Infection of cynomolgus macaques (Macaca fascicularis) and rhesus macaques (Macaca mulatta) with different wild-type measles viruses

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INTRODUCTION

Measles remains one of the most important causes of vaccine-preventable deaths in developing countries (WHO, 2006). A safe and effective live-attenuated measles virus (MV) vaccine is available and has been used successfully to achieve elimination of endemic MV transmission in large parts of the world (WHO, 2004). Effective MV control requires strengthening of routine vaccination programmes, resulting in high vaccination coverage of infants aged 9–15 months, in combination with a second opportunity for vaccination at a later age (Orenstein et al., 2006). In tropical countries with limited infrastructure, mass vaccination campaigns can be highly effective as a method of providing this second opportunity. However, injection safety and safe waste disposal in countries where infections with human immunodeficiency virus and hepatitis B virus are often highly prevalent is difficult to achieve. Therefore, the World Health Organization (WHO) is currently evaluating alternative non-parenteral routes of administration of the existing live-attenuated MV vaccine (Bennett et al., 2002; De Swart et al., 2006).

Measles is associated with transient immunosuppression, which accounts for a large part of the associated morbidity and mortality. Paradoxically, the disease also results in lifelong immunity, resulting in an epidemiological pattern of childhood infections in areas of MV endemicity. The pathogenesis of measles and the associated immunosuppression are still poorly understood (Duke & Mgone, 2003), although the recent identification of signalling lymphocyte activation molecule (SLAM, CD150) as a cellular receptor for MV infection has provided new insights (Tatsuo et al., 2000; Yanagi et al., 2006). Besides SLAM and CD46, which was previously identified as a receptor for laboratory-adapted and vaccine strains of MV (Naniche et al., 1993), it is suspected that other modes of entry and/or other receptors may exist (Hashimoto et al., 2002; Andres et al., 2003).

For measles pathogenesis studies or evaluation of alternative MV vaccination strategies, animal models are required. Rodents are usually not susceptible to infection with wild-type MV strains, with the exception of cotton rats (Wyde et al., 1992; Niewiesk, 2001) and SCID mice xenografted.
with human cells (Auwaerter et al., 1996). Recently, it has been demonstrated that MV can also replicate in CD150 transgenic mice, although the pathogenesis of the associated disease was quite different from measles in humans (Welstead et al., 2005; Sellin et al., 2006). The only animals with a susceptibility to MV infection similar to humans are non-human primates (Van Binnendijk et al., 1995). In recent years, macaque models have been developed and used for vaccination and pathogenesis studies (Van Binnendijk et al., 1994; Kobune et al., 1996; McChesney et al., 1997; Auwaerter et al., 1999; Stittelaar et al., 2002). Although both rhesus and cynomolgus macaques have been used, clinical symptoms like rash and conjunctivitis were especially reported in rhesus macaques (Auwaerter et al., 1999). Although a skin rash has also been reported in cynomolgus macaques (Kobune et al., 1996), this symptom seems to be less prominent in this species.

Due to differences in the preparation of stocks of challenge virus, the origin of animals and experimental procedures, it is often difficult to compare results obtained by different research groups. We therefore decided to study MV infection ‘head to head’ in both animal species. The first objective of the study was to assess clinical, virological and immunological differences between MV infection in rhesus and cynomolgus macaques. The second objective of the study was to compare the differences in pathogenesis of infection with two different wild-type MV isolates. The first wild-type MV strain used was MV-Bil, a genotype C2 virus isolated in The Netherlands in 1991 and used as challenge virus in both cynomolgus and rhesus macaques by us and others (Van Binnendijk et al., 1994; Auwaerter et al., 1999; Polack et al., 1999). The second MV strain was a genotype B3 virus isolated from a severe measles patient in Khartoum, Sudan, in 1997 (El Mubarak et al., 2000). In the present study, clinical parameters, MV replication kinetics and antibody responses were monitored over time.

**METHODS**

**Macaques.** The study was performed in seven rhesus monkeys (Macaca mulatta) and eight cynomolgus monkeys (Macaca fascicularis). The animals were juvenile (2–4 years) and seronegative for MV as determined by virus neutralization. Body temperatures were measured by telemetry (Rimmelzwaan et al., 2001) and the resulting data were first averaged per animal h⁻¹ and subsequently per group h⁻¹. The study was approved by the animal ethics committee and performed according to Dutch guidelines for animal experimentation.

**Viruses.** Two wild-type MV strains were used for experimental infection of macaques: three rhesus and four cynomolgus macaques were infected with MV-Bil (MV/Bilthoven.NET/91, genotype C2), whilst four rhesus and four cynomolgus macaques were infected with MV-Sudan (MV/Khartoum.SUD/34.97/2, genotype B3) (El Mubarak et al., 2000). Both viruses were isolated from peripheral blood mononuclear cells (PBMCs) of a measles patient in human B-lymphoblastoid cell lines (BLCLs). A third passage in BLCLs was used for infection. The macaques were infected intratracheally with 10³ 50% cell culture infectious doses in 5 ml PBS.

**Samples.** EDTA blood samples were collected at days −6, 3, 6, 9, 13, 17, 24 and 30 after infection. Plasma was separated by centrifugation, heat inactivated (30 min 56°C) and stored at −20°C. PBMCs were isolated by density-gradient centrifugation, resuspended in RPMI 1640 supplemented with antibiotics and heat-inactivated fetal bovine serum (R10F), counted and used fresh for virus isolation (see below). Broncho-alveolar lavages (BALs) were collected on days −6, 3, 6, 9, 13 and 17 after infection, by intratracheal infusion of 10 ml PBS through a flexible catheter. Recovered BAL fluid was centrifuged and BAL cells were resuspended in R10F, counted and used fresh for virus isolation.

**MV isolation.** MV was isolated in BLCLs using an infectious centre test, as described previously (Stittelaar et al., 2000). Briefly, 3.2 × 10⁵ PBMCs (first stimulated with phytohaemagglutinin-L for 1 h at 37°C) or BAL cells were transferred to eight wells in the first row of a 96-well round-bottomed plate (each well containing 4 × 10⁴ cells). Subsequently, twofold dilutions were prepared to obtain a cell density gradient from 2 × 10⁴ to 10 cells. Subsequently, BLCLs were added (1 × 10⁴ cells per well) and plates were incubated at 37°C. Cytotoxic changes were monitored by light microscopy after co-cultivation for 3–6 days. The number of cells resulting in 50% of the cultures becoming infected was calculated using the formula of Reed & Muench (1938).

**MV-specific antibody responses.** The levels of MV fusion protein (F) - and haemagglutinin (H)-specific IgM and IgG antibodies were determined in plasma by FACS-measured immunofluorescence using transfected human melanoma cell lines as targets, as described previously (De Swart et al., 1998). FITC-labelled rabbit anti-human IgM or IgG (F(ab')² fragments; Dako) were used, which cross-reacted with macaque antibodies (De Swart et al., 1998). MV nucleoprotein (N)-specific IgM was measured in a capture ELISA, as described previously (El Mubarak et al., 2004). Capturing plates were coated with a polyclonal anti-human IgM serum (Meddens Diagnostics), which cross-reacted with macaque IgM. Specific signals were detected with a recombinant baculovirus-produced purified N preparation (kind gift of Dr T. F. Wild, Lyon, France), which was peroxidase labelled by Meddens Diagnostics. N-specific IgG responses were measured in an indirect ELISA using baculovirus-produced purified N, as described previously (El Mubarak et al., 2004). Horseradish peroxidase-labelled rabbit-anti-human IgG (Dako) was used, which cross-reacted with macaque IgG.

**Haematology.** White blood cell counts were measured using an automated haematology analyser (Sysmex). Thin blood films were prepared from EDTA blood and stained with Giemsa (Merck). Differential cell counts were obtained by counting 500 cells per slide, and the numbers of lymphocytes, neutrophils, eosinophils and monocytes were calculated by multiplying these percentages by the white blood cell counts obtained for the same sample.

**Statistical analysis.** Virus loads were summarized by the area under the curve (AUC, above the detection limit of 3) between days 0 and 17, using the trapezoidal method of numerical integration after natural logarithmic transformation. The effects of animal species and virus strain were estimated using multiple linear regression analysis. The haematological parameters and antibody responses were analysed using a mixed model analysis of variance with the explanatory factors time, species and virus strain, and with the baseline measurement of each variable as the continuous covariate.

**RESULTS**

**Clinical features**

All rhesus monkeys developed a skin rash and conjunctivitis between 1 and 2 weeks after infection. Levels and kinetics differed among animals, but did not seem to be related to the
virus strain used for infection. Monitoring of the kinetics of these symptoms was difficult without capturing and sedating the animals, which was only done on the days of sampling (Fig. 1a, b). A similar skin rash was seen in a minority of the cynomolgus macaques, although less profound and again not related to one particular virus strain.

Measurement of body temperature demonstrated a rhythmic pattern, with mean temperatures fluctuating between 36.5°C at night and 39.5°C during the day (Fig. 1c). Between 8 and 11 days after infection, a slight (~0.5°C) increase in body temperatures was recorded during the night time, especially in the animals infected with MV-Sudan (Fig. 1d).

Peripheral lymphopenia and neutropenia were observed in all animals between days 6 and 13 (Fig. 2, upper panels). The number of neutrophils measured at day 13 was significantly higher ($P=0.042$) in the cynomolgus macaques compared with the rhesus macaques. Interestingly, an increase in the number of peripheral monocytes was observed in all animals on days 13 and/or 17 (Fig. 2, lower right panel), and no significant variation between the different animal species or virus strains was found. No eosinophilia was seen in any of the animals (Fig. 2, lower left panel).

**Virus isolation**

Quantification of MV-infected cells in BAL cells and PBMCs showed that the peak of virus replication was in most cases on day 6 after infection for MV-Sudan and on day 9 for MV-Bil. In addition, a trend was detected of higher numbers of infected cells early (day 3) and/or late (day 13) after infection with MV-Sudan when compared with MV-Bil (Fig. 3). This was also reflected by calculations of the AUC between days 0 and 17 (Table 1). Virus loads in

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**Fig. 1.** Clinical symptoms of measles in macaques. (a, b) Measles rash and conjunctivitis in a rhesus monkey 13 days after infection with wild-type MV strain MV-BIL. (c) Body temperature, as measured by telemetry. Filled symbols represent animals infected with MV-Sudan; open symbols represent animals infected with MV-Bil. Circles represent rhesus macaques and triangles represent cynomolgus macaques. (d) Enlarged part of panel (c).
BAL cells of rhesus macaques infected with MV-Sudan were significantly higher than in rhesus macaques infected with MV-Bil \( (P<0.001) \) and a similar trend was seen in cynomolgus macaques \( (P=0.058) \). Virus levels in BAL cells were higher in rhesus than in cynomolgus macaques infected with MV-Sudan \( (P=0.019) \), but this effect was not found after infection with MV-Bil \( (P=0.32) \). No significant differences were found in the PBMC compartment between the virus strains or macaque species.

**Antibody responses**

MV protein-specific IgM antibodies were detected in all animals from day 13 until day 30 after infection (Fig. 4). N-specific IgM antibodies peaked earlier in the animals infected with MV-Sudan compared with those infected with MV-Bil (days 9 and 13, respectively), but showed similar kinetics in both macaque species (Fig. 4, upper left panel). The same pattern could be observed for IgM responses to F and H, which on day 9 were also higher in the animals infected with MV-Sudan than in animals infected with MV-Bil. However, individual variation was substantially higher in these responses.

The kinetics of MV protein-specific IgG antibodies were largely similar in all animals, with responses being detectable from day 13 onward and reaching maximum levels on day 24 (Fig. 5). Earlier onset of N- and F-specific IgG

**Table 1.** MV loads in BAL cells and PBMCs, as determined from the virus isolation data shown in Fig. 3 (AUC \( 0–17 \), log transformed)

<table>
<thead>
<tr>
<th>Macaque species</th>
<th>Virus</th>
<th>BAL cells</th>
<th>PBMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhesus</td>
<td>MV-Sudan</td>
<td>4.83 ± 0.37</td>
<td>4.40 ± 0.33</td>
</tr>
<tr>
<td>Cynomolgus</td>
<td>MV-Sudan</td>
<td>4.92 ± 0.24</td>
<td>4.21 ± 0.55</td>
</tr>
<tr>
<td>Rhesus</td>
<td>MV-Bil</td>
<td>4.58 ± 0.32</td>
<td>3.86 ± 0.10</td>
</tr>
<tr>
<td>Cynomolgus</td>
<td>MV-Bil</td>
<td>4.45 ± 0.02</td>
<td>3.77 ± 0.32</td>
</tr>
</tbody>
</table>

**Fig. 2.** Numbers of lymphocytes, neutrophils, eosinophils and monocytes in peripheral blood of macaques at different time points after MV infection. Results are shown as means ± SD per group. Symbols are as described in the legend to Fig. 1.

**Fig. 3.** MV loads in BAL cells and PBMCs. Results are expressed as number of infected cells per \( 10^6 \) total cells and plotted as means ± SD per group. Symbols are as described in the legend to Fig. 1.
responses was observed in cynomolgus macaques infected with MV-Sudan, mainly caused by one outlier.

**DISCUSSION**

In the present study, we demonstrated that rhesus and cynomolgus macaques are equally susceptible to infection with wild-type MV. Both macaque species have been used previously for experimental MV infections, but never head to head in one study. Previous reports of a more distinct skin rash and conjunctivitis in rhesus than in cynomolgus macaques (McChesney et al., 1997; Auwaerter et al., 1999) were confirmed, but virological and immunological post-infection parameters were largely similar between the two species. Interestingly, MV-Sudan was found to be more pathogenic than MV-Bil.

Measurement of body temperature in non-human primates using standard methods can be unreliable due to changes in body temperature as a result of the stress associated with capturing the animal. We have previously successfully used telemetry to demonstrate the development of fever after infection of macaques with influenza virus (Rimmelzwaan et al., 2001) or monkeypox (Stittelaar et al., 2005). Indeed, the present study showed changes in body temperature after MV infection, although to different levels in individual animals. Animals infected with MV-Sudan showed the most significant (~0.5°C) increase in night temperature during the days immediately after the peak in virus replication.

Skin rash and conjunctivitis were more evident in rhesus macaques than in cynomolgus macaques, confirming previous observations (McChesney et al., 1997; Auwaerter et al., 1999). This was not accompanied by differences in MV replication kinetics or specific antibody responses, suggesting that both animal species were equally susceptible to MV. In addition, the haematological parameters measured showed lymphopenia and neutropenia during the peak of virus replication in all animals, followed by monocytosis a few days later, essentially confirming previous observations (Auwaerter et al., 1999). The observed clinical differences could result from specific cells (e.g. endothelial cells) being susceptible to MV infection in rhesus macaques but not, or to a lesser extent, in cynomolgus macaques. However, it has been speculated that measles rash and conjunctivitis have an immune-mediated origin, as they are often absent in immunocompromised patients infected with MV (Griffin, 2001; De Swart et al., 2000). Therefore, an alternative explanation is that there may be qualitative or quantitative differences in the MV-specific cellular immune response between rhesus and cynomolgus macaques.

MV-Sudan proved to be more pathogenic in macaques than MV-Bil, as demonstrated by increased levels of virus replication and the more rapid onset of specific IgM
responses. This virus was isolated from a severe measles patient in Khartoum in 1997. Measles-associated morbidity and mortality in Sudan is relatively high, with case fatality rates of between 1 and 10% (Ibrahim et al., 2002). Although MV is a monotypic virus, genetic differences exist and little is known about the biological differences among members of the different clades (WHO, 1998, 2005). The present study suggests that the virus strain circulating in Khartoum is more pathogenic than the European strain MV-Bil, which may at least in part account for the observed clinical severity of measles in Sudan.

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