Measles Virus Infection in Rhesus Macaques: Altered Immune Responses and Comparison of the Virulence of Six Different Virus Strains

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Measles remains a major cause of childhood mortality, with questions about virus virulence and pathogenesis still requiring answers. Rhesus macaques were infected with 5 different culture-adapted strains of measles virus, including 2 from patients with progressive vaccine-induced disease, and a sixth nonculture-adapted strain, Bilthoven. All caused infection detectable by reverse transcriptase-polymerase chain reaction and induction of antibody. Chicago-1 and Bilthoven induced viremias detectable by leukocyte cocultivation. Bilthoven induced Koplik’s spots, conjunctivitis, and rash. Lymphopenia and depressed interleukin (IL)-2 production were followed by monocytesis and eosinophilia. All monkeys, including 41 involved in a primate facility outbreak, showed suppressed responses to phytohemagglutinin. As the rash resolved production of IL-2, IL-1α, tumor necrosis factor-α, IL-6, and IL-5 mRNA increased. Monkeys are useful for studies of measles immunopathogenesis, but virus strains must be carefully chosen. Increased virulence of vaccine strains isolated from immunocompromised infants with fatal infections was not evident.

Measles is an important human disease that causes the death of ~1,000,000 children each year. Most of these deaths are due to secondary infection [1]. This increase in susceptibility to other pathogens is associated with a well-documented measles-induced immunosuppression [2]. This suppression of immune responses is incompletely understood and is probably multifactorial: it is likely that different mechanisms are of primary importance in early and late phases of infection. Human studies of necessity focus on the time of the appearance of the rash and thereafter, because that is when measles is recognized clinically. Studies in primates offer the opportunity to look at all phases of infection. Many of the deaths associated with secondary infection could be prevented by more widespread application of measles immunization. The vaccine against measles is a live attenuated virus with an impressive record of efficacy and safety, although suppression of immune responses is often detectable after immunization [3]. The virus strain (Edmonston) from which most measles vaccines have been derived was isolated by Enders and Peebles in 1954 [4]. This virus was passaged multiple times in primary human kidney and amnion cells and then adapted to eggs and multiply passaged in chick embryo cells to produce the original Edmonston B vaccine, licensed in 1963 [5]. Edmonston B virus caused fever and rash in 30%–60% of infants receiving it [6] and was therefore often given with immune globulin [7]. It was eventually replaced by further attenuated strains [8]. Although live attenuated measles vaccine is not recommended for use in severely immunocompromised individuals, infants with congenital immunodeficiencies are not always recognized before measles immunization is given. This has resulted in a few cases of progressive fatal vaccine-induced disease [9, 10]. The determinants of measles virus (MV) virulence are not known, and it would therefore be of interest to know whether viruses isolated from these children are of increased virulence compared with the originally inoculated vaccine virus.

Studies of measles pathogenesis, the virulence of different strains of MV, and the mechanisms of immune suppression have been hampered by the lack of a small animal model for measles. Nonhuman primates are susceptible to MV infection but may or may not develop clinical manifestations typical of measles in humans [11–17]. Rhesus macaques are most likely to develop clinical measles, and this species potentially provides a means...
of studying virus virulence and the host response to infection [13, 18]. Although many outbreaks of measles in primate colonies have been described, laboratory strains of MV often do not induce disease in primates [12, 14, 18, 19]. In this article we report the clinical features of infection and the immune response of rhesus macaques to measles during a primate facility outbreak [20] and after experimental infection by the respiratory route with 6 different strains of MV: 2 wild-type strains, 1 that had been adapted for growth in Vero cells (Chicago-1) and 1 that had not (Bilthoven), and 4 different Edmonston-derived strains: wild-type Edmonston; Moraten, a vaccine strain; and 2 isolates from immunodeficient children with progressive vaccine-induced disease. We identified lymphopenia followed by eosinophilia, suppression of mitogen-induced lymphoproliferation, and induction of a variety of monocyte and T cell–derived cytokines as a part of the host response to the most virulent of the viruses.

Materials and Methods

Subjects. For experimental infections, 20 male and female 1–2 year-old rhesus macaques (Macaca mulatta; 2.0–2.7 kg) from the Caribbean Primate Research Facility in Puerto Rico, Sabana Seca Field Station, University of Puerto Rico, and 4 male 2-year-old rhesus macaques (2.2–3.0 kg) from the Johns Hopkins University primate facility were studied. Groups of 4 monkeys were inoculated intranasally and intratracheally with 10⁴ pfu (Vero cell–adapted strains) or intratracheally with 10⁴ TCID₅₀ (Bilthoven) of MV. Two monkeys were mock-infected with tissue culture fluid to control for changes associated with repeated chemical restraint and phlebotomy. Monkeys were monitored daily for signs of illness. Monkeys, pretreated with ketamine (10–15 mg/kg), were bled before infection and at frequent intervals after infection. In addition, selected studies were performed on specimens from 41 rhesus macaques that developed measles (confirmed by presence of MV-specific IgM) during a 1996 outbreak at primate facilities in Poolesville and Bethesda, Maryland [20]. Blood was heparinized, and white blood cell (WBC) and differential counts were performed either by a commercial laboratory (MetPath, Rockville, MD) or by the Comparative Medicine Laboratory at Johns Hopkins University School of Medicine. Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Hypaque gradients (density, 1.077; Sigma, St. Louis). Plasma was frozen at −20°C. PBMC were washed, and a portion used to assess spontaneous and mitogen-induced proliferation. The rest was suspended in fetal bovine serum (FBS) containing 7.5% dimethylsulfoxide, frozen in a stepwise fashion and stored in liquid nitrogen for subsequent virus titration.

Viruses. Six strains of MV were used for experimental infection. Bilthoven was isolated in 1991 during an outbreak of measles in Rhesus Macaques.
in Bilthoven, The Netherlands, and is in genotype group C2 [12].

Chicago-1 was isolated in 1989 during an outbreak of measles in
Chicago and is in genotype group D3 [21–23]. Edmonston–wild
type is a derivative of the original Edmonston strain of MV isolated
by Enders and Peebles [4]. The earliest passage available had been
passaged at least 7x in human kidney cells. Edmonston and its
derivatives are in genotype group A. Moraten is a chick embryo
fibroblast-attenuated vaccine strain of MV licensed for use in the
United States. Mvi/Los Angeles USA/89 (LA-89) and Mvi/St.
Louis USA/91 (STL-91) were isolated on HEP-2 cells 3 months
after measles immunization from the lungs of 18-month-old infants
with severe combined immunodeficiency [9]. Both LA-89 and STL-
91 were characterized by sequencing the H, N, and M genes and
found to be identical to Moraten. The virus that caused the primate
facility outbreak is in genotype group D4 [20]. All viruses used for
experimental infection, except Bilthoven, had been or were adapted
to growth in Vero cells (2–8 passages).

**Virus assays.** Stocks of tissue culture–adapted viruses were
grown and assayed by plaque formation in Vero cells. Bilthoven
was grown in human cord blood cells and assayed by syncytium
formation in B95-8 cells, a marmoset lymphoblastoid cell line [19].
Cell lysates for virus stocks were prepared when the cytopathic
effect was maximal and stored as frozen aliquots at −70°C.

Viremia was assessed by cocultivation of PBMC with B95a or
B95-8 cells [19]. For Chicago-1 and the Edmonston strains,
5 × 10^4 PBMC suspended in Dulbecco’s modified Eagle’s medium
supplemented with 10% FBS, penicillin, and streptomycin were
added to each of 2 wells of a 24-well tissue culture plate containing
confluent cells. After 48 h the medium was removed, and the cells
were allowed to dry, and then were fixed with cold 80% acetone
in PBS. Measles foci were identified by staining with a polyclonal
rabbit antisera to MV and quantitated by counting the total
number of foci of syncytia, as described elsewhere [24]. For Bil-
thoven, serial dilutions of PBMC were cocultivated with B95-8 cells
in triplicate, and wells were scored at 72 h for MV-positive syncytia.
Data are reported as number of syncytia/100,000 PBMC.

For detection of viral RNA by reverse transcriptase–polymerase
chain reaction (RT–PCR), total cellular RNA was extracted from
5 × 10^4 washed PBMC by use of the guanidinium acid-phenol
method [25]. MV RNA was detected by RT–PCR by use of H-
and N-specific primers for cDNA synthesis and for amplification
of MV sequences, as described elsewhere [26]. Amplification of β-
actin mRNA served as a control for the RNA extractions.

**Sequence analysis.** Virus was isolated in B95a cells from PBMC
obtained from each animal 4, 7, or 9 days after infection or in Vero
cells for initial characterization of STL-91 and LA-89 [19]. RNA
was extracted. Regions of the H and N genes were amplified, and
the PCR products were sequenced as described elsewhere [27].

**Antibody assays.** Plaque reduction neutralization (PRNT) as-
says were performed by standard procedures described elsewhere
[28] by use of the Chicago-1 strain of MV, Vero cell monolayers,
and serial half-log dilutions of plasma. Three monkeys from Puerto
Rico had levels of MV neutralizing antibody >120 PRNT/mL
prior to infection, and data from these immune monkeys were not
included in the analyses. All other monkeys were seronegative prior
to challenge.

**Lymphoproliferation.** PBMC were diluted to 10^6 cells/mL in
RPMI supplemented with 10% FBS and gentamicin. Cells were
cultured in round-bottomed, 96-well plates (200 µL/well) with or
without phytohemagglutinin (PHA-P, 2.5 µg/mL; Sigma). To assess
spontaneous proliferation, [3H]thymidine ([H]-Tdr, 1 µCi/well) was
added to unstimulated cells for the first 24 h of culture. To assess
PHA-induced lymphoproliferation, [H]-Tdr was added at 48 h, and
the cells were harvested and counted 18 h later. Data are expressed
as counts per minute (cpm) incorporated into PHA-stimulated cells
minus cpm of control cells (Δ cpm). Parallel cultures were
established to assess cytotoxic production. For these assays, cells
and supernatant fluids were harvested at 48 h.

**Cytokine assays.** Interleukin (IL)-1α, IL-6, tumor necrosis factor
(TNF)-α (PharMingen, San Diego, CA), and IL-2 (Amersham,
Arlington Heights, IL) were measured in supernatant fluids of
PHA-stimulated PBMC by enzyme immunoassay according to the
manufacturers’ instructions. IL-5 mRNA was measured by semi-
quantiative RT-PCR essentially as described elsewhere [29]. RNA
was extracted (RNA Stat 60; Teltest, Friendswood, TX) from
PBMC that had been cultured for 48 h with or without PHA. RNA
was treated with DNase and cDNA synthesized by use of avian

![Figure 3. Plaque reduction neutralization (PRNT) antibody responses to measles virus (MV) after infection of monkeys by respiratory route with 10^4 pfu of 6 different strains of MV. Each point is geometric mean of individual values from 3–4 monkeys. Bilt, Bilthoven; Ch-1, Chicago-1; Ed-wt, Edmonston–wild type; Mor, Moraten; LA, Mvi/Los Angeles USA/89; STL, Mvi/St. Louis USA/91.](https://academic.oup.com/jid/article-abstract/180/4/950/848049/180485049.png)
myeloblastosis virus reverse transcriptase. IL-5–specific cDNA was amplified by use of primers designed to cross an intron (5′-AGCTGCTACGTGATGCCATC and 3′-GCTAGGAATTGGTTTACTCTCCC). Amplification of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA served as a control for the quantity and quality of the RNA. Products were detected by Southern analysis and relative amounts of IL-5 mRNA compared with GAPDH mRNA, as described elsewhere [29].

Results

Clinical disease. Monkeys infected with the Bilthoven strain of MV developed Koplik’s spots, conjunctivitis, and a maculopapular rash beginning 8–9 days after infection (figure 1). The rash persisted for ~5 days. None of the monkeys infected with Chicago-1, Edmonston–wild type, Moraten, STL-91, or LA-89 became clinically ill or was noted to develop signs or symptoms characteristic of measles.

Viremia. Appearance of virus in the blood after respiratory tract inoculation was assessed both by cocultivation of PBMC with marmoset lymphoblastoid cells (figure 2) and by RT-PCR (table 1). Monkeys inoculated with the Bilthoven and Chicago-1 strains developed a viremia that was consistently detectable by cocultivation. Virus was isolated from PBMC between 4 and 9 days after infection, and titers peaked on day 7. Virus was intermittently identified at low levels in PBMC of the monkeys inoculated with Edmonston viruses (Edmonston–wild type, LA-89, STL-91, and Moraten) during the same period. By RT-PCR, infected PBMC were detected in all the infected monkeys between 4 and 21 days after infection. By day 29, neither virus nor viral RNA was detectable in any of the monkeys, indicating
complete clearance of infected cells from the blood. To be certain that there was no cross-contamination, one reisolate from each animal was sequenced; in each case, the identity of the infecting strain was confirmed.

Antibody responses. Neutralizing antibody appeared between days 7 and 14 in all groups of monkeys (figure 3). The neutralizing antibody response was highest after infection with the Chicago-1 strain, presumably, as has been observed elsewhere, because this genotype D3 strain was used in the assay for neutralizing antibody [30, 31]. Otherwise, responses were similar among groups.

Changes in circulating leukocytes. Monitoring total numbers of WBCs and differential counts showed that animals were leukopenic on days 7 and 9 after Bilthoven and Chicago-1 infection, coincident with the peak of the viremia (figure 4). This leukopenia was independent of initial WBCs and was primarily due to a decrease in the numbers of circulating lymphocytes, which were lowest on days 7 and 9 and gradually returned toward and above baseline over the next 2 weeks (figure 4B). Mock-infected monkeys developed no significant leukocyte changes. The changes in peripheral lymphocyte numbers were less marked in monkeys infected with the Edmonston-derived viruses, and there were no significant differences among the virus strains in the quality or quantity of the changes in peripheral WBC counts during infection, so these data were grouped to simplify the graphs. The numbers of circulating monocytes and eosinophils increased in Bilthoven and Chicago-1–infected monkeys at the time of the rash, 9 days after infection (figure 4C and 4D). The absolute eosinophil count increased 3–4-fold and had not yet returned to baseline in these animals 21 days after infection.

Proliferation of PBMC. All groups of monkeys showed spontaneous proliferation of PBMC as evidence of immune activation, beginning 4 days after infection and continuing through day 21 (figure 5A). All groups of monkeys also showed suppressed proliferation of PBMC in response to PHA that persisted through day 45 (figure 5B). Suppression was most profound in Bilthoven-infected monkeys on day 7, the time of peak viremia (figure 2).

To gain further insight into how long the proliferative responses to PHA remained suppressed, monkeys naturally infected with MV during a primate facility outbreak were studied at various times after the appearance of the rash (figure 5C). Compared with control uninfected monkeys, the responses of these monkeys remained suppressed for at least 5 weeks after the rash.

Cytokines. To begin to characterize the cytokine responses during measles in macaques, we measured IL-1β, IL-2, IL-6, and TNF-α produced after PHA stimulation of PBMC from 2 monkeys infected with Bilthoven (figure 6). Production of the type 1 T cell cytokine IL-2 was suppressed during the viremia (days 5–9, figure 2), while production of the macrophage-derived proinflammatory cytokines IL-1β, IL-6, and TNF-α were less affected during this period. There was an increased production of all measured cytokines around the time the rash was resolving (days 12–14).

IL-5 is specifically associated with development and terminal differentiation of eosinophils, but reagents are not available for measuring rhesus IL-5. Therefore, IL-5 mRNA expression in PBMC from Bilthoven-infected monkeys was assessed semiquantitatively by RT-PCR (figure 7A). Increased levels were present at day 14 after infection, just prior to the major increase in eosinophil numbers on day 16 (figure 4D). Because PBMC from only 2 experimentally infected monkeys were available, levels of IL-5 mRNA in PBMC from the measles outbreak monkeys were also assessed (figure 7B). IL-5 mRNA was increased in PHA-stimulated PBMC from monkeys studied during the first week after appearance of the rash (estimated to be 10–20 days after infection) and then decreased to baseline 3–5 weeks after the rash.
Figure 6. Changes in cytokine production after infection with measles virus. Supernatant fluids from phytohemagglutinin-stimulated peripheral blood mononuclear cells from monkeys infected with Bilthoven were assessed for levels of interleukin (IL)–1β (A), IL-2 (B), IL-6 (C), and tumor necrosis factor (TNF)–α (D). Each point is average of values from 2 monkeys.

Discussion

Our studies confirm that rhesus macaques are susceptible to MV infection and are useful models for the study of some aspects of the pathogenesis of measles. For instance, lymphocytopenia has been reported in humans with measles, but the induction of monocytosis and eosinophilia has not been noted, because analysis of WBCs is usually confined to the time of clinical illness. Likewise, increased plasma levels of IL-4 and elevation of serum levels of IgE suggestive of predominance of a type 2 cytokine response occur in human measles [32, 33], but IL-5 increases have not been reported. Experimentally infected monkeys allow frequent analysis of leukocyte counts and plasma cytokines from before infection through complete recovery from disease. Such careful monitoring is not easily accomplished in humans with natural infection. RT-PCR amplification of MV RNA in PBMC has not previously been used to follow the viremia in macaques. Use of this adjunctive technique showed in our studies that all virus strains studied induced viremia, although the amount of infectious virus produced was frequently insufficient for detection by cocultivation and that clearance of virus from the blood was not complete until >3 weeks after infection.

Monkeys were a major tool for MV research prior to the isolation of the virus in tissue culture [34–37] and during the development of measles vaccines [38, 39]. These primate studies contributed significantly to our understanding of sites of virus replication, spread of virus in the host, and pathologic changes in response to infection [40]. Vaccine strains of virus were shown to produce less viremia and to protect against subsequent challenge [14]. Rapid advances have been made since that time in our understanding of both the molecular biology of MV and the development of immune responses to viruses. New measles pathogenesis questions have emerged, such as the molecular determinants of virulence and the mechanism(s) of immune suppression, but only a few investigators have returned to the study of MV infection in primates [12, 14, 18].

Measles is generally recognized to be a milder disease in monkeys than in humans. However, naturally acquired infec-
tion with wild-type strains of MV usually causes a recognizable rash and clinical evidence of measles, at least in macaques [13, 17]. The Bilthoven strain, which has not been adapted to Vero cells, produced classic measles and provides a system for further study of measles pathogenesis. Interestingly, this strain does not produce clinical disease in cynomolgus macaques, suggesting that rhesus macaques are generally more susceptible to measles [12]. The absence of recognizable clinical signs in the monkeys inoculated with the recently isolated Chicago-1 strain suggests that adaptation to Vero cells has probably led to attenuation of the virulence of this virus. Clinical disease was experimentally produced in macaques by using a strain of MV (Davis 87) closely related to the Chicago-1 strain used in our studies, which was isolated during an outbreak of measles at a primate center [18], again suggesting that changes that occur during adaptation in vitro significantly alter virulence. Rapid attenuation by adaptation to cell culture is further suggested by studies in cynomolgus macaques that have shown that a wild-type strain of MV isolated and grown in marmoset lymphoblastoid cells produces rash, weight loss, and leukopenia, but when the same strain is adapted to, and grown in, Vero cells it does not produce these clinical signs and symptoms [19, 41]. Sequence analysis of these strains suggests that these differences are determined by nucleotide changes in either the L (polymerase) or P (phosphoprotein, C and V proteins) genes [42]. Similarly, studies in cynomolgus macaques, which do not develop illness after MV infection, indicated that the Bilthoven strain (non–Vero cell–adapted) infected a greater number of PBMC than the Vero cell–adapted Edmonston–wild type or the Schwarz vaccine strains [12].

Although Bilthoven, Chicago-1, and Edmonston–wild type produced differing levels of viremia, we were unable to detect any differences between Edmonston–wild type, the Moraten vaccine strain, and 2 viruses that were isolated from progressive vaccine-induced disease. STL-89 and LA-91 were isolated from infants with severe congenital immunodeficiencies 3 months after immunization with Moraten. The H, N, and M genes of STL-89 and LA-91 are identical to Moraten, but it is not known whether STL-89 and LA-91 acquired mutations in other regions of the genome during their prolonged replication in humans, which could enhance virulence. Whereas STL-91, LA-89, and Moraten could clearly infect rhesus macaques, there were no detectable differences in viremia between the vaccine strain or the vaccine reisolates. These results suggest that either there are no differences in virulence between the vaccine virus and these vaccine reisolates or that rhesus monkeys do not provide a sensitive system for detecting small differences in virulence between related strains. Unfortunately, only Vero cell–passaged viruses were available for this comparison.

Marmosets are much more susceptible to MV infection than macaques but have not been studied extensively because they develop a disease that is not typical of measles in humans [15, 43]. Analysis of MV growth and pathologic effects in human thymic implants in SCID mice has provided a sensitive system for detecting differences in virulence [44]. However, this system does not provide a means of studying host responses. For such investigations, it is necessary to study human or nonhuman primates. Therefore, a variety of approaches is likely to be necessary to significantly advance our knowledge of measles pathogenesis.

Figure 7. Expression of interleukin (IL)-5 mRNA after measles virus infection. Relative levels of IL-5 mRNA in unstimulated and phytohemagglutinin (PHA)–stimulated peripheral blood mononuclear cells were assessed at various times after infection for monkeys (A) experimentally infected with Bilthoven (n = 2) and (B) naturally infected during a measles outbreak in a primate facility (2–4 monkeys per group). Relative amounts of mRNA were calculated by dividing the laser-scanned density of the IL-5 signal by glyceraldehyde phosphate dehydrogenase signal for each specimen. NA, not available.
Interestingly, few studies have carefully examined or reported changes in peripheral WBCs during MV infection. Leukopenia has been noted in experimentally infected monkeys 7–12 days after infection [35, 39], and lymphocytes were the primary cells decreased in cynomolgus macaques infected with wild-type virus [19, 41]. Leukopenia and lymphopenia are a recognized consequence of human measles at the time of the rash, and our studies suggest that lymphopenia is most closely associated with viremia and may be related to MV-induced lymphocyte death.

No previous studies of humans or monkeys have observed eosinophilia. Detection of this change requires serial differential WBC counts on the same individuals, preferably from the time of infection. Eosinophils increased in the blood 9 days after infection, as the immune response and the rash appeared and virus clearance was initiated. This suggests that eosinophilia is a consequence of the immune response and is consistent with the observations of a type 2 skewing of T cell cytokine responses with recovery from infection. Peripheral eosinophilia is generally associated with increases in type 2 cytokines, particularly IL-5 [45–48], and has also been reported to occur in the immunosuppressive phases of human immunodeficiency virus infection [49]. In addition, respiratory viral infections often induce pulmonary eosinophilia, which may contribute to exacerbations of asthma [50–52]. Increases in IgE and type 2 cytokines occur during the recovery phase of natural measles of measles is likely to improve our understanding of the immunopathogenesis of measles.

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References