JOURNAL OF VIROLOGY, Aug. 2005, p. 9854–9861 Vol. 79, No. 15
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.15.9854–9861.2005

Characterization of Immune Responses Induced by Intramuscular Vaccination with DNA Vaccines Encoding Measles Virus Hemagglutinin and/or Fusion Proteins

Man Ki Song,[1,3] Christofer J. Vindurampulle,[1] Alejandra V. E. Capozzo,[1] Jeffrey Ulmer,[2] John M. Polo,[2] Marcela F. Pasetti,[1] Eileen M. Barry,[1] and Myron M. Levine[1]*

Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland[1]; Chiron Vaccines, Emeryville, California[2]; and International Vaccine Institute, Seoul, Korea[3]

Received 22 December 2004/Accepted 18 April 2005

Measles virus (MV) hemagglutinin (MV-H) and fusion (MV-F) proteins induce plaque reduction neutralizing (PRN) antibodies and cell-mediated immune responses that protect against clinical measles. DNA vaccines that encode MV-H and MV-F are being investigated as a new generation of measles vaccine to protect infants too young to receive currently licensed attenuated measles vaccines. However, it is unclear whether DNA vaccines encoding both MV-H and MV-F act synergistically to induce stronger immunity than immunization with plasmids encoding MV-H or MV-F alone. To address this question, we generated Sindbis virus-based pSINCP DNA vaccines that encode either MV-H or MV-F alone or bicistronic or fusion system vectors that encode both MV-H and MV-F (to mimic MV infection where both MV-H and MV-F proteins are expressed by the same mammalian cell). Mice immunized with DNA vaccine encoding MV-H alone developed significantly greater PRN titers than mice immunized with bicistronic constructs. Interestingly, the presence of MV-F in the bicistronic constructs stimulated serum MV-specific immunoglobulin G of reduced avidity. By contrast, mice immunized with bicistronic constructs induced equivalent or higher levels of MV-specific gamma interferon responses than mice immunized with DNA vaccine encoding MV-H alone. These data will help guide the design of DNA-based MV vaccines to be used early in life in a heterologous prime-boost strategy.

Measles virus (MV) remains a major cause of infant morbidity and mortality in many regions of the world. An estimated 700,000 deaths occur worldwide per year (31, 48), the overwhelming majority clustered in developing countries. Given at 9 months of age or above, the existing licensed live attenuated MV vaccines are safe and have been highly effective in eliminating measles where they have been used programmatically in industrialized and middle-income countries (13, 27, 39). In contrast, in many less-developed countries, particularly in Africa, measles has remained a public health problem, despite moderate levels of coverage with attenuated measles vaccine (20, 48, 50). A specific high-risk group has been young infants who are too young (less than 9 months of age) to respond reliably to the current attenuated measles vaccine but who can develop severe, often fatal clinical measles upon exposure to wild-type virus (4, 25, 48).

Three independent strategies are under way to address the problem of measles mortality in developing countries. The first strategy, pursued by the Global Alliance for Vaccines and Immunization and its Vaccine Fund, aims to strengthen the infrastructure for delivering routine infant immunizations, including existing measles vaccine, in the world's 74 least-developed countries (28). The second strategy, undertaken by various international and national agencies (e.g., the World Health Organization, UNICEF, Centers for Diseases Control and Prevention, American Red Cross, U.S. Agency for International Development, etc.) focuses on mass immunization campaigns against measles among children 9 months to 15 years of age in developing countries in Africa where measles is endemic (6, 10, 21, 22, 34). These two strategies indirectly diminish the measles risk for young infants by reducing the overall transmission of measles within communities. The third strategy, spearheaded by initiatives of the Bill and Melinda Gates Foundation, aims to develop a new generation of measles vaccine that will specifically target infants who are too young to receive the current licensed measles vaccines.

DNA vaccines encoding measles antigens induce both humoral and cell-mediated immune responses (42–44, 46, 53). MV-H or MV-F DNA vaccines induce neutralizing antibodies in macaques and confer protection against experimental challenge with wild-type measles virus (44). Notably, an MV DNA vaccine containing the H, F, and nucleoprotein (N) genes induced protective immunity to MV in infant rhesus macaques even in the presence of maternal antibody (46) and protected against experimental challenge (47). However, there is debate as to whether coimmunization with DNA vaccines encoding MV-H and MV-F has an additive or a suppressive effect on the generation of protective immunity against MV and whether the presence of both proteins modulates the immune responses. Coimmunizing with a combination of two DNA vaccines encoding MV-H and MV-F, Polack et al. (44) reported that MV-specific cytotoxic T-lymphocyte responses were slowed and plaque reduction neutralizing (PRN) antibodies were decreased compared to responses elicited by MV-H. These investigators subsequently showed that vaccination with

* Corresponding author. Mailing address: Center for Vaccine Development, University of Maryland School of Medicine, 685 W. Baltimore St., Rm. 480, Baltimore, MD 21201. Phone: (410) 706-7588. Fax: (410) 706-6205. E-mail: mlevine@medicine.umaryland.edu.

DNA vaccines encoding MV-H and MV-F prime Th2 and Th1 cytokine production in macaques, respectively, following challenge with MV (43). Coimmunization with MV-H and MV-F induced higher gamma interferon (IFN-γ) production by phytohemagglutinin-stimulated peripheral blood lymphocytes than vaccination with MV-H alone (43).

A detailed characterization of the immune response to measles DNA vaccines is important, because a formalin-inactivated whole-virus vaccine licensed and used in the 1960s in the United States and Europe sometimes predisposed to the development of severe atypical measles syndrome when vaccinated children were subsequently exposed to wild-type measles virus (5, 18). It had been hypothesized that the atypical measles syndrome resulted from an imbalance in the antibody responses to the viral MV-H and MV-F proteins, as the latter antigen was denatured during manufacture of the vaccine (30, 33). However, based on more recent studies in the rhesus macaque model, it is now believed that the atypical measles syndrome results from aberrant prior priming that leads to low-avidity antibodies with high complement-fixing capacity which favor the deposition of immune complexes, rather than from lack of functional antibody against the fusion protein (40, 41).

To characterize the immune responses elicited following parenteral administration of DNA vaccines encoding MV proteins, we engineered Sindbis virus-based DNA replicon pSINCP constructs to encode MV-H or MV-F alone or both MV-H and MV-F (thereby ensuring expression of both MV-H and MV-F proteins within the same transfected cell and eliminating any possible competition involving separate plasmids). Herein, we present data on the MV-specific immune responses elicited by the measles virus DNA vaccine constructs in mice.

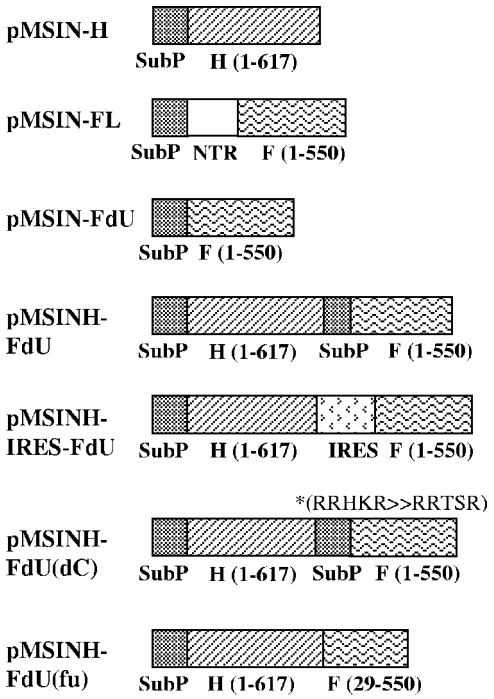

FIG. 1. Schematic diagram of eukaryotic expression vectors. All constructs (not drawn to scale) were based on pSINCP. The pMSIN-H vector contains a full-length MV-H under the control of Sindbis virus subgenomic promoter (SubP). pMSIN-FL and pMSIN-FdU encode MV-F with or without its 5′ NTR, respectively. The bicistronic expression vectors, pMSINH-FdU and pMSINH-IRESFdU, encode MV-F under the control of SubP or the IRES element, respectively. The plasmid pMSINH-FdU(dC) contains MVF with mutations in its cleavage site (RRHKR to RRTSR at amino acids 108 to 112). The fusion construct, pMSINH-FdU(fu), encodes an in-frame fusion of MV-H, lacking its termination codon, with MV-F, lacking its signal sequence. Numbers in parentheses indicate the number of amino acids encoded by each DNA fragment.

MATERIALS AND METHODS

Construction of expression vectors. All PCR amplification reactions were performed using Vent DNA polymerase (Invitrogen Corp., Grand Island, N.Y.). The Sindbis-derived DNA vaccine replicon pSINCP (Chiron Corp., Emeryville, CA) was employed as the backbone for all plasmid constructs (Fig. 1). The vector pMSIN-H encoding full-length measles virus H has been described previously (36). Plasmids pMSIN-FL and pMSIN-FdU were generated to facilitate the expression of MV-F protein in mammalian cells. The former was constructed by direct insertion of a 2.4-kb cDNA gene encoding full-length MV-F with 5′ nontranslated region (NTR) excised from pEF1 (kindly provided by Alexandra Valsamakis, Johns Hopkins University) as a XhoI/NotI fragment and ligated into similarly digested pSINCP, creating pMSIN-FL. Replicon pMSIN-FdU encodes MV-F lacking the 5′ NTR and was constructed with primers 5′XhoIF (5′-ATC CCTCGAGGCAAGATCATCCACAATGTCACCA-3′) and 3′NotIF (5′-GGA TGCGGCCGCATCAGAGCGACCTTACATAGGATTTTG-3′) and cloning the XhoI-NotI-digested PCR product into similarly digested pSINCP.

Vector pMSINH-FdU, which encodes both MV-H and MV-F, was generated by PCR amplifying the subgenomic promoter and MV-F gene lacking its 5′ NTR from pMSIN-FdU with primers 5′subG (5′-GCTCGCGGCCGCGACGACCC GGTATGAGGTAGACAAT-3′) and 3′NotIF (above). The NotI-digested PCR product was cloned into similarly digested pMSIN-H, placing MV-FdU at the 3′ end of MV-H. To generate pMSINH-FdU(dC), which encodes a mutated MV-F lacking its 5′ NTR, site-directed mutagenesis was performed by using PCR with primers 5′-F2SpeI (5′-AAATCTACTAGTTCTCCTACTTGAAGCTACACTC-3′) and 3′-F1SpeI (5′-GAGTGTAGCTTCAAGTAGGAGAACTAGTAGATT T-3′). The SpeI-digested PCR product was cloned into similarly digested pMSINH-FdU, resulting in the change in the cleavage site of MV-F from RRHKR to RRTSR. To construct pMSINH-IRESFdU, which encodes MV-F under the control of an encephalomyocarditis virus internal ribosomal entry site (IRES), the MV-FdU was PCR amplified from pMSIN-FdU with primers 5′XhoIF and 3′NotIF (above) and the NotI-digested PCR product was cloned into similarly digested pIRES (Clontech, Palo Alto, CA). We then PCR amplified the sequence encoding the IRES and MV-FdU (IRESFdU) with primers 5′-IRESNotI (5′-GGCTAGCCTCGAGAATTCACGCGGCCGCCA-3′) and 3′NotIF (above) and inserted the NotI-digested PCR product into similarly digested pMSIN-H. This placed IRESFdU at the 3′ end of MV-H.

The fusion construct pMSINH-FdU(fu) was generated by first PCR amplifying MV-H lacking its termination codon with primers 5′HXhoI (5′-ATCCCTCGA GGCAAGATCATCCACAATGTCACCA-3′) and 3′HNotI (5′-ATCCGCGGC CGCTCTGCGATTGGTTCCATCTTCCCG-3′) and inserting the XhoI-NotI-digested product into similarly digested pSINCP, creating pMSINH(fu). We then PCR amplified MV-FdU lacking its signal sequence from pMSIN-FdU with primers 5′NotIfu (5′-ATCCGCGGCCGCCCATTGGGGCAATCTCTCTAAG ATAGG-3′) and 3′-NotIF (above) and inserted the NotI-digested PCR product into similarly digested pMSINH(fu), creating pMSINH-FdU(fu).

Immunization of mice. Six- to eight-week-old female BALB/c mice (Charles River, Wilmington, Mass.) were immunized intramuscularly (i.m.) with 100 μg of DNA suspended in phosphate-buffered saline (PBS; 50 μl per hind leg) administered via a 25-gauge, 1-ml hypodermic needle (Becton Dickinson, Cockeysville, MD). All mice received a booster dose 28 days after their initial immunization. Sera were obtained from blood samples collected from the retro-orbital plexus prior to each immunization and on day 56.

Western immunoblot analysis. BHK-21 and Vero cells (both obtained from the American Type Culture Collection, Manassas, Va.) were grown in Dulbecco's modified Eagle's medium (Gibco Invitrogen Corp., Grand Island, N.Y.) supplemented with 10% fetal calf serum (Gibco Invitrogen Corp.). Cells were transfected with the various pSINCP-based constructs using Lipofectamine (Gibco Invitrogen Corp.) according to the manufacturer's instructions. Approximately 2 μg of plasmid DNA was used to transfect cells seeded at 5×10^5 cells per well in a six-well tissue culture plate (MULTIWELL 6; Becton Dickinson,

Cockeysville, MD). Cell lysates were obtained after 48 h of incubation by scraping cells from each well and resuspending cell pellets in lysis buffer (150 mM NaCl, 1% Nonidet P-40, and 10 mM Tris [pH 7.4]). Cell lysates were diluted in sample buffer (Bio-Rad, Hercules, CA) and separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis polyacrylamide gels. For Western immunoblot analysis, proteins were transferred from the gels onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Following transfer, membranes were blocked with 10% skim milk and incubated for 1 h at 37°C with a 1:3,000 dilution of mouse anti-MV-H or anti-MV-F monoclonal antibody (kindly provided by Diane Griffin, Johns Hopkins University) suspended in buffer (1× PBS, 0.05% Tween 20, and 5% nonfat dry milk). A 1:3,000 dilution of alkaline phosphatase-conjugated goat anti-mouse antiserum was used to detect protein-antibody complexes, which were subsequently visualized with alkaline phosphatase substrate (Bio-Rad, Hercules, CA).

Measurement of MV-specific serum IgG and PRN antibodies. Total serum immunoglobulin G (IgG) specific for MV was measured by enzyme-linked immunosorbent assay (ELISA) as previously described (36), with brief modifications: ELISA plates were coated with MV lysate (Advanced Biotechnologies Inc., Columbia, MD) at 5 μg/ml in carbonate buffer, pH 9.6, for 3 h at 37°C and blocked overnight with 10% nonfat dried milk (Nestle USA Inc., Glendale, CA) in PBS. Serum samples were tested in twofold dilutions in 10% dried milk in PBST (PBSTM). IgG against MV was revealed with horseradish peroxidase-labeled goat anti-mouse conjugate (Roche Diagnostic Corporation, Indianapolis, Ind.) diluted 1:1,000 in PBSTM. The substrate solution used was TMB Microwell peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). After 15 min of incubation in the dark, the reaction was stopped by the addition of 100 μl of 1 M H_2PO_4 and the optical density at 450 nm was measured in an ELISA microplate reader (Multiskan Ascent; Thermo Labsystem). Sera were run in duplicate. Negative and positive control sera were included in each assay. Linear regression curves were plotted for each serum sample, and titers were calculated (through equation parameters) as the inverse of the serum dilution that produced an optical density of 0.2 above the blank (ELISA units/ml).

Measurement of MV-specific PRN antibodies was also performed as described previously (36). The World Health Organization anti-measles virus serum standard 66/202 was tested in parallel with the samples, thereby allowing PRN titers to be expressed in mIU/ml (17). Samples with undetectable neutralizing antibody (titer of <10 by PRN) were assigned a value of 6.25 mIU/ml, the equivalent of a titer of 5 by PRN. Titers of ≥200 mIU/ml were considered protective (11).

Avidity of MV-specific IgG antibodies. Antibody avidity was measured by ELISA using urea as chaotropic agent to dissociate low-affinity antigen-antibody complexes. Similar assays were used to measure avidity of measles virus antibodies following vaccination or natural infection in monkeys (41) and human sera (14, 15, 35). Briefly, serum samples (in twofold dilutions) were incubated for 1 h at 37°C in ELISA plates coated with MV lysate as described above. Plates were washed with PBST and soaked with 6 M urea for 10 min. After subsequent washes with PBST, bound IgG was detected with anti-mouse IgG conjugate followed by TMB substrate as described above. Endpoint titers for each serum sample were determined as described above, in the presence and absence of urea. Results are expressed as an avidity index, calculated as the percentage of residual activity (endpoint titer) using the following formula: [(endpoint titer in the presence of urea)/(endpoint titer in the absence of urea)] × 100. Positive and negative samples of known antibody titer (calibrated controls) were run in each assay. During the standardization of the assay, several concentrations of urea (6 to 8 M) were tested, and 6 M was selected as optimal; higher urea concentrations completely abrogated antibody binding. To validate the assay, a large number of positive and negative mouse samples were tested simultaneously for measles virus PRN and IgG avidity. PRN titers above the protective threshold (200 mIU/ml) showed avidity indices above 30, as already described in the literature for human samples (14).

IFN-γ ELISPOT assays. IFN-γ responses specific for MV were determined by ELISPOT (mouse IFN-γ-specific ELISPOT kit; BD Biosciences, CA) as described previously. Spleens were collected on day 70 after immunization and homogenized, and single-cell suspensions were prepared. Samples were pooled within each group and restimulated in vitro for 48 h with 5 μg/ml of wild-type MV (Edmonston strain) lysate (Biodesign, Saco, Maine) in triplicate wells, starting at a concentration of 5×10^5 cells per well. Plates were then developed as recommended by the manufacturer, and the spots were enumerated. Results are expressed as the mean spot-forming cells/10^6 splenocytes.

Statistical analysis. Individual mouse antibody titers were log transformed, and group titers are expressed as the geometric mean (GM) ± the standard deviation (SD). Probability values were calculated by one-way analysis of variance (GraphPad Software Inc.) at a 95% confidence interval and involved the Newman-Keuls multiple comparison test.

RESULTS

Construction and identification of various plasmids expressing MV-H and/or MV-F. Recombinant plasmids expressing MV-H and/or MV-F were constructed using the Sindbis virus-based vector pSINCP as a backbone. Replicon pMSIN-H, which encodes full-length MV-H, has been described previously (36). Two plasmids were constructed to facilitate the expression of MV-F with or without its 5′ NTR (Fig. 1, pMSIN-FL and pMSIN-FdU, respectively). To compare MV-F protein expression in BHK-21 cells transfected with either of these constructs, Western immunoblot analysis was conducted with mouse anti-F monoclonal antibody. Cells transfected with pMSIN-FdU were able to express notably more F protein than those transfected with pMSIN-FL (Fig. 2B), demonstrating the inhibitory effect of the 5′ NTR on gene expression. On the basis of this result, we used MV-F lacking its 5′ NTR (MV-FdU) to construct the bicistronic vectors encoding MV-F downstream of MV-H. Replicons pMSINH-FdU and pMSINH-IRESFdU contain MV-FdU under the control of either the Sindbis virus subgenomic promoter or IRES element, respectively. While the duplicated subgenomic promoter is an actual transcription promoter that gives rise to two separate subgenomic RNA transcripts, the IRES is a means to achieve internal translation initiation from the same single subgenomic transcript that encodes both H and F. Western immunoblot analysis of cells transfected with these constructs revealed equivalent levels of MV-H expression (data not shown). However, cells transfected with pMSINH-FdU expressed greater amounts of MV-F than pMSINH-IRESFdU (data not shown). Thus, the Sindbis virus subgenomic promoter appeared to be more efficient than the IRES element in achieving expression of MV-FdU, along with H.

Proteolytic cleavage of the MV-F protein precursor into its F1 and F2 subunits is required for the inhibition of lymphoid cell proliferation. To inhibit cleavage we performed PCR-based mutagenesis to change two amino acids in the cleavage site of MV-F (RRHKR to RRTSR at amino acids 108 to 112), creating pMSIN-H-FdU(dC). Unexpectedly, cells transfected with this plasmid produced MV-F protein that was cleaved as efficiently as the wild-type protein produced by cells transfected with pMSINH-FdU (Fig. 2). To construct a plasmid encoding a fusion of MV-H and MV-F proteins, we utilized PCR to remove the signal sequence (residues 1 to 28) of MV-F and attached the truncated gene to the 3′ end of MV-H lacking a termination codon, thereby creating pMSINH-FdU(fu). MV-H and MV-F are type II and I glycoproteins, which have the transmembrane domain at the N terminus or C terminus, respectively. We designed pMSINH-FdU(fu) with the goal of expressing a large fusion polypeptide at the cell surface with each terminus attached to cell membrane. Cells transfected with this construct produced a protein with a molecular mass greater than 113 kDa that reacted with both anti-MV-H and anti-MV-F monoclonal antibodies (Fig. 2). Smaller proteins corresponding to MV-F1 and MV-F2 cleavage products were not detected.

MV-H- and MV-F-mediated syncytium formation. In natural MV infection, mammalian cells that express both MV-H and MV-F fuse to form syncytia. In this study, whereas Vero cells cotransfected with pMSIN-H and pMSIN-FdU formed syncytia

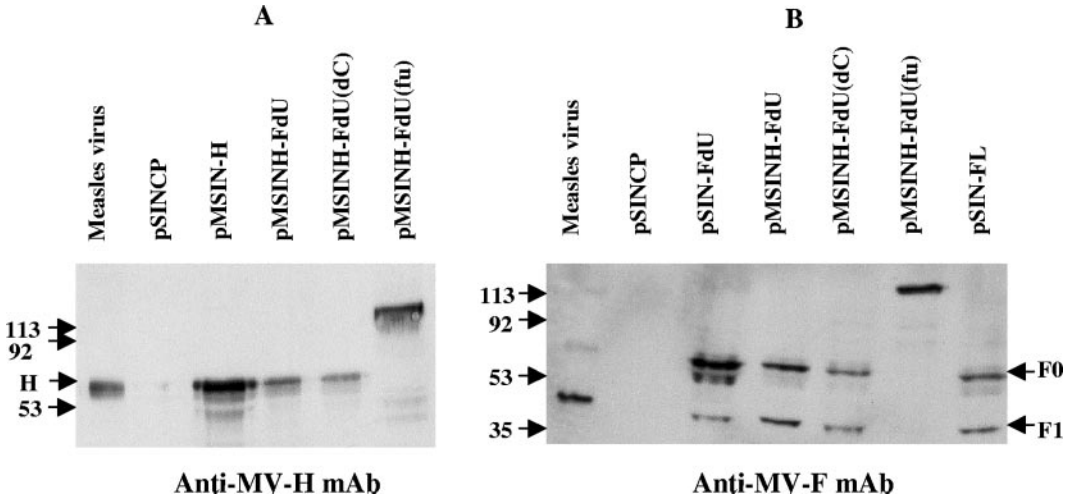

FIG. 2. Identification of the MV-H and MV-F proteins expressed in BHK-21 cells. Cell lysates were prepared from BHK-21 cells 48 h posttransfection with the different plasmid constructs. Cell lysates were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred, and the immunoblot was probed with a mouse monoclonal anti-MV-H antibody (A) or with anti-MV-F antibody (B). Sizes are indicated in kilodaltons.

(Fig. 3), the bicistronic plasmids pMSINH-FdU and pMSINH-IRESFdU induced ~10-fold greater syncytium formation. Interestingly, pMSINH-Fdu(dC), which has the mutation at the cleavage site of MV-F, did not cause any syncytium formation even though the MV-F protein was cleaved as efficiently as the wild-type protein. Lastly, the fusion construct pMSINH-FdU(fu) did not induce syncytium formation, indicating that the correct conformation of each protein was not achieved on the cell surface.

Measles virus-specific antibody responses. Groups of mice were given two spaced immunizations, 28 days apart, with 100 μg i.m. of each DNA construct, and sera collected on days 0, 28, and 56 were analyzed for IgG antibodies specific for MV by ELISA (Fig. 4). As expected, sera from mice immunized with pSINCP did not exhibit any measles virus-specific IgG. By day 56, groups of mice vaccinated with plasmids encoding MV-H and/or MV-F developed IgG-specific anti-measles virus antibodies; the GM titer of each vaccinated group was significantly greater than the control group ($P < 0.01$), but they were equivalent to each other. Interestingly, mice immunized with the fusion construct pMSINH-FdU(fu) failed to elicit any anti-measles virus antibody responses, despite the previous confirmation of expression of both MV-H and MV-F components in vitro (data not shown).

Sera from these mice were subsequently analyzed for their ability to neutralize MV replication in vitro. By day 56, the

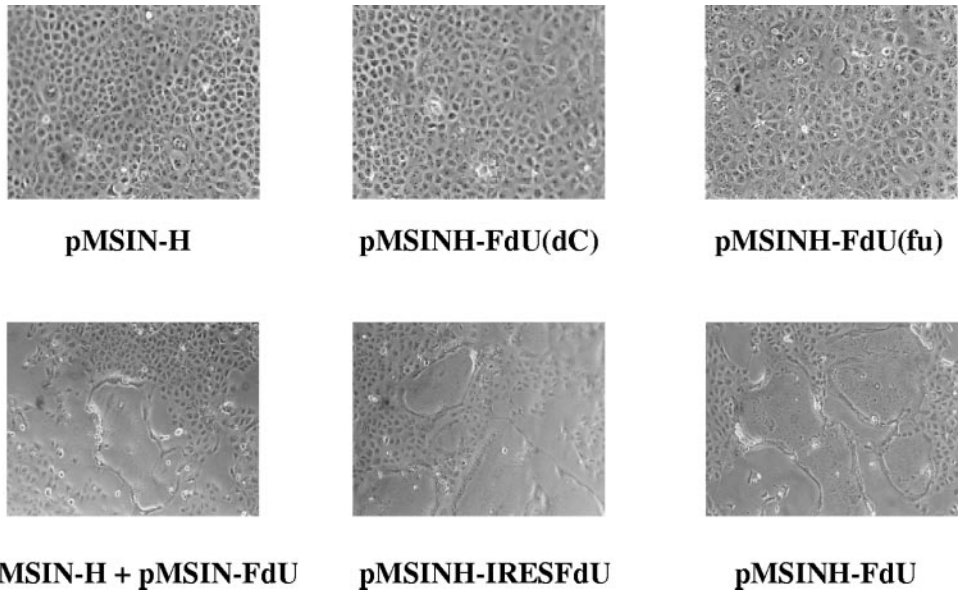

FIG. 3. Syncytium formation in Vero cells transiently expressing MV-H and/or MV-F protein. Vero cells were transfected with the various plasmid constructs as indicated. Cells were photographed 48 h after transfection by phase-contrast microscopy. Magnification, ×100.

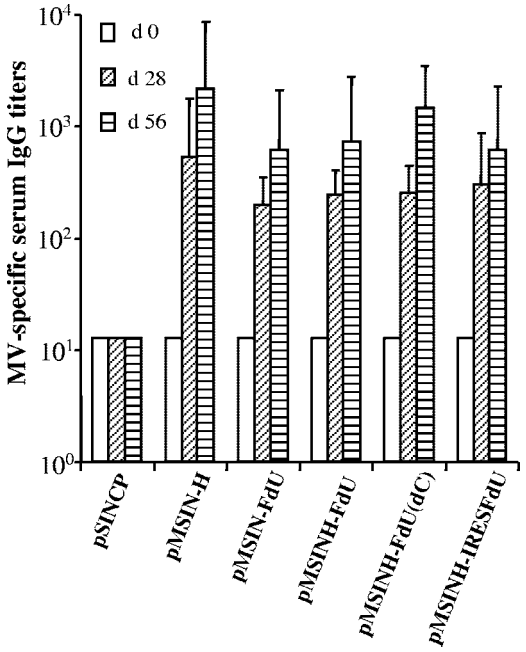

FIG. 4. Induction of MV-specific IgG antibody in mice immunized i.m. with DNA vaccines encoding MV-H and/or -F. Groups of BALB/c mice ($n = 10$) were immunized i.m. on days 0 and 28 with 100 μg of the various plasmid constructs as depicted on the *x* axis. MV-specific IgG titers were measured by ELISA on days 0, 28, and 56. Bars show GM titers (EU/ml) ± the SD.

PRN titers of sera from mice immunized with pMSIN-FdU and the bicistronic constructs were all significantly lower than those of mice immunized with pMSIN-H ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 5); of the bicistronic constructs, pMSINH-FdU gave the strongest PRN responses (Fig. 5). Mice immunized with monocistronic pMSIN-FdU failed to reach the human seroprotective threshold (titer ≥ 200 mIU).

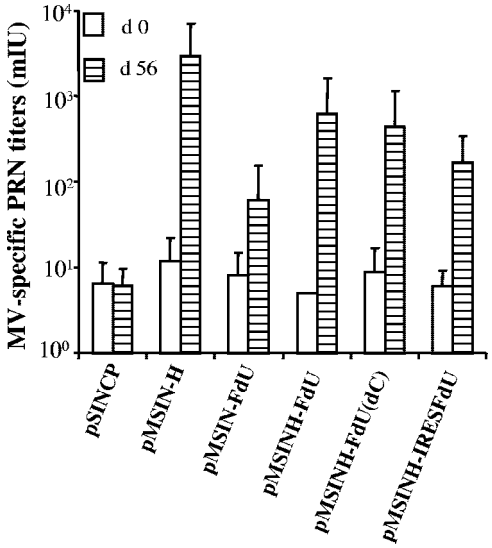

FIG. 5. Serum PRN titers from mice i.m. immunized with DNA vaccines encoding MV-H and/or MV-F. Sera from the experiment outlined in Fig. 4 were assessed for their ability to neutralize MV replication in Vero cells using the plaque reduction neutralization assay. PRN titers were measured using sera prepared on days 0 and 56. Responses are expressed as GM titers (mIU/ml) ± SD.

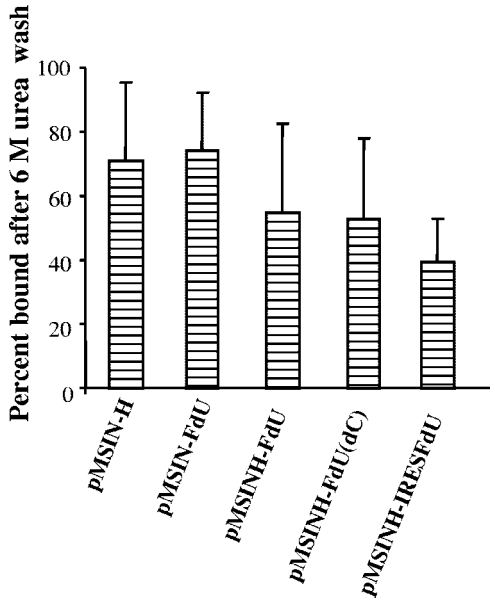

FIG. 6. Avidity of MV-specific serum IgG in mice immunized i.m. with DNA vaccines encoding MV-H and/or MV-F. Sera from day 56 of the experiment outlined in Fig. 4 were assessed for avidity. Bars show the percent binding after urea treatment ± SD from replicate measurements.

Avidity of measles virus antibodies has been used to estimate measles virus vaccine efficacy or vaccine failure in humans (14, 35, 37) and animal models (41). The presence of measles virus B-cell memory is reflected by the induction of high-avidity antibodies (i.e., with stronger antigen binding capacity). We established an ELISA with a urea washing step to measure avidity of vaccine-induced measles virus IgG in mouse sera based on techniques described in the literature. The test was validated in comparison with a PRN assay. Samples with neutralization titers above the protective level (200 mIU/ml) showed avidity indices equal to or greater than 30, in agreement with the parameters indicative of a memory (secondary) response in humans (14). Mice immunized with pMSINH-IRESFdU produced antibodies that had the lowest avidity to MV antigens (Fig. 6). Antibodies with significantly higher avidity were stimulated in mice immunized with pMSIN-H and pMSIN-FdU ($P < 0.01$ for both groups). MV-specific avidities elicited in mice immunized with the other bicistronic constructs, pMSINH-FdU and pMSINH-Fdu(dC), were about 20% lower than that of the groups vaccinated with pMSIN-H or pMSIN-FdU, although the differences were not statistically significant.

Frequencies of IFN-γ-secreting T cells after DNA immunization. To evaluate the frequency of MV-specific systemic effector T cells after DNA vaccine immunization, we measured the frequency of IFN-γ production by spleen cells by using ELISPOT. In comparison to the control group vaccinated with pSINCP, all other groups developed discernible measles virus-specific IFN-γ responses (Fig. 7). Mice immunized with pMSIN-FdU developed the lowest IFN-γ responses compared to mice

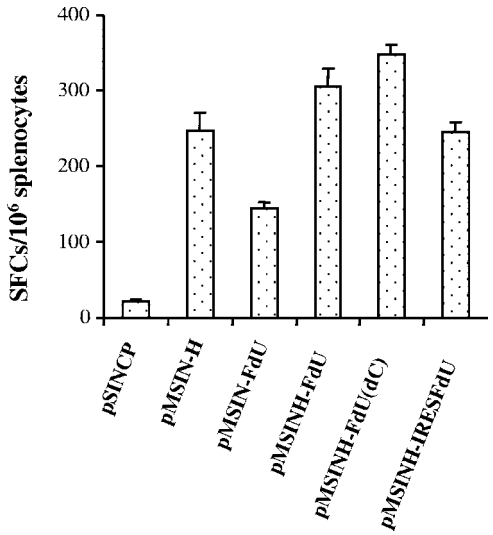

FIG. 7. Measles virus-specific IFN-γ responses. On day 70, mice were sacrificed, their spleens were extracted and homogenized, and splenocytes were isolated. Single-cell suspensions were pooled within each group and restimulated in vitro for 48 h with 5 μg/ml MV lysate in an IFN-γ-specific ELISPOT assay. Results are expressed as the mean IFN-γ spot-forming cells (SFC) ± SD from triplicate wells/10^6 cells. IFN-γ spots in wells containing medium (controls) were subtracted from the spots counted in wells containing MV for all experimental groups.

immunized with pMSIN-H or the bicistronic constructs ($P < 0.01$). Mice immunized with either pMSINH-FdU or pMSINH-FdU(dC) developed equivalent IFN-γ responses. However, only the responses of the latter group were significantly greater than those in mice immunized with pMSIN-H and pMSINH-IRES-FdU ($P < 0.01$). Lastly, the fusion construct pMSINH-FdU(fu) failed to induce any IFN-γ responses (data not shown).

DISCUSSION

To develop a new generation of measles vaccine specifically targeted for infants who are too young to receive the current licensed attenuated measles vaccines, we have proposed to prime young infants with two spaced doses of DNA vaccine encoding measles virus proteins so that they can be subsequently boosted successfully with attenuated measles virus vaccine. Immunization with DNA vaccines is an attractive strategy to immunize young infants despite the presence of maternal antibodies (29, 45–47). Looking to the future, our heterologous prime-boost strategy is to first immunize human infants with measles DNA vaccine during their initial contacts with the Expanded Program on Immunization in developing countries when they receive their first and second diphtheria-pertussis-tetanus (DPT), polio, hepatitis B, and *Haemophilus influenzae* type b immunizations (at 6 and 10 weeks of age in most developing countries). It is expected that infants so immunized would be primed to respond to a boost with attenuated measles vaccine that would be administered at 14 weeks of age (along with the third DPT and related vaccines); this is an age when young infants would not otherwise be expected to respond with neutralizing antibody without successful prior immunologic priming.

The two measles virus proteins that have by far garnered the most attention as DNA vaccine components are MV-H and MV-F (36, 43, 44, 46, 47, 53). While it is a general principle in vaccinology that administration of multiple antigens increases immunogenicity and protection over what can be achieved with each individual antigen (1, 9, 23, 49, 54), measles virus antigens may be special in that preliminary evidence suggests that immunization with both MV-H and MV-F does not result in additive immunogenicity, at least with respect to PRN titers (44). Thus, it is important to characterize the immune responses elicited by MV-H and MV-F proteins individually and in combination in order to rationally develop safe and effective MV DNA vaccines. Whereas DNA vaccines separately encoding MV-H and MV-F have previously been studied in animal models and were shown to elicit PRN antibodies and to confer protection (36, 44, 46, 47), heretofore, single DNA vaccine constructs that concomitantly deliver both MV-H and MV-F to the same eukaryotic cell have not been reported. As a consequence, there has not previously been a comparison of the relative cell biologic (syncytium formation) and immunogenic potential of such constructs versus individual MV-H and MV-F DNA vaccines.

Our first task was to design DNA vaccines that would allow MV-H and MV-F to be codelivered in different configurations, some of which would be predicted to be biologically active. We chose several distinct approaches to engineer plasmids that simultaneously encode both MV-H and MV-F. The first approach was to construct a bicistronic construct, pMSINH-FdU, that utilizes two copies of the Sindbis subgenomic promoter. We employed the IRES element to promote expression of MV-F in a second approach, resulting in construction of pMSINH-IRESFdU. In a third approach, we created a fusion between MV-H and MV-F, pMSINH-FdU(fu), thus bypassing the need for a second promoter.

Proteolytic cleavage of the MV-F protein precursor into its F1 and F2 subunits is required for cell fusion and subsequent inhibition of lymphoid cell proliferation (51, 52). Prior to conducting in vivo immunogenicity experiments, we assessed our constructs for their ability to cause Vero cell fusion in vitro. We attempted to inhibit cleavage by utilizing PCR to mutate the F cleavage site. However, our cleavage site mutant pMSINH-FdU(dC) was cleaved as efficiently as the wild-type protein produced by cells transfected with pMSIN-FdU and pMSINH-FdU, showing F0 and F1 at the same size (Fig. 2). Similar results have been reported with a mutation in MV-F at amino acid residue 112 (Arg to Leu) (3). However, the cleavage products of this Leu-112 MV-F mutant were different in size from those of the wild-type protein, suggesting that the Leu-112 MV-F precursor was cleaved at a new site. It has been reported that the cleavage of F is essential to induce syncytium formation (26). Notably, neither our fusion construct pMSINH-FdU(fu) nor pMSINH-FdU(dC) led to syncytium formation in Vero cells. Thus, our results show that the amino acid residues that reside in the cleavage site are important for the formation of syncytia.

In the mouse model, syncytium formation does not occur because murine cells lack receptors for MV (19, 32). Nevertheless, this model provided a practical and economical starting point to begin assessing the immunogenicity of our Sindbis-based constructs. Overall, mice immunized i.m. with pMSIN-H

developed the highest measles serum IgG titers by day 56. In comparison, mice immunized with pMSIN-FdU developed measles-specific GM titers that were ~3-fold lower. Mice immunized with the bicistronic plasmids encoding both MV-H and MV-F induced strong anti-measles antibody responses, but these were at least twofold lower than those in mice immunized with pMSIN-H alone. Hence, although not statistically significant, it appears that the presence of MV-F in the bicistronic plasmids slightly reduced MV-specific IgG levels. It is not known why mice immunized with the fusion construct pMSINH-FdU(fu) failed to develop MV-specific serum IgG and IFN-γ responses. Although we detected expression of the fusion protein in vitro, it is likely that MV-H and MV-F failed to fold correctly into their respective structures and/or were not secreted to the cell surface.

The impact of MV-F was most clearly observed in the elicitation of PRN antibodies, the strongest immunologic correlate of protection against measles (2, 8). Although they developed strong serum PRN antibody responses, the PRN titers of mice immunized with the bicistronic constructs were significantly lower than PRN titers in mice immunized with pMSIN-H (Fig. 5).

It has been reported that immunization of rhesus macaques with a DNA vaccine encoding MV-H engenders MV-specific antibodies with higher avidity than those immunized with formalin-inactivated MV (41). Moreover, these investigators showed that the neutralizing capacity of antibodies measured in marmoset B958 cells (which express CD150, the cell membrane receptor for many wild-type MVs) correlated with the avidity of the antibodies (16, 41). Accordingly, the mouse sera collected in our study were subjected to an avidity measurement. Antibodies of the lowest avidity were observed in mice immunized with pMSINH-IRESFdU. The avidities of antibodies elicited in mice immunized with the bicistronic constructs pMSINH-FdU and pMSINH-Fdu(dC) were about 20% lower than antibodies from mice immunized with pMSIN-H or pMSIN-FdU, although there was no statistically significant difference among these four groups. Individually, pMSIN-H and pMSIN-F stimulated strong MV-specific IgG responses following i.m. administration in mice. These antibodies were of high avidity, although those induced by the latter construct were poorly neutralizing (Fig. 5). Given these results, it was surprising to observe a decrease in MV-specific serum PRN titers and avidity in mice immunized with bicistronic constructs. The Western blots shown in Fig. 2 suggest that the level of expression of MV-H in cells transfected with the bicistronic constructs was less, lending some support for this explanation. In contrast, despite the decrease in humoral responses, the presence of MV-F in the bicistronic DNA vaccines did not diminish the development of MV-specific cell-mediated immunity. Mice immunized with the bicistronic constructs developed equivalent or greater IFN-γ responses than those immunized with pMSIN-H or pMSIN-F (Fig. 6).

The study reported herein is the first that addresses the immunogenicity of DNA vaccines concomitantly encoding both MV-H and MV-F. Presumably, transfection with these bicistronic constructs mimics natural MV infection wherein both MV-H and MV-F are expressed by the same cell. While it has been shown that coadministration of two separate DNA vaccines by i.m. immunization can result in coexpression of both antigens in the same myocytes and stimulation of immune responses to both antigens (12, 38), other reports indicate a clear superiority in immunogenicity of bicistronic constructs rather than two coadministered plasmids (7, 24). Under any circumstances, other considerations tip the balance towards the bicistronic strategy, since as a vaccine candidate is moved forward into clinical trials as a product, having to deal with production and control of two DNA vaccines rather than one increases the complexity and expense. Based on these considerations and the preliminary data presented herein, the constructs pMSINH-FdU and pMSIN-H were selected to be evaluated further for safety and immunogenicity in juvenile and young infant rhesus macaques, in preparation for undertaking future phase 1 clinical trials in humans. Should the trials with DNA vaccines encoding measles virus H and F yield encouraging results, it is possible that a third antigen, N, may be considered for inclusion to further enhance cell-mediated immune responses.

ACKNOWLEDGMENTS

We are grateful to Diane E. Griffin for providing a plasmid encoding MV-F protein and monoclonal antibodies and to Yu Lim for the technical support of the PRN assay.

This work was supported by a grant from the Bill and Melinda Gates Foundation (to M.M.L.).

REFERENCES

1. **Ad Hoc Group for the Study of Pertussis Vaccines.** 1988. Placebo-controlled trial of two acellular pertussis vaccines in Sweden—protective efficacy and adverse events. Lancet **i:**955–960.
2. **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.
3. **Alkhatib, G., J. Roder, C. Richardson, D. Briedis, R. Weinberg, D. Smith, J. Taylor, E. Paoletti, and S. H. Shen.** 1994. Characterization of a cleavage mutant of the measles virus fusion protein defective in syncytium formation. J. Virol. **68:**6770–6774.
4. **Bosu, W. K., S. Odoom, P. Deiter, and M. Essel-Ahun.** 2003. Epidemiology of measles in the central region of Ghana: a five-year case review in three district hospitals. East Afr. Med. J. **80:**312–317.
5. **Brodsky, A. L.** 1972. Atypical measles. Severe illness in recipients of killed measles virus vaccine upon exposure to natural infection. JAMA **222:**1415–1416.
6. **Centers for Disese Control and Prevention.** 2004. Measles mortality reduction—West Africa, 1996–2002. Morb. Mortal. Wkly. Rep. **53:**28–30.
7. **Chang, S. Y., K. C. Lee, S. Y. Ko, H. J. Ko, and C. Y. Kang.** 2004. Enhanced efficacy of DNA vaccination against Her-2/neu tumor antigen by genetic adjuvants. Int. J. Cancer **111:**86–95.
8. **Chen, R. T., L. E. Markowitz, P. Albrecht, J. A. Stewart, L. M. Mofenson, S. R. Preblud, and W. A. Orenstein.** 1990. Measles antibody: reevaluation of protective titers. J. Infect. Dis. **162:**1036–1042.
9. **Chen, Z., K. Matsuo, H. Asanuma, H. Takahashi, T. Iwasaki, Y. Suzuki, C. Aizawa, T. Kurata, and S. Tamura.** 1999. Enhanced protection against a lethal influenza virus challenge by immunization with both hemagglutinin- and neuraminidase-expressing DNAs. Vaccine **17:**653–659.
10. **Cliff, J., A. Simango, O. Augusto, P. L. Van Der, and R. Biellik.** 2003. Failure of targeted urban supplemental measles vaccination campaigns (1997–1999) to prevent measles epidemics in Mozambique (1998–2001). J. Infect. Dis. **187**(Suppl. 1)**:**S51–S57.
11. **Cutts, F. T.** 1993. The immunological basis for immunization series. World Health Organization, Geneva, Switzerland.
12. **Davis, H. L., C. L. Millan, and S. C. Watkins.** 1997. Immune-mediated destruction of transfected muscle fibers after direct gene transfer with antigen-expressing plasmid DNA. Gene Ther. **4:**181–188.
13. **De Quadros, C. A., J. M. Olive, B. S. Hersh, M. A. Strassburg, D. A. Henderson, D. Brandling-Bennett, and G. A. O. Alleyne.** 1996. Measles elimination in the Americas. Evolving strategies. JAMA **275:**224–229.
14. **de Souza, V. A., C. S. Pannuti, L. M. Sumita, and H. F. de Andrade Junior.** 1997. Enzyme-linked immunosorbent assay-IgG antibody avidity test for single sample serologic evaluation of measles vaccines. J. Med. Virol. **52:**275–279.
15. **El Mubarak, H. S., S. A. Ibrahim, H. W. Vos, M. M. Mukhtar, O. A. Mustafa, T. F. Wild, A. D. Osterhaus, and R. L. de Swart.** 2004. Measles virus

protein-specific IgM, IgA, and IgG subclass responses during the acute and convalescent phase of infection. J. Med. Virol. **72:**290–298.
16. **Erlenhofer, C., W. P. Duprex, B. K. Rima, V. ter Meulen, and J. Schneider-Schaulies.** 2002. Analysis of receptor (CD46, CD150) usage by measles virus. J. Gen. Virol. **83:**1431–1436.
17. **Forsey, T., A. B. Heath, and P. D. Minor.** 1991. The 1st international standard for anti-measles serum. Biologicals **19:**237–241.
18. **Fulginiti, V. A., J. J. Eller, A. W. Downie, and C. H. Kempe.** 1967. Altered reactivity to measles virus. Atypical measles in children previously immunized with inactivated measles virus vaccines. JAMA **202:**1075–1080.
19. **Hahm, B., N. Arbour, D. Naniche, D. Homann, M. Manchester, and M. B. Oldstone.** 2003. Measles virus infects and suppresses proliferation of T lymphocytes from transgenic mice bearing human signaling lymphocytic activation molecule. J. Virol. **77:**3505–3515.
20. **Kambarami, R. A., K. J. Nathoo, F. K. Nkrumah, and D. J. Pirie.** 1991. Measles epidemic in Harare, Zimbabwe, despite high measles immunization coverage rates. Bull. W. H. O. **69:**213–219.
21. **Kambire, C., M. K. Konde, A. Yameogo, S. R. Tiendrebeogo, R. T. Ouedraogo, M. W. Otten, Jr., K. L. Cairns, and P. L. Zuber.** 2003. Measles incidence before and after mass vaccination campaigns in Burkina Faso. J. Infect. Dis. **187**(Suppl. 1)**:**S80–S85.
22. **Kertesz, D. A., K. Toure, A. Berthe, Y. Konate, and F. Bougoudogo.** 2003. Evaluation of urban measles mass campaigns for children aged 9–59 months in Mali. J. Infect. Dis. **187**(Suppl. 1)**:**S69–S73.
23. **Kong, W. P., Y. Huang, Z. Y. Yang, B. K. Chakrabarti, Z. Moodie, and G. J. Nabel.** 2003. Immunogenicity of multiple gene and clade human immunodeficiency virus type 1 DNA vaccines. J. Virol. **77:**12764–12772.
24. **Lee, S. W., J. H. Cho, and Y. C. Sung.** 1998. Optimal induction of hepatitis C virus envelope-specific immunity by bicistronic plasmid DNA inoculation with the granulocyte-macrophage colony-stimulating factor gene. J. Virol. **72:**8430–8436.
25. **Loening, W. E., and H. M. Coovadia.** 1983. Age-specific occurrence rates of measles in urban, peri-urban, and rural environments: implications for time of vaccination. Lancet **ii:**324–326.
26. **Maisner, A., B. Mrkic, G. Herrler, M. Moll, M. A. Billeter, R. Cattaneo, and H. D. Klenk.** 2000. Recombinant measles virus requiring an exogenous protease for activation of infectivity. J. Gen. Virol. **81:**441–449.
27. **Markowitz, L. E., and P. Nieburg.** 1991. The burden of acute respiratory infection due to measles in developing countries and the potential impact of measles vaccine. Rev. Infect. Dis. **13**(Suppl. 6)**:**S555–S561.
28. **Martin, J. F., and J. Marshall.** 2003. New tendencies and strategies in international immunisation: GAVI and The Vaccine Fund. Vaccine **21:**587–592.
29. **Martinez, X., C. Brandt, F. Saddallah, C. Tougne, C. Barrios, F. Wild, G. Dougan, P. H. Lambert, and C. A. Siegrist.** 1997. DNA immunization circumvents deficient induction of T helper type 1 and cytotoxic T lymphocyte responses in neonates and during early life. Proc. Natl. Acad. Sci. USA **94:**8726–8731.
30. **Merz, D. C., A. Scheid, and P. W. Choppin.** 1980. Importance of antibodies to the fusion glycoprotein of paramyxoviruses in the prevention of spread of infection. J. Exp. Med. **151:**275–288.
31. **Murray, C. J., and A. D. Lopez.** 1997. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. Lancet **349:** 1436–1442.
32. **Naniche, D., G. Varior-Krishnan, F. Cervoni, T. F. Wild, B. Rossi, C. Rabourdin-Combe, and D. Gerlier.** 1993. Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. J. Virol. **67:**6025–6032.
33. **Norrby, E., G. Enders-Ruckle, and V. Meulen.** 1975. Differences in the appearance of antibodies to structural components of measles virus after immunization with inactivated and live virus. J. Infect. Dis. **132:**262–269.
34. **Otten, M. W., Jr., J. M. Okwo-Bele, R. Kezaala, R. Biellik, R. Eggers, and D. Nshimirimana.** 2003. Impact of alternative approaches to accelerated measles control: experience in the African region, 1996–2002. J. Infect. Dis. **187**(Suppl. 1)**:**S36–S43.
35. **Pannuti, C. S., R. J. Morello, J. C. Moraes, S. P. Curti, A. M. Afonso, M. C. Camargo, and V. A. Souza.** 2004. Identification of primary and secondary measles vaccine failures by measurement of immunoglobulin G avidity in measles cases during the 1997 Sao Paulo epidemic. Clin. Diagn. Lab. Immunol. **11:**119–122.
36. **Pasetti, M. F., E. M. Barry, G. Losonsky, M. Singh, S. M. Medina-Moreno, J. M. Polo, J. Ulmer, H. Robinson, M. B. Sztein, and M. M. Levine.** 2003. Attenuated *Salmonella enterica* serovar Typhi 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.
37. **Paunio, M., K. Hedman, I. Davidkin, M. Valle, O. P. Heinonen, P. Leinikki, A. Salmi, and H. Peltola.** 2000. Secondary measles vaccine failures identified by measurement of IgG avidity: high occurrence among teenagers vaccinated at a young age. Epidemiol. Infect. **124:**263–271.
38. **Payette, P. J., R. D. Weeratna, M. J. McCluskie, and H. L. Davis.** 2001. Immune-mediated destruction of transfected myocytes following DNA vaccination occurs via multiple mechanisms. Gene Ther. **8:**1395–1400.
39. **Peltola, H., O. P. Heinonen, M. Valle, M. Paunio, M. Virtanen, V. Karanko, and K. Cantell.** 1994. The elimination of indigenous measles, mumps, and rubella from Finland by a 12-year, two-dose vaccination program. N. Engl. J. Med. **331:**1397–1402.
40. **Polack, F. P., P. G. Auwaerter, S. H. Lee, H. C. Nousari, A. Valsamakis, K. M. Leiferman, 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.
41. **Polack, F. P., S. J. Hoffman, G. Crujeiras, 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.
42. **Polack, F. P., S. J. Hoffman, W. J. Moss, and D. E. Griffin.** 2002. Altered synthesis of interleukin-12 and type 1 and type 2 cytokines in rhesus macaques during measles and atypical measles. J. Infect. Dis. **185:**13–19.
43. **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.
44. **Polack, F. P., S. H. Lee, S. Permar, E. Manyara, H. G. Nousari, Y. Jeng, F. Mustafa, A. Valsamakis, R. J. Adams, H. L. Robinson, and D. E. 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.
45. **Premenko-Lanier, M., P. Rota, G. Rhodes, W. Bellini, and M. McChesney.** 2004. Prior DNA vaccination does not interfere with the live-attenuated measles vaccine. Vaccine **22:**762–765.
46. **Premenko-Lanier, M., P. A. Rota, G. Rhodes, D. Verhoeven, D. H. Barouch, N. W. Lerche, N. L. Letvin, W. J. Bellini, and M. B. McChesney.** 2003. DNA vaccination of infants in the presence of maternal antibody: a measles model in the primate. Virology **307:**67–75.
47. **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.
48. **Stein, C. E., M. Birmingham, M. Kurian, P. Duclos, and P. Strebel.** 2003. The global burden of measles in the year 2000—a model that uses country-specific indicators. J. Infect. Dis. **187**(Suppl. 1)**:**S8–S14.
49. **Svennerholm, A. M., and J. Holmgren.** 1976. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect. Immun. **13:**735–740.
50. **Taylor, W. R., R. K. Mambu, M. ma-Disu, and J. M. Weinman.** 1988. Measles control efforts in urban Africa complicated by high incidence of measles in the first year of life. Am. J. Epidemiol. **127:**788–794.
51. **Weidmann, A., C. Fischer, S. Ohgimoto, C. Ruth, V. ter Meulen, and S. Schneider-Schaulies.** 2000. Measles virus-induced immunosuppression in vitro is independent of complex glycosylation of viral glycoproteins and of hemifusion. J. Virol. **74:**7548–7553.
52. **Weidmann, A., A. Maisner, W. Garten, M. Seufert, V. ter Meulen, and S. Schneider-Schaulies.** 2000. Proteolytic cleavage of the fusion protein but not membrane fusion is required for measles virus-induced immunosuppression in vitro. J. Virol. **74:**1985–1993.
53. **Yang, K., F. Mustafa, A. Valsamakis, J. C. Santoro, D. E. Griffin, and H. L. Robinson.** 1997. Early studies on DNA-based immunizations for measles virus. Vaccine **15:**888–891.
54. **Ze, C., T. Kurata, and S. Tamura.** 2000. Identification of effective constituents of influenza vaccine by immunization with plasmid DNAs encoding viral proteins. Jpn. J. Infect. Dis. **53:**219–228.