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Persistent infection of RAW264.7 macrophages with the DA strain of Theiler's murine encephalomyelitis virus: An *in vitro* model to study viral persistence

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Theiler's murine encephalomyelitis virus (TMEV) is a member of the *Picornaviridae* family and causes a virus strain dependent pathology in the central nervous system of mice. The GDVII strain induces an acute and mostly fatal encephalomyelitis. In the few mice that survive, the virus is cleared by the immune system. In contrast, infection with the DA strain leads to a persistent infection, marked by inflammation and demyelination that resembles multiple sclerosis. In the DA-induced disease, macrophages play a crucial role because they contribute to demyelination by the secretion of toxic mediators. Moreover, they represent the main viral reservoir, hereby also underlining their essential role in TMEV persistence. The mechanism of this persistence is not yet understood and tools to investigate it directly, without the complexity imposed by experimental animals, are largely missing. By studying TMEV infection of RAW264.7 macrophages, we found that the DA strain establishes a persistent infection in these cells, in contrast to the neurovirulent GDVII strain. Whereas the GDVII strain was cleared within 4 to 5 days post infection, DA virions were still present after 1 year of cell cultivation. This persistently DA-infected macrophage cell line, which we have called DRAW, provides a model to investigate the interactions between the cellular and viral factors influencing persistence and to screen for anti-TMEV agents. *Journal of NeuroVirology* (2006) **12,** 108–115.

Keywords: *in vitro* model; macrophage; persistent infection; picornavirus; TMEV

Introduction

Theiler's murine encephalomyelitis virus (TMEV) is a member of the *Theilovirus* species belonging to the *Cardiovirus* genus within the *Picornaviridae* family (King *et al*, 2000). It is a natural pathogen of mice, mostly causing an asymptomatic enteric infection (Theiler, 1937). Only occasionally, the virus will spread to the central nervous system (CNS). Depending on the infecting virus strain, mice will either develop an acute and mostly fatal encephalomyelitis (neurovirulent strains GDVII and FA) or a chronic disease marked by inflammation and demyelination (persistent strains, e.g., DA and BeAn) (Lorch *et al*, 1981). Because of its clinical and histopathological resemblance with the human demyelinating disease multiple sclerosis (MS), infection of mice with the persistent strains has been recognized as an excellent animal model for studying MS (Oleszak *et al*, 2004). An important feature of this model is the continuous presence of virions during the animals' lifetime, which is believed to play a decisive role in the

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onset of demyelination (for review, see Brahic, 2002). Recently, Trottier *et al* (2004) showed that TMEV persistence requires active viral replication, thereby triggering Th1 cytokines that contribute to disease progression.

During the persistent infection, viruses can be found in oligodendrocytes and astrocytes, but mainly in macrophages (Lipton *et al*, 1995). The importance of this latter cell type is underlined by the finding that, if mice are treated with mannosylated liposomes to deplete the macrophages, the virus can no longer persist in the majority of mice and no demyelination is observed in these animals (Rossi *et al*, 1997).

The establishment of a persistent infection is the result of a complex interaction between the virus and its host, with the involvement of both viral and host determinants. The influence of the host genotype is highlighted by the fact that certain mouse strains are resistant to the persistent infection/demyelination, e.g., the C57BL/6 strain, whereas others, e.g., the SJL/J strain, are highly susceptible. Susceptibility to persistent infection/demyelination has been found to be multigenic, both involving *H*2 class I genes as well as non-*H*2 susceptibility loci (for review, see Brahic *et al*, 2005). Viral determinants of TMEV persistence have been linked to the capsid proteins VP1 and VP2 (Adami *et al*, 1998; Jarousse *et al*, 1994), and although the TMEV receptor remains to be identified, the neurovirulent and persistent TMEV strains have been found to use different carbohydrate coreceptors (Reddi and Lipton, 2002; Shah and Lipton, 2002), pointing both towards a role for cellular tropism as well as for the viral capsid in the establishment of a persistent infection. The non-structural TMEV proteins L and L* also play a role in persistence. The L protein inhibits the transcription of early interferon (IFN) genes (van Pesch *et al*, 2001) and interferes with the nucleocytoplasmic trafficking of cellular proteins (Delhaye *et al*, 2004), whereas the L* protein facilitates the infection of macrophages (Takata *et al*, 1998) and has an antiapoptotic effect (Ghadge *et al*, 1998). However, the interactions between the host and viral factors directing persistence remain poorly understood and need further clarification.

Here, we describe an *in vitro* model based on the persistent infection of macrophages with the DA strain of TMEV that has the potential to investigate these factors and their interactions more directly, thereby allowing a deeper insight in the mechanism of viral persistence.

Results

Growth characteristics of L929 fibroblasts versus RAW264.7 macrophages after TMEV infection

Because macrophages play an important role in TMEV persistence (Lipton *et al*, 1995; Rossi *et al*, 1997), we compared the effect of infection with the neurovirulent GDVII strain and the persistent DA strain on the growth of L929 fibroblasts (Figure 1A) versus RAW264.7 macrophages (Figure 1B). L929 cells were chosen as comparative cell line, because they are permissive for TMEV and allow a productive infection. Cells were seeded in 96-well plates at 2×10^4 cells/well and infected with GDVII or DA at 10 plaque forming units (PFU)/cell. Phosphate-buffered saline (PBS)-treated cells functioned as mock-infected control, whereas camptothecin treatment of cells, which induces apoptosis, served as an indicator of cytotoxicity. The amount of cells was counted at the times indicated using a Bürker chamber.

As shown in Figure 1A, infection of L929 cells with the GDVII and DA strain, as well as treatment with camptothecin, resulted in a reduction of cells, whereas mock-infected L929 cells displayed a continuous growth. In contrast to L929 cells, TMEV-infected RAW264.7 macrophages showed only restricted cytopathic effects, because the growth rates of GDVII or DA-infected macrophages and the mock-infected ones were comparable (Figure 1B). As with the L929 fibroblasts, camptothecin treatment of the RAW264.7 cells resulted in a progressive decrease of the amount of cells.

These observations demonstrate that TMEV infection of cells has a differential, cell line–dependent outcome. Whereas L929 cells mainly died as a result of TMEV infection, within the observed period, macrophages survived the infection with almost unaffected growth rates as compared to the mock-infected controls.

Metabolic activity of L929 fibroblasts versus RAW264.7 macrophages after TMEV infection

In order to confirm the results obtained by counting the amount of cells (Figure 1A, B), we assessed the metabolic activity of both L929 (Figure 1C) and RAW264.7 cells (Figure 1D) using a cell viability assay (CellTiter-Blue; Promega), which is based on the reduction of resazurin into the fluorescent resorufin. This only occurs in viable cells, whereas nonviable cells will generate no or only low fluorescence due to the loss of their metabolic capacity.

Cells, seeded at 2×10^4 cells/well, were again infected with 10 PFU/cell GDVII or DA and treated with PBS or camptothecin and followed over a period of 4 days. Results were expressed as a percentage of the uninfected, PBS-treated cells. In line with the results of the cell counting (Figure 1A), TMEV-infected and camptothecin-treated L929 cells (Figure 1C) both exhibited decreased cell viability over time.

In RAW264.7 cells, infection with GDVII as well as DA generated increasing fluorescence signals that exceeded the signal of the uninfected controls (Figure 1D). This might reflect an enhanced metabolic state of the RAW264.7 macrophages following infection. After 3 days, the metabolic activity of the infected macrophages equaled the values of the mock-infected controls. Camptothecin treatment

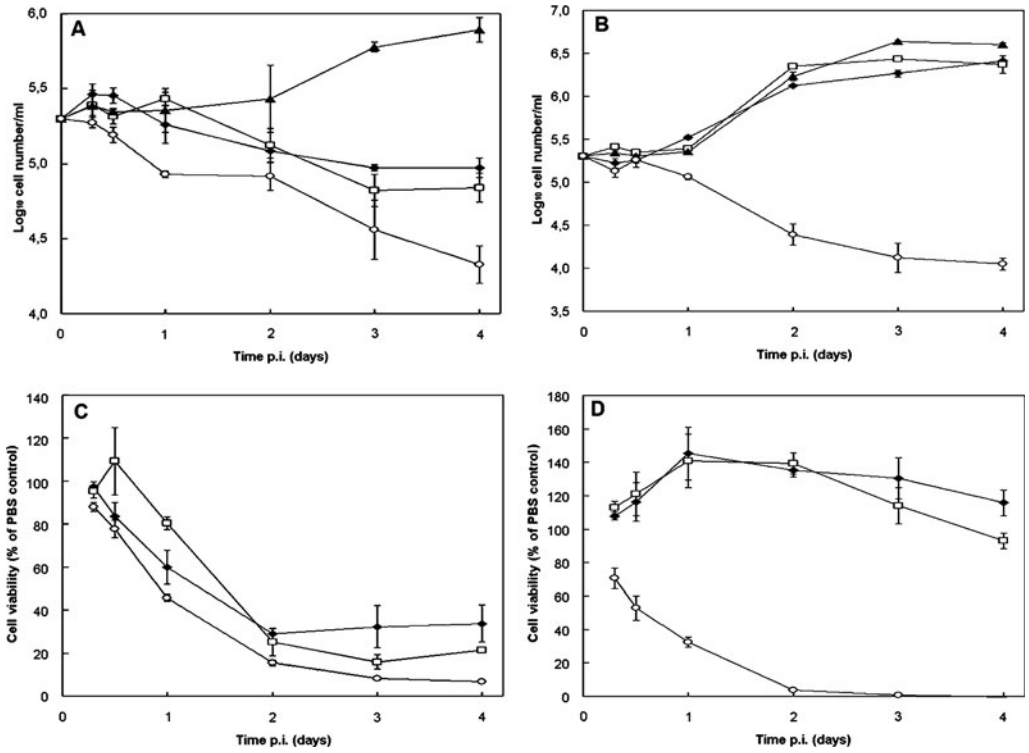

Figure 1 Effect of TMEV infection on cell growth (**A**, **B**) and cellular metabolic activity (**C**, **D**) in L929 fibroblasts (**A**, **C**) and RAW264.7 macrophages (**B**, **D**) as function of time. Cells (2×10^4/well) in 96-well tissue culture plates were infected with GDVII (♦) or DA (□) at 10 PFU/cell, PBS mock-infected (▲), or treated with 10 μM camptothecin (○). Quadruplicate samples were counted using a Bürker chamber (**A**, **B**). Cell viability was assayed using Promega's CellTiter-Blue kit on triplicate samples and expressed as a percent of PBS control values (**C**, **D**). All data are presented as the mean ± standard deviation (error bars).

rapidly decreased cell viability, resulting in a total loss of this function after 3 days.

When compared to infected L929 cells (Figure 1C), infected macrophages (Figure 1D) again displayed a significantly different behavior because they retained a high viability after TMEV infection. These results are in accordance with those from the cell counts.

Viral growth kinetics in RAW264.7 macrophages

Because TMEV-infected RAW264.7 macrophages showed a continuous growth, without the extensive cytopathic effects observed in the infected L929 fibroblasts, our aim was to rule out the possibility that the macrophages didn't succumb after TMEV infection due to a lack of virus replication. Therefore, we infected these cells with the GDVII and DA strain at 10 PFU/cell and determined the amount of infectious virus in function of time via plaque assay (Figure 2). After an initial lag phase as a consequence of uncoating, viral titers rose for both TMEV strains from 8 h post infection (p.i.), which proves that RAW264.7 cells supported their replication. In addition, at 12 h p.i. viral titers were comparable for both strains, indicating that their replication efficiency is not influenced by a different coreceptor use because the neurovirulent strains bind to heparan sulfate, whereas the demyelinating strains bind to sialic acid (Reddi and Lipton, 2002; Shah and Lipton, 2002). However, in case of infection with the GDVII strain, viral replication reached a maximum between 12 and 24 h p.i., with a maximum production of ca. 0.1 PFU/cell. Thereafter, a gradual decrease of infectivity was observed. At 5 days p.i., no infectious virus could be recovered anymore at all. Infection with the DA strain on the other hand, resulted in a continuous production of new virions, thereby reaching higher viral titers than observed for the GDVII strain. Between 48 and 72 h p.i., corresponding with the replication maximum of the DA strain, the DA-infected macrophages produced ca. 1000 times more infectious virus as compared to the GDVII-infected macrophages (1 PFU/cell versus 0.001 PFU/cell).

Establishment of persistently TMEV strain DA-infected RAW264.7 macrophages

In order to investigate how viral titers would evolve over a longer period than 5 days, the DA-infected RAW264.7 macrophages were further passaged. The procedure consisted of detaching the cells by manual shaking and transferring a portion of these cells to new plastic recipients with the subsequent addition of fresh medium. On several occasions, samples were withdrawn from the cultures and submitted to plaque assay. Infectious virus was always present, even after 1 year of passaging the cells. Within this period, viral titers fluctuated depending on the time

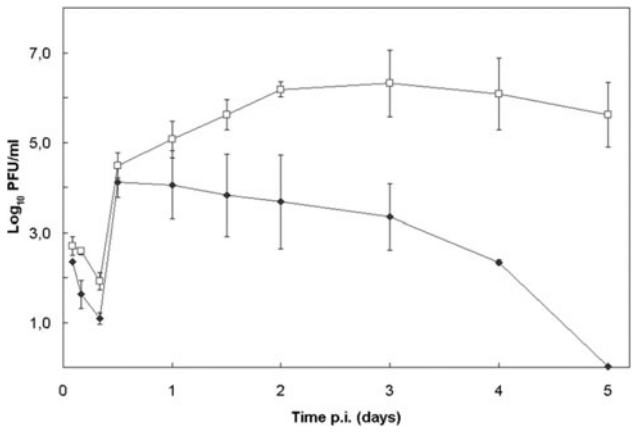

Figure 2 Viral growth kinetics of TMEV strains GDVII (♦) and DA (□) in RAW264.7 macrophages. Cells, cultivated in 96-well plates at a density of 2×10^4 cells/well, were infected at 10 PFU/cell GDVII or DA and incubated for 1 h at 37°C. After adsorption, the cells were washed three times with DMEM containing 2.5% FBS and subsequently transferred to 96-well plates. At the times indicated, samples consisting of medium and cells were harvested and frozen at −50°C. After thawing, the infectivity was determined by a plaque assay on L929 cells. Each point is the average of duplicate samples from two independent experiments ± standard deviation (error bars).

at which the samples were taken. On the average, the cells produced between 0.1 and 1 PFU/cell (results not shown). Because infectious virus could be recovered for as long as the cells were grown, we can conclude that infection of RAW264.7 macrophages with the DA strain results in persistently infected cells, which we have termed DRAW. This was never observed with GDVII-infected macrophages.

Characterization of DRAW cells

To gain more insight in the dynamics of the persistent infection, cell growth (Figure 3A), viral replication (Figure 3B), and the amount of cells containing viral antigen (Figure 3C) were monitored in DRAW cells for 9 days. The experiment was carried out with DRAW cells cultivated for nearly 3 months (20 passages) and seeded at 2×10^4 cells/well in 96-well plates. During the time of the experiment, the medium was not replaced in order to limit the number of external, and thus potentially affecting, manipulations.

Figure 3A shows that after 24 h, DRAW cells proliferated and exhibited growth until the end of the experiment. As shown in Figure 3B, virus replication took place within these cells. A maximum virus production of 6.2 $\log_{10}$ PFU/ml was reached at 48 h and gradually decreased thereafter. The experiment had to be stopped after 9 days because of cell aging. However, even after 9 days of cultivation, the virus titer remained at about 4.3 $\log_{10}$ PFU/ml.

The establishment of a persistent infection often involves a coevolution between the host cell and the infecting virus resulting from changes from both sides (Ahmed and Stevens, 1991). Reported picornaviral changes include genetic mutations resulting

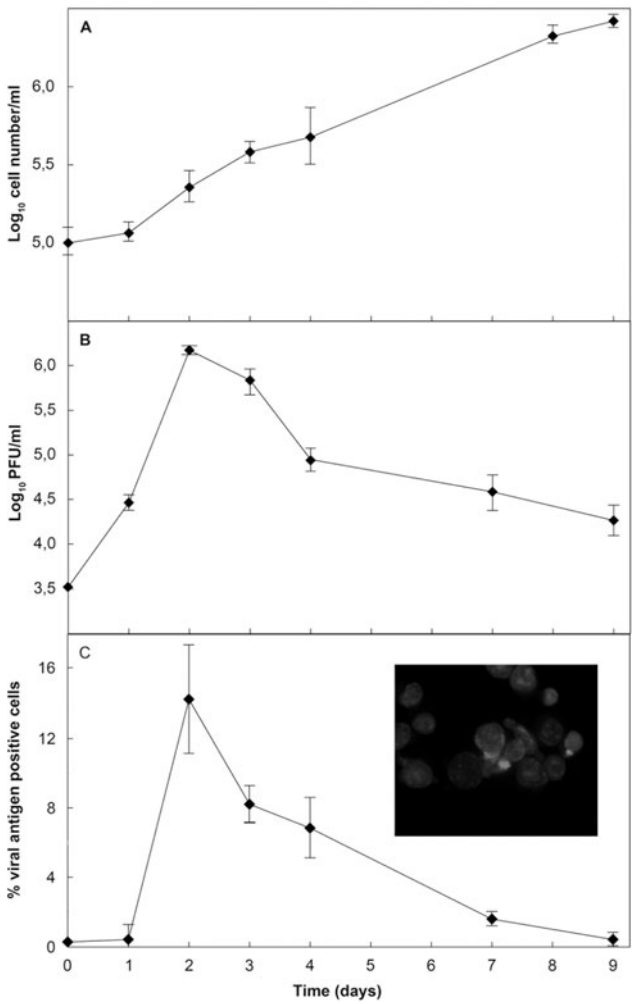

Figure 3 Characteristics of DRAW: a persistently DA-infected RAW264.7 macrophage cell line. DRAW cells, collected after 3 months of cultivation, were seeded at a density of 2×10^4 cells/well in 96-well plates. At the times indicated, the cells were counted in a Bürker chamber (**A**), the amount of infectious virus present in medium and cells was determined by plaque assay (**B**) and the percentage of viral antigen containing cells was determined by counting the amount of positive cells in the population using immunofluorescence microscopy (**C**). At least 400 cells/sample in representative fields were counted. The mean and standard deviation of duplicate samples from two independent experiments are given. (*Insert* is in color in online version.) Immunofluorescent visualization of viral antigen in DRAW cells. Viral antigen (*red*) in DRAW cells was detected using a monoclonal anti-VP1 antibody (1:1000) and a Cy3 labeled anti-mouse IgG secondary antibody (1:400). Nuclei (*blue*) were stained with DAPI. Magnification: 630×.

in a smaller plaque phenotype (de la Torre *et al*, 1985) and reduced infectivity (Yeung *et al*, 1999). None of these have been observed in DRAW cells because the plaque phenotype of viruses derived from naïve RAW264.7 infected with wild-type DA was not different from that of virus resulting from DRAW cells (results not shown) and comparable maximum viral titers were reached at 48 h in both cases (Figures 2 and 3B). However, in the case of DRAW cells, viral titers showed a considerable decrease after 3

days, which was not observed for the DA-infected RAW264.7 cells. As a consequence, we cannot exclude the possibility that this might have resulted from alterations of the virus and/or the host cells.

To determine the amount of cells that contain viral antigen, immunofluorescence microscopy using a monoclonal antibody reacting with the capsid protein VP1 and recognizing (sub)viral particles was performed. Viral antigen (red) and nuclei (blue) were visualized using a Cy3-labeled secondary antibody and DAPI staining, respectively. As expected, viral antigen was localized within the cell's cytoplasm (Figure 3C, *insert*). At the times indicated, samples were quantified by counting the amount of virus-positive cells within the total population (Figure 3C). At the start of the experiment, the percentage of viral antigen containing cells was low. This amount increased over the following 48 h, reaching a maximum of 14% $\pm$ 3% viral antigen–positive cells. Thereafter, a gradual decrease was observed over the next days. At the end of the experiment, the percentage of positive cells was comparable to that found at the start and although low, viral antigens were always detected. These data are in agreement with the viral growth kinetics (Figure 3B), because they exhibit the same pattern. Similar results were obtained when the experiment was done with DRAW cells cultivated for one year (approximately 104 passages).

Superinfection of DRAW cells with TMEV strains GDVII and DA

Because it was not possible to obtain persistently infected RAW264.7 macrophages using the GDVII strain due to its elimination after a few days, we examined what effect superinfection with this strain, as well as with the DA strain would have on DRAW cells. Therefore, we brought 2×10^4 DRAW cells/well in 96-well plates and superinfected them with 10 PFU/cell of strain GDVII or DA. Nonsuperinfected, PBS-treated DRAW cells served as reference. After 1 h, the cell cultures were washed three times to remove unbound virus. Samples were collected after 48 h, corresponding with the maximum virus production in DRAW cells (Figure 3B), and analyzed for infectious virus by plaque assay. Superinfection with the GDVII or DA strain did not result in an increase of cytopathic effects (results not shown). In nonsuperinfected DRAW cells, a titer of 6.34 $\log_{10}$ PFU/ml was obtained. In case of superinfection with DA or GDVII, titers of 6.23 and 5.85 $\log_{10}$ PFU/ml were obtained, respectively. When compared to the PBS-treated DRAW cells, superinfection with the DA strain reduces the number of plaques with 22%, whereas superinfection with the GDVII strain decreases the number of plaques with 68%. From this, it can be concluded that the GDVII strain interferes with the viral replication activity in DRAW cells, whereas this was only to a much lesser account observed with the DA strain.

Discussion

Because TMEV persistence is a major disease determinant and only poorly understood (Brahic, 2002), further identification of viral and cellular factors contributing to persistence and unraveling their interactions remains an important objective. Compared to the number of studies on TMEV persistence *in vivo*, few have been dealing with the establishment of *in vitro* persistent infections. In the present study, we describe the establishment and characterization of a persistent infection with the DA strain of TMEV in a mouse macrophage cell line. Although previous reports have mentioned the establishment of *in vitro* persistent TMEV infections in L929 cells (Roos *et al*, 1982), in cerebrovascular endothelial cells (Sapatino *et al*, 1995), and in a glioma cell line (Patick *et al*, 1990), this report is the first to demonstrate this feature in macrophages. This is of particular relevance because macrophages represent the main virus antigen burden during TMEV persistence and are thought to be the main cells in which the virus persists (Lipton *et al*, 1995; Rossi *et al*, 1997).

By comparing cell growth and viability between RAW264.7 and L929 cells after infection with the TMEV strains GDVII and DA, we have found that the macrophages almost remain unaffected by TMEV infection in contrast to the fibroblasts. This might be related to their function. Macrophages play an important role in the host defense against viruses by clearing virus-infected cells from the tissue. In doing so, the macrophages presumably have to avoid their own virus-induced death (Tyner *et al*, 2005). Therefore, maintenance of the macrophages' viability is of essential importance for effective clearance to take place.

RAW264.7 macrophages both support the replication of TMEV strains GDVII and DA. However, a marked difference in viral propagation between the two virus strains was observed in the macrophages. The GDVII strain showed restricted replication and no infectious virus could be found anymore after 5 days, whereas for the DA strain, infectious titers remained high. To evaluate if clearance of the DA strain would also occur, but needed more time in comparison to the elimination of the GDVII strain, we further passaged the DA-infected RAW264.7 macrophages for more than 1 year and regularly monitored for the presence of infectious virus by plaque assay. Surprisingly, infectious virus was present at any time reaching average titers between 0.1 and 1 PFU/cell. Comparable low TMEV titers in macrophages were also found by Jelachich *et al* (1995, 1999), although the macrophages were not shown to be persistently infected and samples were only taken until 24 to 42 h p.i. From our results, it can be concluded that the DA strain establishes a persistent infection in the RAW264.7 macrophages and we have termed this cell line DRAW. On the other hand, we were unable to induce a persistent infection with the GDVII strain.

We further characterized the DRAW cells by measuring cell growth, viral replication, and quantified the amount of viral antigen containing cells. DRAW cells both exhibited growth and supported viral replication. Even after 9 days of cultivating the cells without changing the medium, infectious virus was recovered. This is of importance because it implicates that host innate immune responses are unable to cure the infection of DRAW cells. Cells normally respond to a viral infection with the production of type I interferons (IFNs), which are potent inhibitors of viral replication. van Pesch *et al* (2001) have shown that the L protein of TMEV inhibits immediate-early alpha/beta IFN production and is important for viral persistence. Indeed, viral down-regulation of the IFN response is a strategy that would promote virus survival and favor the establishment of a persistent infection. In this regard, we have found that less type I IFNs are produced in response to a DA infection of the RAW264.7 macrophages when compared to a GDVII infection (manuscript in preparation).

The percentage of DRAW cells containing viral antigen varied in function of time, but didn't exceed 14% ± 3% of the total cell population. This maximum was reached 48 h after the start of the experiment and coincided with the maximum of infectivity. As found in other persistently TMEV-infected cell lines (Roos *et al*, 1982; Patick *et al*, 1990), the majority of cells didn't contain viral antigen, indicating that infectious virus is only produced by a small percentage of cells. Likely, this restriction contributes to the maintenance of the persistent infection because host defenses and cell death would be less triggered. Interestingly, several reports, both *in vivo* and *in vitro* (Clatch *et al*, 1990; Jelachich *et al*, 1995; Obuchi *et al*, 1997), indicate that TMEV replication is often restricted in macrophages. In addition, macrophages generally are long-lived and mobile cells, features that additionally enhance their attractiveness of being the target for a persistent viral infection.

Superinfection of DRAW cells with the GDVII strain resulted in a strong reduction of virus production in comparison to mock-infected or DA-superinfected DRAW cells. In one experiment we even found no virus anymore after superinfection of the DRAW cells with the GDVII strain. This indicates that the GDVII strain interferes with the replication of the persistent virus in DRAW cells. This interference may be due to a more effective triggering of innate immune responses, e.g., IFN by the GDVII virus, and will be further investigated.

The TMEV mouse model has increased our knowledge on determinants of persistence. However, due to the inherent complexity of an animal model, it is difficult to study virus-host interactions at the molecular level, which is a prerequisite to understand the mechanism of TMEV persistence. Persistent *in vitro* infections can be of valuable importance in this quest because virus-host interactions can be studied more directly and due to a better access, it is easier to manipulate the cellular environment, e.g., in the assessment of the effect of cytokines on viral persistence.

In conclusion, we believe that DRAW cells will be useful to (1) investigate the molecular mechanism of viral persistence through the identification of contributing cellular and viral factors using respectively a mouse whole-genome DNA microarray and mutant viruses; (2) screen for cytokines and other molecules with a modulating activity on viral persistence; (3) search for anti-TMEV compounds. The availability of such antiviral agents is wanted because they could help address fundamental questions as whether they would be more effective than immunomodulatory agents in treating TMEV-induced demyelinating disease in mice or how autoimmunity would progress after viral clearance (Lipton *et al*, 2005).

Ultimately, the presented model might also help in the search for MS treatments as can be illustrated by interferon beta, which is an antiviral agent and a successful MS drug (Billiau *et al*, 2004).

Materials and methods

Cells and viruses

L929 mouse fibroblasts purchased from American Type Culture Collection (ATCC) were grown as monolayer in minimal essential medium (MEM) with Earle's salts and nonessential amino acids as well as 1 mM sodium pyruvate and 5% horse serum.

RAW264.7 cells, a mouse macrophage cell line derived from an Abelson murine leukemia virus–induced tumor and kindly donated by T. Michiels (Christian de Duve Institute of Cellular Pathology, UCL, Belgium), were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2.5% fetal bovine serum.

Persistently DA-infected RAW264.7 cells, designated DRAW, were cultivated in the same way as naïve, uninfected RAW264.7 macrophages.

Experiments were performed in 96-well plates (Greiner Bio-One, Wemmel, Belgium) and the cells were kept at 37°C in a 5% CO_2 atmosphere.

All medium components were purchased from Gibco (Invitrogen, Merelbeke, Belgium). Stocks of TMEV strains DA and GDVII, originally received from T. Michiels, were grown in baby hamster kidney-21 fibroblasts and concentrated by ultracentrifugation.

Plaque assay

The amount of infectious virus present in medium and cells was determined by a standard plaque assay on confluent L929 cells grown in 60-mm Petri dishes (Greiner Bio-One, Wemmel, Belgium). Samples were harvested by collecting the medium, detaching the cells with 100 μl EDTA solution (1.6 mM EDTA, 137 mM NaCl, 7.8 mM $Na_2HPO_4{\cdot}12H_2O$, 1.3 mM $NaH_2PO_4{\cdot}H_2O$, 8.0 mM KCl; pH 7.4) and rinsing the wells with 100 μl DMEM. After three cycles of

freeze-thawing (freezing at −50°C, thawing at 4°C), 200 μl of 10-fold sample dilutions were brought on the cells and incubated for 1 h at 37°C to allow virus adsorption. Subsequently, the cells were covered with 5 ml of MEM containing 0.8% agar (Sigma, Bornem, Belgium) and further incubated at 37°C. After 3 days, the cells were fixed for 1 h with 10% formaldehyde. Following removal of the agar, the plaques were visualized with 0.2% crystal violet in 2% ethanol and counted. Virus infectivity, expressed as $\log_{10}$ plaque forming units (PFU) per milliliter, was determined on duplicate samples from two independent experiments.

Cell counting
Because RAW264.7 macrophages are semiadherent cells, prior to their detachment with 100 μl 0.25% trypsin–0.3% EDTA solution, the cell medium was transferred to microcentrifuge tubes and centrifuged for 5 min at 2000 × *g* in a MSE Micro Centaur centrifuge. After removal of the supernatant, the cell pellet and the detached cells were brought together and carefully resuspended. Quadruplicate samples were counted using a Bürker chamber and a Leitz Orthoplan microscope. The same procedure was followed for the L929 cells.

Cell viability assay
The metabolic activity of cells was measured using the CellTiter-Blue cell viability assay (Promega, Leiden, The Netherlands) according to the instructions of the manufacturer. The assay is based on the reduction of the indicator dye resazurin into the highly fluorescent resorufin, only occurring in metabolically active cells. After 2 hours of incubation of the cells with the indicator dye at 37°C, the fluorescence was measured at an excitation wavelength of 530 ± 25 nm and an emission wavelength of 590 ± 35 nm with a Bio-Tek FL600 microplate fluorescence reader. Triplicate samples were assayed. Camptothecin (Aldrich, Bornem, Belgium), a DNA topoisomerase I inhibitor and inducer of apoptosis (Zhang *et al*, 2000). was used as a control. Results were expressed as a percentage of the PBS control values.

Immunofluorescence microscopy
DRAW samples in 96-well plates were harvested by collecting the medium, detaching the cells with the 1.6 mM EDTA solution, and rinsing the wells with 100 μl DMEM. The samples were centrifuged for 5 min at 2000 × *g* in a MSE Micro Centaur centrifuge and the supernatants were removed. The cells were resuspended in 40 μl DMEM, transferred to glass coverslips, and incubated for 1 h at 37°C in a 5% CO_2 incubator. After incubation, the cells were fixed with 500 μl cold 4% *p*-formaldehyde for 10 min, washed twice with 500 μl PBS, and permeated with 500 μl methanol at room temperature (RT) for 30 s. Permeated cells were washed twice with 500 μl PBS and residual aldehyde groups were blocked with 500 μl 50 mM NH_4Cl (pH 7.2) for 15 min, followed again by washing twice with PBS. After blocking the cells for 10 min with 40 μl PBS containing 0.5% bovine serum albumin (BSA), 40 μl 1:1000 PBS/BSA diluted TMEV anti-VP1 antibody (kindly obtained from M. Brahic, Institut Pasteur, Paris) was added for 1 h at RT. Unbound primary antibody was removed by washing the cells thrice with PBS/BSA for 10 min. Subsequently, 40 μl 1:400 PBS/BSA diluted anti-mouse immunoglobulin (IgG) Fab Cy3 (Jackson Immunoresearch, West Grove, PA) was added for 45 min at RT and washed away by rinsing three times with PBS/BSA for 10 min. The coverslips were brought on glass slides using 7 μl fluorescence mounting medium (DakoCytomation) containing 4′,6-diamidino-2-phenylindole (DAPI) in a ratio 7:1. Viral antigen and nuclei were visualized using a Zeiss Axioskop2M equipped with a digital CCD camera (Orca AG, Hamamatsu) and SmartCapture imaging software (Digital Scientific, Cambridge).

Quantification of virus-positive cells was performed by counting 4 representative fields of at least 100 cells/field. Samples without incubation with TMEV anti-VP1 antibody were included as controls.

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