Modulation of disease, T cell responses, and measles virus clearance in monkeys vaccinated with H-encoding alphavirus replicon particles

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This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected on April 20, 2004.

Contributed by Diane E. Griffin, June 2, 2005

Measles remains a major worldwide problem partly because of difficulties with vaccination of young infants. New vaccine strategies need to be safe and to provide sustained protective immunity. We have developed Sindbis virus replicon particles that express the measles virus (MV) hemagglutinin (SIN-H) or fusion (SIN-F) proteins. In mice, SIN-H induced high-titered, dose-dependent, MV-neutralizing antibody after a single vaccination. SIN-F, or SIN-H and SIN-F combined, induced somewhat lower responses. To assess protective efficacy, juvenile macaques were vaccinated with a single dose of 10^6 or 10^8 SIN-H particles and infant macaques with two doses of 10^8 particles. A dose of 10^8 particles induced sustained levels of high-titered, MV-neutralizing antibody and IFN-γ-producing memory T cells, and most monkeys were protected from rash when challenged with wild-type MV 18 months later. After challenge, there was a biphasic appearance of H- and F-specific IFN-γ-secreting CD4+ and CD8+ T cells in vaccinated monkeys, with peaks ~1 and ~3–4 months after challenge. Viremia was cleared within 14 days, but MV RNA was detectable for ~4–5 months. These studies suggest that complete clearance of MV after infection is a prolonged, phased, and complex process influenced by prior vaccination.

cellular immunity | interferon-γ | vaccine | protective immunity

Measles remains a major worldwide public health problem and is the most common cause of vaccine-preventable death (1). Most measles cases and deaths are in developing countries, but outbreaks continue to occur in developed countries as well (1, 2). The current live attenuated measles vaccine is safe and effective, but problems with delivery and inability to immunize young infants have hampered measles control efforts. This vaccine is given in developed countries between 12 and 15 months of age with seroconversion rates of ~95%. In developing countries, many cases of measles occur in infants under the age of 1 year (3), and the vaccine is given at 9 months of age with seroconversion rates of ~85% (4). In both situations, a second dose is necessary to establish sufficient herd immunity to interrupt endemic transmission (5). A measles vaccine given before the age of 6 months as a part of routine immunization programs could improve measles vaccine coverage and decrease deaths due to measles in many regions of the world.

Problems with previous measles vaccines make development of a new vaccine particularly challenging. A formalin-inactivated vaccine licensed in the 1960s induced short-lived immunity and predisposed vaccinated individuals to atypical measles, an enhanced form of measles characterized by a hemorrhagic rash, high fever, and pneumonitis (6, 7). Atypical measles is associated with anamnestic production of large amounts of nonprotective, low avidity, complement-fixing antibody leading to immune complex deposition in multiple sites (8, 9). A high-titer live attenuated vaccine also proved problematic when given to 4- to 6-month-old infants because of an unexplained increase in all-cause mortality in vaccinated girls (10–12).

Recent advances in vaccinology have offered many new tools for vaccine development (13), and monkeys provide a model system for developing and testing new measles vaccines (14, 15). Previous studies have shown that vaccination with DNA expressing either the hemagglutinin (H) or the fusion (F) protein of measles virus (MV) protected monkeys from measles and did not predispose to atypical measles (16). After DNA vaccination, cytotoxic T lymphocytes are induced, and production of high-avidity neutralizing antibody is sustained. However, amounts of antibody are variable, and the immune response is sometimes insufficient for protection from challenge with wild-type MV (16). Sustained immune responses and protection from measles have also been accomplished by vaccination with MV proteins incorporated into immune stimulating complexes (ISCOMs), with vaccinia virus expressing the H and F proteins, and with DNA encoding the H, F, and nucleoprotein (N) genes (17–20). However, none of these approaches have yet proven sufficiently practical for further development.

A promising new approach that has been applied to development of a number of experimental vaccines is the use of alphavirus vectors expressing heterologous proteins (13, 21, 22). Alphaviruses are small plus-strand RNA viruses that replicate entirely in the cytoplasm of infected cells. The nonstructural proteins are translated from the genomic RNA whereas the structural proteins are translated from the abundant subgenomic RNA. The subgenomic promoter can be engineered to express foreign genes either by duplicating it in the genome or by substituting the foreign gene for the structural proteins (22–24). In the latter replicon system, these RNAs can be used directly for vaccination (25–27) or packaged into particles by supplying the viral structural proteins, selected for delivery to specific target cells, in trans (28–30). The alphaviruses Semliki Forest virus, Venezuelan equine encephalitis virus, and Sindbis virus (SINV)

Abbreviations: EIA, enzyme immunoassay; F, fusion; H, hemagglutinin; i.d., intradermal; MV, measles virus; PBMC, peripheral blood mononuclear cell; SFC, spot-forming cells; SINV, Sindbis virus; SIN-H, SINV replicon to express MV protein H; SIN-F, SINV replicon to express MV protein F; ELISPOT, enzyme-linked immunospot; GMT, geometric mean titer; N, nucleoprotein.

See accompanying Profile on page 11578.

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have all been developed as potential vaccine and gene therapy vectors (13, 31).

To determine immunogenicity, safety, and protective capacity of a measles alphavirus replicon particle vaccine in nonhuman primates, we have developed a SINV replicon to express MV proteins H (SIN-H) and F (SIN-F). In juvenile and infant macaques, SIN-H induced sustained neutralizing antibody, IFN-γ-producing memory T cell responses, and protection against disease after a respiratory challenge with wild-type MV without atypical measles. The MV-specific T cell responses after challenge were biphasic, peaking at 1 and 3–4 months. Although infectious virus was cleared within 2 weeks, MV RNA persisted for 4–5 months, suggesting that the process of complete clearance of MV is prolonged, phased, and immunologically complex.

Materials and Methods

Animals. Female BALB/c mice, 4–6 weeks old, were purchased from Charles River Breeding Laboratories. Rhesus macaques (Macaca mulatta), 4–24 months old, were from the Johns Hopkins Primate Breeding Facility. Monkeys were chemically restrained with ketamine (10–15 mg/kg) during procedures. All animals were maintained within the guidelines, and studies were performed in accordance with experimental protocols approved by the Animal Care and Use Committee for Johns Hopkins University.

Vaccine. SINV-based particle vaccines were produced by subcloning the H (SIN-H) or F (SIN-F) gene from the Edmonston strain of MV into a SINV-derived replicon vector backbone (29) (28). This replicon vector contains the coding region for the SINV nonstructural genes and the subgenomic promoter for the structural genes. Particles for vaccination studies were produced by transfecting replicon RNA into a packaging cell line that expresses the SINV capsid and envelope glycoproteins containing the E2-160 mutation associated with human dendritic cell tropism (21, 28, 29). The packaging cells were infected with seed stocks of replicon particles at a multiplicity of 0.5 and plated in Cell Factories (Nunc). Particle-containing supernatant fluids were harvested ~20 and 32 h after infection, clarified by 0.2-μm filtration, precipitated with polyethylene glycol, resuspended in PBS plus lactose (40 mg/ml) buffer, and repelleted by high-speed centrifugation. The final pellet material was resuspended in PBS plus lactose buffer, filtered, and frozen (~80°C) in aliquots. Replicon particles were titrated by infection of a cell line that expresses a β-galactosidase reporter that responds to the SINV replicon-encoded nonstructural proteins. Test material was compared with a standard curve generated by using SINV replicon particles expressing green fluorescent protein. Preparations of SIN-F replicon particles were ~10-fold lower in titer than SIN-H particles. H and F protein expression was confirmed by Western blot after infection of BHK cells with SIN-H and SIN-F. All materials used for vaccination were confirmed to have endotoxin levels <0.5 endotoxin unit/ml.

Vaccination of Mice. Groups of three mice were used for each vaccine, dose, and route. Single doses of 10^6, 10^7, or 10^8 SIN-H or SIN-F particles were given by the intradermal (i.d.) or i.m. route. Mice were bled at 2 and 4 weeks after vaccination, and sera were collected and stored at ~20°C until use.

Vaccination and Challenge of Monkeys. Four juvenile (all 1 to 2 yr old) and two infant [4 months old (subject 23P) and 5.5 months old (subject 23P)] rhesus macaques were vaccinated. The juvenile monkeys received a single i.d. injection with either 10^6 (8N and 13N) or 10^7 (25N and 29N) SIN-H particles. Monkey 25N died from shigellosis 9 months after vaccination. The infant macaques were vaccinated with 10^6 particles i.d. and were boosted with the same dose 19 weeks later. Two additional juvenile monkeys (13P and 29P) served as unvaccinated controls. Blood was collected at various times, cells were separated, and plasma was stored at ~20°C. For MV challenge, 10^6 50% tissue culture infectious doses of the Bilthoven strain of MV (a gift from A. Osterhaus, Erasmas University, Rotterdam) were instilled intratracheally into anesthetized macaques.

Virus Assays. Viremia was assessed by cocultivation in triplicate of serial dilutions of peripheral blood mononuclear cells (PBMCs) with B95-8 cells in MEM supplemented with 10% FBS, penicillin, and streptomycin. Wells were scored at 72 h for syncytia. Data are reported as number of syncytia per 10^5 PBMC.

Amounts of viral RNA were assessed by isolating total RNA (RNaseasy mini kit, Qiagen, Valencia, CA) from 2 × 10^6 PBMC that had been cultured for 3 days without stimulation. The N gene was amplified by using a TaqMan one-step RT-PCR kit with MV-specific primers (5′-GGGTACCATCTTACG- CCAAATT-3′ and 5′-CGATTCGCTGCGTGCTC-3′) and measured with an N-specific probe (FAM-CTCGCAAGG- CGGTACGACCC-TAMRA) in an Applied Biosystems Prism 7700. Controls included GAPDH amplification (Applied Biosystems) and RNA isolated from cultured PBMCs from naive monkeys. Copy number was determined by construction of a standard curve from 1–10^6 copies diluted from known amounts of N RNA synthesized by in vitro transcription from a plasmid encoding the Edmonston N gene. The sensitivity of the assay was 10 copies. Data were normalized to the GAPDH control and expressed as [(copies of MV N RNA per 10^6 PBMC)/(copies of GAPDH RNA per 10^6 PBMC)] × 100,000.

Antibody Assays. MV-specific neutralizing antibody was measured by reduction of plaque formation of the Chicago-1 strain of MV on Vero cells (14). Data were normalized to a standard serum run with each assay. SINV-specific neutralizing antibody was measured by reduction of plaque formation by the AR339 strain of SINV on BHK cells. Titors are expressed as the reciprocal of the dilution that reduced plaques by 50% (PRNT50).

MV-specific enzyme immunoassays (EIAs) used 96-well Maxisorp plates (Nunc) coated with Edmonston MV-infected Vero cell lysate (Advanced Biotechnologies, Columbia, MD; 1.16 μg of protein per well) and then incubated with serially diluted plasma. For detection of IgG, an alkaline phosphatase-conjugated rabbit antibody against monkey IgG (Biomakor; Accurate Chemicals) was used. For detection of IgM, a horse-radish peroxidase-conjugated goat antibody against monkey IgM (Nordic) (Lausanne, Switzerland) was used. To measure the avidity of MV-specific antibodies, 50 μl of variable concentrations of NaSCN (0.25–3 M) in PBS were added to EIA wells for 10 min after incubation with serially diluted plasma. The plates were washed, and the secondary antibody was added as above. The avidity index is equal to the concentration of NaSCN at which 50% of the bound antibody is eluted (32).

Lymphoproliferation and Enzyme-Linked Immunospot (ELISPOT) Assays. PBMCs were isolated from heparinized blood by gradient centrifugation on Ficoll-Paque (density 1.077; Amersham Pharma- cia), washed, and suspended in RPMI medium 1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin, and streptomycin. For some assays, CD4+ T cells were depleted with anti-human CD4 magnetic beads and then separated by midMACS (Miltenyi Biotec, Auburn, CA). After depletion, the percentage of CD4+ cells was <5%. MV-specific T cell responses were assessed by incorporation of [3H]thymidine [1 μCi per well (1 Ci = 37 GBq)] after stimulation of PBMCs with pooled H or F peptides (20mers overlapping by 11 aa, 10 μg/ml) for 72 h and by ELISPOT assays of cells producing IFN-γ and IL-4 in response to MV antigens. For ELISPOTs, multiscreen
plates (Millipore) were coated with anti-human IFN-γ antibody (2 μg/ml, Pharmingen) or anti-human IL-4 antibody (5 μg/ml, Pharmingen). Plates were washed and blocked with culture medium. PBMCs were added at 1 or 5 × 10^5 cells per well with medium alone, 10 μg/ml of pooled MV H or F peptides, or 5 μg/ml Con A (Sigma). After 40 h at 37°C, plates were washed and incubated for 2 h at room temperature with 1 μg/ml biotinylated anti-IFN-γ antibody (MABTECH, Stockholm) or 2 μg/ml biotinylated anti-IL-4 antibody (Pharmingen). After washing, avidin-conjugated horseradish peroxidase (Amersham Pharmacia) was added for 1 h. Assays were developed with 50 μl of stable dianisobenzidine solution (Invitrogen). The reaction was stopped with tap water, plates were allowed to dry, and wells were scanned in an ImmunoSpot reader and analyzed by using IMMUNOSPOT 2.05 software (Cellular Technology, Cleveland). Data are presented as spot-forming cells (SFC) per 10^6 PBMC minus the medium control (typically 0–2 SFC before and 0–30 after challenge per 10^6 PBMC).

**Statistical Analysis.** Student’s t test on log-transformed data was used for comparison of responses between groups of mice by using STATVIEW software (SAS Institute, Cary, NC).

**Results**

**Antibody Responses in Vaccinated Mice.** To determine the immunogenicity of the vaccine, mice were vaccinated with three different doses of SIN-H or SIN-F i.d. or i.m. (Fig. 1). Neutralizing antibodies were induced by both vaccines, but titers were higher in SIN-H than SIN-F vaccinated mice and, except at the lowest dose, the response to SIN-H plus SIN-F was lower than to SIN-H alone. In general, there were no differences in the responses to the i.d. or i.m. routes of delivery. For SIN-H, the antibody response was better to 10^6 particles [i.d. geometric mean titers (GMT) = 8191] than to 10^5 particles (i.d. GMT = 119; P = 0.0011), and increasing the dose to 10^6 particles led to a further increase in antibody (i.d. GMT = 16,243; P = 0.0044). The neutralizing antibody response to SIN-F was lower than to SIN-H in all but the lowest dose group. The best responses to SIN-H plus SIN-F were in the groups receiving the highest dose (i.d. GMT = 3184; i.m. GMT = 4820), but, at the lowest dose, the response to the combined vaccine was improved over SIN-H alone (i.d. GMT = 1064, P = 0.05).

**Immunogenicity of SIN-H in Juvenile Rhesus Macaques.** Two juvenile rhesus macaques (8N and 13N) were vaccinated with 10^6 SIN-H particles i.d. and two (25N and 29N) with 10^5 SIN-H particles i.d. Both monkeys given 10^5 SIN-H particles generated titers of neutralizing antibody above the generally accepted protective level of 120 (33, 34) within 2–4 weeks after vaccination (Fig. 2A). These responses were sustained for more than a year. One of the monkeys given 10^6 particles (13N) developed a modest neutralizing antibody response that was sustained, whereas the second monkey (8N) developed only a transient response. Avidity matured to good levels over 2–3 months in juvenile macaques receiving 10^6 particles, but avidity was lower and maturation was slower, or did not occur, in those receiving 10^5 particles (Fig. 2B).

Cellular immune responses were assessed by lymphoproliferation and by production of IFN-γ and IL-4. Proliferative responses to stimulation with pooled H peptides were detected in PBMC 7–21 days after vaccination, indicating initial stimulation of T cells (Fig. 2C). To assess memory T cell responses, PBMCs were stimulated with pooled H peptides, and IFN-γ and IL-4 ELISPOT assays were performed. Cells producing IFN-γ (1–18 SFC/10^5 PBMC), but not IL-4, were detected in PBMCs from all vaccinated monkeys 270–310 days after vaccination.

**Immunogenicity of SIN-H in Infant Macaques.** Infant macaques were immunized i.d. with 10^8 SIN-H particles and boosted 19 weeks later. Neutralizing antibody responses developed more slowly than in juvenile macaques (Fig. 2A), but after 4 months reached levels predicted to be protective. There was a steady increase in antibody avidity over 9 months after initial vaccination (Fig. 2B). There was little evidence that the second dose improved the titers of neutralizing antibody, but it may have improved the avidity of the antibody. H-specific IFN-γ-producing memory T cells (1–66 SFC per 10^6 PBMC) were detected in both monkeys 105–483 days after vaccination.

**Development of Antibody to SINV.** To determine responses to the vector, neutralizing antibody to SINV was measured (Fig. 2D) and was detected 2 weeks after vaccination and then steadily declined. Titers were highest in juvenile macaques given 10^6 particles. This antibody response was consistent with previous reports of anti-alphavirus vector antibody after replicon particle vaccination (35–37) and with the short-term stimulation provided by antigens present only in the injected particles. SINV antibody was not boosted by repeat vaccination in the infant macaques.

**Protection from Challenge with Wild-Type MV.** All monkeys, except for 25N (who died of unrelated causes), were challenged with wild-type MV 18 months after vaccination (Fig. 3). Viremia and clinical symptoms were monitored. Two unvaccinated naive monkeys (13P and 29P) and one of the monkeys vaccinated as an infant (37P) developed a rash. Viremia was detected in all monkeys, but the mean peak viremia was 10-fold lower for
monkeys vaccinated with $10^8$ particles [102.6 TCID50 tissue culture infectious doses (TCID50) per 10^6 PBMCs] than for unvaccinated monkeys (103.5 TCID50 per 10^6 PBMCs) (Fig. 3A).

Antibody Response to Challenge. The antibody response to challenge was assessed by neutralization and EIA. Only the monkey with the lowest antibody response to vaccination (SN) and the two naive monkeys (13P and 29P) developed significant levels of MV-specific IgM, indicative of a primary immune response, after challenge (Fig. 3A). Neutralizing antibody titers increased substantially in all monkeys (Fig. 4A). For 13N and 29N, the surviving juvenile monkeys with the best responses to vaccination, increases in neutralizing antibody were detected within 8 days after challenge. IgG responses to MV antigens not included in the vaccine, in addition to H that was in the vaccine, were measured by EIA and showed similar increases after challenge in all groups (Fig. 4B).

Avidity of antibody after challenge will reflect the combined avidity of the antibodies to H primed by vaccination plus the avidity of the antibodies being formed to other MV antigens encountered for the first time (Fig. 4C). Avidity matured more rapidly after challenge in all vaccinated monkeys, except SN, compared with avidity maturation in unvaccinated naive monkeys.

T Cell Response to Challenge. To monitor the cell-mediated immune responses to viral challenge, IFN-γ production by PBMC in response to pools of MV-H (Fig. 5A) or MV-F (Fig. 5B) peptides was measured by ELISPOT assay. H-specific IFN-γ-producing cells were detected in circulation 7 days after challenge in the high-dose vaccinated monkey (29N). These cells were not detected during the viremia, but reappeared soon after infectious virus was no longer detectable in PBMCs (Fig. 3A) and reached a peak (120 SFC per 10^6 PBMC) on day 25. For the two low-dose vaccinated juvenile monkeys, H-specific IFN-γ-producing cells were undetectable until the viremia was cleared and then quickly increased. The numbers of IFN-γ SFCs peaked for 8N (n = 30) and 13N (n = 160) on day 25 after challenge and decreased over the next 3 weeks. Beginning ~90 days after challenge, the H-specific IFN-γ responses rose again to a second peak at 110–125 days and were still detectable at 175 days after challenge.

For monkeys vaccinated as infants, 23P showed an H-specific IFN-γ response before and early after challenge, but these cells were not detected in PBMC after the onset of viremia until day 97 after challenge, similar to the timing of the second peak in the juvenile monkeys (Fig. 5A). The numbers of IFN-γ SFCs for 23P and 37P peaked at 220 and 180 SFCs per 10^6 PBMCs on day 97 after challenge. Only one (13P) of the two naive monkeys demonstrated a detectable H-specific IFN-γ response, and that was at 25 days after challenge.

Numbers of primary F-specific IFN-γ SFCs were also higher in the high-dose vaccinated monkey 29N than in low-dose
the peaks were CD4+ mainly CD8+ assayed (Fig. 5).

This period. Monkeys, Con A-induced SFC decreased early after challenge

biphasic responses were detectable in low-dose vaccinated monkeys. A maximum response at d110, and 23P showed F-specific IFN-γ-producing memory T cells after a single dose of 10^8 particles in juvenile macaques and after two doses in infant macaques. These responses protected most monkeys against rash after challenge with wild-type virus 18 months after vaccination, but only partially protected against infection. After challenge, infectious virus was cleared rapidly and viral RNA was reduced, but complete clearance of viral RNA was accomplished only after several months. During that time, levels of antibody rose rapidly and remained high whereas the kinetics of the CD4+ and CD8+ T cell IFN-γ responses to both the H and F proteins were biphasic.

Alphavirus replicon particle vaccines have been successfully used to induce immune responses in small animals to a variety of exogenous viral proteins and usually induce antibody and T cell responses that protect from virus challenge (22, 28, 38–43). For instance, SINV replicon particle vaccines protect mice from mucosal challenge with vacinia virus expressing HIV gag (38) and from herpes simplex virus infection (28). However, success in small animals does not always predict success in primates (44).

Encouragingly, Semliki Forest virus and Venezuelan equine encephalitis virus replicon particle vaccines provide complete or partial protection in monkeys against disease due to simian immunodeficiency virus, chimeric simian-HIV, and Marburg virus (31, 37, 42, 45). However, these studies have used four- to six-dose regimens spread over 6–12 months or combined prime-boost strategies (35, 36) to induce protective immunity, and challenges have been performed within 1–12 weeks after completion of the vaccination schedule. For a new measles vaccine to provide a substantial advantage over the current approach, induction of protective immunity with one or two doses is desirable, and protection needs to be long-lasting. This study has shown that a single dose of 10^8 particles, similar to the doses used in multidose alphavirus replicon particle vaccine studies, induced a substantial and sustained immune response to the MV H protein that provided protection from disease for at least 18 months.

After vaccination, levels of neutralizing antibody rose continuously over 3–12 weeks in juvenile monkeys and over 25 weeks in infant monkeys. Avidity maturation was also a protracted process, particularly in young animals. The reason for the slow development of antibody is not clear, but suggests activation of a program of progressive differentiation after a single exposure to antigen (46). Immaturity of the immune system plays an important role in the antibody response to measles vaccines in humans (47), but it is not known whether this limitation of response is related to problems with immune recognition of specific MV antigens or to other factors. As with DNA vaccines and the live attenuated virus vaccine, SIN-H induced a sustained antibody response consistent with the development of long-lived plasma cells secreting MV-specific antibody. In contrast, the

generated, both subsets of T cells participated in both phases of the biphasic response to H and F proteins after MV challenge.

Persistence of MV RNA. To determine whether the biphasic T cell response was potentially driven by persistence of MV, a quantitative RT-PCR assay was established to detect N gene sequences in PBMCs (Table 1). Low levels of MV RNA were detected at 50 and 125 days after challenge in all monkeys and at 150 days in one vaccinated and one unvaccinated monkey. Levels of MV RNA during viremia were not available for these monkeys, but were ~2 x 10^5 copies per 10^6 PBMCs in comparable animals at day 9, indicating substantial control of virus replication with appearance of the immune response. MV RNA was no longer detectable by 165 days after challenge.

Discussion

These studies have shown that SINV replicon particles encoding the MV H protein can elicit sustained levels of MV-neutralizing antibody and IFN-γ-producing memory T cells after a single dose of 10^8 particles in juvenile macaques and after two doses in infant macaques. These responses protected most monkeys against rash after challenge with wild-type virus 18 months after vaccination, but only partially protected against infection. After challenge, infectious virus was cleared rapidly and viral RNA was reduced, but complete clearance of viral RNA was accomplished only after several months. During that time, levels of antibody rose rapidly and remained high whereas the kinetics of the CD4+ and CD8+ T cell IFN-γ responses to both the H and F proteins were biphasic.

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antibody induced by the formalin-inactivated measles vaccine is of low avidity and is transient, suggesting development of only short-lived plasma cells that secrete MV antibody (6, 9, 48). It is likely that this difference is related to the adequacy of T cell help for B cells, which may not be efficiently induced by the inactivated vaccine.

After challenge, most monkeys were protected from rash, and, in the one monkey that developed a rash, it was mild. Therefore, the SIN-H vaccine did not predispose to atypical measles even when protection was incomplete. This level of protection is similar to that reported for most other experimental MV vaccines in monkeys and probably represents the combined effects of neutralizing antibody and cellular responses (16, 17, 20). After challenge with MV, there was rapid control and clearance of infectious virus with lower viremia and no induction of IgM in most vaccinated animals. Greater protection from viremia is provided by the live attenuated vaccine (16) and by vaccines in monkeys and probably represents the combined effects of neutralizing antibody and cellular responses (16, 17, 20). After challenge with MV, there was rapid control and clearance of infectious virus with lower viremia and no induction of IgM in most vaccinated animals. Greater protection from viremia is provided by the live attenuated vaccine (16) and by vaccines in monkeys and probably represents the combined effects of neutralizing antibody and cellular responses (16, 17, 20). After challenge with MV, there was rapid control and clearance of infectious virus with lower viremia and no induction of IgM in most vaccinated animals. Greater protection from viremia is provided by the live attenuated vaccine (16) and by vaccines in monkeys and probably represents the combined effects of neutralizing antibody and cellular responses (16, 17, 20).

**Table 1. Quantitative RT-PCR for MV RNA in PBMCs from nonvaccinated and SIN-H-vaccinated monkeys after challenge:**

<table>
<thead>
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<th>Day</th>
<th>13N*</th>
<th>29N</th>
<th>23P</th>
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*The labels 13N, 29N, etc. are subject identifiers.*

neutralizing antibody is the best correlate of protective immunity (16, 34) and passive antibody can protect from disease (49), in these studies, H-specific neutralizing antibody was not sufficient to protect from infection. It is not clear whether this lack of protection was due to the quality (e.g., avidity), quantity (e.g., titer), or specificity (e.g., epitopes recognized) of the antibody induced or to inadequate CD4+ or CD8+ T cell responses.

H-specific T cell immunity was induced by vaccination, but numbers of memory T cells producing IFN-γ were low. CD4+ T cell help is important for establishing effective CD8+ memory T cell protection (50–53), and the size of the memory T cell pool depends on the strength of the antigen stimulation (54–56). Initial levels of antigen produced by the single round of SIN-H particle infection may have been limiting and may not have fully engaged the available H-specific CD4+ and CD8+ T cells. Alternatively, humoral and cellular responses to other viral proteins may be important for complete protection. For instance, neutralization may be enhanced with antibody to F (57), and F is also more potent than H in priming for a type 1 IFN-γ response (58). CD4+ and CD8+ T cells directed to different viral epitopes can have different functions in protection (59), and individual proteins may be more or less likely to contain epitopes efficiently presented by major histocompatibility complex class I and class II to CD8+ and CD4+ T cells in these outbred animals.

Once infection was initiated, the process of control and then elimination of the virus was immunologically complex. Clearance of RNA viruses that cause systemic infection is a multistep process. Neutralizing antibody contributes to the elimination of infectious virus, particularly cell-free virus, but elimination of virus-infected cells requires virus-induced or immune-mediated cytotoxicity. Infected cells that have not been eliminated through cytotoxic mechanisms need to be cleared of replicating virus and viral RNA through nontoxic processes. In immunologically normal individuals, MV rarely causes disease due to persistent infection (60). Primary target cells for MV are monocytes and endothelial and epithelial cells, all of which have a finite lifespan (61). When persistent infection occurs, as in subacute sclerosing
panencephalitis, there is infection of the nervous system (62), a site known to harbor viral RNA for long periods of time (63, 64). We were surprised to detect MV RNA for 4–5 months after challenge in the PBMCs of both previously vaccinated and unvaccinated animals. MV RNA has been detected by routine RT-PCR for 4–10 weeks after infection, but not later, in previous studies of monkeys (14) and children with measles (65). Our assay that used quantitative RT-PCR on PBMCs cultured for 3 days may have increased the sensitivity of detection by allowing some amplification of the virus present and detection at times beyond that previously reported.

The critical factors for clearance of MV are not known. Individuals with compromised cellular immune responses clear virus slowly (65) and are at risk for progressive infection leading to giant cell pneumonia or measles inclusion body encephalitis (66, 67). CD8+ T cells are induced by infection (68, 69) and infiltrate sites of MV replication (8, 70, 71). Clearance of infectious virus is delayed in monkeys depleted of CD3+ CD8+ T cells, and, during the early phase of control, large numbers of LCMV-specific CD8+ T cells producing IL-2, IFN-γ, and TNF-α are produced in the spleen. Subsequently, CD8+ T cells progressively lose cytokine production and then eventually regain the ability to produce IFN-γ and TNF-α coincident with the final phase of virus clearance. CD4+ T cells do not show a similar biphasic pattern (78). A more compressed biphase CD8 T cell response has also been observed after challenge of previously immunized mice with influenza virus (79).

The biphase response observed after MV challenge in SIN-H vaccinated animals included CD4+ and CD8+ T cells specific for both H, for which the animals were primed, and for H, encountered first at the time of challenge. All of the MV-specific T cells detected produced IFN-γ, but the cells appearing ~1 month after infection may have different properties than those appearing 3–4 months after infection. Low levels of IFN-γ-producing T cells between 1 and 3 months after infection may represent deceased capacity of MV-specific cells to produce IFN-γ in response to antigen stimulation without a change in the numbers of MV-specific T cells or the expected contraction of the T cells (55) followed by the emergence of a new population of late-responding cells induced by continued presence of MV RNA. Memory CD4+ and CD8+ T cells can be functionally and anatomically subdivided into effector memory and central memory cells (80, 81). On challenge, these cells are induced to proliferate at different rates, differ in cytokine profiles and protective capacity, and may be sequentially mobilized from nonlymphoid and lymphoid tissues (82, 83). Improving ability to identify these populations should allow clarification of these issues in future experiments. In summary, the alphavirus replicon particle vaccine shows promise for future MV vaccine development and has provided insights into immune mechanisms of protection from, and clearance of, MV.

The technical assistance of Jun Yang and helpful discussions of many members of the measles virus laboratory at The Johns Hopkins University is appreciated. This work was supported by research grants from the Bill and Melinda Gates Foundation and the National Institutes of Health (R01 AI35149) and training grants from the National Institutes of Health (T32 AI07417 and AI07541).