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A Novel, Killed-Virus Nasal Vaccinia Virus Vaccine▽

Anna U. Bielinska,[1] Alexander A. Chepurnov,[1] Jeffrey J. Landers,[1] Katarzyna W. Janczak,[1] Tatiana S. Chepurnova,[1] Gary D. Luker,[2] and James R. Baker, Jr.[1]*

Michigan Nanotechnology Institute for Medicine and Biological Sciences (MNIMBS), University of Michigan, Ann Arbor, Michigan 48109,[1] and Department of Radiology, University of Michigan, Ann Arbor, Michigan 48109[2]

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Live-virus vaccines for smallpox are effective but have risks that are no longer acceptable for routine use in populations at minimal risk of infection. We have developed a mucosal, killed-vaccinia virus (VV) vaccine based on antimicrobial nanoemulsion (NE) of soybean oil and detergent. Incubation of VV with 10% NE for at least 60 min causes the complete disruption and inactivation of VV. Simple mixtures of NE and VV (Western Reserve serotype) (VV/NE) applied to the nares of mice resulted in both systemic and mucosal anti-VV immunity, virus-neutralizing antibodies, and Th1-biased cellular responses. Nasal vaccination with VV/NE vaccine produced protection against lethal infection equal to vaccination by scarification, with 100% survival after challenge with 77 times the 50% lethal dose of live VV. However, animals protected with VV/NE immunization did after virus challenge have clinical symptoms more extensive than animals vaccinated by scarification. VV/NE-based vaccines are highly immunogenic and induce protective mucosal and systemic immunity without the need for an inflammatory adjuvant or infection with live virus.

Smallpox is a dreaded human disease that was eradicated due to the efforts of the World Health Organization's vaccination program in 1980 (38). Ending compulsory smallpox vaccination was welcomed because the live-, attenuated-virus (vaccinia virus [VV]) vaccine Dryvax (Wyeth Laboratories) was associated with serious adverse effects, including the inadvertent transfer of replicating VV and mortality (24). Unfortunately, concerns about the use of smallpox as a biological weapon have led to a consideration of the reintroduction of live-vaccine vaccination (33), despite the current risk of the disease and the known risks of the vaccine. The vaccination program undertaken in the United States before the Iraq War demonstrated significant safety issues with the live VV vaccine (2, 33).

Given the aforementioned discussion, there is substantial interest in new vaccines for orthopoxviruses that represent potential pathogens for humans, including monkeypox, cowpox, and variola viruses. An optimal smallpox vaccine would be safer by employing inactivated virus or recombinant viral antigens while retaining the efficacy of the live vaccine. Also, some form of rapid mucosal administration would be advantageous. Unfortunately, current formulations of killed-virus vaccines for mucosal application are poorly immunogenic or use bacterial toxins as adjuvants, which have resulted in inflammation and autoimmunity (4, 6, 11). We used a nanoscale (<400-nm) oil-in-water emulsion as a formulation for a killed-virus mucosal smallpox vaccine. The antimicrobial spectrum of nanoemulsions (NE) is broad and includes enveloped viruses, bacteria, fungi, and spores (7, 17, 18, 30). Formulations similar to the one used for vaccine have been demonstrated as safe and effective in animal tests and in human trials for herpetic cold sores (23). This lack of toxicity is partly due to the emulsion particles being too large to effectively penetrate the tissue matrix and disrupt organized tissues.

Previously, we reported that an antimicrobial NE mixed with an enveloped virus (influenza virus) disrupted the virus and produced efficient immunization when applied topically to the nares (29). This present study evaluates the immunogenicity and effectiveness of a potential smallpox vaccine, based on an NE adjuvant mixed with VV (VV/NE) purified from tissue culture. Our findings indicate that the NE inactivates VV and that this mixture results in protective mucosal and systemic immunity when applied to the nares of mice.

MATERIALS AND METHODS

Animals. Pathogen-free 5- to 6-week-old female BALB/c mice were purchased from Charles River Laboratories. Vaccination groups were housed separately, with five animals to a cage, in accordance with the American Association for Accreditation of Laboratory Animal Care standards. All procedures involving mice were performed according to the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

Viruses. Two VV strains were used in these studies: VV Western Reserve strain (VV_{WR}) and $VV_{WR\text{-}Luc}$. VV_{WR} (NIH tissue culture adapted) was obtained from the American Type Culture Collection (ATCC). The recombinant VV ($VV_{WR\text{-}Luc}$) is the same virus but expresses firefly luciferase from the pH 7.5 early/late promoter and has been described previously (27). $VV_{WR\text{-}Luc}$ is not attenuated in vitro or in vivo because the virus was constructed using a method that does not require the deletion or disruption of any viral genes (1), as confirmed in prior studies (27).

Stocks of all viruses were generated using the method of Lorenzo et al. (25) with some modifications. Virus was propagated on Vero cells infected at a multiplicity of infection of 0.5. Cells were harvested at 48 to 72 h, and virus was isolated from culture supernatants and cell lysates. Cell lysates were obtained by rapidly freeze-thawing the cell pellet followed by homogenization in a Dounce homogenizer in 1 mM Tris, pH 9. The virus preparations contained both intracellular mature virus and extracellular mature virus virions. Cell debris was removed by centrifugation at 2,000 rpm. The purified virus stocks were obtained from the clarified supernatants by layering on 4% to 40% sucrose gradients, which were centrifuged for 1 h at 25,000 × *g*. Turbid bands, containing viral particles, were collected, diluted in 1 mM Tris, pH 9, and then concentrated by 1 h of centrifugation at 25,000 × *g*. Viral pellets were resuspended in 1 mM Tris, pH 9, and stored frozen at −80°C. The virus stocks were sonicated and subjected to titer determination on Vero cells before use (29).

* Corresponding author. Mailing address: Michigan Nanotechnology Institute for Medicine and Biological Sciences (MNIMBS), University of Michigan, 9220 MSRB III, 1150 West Medical Center Drive, Ann Arbor, MI 48109-5648. Phone: (734) 647-2777. Fax: (734) 936-2990. E-mail: jbakerjr@umich.edu.

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VV_{WR-Luc} has surface proteins identical to those of the native strain but expresses luciferase protein during infection (27). This allows for a sensitive cytotoxicity and morbidity assessment and monitoring of the viral infection in animals with imaging techniques. Comparison of the serological responses of VV_{WR}-immunized animals either by enzyme-linked immunosorbent assay (ELISA), Western blotting, or virus neutralization assay showed no difference in titers to VV_{WR} and VV_{WR-Luc}.

NE. NE ($W_{20}5EC$ formulation) was supplied by NanoBio Corporation, Ann Arbor, MI. This NE is manufactured by emulsification of cetyl pyridum chloride (1%), Tween 20 (5%), and ethanol (EtOH) (8%) in water with soybean oil (64%) by use of a high-speed emulsifier. Resultant droplets have a mean particle size of 300 ± 25 nm in diameter. $W_{20}5EC$ has been formulated with surfactants and food substances considered "generally recognized as safe" by the FDA. $W_{20}5EC$ can be economically manufactured under good manufacturing practices and is stable for at least 18 months at 40°C.

Virus inactivation assays. The virucidal activity of NE was determined using both a standard plaque reduction assay (PRA) (31) for VV_{WR} and the inhibition of luciferase activity in addition to PRA for the recombinant VV_{WR-Luc}. Generally, 10-μl samples of VV_{WR} or VV_{WR-Luc} were mixed with a 1% to 10% concentration of NE and incubated for 1, 2, or 3 h at 37°C. Subsequently, undiluted and serially diluted samples were used for the infection of Vero cell monolayers. No cellular toxicity was observed even with samples containing 10% NE, since the emulsion was diluted extensively before addition to the cells (the final alcohol and detergent concentrations in the cell culture medium were 0.01% and 0.016%, respectively). For PRA, cells were fixed and stained with 1% crystal violet to visualize plaques. Luciferase expression allowed an assessment of replicating virus in cells that was more sensitive than that achieved with plaque-based assays. Luciferase activity was determined by measuring the light emission in Vero cell lysates incubated with a luciferin substrate (Promega, Madison, WI). Light emission was measured in relative light units by use of an LB96P chemiluminometer (EG&G Berthold) and adjusted to the protein concentration of the sample. The protein concentration in the cell lysates was measured in a standard protein assay (BCA protein assay; Pierce, Rockford, IL).

PCR detection of viral DNA. Primers for conserved regions of the hemagglutinin gene of orthopoxviruses (the 5′ region from the start codon to residue 19 and the 3′ region segment proximal to the stop codon) were synthesized by Integrated DNA Technologies (Coralville, IA). The sequences of the forward primer (5′-ATG ACA CGA TTG CCA ATA C 3′) and the reverse primer (5′-CTA GAC TTT GTT TTC TG 3′) were obtained from a prior report (37). DNA was isolated from Vero cells or from lung tissue homogenates with TriReagent according to the manufacturer's protocol (MRC, Cincinnati, OH). To optimize virus detection and increase sensitivity, cells and lung tissue were collected from 2 to 4 days after infection, when viral replication was highest in control animals. PCR amplification was performed with 10 μg of total cell or lung DNA by use of 0.5 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 2.5 mM of $MgCl_2$, and 0.1 U/μl of *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). The reaction was carried out in a volume of 20 μl, and the mixture was incubated at 94°C for 1 min, followed by 25 cycles with annealing at 55°C, extension at 72°C, and denaturation at 94°C. The PCR products were analyzed by electrophoresis on a 1% agarose gel in Tris-borate buffer for electrophoresis and with ethidium bromide for DNA staining. The purified VV DNA (1 ng) mixed with lung DNA served as a positive control. Gel analysis was performed using a photoimaging camera and software from Bio-Rad (Hercules, CA).

Bioluminescence imaging. Bioluminescence imaging was performed with a cryogenically cooled charge-coupled-device camera (IVIS) as described previously (26). Data for photon flux were quantified by region-of-interest analysis of the heads, chests, and abdomens of infected mice. The background photon flux from an uninfected mouse injected with luciferin was subtracted from all measurements.

Preparation of the NE-based vaccine. VV inactivation studies indicated that a minimum of 1 h of incubation with either 10% NE or 0.1% formalin was needed to completely inactivate the virus (a greater-than-6-log VV titer reduction in plaque and luciferase assays). Based on these results, for NE-killed vaccine, the aliquots of 1×10^6 PFU of VV_{WR} were incubated for 3 h at 37°C in 10% NE. Nasal instillation killed virus was diluted to obtain either 1×10^3 PFU or 1×10^5 PFU per dose in 1% NE. Vaccine formulations containing formalin-killed virus (Fk) were prepared by incubation of VV (>10^8 PFU/ml) with 0.1% formalin (Sigma) at room temperature for 3 h. This mixture was then diluted in either saline or 1% NE to 1×10^3 or 1×10^5 PFU per dose to reduce the formalin to nontoxic concentrations for intranasal (i.n.) immunization. For every formulation in every experiment, virus inactivation by either NE or formalin was confirmed in vitro by infecting Vero cells, followed with two subsequent passages of culture supernatants after 3 to 4 days of incubation. None of the control infections showed the presence of viral plaques. Additionally, a PCR assay for viral DNA in Vero cells and in the lungs of animals harvested 2 to 4 days after vaccination showed an absence of viral DNA (PCR limit of detection, <0.001 ng viral DNA).

Immunizations. Samples of preimmune sera were collected from the mice prior to initial immunization. For i.n. immunization, mice were anesthetized with isoflurane and vaccinated with 10 to 15 μl of vaccine formulation per naris by use of a pipette tip. Emulsion was applied slowly to minimize bronchial distribution and swallowing of the material. After immunization, animals were carefully observed for adverse reactions. Specific anti-VV antibody response was measured in blood samples 3 weeks after the initial immunization and at 2- to 3-week intervals after the second and third immunizations (in those cases where multiple immunizations were performed).

Immunization was performed in the anesthetized mice by superficial scarification at the base of the tail. Before the procedure, hair was removed by a clipper to expose approximately 0.5 to 0.7 cm^2, and the naked skin was disinfected with 70% EtOH. The alcohol was allowed to completely dry (time, 10 to 15 min) before scarification. A sterile bifurcate needle was used to superficially abrade the epidermis and apply a 1×10^5-PFU dose of live VV_{WR} in 10 μl phosphate-buffered saline (PBS). Animals were held still for up to 10 min to ensure virus absorption into the skin.

Collection of blood, bronchial alveolar lavage (BAL) fluids, and splenocytes. **(i) Blood.** Blood samples were obtained from the saphenous vein at various time points during the course of the trials. The final sample was obtained by cardiac puncture from euthanized, premorbid mice. Serum was obtained from the blood by centrifugation at 1,500 × *g* for 5 min after it coagulated for 30 to 60 min at room temperature. Serum samples were stored at −20°C until needed.

(ii) BAL fluids. BAL fluids were obtained at 16 weeks from mice euthanized by isoflurane inhalation. After the trachea was dissected, a 22GA catheter (Angiocath; B-D) attached to a 1-ml syringe was inserted into the trachea. The lungs were infused twice with 0.5 ml of PBS containing 10 μM dithiothreitol and 0.5 mg/ml aprotinin. Approximately 1 ml of aspirate was recovered with a syringe. BAL fluid samples were stored at −20°C until analyzed.

(iii) Splenocytes. Murine splenocytes were mechanically isolated to obtain single-cell suspensions in PBS. Red blood cells were removed by lysis with ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA), and the remaining cells were washed twice in PBS. For the antigen-specific proliferation or cytokine expression assays, splenocytes were resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum, 200 nM L-glutamine, and penicillin-streptomycin (100 U/ml and 100 μg/ml).

Specific antivirus IgG and IgA determination. Mouse anti-VV antibodies were determined by ELISA. Microtiter 96-well flat-bottom NUNC-PolySorp polystyrene plates were coated with a dilution of infected Vero cell lysate containing at least 5×10^4 PFU/well of VV in PBS. Plates were incubated overnight at 4°C and fixed with a 50% mixture of EtOH-acetone for 1 h at −20°C. After the fixing solution was removed, plates were washed twice with PBS containing 0.001% Tween 20 and then blocked for 1 h at 37°C with 1% nonfat dry milk in PBS containing 0.2% Tween 20. Mouse sera or BAL fluids were serially diluted in PBS with 0.1% bovine serum albumin, 100-μl aliquots were added to wells, and the plates were incubated for 2 h at 37°C. Plates were washed three times with PBS-0.05% Tween 20, incubated for 1 h with either anti-mouse immunoglobulin G (IgG) or anti-mouse IgA alkaline phosphatase (AP)-conjugated antibodies (Rockland Immunochemicals), and then washed three times. The colorimetric reaction was performed with the AP substrate SigmaFast (Sigma, St. Louis, MO) according to the manufacturer's protocol. Spectrophotometric readouts were done using the Spectra Max 340 ELISA reader (Molecular Devices, Sunnyvale, CA) at 405 nm and with a reference wavelength of 690 nm. The endpoint titers and antibody concentrations were calculated as the serum dilutions resulting in an absorbance greater than 2 standard deviations above the absorbance in control wells. To aid comparisons in some experiments, the IgG antibody concentration was calculated using the logarithmic transformation of the linear portion of the standard curve generated with dilutions of a mouse monoclonal antibody (clone B808M; Biodesign) multiplied by the serum dilution factor. The serum antibody concentrations are presented as mean ± standard error of the mean (SEM). Serum from the naive mice was used as a control for nonspecific absorbance. Since commercial mouse VV-specific IgA antibodies were not available, the concentration of anti-VV IgA in BAL fluid was inferred from the logarithmic transformation of the linear portion of the standard curve generated with IgA and detected by anti-IgA AP conjugate. BAL fluid from the naive mice was used as a control for nonspecific absorbance.

Anti-VV IgG antibody activity targeted toward alcohol-denatured versus formalin-alkylated viral epitopes was measured using ELISA as described above with a few modifications. The 96-well plates were coated with 1×10^5 PFU/well

of purified VV and incubated overnight at 4°C. After virus was removed, wells were treated for 1 h with either 50% EtOH-acetone at −20°C or with a 1% formalin solution in PBS at 4°C. Plates were washed and blocked as described above. Pooled sera from mice immunized with various formulations of vaccine (VV/NE, VV/Fk/NE, VV/Fk) and sera from mice which survived sublethal infection with live VV were serially diluted in 0.1% bovine serum albumin, and 100-μl aliquots were added to EtOH-acetone and to the formalin-fixed wells. The assay was performed as described above for the anti-VV IgG determination. The values for optical density at 405 nm were compared between EtOH-acetone and formalin-fixed viral antigens. The differences in the activity of anti-VV antibodies were evaluated by the ratio of IgG titers on EtOH-acetone versus formalin at the same value for optical density at 405 nm.

Virus-neutralizing antibodies. Neutralizing antibodies were determined using both a standard PRA (31) and the inhibition of luciferase activity using the recombinant $VV_{WR\text{-}Luc}$. The PRA was performed in duplicate by mixing 10 μl of heat-inactivated mouse serum in serial twofold dilutions with 10 μl of serum-free RPMI medium containing 200 to 300 PFU of VV. Sera were incubated 6 h at 37°C and subsequently placed in 0.5 ml of serum-free medium and overlaid on Vero cell monolayers. After 1 h of incubation, the virus-serum inocula were removed and fresh medium was placed on the cell monolayers. After 48 to 72 h, the cells were fixed and stained with 0.1% crystal violet. Plaques were counted, and the 50% neutralization titer (NT_{50}) was calculated using nonimmune serum as a control. For the assessment of NT_{50} with $VV_{WR\text{-}Luc}$, 10 μl of heat-inactivated mouse serum in serial twofold dilutions was mixed with 10 μl of serum-free RPMI medium containing 2×10^3 PFU of virus. As in the PRA-based neutralization assay, samples were incubated for 6 h at 37°C and then added to 100 μl of serum-free RPMI and incubated for 1 h with Vero cells in 24-well plates. After 24 to 36 h, infected cells were lysed and virus-dependent luciferase activity was assessed by the luciferase assay as described above. NT_{50}s were calculated from the luciferase inhibition curves by use of nonimmune sera and virus in PBS as controls. Correlations between PRA and luciferase inhibition activity were made for each sample.

VV-specific cytokine expression in splenocytes. Spleens from vaccinated mice were harvested 12 weeks after initial vaccination. Splenocytes were obtained from mechanically disrupted spleens and suspended at 3×10^6 cells/ml in RPMI 1640 supplemented with 5% fetal bovine serum, L-glutamine, and penicillin-streptomycin. Cells were incubated with either 1×10^3 or 1×10^4 PFU per well of VV for 72 h at 37°C. Cell culture supernatants were harvested and analyzed for cytokine production. Phytohemagglutinin-P (1 μg per well) was incubated with the cells as a positive control. The gamma interferon (IFN-γ) concentrations in splenocyte supernatants were determined using Quantikine M ELISA kits (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's directions.

Induction of VV-specific T-cell responses. Detection of intracellular cytokine expression in splenocytes was preformed using a modification of the technique described by Harrington et al. (19). Splenic lymphocytes were obtained 10 days after BALB/c mice were either given a second i.n. immunization of 1×10^5 PFU NE-killed virus or subjected to scarification with 1×10^5 PFU live VV (scarification with live virus [VV/scar]). Splenocytes were stimulated with the syngeneic *H-2*^{d} haplotype A-20 B-cell lymphoma cell line expressing both major histocompatibility complex I (MHC class I) and MHC class II antigens or the cl7 fibroblast line expressing only MHC class I antigens. The A-20 cells were maintained in complete RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, and 1% penicillin-streptomycin, while the cl7 cells were maintained in Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1% penicillin-streptomycin. Both cell lines were infected with VV_{WR} at a multiplicity of infection of approximately 0.7, harvested after 12 h of incubation at 37°C, washed, and resuspended in complete RPMI 1640. Single-cell splenocyte suspensions from immunized mice (3×10^6 cells/ml) were incubated with approximately 6×10^5 A-20 or cl7 cells, with noninfected A-20 cells serving as controls. All stimulations were performed for 5 h at 37°C in complete RPMI 1640 medium in the presence of monensin (GolgiStop; BD Bioscience Pharmingen) according to the manufacturer's specifications.

Cell surface and intracellular staining for the detection of CD antigens and cytokines. Cell surface staining and intracellular cytokine detection was performed with antibodies specific for mouse CD4 and CD8a and for IFN-γ (BD Bioscience Pharmingen) according to the manufacturer's manual (BD Cytofix/Cytoperm Plus fixation/permeabilization kit using BD GolgiStop monensin protein transport inhibitor). First, the cells were stained with anti-CD8a or anti-CD4 Cy5-conjugated antibodies, and then they were fixed and permeabilized with EtOH. Subsequently, intracellular staining was performed with anti-IFN-γ fluorescein isothiocyanate-conjugated antibody. Samples were then washed, fixed with 2% paraformaldehyde in PBS, and resuspended in PBS with 0.1% bovine serum albumin for analysis. Flow cytometry was performed using a Coulter EPICS-XL MCL Beckman-Coulter flow cytometer, and data were analyzed with Expo32 software (Beckman-Coulter, Miami, FL).

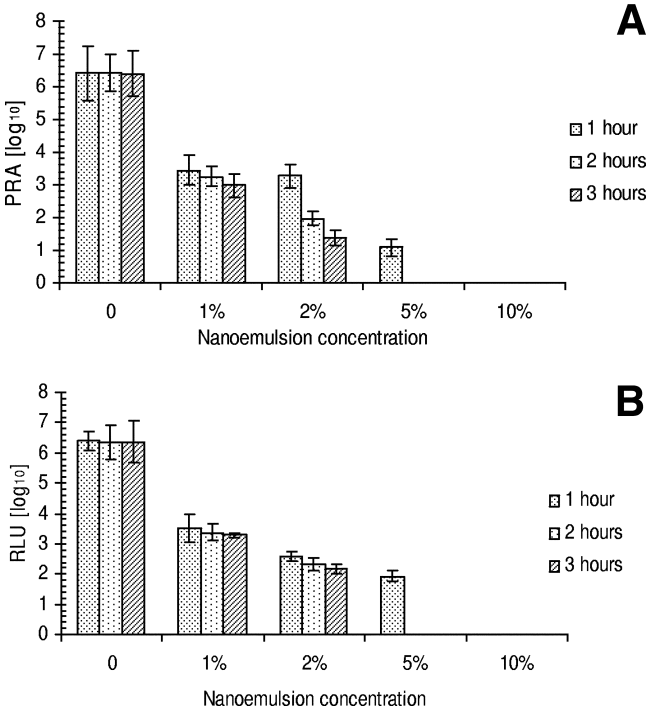

FIG. 1. Complete virus inactivation with NE. (A) PRA of VV_{WR}. (B) Luciferase assay of $VV_{WR\text{-}Luc}$. Luciferase activity is presented in relative light units (RLU). In standardized assays, the limit of virus detection was <10 PFU for PRA and <4 PFU for the luciferase assay. (C) PCR analysis of lung DNA. Lanes: 1, DNA size marker; 2, primers, no DNA; 3, no *Taq*; 4, 10^5/Fk lung DNA; 5 to 7, 10^5/Fk/NE lung DNA; 8 to 10, 10^5/NE lung DNA; 11, control (VV DNA mixed with lung DNA collected from tissue harvested 4 days after vaccination). Arrows indicate amplified viral template and primers. The limit of detection of this assay was <0.001 ng viral DNA. (D) In vivo bioluminescence imaging of mice after i.n. infection with live $VV_{WR\text{-}Luc}$ and with 10^5 PFU of NE-killed virus. Circles visible in some images indicate the regions of interest used for the photon flux analysis (Table 2).

VV challenge. Immunized mice were challenged with live VV to evaluate the effectiveness of the vaccine. Serum samples were collected 2 days before the VV challenge, and animals were weighed on the day of the challenge. Aliquots of purified recombinant $VV_{WR\text{-}Luc}$ or VV_{WR} (sonicated and subjected to titer determination before use) were thawed and diluted in saline on the day of the challenge. Mice were anesthetized by inhalation of isoflurane and challenged i.n. with a 20-μl suspension of 2×10^6 PFU live $VV_{WR\text{-}Luc}$, corresponding to 10 times the 50% lethal dose (LD_{50}), or with live VV_{WR} doses ranging from 1×10^7 to 3.2×10^3 in fivefold dilutions. Weight and body temperature were measured daily for 3 weeks following challenge. Mice that demonstrated a 30% loss in initial body weight were euthanized. LD_{50} and 50% infectious dose (ID_{50}) calculations were based on the animals' death rates and on the core body temperature and body weight loss, respectively (36). The index of protection against lethal challenge (IP_{LD}) was calculated as follows: $IP_{LD} = \log_{10}$ maximum-dose value $- \log_{10} LD_{50}$ control value. Similarly, the index of protection against infection (IP_{ID}) was calculated as follows: $IP_{ID} = \log_{10} ID_{50}$ vaccinated value $- \log_{10} LD_{50}$ control value.

Statistical analysis. Statistical analysis of the results was preformed using analysis of variance and Student's *t* test for the determination of the *P* value, with a Bonferroni's correction for multiple comparisons where appropriate.

RESULTS

NE inactivation of VV. To evaluate the virucidal activity of NE in vitro, a range of NE concentrations was mixed with

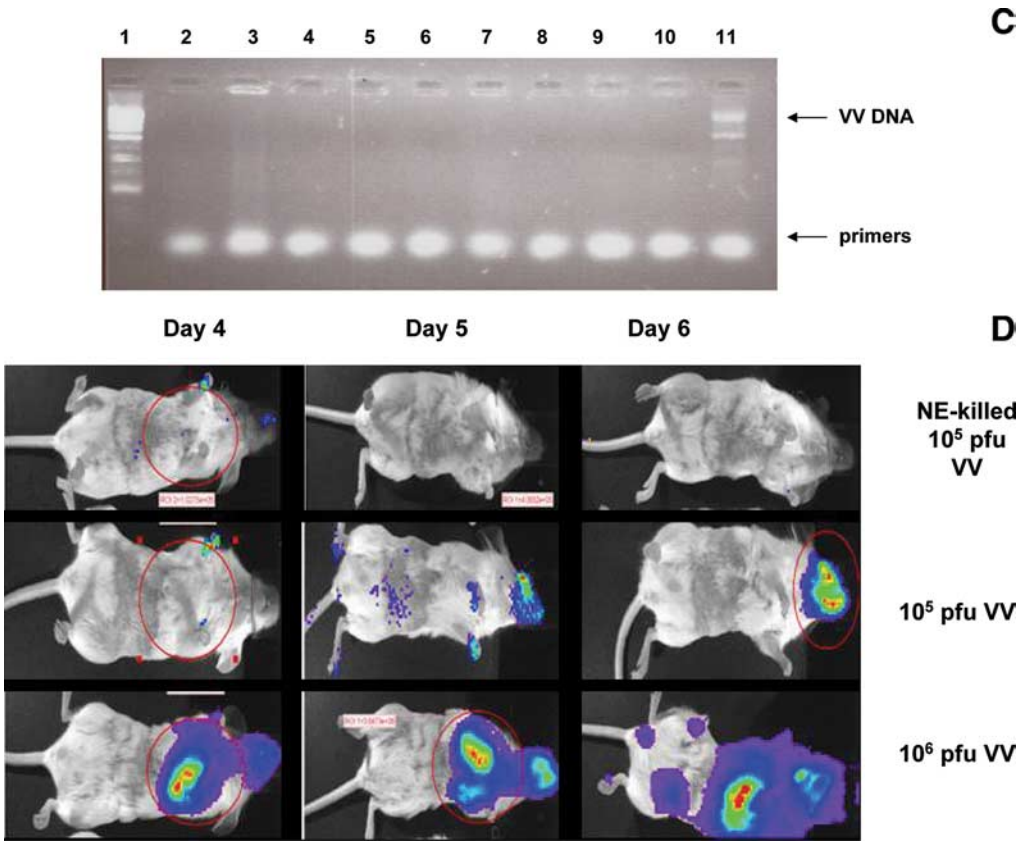

FIG. 1—*Continued*.

either VV_{WR} or $VV_{WR\text{-}Luc}$, and the mixtures were incubated for 1 to 3 h at 37°C. Results of both PRA and luciferase bioluminescence assay indicated NE concentration-dependent inactivation of both viruses. NE at 10% completely inactivates more than 10^6 PFU of VV within 1 h of incubation (Fig. 1A and B). Three subsequent passages of the culture supernatants from cells infected with VV inactivated with 10% NE showed no evidence of surviving virus (data not shown).

The complete inactivation of virus in the NE preparations was further demonstrated in vivo after i.n. administration of inactivated VV_{WR} by use of PCR amplification of DNA isolated from mouse lungs after administration. No viral DNA was detected in any of the treated mice (Fig. 1C), while a control PCR (lung DNA spiked with VV_{WR} DNA) resulted in a product of the expected size of >950 bp. In addition, in vivo bioluminescence imaging of mice also indicated an absence of viral infection and no evidence of virus amplification after the administration of 10^5 PFU of NE-inactivated $VV_{WR\text{-}Luc}$, compared to a strong signal from mice nasally infected by either 1×10^5 or 1×10^6 PFU of live $VV_{WR\text{-}Luc}$ (Fig. 1D). Taken together, the in vitro and in vivo results indicated that incubation with 10% NE for at least 60 min causes the complete inactivation of VV. Thus, all virus inactivations were performed with 10% NE for 3 h and subsequently with a 1% NE dilution for immunization.

VV/NE vaccine induces potent immune responses. The evaluation of NE efficacy as a mucosal adjuvant for VV immunization was based on a previous study which demonstrated that NE mixed with influenza virus and applied to the nares induces anti-influenza virus serum IgG, mucosal IgA antibodies, serum virus neutralization, and specific splenocyte responses (29). Mice ($n = 5$) were immunized i.n. with six formulations containing either 10^5 or 10^3 PFU doses of VV_{WR} killed with NE (10^5/NE and 10^3/NE, respectively), Fk mixed with 1% NE (10^5/Fk/NE and 10^3/Fk/NE, respectively), and Fk in saline (10^5/Fk and 10^3/Fk). Control mice were treated with 1% NE alone. The weak anti-VV antibody responses were detected at 3 weeks after single administration, and immunity was boosted with subsequent administrations at 5 and 9 weeks (Fig. 2A). Increased anti-VV IgG levels were detected after booster immunization in sera from mice vaccinated with either 10^5/NE or 10^5/Fk/NE, with mean anti-VV IgG concentrations of 1.5 μg/ml and 1 μg/ml, respectively. After a second boost, anti-VV antibody concentrations increased in all groups, and at the conclusion of the experiment (at 16 weeks), immunization with 10^3/NE and 10^5/NE produced the highest responses, with mean concentrations of 44 μg/ml and 70 μg/ml of anti-VV IgG, respectively, followed by 10^5/Fk/NE (17 μg/ml). Immunizations with 10^3/Fk/NE and either a 10^5/Fk or a 10^3/Fk formulation of vaccine consistently produced low levels of anti-VV antibodies, which did not increase significantly after booster administrations (Fig. 2A). A comparison of a single dose with a three-dose schedule of immunization with 10^5/NE shows that a single dose of vaccine produced a significant (~4-μg/ml) concentration of serum anti-VV IgG at 12 weeks after immunization, albeit lower than that seen for the three-dose sched-

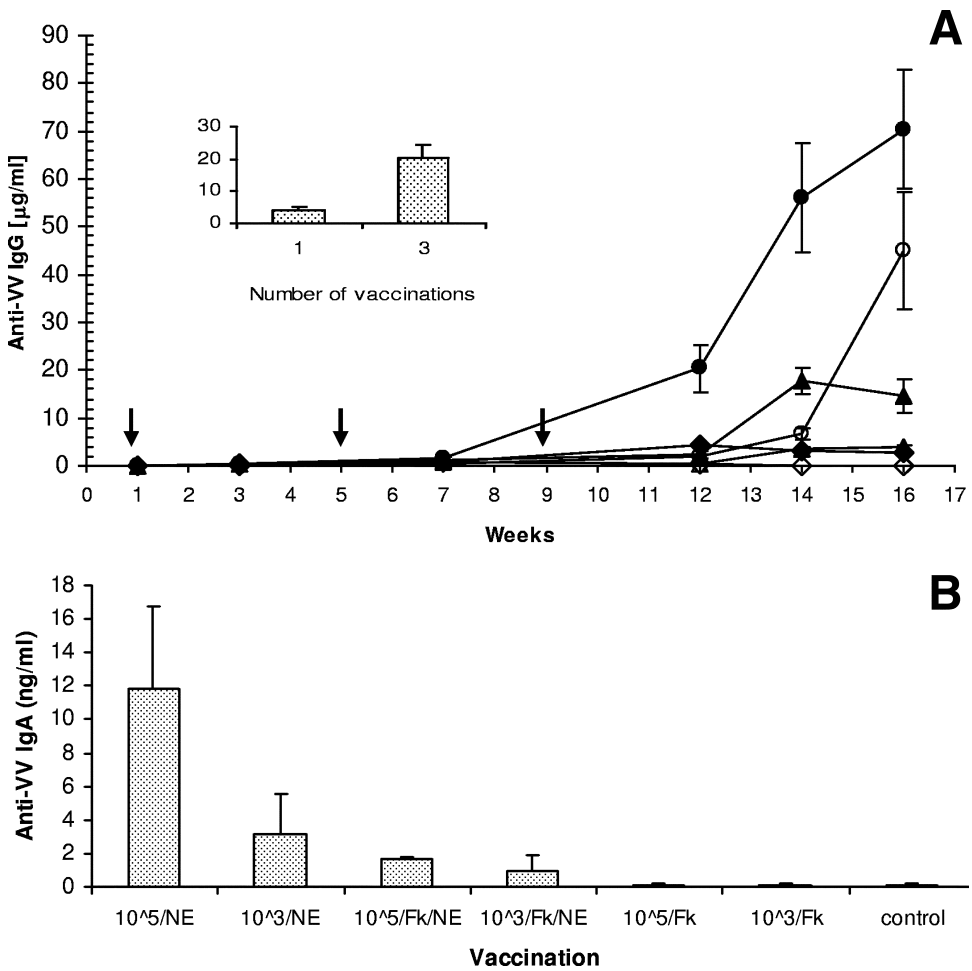

FIG. 2. Immunogenicity of mucosal NE vaccine in mice. (A) Development of serum anti-VV IgG antibody response in mice vaccinated with various formulations of killed-virus vaccine: 10^5/NE (●), 10^3/NE (○), 10^5/Fk/NE (▲), 10^3/Fk/NE (△), 10^5/Fk (◆), and 10^3/Fk (◇). Arrows indicate i.n. administrations of the vaccine. (Inset) Comparison of serum anti-VV IgG levels after one or three vaccinations with 10^5/NE. Data are presented as mean individual anti-VV IgG concentrations ± SEM. (B) Secretory anti-VV IgA antibody in BAL fluid. Results are presented as mean concentrations (± SEM) of IgA obtained in assays performed with individual and pooled BAL fluids collected at 16 weeks.

ule. This suggests that a single dose of VV/NE vaccine may be sufficient to initiate mucosal responses, which are enhanced by subsequent immunization (Fig. 1A). No specific anti-VV antibodies were detected for any of the control mice.

Analysis of cross-reactive anti-VV antibodies indicated that serum IgG from mice immunized with NE-killed virus reacted both with formalin-cross-linked (alkylated) and with alcohol-fixed (denatured but not alkylated) viral proteins. Vaccination with VV/NE vaccine produced anti-VV IgG with 25-fold-higher reactivity to the native, nonalkylated epitopes (similar to sera from mice exposed to a live virus), and those antibodies were also effective in recognizing formalin-fixed viral proteins. In contrast, sera from animals vaccinated with Fk, either alone or mixed with NE, did not have increased reactivity with native VV epitopes (data not shown).

Mucosal immunity was assayed by VV-specific secretory IgA antibody in BAL fluids. Anti-VV IgA was detected in BAL fluids from animals immunized with either 10^3/NE or 10^5/NE. Animals vaccinated with formulations containing Fk, whether diluted in saline or in NE, did not produce measurable mucosal responses despite the presence of serum anti-VV IgG (Fig. 2B).

The biological relevance of the anti-VV antibody response was assessed in virus neutralization assays. Neutralizing activity was detected in the sera of some mice after a single vaccination (Fig. 3A). However, consistent titers of serum neutralizing antibodies were present after two immunizations with 10^3/NE, 10^5/NE, or 10^5/Fk/NE. The mean NT_{50} for each of these groups was ≥20. In contrast, for animals vaccinated with 10^3/Fk/NE, 10^3/Fk, or 10^5/Fk, virus neutralization was observed only in the lowest serum dilution. Subsequent immunization produced a greater-than-10-fold increase in NT_{50} titers only in the mice vaccinated with NE-killed virus (10^3/NE and 10^5/NE [NT_{50} = 180 and NT_{50} = 500, respectively]). A third vaccination with any of the formulations containing Fk did not significantly increase VV neutralization. Significant neutralizing activity was also detected in BAL fluids from mice vaccinated with either 10^3/NE or 10^5/NE and was lower in BAL fluids from mice immunized with either 10^3/Fk/NE or 10^5/Fk/NE (Fig. 3, inset). Neutralizing activity was absent from the BAL

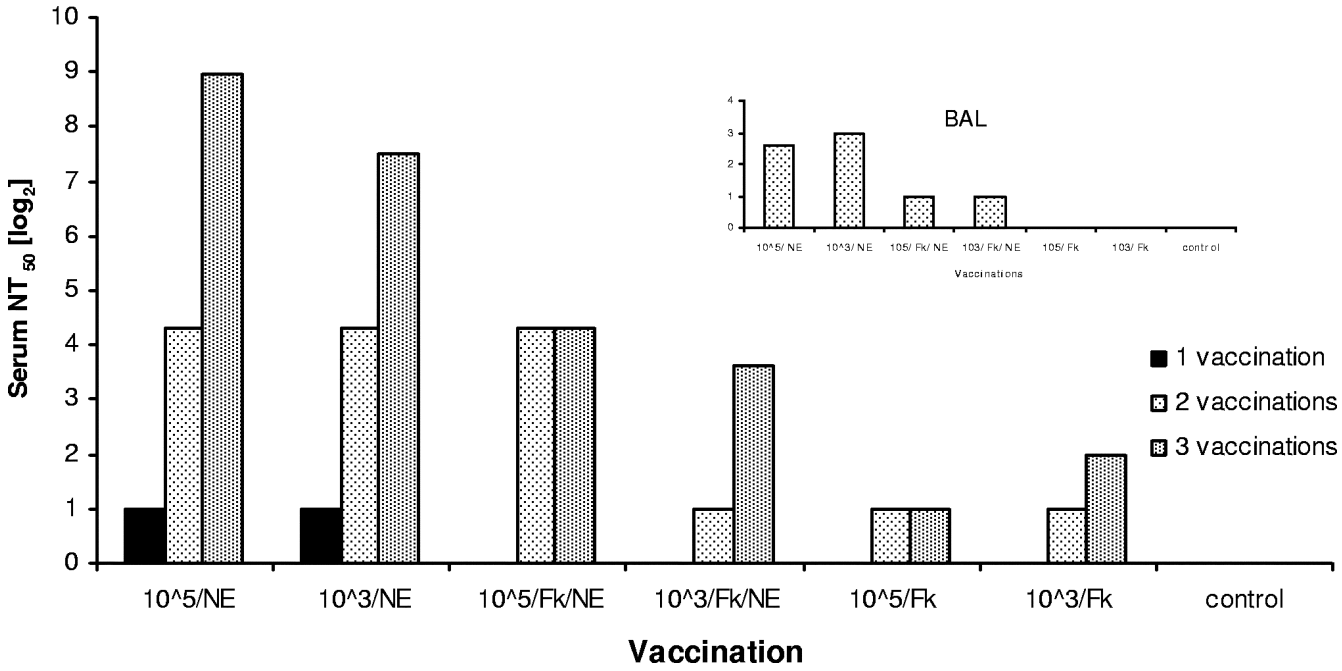

FIG. 3. Virus-neutralizing antibodies. Assays for virus neutralization were performed with both individual and pooled sera obtained after one, two, and three vaccinations. (Inset) Detection of virus-neutralizing activity in BAL fluids. Assays were performed with individual and pooled BAL fluids collected at the conclusion of the experiment at 16 weeks. Results were normalized and presented as NT_{50}s for the viral PRA.

fluids of mice immunized with Fk diluted in saline and in the control nonvaccinated animals.

VV-specific cellular responses. The effect of NE-based vaccine on cellular response was explored using an in vitro cytokine expression assay with splenocytes. Individual cultures of mouse splenocytes were stimulated with 10^3 and 10^4 PFU of live VV. VV-specific cellular immune responses were demonstrated by IFN-γ expression in vitro in splenocytes from animals immunized with either 10^3/NE or 10^5/NE. In contrast, no increase VV-specific IFN-γ production was observed for splenocytes from animals immunized with Fk, even when was it was mixed with NE. The production of IFN-γ in cells from mice treated with VV/NE vaccine indicates a Th1 polarization of cellular response. No antigen-specific cytokine expression was detected in control splenocyte cultures (Fig. 4).

To assess the VV-specific IFN-γ expression in effector CD4 and CD8 T cells, the splenocytes from VV/NE- and VV/scar-immunized mice were stimulated in vitro with A-20 cells infected with VV_{WR}. As shown in Table 1, nasal immunization with NE-killed virus elicited a potent VV-specific CD8 T-cell response in the animals' splenocytes. Over 40% of CD8 cells produced IFN-γ after stimulation with VV-infected A-20 cells, compared to 7.9% of CD8 cells after stimulation with noninfected A-20 controls. Analysis of virus-specific CD4 T cells indicated that approximately 20% of the CD4 cells were positive for IFN-γ production following stimulation with VV-infected A-20 cells (Table 1). Analysis of either VV/NE- or VV/scar-vaccinated mice showed that both techniques elicited strong virus-specific CD8 and CD4 T-cell responses (Table 1 and Fig. 5).

VV/NE immunization protects against live-virus challenge. Protective immunity produced by mucosal immunization was

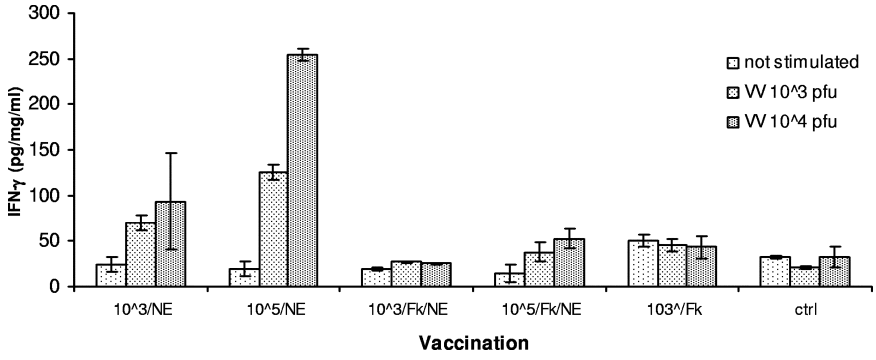

FIG. 4. VV-specific cellular immune responses. The IFN-γ expression in vitro in splenocytes stimulated with 10^3 and 10^4 PFU of live VV_{WR}. The data show a specific IFN-γ response to the virus in splenocytes from animals immunized with VV inactivated by NE.

TABLE 1. T-cell subset response to VV in immunized animals[a]

Vaccination	Stimulation	No. of T cells (SEM)			
		$CD8^+$	$CD8^+/IFN\text{-}\gamma^+$	$CD4^+$	$CD4^+/IFN\text{-}\gamma^+$
VV/NE	A-20 (infVV)	836.0 (± 87)	282.0 (± 14)	1,941.5 (± 47.5)	271.4 (± 25)
	A-20	825.0 (± 157)	40.5 (± 17.5)	2,073.0 (± 170)	75.3 (± 14)
	cl7 (infVV)	815.4 (± 91.2)	212.6 (± 73)	NA	NA
	cl7	1,086.3 (± 113.5)	73.0 (± 24)	NA	NA
VV/scar	A-20 (infVV)	475.1 (± 43)	135.6 (± 19.6)	1,987.2 (± 244)	303.5 (± 65.5)
	A-20	594.3 (± 17)	52.0 (± 10.4)	2,209.2 (± 365)	81.3 (± 12.7)
	cl7 (infVV)	589.5 (± 33.5)	173.0 (± 22)	NA	NA
	cl7	679.0 (± 32.6)	81.5 (± 12.5)	NA	NA
Nonvaccinated controls	A-20 (infVV)	644.0 (± 48.6)	75.5 (± 15.5)	1,934.0 (± 50)	82.5 (± 9.6)
	A-20	667.5 (± 75.5)	42.5 (± 14.8)	1,767.0 (± 56)	54.5 (± 15.4)
	cl7 (infVV)	518.5 (± 10.6)	61.5 (± 12.1)	NA	NA
	cl7	691.5 (± 22.1)	44.0 (± 25.7)	NA	NA

[a] Splenocytes were stimulated with A-20 cells infected with VV_{WR} (infVV), with uninfected A-20 cells serving as a background control. VV-specific T cells expressing IFN-γ were detected by intracellular staining and enumerated by flow cytometry, and percentages of $CD8^+$ and $CD4^+$ cells that were IFN-γ^+ are indicated. A-20 is a syngeneic *H-2*^{d} haplotype B-cell lymphoma cell line expressing both MHC class I and MHC class II molecules, and cl7 is a syngeneic *H-2*^{d} haplotype fibroblast line expressing only the MHC class I molecule. These were used as stimulator cells to show that different populations of cells could be stimulated based on CD4/CD8 expression. IFN-γ-producing CD4 and CD8 cells were specifically generated in response to VV stimulation in animals vaccinated with either scarification or i.n. NE administration.

evaluated in the challenge studies. Three groups of mice (n = 5) were nasally immunized with three doses of either 10^5/NE, 10^5/Fk/NE, or 10^5/Fk vaccine. Control animals (n = 5) were treated with saline. At 12 weeks, mice were challenged with 10 times the LD_{50} (2×10^6 PFU) of live $VV_{WR\text{-}Luc}$. Body weight and temperature were measured two times a day, and animals were imaged for $VV_{WR\text{-}Luc}$ luminescence once a day. All 10^5/NE-vaccinated mice survived viral challenge (Fig. 6A). Mice vaccinated with 10^5/Fk/NE and 10^5/Fk had 40% and 20% survival rates, respectively. Although not fully protective, vaccination with 10^5/Fk/NE also extended the mean time to death from 5 to 7 days. None of the control mice survived the challenge. Bioluminescence imaging used for the assessment of viral infection demonstrated that two of the five 10^5/NE-immunized mice had minimally detectable virus replication which did not affect their weight and body temperature, while the other three had more-progressive replication that resolved within 6 days after challenge (Fig. 6B). However, none of these animals had clinical evidence of infection. In contrast, all nonvaccinated controls became ill and died or were humanely euthanized within 4 to 7 days of virus challenge. These animals had massive virus replication and spreading of the infection throughout the nasopharyngeal passage, lung, and abdomen, as presented in photon flux data. In 10^5/NE-vaccinated mice, a low-grade infection after i.n. challenge was limited to the heads (noses) of vaccinated animals, without spreading to the chest or abdomen (Table 2). Taken together, the imaging studies suggested an inverse correlation between the dissemination of infection and survival. The presence of a self-limiting infection in some immunized mice correlated with the levels of neutralizing antibodies in the individual animals.

To further investigate the effectiveness of the mucosal NE-based vaccine, the i.n. immunization with three doses of 10^5/NE was compared with vaccination by scarification with

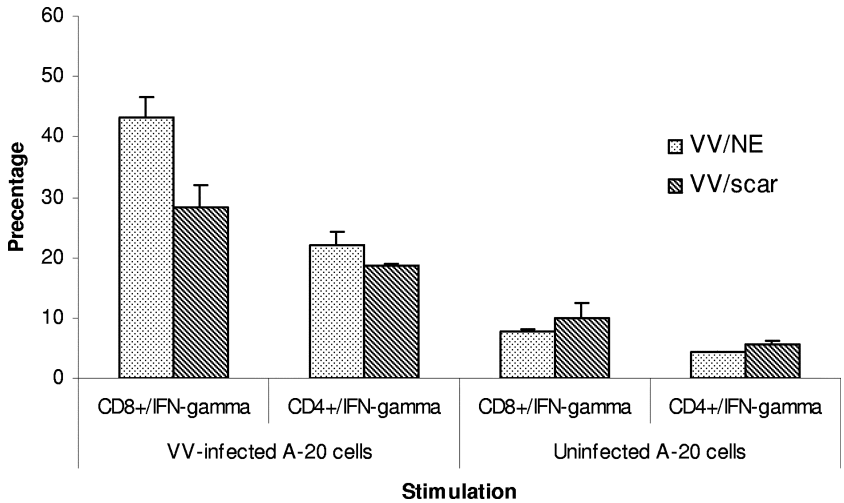

FIG. 5. VV-specific CD8 and CD4 responses. Splenocytes from mice immunized i.n. with NE-killed vaccine were analyzed for VV-specific responses 10 days after the second booster administration of vaccine. Frequencies of IFN-γ^+ VV-specific $CD8^+$ and $CD4^+$ cells. Data are presented as mean values obtained from splenocytes from different groups of mice (n = 3) immunized with either nasal VV/NE or VV/scar ± SEM.

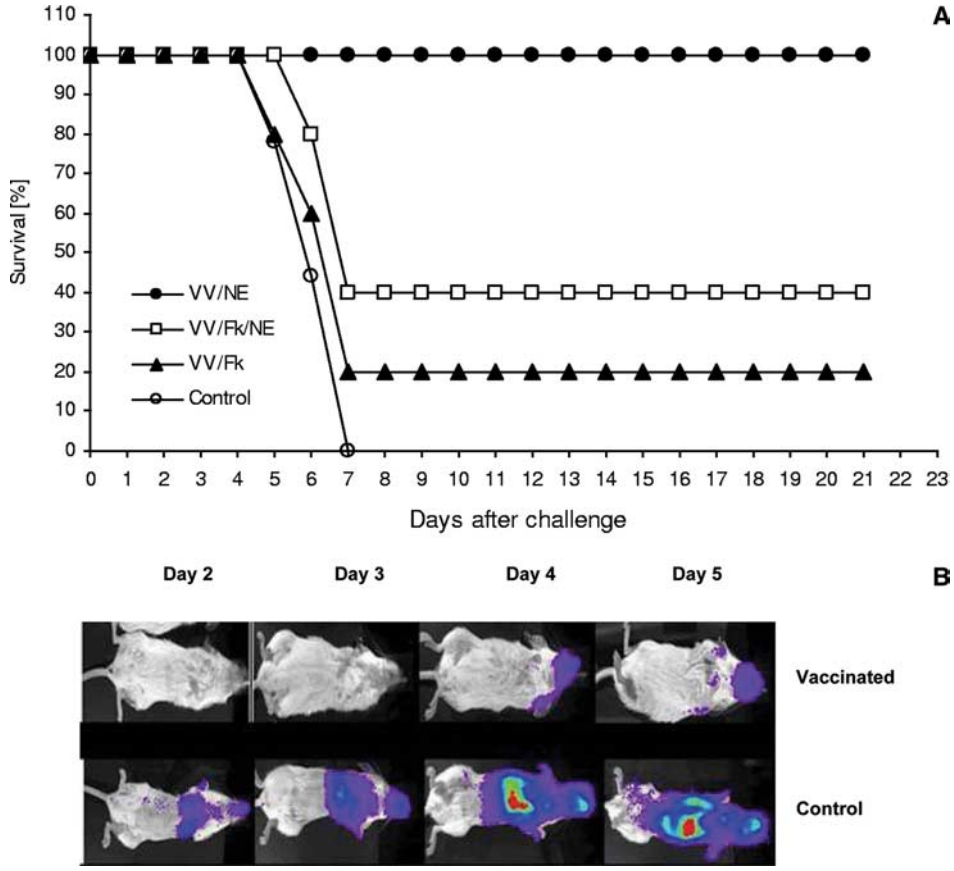

FIG. 6. i.n. challenge with live VV. (A) Survival curves for mice vaccinated with 10^5 PFU of killed VV_{WR} in various vaccine formulations, VV/NE, VV/Fk/NE, and VV/Fk, after i.n. challenge with 10 times the LD_{50} of $VV_{WR\text{-}Luc}$. Significant protection was observed for the vaccinated animals compared to what was seen for control mice vaccinated with either NE alone or PBS. (B) Bioluminescence images of representative vaccinated (top) and control (bottom) mice challenged with a luciferase-producing VV. Images were recorded 2 to 5 days after challenge.

TABLE 2. Photon flux analysis of viral luciferase expression in vaccinated and control mice

Area	Day	Fold photon flux increase for[a]:			
		Vaccinated mice		Controls	
		Avg	SEM	Avg	SEM
Head	2	7.0	3.3	33.8	12.0
	3	26.1	13.1	135.9	86.1
	4	75.2	16.5	431.0	252.0
	5	56.6	17.1	924.7	355.6
Chest	2	8.2	1.8	24.0	3.8
	3	13.3	2.0	119.8	25.0
	4	22.1	6.3	216.3	62.0
	5	16.6	1.3	618.2	135.8
Abdomen	2	12.1	1.0	19.2	5.1
	3	13.7	1.3	23.8	8.2
	4	23.0	2.8	28.3	10.0
	5	14.8	1.8	32.1	6.9

[a] Results are presented as mean photon flux increase over normalized 1×10^4 background values in the individual mice ($n = 5$ per group). Photon flux from an uninfected mouse injected with luciferin was subtracted from all measurements.

live VV_{WR} (10^5/scar). Serum anti-VV antibody response measured at 3 weeks indicated that a higher titer of antibody was achieved with scarification (Fig. 7A). However, a repeated study examining multiple time points showed no difference in titer at either 3 weeks or after the first booster (5 weeks) and no difference in subsequent titers out to 11 weeks after scarification (Fig. 7B). End titers were equivalent to those seen for animals that survived infection with an LD_{50} of virus (Fig. 7B).

We i.n. challenged mice immunized three times with VV/NE with escalating doses of live VV_{WR} at 12 weeks after initial immunization. Survival data indicate that mucosal vaccination produced protective immunity equal to vaccination by scarification (Table 3), with all mice surviving i.n. challenges with the maximal dose of 1×10^7 PFU of VV_{WR} (77 times LD_{50}). The IP_{LD} was 1.9 for both the NE-based vaccine and scarification. All control nonvaccinated animals died after challenge with only 15 times the LD_{50} of VV_{WR}. The high level of protection attained with i.n. immunization was also seen in a weight loss analysis of surviving mice. Although mucosal vaccination did not completely protect mice against respiratory infection with high doses of VV_{WR} (Table 4), animals immunized with NE vaccine did not have clinical evidence of illness and had an average weight loss of 10% or less, whereas surviving mice in control groups lost more than 25% of their weight at much

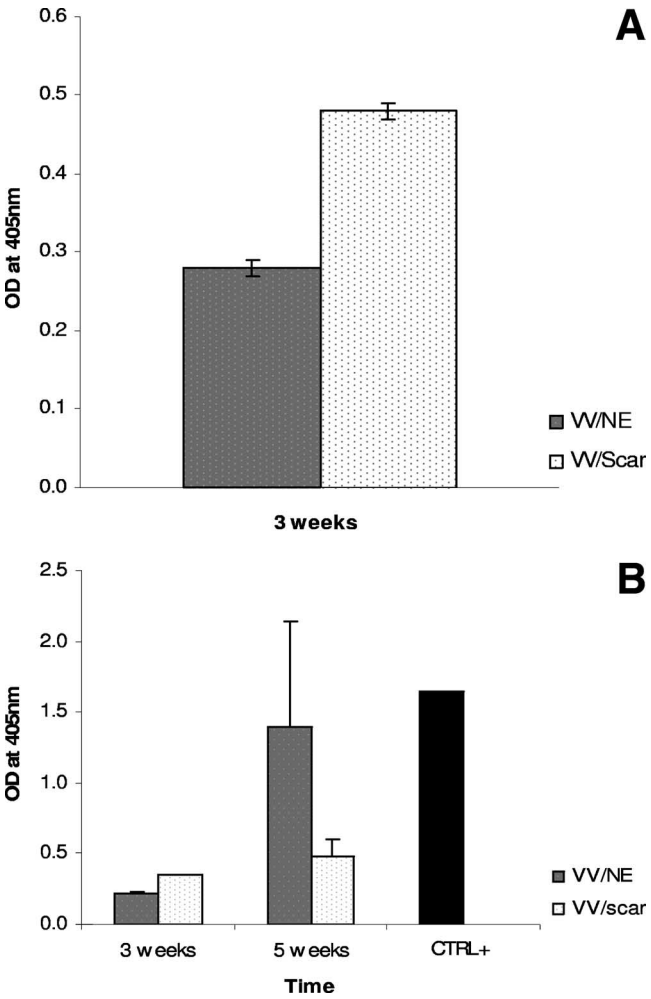

FIG. 7. Comparison of IgG serum anti-VV titers with nasal vaccination using NE-killed virus and VV/scar. Mice were vaccinated with either three doses of VV/NE vaccine at 0, 3, and 8 weeks (VV/NE) or one VV/scar at week 0. VV/NE serum samples were analyzed at 3, 5, and 11 weeks, and VV/scar samples were analyzed at 3 weeks. Titers were assessed by ELISA at a 1:1,000 serum dilution to assure analysis in the linear range of the assay. (A) Titers in the first assay showed higher initial antibody concentrations in the mice immunized with scarification ($P < 0.5$). (B) Subsequent serial studies again showed a higher titer for scarification at 3 weeks but no significant difference at later time points (up to 11 weeks) after booster immunization with the VV/NE vaccine. Overall titers after week 5 were similar to those seen for naive animals who survived an LD_{50} challenge with live virus (CTRL+, black bar).

lower doses of VV_{WR}. Statistical analysis indicated differences with P values of <0.01 between the body weights of immunized and control mice. The IP_{ID} values were 1 for VV/NE vaccination and 2.2 for scarification.

DISCUSSION

The currently licensed smallpox vaccines use live VV obtained from infected calf skin and lymph nodes or tissue culture (Dryvax package insert; Wyeth Pharmaceuticals Inc., Madison, NJ). These vaccines produce infectious skin pustules (pox) and infrequent but severe side reactions that limit their use in individuals (and their close contacts) with immunodeficiency, eczema, atopic dermatitis, allergy, or pregnancy (10). Although these vaccines confer a long-lasting immunity against several different orthopoxviruses, the adverse reactions argue for the development of new, safer human vaccines (33). In contrast to the eradication of natural infection, for which the current vaccines were developed, the most likely future use of a smallpox vaccine would be for protection against either a bioterrorist attack or a natural outbreak of another rare orthopoxvirus infection, such as monkeypox (3, 9). These scenarios suggest that the risk/benefit ratio of vaccination might require new smallpox vaccines with greater safety. Furthermore, a safe mucosal vaccine could be of aid in emergent public health situations. However, safe and effective mucosal vaccines for most infectious agents based either on killed viruses or on purified antigens have not been achieved (15).

These studies document the development of antiviral immunity after mucosal immunization with a unique inactivated VV preparation. NE are surface-active antimicrobials that when simply mixed with highly purified, cell culture-derived VV pro-

TABLE 3. Comparison of protective immunity after intranasal vaccination with 10^5/NE or scarification with live VV_{WR}[a]

Challenge dose (PFU)	LD_{50}[c]	Survival[b]		
		NE vaccine	Scarification	Control
1.0E+07	77	5/5	5/5	0/5
2.0E+06	15	5/5	5/5	0/5
4.0E+05	3	5/5	5/5	1/5
8.0E+04	0.62	5/5	5/5	3/5
1.6E+04	0.12	5/5	5/5	5/5
3.2E+03	0.02	5/5	5/5	5/5

[a] Survival after challenge with escalating doses of VV_{WR}. Three doses of NE-killed vaccine (10^5/NE) were administered and compared with a single vaccination by scarification with 10^5 PFU of live VV_{WR}. Viral challenge was performed at 12 weeks after the initial vaccination. No difference in survival was observed for animals based on their immunization protocols.
[b] Presented as a ratio of surviving mice to all mice.
[c] Calculated value for 1× LD_{50} was 1.3×10^5 PFU of VV_{WR}.

TABLE 4. Protection against viral infection after intranasal vaccination with 10^5/NE or scarification with live VV_{WR}[a]

Challenge dose (PFU)	LD_{50}[c]	Protection[b]		
		NE vaccine	Scarification	Control
1.0E+07	77	0/5	3/5	0/5
2.0E+06	15	0/5	5/5	0/5
4.0E+05	3	1/5	5/5	0/5
8.0E+04	0.62	2/5	5/5	0/5
1.6E+04	0.12	4/5	5/5	0/5
3.2E+03	0.02	5/5	5/5	5/5

[a] Protection against viral infection was assessed for mice vaccinated with three doses of NE-killed vaccine (10^5/NE) compared to a single scarification with 10^5 PFU live VV_{WR}. Viral challenge was performed at 12 weeks after initial vaccination. Mice were observed twice daily for changes in body weight and core body temperature after challenge with live virus. Significantly more changes in body weight and temperature were observed for the VV/NE-immunized animals than for those immunized with scarification, especially at higher virus challenge doses ($P > 0.5$).
[b] Presented as a ratio of mice which did not have decreased body weight or temperature at any time after challenge to all mice.
[c] Calculated value for 1× LD_{50} was 1.3×10^5 PFU of VV_{WR}.

duce a stable adjuvant formulation for mucosal immunization. This formulation has adjuvant activity without the addition of any specific proinflammatory materials, toxins, or cytokines. This activity appeared to result from the ability of the NE to penetrate the nasal mucosa and be readily endocytosed by dendritic cells and macrophages (22, 40). VV/NE immunization resulted in high-titer mucosal and systemic antibody responses and specific Th1 cellular immunity. Further, after three administrations, vaccinated mice were fully protected against an instillation challenge with 10 to 77 times the LD_{50} of VV. In a direct comparison, three-dose immunization with NE-killed vaccine attained a degree of protection against VV mortality similar to that for the scarification technique typically used for human smallpox vaccination. However, differences in the IP_{ID}s between these approaches indicated that vaccination with live virus was more protective against challenge with higher viral loads than was the nasal NE-killed vaccine. Overall viral infection, measured both by virus replication and by changes in body weight and temperature in vaccinated animals, was markedly attenuated with both approaches compared to what was seen for control mice. A comparison of antibody responses after nasal vaccination with NE-killed virus and scarification with live virus indicated that although the NE-killed vaccine required repeated doses, it was capable of producing similar immune responses. In addition, we found that after a single immunization with the virus-NE formulation, serum concentrations of anti-VV IgG developed in 10 to 12 weeks were comparable to the serological response in mice immunized by intramuscular injection with live VV (Dryvax; Wyeth) (5). This suggests that multiple immunizations with the VV/NE vaccine significantly enhance the immune response and are necessary for full immunity. This is in accord with recent reports on the development of safer parenteral recombinant or attenuated smallpox vaccines, which also indicate that multiple administration might be necessary to achieve optimal immune responses and protective immunity (12, 32).

In contrast to these results, protective immunity has been reported not to be achieved with several other formulations of live or inactivated VV. i.n. application of formalin-killed VV, either with or without NE, produced inconsistent, low serum antibody responses that did not significantly augment even after a third immunization. A similar absence of virus-neutralizing activity was detected in sera and BAL fluids of mice vaccinated intramuscularly with Fk. In addition, neutralizing activity was not detected in BAL fluids of animals vaccinated with either intraperitoneal or subcutaneous injections of a live virus (data not shown). The reason for the difference in activity is not entirely clear but may involve several mechanisms. Our studies examining anti-VV IgG activity suggest that in contrast to that with formalin, NE inactivation may preserve the critical viral epitopes important in neutralizing the virus. In addition, parenteral injections of VV/NE formulations failed to induce neutralizing antibodies in bronchial secretions, suggesting benefits from mucosal immunity. The ability to produce mucosal immunity without the addition of proinflammatory materials is unique to NE formulations, since other vaccines use inflammatory toxins or other immune stimuli to induce mucosal responses (20). It is possible that this material may act as a "physical adjuvant" that transports the antigens across the mucosa and enhances VV protein presentation to the immune system via dendritic cells. This has been reported to occur with NE used for cosmetic or drug delivery applications (41, 42).

Work on a new generation of smallpox vaccines is hindered by the fact that the exact mechanism of immune protection against smallpox is not completely understood. This is due in part to the absence of human smallpox infections in the "modern" era of immunology. However, it is thought that both cellular and humoral immunities play a role in protection against orthopoxviruses, and both were induced with the NE formulations. VV-specific antibody titers are considered the most direct estimate of protective immunity in human and animal models of vaccination (16). Several studies have identified proteins important for the elicitation of neutralizing antibodies (13, 21). A recent trial with dilutions of the licensed smallpox vaccine Dryvax in human volunteers confirmed that pustule formation strongly correlated with the development of both the specific antibodies and with the induction of cytotoxic T lymphocytes (CTL) and elevated IFN-γ cell responses (14). Neither these studies nor our own work have fully characterized the CTL responses, but an assessment of virus-specific T cells expressing IFN-γ in mice vaccinated with VV/NE clearly indicated that NE nasal vaccination induces a potent activation of VV-specific MHC class I-restricted $CD8^+$ T cells. These cells have been implicated in the recognition and clearance of VV-infected cells and in the maintenance of immunity after vaccination (8, 9, 16). The VV-specific CD8 and CD4 T-cell responses were comparable between nasal vaccination with NE-killed virus and scarification with live virus and were similar to the CTL results reported previously for VV-infected animals (19, 34, 35, 39). Live-virus stimulation of splenocytes from mice vaccinated with Fk failed to significantly induce IFN-γ production, again suggesting that the NE formulations are unique in inducing CTL responses. Finally, while it is not proven, a key benefit to this candidate vaccine may be mucosal application, providing barrier immunity at sites where many VV infections initiate (28). Thus, a combination of systemic serological and cellular immunity and the mucosal immunity achieved with virus-NE immunization suggests the potential for protection against lethal mucosal exposure to VV and is probably important to yield an effective smallpox vaccine.

In summary, we have documented that a new formulation of NE-inactivated VV serves as an effective mucosal vaccine that induces both mucosal and systemic antibody and TH1 cellular immunity. This prototype vaccine is easily produced by simply mixing the NE with purified virus and may provide an additional margin of safety over live-virus vaccination.

ACKNOWLEDGMENT

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