Measles virus (MV)-induced immune suppression during acute measles often leads to secondary viral, bacterial and parasitic infections which severely complicate the course of disease. Previously, we have shown that cotton rats are a good animal model to study MV-induced immune suppression, where proliferation inhibition after ex vivo stimulation of cotton rat spleen cells is induced by the viral glycoproteins (fusion and haemagglutinin proteins). We have now tested a variety of putative mechanisms of MV-induced immune suppression in this animal model. Proliferation inhibition is not due to fusion mediated by the MV glycoproteins and subsequent lysis of cells. Other putative mechanisms like classical anergy (unresponsiveness towards IL-2) or apoptosis do not seem to play a role in MV-induced immune suppression. In contrast, it was shown that spleen cells from infected animals preferentially accumulate in the G0/G1 phase and progress more slowly through the cell cycle after mitogen stimulation in comparison to cells from non-infected animals. These data indicate a retardation of the cell cycle which is correlated with proliferation inhibition and might have severe consequences in mounting an effective immune response.

**Introduction**

During and for weeks after acute measles a severe immune suppression is observed in infected individuals. The tuberculin reaction disappears (von Pirquet, 1908) during the acute disease, ex vivo peripheral blood lymphocytes (PBL) show reduced responses towards antigen and mitogen stimulation and individuals are highly susceptible to secondary infections (for review see Griffin, 1995). As only few lymphocytes are infected during natural measles in vivo (Esolen et al., 1993; Nakayama et al., 1995) indirect mechanisms rather than virus-mediated destruction of lymphocytes seem a likely explanation. For the molecular basis of measles virus (MV)-induced immune suppression experimental evidence for a variety of putative mechanisms has been obtained. Ex vivo studies with PBL from MV-infected patients have shown that supplementation of interleukin-2 (IL-2) led to a reversal of proliferation inhibition indicating classical anergy as the cause of immune suppression (Ward & Griffin, 1991; Griffin et al., 1987). In tissue culture experiments, human PBL were inhibited in their response to mitogen after contact with MV-infected cells (Sanchez-Lanier et al., 1988). To induce this unresponsiveness expression of the haemagglutinin (H) and fusion (F) proteins is necessary and sufficient (Schlender et al., 1996). Expression of H and F on human cells leads to cell fusion (Nussbaum et al., 1996) and induces cell cycle arrest (Schnorr et al., 1997) in lymphocytes. This cell cycle arrest has been analysed in great detail in MV-infected B cells and T cells (McChesney et al., 1987, 1988; Yanagi et al., 1992). After mitogenic activation infected cells increase in volume, upregulate their mRNA synthesis and express cell surface activation markers such as MHC class II, CD71 (transferrin receptor) and CD25 (IL-2-R) (on T cells). After 48 to 72 h synthesis of total mRNA as well as the level of mRNA for histone 2B (a gene up-regulated during the S phase of the cell cycle) is reduced in comparison to uninfected cells. These data indicate an arrest in the late G1 phase of the cell cycle. However, the arrest is incomplete as a low frequency of T cells synthesizes DNA (Yanagi et al., 1992). In addition to induction of cell cycle arrest, MV infection has also been shown to induce apoptosis in tissue culture (Esolen et al., 1995; Fugier-Vivier et al., 1997) as well as in a SCID-hu mouse model (Auwaerter et al., 1996).

We use the cotton rat (*Sigmodon hispidus*) model to study MV-induced immune suppression (Niewiesk et al., 1997a) because these animals are the only rodents in which after
intranasal infection MV replicates in the respiratory tract. In cotton rats, infection with MV leads to inhibition of mitogen-stimulated proliferation of spleen cells ex vivo (Niewiesk et al., 1997a). Proliferation inhibition correlates with viral titres in lung tissue homogenates and is induced by MV glycoproteins. This has been demonstrated by injection of human fibroblast cells expressing both the MV H and fusion F proteins as well as by infecting cotton rats with a recombinant MV where the viral glycoproteins are replaced by the G protein of vesicular stomatitis virus (Niewiesk et al., 1997a). In this animal model we have addressed the question whether contact-mediated lysis, IL-2 deficiency, apoptosis or cell cycle arrest are the cause of ex vivo inhibition of mitogen-driven proliferation of cotton rat lymphocytes.

**Methods**

- **Animals.** Cotton rats (inbred strain COTTON/Nico) were obtained from Ifa Credo, France. Animals from 3 weeks up to 7 months of age were used. The animals were bought specific pathogen free according to the breeder’s specification. CD46 transgenic rats were produced as described (Niewiesk et al., 1997a). All animals were maintained in a barrier system. Sentinel mice or rats were examined serologically. Animals were kept under controlled environmental conditions of 22 ± 1 °C, 50 ± 10% humidity and a 12 h light cycle.

- **Cells, viruses and plasmids.** Vero cells (African green monkey) were grown in minimal essential medium (MEM) with 5% foetal calf serum (FCS), human osteosarcoma cells TK+ (Schlender et al., 1988) were grown and titrated according to standard protocols. MV strain Edmonston was passaged and titrated on Vero cells (African green monkey) and vaccinia virus on 143B (TK+) cells. All cells and virus stocks were serologically. Animals were kept under controlled environmental conditions of 22 ± 1 °C, 50 ± 10% humidity and a 12 h light cycle.

- **Transfection.** 293-F cells were transfected with Lipofectin (Gibco BRL) according to the manufacturer’s recommendations: 5 × 10⁶ cells, 5 µg plasmid (pMV87), 10 µl Lipofectin and 1 ml Opti-MEM were mixed by gentle pipetting and left for 4 h. Afterwards, MEM containing 10% FCS was added and cells incubated overnight. Cells were injected into cotton rats when 80% showed cell fusion as estimated by light microscopy examination. Aliquots were stained with monoclonal antibody L77 (H specific) and monoclonal antibody A 504 (F specific) and a secondary FITC-labelled donkey anti-mouse serum and analysed by flow cytometry. Usually, more than 80% of the cells expressed H and F.

- **Infection of cotton rats.** For intranasal (i.n.) and intraperitoneal (i.p.) infection MV (Edmonston strain) was given in PBS to ether-anesthetized cotton rats. In. inoculations of MV were administered in a volume of not more than 100 µl and for i.p. delivery, MV was injected in a 1 ml volume. For i.p. infection, 10⁸ p.f.u. virus was used and for i.p. injection 10³ 293 cells were injected in 1 ml PBS. It had previously been shown that immune suppression caused by MV can be induced by i.p. or i.n. infection as well as by injection of cells expressing both the MV H and F proteins (Niewiesk et al., 1997a). Four days later, animals were asphyxiated using CO₂; spleens were removed and spleen cells tested in a proliferation assay. For mock-infection PBS was used. No difference between mock-infected and non-infected animals was observed.

- **Proliferation assay.** Spleen cells from infected and mock-infected animals were plated in triplicate at 5 × 10³ cells per well in a 96-well-plate in RPMI 1640 with 10% FCS and were left untreated (medium control) or stimulated with mitogen [Concanavalin A (Con A); 2.5 µg/ml]. Where indicated IL-2 was added. After 40 h 0.5 µg [³H]thymidine per well was added and 16–20 h later cells were harvested onto glass-filters and counted with a Betaplate Counter (Wallac). The stimulation index (SI) was calculated as the mean of proliferation of mitogen-stimulated cells in c.p.m./proliferation of cells in medium in c.p.m.. The percentage of proliferation inhibition is expressed by comparing the stimulation indices of an infected to a mock-infected animal. Mock-infected animal are set as 100% and proliferation of cells from infected animals expressed accordingly.

- **Production and testing of IL-2.** Rat and cotton rat IL-2 were produced from spleen cells (10⁸/ml) incubated in RPMI-10% FCS with 5 × 10⁵ M β-mercaptoethanol and Con A (5 µg/ml for rat and 2.5 µg/ml for cotton rat cells). After 36 h cells were centrifuged, and to the harvested supernatant α-methylmannoside (10 mg/ml) was added. IL-2 content was measured using the IL-2-dependent CTLL clone 3 cell line and the optimal concentration (just enough to reach the plateau of the growth curve) was used. Human IL-2 was purchased from Eurocetus, Frankfurt, Germany.

- **Cell cycle analysis.** Mitogen-stimulated cotton rat spleen cells were mixed with detergent solution (0.1% Triton-X 100, 0.15 NaCl, 0.1 M HCl), centrifuged, resuspended in 50 µl RΝase (RΝase A 100 µg/ml, 1% trisodium citrate) and incubated at 37 °C for 15 min. Cells were washed in 0.1 M Tris–HCl pH 7.4 and stained for 10 min (50 µg/ml propidium iodide in 1% trisodium citrate) (Taylor & Milthorpe, 1980). For CFSE [5(6)-carboxyfluorescein diacetate succinimidyl ester] staining, mitogen-stimulated cotton rat spleen cells were resuspended at 5 × 10⁷/ml in RPMI with no protein. A 5 mM stock solution of CFSE in DMSO (stored at −20 °C) was added to a final concentration of 5 µM and incubated at 37 °C for 8 min. At the end of the incubation period, cells were immediately washed three times with RPMI–10% FCS (Lyons & Parish, 1994). After propidium iodide and CFSE staining lymphocytes were analysed by flow cytometry.

- **Fusion and lysis assay.** For the fusion assay P815 cells (mouse mastocytoma cell line) were infected with vaccinia virus recombinants expressing MV H and F or the HIV reverse transcriptase (m.o.i. 10). For 24 h after infection no fusion between infected P815 cells was observed. After overnight infection P815 cells were incubated at the indicated ratios with lymphocytes from CD40-transgenic rats, non-transgenic rats or cotton rats. After 6 h fusion was observed by light microscopy. For the lysis assay infected P815 cells were labelled with 37 MBq Na¹¹⁵CrO₄ (DuPont) for 80 min at 37 °C and washed twice. 10⁶ labelled target cells in a volume of 100 µl were added to varying numbers of spleen cells in 100 µl volumes in U-bottomed microtitre plates. After 6 h incubation at 37 °C, 100 µl supernatant was harvested and counted. The percentage of lysis was calculated as: 100 x (experimental — spontaneous release)/(total — spontaneous release).

- **Apoptosis assay.** For the inhibition of apoptosis, 100 µM Z-VAD-fmk (Enzyme Systems Product, Dublin, CA, USA) was dissolved in PBS-0.5% DMSO and added on day 0 to spleen cells for a proliferation assay.
As a control the same volume of PBS–0.5% DMSO was added to spleen cells.

Results

We have shown previously (Niewiesk et al., 1997a) that injection of cells expressing the MV H and F proteins induces proliferation inhibition in cotton rat spleen cells. This effect is dose dependent. Injection of $10^5$ cells induced a good proliferation inhibition; injection of $10^6$ cells induced less inhibition and $3 \times 10^5$ cells none at all (data not shown). To define the underlying mechanism we tested whether contact-mediated lysis, IL-2 deficiency, apoptosis or cell cycle arrest correlate with MV-induced proliferation inhibition.

Contact-mediated lysis does not contribute to MV-induced proliferation inhibition

MV-infected cells expressing the H and F proteins of a vaccine strain are able to fuse with non-infected cells expressing the receptor for MV vaccine strains, the human CD46 molecule (Nussbaum et al., 1995). Flow cytometry analysis with monoclonal antibodies as well as polyclonal antisera specific for human CD46 did not reveal a homologous structure on cotton rat lymphocytes (data not shown). However, it is not possible to exclude a functional homologue of human CD46 by antibody staining. In order to test for a functional homologue we incubated P815 cells (mouse mastocytoma cell line) expressing MV H and F proteins from a vaccinia virus recombinant with lymphocytes from a CD46-transgenic rat (Niewiesk et al., 1997b), lymphocytes from non-transgenic rats or cotton rat lymphocytes. After 6 h fusion between P815 cells expressing H and F and CD46-expressing lymphocytes occurred (data not shown). No fusion was seen between P815 cells expressing H and F and lymphocytes from non-transgenic rats or cotton rats. Neither did control P815 cells expressing the reverse transcriptase of HIV fuse with CD46-expressing rat lymphocytes (data not shown). HIV infection mediates fusion between infected and non-infected cells leading to unstable cell aggregates which rapidly undergo cell lysis (Ohnimus et al., 1997). We therefore incubated lymphocytes from CD46-transgenic or non-transgenic rats or cotton rats with chromium-labelled H- and F-expressing target cells. Only coculture of CD46-expressing lymphocytes resulted in lysis of H- and F-expressing P815 cells in a dose-dependent manner (Fig. 1). These data indicate that proliferation inhibition is not due to a direct lytic effect of infected cells or virus on cotton rat lymphocytes.

Proliferation inhibition in cotton rat spleen cells is not relieved after addition of IL-2

As expression of H and F is crucial for induction of proliferation inhibition, the contact of H and F with cotton rat lymphocytes might lead to anergy. Classically, anergic T cells are defined as being non-responsive to IL-2 (for review see Paul, 1993). Addition of recombinant human IL-2 (100 U/ml) or optimal concentrations of IL-2-containing rat Con A supernatant to mitogen-stimulated cotton rat lymphocytes did not relieve the difference in proliferation of spleen cells from infected versus mock-infected animals (Fig. 2). To exclude the possibility that specifically cotton rat IL-2 is needed we produced IL-2-containing Con A supernatant from cotton rat lymphocytes. Optimal concentrations of cotton rat IL-2 had no effect on MV-induced proliferation inhibition (Fig. 2). It is possible that lymphocytes from infected cells are no longer able to respond to IL-2 (because of, e.g., lack of IL-2 receptor). To test this possibility we used the fact that lymphocytes respond to stimulation with a suboptimal concentration of mitogen only in the presence of IL-2. As shown in Fig. 3 lymphocytes did not respond to suboptimal concentrations of Con A alone, but did so after addition of IL-2 (either human, rat or cotton rat). This indicates that spleen cells from infected animals were able to bind and respond to IL-2. However, the difference in proliferation between spleen cells from infected and non-infected animals remained. These data demonstrate that MV-induced proliferation inhibition is not overcome by addition of IL-2.

Apoptosis is not involved in MV-induced proliferation inhibition of cotton rat lymphocytes

Apoptosis has been observed in tissue culture after infection with MV (Esolen et al., 1995) as well as in hu-SCID mice (Auwaerter et al., 1996). It therefore seemed a possible
Addition of IL-2 does not relieve proliferation inhibition. Spleen cells from animals injected with $10^7$ 293–F cells or $10^7$ 293–F + H cells were taken on day 4 and stimulated with Con A (2.5 µg/ml) with or without addition of IL-2 from either humans, rats or cotton rats. Proliferation was measured as thymidine incorporation on day 3. The experiment shown is representative of six experiments with transfected 293 cells or MV infection ($2–3 \times 10^6$ p.f.u.).

Proliferation inhibition is not due to unresponsiveness to IL-2. Spleen cells from infected ($2–3 \times 10^6$ p.f.u.) and non-infected animals were taken on day 4 and stimulated with an optimal (2–5 µg/ml) or suboptimal (0–5 µg/ml) dose of Con A, IL-2 alone or IL-2 with a suboptimal (0–5 µg/ml) dose of Con A. Proliferation was measured as thymidine incorporation on day 3. The experiment shown is representative of three experiments.

Peptide inhibition of apoptosis does not relieve proliferation inhibition. Spleen cells from i.n. and i.p. infected ($2 \times 10^6$ p.f.u.) and non-infected animals were taken on day 4 and a proliferation assay was performed with or without addition of 100 µM ZVAD-fmk. The data shown represent the average of four experiments. Mechanism to explain MV-induced proliferation inhibition. However, the numbers of spleen cells isolated from infected versus non-infected animals did not differ. The caspase inhibitor Cbz-Val-Ala-Asp(Ome)-fluoromethyl ketone (ZVAD-fmk) has been shown to prevent apoptosis by blocking the activation of caspases in vivo (Rodriguez et al., 1996) and in vitro (Sarin et al., 1996). Addition of ZVAD-fmk to a proliferation assay increased c.p.m. from unstimulated cells or cells stimulated with mitogen by two to three fold. This was true for cells from infected and uninfected animals and indicates that apoptosis occurred in cell cultures of both. However, the stimulation indices (the ratio of c.p.m. stimulated/unstimulated cells) did not differ and therefore the difference in proliferation was unaffected (Fig. 4). Similarly, with the DNA fragmentation assay and the poly(ADP-ribose) polymerase (PARP) cleavage assay (Ohnimus et al., 1997) we found no difference in apoptosis between spleen cells from infected versus non-infected animals after removal of the spleen or on day 1, 2 and 3 after mitogen stimulation (data not shown).

MV infection induces cell cycle retardation in cotton rat spleen cells

In tissue culture MV infection leads to cell cycle arrest in the G$_0$/G$_1$ phase (McChesney et al., 1987, 1988; Yanagi et al., 1992). We attempted to correlate the proliferative capacity of spleen cells from either MV- or mock-infected cotton rats with the number of cells in the G$_0$/G$_1$ phase. After propidium iodide staining we analysed the DNA content of mitogen-stimulated lymphocytes by flow cytometry. The percentage of cells in the G$_0$/G$_1$ phase was higher in cells from infected animals (Fig. 5 a). Cells from mock-infected animals differed significantly from i.n. infected animals on day 3 and 4 and from i.p. infected animals on day 2 and 3 (Fig. 5 b). Proliferation inhibition of 40–60% was measured on day 3 and correlated with changes in the cell cycle. To investigate whether all cells replicate slowly or only some cells are arrested in the G$_0$/G$_1$ phase we used the fluorescent dye CSFE, which binds to cytosolic proteins and allows cell division over time to be followed (Lyons & Parish, 1994). Flow cytometry analysis showed that all cells from infected animals go through the cell cycle.
However, they progress more slowly in comparison with cells from non-infected animals (Fig. 6). This was also true if instead of Con A the superantigen SEC 3, which stimulates only a subpopulation of T cells, was used (data not shown).

**Discussion**

Immune suppression during acute measles is a well-documented phenomenon in man. *Ex vivo*, proliferation of PBL in response to mitogen and to antigen from infected individuals is inhibited (for review see Griffin, 1995). Addition of IL-2 has in some instances relieved this proliferation inhibition (Ward & Griffin, 1991; Griffin *et al.*, 1987). So far, no other data have been obtained from patients in respect to the mechanism of MV-induced immune suppression. In a SCID mouse model secretion of immunoglobulins by human B cells is reduced in infected animals and cell cycle arrest has been suggested to be responsible (Tishon *et al.*, 1996). An alternative mechanism has been found in SCID mice with implants of human foetal tissue (Auwaerter *et al.*, 1996). Infection of thymic epithelium with a strongly replicating wild-type strain of MV induced apoptosis in the implanted human thymocytes. Most putative mechanisms of MV-induced immune suppression are derived from tissue culture experiments. In contrast to the *in vivo* situation where virus is relatively scarce (Esolen *et al.*, 1993; Nakayama *et al.*, 1995), *in vitro* cultures are used where most or all of the cultured PBLs are infected (McChesney *et al.*, 1987, 1988; Yanagi *et al.*, 1992; Esolen *et al.*, 1995; Fugier-Vivier *et al.*, 1997). In an alternative approach UV-inactivated MV or inactivated MV-infected cells have been used to investigate immune suppression (Sanchez-Lanier *et al.*, 1988; Schlender *et al.*, 1996). With all these tissue culture systems various mechanisms such as contact-mediated lysis, IL-2 deficiency, apoptosis and cell cycle arrest have been observed and suggested to play a role *in vivo*.

*In vitro*, the presence of CD46, F and H are necessary to mediate fusion (Nussbaum *et al.*, 1995) and lysis (this paper). However, in a tissue culture system with human cells it was shown that CD46-negative cells are also susceptible to MV-induced immune suppression (Schlender *et al.*, 1996). Similarly, MV induces proliferation inhibition in cotton rats, which do not express molecules structurally similar to human CD46 on
Apoptosis is enhanced by contact with T cells and T cells become apoptotic themselves without being infected. Cultured Vero cells infected with MV become apoptotic, too (Esolen et al., 1995). In SCID mice with implants of human fetal thymic tissue infection of thymic epithelium with MV leads to thymocytes undergoing apoptosis (Auwaerter et al., 1996). So far, it is not clear whether apoptosis is an effect induced by MV in particular in contrast to other viruses or whether apoptosis of thymocytes, activated T cells and DCs is a general regulatory mechanism of the immune response. However, in cotton rats apoptosis does not seem to be responsible for MV-induced proliferation inhibition.

Lymphocytes have been demonstrated to arrest in the G0/G1 phase of the cell cycle after infection with MV (Mcchesney et al., 1987, 1988; Yanagi et al., 1992) or contact with MV-infected cells (Schnorr et al., 1997). In cotton rats, all spleen cells from infected animals divide more slowly than those from non-infected animals. This indicates that the observed ‘arrest’ is, rather, a retardation of cells in the G0/G1 phase. So far it is not known how MV affects the cell cycle. In vitro experiments with a two-chamber system have shown that MV-induced proliferation inhibition is induced by direct contact between lymphocytes and infected cells or cells expressing the viral glycoprotein (Schlender et al., 1996) and that soluble factors smaller than 70 kDa do not play a role. However, inhibition of antigen-specific T cell lines in vitro seems to be mediated by an unidentified 100 kDa protein (Sum et al., 1998). Therefore, the mechanism underlying the cell cycle retardation remains to be solved. Most likely the cell cycle retardation of T cells described here is only one of the factors contributing to MV-induced immune suppression in vivo. At least in vitro, the nucleocapsid protein inhibits B cell responses (Ravanel et al., 1997) and MV infection has been shown to induce aberrant cytokine expression in macrophages (Karp et al., 1996) and to reduce their ability to present antigen (Leopardi et al., 1993).

In summary, we have shown that the proliferation inhibition of lymphocytes from MV-infected cotton rats is due a retardation of the cell cycle and not to virus-mediated lysis, apoptosis or IL-2 deficiency.

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