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A vaccine that would protect young infants against measles could facilitate elimination efforts and decrease morbidity and mortality in developing countries. However, immaturity of the immune system is an important obstacle to the development of such a vaccine. In this study, DNA vaccines expressing the measles virus (MeV) hemagglutinin (H) protein or H and fusion (F) proteins, previously shown to protect juvenile macaques, were used to immunize groups of 4 newborn rhesus macaques. Monkeys were inoculated intradermally with 200 μg of each DNA at birth and at 10 months of age. As controls, 2 newborn macaques were similarly vaccinated with DNA encoding the influenza virus H5, and 4 received one dose of the current live attenuated MeV vaccine (LAV) intramuscularly. All monkeys were monitored for development of MeV-specific neutralizing and binding IgG antibody and cytotoxic T lymphocyte (CTL) responses. These responses were poor compared to the responses induced by LAV. At 18 months of age, all monkeys were challenged intratracheally with a wild-type strain of MeV. Monkeys that received the DNA vaccine encoding H and F, but not H alone, were primed for an MeV-specific CD8+ CTL response but not for production of antibody. LAV-vaccinated monkeys were protected from rash and viremia, while DNA-vaccinated monkeys developed rashes, similar to control monkeys, but had 10-fold lower levels of viremia. We conclude that vaccination of infant macaques with DNA encoding MeV H and F provided only partial protection from MeV infection.

Measles remains an important cause of vaccine-preventable morbidity and mortality, with the highest mortality in young infants (1). Although measles cases and deaths decreased substantially through 2007, this decline has not continued, and measles was responsible for an estimated 139,000 deaths in 2010 (2–6). The currently available live attenuated vaccine (LAV) is safe and efficacious when administered to children older than 9 months of age, but seroconversion rates are low in younger infants, and this leads to a window of susceptibility before routine vaccination at 9 to 15 months of age (7–11). Development of an efficacious vaccine for young infants would decrease morbidity and mortality in this age group, facilitate elimination efforts, and increase delivery by coinciding with the earlier schedule for other vaccines in the World Health Organization’s Expanded Program on Immunization. Vaccine delivery at birth would be ideal (7).

The poor responses of young infants to LAV have been attributed primarily to interference of maternal antibodies with replication of vaccine virus (8), but even in the absence of maternal antibody, responses are less robust in younger than older infants (10, 12). Use of a high-titer vaccine to increase the dose of LAV elicited better antibody responses to measles virus (MeV) in young infants but resulted in an unexpected increase in mortality in female vaccine recipients (13, 14). Thus, development of new vaccines to protect young infants against MeV infection will require a different vaccine strategy.

The ideal measles vaccine should be inexpensive, safe, and heat stable. DNA vaccines have these characteristics and theoretically could elicit antibody in the presence of passively acquired maternal antibody and be delivered at birth. Macaques provide an excellent nonhuman primate model for study of measles pathogenesis and vaccine-induced protective immunity (15–20), and they have been used to test a number of different formulations of measles DNA vaccines (21, 22). In general, these studies have demonstrated that naked DNA vaccines encoding the MeV glycoproteins hemagglutinin (H) and/or fusion (F), with and without the nucleoprotein (N), prime humoral and cellular immune responses associated with complete or partial protection from rash and viremia after challenge in juvenile macaques (23–26). Attempts to improve responses and the level of protection have included codon optimization, changes in the DNA vectors and delivery, inclusion of adjuvants, and a variety of prime-boost strategies (26–29). In general, protection has been correlated with the levels of neutralizing antibody present at the time of challenge.

To begin to determine whether MeV DNA vaccines encoding the MeV glycoproteins are immunogenic in younger infants and can protect from measles, the immune responses of newborn ma-
cacaes administered DNA vaccines encoding H and F (ptermed H+F) were assessed and compared to LAV after vaccination and after challenge with wild-type MeV.

MATERIALS AND METHODS
Vaccines. Plasmids expressing H and F glycoproteins of MeV were prepared by cloning the cDNA for the H and F proteins of the Edmonston strain of MeV into an expression plasmid, as previously described (24), to produce pGA-H and pGA-F. The pGA expression plasmid was constructed for human use and is an updated version of the previously used research-grade vector pJW4303 (24). pJW4303 included a number of miscellaneous sequences, while pGA includes only the essential elements for replication in bacteria and for high levels of expression in eukaryotic cells. The transcriptional control elements in the two vectors are the same: cytomegalovirus immediate-early promoter, including intron A, and the bovine growth hormone polyadenylation sequence. A plasmid expressing the H5 glycoprotein of influenza virus was used as a control (30). The LAV Attenuvax, currently licensed in the United States, was obtained from Merck (West Point, PA) and reconstituted according to the manufacturer’s instructions.

Animals, immunization, and virus challenge. A total of 14 newborn rhesus macaques (Macaca mulatta) from the Yerkes primate facility born to MeV-seronegative mothers were immunized intradermally with 200 µg DNA of the MeV H plasmid alone (n = 4), 200 µg each of the MeV H and F plasmids (n = 4), 200 µg of the influenza virus H5 plasmid (n = 2), or intramuscularly with 0.5 ml LAV (n = 4). One LAV-immunized monkey died at 10 weeks of age. DNA-vaccinated monkeys were boosted at 10 months of age. Heparinized blood was taken at 2, 4, 6, 10, 12, and 18 months.

Approximately 18 months after initial vaccination, all monkeys were transferred to the Johns Hopkins University primate facility and challenged intratracheally with 106 median tissue culture infectious doses (TCID50) of the Bilthoven strain of MeV (a gift from A. Osterhaus, Erasmus University, Rotterdam, Netherlands). Macaques were shaved and examined every 2 to 3 days for a rash. White blood cell counts and differential counts were performed with each bleed. For all procedures, monkeys were chemically restrained with ketamine (10 mg/kg of body weight). All studies were performed in accordance with protocols approved by the Animal Care and Use Committees at Emory University and Johns Hopkins University.

Viremias and antibody assays. Peripheral blood mononuclear cells (PBMCs) were isolated on Ficoll-Hypaque (density, 1.077; Sigma) gradients and used fresh or after cryopreservation. Plasma was stored frozen until use. Viremia was assessed by cocultivation in triplicate of serial dilutions of PBMCs with B95-8 cells (31) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Cocultured cells were scored for syncytia at 96 h, and data are reported as number of syncytia/106 BMC.

MeV neutralizing antibody in plasma was measured by reduction of plaque formation in Vero cells as previously described (24, 32). Titers are reported as the highest dilution of plasma providing 50% plaque reduction. After vaccination, the lowest dilution of plasma tested was 1:16 and the highest dilution was 1:640. Before and after challenge, endpoint titers were calculated using the Karber method (33).

Enzyme immunoassays (EIAs) used 96-well Maxisorp plates (Nunc) coated with MeV-infected Vero cell lysate (1.16 µg protein/well; Advanced Biotechnologies). Serially diluted plasma samples were added, and plates were incubated overnight at 4°C. Alkaline phosphatase-conjugated rabbit antibody to monkey IgG (Biomakor; Accurate Chemicals) was used to detect bound IgG. Horseradish peroxidase-conjugated goat antibody to monkey IgM (Nordic) was used to detect bound IgM.

To measure avidity of MeV-specific antibodies, 50 µl of increasing concentrations of ammonium thiocyanate (NH4SCN; 0.25 to 3 M) was added to ELISA wells for 10 min after incubation with plasma that had been diluted 1:100. Plates were washed and the secondary antibody added as described above. The avidity index was calculated as the concentration of NH4SCN at which 50% of the bound antibody was eluted (12).

Cytotoxic T-lymphocyte assay. Herpesvirus papio-transformed autologous B-lymphoblastoid cell lines (B-LCLs) were established from individual monkeys. Cytotoxic activity of PBMCs was measured either directly ex vivo or after in vitro stimulation. For in vitro stimulation of MeV-specific effector cells, B-LCLs were infected with the Edmonston strain of MeV (multiplicity of infection, 5) for 24 h, treated for 10 min with 10 µg psoralen/ml (Sigma), irradiated for 5 min with long-wavelength UV light, and washed three times in 2% FBS in phosphate-buffered saline (PBS). Thawed PBMCs were incubated for 48 h with 2.5% T-STEM (Becton, Dickinson) and then cocultured at a ratio of 1:1 with infected, psoralen-treated autologous B-LCLs for 9 to 12 days in RPMI 1640, supplemented with 10% FBS, glutamine, penicillin, streptomycin, and 1 mM β-mercaptoethanol, in 24-well plates at a density of 3.0 × 105 to 3.5 × 106 per well. Cocultures were supplemented every 3 days with human recombinant interleukin-2 (IL-2; 20 U/ml; Sigma).

For the cytotoxicity assay, 106 autologous mock-infected or MeV-infected B-LCLs were labeled with 100 µCi 51Cr (Amer sham). 51Cr-labeled target cells (104) and unlabeled, uninfected autologous B-LCL cold target cells (1.5 × 105 to 2 × 105) were cultured in triplicate with PBMCs at ratios of effector to target cells (E:T) ranging from 1:1 to 90:1 in 24-well plates at a density of 3.0 × 105 to 3.5 × 106 per well. Cocultures were supplemented every 3 days with human recombinant interleukin-2 (IL-2; 20 U/ml; Sigma).

51Cr release was assessed after 4 to 5 h. Target cells cultured with media alone were used to determine spontaneous 51Cr release, and target cells in 1% Nonidet P-40 were used to determine maximum 51Cr release. Percent release was calculated by the equation [experimental 51Cr release − spontaneous 51Cr release]/(maximum 51Cr release − spontaneous 51Cr release) × 100. MeV-specific lysis was calculated as percent lysis of virus-infected target cells minus percent lysis of mock-infected target cells. For experiments to determine the nature of the cytotoxic cells, stimulated PBMCs were depleted using magnetic beads coated with antibody to CD4 or CD8 (Dynal).

Statistical analysis. Groups were compared at the indicated selected times using one-way analysis of variance (ANOVA) (for 3 or more groups) or Student’s unpaired t test (2 groups). Student’s paired t test was used to compare data from different times within a group. P < 0.05 was considered significant.

RESULTS
Response to immunization. Newborn rhesus macaques were immunized with MeV H DNA alone, MeV H+F DNA, influenza virus H5 DNA (control), or LAV at birth. Little neutralizing antibody was produced in response to the DNA vaccines, while there
was a robust antibody response to LAV (4 months; \( P = 0.0074 \)) (Fig. 1A). DNA-vaccinated monkeys were boosted at 10 months, and 2 of 4 macaques immunized with H+F responded with increased levels of neutralizing antibody (mean peak 50% plaque reduction on Vero cells, 240), but antibodies waned and returned to low levels by the time of challenge at 18 months. In contrast, the neutralizing antibody responses to a single dose of LAV were sustained.

Sufficient PBMCs were available to assess cytotoxic T lymphocyte (CTL) responses 4 and/or 6 months after immunization from 3 monkeys immunized with H+F DNA (1 or 2 time points), 1 monkey immunized with LAV (4 and 6 months), and 2 monkeys immunized with influenza H5 DNA (6 months) (Fig. 1B). Memory CTLs were easily detectable in the LAV-immunized monkey but were present at lower levels in MeV H+F DNA-immunized monkeys \( ( P = 0.0165) \).

**Response to challenge.** Intratracheal challenge with wild-type MeV was performed 18 months after the first dose of vaccine (8 months after the boost for DNA-vaccinated monkeys). LAV-vaccinated monkeys were completely protected from rash and viremia. All MeV DNA-vaccinated monkeys developed rashes that were similar in severity to those of control influenza virus DNA-vaccinated monkeys. These monkeys also developed viremias, but the peak levels were less than those in control monkeys (Fig. 2A) \( ( P = 0.06) \). An increase in IgM indicating response to infection was also observed in all monkeys except those vaccinated with LAV (day 11; \( P = 0.014 \)) (Fig. 2B). Total leukocyte counts varied (Fig. 2C), but all challenged animals had decreased lymphocyte counts 1 week after challenge, at the time of viremia in DNA-vaccinated animals (significance of comparisons of day 0 to 2 to day 7 were the following: H; \( P = 0.023 \); H+F, \( P = 0.017 \); LAV, \( P = 0.047 \)) (Fig. 2D).

Neutralizing and binding antibody responses after challenge showed no evidence of priming in the MeV DNA-vaccinated monkeys, as the timing, level, and avidity of responses were similar to those of the control animals (Fig. 3A to C). However, there was evidence of H+F DNA priming of the T cell response with CTL activity detected in H+F DNA-vaccinated as well as LAV-vaccinated monkeys earlier than in control or H DNA-vaccinated monkeys (Fig. 4A and B).

**CTls induced in response to MeV are primarily CD8\(^+\) T cells.** Previous studies have shown that MeV infection induces cytotoxic CD4\(^+\) as well as CD8\(^+\) T cells (34–36). To determine the contributions of CD8\(^+\) and CD4\(^+\) T cells to the CTL response to MeV in young macaques, CD4\(^+\) or CD8\(^+\) T cells were depleted from in vitro-stimulated PBMCs obtained from immunized and control monkeys approximately 2 months after MeV challenge (Fig. 5). Depletion of CD4\(^+\) T cells slightly increased CTL activity compared to that of bulk PBMCs, while CD8\(^+\) T cell depletion substantially decreased cytotoxicity.

**DISCUSSION.** This study has shown that newborn macaques do not develop protective antibody or CTL responses to MeV naked H or H+F DNA vaccines delivered intradermally at a dose of 200 \( \mu \)g. H+F DNA primed the CTL response but not the antibody response, while H DNA appeared to prime neither the antibody nor the CTL response. However, lower viremias after challenge in both groups of DNA-vaccinated macaques suggested partial protection. These studies emphasize the role of immune system immaturity in vaccine responses and also confirm previous observations that T cell responses are more readily induced in infants than antibody responses.

Age is a critical factor influencing the immune responses to many vaccines (37). Most juvenile macaques immunized with the
same MeV H or H+F DNA vaccines either intradermally (500 μg) or epidermally by gene gun (8 μg) developed CTL responses and durable neutralizing antibody, although not all animals were protected from rash and viremia after challenge (24). The amount of DNA used for intradermal immunization (200 μg) in the current study may have played a role in the lower response. Immunization of newborn macaques using similar DNA constructs encoding MeV H, F, and N (100 μg of each intradermally; termed H+F+N) also elicited weak neutralizing antibody responses and variably primed T cells for IFN-γ production (38, 39). The H+F+N DNA vaccine also primed the immune system of some monkeys for anamnestic production of antibody, while our H+F vaccine did not.

In that study, responses were sufficiently improved by administration of an IL-2/IgG plasmid to partially protect from viremia when the challenge with wild-type MeV was conducted 3 months after the vaccine boost (38, 39). In our study, challenge was conducted 8 months after the boost, and during this time there was a substantial decline in antibody titers. It is possible that protection would have been greater at earlier times after boosting before antibody declined. Lack of durability of the antibody response in young animals has been attributed to a lack of factors required to sustain long-lived plasma cells in the bone marrow (40).

Immune immaturity is poorly understood in both human infants and rhesus macaques. The gestation period in rhesus monkeys is 5 months, with hallmarks of fetal development that are similar to those of humans (41). The nature of the vaccine is important, as several vaccines are successfully delivered at birth (7). Immunization of 2-day-old macaques with vaccinia virus vectors expressing MeV H and F induced neutralizing antibody titers and CTL responses that protected from viremia and rash (42). In the current study, newborn macaques without maternal antibody responded well to LAV with both antibody and CTL responses. However, in a study of 6-month-old human infants without detectable passively acquired antibodies, neutralizing, but not EIA, antibody responses to LAV were deficient compared to the responses of 9- and 12-month-old infants (10, 12). Poor antibody responses in early life have been attributed to low levels of complement component C3, altered cytokine production, and immature architecture of secondary lymphoid tissue that limits germinal center formation (37, 40).

Despite poor antibody responses, LAV primed T cells in 6-month-old infants both in the presence and absence of maternal antibody, as determined by specific T cell proliferation and IFN-γ production after MeV stimulation (11). Better T cell responses than antibody responses were also induced by mumps virus vaccine in 6-month-old infants (43). The CD4+ T cell IFN-γ response to Bacille Calmette-Guerin (BCG) is similar to that of...
adults (44). Some newborn macaques vaccinated with recombinant BCG expressing MeV N developed a lymphoproliferative response to MeV antigens but did not develop antibody, and most had no evidence of a CTL response. However, there was partial protection from challenge with reduced lung pathology and a suggestion that viremia resolved more rapidly (45).

Most studies of the effect of age on responses to DNA vaccines have been performed in mice, and these studies have shown that DNA vaccines can induce IFN-γ-producing CD8+ T cells in neonatal mice similar to those of adults (46, 47). The reason for the different response to LAV between rhesus and human infants is not clear, but it highlights the fact that although nonhuman primates are often the best model system for preclinical testing of vaccines, differences in responses exist between humans and macaques.

As for DNA vaccine administration to young infants, the current study suggests that a potential benefit for T cell priming resides in immunizing with both H and F glycoproteins instead of using H alone. The role of CD8+ T cells in MeV immunity and pathogenesis of adult macaques has been explored by depletion with an antibody to CD8 (48). Seven- to 11-year-old rhesus macaques depleted of CD8+ T lymphocytes at the time of infection with WT MeV had a more extensive rash, higher viral loads at the peak of virus replication, and viremia of a longer duration than that of control animals (48). In addition, CTL responses appear to play a role in preventing atypical measles (32). Therefore, a better understanding of MeV-specific CTL responses is important. Our study confirmed the critical role of CD8+ T cells in MeV-specific cytotoxicity, with a limited contribution from CD4+ T cells, in young monkeys.

In summary, this study demonstrates that MeV DNA vaccines similar to those that are immunogenic and elicit protective responses in juvenile macaques do not elicit strong antibody and CTL responses in newborn monkeys, and it confirms previous observations that successful approaches to immunization for older vaccine recipients often fail to elicit the desired response in very young individuals (10). However, DNA vaccines encoding F and H primed T cell responses and resulted in lower levels of viremias than those in control monkeys. New-generation DNA vaccines, better adjuvants, or alternative strategies are needed to overcome the immaturity of the immune system and obtain a safe and immunogenic vaccine to protect young infants against measles. In the meantime, there is substantial evidence that effective delivery of 2 doses of LAV at older ages can protect young infants from infection by decreasing exposure to MeV in the community through improved herd immunity (4, 49).

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