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

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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 programatically 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

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DNA vaccines encoding MV-H and MV-F prime Th2 and Th1 cytokine production in macaques, respectively, following challenge with MV (43). Coinmunization with MV-H and MV-F induced higher gamma interferon (IFN-\(\gamma\)) 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 has 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.

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′ untranslated (UTR) and was constructed with primers 5′-AAATCTACTAGTTCTCCTACTTGAAGCTACACTC-3′-F2SpeI (5′-GGCGGCCGCATCAGAGCGACCTTACATAGGATTTG-3′) and cloning digested product into similarly digested pSINCP, creating pMSINH(fu). Replicon pMSIN-FdU encodes MV-F lacking the 5′ UTR and was constructed with primers 5′-XhoIF (5′-ATCCCTCGAGCTAGATC-3′) and 3′NotIF (5′-GGCTAGCCTCGAGAATTCACGCGGCCGCCA-3′) and NotIfu (5′-GGCGGCCG CATCAGAATCTCTCTAAGGGCAAGATCATCCACAATGTCACCA-3′) and 3′NotIF (above) and inserted the NotI-digested PCR product into similarly digested pSINCP, creating pMSINH(fu). We then fixed the sequence encoding the IRES and MV-FdU (IRESFdU) with primers 5′-IRESNol (5′-GGGTGAGCTCTTAGAGAATTCACGCGGCCGCCA-3′) and 3′NotIF (above) and inserted the NotI-digested PCR product into similarly digested pSINH-fu. 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′-XhoInol (5′-ATCCCTCGAGCTAGATC-3′) and 3′NotIF (above) and inserted the NotI-digested PCR product into similarly digested pSINCP, creating pMSINH(fu). We then PCR amplified MV-FdU lacking its signal sequence from pMSIN-FdU with primers 5′-NotIF (5′-ATCCCTCGAGCTAGATC-3′) and 3′NotIF (above) and inserted the NotI-digested PCR product into similarly digested pSINH-fu, creating pMSINH-FdU(fu).

Immunization of mice. Six- to eight-week-old female BALB/c mice (Charles River, Wilminton, 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. Approximtately 2 μg of plasmid DNA was used to transfet cells seeded at 5 × 10⁵ cells per well in a six-well tissue culture plate (MULTIWELL 6, Becton Dickinson, Vol. 79, 2005 BICISTRONIC MEASLES DNA VACCINE 9855
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 1:1,000 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 1:1,000 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 PBST. 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 H2PO4 and the optical density at 450 nm was measured in an ELISA microplate reader (Multiskan Ascent; Thermo Labystems). 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 were determined as described above, in the presence and absence of 6 M 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 (Biodies, Saco, Maine) in triplicate wells, starting at a concentration of 5 × 106 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/106 splenocytes ± 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 pMSINH-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 pMSIN-H-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 pMSIN-H-FdU(uc). 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(uc) 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. 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
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, pMSIN-H-FdU gave the strongest PRN responses (Fig. 5). Mice immunized with monocistronic pMSIN-FdU failed to reach the human seroprotective threshold (titer ≥ 200 mIU).

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 pMSINH-FdU developed the lowest IFN-γ responses compared to mice
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 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-IRES-FdU. 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 pMSINH-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-mebes antibodies 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 elevation 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.

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REFERENCES
