Measles Virus-Induced Immune Suppression in the Cotton Rat (Sigmodon hispidus) Model Depends on Viral Glycoproteins

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Received 5 May 1997/Accepted 27 June 1997

Measles virus (MV) infection is still among the primary causes of infant death in the third world. The acute infection with fever and rash is often complicated by respiratory symptoms (32) such as laryngotracheitis and pneumonia, diarrhea (25), and postinfectious encephalitis (16). During infection, a marked immune suppression is observed which leads to a high incidence of bacterial and parasitic superinfections with often lethal outcome (see reference 9 for a review). Delayed-type hypersensitivity reactions have been reported to disappear (28, 38), and this correlates with evidence obtained in vitro that the proliferative capacity of peripheral blood lymphocytes (PBL) in response to mitogens and antigens is also impaired (2, 13–15, 36).

The study of MV pathogenesis and immune suppression has been hampered by the lack of a suitable animal model. Although accidental and experimental infection of monkeys reproduces acute measles (1, 4, 12, 22, 40), their use is restricted by high costs, the lack of an inbred population, and limited availability. In hamsters (6), mice (10, 27), and rats (20), encephalitis can be induced with a rodent-adapted neurotropic strain of MV but the infection does not spread to the periphery (10, 24). In severe combined immunodeficiency (SCID) mice lacking a functional immune system, engrafted human thymus tissue and transferred human PBL survive. These so-called SCID-hu mice have been used to study MV-induced apoptosis (3) and immune suppression (39) in vivo. However, no antigen-specific responses are generated in these animals, as human lymphocytes are not able to interact with (stromal) tissue in vivo. The cotton rat (Sigmodon hispidus) has been described by Wyde and colleagues (43) as an alternative small-animal model which is susceptible to MV infection but is immunologically ill defined. They have used outbred cotton rats which were infected intranasally with MV (strain Edmonston) and recovered virus from lung tissue and bronchial lavage cells for 7 days. Histologically, interstitial infiltrates have been observed. By using inbred cotton rats, we were able to demonstrate that acute MV infection induces immune suppression in this animal model. As in humans, the proliferation of spleen cells in response to mitogens is inhibited. The extent of proliferation inhibition correlates with viral titers recovered from lung tissue. Proliferation inhibition is linked to the expression of the MV glycoproteins fusion protein and hemagglutinin.

MATERIALS AND METHODS

Animals. Cotton rats (inbred strain COTTON/Nico) were obtained from Iffa Credo. Animals 3 weeks to 7 months of age of both sexes were used. The animals were specified as pathogen free according to the breeder; monitored for the presence of mouse hepatitis virus, Thiel’s virus, reovirus 3, pneumonia virus of mice, Sendai virus, and minute virus of mice; and maintained in a barrier system. Animals were kept under controlled environmental conditions of 22 ± 1°C, 55 to 60% humidity, and a 12-h light cycle.

Cells, viruses, and plasmids. African Green monkeys (Cercopithecus aethiops) were grown in minimal essential medium (MEM) with 5% fetal calf serum (FCS), and 293 (human transformed primary embryonal kidney) cells were grown in MEM–10% FCS. 293 cells stably expressing T7 polymerase H (293-H), or F (293-F) were made as described previously (35). The MV HU2 strain was isolated from a child with measles-induced encephalitis after vaccination with Schwarz vaccine (from B. Rima, Belfast, Ireland) (31). The rodent-adapted neurotropic CAM/RBH strain has been used in rats (20) and mice (27) to induce encephalitis and has a sequence similar to that of the Edmonston vaccine strain, except for the H protein. The recombinant MV in which the H and F glycoproteins are replaced with the G protein of vesicular stomatitis virus (Mov) (37) and the molecularly cloned Edmonston (EdTag) strain (28) were kindly provided by M. Billeter. All Mov strains were passaged and titrated on Vero cells. All cells and virus stocks were free of mycoplasma (staining with Hoechst dye 33258).

The hemagglutinin gene from CAM/RBH was cloned into the BamHI site of pMV100 (42) under the control of the cytomegalovirus major immediate-early promoter (pMV87), and the fusion gene from the Edmonston strain was cloned into the XbaI site (pMV93). Expression was verified by immunofluorescence assay.

Virus purification. Virus and viral glycoproteins were purified on a sucrose gradient as described previously (31). Purified virus was UV inactivated (5 J/cm² for 5 min), and 100 µg of virus protein did not contain infectious virus as tested on Vero cells.

Transfection. 293-F and 293-H cells were transfected with Lipofectin (Gibco BRL) in accordance with the manufacturer’s recommendations. Five million cells, 5 µg of plasmid, 10 µl of Lipofectin, and 1 ml of Opti-MEM were mixed by gentle pipetting and left for 4 h. Afterwards, MEM containing 10% FCS was added and cells were incubated overnight. Cells were infected into cotton rats when 80% showed cell fusion as estimated by light microscopy examination.

Infection of cotton rats and virus titration. For intranasal (i.n.) and intraperitoneal (i.p.) infection, MV was given in phosphate-buffered saline (PBS) to ether-anesthetized cotton rats. For i.n. inoculations, virus was administered in a volume of not more than 100 µl and for i.p. delivery, MV was injected in a 1-ml volume. Four days later, animals were asphyxiated by using CO₂ and lungs were
removed and weighed. Lung tissue was minced with scissors and dounced with a glass homogenizer. Serial 10-fold dilutions of virus-containing supernatant were assessed for the presence and levels of infectious virus in a 48-well microassay using Vero cells and a cytopathic effect as the end point. Plates were scored microscopically for a cytopathic effect after 7 days. The amount of virus in inocula was expressed as the quantity of virus that could infect 50% of inoculated tissue culture monolayers (TCID₅₀). TCID₅₀ was calculated as described by Reed and Muench (29). For i.p. infection, 10⁶ PFU of virus was used, and for i.p. inoculation 10⁷ and 10⁸ PFU were injected in 1 ml of PBS. Four days later, spleen cells were tested in a proliferation assay.

**Proliferation assay.** Spleen cells from infected and mock-infected animals were plated in triplicate at 5 × 10⁶ cells/well in a 96-well plate in RPMI 1640 with 10% FCS and were left untreated (medium) or mitogen was added. After 48 h, 0.5 μCi of [³H]thymidine/well was added and 16 to 20 h later cells were harvested onto glass filters and counted with a Betaplate Counter (Wallac). Variation between wells usually did not exceed 10%. Counts of Background wells (medium) ranged from 500 to 3,000 cpm, and those of mitogen-stimulated cells ranged from 80,000 to 250,000 cpm. The stimulation index was calculated by dividing the mean of proliferation of mitogen-stimulated cells (in counts per minute) by the proliferation of cells in medium (in counts per minute). The percentage of proliferation inhibition was determined by comparing the stimulation indices of an infected animal with that of a mock-infected animal. 293 cells were plated out at 5 × 10⁶ cells/well, and 5 × 10⁶ cotton rat spleen cells were added with and without mitogen. Optimal mitogenic concentrations in the cotton rat were determined for concanavalin A (ConA; 2.5 μg/ml), lectin from Vitis sativa (5 μg/ml), Phascolarctos vulgatus phytohemagglutinin (PHA; 10 μg/ml) (all T-cell mitogens), Phytophthora americana lectin (pokeweed mitogen [PWM]; 0.5 μg/ml) (T- and B-cell mitogen), and lipopolysaccharide (LPS) from Salmonella typhosa (10 μg/ml) (B-cell mitogen). All mitogens were purchased from Sigma.

**Reverse transcriptase (RT)-PCR.** mRNA was extracted from organs with an RNeasy Total RNA Kit (Qiagen) as specified by the manufacturer, and cDNA synthesis and PCR were performed by standard procedures. A primary amplification used F (plus strand, 5'-GGTTTAACGAGCACTAGCAT-3') and N (minus strand, 5'-CCCTTGTTCTCGAACCATCC-3') primers used for the amplification of N (plus strand, 5'-GGAGTCTCCAGGTCAATTGA-3') and N (minus strand, 5'-GGTTTGTTCTCGAACCATCC-3') and with 20 cycles of 95°C for 5 min, 55°C for 2 min, and 72°C for 1.5 min was used. A secondary amplification with 40 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 1.5 min was used for the amplification of N (plus strand, 5'-GGAGTCTCCAGGTCAATTGA-3'; minus strand, 5'-GGTTTGTTCTCGAACCATCC-3'). The primers used for fusion gene RT-PCR are able to detect 1 MV (strain Edmonston)-infected 293 cell in 10³; the nested primer set specific for the nucleocapsid RNA detects up to 1 cell in 10⁶.

**RESULTS**

Minimal infectious dose, kinetics of MV replication and dissemination in inbred cotton rats. To confirm the results obtained by Wyde et al. (43) with outbred cotton rats after MV infection, we used the COTTON/Nico inbred strain. First, we studied virus dissemination within inbred cotton rats, kinetics of viral replication, and the minimal infectious dose. Animals were infected i.n. with inocula ranging from 2 × 10³ to 4 × 10⁶ PFU of the Edmonston strain of MV. We analyzed spleen, blood, bone marrow, and brain on days 4 and 7 by RT-PCR for the presence of MV RNA and by cocultivation of homogenates for the presence of virus. With the less sensitive F primers, only lung tissue was PCR positive, but with the N-specific primers, positive signals were found in the organs tested (spleen, bone marrow, brain, and lung). However, after perfusion with PBS the signals disappeared. This indicates that these organs were not productively infected but that the virus is disseminated by leukocytes. Lung tissue remained PCR positive (with the F primer) after perfusion, and after cocultivation, virus could be recovered from lung tissue but not from other organs. The lung is therefore the main locus of virus replication. The threshold of detection of the F and N primer set indicates that between 1 cell in 10⁶ and 1 in 10⁴ cells is infected. After intranasal inoculation of 2 × 10⁶ to 4 × 10⁶ PFU of the Edmonston strain, virus titers reached a peak in lung tissue on days 4 to 5 and dropped toward day 10 (Fig. 1a). A minimal infectious dose of 5 × 10⁴ PFU was needed to recover virus from lung homogenates on day 4 in a reproducible fashion, although inocula as small as 10³ PFU resulted in virus recovery in individual animals.

Infection with different MV strains. We compared various MV isolates for the capacity to grow in cotton rat lung tissue. In agreement with Wyde and colleagues (43), we found that wild-type MV isolates such as Chicago 1 and WTF could rarely be reisolated from lung tissues of cotton rats (data not shown). We therefore compared various MV isolates (HU2, Edmonston, and CAM/RB) belonging to the same lineage group as the Edmonston strain in the cotton rat system. After i.n. infection (10⁶ PFU) with the various MV strains, titers in lung tissues from HU2-, Edmonston-, and CAM-infected animals were similar (6.05 ± 0.4, 6.03 ± 0.2, and 6.07 ± 0.3 TCID₅₀/g of lung tissue [mean ± standard deviation]).

**Impaired proliferation of spleen cells in MV-infected cotton rats.** During MV infection of humans, the response of PBL to mitogens is reduced. We examined the mitogenic response of spleen cells from cotton rats on various days postinfection.
by PCR (N primers) on day 4 and after injection, marked
or lung tissue. However, the spleen was positive for viral RNA
By cocultivation, no infectious virus was isolated from spleen
ulated. Spleen cells (5 \times 10^5/well) from an animal infected with 5 \times 10^6 PFU of the Edmonston strain (ED) and a mock-infected animal were stimulated with
ConA (2.5 \mu g/ml), lectin from V. sativa (V.S.; 5 \mu g/ml), and P. vulgaris PHA (10 
\mu g/ml), which stimulate T cells, or the P. americana (P.A.) lectin PWA (0.5 
\mu g/ml) (B- and T-cell mitogen) and S. typhosa LPS (10 \mu g/ml) (B-cell mitogen). Results of one representative experiment of four independent experiments are shown. SI, stimulation index.

Starting on day 3, the proliferative capacity of lymphocytes was inhibited. This suppression was marked on day 4 and was undetectable by day 10 (Fig. 1b). The extent of proliferation inhibition correlated with viral titers in lung tissue. To establish the minimum virus dose for proliferation inhibition, we infected cotton rats with inocula of 5 \times 10^3, 5 \times 10^4, 5 \times 10^5, and 1 \times 10^6 to 4 \times 10^6 PFU of MV (strain Edmonston) and tested the proliferative capacity of their spleen cells on day 4. The titer of the inoculum had to be at least 10^6 PFU (to reach a significance level of \( P < 0.05 \)) to inhibit mitogen-dependent proliferation (data not shown). Titer of 2 \times 10^6 to 4 \times 10^6 PFU reached \( P \) values of 0.01 and lower.

Inhibition of responses to different mitogens. We wanted to know whether the proliferation inhibition is a peculiarity of the mitogen ConA or a specific lymphocyte subset. Various B- and T-cell mitogens were tested for the capacity to stimulate cotton rat spleen cells. LPS and PHA were poor stimulators, whereas stimulation with PWA and V. sativa lectin resulted in good proliferation. Independent of the extent of proliferation with the various mitogens, proliferation inhibition was seen with spleen cells from infected animals (Fig. 2). This indicates that the proliferative capacity of all lymphocyte subsets is suppressed.

Induction of proliferation inhibition by inactivated virus. We wanted to know whether live virus and virus replication are needed to induce proliferation inhibition. To investigate this, we used UV-inactivated Edmonston virus to inoculate cotton rats i.n. This resulted in marked, dose-dependent proliferation inhibition (Fig. 3). Another approach used to assess the necessity of virus replication for the induction of proliferation inhibition was i.p. injection of 10^6 PFU of the Edmonston strain.

FIG. 2. Proliferation inhibition is independent of the lymphocyte subset stimulated. Spleen cells (5 \times 10^5/well) from an animal infected with 5 \times 10^6 PFU of the Edmonston strain (ED) and a mock-infected animal were stimulated with
ConA (2.5 \mu g/ml), lectin from V. sativa (V.S.; 5 \mu g/ml), and P. vulgaris PHA (10 
\mu g/ml), which stimulate T cells, or the P. americana (P.A.) lectin PWA (0.5 
\mu g/ml) (B- and T-cell mitogen) and S. typhosa LPS (10 \mu g/ml) (B-cell mitogen). Results of one representative experiment of four independent experiments are shown. SI, stimulation index.

Proliferation inhibition was seen (Fig. 4). The extent of proliferation inhibition was more pronounced (40 to 50% inhibition) than after i.n. (10 to 20% inhibition) application of 10^6 PFU.

M. V. hemagglutinin and fusion proteins induce proliferation inhibition in cotton rats. In a recent study (35), we demonstrated that in tissue culture the mitogen-dependent proliferation of human PBL was inhibited after contact with MV-infected cells. The coexpression of MV glycoproteins in transfected cells was sufficient to induce proliferation inhibition (35). To assess the importance of MV glycoproteins for the induction of proliferation inhibition in vivo, we infected cotton rats i.p. with MGV, an Edmonston strain-derived recombinant MV in which the glycoproteins were replaced with the vesicular stomatitis virus G protein. MGV did not induce proliferation inhibition, whereas molecularly cloned complete

FIG. 3. Proliferation inhibition induced by UV-inactivated MV. Cotton rats were inoculated i.n. with various amounts of UV-inactivated MV (Edmonston strain). Four days later, spleen cells were stimulated with ConA. The lymphocyte proliferation of animals inoculated with 0 \mu g represents 100%. Inoculations of 0 and 400 \mu g represent the mean (\pm the standard deviation) of five animals, and inoculation of 80 \mu g represents the mean (\pm the standard deviation) of four animals. In a paired two-tailed \( t \) test, the difference between inoculations of 0 and 80 \mu g is not significant whereas inoculation of 400 \mu g differs significantly from both (\( P < 0.0002 \) and \( P < 0.01 \)). Inoculation of 80 \mu g of purified MV glycoproteins never reduced proliferation, whereas inoculation of 400 \mu g resulted in 15% \pm 5% proliferation inhibition.

FIG. 4. Recombinant MV lacking the H and F proteins (MGV) does not induce proliferation inhibition. Cotton rats were injected i.p. with PBS (mock infection), 10^6 PFU of MGV, molecularly cloned strain Edmonston (EDtag), and strain Edmonston (laboratory strain). Four days later, spleen cells were stimulated with ConA. Lymphocyte proliferation in mock-infected animals was set at 100%. Each bar represents the mean (\pm the standard deviation) of five animals. In a paired two-tailed \( t \) test, the difference between EDtag and mock infection (\( P < 0.0001 \)), as well as the difference between the Edmonston strain and mock infection (\( P < 0.0005 \)), is significant.
Edmonston virus or normal Edmonston virus had the same immunosuppressive capacity after i.p. infection (Fig. 4). To further characterize the immunosuppressive capacity of MV, glycoproteins were separated on a sucrose gradient (30) and inoculated i.n. In comparison with UV-inactivated MV, the inhibitory effect was small (data not shown). In a different approach, we used 293 cells which were stably transfected and expressed a single glycoprotein or both the H and F proteins after cotransfection. Injection of 293 cells singly expressing the F protein or untransfected, or 293-F cells, coexpressing the H protein or untransfected, for four days later, spleen cells were stimulated with ConA. Animals injected with 293-T7 pol. cells were set at 100%. Results are given as means of three to five animals (± the standard deviation). In a paired two-tailed t test, the differences between injection of 293-T7 pol. cells with and without MV infection (P < 0.002), 293-F cells and 293-F cells cotransfected with H (P < 0.005), and 293-H cells and 293-H cells cotransfected with F (P < 0.004) were significant. (b) 293-T7 pol. cells (5 × 10⁵/well), either infected or uninfected; 293-H cells, coexpressing the F protein or protein, or 293-F cells, untransfected or coexpressing the H protein, were plated onto a 96-well plate. After adhesion, cells were UV irradiated, 5 × 10⁵ spleen cells from a naive cotton rat were added, and a proliferation assay was performed. Coincubation with 293-T7 pol. was set at 100%. Results are given as the mean of three animals (± the standard deviation). In a paired two-tailed t test, the differences between coincubation of 293-T7 pol. cells with and without MV infection (P < 0.01), 293-F cells and 293-F cells cotransfected with H (P < 0.02), and 293-H cells and 293-H cells cotransfected with F (P < 0.005) were significant.

Immune suppression during MV infection was already established in 1908, when von Pirquet observed the disappearance of the tuberculin reaction during MV infection and exacerbation of tuberculosis (38, 41). Ex vivo, PBL isolated from measles patients show a 20 to 80% reduced ability to proliferate in response mitogenic and antigenic stimulation (2, 13–15, 36). In the face of such strong immune suppression, it is surprising that only a very small proportion of peripheral blood mononuclear cells (PBMC) are infected (26). Recent work suggests that the majority of infected PBMC are monocyte-macrophages as determined by virus-specific RT-PCR (7). In the cotton rat model, similar data were obtained after MV infection. The observed proliferation inhibition of isolated lymphocytes was very strong, and the number of infected leukocytes was low, between 1 in 10³ and 1 in 10⁶ PBMC. This suggests that in cotton rats the immune suppression after MV infection is probably not directly related to the destruction of infected PBL by virus replication but rather is caused by an indirect mechanism(s).

In the past, the mechanisms by which MV infection leads to immune suppression have been studied intensively and the data obtained are controversial and support both direct and indirect effects of the virus on lymphocyte function (reviewed by Borrow and Oldstone [5]). Evidence has been presented that direct killing of activated T lymphocytes by MV may explain some of the observed immune suppression (33). On the other hand, viral replication may stimulate the production of immune regulatory molecules which interfere with the immunological functions of lymphocytes (18, 19). In addition, the interaction of MV with cell surface molecules of lymphocytes may induce intracellular events which block immune reactions (34, 35, 44). In this context, the observation of Karp et al. (17) is of interest. Isolated macrophages infected with MV and stimulated with LPS showed reduced secretion of interleukin 12. In addition, the cross-linking of CD46 molecules by C3b-complement was sufficient to induce this phenomenon. If it occurs in vivo, the reduced secretion of interleukin 12 might bias an MV-specific immune response toward a TH2 response, which has been speculated to be at the root of immune suppression (11). However, if simple cross-linking of CD46 (e.g., activation of complement) led to a TH2 response, one would expect this to occur also in bacterial infections.

The last two suggested pathways of immune suppression are based upon the expression and engagement of CD46 by hemagglutinin. As no protein homologous to CD46 is expressed in cotton rats (26a), an alternative mechanism is apparently used. Moreover, results obtained with recombinant MV lacking the viral glycoproteins and 293 cells expressing the viral glycoproteins demonstrate that both the MV H and F proteins are needed to induce immune suppression. A similar mechanism of MV-induced impairment of lymphocyte function was recently observed by us using an in vitro tissue culture system (35). Human PBL cocultured with cells infected with UV-inactivated MV showed reduced proliferation after mitogen-induced stimulation in the absence of soluble mediators. The same effect could be induced by cocultivation of PBL with transfected cells expressing MV glycoproteins. Besides inhibi-
tion of signalling pathways, apoptosis is another possible mechanism for proliferation inhibition. In tissue culture experiments, apoptosis has been observed in MV-infected cells (8). In vivo, in SCID-hu mice engrafted with human embryonic thymic tissue, MV-infected thymic epithelial cells induce apoptosis of thymocytes (3). In contrast, in SCID-hu mice supplemented with human PBL, no cell loss was observed but MV infection impaired immunoglobulin secretion of B cells from (preferentially) neonates (39). As a possible explanation, the investigators suggested a cell cycle arrest in the G0/G1 phase, as has been observed for MV-infected T and B cells in vitro (21, 23, 44).

In summary, we have shown that the expression of MV glycoproteins on cell surfaces is sufficient to induce proliferation inhibition of lymphocytes in inbred cotton rats. This observation supports the hypothesis that in acute measles the interaction of a relatively small number of infected cells with a large population of uninfected lymphocytes has a strong inhibitory effect on the immune response. With this animal model, we wish to investigate the influence of immune suppression on antigen-specific immune responses, as well as its consequences for secondary infections.

ACKNOWLEDGMENTS

We thank Martin Billetter and Hussein Y. Naim for providing MGV and information about it prior to publication. We thank Roberto Cattaneo for providing the transfected 293 cells. We are grateful to Michaela Götzellmann for technical assistance.

S.N. was supported by the Stiftungskrank insprogramm Infektionsforschung (Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie). This work was in part supported by the Deutsche Forschungsgemeinschaft, the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie, the Pfleger-Stiftung, and the World Health Organization.

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