Poor Immune Responses of Newborn Rhesus Macaques to Measles Virus DNA Vaccines Expressing the Hemagglutinin and Fusion Glycoproteins

Fernando P. Polack, Johns Hopkins University
Shari L. Lydy, Emory University
Sok-Hyong Lee, Johns Hopkins University
Paul Rota, Emory University
William J. Bellini, Centers for Disease Control and Prevention
Robert J. Adams, Johns Hopkins University
Harriet Robinson, Emory University
Diane E. Griffin, Johns Hopkins University

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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.
Macaques administered DNA vaccines encoding H and F (termed 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 plasmids expressing H and F glycoproteins of MeV were 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 of 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-lymphotblastoid 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 mg 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-STIM (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 × 10⁶ 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 × 10⁶ to 2 × 10⁶) were cultured in triplicate with PBMCs at ratios of effector to target cells (E:T) ranging from 1:1 to 90:1. ⁵¹Cr lysis was calculated as the percentage 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 detectable level of MeV neutralizing antibody in plasma was measured by reducing plaque formation in Vero cells as previously described (24, 32). Titters 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. Titters below 1:16 are reported as 1:8 and those above 1:640 as 1:1,280. Before and after challenge, endpoint titers were calculated using the Karber method (33).
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 were present at lower levels in MeV H+F DNA-immunized monkeys (\( P = 0.017 \)).

**Cytolytic activity.** Intracellular staining of PBMCs with B95-8 cells and reported as syncytia/10^6 PBMCs. The dashed line indicates the limit of detection. \( P = 0.0165 \) by one-way ANOVA. (B) MeV-specific IgM as measured by EIA and expressed as optical density (OD). \( P = 0.014 \) on day 11 by one-way ANOVA. (C) Total leukocytes/μl blood. (D) Total lymphocytes/μl blood. Significance in comparison of data from day 0 or 2 to day 7 by Student’s paired t test was the following: H, \( P = 0.023 \); H+F, \( P = 0.017 \); LAV, \( P = 0.047 \).

**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 μg. 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 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

FIG 4 MeV-specific cytotoxic T lymphocyte activity after challenge. Percent MeV-specific lysis of MeV-infected autologous B-LCLs by PBMCs after challenge measured directly ex vivo (A) and after in vitro stimulation (B) with psoralen-treated, MeV-infected autologous B-LCLs for 9 to 12 days. The effector/target ratio was 30:1. Results are means ± SEM.

FIG 5 Role of CD8+ T lymphocytes in MeV-specific cytotoxicity. Cytotoxic activity of PBMCs from monkeys immunized with H+F DNA, LAV, or H5 DNA (naive) and then challenged with wild-type MeV. Two months after challenge, PBMCs were stimulated in vitro with MeV-infected, psoralen-treated autologous B-LCLs. Cytotoxicity was measured before (bulk; black bars) and after depletion of CD8+ (open bars) or CD4+ (gray bars) T cells. The effector/target ratio was 30:1. Results are means ± SEM.
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 CD8+ sides in immunizing with both H and F glycoproteins. The study suggests that a potential benefit for T cell priming vaccines, differences in responses exist between humans and macaques. In the meantime, there is substantial evidence that effective vaccines can induce IFN-γ. H11001

REFERENCES


