Dose-Dependent Protection against or Exacerbation of Disease by a Polylactide Glycolide Microparticle-Adsorbed, Alphavirus-Based Measles Virus DNA Vaccine in Rhesus Macaques

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Measles remains one of the most important vaccine-preventable child mortality. Development of a low-cost, heat-stable vaccine for infants under the age of 6 months could improve measles control by facilitating delivery at the time of other vaccines and by closing a window of susceptibility prior to immunization at 9 months of age. DNA vaccines hold promise for development, but achieving protective levels of antibody has been difficult and there is an incomplete understanding of protective immunity. In the current study, we evaluated the use of a layer of alphavirus DNA/RNA vector encoding measles virus II (SINCP-H) adsorbed onto polylactide glycolide (PLG) microparticles. In mice, antibody and T-cell responses to PLG-formulated DNA were substantially improved compared to those of naked DNA. Rhesus macaques received two doses of PLG/SINCP-H delivered either intramuscularly (0.5 mg) or intradermally (0.5 or 0.1 mg). Antibody and T-cell responses were induced but not sustained. On challenge, the intramuscularly vaccinated monkeys did not develop rashes and had lower viremias than vector-treated control monkeys. Monkeys vaccinated with the same dose intradermally developed rashes and viremia. Monkeys vaccinated intradermally with the low dose developed more severe rashes, with histopathologic evidence of syncytia and intense dermal and epidermal inflammation, eosinophilia, and higher viremia compared to vector-treated control monkeys. Protection after challenge correlated with gamma interferon-producing T cells and with early production of high-avidity antibody that bound wild-type H protein. We conclude that PLG/SINCP-H is most efficacious when delivered intramuscularly but does not provide an advantage over standard DNA vaccines for protection against measles.

Measles remains one of the most important vaccine-preventable childhood diseases and was associated with approximately 450,000 deaths in 2004 (9). A live attenuated measles virus (MV) vaccine, introduced in 1963, is widely used and safe and provides long-term protection from measles. Vaccination at 12 months of age results in approximately 95% seroconversion (51), but children below 9 months of age are less likely to respond due to persistence of maternal antibodies and immaturity of the immune system (1, 19). Because the time to loss of maternal antibodies depends on the amount of antibody transferred and the rate of decay, children spend a variable time at risk of infection before receiving routine vaccination (4, 5, 11). This window of susceptibility may be particularly important for children born to human immunodeficiency virus (HIV)-infected mothers because they are born with relatively low levels of maternal antibody and are at increased risk of acquiring measles at an early age (17, 35). An MV vaccine that could be given before the age of 6 months would help to close this window of susceptibility and would allow delivery of the vaccine in conjunction with other early childhood vaccines.

To develop a new vaccine requires a thorough understanding of the correlates of protective immunity. Information on these correlates often comes from comparative studies of successful and unsuccessful vaccines. Young age affects the quality and quantity of antibody responses to the current live attenuated vaccine but has less of an effect on T-cell responses (19, 20, 53, 60). Increasing the dose of vaccine improved the antibody responses in young infants but resulted in an unexpected increase in mortality for girls, so this is not a viable approach to lowering the age of vaccination (22, 26, 29). Adverse responses also occurred in children who were vaccinated with an early formalin-inactivated MV vaccine. This vaccine provided only short-term protection, and subsequent infection with wild-type MV was often associated with atypical measles, a more severe form of disease characterized by high fever, hemorrhagic or vesicular rash, and pneumonitis (50). Studies with monkeys have indicated that atypical measles is associated with production of large amounts of low-avidity antibody after challenge that cannot neutralize wild-type virus, leading to immune complex formation, vasculitis, and pneumonitis (45, 46). Therefore, evaluation of the responses to different MV vaccines and their ability to protect against challenge is a paradigm for understanding protective immunity. Any attempts to develop a new MV vaccine require careful assessment of safety, as well as immunogenicity.

Several animal models, including cotton rats, mice, and non-
human primates, have been used for testing potential new MV vaccines. Only monkeys develop a disease similar to that of humans and offer the opportunity for assessing both protection from wild-type MV challenge and priming for enhanced disease (3, 45, 48, 59). MV encodes six structural proteins: hemagglutinin (H), fusion (F), matrix, nucleoprotein (N), phosphoprotein, and large polymerase protein. Vaccine-induced protection from measles correlates best with the quality and quantity of neutralizing antibody induced (10, 48). Antibody that inhibits MV infection is directed primarily against the H protein, with some contribution from F (14, 32, 48). Interestingly, immunization with H plus F often results in lower neutralizing antibody titers than immunization with H alone, which perhaps is related to differential T-cell responses to these proteins (47, 48). H also contains important CD8+ T-cell epitopes (36, 40). Therefore, experimental vaccines have generally used H alone or H plus F for induction of MV protective immunity. A variety of experimental MV vaccines and routes of vaccination, including immunostimulatory complexes (iscoms) (15, 56), recombinant viral vectors (42, 57, 58, 62), recombinant bacterial vectors (61), and DNA vaccines (48, 55), have been tested in primates. Iscoms, recombinant viral vectors, and DNA vaccines have all demonstrated at least some protection against MV challenge. In addition, characterization of the responses to these experimental vaccines and subsequent challenge has provided essential information on the important features of protective immunity.

DNA vaccines are attractive for development because they offer the potential for induction of immunity in the face of maternal antibody, as well as heat stability and inexpensive manufacture. In previous studies, we showed that both sustained neutralizing antibody and cytotoxic T-lymphocyte responses were induced by naked plasmid DNA vaccines carrying the genes for H, F, or H plus F (48). However, the titers of neutralizing antibody were often low and there was only partial protection from challenge, indicating that the immunogenicity of this candidate MV vaccine required improvement.

Several approaches to improvement of DNA vaccines, such as addition of adjuvants and optimization of DNA plasmids, are under study in many laboratories (24, 27, 49). Adsorption of DNA onto biodegradable cationic poly lactide coglycolide (PLG) microparticles is one approach to improving the immunogenicity of DNA vaccines (12, 37). PLG microparticles deliver the DNA vaccine to and activate antigen-presenting cells, increase DNA persistence, and recruit mononuclear cells to the site (13). An improvement over naked DNA has been achieved after intramuscular (i.m.) injection for induction of both antibody and T cells in rodents and primates in response to other candidate vaccines (34, 37, 38, 41).

A novel approach to improved plasmid-based expression of the protein antigen has been to use alphavirus DNA vectors (16). In this approach, the DNA used for immunization consists of the genes coding for the alphavirus nonstructural proteins followed by the subgenomic promoter and the gene encoding the antigen of interest. When the DNA is transcribed to mRNA, the nonstructural proteins are translated, replicate the RNA, and produce abundant subgenomic mRNAs that code for the antigen. Thus, there is amplification of the mRNAs so that large amounts of protein are synthesized (6, 16, 25). In addition, these vaccines activate innate immune pathways to increase the immunogenicity of the antigens encoded (30). Sindbis virus-based replicon plasmid (SINCP)-based vaccines have been used successfully to immunize mice, cotton rats, or monkeys against herpes simplex virus, HIV, and MV (8, 23, 37, 41, 43).

In this study we have combined these two approaches to develop a SINCP-based vaccine encoding the MV H protein adsorbed onto PLG to form microparticles (PLG/SINCP-H). The immunogenicity was tested at various doses in both mice and rhesus macaques. Antibody and T-cell responses in mice were improved compared to those with naked DNA, and 500 µg PLG/SINCP-H delivered i.m. elicited partially protective immune responses in rhesus macaques. Intradermal (i.d.) delivery was less effective, and monkeys immunized with a low (100-µg) dose developed more severe rashes and higher viremia after challenge than control monkeys.

**MATERIALS AND METHODS**

**Animals.** Six-week-old female BALB/c mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). Eight rhesus macaques (Macaca mulatta), 1 to 2 years old, were from the Johns Hopkins Primate Breeding Facility. Monkeys were chemically restrained with ketamine (10 to 15 mg/kg) during procedures. All animals were maintained and studies were performed in accordance with experimental protocols approved by the Animal Care and Use Committee for Johns Hopkins University.

**Vaccine.** The H gene from the Edmonston strain of MV was subcloned into a Sindbis virus-based replicon vector (pSINCP) (16, 21, 41) to produce an MV DNA vaccine (SINCP-H). This replicon vector contains a cytomegalovirus promoter, the coding region for the Sindbis virus nonstructural proteins, the subgenomic promoter, and the H gene. Plasmid DNA was purified with a Qiagen Plasmid Maxi kit (Qiagen, Inc.) and redissolved in normal saline before use. H protein expression was confirmed by Western blotting after transfection of BHK cells with pSINCP-H. Preparation of MV PLG/DNA vaccine was by a previously described procedure (37). Briefly, cationic microparticles were prepared by emulsifying 10 ml of a 5% (wt/vol) PLG polymer solution in methylene chloride with 1 ml of phosphate-buffered saline (PBS) at high speed using an IKA homogenizer. The primary emulsion was then added to 50 ml of distilled water containing cetyl trimethylammonium bromide (0.5% wt/vol), resulting in the formation of a water-in-oil-in-water emulsion, which was stirred at 6,000 rpm for 12 h at room temperature (RT), allowing the methylene chloride to evaporate. The resulting microparticles were washed twice in distilled water by centrifugation at 10,000 × g and freeze-dried. SINCP-H plasmid DNA was adsorbed onto the microparticles by incubating 100 µg of cationic microparticles in a 1-mg/ml solution of DNA at 4°C for 6 h. The microparticles were then separated by centrifugation, the pellet was washed with Tris-EDTA buffer, and the microparticles were freeze-dried.

**Viruses and virus assays.** The Chicago-1 (wild type, tissue culture adapted) (52) and Moraten (vaccine) strains of MV were grown and assayed by plaque formation in Vero cells. The wild-type Bihloven strain of MV (a gift from A. Osterhaus, Erasmus University, Rotterdam) was grown in phytohemagglutinin-stimulated cord blood mononuclear cells and assayed by syncytium formation in B95-8 cells. Viremia was assessed by cocultivation of peripheral blood mononuclear cells (PBMCs) with B95-8 cells in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Cultures were scored for syncytia at 96 h, and data are reported as number of syncytia/106 PBMCs.

Numbers of MV-infected PBMCs were also assessed by flow cytometry. A total of 5 × 106 PBMCs were suspended in staining buffer (0.05% bovine serum albumin in PBS, 0.002% sodium azide) and blocked with 1% human AB serum and 10 µg/ml of mouse immunoglobulin G (IgG) for 20 min. Cells were stained with phycoerythrin-conjugated anti-CD20 and allophycocyanin-conjugated anti-CD3 monoclonal antibodies (BD Pharmingen, San Diego, CA). After washing, Cytofix and CytoPerm/wash buffer (BD Pharmingen) were added and the cells were incubated for 30 min at 4°C with fluorescein isothiocyanate-conjugated monoclonal antibody to the MV N protein (Chemicon). Stained cells were analyzed using a FACSCalibur flow cytometer (BD) and FlowJo software (version 6.4.7, Treestar, CA).
Vaccination of mice. Groups of five mice were used for each vaccine and dose. Two doses of 10 or 1 µg of PLG/SINCP-H, 10 µg of naked pSINCP-H, or 10 µg of pSINCP vector were used for vaccination by the i.m. route. Mice were bled at various times after immunization, and sera were collected and stored at −20°C until use.

Vaccination and challenge of monkeys. Eight 1- to 2-year-old rhesus macaques were immunized and boosted 11 weeks later. Four monkeys were injected with a high dose (0.5 mg in 0.5 ml) of PLG/SINCP-H i.d. (0.1 mg in each of five sites) (monkeys 4P and 8P) or i.m. (26P and 28P). Two monkeys received a low dose (0.1 mg) of PLG/SINCP-H i.d. in a single site (12P and 14P). As controls, two monkeys (11P and 27P) received 0.5 mg of PLG/SINCP vector DNA i.d. (0.1 mg/0.1 ml in each of five sites). Heparinized blood was collected at various time points for evaluation of the immune response.

For MV challenge, 10^7 50% tissue culture infectious doses (TCID50) of the Biltoven strain of MV was instilled intratracheally into anesthetized macaques. After challenge, monkeys were monitored every 48 to 72 h for development of a measles rash and blood was collected to assess viremia, leukocyte counts, and immune responses.

Histology and immunohistochemistry. Punch biopsies were taken of rashes and normal skin from two monkeys (11P and 14P) 10 days after challenge and fixed in 10% formalin. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Deparaffinized sections were stained for MV protein using a mouse monoclonal antibody to MV N protein (Chemicon), horse-radish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Dako), and diaminobenzidine as the chromogen.

Antibody assays. Neutralizing antibody was measured by the ability of serially diluted plasma to reduce plaque formation by the Chicago-1 strain of MV on Vero cell monolayers (plaque reduction neutralization test [PRNT]) (2). The international standard serum 66/202 was included in all assays, and data were normalized to that standard to calculate international units (IU) (18).

Enzyme immunoassays (EIA). Punch biopsies were taken of rashes and normal skin from two monkeys (11P and 14P) 10 days after challenge and fixed in 10% formalin. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Deparaffinized sections were stained for MV protein using a mouse monoclonal antibody to MV N protein (Chemicon), horse-radish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Dako), and diaminobenzidine as the chromogen.

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Enzyme immunoassays (EIA) were performed using 96-well Maxisorp plates (Nunc, Rochester, NY) coated with MV-infected Vero cell lysates (Advanced Biotechnologies, Columbia, MD; 1.16 µg of protein/well). Plates were incubated with serially diluted serum samples overnight at 4°C. For mice, HRP-conjugated goat antibody (HRP-conjugated goat anti-mouse IgG (Amersham) was the secondary antibody at a dilution of 1:5,000. For monkeys, alkaline phosphatase-conjugated rabbit antibody to mouse IgG (Biorad) were used. For monkeys, alkaline phosphatase-conjugated rabbit antibody to mouse IgG (Biorad) were used. For mice, an ELISA unit (EU) was measured by the dilution needed to achieve half of the maximum optical density (OD) of a laboratory standard serum.

To measure avidity of MV-specific antibodies, 50-µl portions of increasing concentrations of ammonium thiocyanate (NH4SCN) (0.25 M to 3 M) were added to ELISA wells for 10 min after incubation with serially diluted plasma. Plates were washed and antibody to IgG was added as described above. The avidity index was calculated as the concentration of NH4SCN at which 50% of the bound antibody was eluted (60).

For radioimmunoprecipitation assay (RIPA), Vero-SLAM cells (39) were infected with either the Moraten vaccine or Biltoven wild-type strain of MV at a multiplicity of infection of 0.1. At 48 h after infection, the medium was replaced with methionine- and cysteine-free RPMI 1640 containing 2% dialyzed FBS (Gibco) and 100 µCi/ml of Trans35S-label (NCL), incubated for 2 h, rinsed with PBS and harvested with lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1 mM EDTA) containing a cocktail of protease inhibitors (Sigma, St. Louis, MO). Serum (50 µl) was incubated with protein G-Sepharose (Sigma) at RT for 1 h and washed three times with RIPA buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.1% sodium deoxycholate, and then the 35S-labeled cell lysate was added and incubated at 4°C for 2 h. The resulting Sepharose-bound immune complexes were washed three times with RIPA buffer, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiographed at 70°C.

ELISPOT assays. MV-specific production of gamma interferon (IFN-γ) and interleukin-4 (IL-4) was measured by enzyme-linked immunospot (ELISPOT) assay. For mice, splenocytes were incubated with RBC lysis buffer (Sigma), washed, and suspended in RPMI supplemented with 10% FBS, 2 mM l-glutamine, penicillin, and streptomycin. Multiscreen plates (Millipore) were coated with antibody to mouse IFN-γ or IL-4 (5 µg/ml; BD Pharmingen). After the plates were washed and blocked with culture medium, 10^5 or 5 × 10^5 splenocytes were added along with 1 µg/ml pooled MV H or F peptides (20-mer overlapping by 11 amino acids) or 5 µg/ml concanavalin A (Sigma). After 4 h of incubation, plates were washed and incubated with 2 µg/ml biotinylated anti-mouse IFN-γ or IL-4 for 2 h at 37°C. For monkeys, Fresh PBMCs were added to plates coated with anti-human IFN-γ antibody (2 µg/ml) or anti-human IL-4 antibody (5 µg/ml; BD Pharmingen) in the presence of pooled MV H or F peptides (40, 42), concanavalin A, or medium alone. After 48 h at 37°C, plates were washed and incubated for 2 h at RT with biotinylated antibody to IFN-γ (1 µg/ml; Mabtech) or IL-4 (2 µg/ml; BD Pharmingen). After washing, 50 µl of HRP-conjugated avidin (1:2,000 in PBS–1% FBS; Research Laboratory Inc.) was added and incubated for 1 h at 37°C. The assay was developed with 50 µl of stable diaminobenzidine solution (Invitrogen, Carlsbad, CA) for 10 min. The reaction was stopped with tap water, plates were allowed to dry, and wells were scanned in an ImmunoSpot reader and analyzed using ImmunoSpot 2.0.5 software (C.T.L., Cleveland, OH). Data are presented as spot-forming cells (SFC)/10^5 splenocytes or PBMCs.

Statistical analysis. Student’s t test on log-transformed data was used for comparison of responses between groups using Statview software (SAS, Cary, NC).

RESULTS

Immune responses to SINCP-H and PLG/SINCP-H in mice. To determine whether the PLG microparticle adsorption of SINCP-H improved immunogenicity, groups of BALB/c mice were immunized i.m. with two doses of 10 µg SINCP-H DNA, 10 µg PLG/SINCP-H, 1 µg PLG/SINCP-H, or 10 µg control SINCP vector at 0 and 4 weeks. MV-specific IgG was induced in all SINCP-H-vaccinated mice, with the highest titers being in those vaccinated with 10 µg PLG/SINCP-H (Fig. 1A). Antibody responses were sustained through 25 weeks after vaccination. Titers in mice given 1 µg PLG/SINCP-H were similar to those in mice given 10 µg naked SINCP-H. The average peak titers were 431 ± 98 EU/ml for 10 µg PLG/SINCP-H, 198 ± 42 EU/ml for 1 µg PLG/SINCP-H, and 123 ± 90 EU/ml for 10 µg naked SINCP-H. The MV-specific antibodies induced by 10 µg PLG/SINCP-H were at higher levels (P < 0.005) and induced earlier (week 6 versus week 8) than those induced by 10 µg SINCP-H without PLG.

Splenocytes producing IFN-γ and IL-4 in response to H peptides were measured by ELISPOT 4 weeks after the second dose of vaccine (Fig. 1B). High IFN-γ and low IL-4 production were found for all groups. IFN-γ responses were higher after both 10 µg (159 ± 9.8 SFC) and 1 µg (140 ± 5.6 SFC) PLG/SINCP-H than after 10 µg SINCP-H (61 ± 7.1 SFC) (P < 0.005). At week 25, MV-specific IFN-γ production was...
still evident in the group vaccinated with 10 μg PLG/SINCP-H (60 ± 3.5 SFC), indicating that a memory T-cell response was established.

**Immunogenicity of PLG/SINCP-H in rhesus macaques.** We further tested the immunogenicity of PLG/SINCP-H vaccine in pairs of rhesus monkeys (Fig. 2). All PLG/SINCP-H-vaccinated monkeys seroconverted to MV with fourfold or greater increases in PRNT values. Levels of neutralizing antibody above 120 mIU/ml, which is generally considered the protective level, were present 4 to 6 weeks after vaccination in monkeys given 0.5 mg i.m. and i.d. but not in those given 0.1 mg PLG/SINCP-H i.d. or the vector control (Fig. 2A). The mean peak PRNT titers were 350 mIU/ml (high dose, i.m.), 225 mIU/ml (high dose, i.d.), and 20 mIU/ml (low dose, i.d.). However, neutralizing antibodies waned quickly and were only modestly and transiently improved after the boost at 11 weeks. Low levels of MV-specific IgM were detected by EIA in vaccinated monkeys within 6 weeks after immunization and likely contributed to the neutralizing capacity of the plasma. MV-specific IgG measured by EIA was not detectable except in the monkeys given the high dose i.m. after the boost (Fig. 2B).

Monkeys receiving 0.5 mg PLG/SINCP-H i.m. developed an IFN-γ response to H peptides that was highest 2 weeks after the first dose of vaccine. After the boost, all PLG/SINCP-H-immunized monkeys demonstrated H-specific IFN-γ production (Fig. 2C). The monkeys given the high dose i.m. averaged 101 ± 28 SFC/10⁶ PBMCs, compared to control values of 21 ± 17 SFC (P < 0.05). No H-specific IL-4 production was detected (data not shown), suggesting a predominantly type 1 T-cell cytokine response. MV F peptides were also tested in the ELISPOT assay, and no F-specific IFN-γ responses were detected (data not shown).

**Vaccine-induced disease protection or exacerbation after challenge with wild-type MV.** To test the protective efficacy of the PLG/SINCP-H vaccine and the durability of the vaccine-induced immune responses, monkeys were followed for approximately 1 year after vaccination and then challenged with wild-type MV (Fig. 3). At the time of challenge, neutralizing antibody titers had fallen to <50 mIU/ml for all monkeys, and memory IFN-γ-producing T cells were not detected. After challenge, monkeys vaccinated i.m. with 0.5 mg PLG/SINCP-H were protected from rash. All other monkeys developed rashes 10 to 14 days after challenge, and the two monkeys vaccinated with the low dose of PLG/SINCP-H had more severe rashes (Fig. 3B and C) than the monkeys given the vector control (Fig. 3A).

Histopathology of the rash developed by the monkeys given the vector control showed dermal vascular dilatation, thickening of the epidermal basement membrane, and perivascular and superficial dermal infiltrates of neutrophils, eosinophils, and macrophages (Fig. 3E), compared to normal skin (Fig. 3D). Few epithelial cells were positive for MV antigen (Fig. 3E, inset), and no syncytia were noted. Histopathology of the more severe rash developed by the low-dose-vaccinated monkeys showed epidermal necrosis with crusts, epithelial hyperplasia, vascular dilatation, intraepithelial microabscesses, and perivascular, superficial dermal and intraepithelial infiltrates of neutrophils, eosinophils, macrophages, and lymphocytes (Fig. 3F). Multiple syncytia were present, predominantly in follicular epithelium. Staining for MV antigen showed multiple positive epithelial cells (Fig. 3F, inset).

**Viremia.** The cell-associated viremia detected by cocultivation of PBMCs with B95-8 cells was apparent at 7 days, peaked at 10 days, and was no longer detectable by 18 days after challenge (Fig. 4A). Titers were lowest (10⁴⁻⁵ TCID₅₀/10⁶ PBMCs) in monkeys previously immunized i.m. with the high dose of PLG/SINCP-H (P < 0.05) and were highest in monkeys previously immunized i.d. with the low dose of PLG/SINCP-H (10⁴⁻⁸ TCID₅₀/10⁶ PBMCs). Monitoring the infection of PBMCs by intracellular staining for the N protein showed similar patterns, but infected cells could be detected for a longer period of time (Fig. 4B). N-expressing PBMCs increased from day 7, reached a peak at day 10, and were cleared by day 27 after challenge. The highest percentages of N-positive lymphocytes were present in monkeys previously immunized i.d. with the low dose of PLG/SINCP-H.

Double staining of PBMCs with antibodies to lymphocytes and MV N protein was performed to determine the types of
FIG. 3. Clinical disease and skin rash histopathology. All monkeys were challenged approximately 1 year after immunization and monitored for development of a rash. (A to C) SINCP-immunized vector control monkeys (e.g., monkey 11P) developed a typical rash (A), while monkeys given the low dose of PLG/SINCP-H i.d. (12P and 14P) developed severe rashes (B and C). (D to E) Skin biopsies were taken and sections stained with hematoxylin and eosin; skin without a rash (D), skin with a typical rash (E), and skin with a severe rash (F) are shown. Insets show the results of immunohistochemical staining for MV N protein (brown color) in skin with a typical rash (E) and with a severe rash (F). Bars, 100 μm. Arrows, inflammatory infiltrates; arrowhead, MV-positive cell.
cells infected (Fig. 4C). For all groups of monkeys, CD20+ T cells had the highest percentage of N-expressing cells. At 10 days after infection, the percentage of CD20+ cells that were MV infected (means of 0.36% for high dose i.m., 4.4% for high dose i.d., 3.5% for low dose i.d., and 5.1% for control) was 2 to 6 times higher than the percentage of CD3+ T cells infected (means of 0.16% for high dose i.m., 1.3% for high dose i.d., 1.4% for low dose i.d., and 0.8% for control). However, because B cells are less abundant than T cells in the peripheral blood, this would result in similar numbers of infected B and T cells.

**White blood cell changes.** All MV DNA-vaccinated monkeys developed a lymphopenia during the viremia that was restored to prechallenge levels or higher after day 18 (Fig. 5A). The extents of the lymphopenia were similar in monkeys given the low and high doses (50 to 67% decrease). The eosinophil count was little changed during the viremia but increased up to eightfold on day 18, as the viremia was cleared. The change in eosinophil counts in the monkeys given the low dose i.d. (7.9±0.5-fold increase) was higher than that observed for those given the high dose i.d. and the vector control with typical rashes (3.9±1.5) (P=0.004) (Fig. 5B).

**Antibody responses to challenge.** After challenge, neutralizing antibodies increased in PLG/SINCP-H-vaccinated monkeys beginning on day 10, reached a peak at day 14, and were sustained at high levels for months (Fig. 6A). In control monkeys, neutralizing antibody was first detected at day 14. All monkeys had an increase in IgM after challenge, with the lowest IgM responses in monkeys given the high dose of PLG/SINCP-H i.m. (Fig. 6B). The monkeys given the low dose PLG/SINCP-H i.d. had a faster and higher (P=0.004 at day 10) IgM response than the other groups. The peak (day 14) mean OD reading was 0.36 for high dose i.m., 0.8 for high dose i.d., 1.0 for low dose i.d., and 0.8 for controls. MV-specific IgG responses were detected by EIA between days 10 and 14 and reached a peak at day 25 (Fig. 6C). All monkeys developed similar levels of MV-specific IgG as measured by EIA.

The quality of the antibody was investigated by determining the avidity in EIA and the capacity to immunoprecipitate H protein. Protected monkeys given the high dose i.m. had antibody with higher avidity at day 14 than the other monkeys (Fig. 6D). The monkeys given the high and low doses of PLG/SINCP-H i.d. and the control monkeys given PLG/SINCP began to show an increase in avidity at day 25, despite the presence of levels of neutralizing and EIA-measured antibodies comparable to those in i.m. vaccinated monkeys at earlier times. To determine the ability of the antibody produced to bind to native H protein, we assessed the ability to immunoprecipitate H from Moraten (vaccine) and Bilthoven (challenge) strain MVs. No MV protein was precipitated by serum collected before day 14. Five of six vaccinated monkeys showed vaccine strain H protein immunoprecipitation on day 14, but only the protected monkeys given the high dose i.m. (monkeys 26P and 28P) immunoprecipitated challenge wild-type Bilthoven strain H protein (Fig. 6E). By day 25, all monkeys, including controls, demonstrated similar patterns for both the vaccine and wild-type challenge strains of MV.

**Cellular immune responses after challenge.** To monitor the cell-mediated immune responses to viral challenge, IFN-γ production by PBMCs was measured by ELISPOT assays. Mon-
keys vaccinated with the high dose of PLG/SINCP-H i.m. or i.d. showed earlier H-specific IFN-γ responses than monkeys given the low dose and control monkeys (Fig. 7A). The IFN-γ response increased at 7 days after challenge, reached a peak at day 25, and was highest for the two protected monkeys given the high dose i.m. \((P = 0.011)\). Monkeys given the vector control developed a slower but similar level of H-specific IFN-γ-producing T cells by day 35 after challenge. The lowest and slowest IFN-γ responses to challenge were in the monkeys vaccinated with PLG/SINCP-H i.d.

All monkeys generated a primary IFN-γ response to F (not in the vaccine) peptide stimulation, with a peak at day 35 (Fig. 7B). The high-dose-vaccinated monkeys showed the lowest F-specific IFN-γ response, perhaps because the viral load stimulating this response was lower. The mean numbers of IFN-γ SFCs/10⁶ PBMCs were 54 for high dose i.d., 68 for high dose i.m., 80 for low dose i.d., and 163 for controls.

**DISCUSSION**

These studies have shown that a PLG-formulated SINCP DNA plasmid encoding the MV H protein induced better

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**FIG. 6. Antibody responses after challenge. (A) Neutralizing antibody titers were measured by plaque neutralization assay. (B and C) MV-specific IgM (B) and IgG (C) were measured by EIA. The changes in OD from baseline at 1:200 (IgM) and 1:400 (IgG) dilutions of monkey plasma are shown. The mean and standard deviation for each group are shown. (D) MV-specific IgG avidity was measured by ammonium thiocyanate disruption of antibody binding in the EIA assay. The avidity index is the thiocyanate concentration required to remove 50% of the bound IgG. (E) The capacity to immunoprecipitate the native MV Moraten and Bilthoven H proteins was measured by RIPA.**

**FIG. 7. T-cell responses after challenge. IFN-γ responses to stimulation by peptide pools of MV H protein (A) or F protein (B) were assayed by ELISPOT. The number of SFC per million PBMCs is shown. The mean and standard deviation for two monkeys in each pair are indicated.**
antibody and T-cell responses in mice than the same vaccine given as naked DNA. PLG/SINCP-H given i.m. elicited both short-lived neutralizing antibody and MV-specific memory T cells in juvenile rhesus macaques. These responses protected against rash, but not viremia, after wild-type MV challenge. The PLG/SINCP-H vaccine was less effective when given i.d., with lower antibody and T-cell responses than after i.m. inoculation. Monkeys vaccinated i.d. were not protected from rash or viremia after challenge. Monkeys vaccinated with the low dose of PLG/SINCP-H i.d. had higher viremias, more severe rashes, and higher eosinophil counts than monkeys that received the control vaccine, suggesting exacerbated disease.

Previous studies with monkeys of candidate DNA vaccines for measles given either i.d. or by gene gun have shown good T-cell responses, modest antibody responses that are sustained, and partial protection from challenge (47). The PLG/SINCP-H vaccine in the current studies differed from earlier vaccines in that the DNA was adsorbed onto PLG microparticles and the SINCP DNA plasmid used a layered DNA-RNA approach to protein expression. Improved antibody and T-cell responses are achieved in mice with DNA adsorbed to cationic PLG microparticles compared to naked DNA (34, 37, 38, 54), and we confirmed this observation for SINCP DNA expressing H. Antibody and T-cell responses to 1 μg PLG/SINCP-H were equivalent to or better than those induced by 10 μg naked SINCP-H. Similar improvement has been seen with experimental priming DNA vaccines for HIV in rhesus macaques (37, 41). The improvement appears to be due to the ability of PLG microparticles to retain DNA, recruit mononuclear phagocytes, and activate antigen-presenting cells at the injection site (13).

The layered alphavirus-based DNA-RNA approach for expression of proteins has also shown advantages in immunogenicity over conventional plasmid DNA vector approaches (16). The RNA expressed from the DNA plasmid is self-amplifying in the cytoplasm and generates large amounts of mRNA for the protein antigen in cells in which sufficient replicon RNA reaches the cytoplasm (6). A related alphavirus replicon approach in which the RNA expressing MV H is packaged into virion particles for delivery protects monkeys from rash but not from viremia (42). In mice, replicon-encoding DNA vaccines activate innate immune pathways, resulting in substantial improvements in the induction of immune responses using small amounts of DNA compared to conventional plasmid DNA (23, 28, 30, 31). Furthermore, a Sindbis virus replicon-based DNA vaccine encoding MV H was able to induce antibody and T-cell responses in neonatal mice in the presence of maternal antibodies (8).

However, in monkeys, no improvement was seen in the antibody response to HIV proteins after immunization with SINCP compared to a conventional plasmid (41). Adsorption of the same DNAs to PLG microparticles significantly improved the antibody responses to both SINCP and conventional DNA vaccines. However, like the antibody responses that we observed after i.m. PLG/SINCP-H vaccination, titers decreased substantially beginning 5 to 10 weeks after vaccination and were not boosted by reimmunization (41). The reason for the short-lived response is not clear but may reflect inadequate induction of T-cell help for development of long-lived antibody secreting plasma cells. However, the memory response elicited with challenge consisted of both high-avidity antibody that could react with H protein from the wild-type vaccine viruses and IFN-γ-producing T cells.

The route of immunization had a major effect on the response to PLG/SINCP-H. Previous experience with this platform has focused on i.m. inoculation, and we observed partially protective responses in macaques vaccinated by that route. However, macaques receiving the same dose of DNA by i.d. inoculation were not protected, and those receiving a lower dose developed enhanced disease. The i.d. route for vaccination has been attractive because lower doses of DNA are often required (7) and because there is a prospect of vaccine delivery without the requirement for use of a needle and syringe (33). The i.d. route was effective for delivery of the same dose of a conventional naked plasmid expressing H, with two of three monkeys protected from rash (48). It is not known whether the i.d. route was inferior for PLG/SINCP vaccination because of the PLG or plasmid formulation, but it is presumed that the DNA was not efficiently delivered to antigen-presenting cells in the skin or that these cells did not respond to the delivered DNA in a way that resulted in induction of sufficient immunity for protection.

Monkeys vaccinated i.d. developed poor IFN-γ responses to vaccination and had lower IFN-γ responses after challenge than control monkeys, suggesting that T cells were poorly induced. The antibody produced was of low avidity and matured over a time course that was similar to that seen in control animals. This antibody reacted with and neutralized vaccine H but did not react with wild-type H protein in an immunoprecipitation assay. Enhanced disease after challenge associated with increased viremia, higher levels of IgM, eosinophilia, and a more severe rash was observed in the two monkeys that were vaccinated with a low dose of PLG/SINCP i.d. The higher viremia and abundant syncytia in the skin biopsy suggest that this was due to higher virus replication. Low-avidity antibody has previously been associated with immune complex deposition but not increased virus replication (45, 46). The immune response to challenge consisted of a poor IFN-γ T-cell response and substantial eosinophilia, suggesting type 2 skewing of the immune response. Previous studies have shown that CD8+ T cells are a primary mechanism for controlling virus replication after challenge (44), suggesting that the altered T-cell response in monkeys primed with the low dose of vaccine i.d. contributed to higher levels of virus in blood and skin after challenge.

These studies have furthered our understanding of the parameters that are important for protective immunity against MV and suggest that PLG/SINCP-H does not provide an advantage over standard DNA vaccines for this disease.

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REFERENCES

1. Albrecht, P., F. A. Ennis, E. J. Saltzman, and S. Krugman. 1977. Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure. J. Pediatr. 91:715–718.
2. Albrecht, P., K. Herrmann, and G. R. Burns. 1981. Role of virus strain in conventional and enhanced measles plaque neutralization test. J. Virol. Methods 3:251–260.
Schlesinger, and J. A. Wolff. 1995. A plasmid-based self-amplifying Sindbis virus vector. Hum. Gene Ther. 6:1161–1167.

20. Holt, E. A., L. H. Moulton, G. K. Siberry, and N. A. Halsey. 1993. Differential mortality by measles vaccine titer and sex. J. Infect. Dis. 168:1087–1096.

21. Dye, C., T. W. C. Huffman, L. H. Moulton, H. J. Jiang, L. He, J. M. Hardwick, S. Kumar, and T. C. Wu. 2003. Enhancing DNA vaccine potency by coadministration of DNA encoding antiapoptotic proteins. J. Clin. Investig. 112:109–117.

22. Kirman, J. R., T. Turon, H. Su, A. Li, C. Kraus, J. M. Polo, J. Belisle, S. Munoz, and R. A. Seder. 2005. Enhanced immunogenicity of Mycobacterium tuberculosis by vaccination with an alphavirus plasmid replicon expressing antigen 85A. Infect. Immun. 71:575–579.

23. Knudsen, K. M., P. Aaby, H. Whittle, M. Rowe, B. Samf, S. Simonzon, J. Sterner, and P. Fine. 2007. The impact on mortality following standard, medium, or high titer measles immunization in West Africa. Int. J. Epidemiol. 36:565–673.

24. Leitner, W. W., L. N. Hwang, M. j.deVeere, A. Zhou, R. H. Silverman, B. R. Williams, T. W. Dubensky, H. Ying, and N. P. Restifo. 2003. Alphavirus-based DNA vaccine breaks immunological tolerance by activating innate antiviral pathways. Nat. Med. 9:33–39.

25. Leitner, W., H. Ying, and N. Restifo. 1999. DNA and RNA-based vaccines: principles, progress, and perspectives. Annu. Rev. Immunol. 17:553–68.

26. Malvoisin, E., and F. Wild. 1990. Contribution of measles virus fusion protein to protective immunity: anti-F monoclonal antibodies neutralize virus infectivity and protect mice against challenge. J. Virol. 64:5160–5162.

27. Mitragotri, S. 2005. Immunization without needles. Nat. Rev. Immunol. 5:22–31.

28. Mollenkopf, H. J., G. Dietrich, J. Fensterle, L. Grode, K. D. Diehl, B. Knapp, M. Singh, D. T. O’Hagan, J. B. Ulmer, and S. H. Kaufmann. 2004. Enhanced protective efficacy of a tuberculosis DNA vaccine by adsorption onto cationic microparticles. Vaccine 22:2690–2695.

29. Moss, W. J., M. J. Ony, T. C. Quinn, D. E. Griffin, and F. Cutts. 2004. Prospective study of measles in hospitalized, human immunodeficiency virus (HIV)-infected and HIV-uninfected children in Zambia. Clin. Infect. Dis. 38:1189–1196.

30. Oh, S., B. Stegman, C. D. Pendleton, M. O. Ota, C. H. Pan, D. E. Griffin, D. S. Burke, and J. A. Berzofsky. 2006. Protective immunity provided by HLA-A2 epitopes for fusion and hemagglutinin proteins of measles virus. J. Virol. 80:39–39.

31. O’Hagan, D., M. Singh, M. Ugozzoli, C. Wild, S. Barnett, M. Chen, S. M. Schaefer, B. Doe, G. Otten, and J. B. Ulmer. 2001. Induction of potent immune responses by cationic microparticles with adsorbed human immunodeficiency virus DNA vaccines. J. Virol. 75:9037–9043.

32. O’Hagan, D. T., M. Singh, C. Dong, M. Ugozzoli, K. Berger, E. Glazer, M. Selby, M. Wineringer, P. Ng, K. Crawford, X. Palliard, S. Coates, and M. Houghton. 2004. Cationic microparticles are a potent delivery system for an HCV DNA vaccine. Vaccine 23:672–680.

33. Ono, N., H. Tatsu, Y. Hidaka, T. Aoki, and H. Minagawa. 2001. Measles viruses on throat swabs from measles patients using sampling lymphocytic activation molecule (CD150) but not CD46 as a cellular receptor. J. Virol. 75:4409–4411.

34. Ota, M. O., Z. Ntshlovu, S. Oh, S. Piyasirisilp, J. A. Berzofsky, W. J. Moss, and D. E. Griffin. 2007. Hemagglutinin protein is a primary target of the measles virus-specific HLA-A2-restricted CD8+ T cell response during measles and after vaccination. J. Virol. 81:6267–6275.

35. Otto, G. R., M. Schaefer, D. B. Doe, H. Liu, I. Srivastava, J. Meregde, J. Kazzaz, Y. Lian, M. Singh, M. Ugozzoli, D. Montefiori, M. Lewis, D. A. Driver, T. Dubensky, J. M. Polo, J. Donnelly, D. T. O’Hagan, S. Barnett, and J. B. Ulmer. 2005. Enhanced potency of plasmid DNA microparticle human immunodeficiency virus vaccines in rhesus macaques by using a priming-booster regimen with recombinant proteins. J. Virol. 79:8199–8200.

36. Pan, C. H., A. Valsamakis, T. Colella, N. Nair, R. J. Adams, F. P. Polack, C. E. Greer, S. Perri, J. M. Polo, and D. E. Griffin. 2005. Modulation of disease by T cell responses, and measles virus clearance in monkeys vaccinated with H-encoding alpha virus replicon particles. Proc. Natl. Acad. Sci. USA 102:11581–11588.

37. Pasetti, M. F., E. M. Barry, G. Losonsky, Singh, M. S. Medina-Moreno, J. Polo, J. Ulmer, H. M. Young, and D. E. Griffin, and M. M. Levine, 2003. Attenuated Salmonella enterica sekorov Typbi and Shigella flexneri 2a strains mucosally deliver DNA vaccines encoding measles virus hemagglutinin, inducing specific immune responses and protection in cotton rats. J. Virol. 77:5209–5217.

38. Pernar, S. R., S. A. Klumpp, K. G. Mansfield, W. K. Kim, D. A. Gorgone, M. A. Lifton, K. C. Williams, J. E. Schmitz, K. A. Reiman, M. K. Axthelm, F. P. Polack, D. E. Griffin, and N. L. Letvin. 2003. Role of CD4+ lymphocytes in control and clearance of measles virus infection of rhesus monkeys. J. Virol. 77:4396–4400.

39. Polack, F. P., F. P. Auwaerter, S.-H. Lee, C. H. Ncouri, A. Valsamakis, K. M. Lefierman, A. Diwan, R. J. Adams, and D. E. Griffin. 1999. Production of atypical measles in rhesus macaques: evidence for disease mediated by immune complex formation and eosinophils in the presence of fusion-inhibiting antibody. Nat. Med. 5:629–634.

40. Polack, F. P., S. J. Hofman, G. Crieur, and D. E. Griffin. 2003. A role for...
nonprotective complement-fixing antibodies with low avidity for measles virus in atypical measles. Nat. Med. 9:1209–1213.

47. Polack, F. P., S. J. Hoffman, W. J. Moss, and D. E. Griffin. 2003. Differential effects of priming with DNA vaccines encoding the hemagglutinin and/or fusion proteins on cytokine responses after measles virus challenge. J. Infect. Dis. 187:1794–1800.

48. Polack, F. S. Lee, S. Permar, E. Manyara, H. Nousari, Y. Jeng, F. Mustafa, A. Valsamakis, R. Adams, H. Robinson, and D. Griffin. 2000. Successful DNA immunization against measles: neutralizing antibody against either the hemagglutinin or fusion glycoprotein protects rhesus macaques without evidence of atypical measles. Nat. Med. 6:776–781.

49. Premenko-Lanier, M., P. A. Rota, G. H. Rhodes, W. J. Bellini, and M. B. McChesney. 2004. Protection against challenge with measles virus (MV) in infant macaques by an MV DNA vaccine administered in the presence of neutralizing antibody. J. Infect. Dis. 189:2064–2071.

50. Rauh, L. W., and R. Schmidt. 1965. Measles immunization with killed virus vaccine. Am. J. Dis. Child. 109:232–237.

51. Redd, S. C., G. E. King, J. L. Heath, B. Forghani, W. J. Bellini, and L. E. Markowitz. 2004. Comparison of vaccination with measles-mumps-rubella vaccine at 9, 12, and 15 months of age. J. Infect. Dis. 189(Suppl. 1):S116–S122.

52. Rota, J. S., K. B. Hummel, P. A. Rota, and W. J. Bellini. 1992. Genetic variability of the glycoprotein genes of current wild-type measles isolates. Virology 188:135–142.

53. Siegrist, C. A. 2001. Neonatal and early life vaccinology. Vaccine 19:3331–3346.

54. Singh, M., M. Briones, G. Ott, and D. O’Hagan. 2000. Cationic microparticles: a potent delivery system for DNA vaccines. Proc. Natl. Acad. Sci. USA 97:811–816.

55. Stittelaar, K. J., R. L. de Swart, H. W. Vos, G. van Amerongen, N. Sixt, T. F. Wild, and A. D. Osterhaus. 2002. Priming of measles virus-specific humoral- and cellular-immune responses in macaques by DNA vaccination. Vaccine 20:2022–2026.

56. Stittelaar, K. J., H. W. Vos, G. van Amerongen, G. F. Kersten, A. D. Osterhaus, and R. L. de Swart. 2002. Longevity of neutralizing antibody levels in macaques vaccinated with Quil A-adjuvanted measles vaccine candidates. Vaccine 21:155–157.

57. Stittelaar, K. J., L. S. Wyatt, R. L. de Swart, H. W. Vos, J. Groen, G. van Amerongen, R. S. Van Binnendijk, S. Rozenblatt, B. Moss, and A. D. Osterhaus. 2000. Protective immunity in macaques vaccinated with a modified vaccinia virus Ankara-based measles virus vaccine in the presence of passively acquired antibodies. J. Virol. 74:4236–4243.

58. Van Binnendijk, R. S., M. C. M. Poelet, G. van Amerongen, P. de Vries, and A. D. M. E. Osterhaus. 1997. Protective immunity in macaques vaccinated with live attenuated, recombinant, and subunit measles vaccines in the presence of passively acquired antibodies. J. Infect. Dis. 175:524–532.

59. Van Binnendijk, R. S., R. W. J. van der Heijden, and A. D. M. E. Osterhaus. 1995. Monkeys in measles research. Curr. Top. Microbiol. Immunol. 191:135–148.

60. Xiang, Z., A. J. Cutler, R. J. Brownlie, K. Fairfax, K. E. Lawlor, E. Severinson, E. U. Walker, R. A. Manz, D. M. Tarlinton, and K. G. Smith. 2007. FcgammaRIIb controls bone marrow plasma cell persistence and apoptosis. Nat. Immunol. 8:419–429.

61. Zhu, Y., G. Fennelly, C. Miller, R. Tarara, I. Saxe, B. Bloom, and M. McChesney. 1997. Recombinant Bacille Calmette-Guerin expressing the measles virus nucleoprotein protects infant rhesus macaques from measles virus pneumonia. J. Infect. Dis. 176:1445–1453.

62. Zhu, Y., P. Rota, L. Wyatt, A. Tamin, S. Rozenblatt, N. Lerche, B. Moss, W. Bellini, and M. McChesney. 2000. Evaluation of recombinant vaccinia virus–measles vaccines in infant rhesus macaques with preexisting measles antibody. Virology 276:202–213.