

Infection of Macrophages by Theiler's Murine Encephalomyelitis Virus Is Highly Dependent on Their Activation or Differentiation State

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Received 27 April 1997/Accepted 16 July 1997

Macrophages are the main targets of Theiler's murine encephalomyelitis virus (TMEV) during persistent demyelinating infection of mice. Replication of TMEV in macrophages was previously shown to depend on their activation state. Here, we show that the quality of the serum used for culture drastically influences viral entry in RAW264.7 macrophages.

Theiler's murine encephalomyelitis virus (TMEV) belongs to the picornavirus family (15). On the basis of the disease they induce in mice after intracranial inoculation, the various TMEV isolates can be divided into two groups. The GDVII and FA strains are highly neurovirulent and induce fatal encephalitis (16). The DA and BeAn strains persist in the central nervous system of susceptible mice and induce a chronic demyelinating disease (2, 8). Persistent virus is almost exclusively detected in the white matter of the spinal cord. Several cell types, including macrophages, astrocytes, and oligodendrocytes, have been shown to be infected (1). However, the predominant virus antigen burden is found within macrophages infiltrating demyelinating lesions (9). Accordingly, depletion of macrophages from infected mice almost completely clears the virus and suppresses the disease (13). These results underline the essential role played by macrophages during chronic infection. *In vitro*, the level of replication of TMEV genomes in macrophage cell lines was found to be lower than in the highly permissive BHK-21 cell line. Restriction of infection was also found to be correlated with the activation state of the macrophage and to depend on the viral strain used for infection (6, 11).

In this report we show that a change in the type of serum used in the culture medium of the RAW264.7 cell line has a spectacular effect on the susceptibility of this cell line to GDVII and DA infection. In this case, permissiveness appears to be controlled at the level of virus entry rather than at the level of viral replication.

RAW264.7 cells were cultured with complete Dulbecco's modified Eagle's medium supplemented with either myclone fetal calf serum (MFCS) (Myclone Super Plus bovine serum, catalog no. 10081-071, batch no. 30Q7351A; GIBCO) or standard fetal calf serum (FCS) (fetal bovine serum, catalog no. 10270-031, batch no. 40F8550J; GIBCO). These two sera differ in their quality and endotoxin levels. After several passages in media containing the different sera, cells were infected at a multiplicity of infection of 5 with the neurovirulent (GDVII) or the persistent (DA) strain of TMEV. Viral RNA replication and viral titers were monitored 24 and 48 h after infection. The

neurovirulent GDVII strain induced a clear cytopathic effect 24 h after infection for cells cultured with the standard FCS (RAW-FCS) but not for cells cultured with the myclone serum (RAW-MFCS) (Fig. 1). Forty-eight hours after infection, viral RNA, quantified by dot blot, was up to 192 times more abundant in RAW-FCS cells than in RAW-MFCS cells and viral titers reached a difference of 400 times. This experiment clearly demonstrates that the type of serum used in the culture medium of the RAW264.7 cell line has a strong influence on the susceptibility of these cells to GDVII infection. We then investigated whether our observations could also apply to the persistent DA strain of TMEV. As in the case of GDVII, 24 h after infection, a strong cytopathic effect and high viral titers were seen only for cells grown with FCS (Fig. 2). However, 48 h after infection, the viral load in cells grown with MFCS had significantly increased. These results show that a change of serum in the culture medium of the RAW264.7 cell line affects both persistent and neurovirulent strains of TMEV, but in different manners. While culture with myclone serum protects cells against GDVII infection, it only delays infection by the DA strain.

A change of serum in the culture medium of the RAW264.7 cell line induced progressive morphological changes in the cells. After several weeks of culture with the standard serum, cells were elongated, adhered to plastic, and grew slowly compared to cells grown with myclone serum, indicating that they were probably activated. This possibility was tested by measuring the tumor necrosis factor alpha (TNF- α) concentration in supernatants of cells cultured with the two sera (3, 17). Five to 54 times more TNF- α was produced by cells grown with standard serum, depending on what day after cell passage samples were taken. RAW264.7 cells cultured for 5, 10, 15, or more than 30 days after the change of serum were infected with the GDVII strain of TMEV, and viral RNA was quantified by dot blot hybridization (data not shown). After 5 days, there was a slight increase in the viral load of cells cultured with standard serum compared to that of cells cultured with myclone serum. The susceptibility of these cells grew with time and finally resulted in an important change of phenotype: cells initially resistant to viral infection became clearly susceptible after a month of culture with FCS. Conversely, culture of susceptible cells with myclone serum totally reverted the phenotype to resistant (passage from a cytopathic to a noncytopathic effect). In our hands, this reversion was even easier to obtain than the original change. Thus, after a change from myclone to stan-

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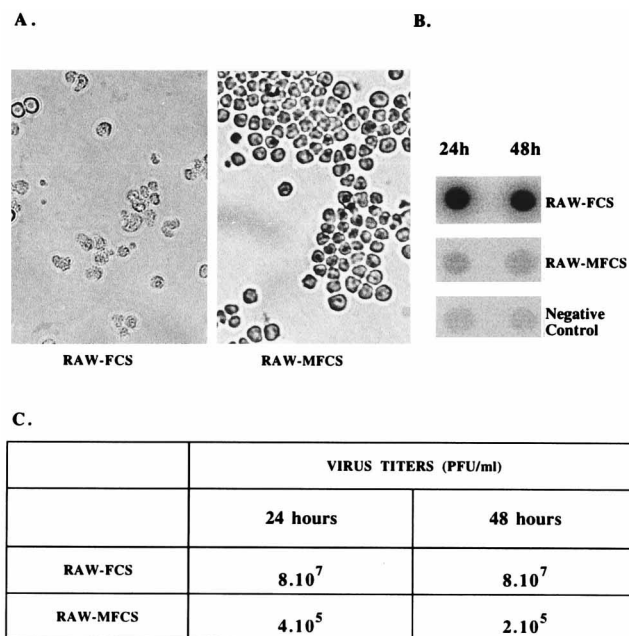


FIG. 1. Infection of RAW264.7 cells by GDVII virus. RAW-FCS and RAW-MFCS cells were infected with GDVII virus. Twenty-four hours after infection, RAW-FCS, but not RAW-MFCS, cells showed a cytopathic effect (A). Twenty-four and 48 h after infection, viral RNA was quantified by dot blot hybridization (B) and viral titers were determined by plaque assay (C).

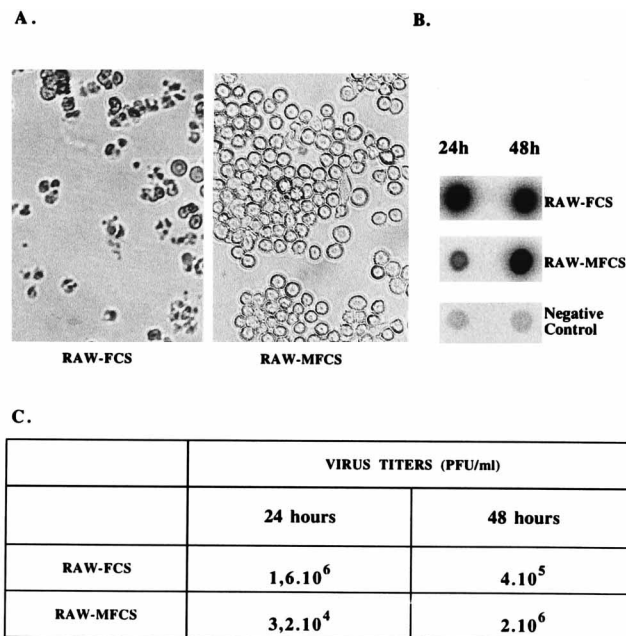


FIG. 2. Infection of RAW264.7 cells by DA virus. RAW-FCS and RAW-MFCS cells were infected with DA virus. Twenty-four hours after infection, RAW-FCS, but not RAW-MFCS, cells showed a cytopathic effect (A). Twenty-four and 48 h after infection, viral RNA was quantified by dot blot hybridization (B) and viral titers were determined by plaque assay (C).

dard serum, the sensitization of cells was progressive and clearly paralleled the activation state of the cells as judged by their morphology. At this point, we cannot tell if activation or differentiation of the cells is responsible for susceptibility to viral infection, because of the lack of clear definition of these states for macrophages. For the sake of clarity and because RAW-FCS cells do produce a certain amount of $\text{TNF-}\alpha$, which is a marker of macrophage activation, we will use the term "activated."

We next examined which step of the virus cycle was affected by the change of serum. The first step following uncoating is the translation of genomic RNA. Translation of picornavirus RNA genomes is initiated by means of an internal ribosome entry site (IRES) (5, 12). We compared the viral translation efficiencies of RAW264.7 cells cultured with FCS or MFCS. For this purpose, we made a bicistronic construct called pCJ28, a pHMG derivative (10) in which translation of the CAT gene occurs through the cap-dependent classical mechanism while translation of the luciferase gene is dependent on IRES activity. As these two proteins are expressed from the same messenger RNA, IRES activity can be evaluated by the LUC/CAT ratio. pCJ28 was stably transfected in RAW264.7 cells cultured with MFCS. Three individual clones were obtained. Their cultures were split and passaged for several weeks with culture media containing either FCS or MFCS. We infected one of the clones with DA and GDVII to ensure that a difference in infectivity could still be observed with a change of serum. The activity of the IRES, and thus translation efficiency, was found to be similar whether cells were cultured with FCS or MFCS (Table 1). These results indicate that translation of the viral genome by internal initiation does not seem to be an important step in the resistance of RAW-MFCS cells to viral infection.

RAW-MFCS cells did not show a cytopathic effect after infection, although virus titers of 4×10^5 PFU/ml were produced (Fig. 1). These viruses could be produced by a high

number of infected cells in which replication was restricted or by a low percentage of permissively infected cells. To monitor the percentage of infected cells, RAW264.7 cells cultured with standard or myoclonal serum were infected with GDVII virus. Twelve and 36 h after infection, cells were stained by immunocytochemistry (kit LSAB2; Dako) with the F12B3 monoclonal antibody directed against the protein VP1. Twelve hours after infection, 3.41% of RAW-FCS cells were stained compared to 0.41% of RAW-MFCS cells, and this difference increased at 36 h (Fig. 3). The majority of RAW-MFCS cells did not show any signal at all, meaning that either the virus did not enter the cells or it replicated at undetectable levels. The few heavily infected cells observed probably account for the virus titers. We stained RAW264.7 cells equally after DA infection (data not shown). This experiment confirmed the serum effect for this strain of virus. Again, in contrast to GDVII infection, where few isolated positive RAW-MFCS cells were seen 12 and 36 h after infection, clusters of 10 to 20 positive cells appeared 36 h after DA infection. Thus, as was found in the

TABLE 1. Comparison of IRES activity in and $\text{TNF-}\alpha$ production by RAW-FCS and RAW-MFCS cells

RAW264.7 clone ^a	IRES activity (LUC/CAT) ^b in:		Relative $\text{TNF-}\alpha$ production in:	
	FCS	MFCS	FCS	MFCS
1	32,067	28,542	6.2	1
2	41,473	30,335	5.1	1
3	67,864	32,846	5	1

^a Clones 1, 2, and 3 are RAW264.7 cells stably transfected with the pCJ28 bicistronic construct.

^b The luciferase and CAT proteins were quantified by a luminometry assay and a CAT enzyme-linked immunosorbent assay (Boehringer Mannheim), respectively.

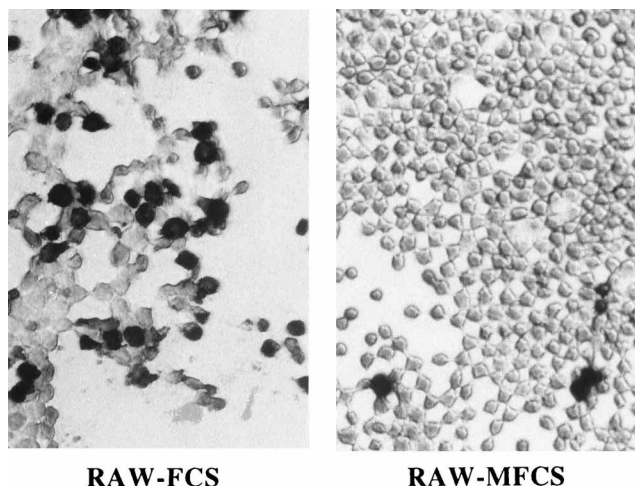


FIG. 3. RAW-FCS and RAW-MFCS cells were infected with GDVII virus. Viral VP1 antigen was detected by immunocytochemistry 36 h after infection. Efficiency of infection was generally much lower in 24-well plates than in 6-cm-diameter petri dishes, probably due to adsorption of the virus on the sides of the wells.

initial experiments, DA seems to be capable of slowly spreading to neighbor cells.

To bypass viral entry and thus to study viral replication alone, we followed the replication of a viral genome introduced into RAW264.7 cells by electroporation. About 10 to 20 μ g of GDVII RNA, obtained by *in vitro* transcription from plasmid pTMGDVII (14), were electroporated in 10^7 RAW-FCS or RAW-MFCS cells. Electroporation took place in 250 μ l of RPMI medium in 0.4-cm-gap cuvettes with settings of 750 V/cm, 960 μ F, and 2,310 Ω (Equibio electroporator). Viral replication was quantified 10 to 12 h after transfection by analyzing the amount of intracellular VP1 antigen by flow cytometry with a monoclonal mouse anti-VP1 antibody (F12B3) and a polyclonal donkey anti-mouse immunoglobulin G fluorescein isothiocyanate-conjugated antibody (Jackson ImmunoResearch Laboratories, Inc.). A GDVII deletion mutant (pTM489) incapable of replicating was used as a negative control. To monitor the efficiency of RNA transfection in cells cultured with the two sera, we electroporated an RNA encoding the luciferase protein (pCJ8). From this RNA, luciferase is translated by means of an IRES and is expressed directly upon entry of the RNA into a cell. Transfection efficiency was low and was found to be 3 to 12 times higher for activated RAW-FCS cells than for RAW-MFCS cells. Taking into account these differences in transfection efficiency, the same proportion of VP1 antigen-positive cells was found for RAW-FCS and RAW-MFCS cells (Fig. 4). Similar results were obtained in four separate experiments. This indicates that GDVII RNA replicates in RAW-FCS cells and in resistant RAW-MFCS cells. Mean fluorescence was up to 2.5 times lower for RAW-MFCS cells, indicative of a slight difference in the level of viral replication. The difference in the replication level is certainly not sufficient to account for the difference in viral susceptibility observed between RAW-FCS and RAW-MFCS cells and to explain the absence of signal in RAW-MFCS cells after immunostaining (Fig. 3). Therefore, our data suggest that resistance to infection is associated with a blockade at the viral entry step rather than at the viral replication level.

Activation of RAW264.7 cells by lipopolysaccharide (LPS) was reported to render these cells resistant to TMEV infection (6, 11). These results contrast with those presented above in

the case of a change of serum, where RAW264.7 cell activation correlated with sensitization to viral infection. To verify that this difference was indeed due to the stimulus, we compared the infection of cells activated by LPS or serum. In most cases, cells activated by LPS were resistant to TMEV infection (data not shown). This resistance was moderate despite the highly activated state of the cells (more than a 1,000-fold increase in TNF- α secretion) and occurred rapidly after the addition of LPS. These observations show that RAW264.7 cell activation can correlate with either resistance or permissiveness to TMEV infection, depending on the stimulus involved. In the case of serum activation, the factor responsible for susceptibility might be unrelated to endotoxin. Alternatively, LPS might be the stimulus. In this case, the dose of the stimulus would totally modify the outcome of the infection.

The macrophage is the predominant cell type that harbors TMEV during chronic infection of mice (9, 13). Large phagocytic cells infiltrating the white matter of the spinal cord were shown to contain either a heavy or a light viral load. Restriction of viral replication in some cells could provide a way for the virus to escape the host's immune response and thus allow viral persistence. *In vitro* studies showed that TMEV replication was restricted in macrophage cell lines compared to that in BHK-21 cells. Replication in macrophages was also found to

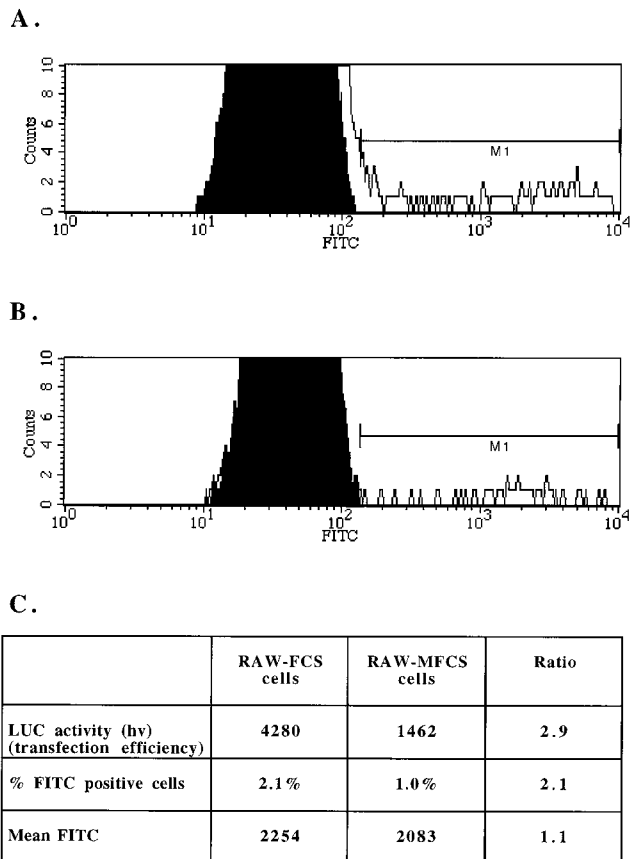


FIG. 4. Detection of intracellular VP1 antigen by fluorescence-activated cell sorter analysis after electroporation of RAW264.7 cells by pTMGDVII RNA (open curve) or control pTM489 RNA (solid curve). (A) RAW-FCS cells. (B) RAW-MFCS cells. (C) Comparison of transfection efficiency, percentage of fluorescein isothiocyanate-positive cells, and average fluorescence levels between cells cultured with the two sera. Transfection efficiency was determined for RAW-FCS and RAW-MFCS cells by electroporation of pCJ8 RNA and measurement of luciferase activity 2 h later.

depend on the activation or differentiation state of the cell and on the viral strain used for infection (6, 11). Here, we showed that a change in the type of serum has an important effect on the susceptibility of RAW264.7 cells to TMEV infection. Cells cultured with standard serum are activated and/or differentiated and show a clear cytopathic effect 24 h after infection with GDVII or DA virus. By contrast, when myoclone serum is used, cells are less activated and resist viral infection. The influence of the activation or differentiation state of macrophages on susceptibility to viral infection has been described for a number of viruses (7). In most cases, viruses were shown to enter resistant cells. Their replication was blocked at a stage dependent on the macrophage-virus combination under study. We observed that the genome of GDVII readily replicated in resistant cells when introduced by electroporation. We cannot totally exclude the possibility that the electroporation process itself activated the replication in MFCS cells; however, this seems very unlikely. Thus, viral entry appears to be the main step controlling RAW264.7 cell resistance or susceptibility to GDVII infection. At this stage, we cannot determine precisely if GDVII infection is arrested at the level of binding to the receptor, internalization, or even uncoating. We found, though, that GDVII surprisingly bound resistant cells even better than sensitive cells (data not shown). However, as the receptor of the virus is unknown to date, we cannot assess the specificity of binding. The infection of RAW264.7 cells by the persistent DA strain of TMEV was equally affected by a change of serum. It seemed, though, that in contrast to the complete blockade of GDVII infection in the majority of cells, infection by DA virus was only slowed down when myoclone serum was used for RAW264.7 cell culture. It has been suggested that the DA and GDVII viruses use the same receptor in different ways (4). If this is the case, one could imagine the existence of a low-affinity receptor on nonactivated RAW264.7 cells that is exclusively recognized by the DA strain. Upon cell activation, either this receptor could undergo conformational changes and become a high-affinity receptor for the DA and GDVII strains or a newly expressed factor could allow efficient entry of both viruses.

In conclusion, these results indicate that the situation *in vivo* is probably more complex than expected. Indeed, on the one hand, macrophage activation can correlate with either restriction or permissiveness of viral infection. On the other hand, resistance can occur at the level of viral entry or replication. Macrophages are often selected by viruses for persistence. This type of cell has the advantage of being mobile, thus easily enabling the spread of viruses to distant tissues. It is also probably present *in vivo* in a large variety of activation and differentiation states. This could allow a virus to find, in a single cell type, a resistant state sheltering the virus from the immune response and a permissive state allowing viral replication. Thus, persistent viruses could have selected macrophages for their variability.

We thank Véronique Kruys for the RAW264.7 cells and for excellent advice on their handling. We are grateful to Nadine Jarousse and Patrick Nedellec for communicating their results on RAW264.7 cell infection, to André Tonon for help with fluorescence-activated cell sorter analysis, and to Michel Brahic, Karima Jnaoui, and Pierre-Emmanuel Lobert for critical reading of the manuscript. Majid Mehtali and Michel Brahic kindly provided the plasmids pHMG and pTMGDVII, respectively.

T.M. is a Senior Research Assistant of the Belgian FNRS (National Fund for Scientific Research). C.S.-J. is the recipient of a scholarship from the FRIA. This work was supported by conv.3.4573.94F of the FRSM, crédit aux chercheurs FNRS 1.5.185.96F, the French Association pour la Recherche sur la Sclérose En Plaques (ARSEP), PAI 44, and Fonds de Développement Scientifique (FDS) from the University of Louvain.

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