-week-old female BALB/c mice were vaccinated and treated with vehicle or ST-246 as described above. At 180 days post-vaccination, mice were lethally challenged with VV-WR by the intranasal route. An LD_{50} titration was performed immediately prior to challenge in identically aged mice. The challenge dose at day 180 post-vaccination was calculated to be 10 LD_{50} based on this relevant titration experiment. Following challenge, the mice were then monitored 14 days for evidence of disease, weight loss, and failure to maintain body temperature. The naive mice steadily lost weight and failed to maintain temperature from day 4 on, reaching a nadir on day 8 post-challenge, at which time three of the five mice in that group were euthanized for humane reasons. Two of the five mice in the naïve group survived the challenge although it should be noted that they exhibited severe disease and weight loss

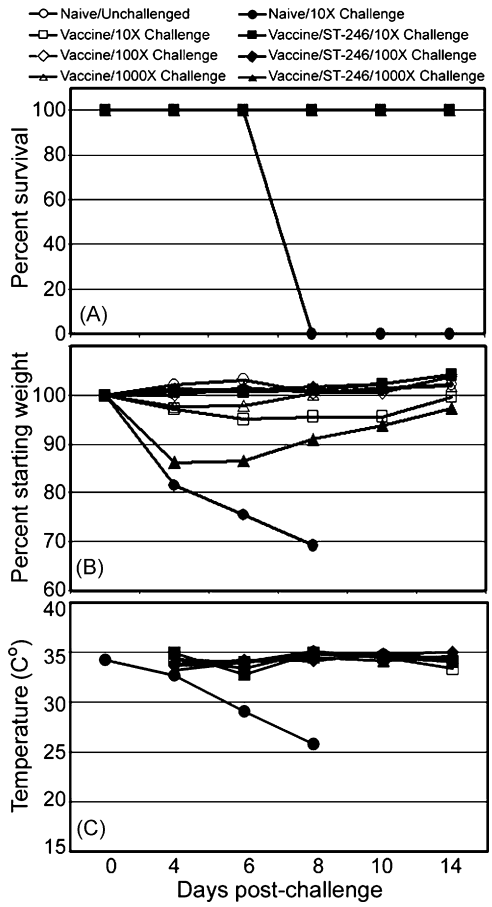

Figure 3 Vaccination in combination with ST-246 and protection from lethal challenge. Thirty mice were vaccinated with 5×10^5 pfu Dryvax by dermal scarification 21 days prior to challenge. One group of 15 vaccinated mice was treated with vehicle while another group of 15 mice was treated with ST-246. On the day of challenge, vaccine/vehicle-treated mice were divided into three groups of 5 mice that were challenged intranasally with VV-WR at 10 LD_{50} (□), 100 LD_{50} (◊), or 1000 LD_{50} (△) doses. Also, on the day of challenge, vaccine/ST-246-treated mice were divided into three groups of 5 mice that were challenged intranasally with VV-WR at 10 LD_{50} (■), 100 LD_{50} (♦), or 1000 LD_{50} doses (▲). As controls, naïve mice were challenged with a 10 LD_{50} dose of VV-WR (●) or remained naïve (○). Mice were monitored for 14 days post-challenge for survival (A), maintenance of starting weight (B), and temperature (C). In A, all experimental groups had 100% survival with the exception of the Naïve/10X Challenge group. Error bars are omitted in B and C to prevent obscuring the data. Individual data points did not deviate from the plotted means by more than 4% at all points with the exception of the ''Vaccine/ST-246/1000X Challenge'' group in B, which had standard deviations of 9.85, 10.81, 10.15, 6.7, and 3.96 on days 4, 6, 8, 10 and 14, respectively, and the ''Vaccine/10X Challenge'' group in B, which had standard deviations of 6.75, 8.52, 9.57, 8.77, and 5.95 on days 4, 6, 8, 10 and 14, respectively.

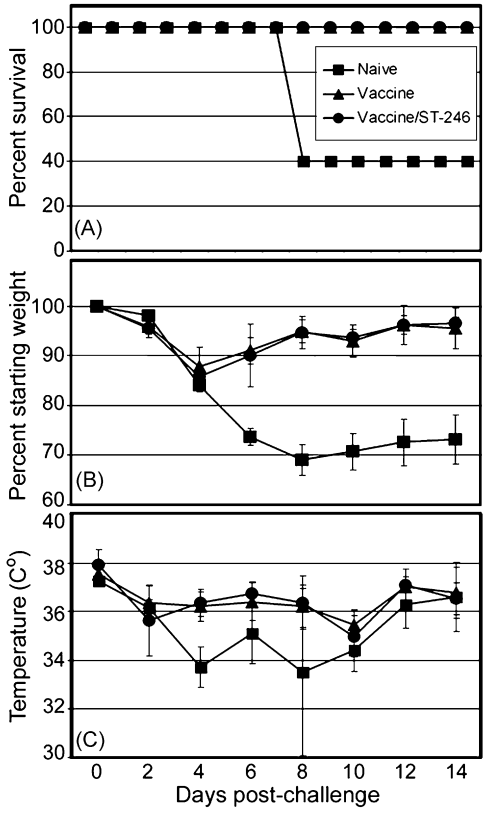

Figure 4 Long-term protection from lethal challenge. Mice were vaccinated with 5×10^5 pfu Dryvax 180 days prior to challenge. One group of vaccinated mice was treated with vehicle (▲) while another group of vaccinated mice was treated with ST-246 (●). Naïve age-matched mice were treated with vehicle and were maintained as controls (■). On day 180, all mice were challenged intranasally with a 10 LD_{50} dose of VV-WR. Mice were monitored for 14 days post-challenge for survival (A), maintenance of starting weight (B), and temperature (C).

from which they never fully recovered. In most experiments, lethally challenged mice are euthanized for humane reasons when their weight loss exceeds 30% of their starting weight. The two surviving unvaccinated mice lost 32% and 28% of their weight respectively but maintained near normal body temperature. For this reason they remained on study. Both groups of vaccinated mice (±ST-246) survived the challenge (Fig. 4A) and lost an equivalent amount of weight (~10% for both groups) on days 2 and 4 post-challenge, then steadily regained it until the end of the monitoring period (Fig. 4B). After an initial drop in temperature on day 2 post-challenge (that may be due to the slightly higher than normal temperatures recorded for all mice on day 0) the mice maintained temperatures throughout the monitoring period (Fig. 4C). From this we conclude that long-term immunity is equally protective between the two treatment groups and is not affected by vaccination in combination with ST-246.

Comparison of IgG responses to vaccination ±ST-246

The correlates of protective immunity elicited by the smallpox vaccine are not clearly defined. In humans, the smallpox

Table 1 Average reciprocal endpoint ELISA IgG titers and IC_{50} neutralization titers

Antigen	Days post-vaccination (± ST-246)										
	Day 7[a]		Day 14			Day 33[a]			Day 180		
	–	+	–	+	*P*[b]	–	+	*P*	–	+	*P*
Virus	LOD[c]	LOD	12,829	7,245	0.58	204,006	256,087	0.54	119,565	23,846	0.17
A27L	<100	<100	914	529	0.38	10,732	10,530	0.88	6,070	1,212	0.08
A33R	<25	<25	458	56	0.27	3,224	2,359	0.57	2,556	1,963	0.73
B5R	<25	<25	338	119	0.48	1,276	1,384	0.93	422	1,658	0.34
D8L	<25	<25	20,676	15,221	0.58	19,820	21,668	0.89	20,030	4,760	0.006
L1R	<50	<50	290	81	0.35	1,700	1,349	0.68	2,688	1,126	0.20
IC_{50}	<100	221	238	231	0.47	4,850	10,111	0.21	2,727	884	0.22

[a] The data shown for days 7 and 33 are representative of three separate experiments using five mice per group in each experiment. The data from days 14 and 180 are from a single experiment using five mice per group.
[b] *P* value; endpoint titers and IC_{50} titers were determined for each individual mouse ($n = 5$). Student's *t*-test were used to determine the level of significance between the two sample groups.
[c] LOD, limit of detection; in three repeat experiments, results varied from both groups being below the limit of detection to one or the other group with detectable but low levels of IgG, while the other was below the limit of detection.

vaccine induces robust, long-lasting humoral and cellular responses [22,23]. Both have been demonstrated to be protective in animal models [24,25]. So, while it is clear that vaccination in combination with ST-246 elicits comparable protective immunity to lethal challenge, the immune responses may not be the same. For that reason, we examined both cellular and humoral responses to vaccination with and without ST-246. To test humoral responses, 7-week-old female BALB/c mice were vaccinated and treated with ST-246 or vehicle as described above. On days 7, 14, 33, and 180 post-vaccination, animals (5 mice/group/timepoint) were sacrificed and blood was obtained by cardiac puncture. Sera samples were tested individually for IgG reactivity towards intact UV-inactivated vaccinia and bacterially expressed vaccinia antigens, D8L, L1R, A27L, B5R, and A33R and endpoint titers were determined by ELISA. Experiments examining immune responses to vaccine on days 7 and 33 post-vaccination were repeated three times. Data from days 14 and 180 post-vaccination are from single experiments, using 5 mice/group analyzed individually. The results of a representative set of experiments are summarized in Table 1, listing the average titers from each group. At day 7 post-vaccination, reactivity to intact virus or individual vaccinia antigens was at or below the level of detection, respectively. We also tested IgM responses at day 7 but were unable to detect specific reactivity above high background reactivity (data not shown). At day 14 post-vaccination, IgG responses were uniformly higher in the vaccine/vehicle-treated group compared to the vaccine/ST-246-treated group although this was not statistically significant. The lack of significance is likely due to the variation in responses within experimental groups. At day 33, IgG responses were very similar regardless of vaccination with or without ST-246. The IgG responses are lower for nearly all of the antigens by day 180 in both treatment groups although the only statistically significant difference was in the response to D8L. The mice that were vaccinated and treated with ST-246 have lower titers to all antigens except for B5R. The lack of significance is likely due to the variation in responses within experimental groups. It should be noted that when vaccinating these animals, five of five mice in the vaccine/vehicle group formed lesions while only three of five mice in the vaccine/ST-246 group formed lesions. Considering that vaccine take in the presence of ST-246 is generally very efficient (i.e., 93 of 95 vaccinated mice formed lesions) we cannot account for this apparent discrepancy although we consider it possible that a technical error was made in the delivery of the vaccine to these mice resulting in the delivery of a substandard dose. In later *ex vivo* assays, these mice exhibited vaccinia-specific splenic cellular responses confirming that they were exposed to vaccinia even though no lesion was evident. Therefore, these mice are included in all analyses of immune responses even though there is a possibility they were not efficiently vaccinated. The two mice that did not form lesions have distinctly lower IgG responses to all antigens tested and also have much lower virus neutralizing titers but have roughly equivalent cellular responses. Therefore, the titers reported in Table 1 are the average of all five mice in the group. In performing analyses of the data excluding the two non-responding mice, the titers are still generally lower for all antigens except for B5R where vaccine/vehicle-treated mice have a titer of 422 and vaccine/ST-246-treated mice have a titer of 3161 and A33R where vaccine/vehicle-treated mice have a titer of 2556 and vaccine/ST-246-treated mice have a titer of 3914. These data suggest that the IgG response to vaccination when combined with ST-246 treatment may develop more slowly, reaching equivalent titers in the short-term and possibly waning to a lower yet still protective titer in the long-term.

MV and EV neutralizing serum responses

Antibodies generated in response to vaccination are thought to primarily provide protection by neutralizing both the MV and EV forms of virus. Neutralization of the MV form of the virus was measured using a plaque-reduction assay. Dilutions of heat-inactivated serum (from individual mice) were incubated with a fixed amount of MV prior to infection

of cells. The mean inhibitory concentration of serum resulting in a 50% reduction in the number of plaques observed when the virus was incubated with naïve serum, termed IC_{50}, was calculated by fitting the data to a four parameter logistic model (variable slope and non-linear regression model) using curve-fitting software. The IC_{50} was determined for sera on days 7, 14, 33 and 180 post-vaccination (Table 1). On day 7 post-vaccination, the IC_{50} for sera from vaccine/vehicle-treated mice was below the limit of detection while the IC_{50} for sera from vaccine/ST-246-treated mice was 221. On day 14, the IC_{50} was similar for both groups (231 vs. 238), while on day 33, the IC_{50} for vaccine/vehicle-treated mice was 4,850 and the IC_{50} for vaccine/ST-246-treated mice was 10,111. By day 180, the IC_{50} for vaccine/vehicle-treated mice had dropped to 2727 and the IC_{50} for vaccine/ST-246-treated mice was 884.

Neutralization of the EV form of the virus was measured using a comet-inhibition assay, a qualitative assessment. The IHD-J strain of vaccinia is a variant that produces relatively large amounts of EV that are released by the cell. As a result of infecting cells downstream in the microcurrents of cell cultures, comet-shaped plaques are formed instead of typical round plaques. Antibody neutralization of EV causes the aggregation of EV on the surface of infected cells preventing the formation of comet-shaped plaques. Normal cell-to-cell spread of virus is not inhibited. Therefore, round-shaped plaques form. IHD-J was diluted such that upon infection, approximately 20 plaques would form per well. Following the infection period, the viral inoculum was aspirated and replaced with fresh media containing a 1:50 dilution of serum (pooled from 5 mice/group/timepoint). The virus was cultured for 36 h before staining the cell monolayer. The sera from the treatment groups were compared to sera from age-matched naïve mice (Fig. 5). The day 7 sera from vaccine/vehicle-treated mice did not inhibit comet formation while the sera from vaccine/ST-246-treated mice completely inhibited plaque-formation altogether. HPLC analysis of the day 7 sera confirmed that the ST-246-treated mice still had sufficiently high concentrations of ST-246 in their blood and that even with a 1:50 dilution, the level of ST-246 was above the tissue culture EC_{50} (data not shown). This likely also accounts for the apparently higher IC_{50} observed for the day 7 sera from vaccine/ST-246-treated mice. On day 14, the sera from both treatment groups partially inhibited comet-formation and by day 33, comet-formation was completely inhibited by sera from both treatment groups. The day 180 sera from both treatment groups were still capable of inhibiting comet formation although not as efficiently as the day 33 sera. While typical comets did not form, there were more satellite plaques visible. These data indicate that ST-246 does not affect the development of neutralizing antibody responses.

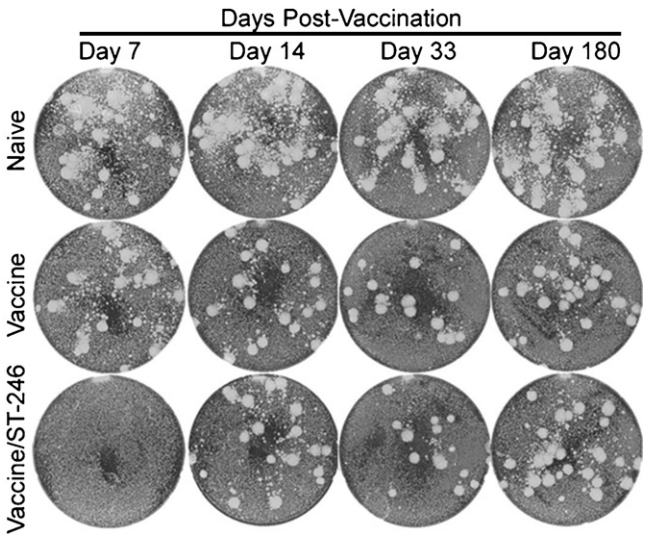

Figure 5 Serum inhibition of IHD-J comet formation. Mice were vaccinated with Dryvax by dermal scarification and treated with vehicle or ST-246, as detailed in Materials and Methods. On days 7, 14, 33, or 180 post-vaccination, pooled sera from each experimental group were tested for the ability to inhibit comet formation by vaccinia IHD-J. BSC40 cell monolayers were infected with IHD-J at a low multiplicity of infection. Following infection, medium containing a 1:50 dilution of pooled sera from naïve mice or vaccinated mice treated either with vehicle or ST-246 was added to the cells. The monolayers were stained with crystal violet at 36 h post-infection.

Cellular immune responses

Mice were vaccinated and treated with vehicle or ST-246 as described above. The mice were sacrificed on days 7, 14, 33, and 180 post-vaccination and splenic cellular immune responses were analyzed by cytometric bead array for cytokine release, intracellular cytokine stain (ICCS) for IFN-γ production by $CD4^+$ and $CD8^+$ T cells, and for proliferation in response to restimulation with vaccinia. The cytometric bead array mouse Th1/Th2 kit (TNF-α, IFN-γ, IL-2, IL-4 and IL-5) was used to analyze cytokine release in response to vaccinia. Splenocytes from vaccinated ($\pm$ST-246) or naïve mice were stimulated immediately *ex vivo* with mock-infected or VV-WR-infected A-20 cells. VV-WR infected naïve splenocytes were used as stimulator cells in some experiments. A-20 cells are syngeneic to the BALB/c background and express both class I and class II molecules and are therefore capable of stimulating both $CD4^+$ and $CD8^+$ T cells. Supernatants were collected at 48 h and the cytokine concentrations were determined (Fig. 6). Splenocytes from all treatment groups produced background levels near zero of all cytokines tested when stimulated with mock-infected cells. Naïve splenocytes produced very little cytokines above background in response to stimulation with vaccinia. Vaccinia-stimulated splenocytes from vaccinated mice ($\pm$ST-246) produced TNF-α, IFN-γ, and IL-2 well above background at all time points tested (Fig. 6), while IL-4 and IL-5 were produced at levels near the limits of detection for the assay (20 pg/ml, data not shown). In general, the production of TNF-α, IFN-γ, and IL-2 was slightly enhanced by vaccination in combination with ST-246 at days 7, 14 and 33 post-vaccination. While this was most pronounced at day 7, it was not statistically significant. In comparing the vaccine/vehicle-treated group to the vaccine/ST-246-treated group, the *P* values for TNF-α and IFN-γ were 0.1 and 0.07, respectively. At day 180 post-vaccination, the cytokine response to vaccine given in combination with ST-246 is slightly reduced in comparison

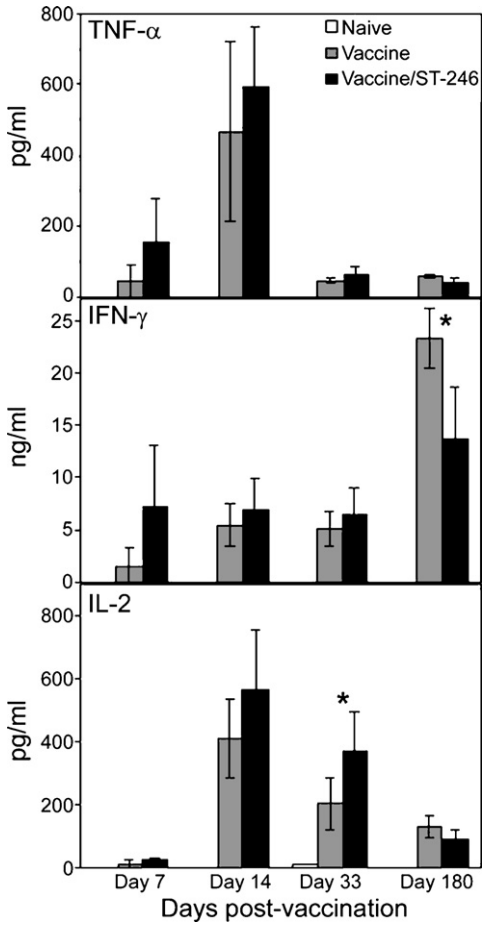

Figure 6 Vaccinia-specific cytokine release. Mice were vaccinated with Dryvax by dermal scarification and treated with vehicle or ST-246, as detailed in Materials and Methods. On days 7, 14, 33, and 180 post-vaccination, splenic T-cell responses were measured. Splenocytes from individual mice (5 mice/group/timepoint) were analyzed for vaccinia-specific cytokine responses. Splenocytes were stimulated with vaccinia-infected naive splenocytes (days 7 and 14) or vaccinia-infected A-20 cells (days 33 and 180) for 48 h and then the supernatants were tested for TNF-α, IFN-γ, and IL-2 release by cytometric bead array. The graphs show the mean response and error bars represent the standard deviation of five individually analyzed mice. Asterisks indicate significant differences (>95% confidence interval, two-tailed *t*-test).

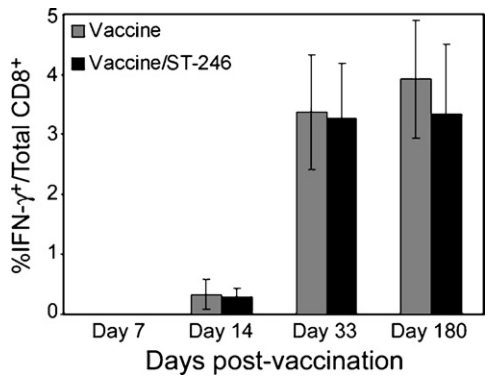

Figure 7 Intracellular cytokine staining of vaccinia-specific $CD8^+$ T cells. Mice were vaccinated with Dryvax by dermal scarification and treated with vehicle or ST-246, as detailed in Materials and Methods. On days 7, 14, 33, and 180 post-vaccination splenic $CD8^+$ T-cell responses were measured. Mice were individually analyzed (5 mice/group/timepoint). Splenocytes from naïve or vaccinated mice (±ST-246) were stimulated with vaccinia-infected naive splenocytes (days 7 and 14) or vaccinia-infected A-20 cells (days 33 and 180) for 12 to 16 h in the presence of brefeldin A and IL-2 and then stained for extracellular CD8α and intracellular IFN-γ. The cells were analyzed by flow cytometry: the $CD8^+$/IFN-γ^+ is plotted as a percentage of total $CD8^+$ T cells (mean of five individually analyzed mice). Background staining (vaccinia stimulation of naïve splenocytes) is subtracted from all values. Error bars represent the standard deviation of five individually analyzed mice.

to the vaccine-vehicle-treated animals. This is most pronounced in the IFN-γ response where the differences are statistically significant ($P = 0.005$).

For the ICCS, splenocytes from vaccinated (±ST-246) or naïve mice were stimulated immediately *ex vivo* with VV-WR-infected A-20 cells or VV-WR infected naïve splenocytes. The splenocytes were stimulated for a total of 12—16 h in the presence of brefeldin A and human IL-2 prior to staining with anti-CD8α, anti-CD4 and anti-IFN-γ antibodies. In three repeats of this experiment, we never observed $CD4^+$ or $CD8^+$ T cell IFN-γ responses at day 7 post-vaccination (Fig. 7). This is in contrast to numerous reports in which robust cellular responses have been observed 7—9 days post-infection with vaccinia [17,26—28]. It is likely that the differences in our observations and those of others are due to the virus used (Dryvax vs. VV-WR), the dose used and the different routes of viral challenge (dermal scarification vs. intraperitoneal). At day 14 post-vaccination, the $CD4^+$/IFN-γ response was still below the limits of detection while there was a low but detectable $CD8^+$/IFN-γ response in both treatment groups that was similar in magnitude. At day 33 post-infection there was a clearly detectable IFN-γ response specific for vaccinia. The percentage of $CD4^+$ T cells producing IFN-γ was low but significantly above background. The responses were similar regardless of vaccination with or without ST-246 and in all repeats of the experiment ranged from 0.35 to 0.5% of total $CD4^+$ T cells (data not shown). The $CD8^+$ responses in each treatment group were also similar at day 33. In three repeats of the experiment, 3.37, 3.86 and 4.87% of $CD8^+$ T cells stained positive for IFN-γ in the vaccine/vehicle group while 3.26, 5.06 and 5.61% of $CD8^+$ T cells stained positive for IFN-γ in the vaccine/ST-246 group. In the first two experiments, pooled splenocytes from 5 mice/group were used. In the third experiment, 5 mice/group were individually analyzed and the mean response is reported here. On day 180 post-vaccination the percentages of vaccinia-specific $CD4^+$ (data not shown) and $CD8^+$ T cells staining positive for IFN-γ were essentially the same as that observed on day 33. In analyzing the cytokine responses to restimulation, it is apparent that there is a relatively robust vaccinia-specific TNF-α and IFN-γ response at day 7 post-vaccination. By the ICCS it is also apparent that at least the IFN-γ is not being produced by $CD8^+$ or $CD4^+$ T cells. This raises the possibility of involvement of other immune cells, such as those involved in the innate response, at early time points post-vaccination.

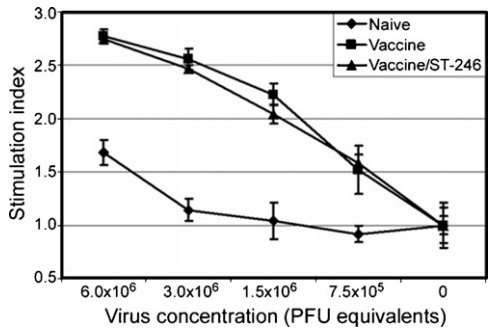

Figure 8 Vaccinia-specific proliferative responses. Mice were vaccinated with Dryvax by dermal scarification and treated with vehicle or ST-246 as detailed in Materials and Methods. On day 33 post-vaccination splenic proliferative responses were measured. Pooled splenocytes (5 mice/group) were stimulated with various dilutions of heat-killed vaccinia (as indicated on the *x*-axis) for 4 days and then proliferation was measured using the CellTiter 96® AQ_{ueous} One Solution. The stimulation index was calculated using the formula: (test absorbance − background absorbance)/(no antigen control absorbance − background absorbance). Error bars indicate standard deviations from triplicate wells.

Although we have not identified the cells involved in this early response, it appears that ST-246 treatment may slightly enhance this response.

Splenocytes were also tested for proliferative responses to vaccinia antigens at day 33 post-vaccination. Heat-killed whole virus was used as the source of antigen. Various dilutions of the heat-killed virus were incubated with a fixed number of splenocytes. Proliferative responses were measured after 4 days incubation. The stimulation index was calculated for each experimental group using control wells that were incubated in the absence of antigen. The stimulation index for naïve mice was at or near 1 at all dilutions of antigen. The stimulation index for vaccine/vehicle- and vaccine/ST-246-treated groups was greater than 2.5 at the highest concentration of antigen and titrated in a linear fashion with each dilution of antigen (Fig. 8). The stimulation index for the vaccine/vehicle and the vaccine/ST-246 groups was significantly higher ($P < 0.05$) than the stimulation index of naïve splenocytes at every dilution of antigen. There were no statistically significant differences in stimulation between vaccine/vehicle- and vaccine/ST-246-treated groups at any dilution of antigen. The data in Fig. 8 is representative of three independently performed experiments examining proliferation at day 33 post-vaccination with similar results. We also tested for proliferative responses to vaccination at days 14 and 180 post-vaccination (data not shown) but were unable to detect responses above a relatively high background. This may represent a limitation to the use of the non-radioactive detection reagents used in this assay, since we anticipated proliferative responses at these times. Alternatively, the proliferative response to vaccination may be genuinely undetectable at these times.

Discussion

There is a need for a safer smallpox vaccine. The current U.S.-approved smallpox vaccine has the highest adverse event rate of any vaccine being used [29]. Historically 1000 out of a million vaccinees suffer complications from smallpox vaccination. More than 50 out of a million events will be life-threatening and 1—2 out of a million will result in death [5]. Life-threatening vaccine-related complications most often arise in individuals with a disease, condition, or treatment resulting in a disrupted skin barrier or compromised immunity. Approximately, 25% of the U.S. population is at risk or would place a close contact at risk of complication from the vaccine [29].

Current efforts to generate safer smallpox vaccines include the use of live but attenuated strains of vaccinia such as Modified Vaccinia Ankara (MVA) or defective Lister LC16m8 [21,30—37]. Alternatives to the live virus approach to smallpox vaccine development include the use of subunit DNA [38—40], peptide [25], or protein vaccines [41,42] and bacterial vectors [43]. These alternatives are in the early stages of development and significant developmental, clinical and regulatory hurdles are likely to be encountered prior to approval for wide-spread clinical use. It should be noted that in the U.S., the Dryvax vaccine will very likely be replaced by ACAM2000 (Acambis plc, Cambridge, UK), a new live virus vaccine derived from Dryvax. While this vaccine has been demonstrated to be as effective as Dryvax, the risk of adverse events due to vaccination apparently remains the same. Immune responses to this vaccine with and without ST-246 as an adjunct should also be tested also.

We propose an alternative approach to the new vaccines in development; one in which the Dryvax vaccine (or ACAM2000), which is proven to be effective but not entirely safe, is used in combination with ST-246, a novel antiviral drug in development as a smallpox therapeutic, to improve its safety. In previous animal studies [13], it has been demonstrated that ST-246 protects animals from lethal poxvirus challenge and suggests that protective immunity is elicited even in the presence of the drug. Previous to this report, a detailed comparative analysis of immune responses elicited by smallpox vaccination in the presence of ST-246 has not been performed.

The data presented in this study demonstrate that protective immunity elicited by the smallpox vaccine is not compromised by concurrent treatment with ST-246. ST-246 reduces the severity of lesion formation due to vaccination with VV-WR but has no effect on the less severe lesion formation due to Dryvax vaccination (Figs. 1 and 2). This suggests that ST-246, if used as an adjunct to the smallpox vaccine in humans, will not inhibit the vaccine ''take''—the lesion formed as a result of vaccination that is often used as evidence of vaccine-induced protective immunity. We consider this to be significant considering that in the event of a smallpox outbreak the vaccine will be administered to all suspected of exposure. In the absence of a lesion, serological testing would be necessary to confirm vaccine-induced protective immunity.

The IgG responses to whole virus or individual virion proteins as determined by ELISA are slightly reduced by ST-246 (Table 1), but the virus-neutralizing serum responses are unaffected by the drug (Table 1 and Fig. 5). Cellular immune responses were not significantly affected by ST-246: the cytokine release assays demonstrate that TNF-α, IFN-γ, and IL-2 responses are slightly enhanced by ST-246 (Figs. 6 and 7) at early times post-vaccination, while the

proliferative responses to vaccinia antigens are unaffected (Fig. 8). Our observations that early cellular responses may be slightly enhanced by ST-246 were initially considered to be the result of normal experimental variability, but in all repeats of the experiment, the trend remained consistent. Additional studies will be necessary to further confirm the reproducibility of this phenomenon and understand the mechanism. Considering our results, it is possible that vaccination in combination with ST-246 enhances natural killer cell activity, or NK1.1 or $\gamma\delta$ T cell activation at early times post-infection since cytokine production did not appear to be due to detectable $CD4^+$ or $CD8^+$ T cell activation although the response was virus-specific. We will address these possibilities in future studies. It was intriguing that the IFN-γ response on day 180 post-vaccination as determined by the CBA assay was elevated relative to earlier time-points. To our knowledge there is no precedent in the literature for this observation. These assays were necessarily performed at different times (although the assay conditions were identical) so it is possible that the results reflect inter-assay variability. We consider this unlikely in light of the fact that the TNF-α and IL-2 responses, measured from the same sample, as well as our negative control samples, are not correspondingly elevated. Analysis of the IFN-γ response by ICCS at these same time-points using the same preparation of splenocytes and stimulator cells demonstrates that the number of cells producing IFN-γ in response to restimulation is very similar at days 33 and 180 post-vaccination. It is possible that at day 180 post-vaccination, T-cell avidity is higher, thus enabling a more robust recall response to restimulation or, alternatively, non-$CD4^+$ or -$CD8^+$ antigen-specific responses are contributing to the overall response similar to what was observed on day 7 post-vaccination.

ST-246 is in development as a post-exposure therapeutic for smallpox. It has been proven to be extremely efficacious in preventing poxvirus disease in a post-exposure setting. Considering this, it is reasonable to think that ST-246 may be used to prevent or treat smallpox vaccine-related adverse events. It is debatable whether a 14-day treatment regimen is feasible under the conditions in which smallpox vaccination would be given (i.e., prior to deployment for military personnel, prophylactic vaccination of ''first responders'', or emergency vaccination of the civilian population in the event of a smallpox outbreak). This issue cannot be specifically addressed at this time considering that ST-246 is still under development as a drug and that final formulations and dosing requirements are unknown.

In this study, we demonstrate that protective immune responses to the vaccine are not inhibited by ST-246. This study was performed in immunocompetent mice so that a direct comparison of robust immune responses could be made. The risk of adverse events in humans is elevated in immunocompromised individuals or those with a history of eczema (atopic dermatitis) or other exfoliative skin disorders amongst other contraindications. Murine models for these situations exist. Future studies will be focused on testing the pathogenicity of the smallpox vaccine and prophylactic and therapeutic efficacy of ST-246 in these models Additionally, we will begin to address the in vivo mechanism of action of ST-246 on poxvirus replication and dissemination both at the site of inoculation (the ''pock'') and at in distal tissues as well as dissemination between hosts. Based on our previous data [13], the observations of others [14,19] and findings presented here, we hypothesize that ST-246 will be effective in preventing or treating smallpox vaccine-related adverse events in these models without compromising immune responses. This raises the possibility that ST-246 may be used as an adjunct to the smallpox vaccine in individuals, for whom the vaccine is currently contraindicated, protecting them from serious adverse events, without inhibiting the generation of protective immunity.

Acknowledgements

This work was supported, in part, by National Institutes of Health SBIR grant 2R44AI056409-05. All authors are employees of Siga Technologies Inc. In addition to the authors (DG, AB, DK, KJ, RW and RJ), Kady Honeychurch (SIGA Technologies) assisted in evaluating tail lesion formation for the images shown in Fig. 2.

References

[1] Sharma DP, Ramsay AJ, Maguire DJ, Rolph MS, Ramshaw IA. Interleukin-4 mediates down regulation of antiviral cytokine expression and cytotoxic T-lymphocyte responses and exacerbates vaccinia virus infection in vivo. J Virol 1996;70(10):7103—7.

[2] Andrew ME, Coupar BE. Biological effects of recombinant vaccinia virus-expressed interleukin 4. Cytokine 1992;4(4):281—6.

[3] Ligon BL. Monkeypox: a review of the history and emergence in the Western hemisphere. Semin Pediatr Infect Dis 2004;15(4):280—7.

[4] Shchelkunov SN, Totmenin AV, Babkin IV, Safronov PF, Ryazankina OI, Petrov NA, et al. Human monkeypox and smallpox viruses: genomic comparison. FEBS Lett 2001;509(1):66—70.

[5] Lane JM, Ruben FL, Neff JM, Millar JD. Complications of smallpox vaccination, 1968: results of ten statewide surveys. J Infect Dis 1970;122(4):303—9.

[6] Moss B. Vaccinia virus: a tool for research and vaccine development. Science 1991;252(5013):1662—7.

[7] Payne LG. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J Gen Virol 1980;50(1):89—100.

[8] Smith GL, Vanderplasschen A, Law M. The formation and function of extracellular enveloped vaccinia virus. J Gen Virol 2002;83(Pt 12):2915—31.

[9] Blasco R, Moss B. Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-Dalton outer envelope protein. J Virol 1991;65(11):5910—20.

[10] Gurt I, Abdalrhman I, Katz E. Pathogenicity and immunogenicity in mice of vaccinia viruses mutated in the viral envelope proteins A33R and B5R. Antiviral Res 2005:158—64.

[11] McIntosh AA, Smith GL. Vaccinia virus glycoprotein A34R is required for infectivity of extracellular enveloped virus. J Virol 1996;70(1):272—81.

[12] Wolffe EJ, Isaacs SN, Moss B. Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination. J Virol 1993;67(8):4732—41.

[13] Yang G, Pevear DC, Davies MH, Collett MS, Bailey T, Rippen S, et al. An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects

mice from lethal orthopoxvirus challenge. J Virol 2005;79(20): 13139—49.
[14] Quenelle DC, Buller RM, Parker S, Keith KA, Hruby DE, Jordan R, et al. Efficacy of delayed treatment of ST-246 given orally against systemic orthopoxvirus infections in mice. Antimicrob Agents Chemother 2007;51(2):689—95.
[15] Earl PL, Cooper N, Wyatt S, Moss B, Carroll MW. Preparation of cell cultures and vaccinia virus stocks. In: Ausubel FM, Brent REKR, Moore DD, Seidman JG, Smith JA, et al., editors. Current protocols in molecular biology. New York: John Wiley and Sons; 1998. p. 16.16.1—3.
[16] Reed LJ, Muench LH. A simple method of estimating fifty percent endpoints. Am J Hygeine 1938;27:493—7.
[17] Harrington LE, Most Rv R, Whitton JL, Ahmed R. Recombinant vaccinia virus-induced T-cell immunity: quantitation of the response to the virus vector and the foreign epitope. J Virol 2002;76(7):3329—37.
[18] Newman FK, Frey SE, Blevins TP, Mandava M, Bonifacio Jr A, Yan L, et al. Improved assay to detect neutralizing antibody following vaccination with diluted or undiluted vaccinia (Dryvax) vaccine. J Clin Microbiol 2003;41(7):3154—7.
[19] Sbrana E, Jordan R, Hruby DE, Mateo RI, Xiao SY, Siirin M, et al. Efficacy of the antipoxvirus compound ST-246 for treatment of severe orthopoxvirus infection. Am J Trop Med Hyg 2007;76(4):768—73.
[20] Davies DH, McCausland MM, Valdez C, Huynh D, Hernandez JE, Mu Y, et al. Vaccinia virus H3L envelope protein is a major target of neutralizing antibodies in humans and elicits protection against lethal challenge in mice. J Virol 2005;79(18):11724—33.
[21] Wyatt LS, Earl PL, Eller LA, Moss B. Highly attenuated smallpox vaccine protects mice with and without immune deficiencies against pathogenic vaccinia virus challenge. Proc Natl Acad Sci USA 2004;101(13):4590—5.
[22] Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting edge: long-term B cell memory in humans after smallpox vaccination. J Immunol 2003;171(10):4969—73.
[23] Demkowicz Jr WE, Littaua RA, Wang J, Ennis FA. Human cytotoxic T-cell memory: long-lived responses to vaccinia virus. J Virol 1996;70(4):2627—31.
[24] Edghill-Smith Y, Golding H, Manischewitz J, King LR, Scott D, Bray M, et al. Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. Nat Med 2005;11(7):740—7.
[25] Snyder JT, Belyakov IM, Dzutsev A, Lemonnier F, Berzofsky JA. 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 2004;78(13):7052—60.
[26] Tscharke DC, Woo WP, Sakala IG, Sidney J, Sette A, Moss DJ, et al. Poxvirus CD8+ T-cell determinants and cross-reactivity in BALB/c mice. J Virol 2006;80(13):6318—23.
[27] Tscharke DC, Karupiah G, Zhou J, Palmore T, Irvine KR, Haeryfar SM, et al. Identification of poxvirus CD8+ T cell determinants to enable rational design and characterization of smallpox vaccines. J Exp Med 2005;201(1):95—104.
[28] Mathew A, Terajima M, West K, Green S, Rothman AL, Ennis FA, et al. Identification of murine poxvirus-specific CD8+ CTL epitopes with distinct functional profiles. J Immunol 2005;174(4):2212—9.
[29] Kemper AR, Davis MM, Freed GL. Expected adverse events in a mass smallpox vaccination campaign. Eff Clin Pract 2002;5(2):84—90.
[30] Coulibaly S, Bruhl P, Mayrhofer J, Schmid K, Gerencer M, Falkner FG. The nonreplicating smallpox candidate vaccines defective vaccinia Lister (dVV-L) and modified vaccinia Ankara (MVA) elicit robust long-term protection. Virology 2005;341(1):91—101.
[31] Earl PL, Americo JL, Wyatt LS, Eller LA, Whitbeck JC, Cohen GH, et al. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. Nature 2004;428(6979):182—5.
[32] Empig C, Kenner JR, Perret-Gentil M, Youree BE, Bell E, Chen A, et al. Highly attenuated smallpox vaccine protects rabbits and mice against pathogenic orthopoxvirus challenge. Vaccine 2006:3686—94.
[33] Kidokoro M, Tashiro M, Shida H. Genetically stable and fully effective smallpox vaccine strain constructed from highly attenuated vaccinia LC16m8. Proc Natl Acad Sci USA 2005;102(11):4152—7.
[34] McCurdy LH, Larkin BD, Martin JE, Graham BS. Modified vaccinia Ankara: potential as an alternative smallpox vaccine. Clin Infect Dis 2004;38(12):1749—53.
[35] Morikawa S, Sakiyama T, Hasegawa H, Saijo M, Maeda A, Kurane I, et al. An attenuated LC16m8 smallpox vaccine: analysis of full-genome sequence and induction of immune protection. J Virol 2005;79(18):11873—91.
[36] Stittelaar KJ, van Amerongen G, Kondova I, Kuiken T, van Lavieren RF, Pistoor FH, et al. Modified vaccinia virus Ankara protects macaques against respiratory challenge with monkeypox virus. J Virol 2005;79(12):7845—51.
[37] Vollmar J, Arndtz N, Eckl KM, Thomsen T, Petzold B, Mateo L, et al. Safety and immunogenicity of IMVAMUNE, a promising candidate as a third generation smallpox vaccine. Vaccine 2005:2065—70.
[38] Hooper JW, Custer DM, Schmaljohn CS, Schmaljohn AL. DNA vaccination with vaccinia virus L1R and A33R genes protects mice against a lethal poxvirus challenge. Virology 2000;266(2):329—39.
[39] Pulford DJ, Gates A, Bridge SH, Robinson JH, Ulaeto D. Differential efficacy of vaccinia virus envelope proteins administered by DNA immunisation in protection of BALB/c mice from a lethal intranasal poxvirus challenge. Vaccine 2004;22(25/26):3358—66.
[40] Hooper JW, Thompson E, Wilhelmsen C, Zimmerman M, Ichou MA, Steffen SE, et al. Smallpox DNA vaccine protects nonhuman primates against lethal monkeypox. J Virol 2004;78(9):4433—43.
[41] Fang M, Cheng H, Dai Z, Bu Z, Sigal LJ. Immunization with a single extracellular enveloped virus protein produced in bacteria provides partial protection from a lethal orthopoxvirus infection in a natural host. Virology 2006;345(1):231—43.
[42] Fogg C, Lustig S, Whitbeck JC, Eisenberg RJ, Cohen GH, Moss B. Protective immunity to vaccinia virus induced by vaccination with multiple recombinant outer membrane proteins of intracellular and extracellular virions. J Virol 2004;78(19):10230—7.
[43] Wilson RL, Hruby DE. Commensal bacteria as a novel delivery system for subunit vaccines directed against agents of bioterrorism. Adv Drug Deliv Rev 2005;57(9):1392—402.