Successful mucosal immunization of cotton rats in the presence of measles virus-specific antibodies depends on degree of attenuation of vaccine vector and virus dose

Bernd Schlereth,1 Linda Buonocore,2 Annette Tietz,1 Volker ter Meulen,1 John K. Rose2 and Stefan Niewiesk1

Correspondence
Stefan Niewiesk
niewiesk@vim.uni-wuerzburg.de

1Institute of Virology and Immunobiology, University of Wuerzburg, Versbacher Str. 7, 97078 Wuerzburg, Germany
2Departments of Pathology, Yale University School of Medicine, 310 Cedar Street, New Haven, CT 06510, USA

INTRODUCTION

Measles virus (MV) infection is still one of the most deadly infectious diseases worldwide. Efficient immunization of neonates is hampered by the fact that maternal antibodies inhibit successful immunization and subsequent protection against disease. To test vaccine vectors in the presence of maternal antibodies, we have used the cotton rat (Sigmodon hispidus) model. Cotton rats are the only rodents in which, after intranasal (i.n.) infection, MV replicates in the respiratory tract (Niewiesk et al., 1997; Wyde et al., 1992, 1999). In these animals, maternal antibodies interfere with vaccine-induced seroconversion and subsequent protection (Schlereth et al., 2000b). To be able to follow simultaneously the decline of maternal antibodies and the generation of actively induced antibodies, we transferred a human serum containing MV-specific antibodies as a substitute for maternal antibodies and distinguished them by ELISA (Schlereth et al., 2000a, b). Human MV-specific antibodies inhibit vaccine-induced seroconversion and subsequent protection in the same way as naturally occurring maternal antibodies. In this animal model, a recombinant vesicular stomatitis virus expressing the MV haemagglutinin (VSV-H) was tested as a vaccine vector. In tissue culture, replication of VSV-H is only slightly inhibited by MV-specific antibodies (Schlereth et al., 2000b). In vivo, i.n. immunization with VSV-H led to the generation of neutralizing antibodies and protection against i.n. challenge with MV. Inoculations of UV-inactivated VSV-H or intraperitoneal (i.p.) inoculation of live virus (leading to abortive infection) were not able to immunize in the presence of MV-specific antibodies. This indicates that some virus replication is necessary to obtain protection (Schlereth et al., 2000b).

In BALB/c mice, i.n. infection with wild-type VSV leads to infection of the brain via the olfactory nerve and fatal encephalitis (Reiss et al., 1998). In contrast, recombinant VSV, derived from an infectious clone, is attenuated and does not cause lethal encephalitis (Roberts et al., 1998).
Furthermore, a VSV recombinant expressing the haemagglutinin protein of influenza virus given i.n. protects mice against influenza virus challenge (Roberts et al., 1998). However, residual pathogenicity (measured as weight loss) is observed after i.n. immunization of mice with VSV recombinants. The replication potential of VSV can be reduced by truncating the cytoplasmic domain of the envelope G protein. A mutant with a cytoplasmic tail of 29 aa truncated to 9 aa (CT9) grows in vitro as well as recombinant VSV, whereas a mutant with a truncation to 1 aa (CT1) is reduced 10- to 100-fold in virus production in vitro (Roberts et al., 1999; Schnell et al., 1998). In mice, immunization with VSV-HA (expressing the haemagglutinin of influenza virus A) results in weight loss but mice recover fully and are protected against influenza (Roberts et al., 1998). A VSV CT9-HA recombinant causes less weight loss, mice recover fully and are protected against influenza (Roberts et al., 1998). Both CT1-HA and CT9-HA induced immunity to influenza challenge (Roberts et al., 1999).

In the present study, we tested the pathogenicity of wild-type VSV and the stepwise-attenuated recombinant VSVs expressing the MV haemagglutinin. We also investigated their protective capacity in the absence and presence of passively transferred MV-specific antibodies.

**METHODS**

**Cotton rats: infection, immunization, serum transfer and virus titration.** Cotton rats (inbred strain COTTON/Nico) were obtained from Iffa Credo, France. Animals were kept under controlled environmental conditions and used at the age of 6–8 weeks (60–70 g). Intracerebral (i.c.), i.n. and i.p. infection or serum injection and retroorbital blood sampling were done under ether narcosis. Intratracheal (i.t.) infection was done under methoxyfluorane narcosis using a holding apparatus and polythene tubing, as described (Waynforth & Flecknell, 1992). To mimic maternal MV-specific antibodies, 1 ml human serum (16 IU ml–1) was coated in 200 mM NaCO3 buffer (pH 9.6) at 4 °C overnight, blocked with PBS/10 % FCS/0.05 % Tween 20 and incubated with diluted cotton rat serum (1:100) at 4 °C for 1 h. After washing, the plate was incubated with rabbit serum specific for cotton rat IgG (Virion Systems) for 1 h at room temperature. After washing, the plate was incubated with horseradish peroxidase-coupled goat serum specific for rabbit IgG (Zymed) for 45 min at room temperature and was subsequently developed with 0.5 mg o-phenylenediamine ml–1 in buffer (35 mM citrate/66 mM Na2HPO4, pH 5.2) and 0.01 % H2O2. The plate was read at a wavelength of 490 nm against a reference reading at 405 nm.

**Neutralization tests.** Serum 2-fold dilutions were incubated with 50 p.f.u. MV strain Edmonston for 1 h at 37 °C and plated in duplicate onto 104 Vero cells per well of a 96-well plate. At 5 days later, infection of wells (>50 %) was determined microscopically. The titre was defined as the reciprocal of the last protective serum dilution, as calculated from duplicate measurements.

**RESULTS**

Replication in the central nervous system is not crucial for inducing immunity in the presence of MV-specific antibodies

To determine the degree of attenuation of recombinant VSV, we compared its virulence to that of wild-type VSV (strain Indiana) in cotton rats. In mice, VSV (strain Indiana) is neurotropic after i.n. infection (104.5 p.f.u.), leading to fatal encephalitis (Reiss et al., 1998). To test the virulence of VSV (strain Indiana) in cotton rats, we infected groups of three to five animals with various doses of VSV (strain Indiana). After inoculation with 104 p.f.u., animals lost weight and succumbed to encephalitis within 5 days, whereas an inoculum of 103 p.f.u. did not induce disease. In comparison, i.n. infection of cotton rats with recombinant VSV and VSV-H at a titre of 5 x 105 p.f.u. resulted sometimes (depending on the virus preparation used) in clinical inoculated tissue culture monolayers (TCID50). TCID50 was calculated according to Reed and Muench (Reed & Muench, 1938). The threshold of virus detectable was 103 TCID50.
signs and weight loss. A titre of $2 \times 10^7$ p.f.u. was always well tolerated and did not result in weight loss. In spite of the absence of clinical signs, virus was isolated from lung and brain tissue but not from mediastinal lymph nodes, thymus, liver or spleen. In brain tissue, virus replication was observed from day 3 to 6, with peak titres of $10^{5.5 \pm 1.5}$ TCID$_{50}$ g$^{-1}$ (Fig. 1). Titres of VSV-H recovered from lung tissue after i.n. infection declined earlier and were lower. This raised the question of whether the virus of VSV-H to stimulate an immune response in the presence of MV-specific antibodies was due to 'hibernation' in the brain or stimulation of the mucosa-associated immune system of the lung. To address this question, cotton rats were immunized i.c. with VSV-H. Non-lethal inocula did not induce antibody responses to either VSV or MV (data not shown). After i.t. inoculation with $2 \times 10^7$ p.f.u. VSV-H, virus load was measured in brain and lung tissue on day 5. VSV-H titres in lung tissue did not differ significantly from animals inoculated i.n. but no virus was found in brain tissue (data not shown). After i.t. and i.n. immunization with $2 \times 10^7$ p.f.u. in the presence of 16 IU MV-specific antibodies, the induction of antibody responses and protection against i.n. challenge were compared. Both routes of immunization induced similar amounts of MV-specific and -neutralizing antibody titres and conferred the same degree of protection against i.n. challenge with MV (Fig. 2). These data indicate that only stimulation of the immune system through the mucosa but not replication of virus in the brain is required to induce active immunity in the presence of maternal antibodies.

**Fig. 1.** Replication of VSV-H, CT9-H or CT1-H in lung and brain tissue after i.n. infection. Cotton rats were inoculated i.n. with $2 \times 10^7$ p.f.u. VSV-H or CT9-H and virus reisolated from lung (above) and brain (below) tissues from days 3 to 8. Each time-point represents the average of three animals ($\pm$SD). Statistical significance was tested by a two-sided Student t-test. After i.n. inoculation of $2 \times 10^7$ p.f.u. CT1-H, no virus was found on day 5 in either lung or brain tissue.

**Fig. 2.** Comparison of i.n. and i.t. immunization with VSV-H in the presence of 16 IU MV-specific antibodies. At 1 day after transfer of 16 IU MV-specific antibodies, cotton rats (five per group) were immunized i.n. (△) or i.t. (●) with $2 \times 10^7$ p.f.u. VSV-H and serum samples were taken at weekly intervals. At no time-point were antibody titres significantly different from each other. The same was true for virus titres in lung tissue of immunized animals, which differed significantly ($P<0.0008$; two-sided Student t-test) from virus titres in lung tissue of non-immune animals. For plotted values, the mean $\pm$SD is given.
Recombinant VSVs CT9-H and CT1-H are attenuated in vitro and in vivo

Whereas VSV-H is able to induce protection in the presence of maternal antibodies, UV-inactivated VSV-H is not (Schlereth et al., 2000b), indicating that virus replication is essential. To answer the question of whether further attenuation of the vaccine vector would still generate protection in the presence of maternal antibodies, we used VSV mutants that have been demonstrated to be stepwise-attenuated in vitro and in the mouse (Schnell et al., 1998; Roberts et al., 1999). On the backbone of VSV CT9 (cytoplasmic tail of the G protein of 9 aa) and VSV CT1 (cytoplasmic tail of the G protein of 1 aa), two viruses were produced which express MV haemagglutinin (CT9-H and CT1-H, respectively). The ratio of MV-H to VSV-G expressed by both recombinant viruses was shown to be 1 : 3 (Fig. 3), the same ratio obtained previously for VSV-H and VSV-G (Schlereth et al., 1996). In tissue culture, the degree of attenuation of CT9-H and CT1-H was comparable to that of the recombinant viruses expressing the influenza virus HA (Roberts et al., 1999). CT9-H was only slightly attenuated in vitro, whereas CT1-H was much more attenuated, with a 100-fold reduction in virus titre and a doubling of replication time (from 16 to 24 h for VSV-H to 48 h). In vivo, CT9-H and CT1-H were tested at a high (2 × 10^6 p.f.u.) and low (5 × 10^5 p.f.u.) inoculum dose. After i.n. infection with 5 × 10^6 p.f.u. CT9-H and CT1-H, no weight loss or clinical signs were observed and no virus was recovered from lung or brain tissue. After i.n. infection with 2 × 10^7 p.f.u. CT1-H, again no weight loss or clinical signs were observed and no virus was recovered from lung or brain tissue. In contrast, after i.n. infection with 2 × 10^7 p.f.u. CT9-H, virus was found in brain and lung tissue. Virus titres in lung tissue declined rapidly, whereas in brain tissue, virus replication was observed from day 4 to 6 (Fig. 1). However, in comparison to VSV-H, CT9-H was attenuated, with peak titres on day 5 of 10^4.9 ± 0.9 TCID_{50} g^{-1} (instead of 10^{6.3 ± 1.5} TCID_{50} g^{-1}) and virus replication in brain for 3 days (instead of 4 days).

Differences in the ability of CT9-H and CT1-H to induce protective immune responses in the absence and presence of maternal antibodies

In vitro, VSV-H replication is not inhibited by MV-specific antibodies and this correlates with its capacity to immunize in the presence of MV-specific antibodies in vivo. Therefore, virus replication of CT9-H and CT1-H was tested in the presence of MV-specific antibodies in vitro. Similar to VSV-H, replication of both viruses was found to be only slightly delayed by MV-specific antibodies in vitro (data not shown).

To test the immunogenicity and protective capacity of CT9-H and CT1-H, naive cotton rats were immunized i.n. with 5 × 10^5 and 2 × 10^7 p.f.u. L.n. immunization of seronegative animals with 2 × 10^7 p.f.u. CT9-H and CT1-H resulted in titres of MV-neutralizing antibodies and reduction in virus titres in lung tissue (Table 1) comparable to those after immunization with VSV-H (Schlereth et al., 2000b). As with VSV-H, CT9-H induced neutralizing antibodies in the presence of MV-specific antibodies and protection against challenge after i.n. immunization (Table 1). In contrast, i.n. immunization with CT1-H in the presence of MV-specific antibodies resulted in lower titres of MV-neutralizing antibodies (P < 0.0001 compared to CT9-H). Immunization of seronegative animals with 5 × 10^5 p.f.u. CT9-H and CT1-H was clearly different in efficiency. The induction of MV-neutralizing antibodies by CT9-H (NT titre 610 ± 300) and protection against challenge were comparable to immunization with the high-dose inoculum (2 × 10^7 p.f.u.). In contrast, after immunization with 5 × 10^5 p.f.u. CT1-H, the titre of neutralizing antibodies was clearly reduced (NT titre 130 ± 90), as was protection (P < 0.02 compared to CT9-H). L.n. immunization with 5 × 10^5 p.f.u. CT1-H in the presence of MV-specific antibodies did not induce MV-specific or -neutralizing antibodies, nor did it induce protection against i.n. challenge with MV (Table 1 and Fig. 4). In contrast, immunization with 5 × 10^5 p.f.u.
CT9-H induced MV-specific and -neutralizing antibodies and protection against i.n. challenge.

**DISCUSSION**

All live vaccine viruses used currently have been derived from pathogenic viruses via tissue culture passages and tested for their appropriate attenuation in patients. For some viruses, like respiratory syncytial virus, the search for a variant that proves to be both protective against disease and not pathogenic was not successful so far. For MV, passaging first resulted in a live attenuated vaccine with residual virulence and this vaccine virus was subsequently attenuated further (Katz, 1995). In seronegative individuals, this vaccine is effective and, with the highly sensitive plaque reduction neutralization assay (PRNT), a titre of > 120 was shown to be protective (Chen et al., 1990). For the sake of a rapid diagnostic evaluation of sera, protective antibody levels are often expressed as ELISA units (IU ml$^{-1}$), even if neutralizing antibodies are not measured. In experimental systems that evaluate new vector systems, the two assays should be used separately because, for example, a non-protective nucleocapsid-specific serum has been shown to have high ELISA units but no NT titre (Schlereth et al., 2000a). The titres obtained by PRNT are 10-fold higher than titres obtained by NT (Chen et al., 1990). NT titres of 10 and above have proven to be protective in cotton rats (Schereth et al., 2000b), similar to the situation in humans. In cotton rats (as in humans) (Chen et al., 1990), there is not a linear increase in protection with increasing antibody titre and residual virus is always found in lung tissue (Schlereth et al., 2000a, b).

So far, neutralizing antibodies are the only proven entity of the immune system to protect against measles, although it has always been assumed that T cell responses play a part because patients with defects in the T cell response have not been able to clear the virus (reviewed by van Els & Nanan, 2002). However, in cotton rats, the induction of a strong CD4 T cell response against the nucleocapsid by plasmid immunization did not lead to protection (Schlereth et al., 2000a). Immunization in the presence of MV-specific antibodies inhibits completely the generation of neutralizing antibodies in cotton rats (Schlereth et al., 2000b) and strongly reduces the CD4 T cell response. Although reduced, the T cell response is still present but is not protective against i.n. infection with MV (unpublished results). For this reason, we have concentrated our efforts on a vector system known to induce high levels of neutralizing antibodies.

In humans, the attenuated vaccine virus does not induce protection in the presence of maternal antibodies (Katz, 1995). To overcome this problem, MV proteins have been expressed in vector systems derived from attenuated viruses (Durbin et al., 2000; Wyde et al., 2000; Stittelaar et al., 2000; Weidinger et al., 2001) and plasmids (Schlereth et al., 2000a; Polack et al., 2000). In experimental models, like monkeys and cotton rats, these vectors induced protective immunity in seronegative animals. In the presence of maternal antibodies, however, they were either not tested or failed to induce a good immune response after single immunization (Stittelaar et al., 2000; Weidinger et al., 2001; Schlereth et al., 2000a). The only system in our hands to overcome this problem was a recombinant VSV expressing MV-H as a passenger protein (Schlereth et al., 2000b). In seronegative animals, a good immune response was induced by live virus. In contrast, UV-inactivated virus was not able to induce an immune response at all. Although live VSV-H is able to induce active immunity in the presence of MV-specific antibodies, the generation of neutralizing antibodies

<table>
<thead>
<tr>
<th>Virus dose (p.f.u.)</th>
<th>Virus</th>
<th>Transfer of human MV-specific antibodies (IU)</th>
<th>NT assay (titre ± SD)</th>
<th>MV titre (log$<em>{10}$ TCID$</em>{50}$ g$^{-1}$ of lung tissue ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>0</td>
<td>&lt;10</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>2 x 10$^7$</td>
<td>VSV-H</td>
<td>0</td>
<td>410 ± 70</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>2 x 10$^7$</td>
<td>CT9-H</td>
<td>0</td>
<td>410 ± 190</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>2 x 10$^7$</td>
<td>CT1-H</td>
<td>0</td>
<td>540 ± 330</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>2 x 10$^7$</td>
<td>VSV-H</td>
<td>16</td>
<td>60 ± 30</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>2 x 10$^7$</td>
<td>CT9-H</td>
<td>16</td>
<td>60 ± 20</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>2 x 10$^7$</td>
<td>CT1-H</td>
<td>16</td>
<td>10 ± 5</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>5 x 10$^5$</td>
<td>CT9-H</td>
<td>0</td>
<td>610 ± 300</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>5 x 10$^5$</td>
<td>CT1-H</td>
<td>0</td>
<td>130 ± 90</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>5 x 10$^5$</td>
<td>VSV-H</td>
<td>16</td>
<td>18 ± 4</td>
<td>ND</td>
</tr>
<tr>
<td>5 x 10$^5$</td>
<td>CT9-H</td>
<td>16</td>
<td>33 ± 11</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>5 x 10$^5$</td>
<td>CT1-H</td>
<td>16</td>
<td>&lt;10</td>
<td>4.9 ± 0.2</td>
</tr>
</tbody>
</table>

**Table 1. Comparison of immunization with VSV-H and CT9-H in the absence and presence of MV-specific antibodies**

Cotton rats (three to five per group) were immunized i.n. with either 2 x 10$^7$ or 5 x 10$^5$ p.f.u. VSV-H, CT9-H or CT1-H in the absence of MV-specific antibody or 1 day after transfer of 16 IU human MV-specific antibodies. At 8 weeks after immunization, serum samples were taken to determine neutralizing antibodies and animals challenged i.n. with MV (strain HU2). After 5 days, virus titres were determined from lung tissues. ND, Not done.
is reduced 10-fold. In addition, the route of immunization is important. i.n. immunization, but not i.p. (Schlereth et al., 2000a) or oral immunization, in the presence of MV-specific antibodies (unpublished results) led to protection against measles.

Because VSV-H at high titres was still able to infect the brain and induce weight loss, the contribution of brain versus lung infection in inducing immunity in the presence of MV-specific antibodies was evaluated. After i.c. inoculation of small volumes of virus, no immune response was observed. This is in line with previous observations that inoculation of virus into the draining cerebrospinal fluid induces immune responses, whereas inoculation into brain parenchyma does not (Stevenson et al., 1997). In contrast, i.t. immunization induced immune responses comparable to i.n. immunization. Why replication of VSV in lung tissue is able to induce a reduced (in comparison to naive animals) but protective immune response in the presence of MV-specific antibodies is not clear. It is possible that the lung is a very conducive environment in that the virus here is able to target dendritic cells at a high frequency and that local replication induces a number of immune stimulatory molecules (e.g. interferons).

In addition, there might be an advantage for the virus to be relatively inaccessible to serum IgG. The delayed appearance of neutralizing antibodies in the circulation might also be explained by the induction of the immune response at the respiratory mucosa.

As immunization via the respiratory mucosa was important and this may lead to infection of the brain via the olfactory nerve, VSV-H was attenuated further by molecular means. It has been shown that the G protein of VSV directs budding (and thereby replication) efficiency of the virus. There do not appear to be specific interactions of the cytoplasmic tail in this process but mutants with a truncated cytoplasmic tail (9 or 1 aa instead of 29 aa) produce fewer virus particles per infected cell (Schnell et al., 1998). CT9-H never induced weight loss or clinical signs in cotton rats. After inoculation at high titre, CT9-H grew in brain tissue but was not found in the brain after low-dose inoculation. For cotton rats, CT1-H was not found in the brain but was slightly overattenuated in that it did not induce as good an immunity at low doses in seronegative animals as CT9-H. Immunization in the presence of MV-specific antibodies always leads to a strong reduction in immune responses (Schlereth et al., 2000b). In the presence of MV-specific antibody, a low dose of CT1-H was not able to induce immunity to MV but a higher dose did yield partial protection. Thus, CT1-H is overattenuated as a MV vaccine vector when used in the presence of MV-specific antibodies. However, this problem can be overcome, at least partially, by increasing the virus dose.

VSV has not only been used to efficiently express MV-H but also for a variety of different viral proteins that proved to be immunogenic in rodents. In addition, VSVs expressing proteins of simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV) have been successful in protecting rhesus macaques against AIDS after challenge with the highly pathogenic SIV/HIV chimera, SHIV 89.6P (Rose et al., 2001). There has been no evidence in this model

![Fig. 4. Comparison of immunization with a low-dose inoculum (5 x 10^5 p.f.u.) of CT9-H and CT1-H in the presence of MV-specific antibodies. Cotton rats were immunized i.n. with 5 x 10^5 p.f.u. CT9-H or CT1-H at 1 day after transfer of 16 IU human MV-specific antibodies. Serum samples were taken at weekly intervals and 8 weeks after immunization animals were challenged i.n. with MV (strain HU2). After 5 days, virus titres were determined from lung tissues. The difference in virus titres from animals immunized with CT1-H or CT9-H was significant (P<0.001).](image-url)
for brain infection in adult monkeys after i.n. inoculation of VSV vectors.

Based on our results in the cotton rat model, we feel that studies in a non-human primate model for measles (such as the infant rhesus macaque model) (Zhu et al., 2000) should be undertaken with VSV-H and the more attenuated CT1-H and CT9-H mutants as well. If these studies show protection in the presence of maternal antibody to measles, the vector system should be moved to human clinical trials.

ACKNOWLEDGEMENTS

This work was supported in part by Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie, Deutsche Forschungsgemeinschaft and US National Institutes of Health Grant AI24345 to J. K. R.

REFERENCES


