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Note Added in Proof

The astrovirus strain from this outbreak reacted in a serotyping EIA (Noel J, personal communication) with rabbit antisera raised to serotype 6 provided by J. B. Kurtz (Lee TW, Kurtz JB. Prevalence of human astrovirus serotypes in the Oxford region 1976-1992, with evidence of two new serotypes. *Epidemiol Infect* 1994;112:187-93).

Viral Replication and Development of Specific Immunity in Macaques after Infection with Different Measles Virus Strains

Robert S. van Binnendijk, Roger W. J. van der Heijden, Geert van Amerongen, Fons G. C. M. UytdeHaag, and Albert D. M. E. Osterhaus

Department of Virology, Erasmus University, Rotterdam; Central Animal Laboratory, National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands

Cynomolgus monkeys (*Macaca fascicularis*) were experimentally infected with a wild type measles virus (MV) strain (MV-BIL). Following intratracheal inoculation with different infectious doses, the virus could be isolated from peripheral blood mononuclear cells (PBMC), lung lavage cells, and pharyngeal cells. The kinetics of the cell-associated viremia was similar in all infected animals. They developed specific serum IgM, IgG, and neutralizing antibody responses as well as MV-specific T cell-mediated immunity. Monkeys infected intratracheally or intramuscularly with the wild type MV-Edmonston or the attenuated MV-Schwartz strain showed a lower level of PBMC-associated viremia and less pronounced specific IgM responses. Nine months after infection with MV strains, all of the monkeys were protected from intratracheal reinfection with MV-BIL. This monkey model is suitable for study of new generations of vaccines and vaccination strategies for measles.

In humans, measles is believed to confer lifelong immune protection against reexposure to measles virus (MV). This protection appears largely to be based on persistence of MV neutralizing antibodies and the presence of MV-specific cytotoxic T lymphocytes (CTL) [1, 2]. Vaccination against measles with live attenuated measles vaccines likewise aims at the induction of lifelong immune protection based on the same immune defense mechanism. However, even in industrialized countries where measles has largely disappeared as a consequence of vaccination with live attenuated measles vaccines, outbreaks of measles continue to occur [3-5]. Of more importance, due to low vaccination coverage and the interference of transplacentally acquired maternal antibodies with replication of vaccine virus, measles remains a major cause of serious disease and mortality in developing countries, annually killing ~1.5 million children [6, 7]. Thus, MV continues to circulate in developing countries as well as

in countries with high vaccination coverage rates. As a consequence, the World Health Organization has reevaluated strategies for control and eradication of measles [7].

The present study was aimed toward development of a suitable animal model that would provide more insight into the pathogenesis of measles, functional aspects of humoral and cellular immune responses, and possible molecular correlates of virus attenuation. Apart from humans, several non-human primate species can be experimentally infected with MV or can contract measles when exposed to infected humans, thereby spreading the virus from monkey to monkey [8]. Among these, marmosets (*Saguinus mystax*) are the most susceptible to infection with wild type MV. However, clinical signs and gross pathologic findings that accompany the usually fatal disease in these animals, lack the characteristic features of measles in humans [9]. In a number of other simian species, such as cynomolgus and rhesus monkeys (*Macaca fascicularis* and *Macaca mulatta*, respectively), natural and experimental infection with wild type MV causes disease of varying severity but with a pathogenesis similar to that of measles in humans [8]. These species may be suitable candidates in which to study the pathogenesis of wild type and attenuated MV infections and the mechanisms of immune-mediated protection they induce. In this study, we evaluated cynomolgus monkeys for possible use as an animal model for study of MV.

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Reprints or correspondence: Prof. A. D. M. E. Osterhaus, Dept. of Virology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, Netherlands.

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Materials and Methods

Wild type MV-BIL isolate. During an outbreak of measles at a high school in Bilthoven, Netherlands, MV was isolated from peripheral blood mononuclear cells (PBMC) and nasopharyngeal washings from patients. MV-BIL was isolated in a continuously growing human B lymphoblastoid cell line (B-LCL) from PBMC of a patient in the outbreak [10]. Viruses isolated in the course of these studies were characterized as MV by negative-contrast electron microscopy and by immunofluorescence staining using monoclonal and rabbit polyclonal anti-MV antisera as described [11, 12]. On the basis of nucleotide sequence variations in the *N* gene between nucleotides 1231 and 1686, a number of different lineages of MV have been identified, several of which can cocirculate in geographically restricted areas [13].

We compared the nucleotide sequence of the region corresponding to the *N* region of 1 MV-BIL isolate by polymerase chain reaction amplification of cDNA obtained from MV-BIL-infected B-LCL. On the basis of this analysis, MV-BIL appeared to be closely related to wild type MVs originating from lineages previously shown to circulate in Europe and the United States (data not shown). MV-BIL was subsequently cloned by a two-fold limiting dilution of cell-free virus in B-LCL (B-LCL JP) [10]. Virus stocks of MV-BIL, containing 2×10^4 TCID₅₀/mL, were prepared in the same cell line and kept at -135°C until use.

Infection of cynomolgus monkeys with different MV strains and reisolation of viruses. MV-seronegative cynomolgus monkeys (ages 20–30 months) were inoculated intratracheally (int) or intramuscularly (im) with the following preparations: MV-BIL ($10^{-1.0}$, 10^0 , $10^{1.0}$, $10^{2.0}$, $10^{3.0}$, and $10^{4.0}$ TCID₅₀ int; 3 monkeys/inoculum), the Edmonston strain of MV (MV-Edmonston; $10^{3.0}$ TCID₅₀ int; 2 monkeys), and attenuated Schwartz measles vaccine (MV-Schwartz, Rouvax, lot G0334; 1 dose/animal int or im; 2 monkeys each; vaccine provided by Institut Mérieux, Lyon, France). MV-Edmonston had a history of six passages in human embryo kidney cells and three additional passages in Vero cells in the laboratory of P. Albrecht (Food and Drug Administration, Bethesda, MD), who donated this virus. All monkeys were monitored daily for development of clinical symptoms. At days 3, 5, 7, 9, 11, 14, and 18 after infection, nasopharyngeal washings and heparinized peripheral blood and lung lavages were collected for MV isolation by the following procedures.

PBMC were isolated from heparinized blood by centrifugation onto a gradient containing 4% (wt/vol) dextran 500 (Sigma, St. Louis) and 32% (vol/vol) of a standard metrizoate solution containing 32.8% (wt/vol) sodium-metrizoate (Nycomed, Oslo). PBMC were cultured in 96-well round-bottomed plates (8 wells/sample) at a density of 8×10^4 cells/well in 150 μL of RPMI 1640, supplemented with 10% (vol/vol) fetal calf serum and antibiotics (FCS-medium) in the presence of 50 $\mu\text{g}/\text{mL}$ phytohemagglutinin (PHA-M; Boehringer, Mannheim, Germany). After culture for 3 h at 37°C , logarithmic dilutions of these cultures were cocultivated with B-LCL JP in round-bottomed wells at a density of 10^4 B-LCL per well. Lung lavages were centrifuged, and the cells were resuspended in FCS-medium. The cells were cultured in 24-well flat-bottomed microtiter

plates (Greiner Labor Technik, Nürtingen, Germany) at a density of 2×10^5 cells/well (6 wells/sample) and cocultivated with 10^5 B-LCL JP/well. Cells were collected from nasopharyngeal washings and cocultured with human B-LCL JP as described for lung lavage cells. All cultures were visually monitored for cytopathic changes over 10 days. The presence of MV-infected B-LCL in cultures was demonstrated by immunofluorescence staining and by use of a T cell proliferation assay with MV-specific human T cell clones as previously described [10, 11].

Serologic assays. Plasma samples from heparinized blood were heat-inactivated for 30 min at 56°C and tested for the presence of MV-specific IgM and IgG antibodies in ELISAs and in virus-neutralizing assays. IgG-specific ELISAs and virus-neutralizing assays were done as described [12]. IgG titers were expressed as the reciprocals of dilutions of individual serum samples showing 50% of the maximal optical density value at 450 nm and virus-neutralizing antibody titers as the reciprocals of dilutions of sera showing 50% inhibition of MV infection on Vero cells.

Specific IgM antibodies were detected by an antibody capture ELISA as described [10] with minor modifications. Briefly, microtiter plates (no. 3590; Costar, Cambridge, MA) were coated with 1:500 diluted F(ab)₂ goat anti-human IgM antibodies (Organon Teknica-Cappel, West Chester, PA). Anti-IgM-coated plates were then incubated with 1:100 dilutions of individual monkey serum samples followed by an incubation with MV antigen (20 $\mu\text{g}/\text{mL}$ purified and β -propiolacton-inactivated MV) [12]. Plates were subsequently incubated with 1:1000 diluted rabbit anti-MV antiserum [12] followed by an incubation with 1:500 diluted horseradish-peroxidase-conjugated swine anti-rabbit IgG (Amersham Laboratories, Amersham, UK). The latter two incubations were done in the presence of 2% (vol/vol) normal monkey serum. Plates were developed and the absorbance was read at 450 nm. IgM titers were expressed as optical density values.

Results

Virus isolation from MV-BIL-infected cynomolgus monkeys. Human and marmoset B lymphoblastoid cell lines have been shown to be sensitive for isolation and propagation of MV from clinical specimens of measles patients [10, 14]. In a preliminary experiment, 1 isolate (MV-BIL) developed a rapid and extensive cell-associated viremia in cynomolgus monkeys after intravenous inoculation with cells infected with MV-BIL [10]. To study the susceptibility of cynomolgus monkeys to infection with cell-free MV-BIL via the respiratory route, 12 MV-seronegative animals were inoculated int with serial 10 log dilutions of biologically cloned MV-BIL ($10^{-1.0}$ – $10^{4.0}$ TCID₅₀). Successful MV infection was demonstrated in all MV-BIL-infected animals by virus isolation through cocultivation of PBMC, lung lavage cells, and pharyngeal epithelial cells with human B-LCL JP (figure 1).

To obtain an indication of the minimal dose of MV-BIL infectious to cynomolgus monkeys, 2 additional animals were inoculated with 10^0 and $10^{-1.0}$ TCID₅₀ MV-BIL, respec-

tively. The monkey inoculated with 10 TCID₅₀ MV-BIL became infected (figure 1). Animals infected with 10^{3.0} and 10^{4.0} TCID₅₀ MV-BIL had peaks of infectivity ~7 days after infection (3–10 × 10^{3.0} MV-infected cells/10⁶ PBMC; figure 1). Although animals infected with lower infectious doses (10–10^{1.0} TCID₅₀) showed similar kinetics of PBMC-associated viremia, the peak of infectivity was delayed by 3–6 days. PBMC-associated viremia largely coincided with successful isolation of MV from lung lavage cells from an infected animal (figure 1). The presence of MV infection in pharyngeal epithelial cells could be demonstrated during and shortly after the peak of PBMC-associated viremia in most of the infected animals (figure 1).

Comparison of virus isolation and serum antibody response in MV-BIL-, MV-Edmonston-, and MV-Schwartz-infected cynomolgus monkeys. Two MV-seronegative monkeys were inoculated int with 10^{3.0} TCID₅₀ MV-Edmonston. Four animals were inoculated with 10^{3.0} TCID₅₀ MV-Schwartz, either int or im. Subsequently, reisolation of the viruses was done as described above. Isolates from animals infected with MV-Edmonston and attenuated MV-Schwartz were compared with those of 2 MV-BIL-infected animals. Monkeys infected with MV-Edmonston had kinetics of MV viremia similar to those of the MV-BIL-infected animals, but they had fewer MV-infected PBMC on day 7 after infection (10–150/10⁶ PBMC; figure 2). Because MV-Edmonston had a

passage history on Vero cells, PBMC from MV-Edmonston-infected monkeys were also cocultivated with Vero cells. Results were identical to cocultivation of PBMC with B-LCL (data not shown). Vaccination (im) of 2 monkeys with MV-Schwartz resulted in low but detectable numbers of MV-infected PBMC in both monkeys (10–50/10⁶ PBMC on days 9–11; figure 2). Infection (int) of 2 other monkeys with MV-Schwartz resulted in low numbers of MV-infected PBMC at day 7 after infection in 1 of the 2, but MV could be isolated from the lung lavage cells of both animals on days 5–7 after infection (data not shown). No pharyngeal epithelial cells could be isolated from MV-Edmonston- and MV-Schwartz-infected monkeys (data not shown).

Monkeys infected with MV-BIL, MV-Edmonston, and MV-Schwartz developed high, moderate, and moderate-to-low levels of specific serum IgM antibodies, respectively, which were detected over ~3 weeks, the onset of which coincided with the peaks of infectivity in individual animals (figure 2). The presence of MV-specific IgG serum antibodies, coinciding with the presence of MV neutralizing antibodies, was seen shortly after the appearance of specific IgM serum antibodies and gradually increased to plateau levels within ~6 weeks. MV neutralizing antibody titers of MV-BIL-infected monkeys remained stable (512–2048) over the 9-month observation period (figure 2). One of the 2 MV-Edmonston-infected monkeys also developed a neutralizing

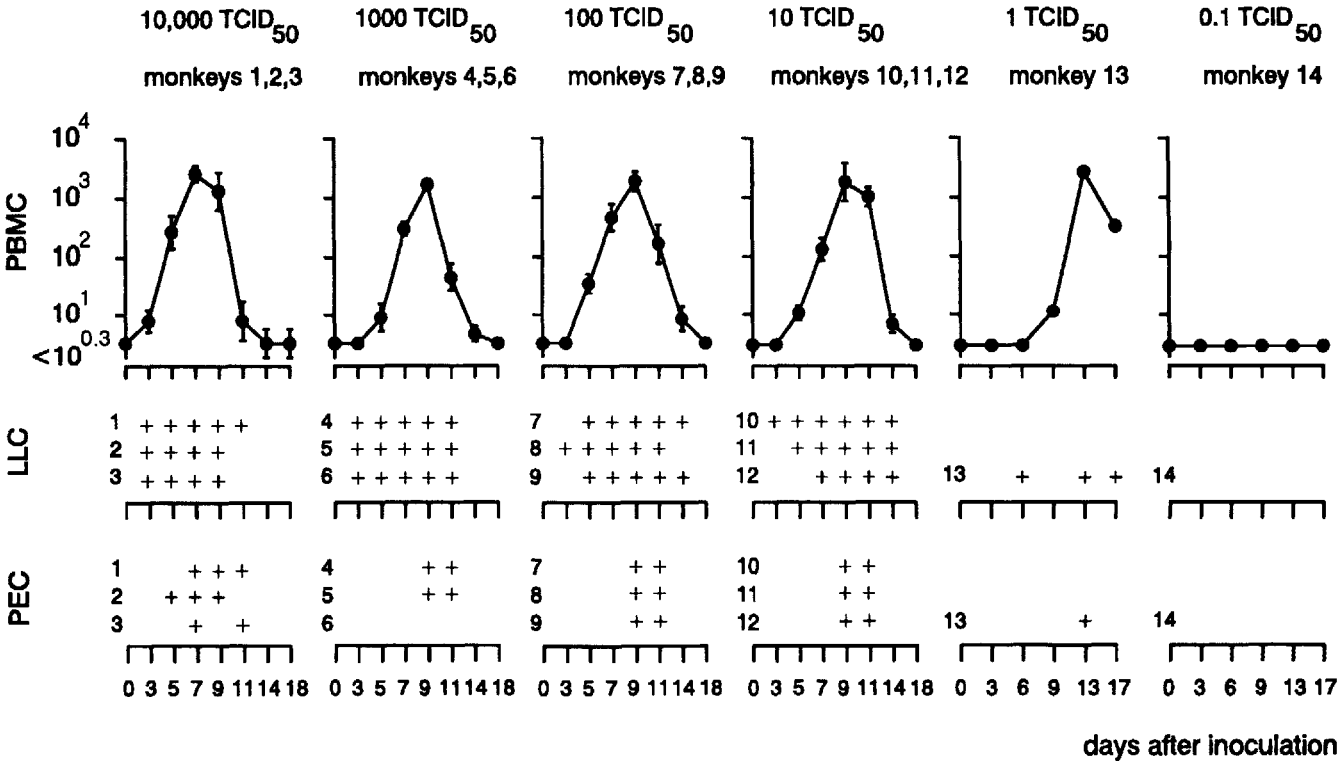


Figure 1. Measles virus (MV)-infected cells in peripheral blood mononuclear cells (PBMC), lung lavage cells (LLC), and pharyngeal epithelial cells (PEC) of 14 cynomolgus monkeys infected intratracheally with different doses of wild type MV strain (MV-BIL). Graphs represent average numbers (±SD) of MV-infected cells/10⁶ PBMC per inoculum. + indicates successful MV isolations from LLC and PEC.

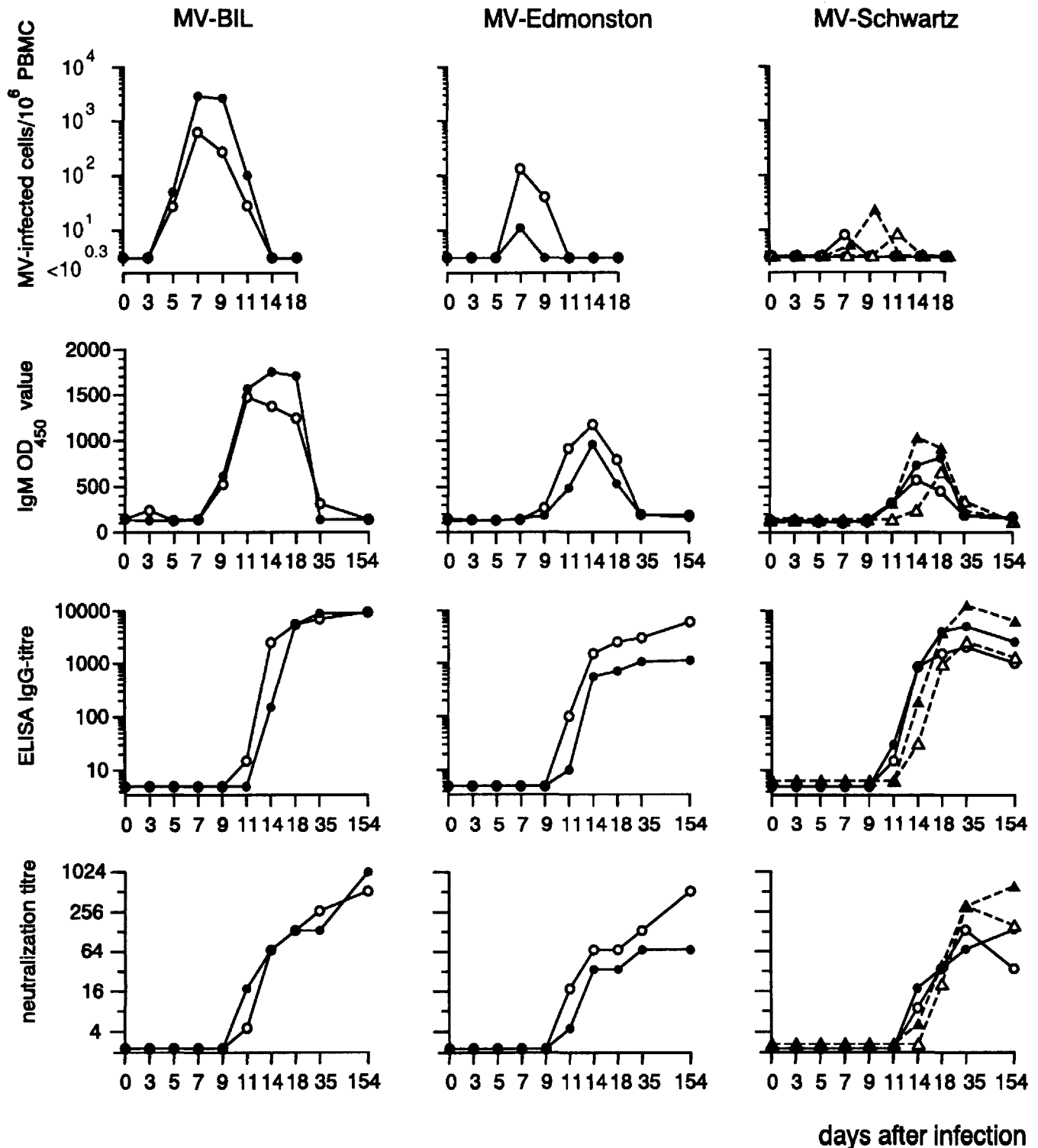


Figure 2. Development of measles virus (MV) viremia and specific serum IgM, IgG, and MV neutralizing antibody responses in 6 monkeys infected intratracheally with $10^{3.0}$ TCID₅₀ MV-BIL, MV-Edmonston, or MV-Schwartz (solid lines) and in 2 monkeys infected intramuscularly with MV-Schwartz (dashed lines). Open and closed symbols represent individual monkeys within each group. PBMC, peripheral blood mononuclear cells.

antibody titer of 1024, whereas the other and all MV-Schwartz-infected monkeys developed lower MV neutralizing antibody titers (32–256).

Protection from reinfection with MV-BIL after infection with different MV strains. Two MV-seronegative control animals and all of the MV-infected monkeys shown in figure 2 were challenged int with $10^{3.0}$ TCID₅₀ MV-BIL 9 months after initial MV infection. They were monitored for development of PBMC-associated viremia, infected cells in lung lavages and pharyngeal washings, and changes in MV-specific IgM and IgG serum antibody titers. No MV-infected cells were seen in any of the monkeys in figure 2 in the 3 weeks after challenge. Accordingly, none of these animals showed specific serum antibody titer rises. The 2 controls included in this challenge experiment had patterns of MV isolation and serology similar to the other monkeys infected int with $10^{3.0}$ TCID₅₀ MV-BIL (not shown).

Discussion

Our results show that cynomolgus monkeys can be infected with MV-BIL, a recently isolated strain that belongs to a cluster of wild type MVs now circulating in the United States and Europe [10, 13]. Monkeys infected with MV-BIL developed higher levels of PBMC-associated viremia than did monkeys infected with MV-Edmonston; MV-infected cells were also found in lung lavages and pharyngeal swabs at different times after infection.

Previous studies have shown that rhesus and cynomolgus monkeys have limited susceptibility to experimental infection with certain wild type MVs, such as MV-Edmonston, when propagated in Vero cell lines [9, 14, 15]. Furthermore, MV-Edmonston completely loses its pathogenicity for rhesus monkeys when propagated in chicken embryo cell cultures [15]. The latter procedure also is the basis for the preparation of live attenuated measles vaccines [15]. It is therefore not unlikely that the observed biologic differences between MV-BIL and MV-Edmonston are due to differences in passage history rather than genuine strain-related differences. Thus, we decided to isolate and biologically clone MV-BIL in human lymphoid cells only, before using this virus for the development of a suitable monkey model for measles. MV-BIL could not be isolated by cocultivation of clinical specimens with Vero cell lines (data not shown).

The dose of MV-BIL used to infect monkeys did not influence the level of PBMC-associated viremia, but with lower doses a delay of some days was observed in the kinetics of the infection. We found that a dose as low as 1.0 TCID₅₀, as measured in human B-LCL, corresponded with ~1 monkey infectious dose (figure 1). This confirms the high infectious nature of MV, which has also been seen in humans under natural conditions [1, 7]. Infection of monkeys via different routes (int or im) with MV-Schwartz, an attenuated vaccine strain, resulted in lower levels of viremia than observed with

MV-BIL and MV-Edmonston (figure 2). The im route was included in these experiments to mimic the normal route of vaccination [7].

It may be speculated that the levels of PBMC-associated viremia and the presence or absence of MV-infected cells in lung lavages and in pharyngeal washings measured in macaques are parameters of MV virulence. In this respect, even a low passage of MV-Edmonston could be regarded as indicating an attenuated MV strain that would not be predicted on basis of its biologic properties in vitro (our findings and [14]). However, as none of the infected animals developed major clinical symptoms (only MV-BIL-infected monkeys showed a transient and mild lethargy and temperature rise; data not shown), we cannot yet conclude whether the biologic properties of the different MV strains will correlate in humans and macaques.

Comparison of serum antibody responses following infection with MV-BIL, MV-Edmonston, or MV-Schwartz showed that the IgM responses had the same trends as seen in PBMC-associated viremia. These differences were less pronounced for MV-specific serum IgG levels and MV neutralizing antibodies (figure 2). MV neutralizing antibody titers of MV-BIL-infected monkeys were similar to those seen in sera from children after infection with MV-BIL (figure 2; data not shown). MV-BIL infection in the children also coincided with strong MV-specific proliferative CD4⁺ and CD8⁺ T cell responses in their PBMC [2, 10, 11]. By using similar techniques, we demonstrated the expansion of specific CD4⁺ and CD8⁺ T cell populations in the PBMC of monkeys previously infected with MV-BIL and MV-Schwartz (unpublished data). These data indicate that MV-specific humoral and cell-mediated immune responses can be studied in this macaque model. The kinetics and specificities of these responses are now being investigated.

After natural infection and vaccination with attenuated MV vaccine strains, a solid immune-mediated protection against measles is observed in humans. We demonstrated that monkeys infected with MV strains were also protected against viremia and viral replication in the lungs and pharynx when they were reinfected with MV-BIL. Although the mechanism and longevity of this protection is not known, this monkey model allows investigation of the mechanisms involved in this apparently immune-mediated protection. In addition, new generations of measles vaccines and novel vaccination routes and strategies may be evaluated in cynomolgus monkeys, both in the presence and absence of maternally derived or passively transferred antibodies.

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