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Theiler's Virus Replication in Brain Macrophages Cultured In Vitro

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Infection of the mouse with Theiler's virus is one of the best animal models for the study of multiple sclerosis, a chronic demyelinating disease of the human central nervous system. The identification of the virus target cell(s) is fundamental to an understanding of the viral persistence as well as the inflammation and demyelination observed in the chronic phase of the disease. This paper reports that a small fraction of brain macrophages grown in vitro can be efficiently infected with Theiler's virus without significant cytolytic effect. Viral replication as well as continuous production of infectivity were observed in these cultures.

Mice persistently infected with Theiler's virus, a murine picornavirus, present tissue lesions closely resembling those of multiple sclerosis (MS), a chronic demyelinating disease of the human central nervous system (CNS) (12, 25, 39). The cause of MS seems to be multifactorial (13, 23). Viral infections may play an important role in the onset of the disease (41). Therefore, study of the mechanisms leading to viral persistence and to demyelination in animal models is of importance for understanding the pathogenesis of MS. In this respect, Theiler's virus, which can persist in the CNS of the host throughout the life of the animal, is one of the best model for these studies (5, 27). Persistence of Theiler's virus has been associated with a restriction of viral RNA and capsid proteins synthesis in infected cells (8–10).

The identification of the cell(s) in which Theiler's virus persists is crucial for an understanding of both the inflammation and the demyelination observed in the late phase of the disease. The nature of the principal target cell for chronic Theiler's virus infection is, however, still controversial. Indeed, looking at demyelinating lesions of adult mice, viral antigens (4, 5, 34), viral RNA (1, 4, 5), and virions (3) have been detected in all types of cells found in the CNS.

We are currently examining the effects of Theiler's virus infection on cultures of the different cell populations of the mouse CNS. This study reports that pure cultures of brain macrophages can be efficiently infected with the DA strain of Theiler's virus without significant cytolytic effect. Viral replication takes place in a minority of the cells. Infectious virus is continuously produced at a relatively low level.

Characterization of brain macrophages in culture. Cultures were prepared from newborn mice as previously described (20, 22). Whole brains were removed aseptically and placed into a sterile 100-mm plastic petri dish containing Dulbecco's modified Eagle's medium supplemented with 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 33 mM D-glucose, 2 mM L-glutamine, 3 mM $NaHCO_3$, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. This medium was used in all studies reported in this article. The meninges were removed, and brains were mechanically dissociated in the presence of 0.25% trypsin (GIBCO, Grand Island, N.Y.) (1 min at room temperature) and then DNase I (1 mg/ml; Sigma, St. Louis, Mo.) (5 min). Dissociated cells were then centrifuged (500 × *g*, 5 min), resuspended in fresh medium, and washed twice. Cells were plated on polyornithine-coated 100-mm culture dishes at 4×10^4 cells per cm^2 and kept for several weeks in 8% fetal calf serum (FCS)-containing medium. The medium was changed on days 6, 10, and 15 after seeding and twice a week thereafter. On day 10, supernatant from interleukin-3 (IL-3)-producing cell line $WEHI_3D^-$ was added to the cell cultures at a final dilution of 1:500 (14). Brain macrophages were separated from confluent astrocytic monolayers at various times of culture. Culture medium was supplemented with phosphate-buffered saline (PBS) containing 2 mM EDTA, and the cell dishes were shaken for 30 min at room temperature. The medium containing detached cells was then centrifuged (500 × *g*, 5 min), and the pellet was resuspended in 5% FCS-containing medium. Cells were counted and plated on 12-mm-diameter glass slides at a density of 1.5×10^4 to 2.5×10^5 cells per cm^2.

The purity of these cultures was determined with a rat monoclonal antibody (F4/80) which is specific for mature mouse macrophages (2). The antigen recognized by this antibody belongs to a 160,000-molecular-weight glycoprotein which is not expressed on any other cell type (38). All cultured cells were labeled with this antibody (Fig. 1A). To detect astrocytes which might contaminate the cultures, immunohistochemistry was also performed with a rabbit antiserum against glial fibrillary acidic protein. No staining was observed with this antiserum during the first 6 days of culture (Fig. 1B).

Effect of Theiler's virus infection on cultured brain macrophages. The DA strain of Theiler's virus was plaque purified three times on BHK cells. Pure brain macrophage cultures were infected at a multiplicity of infection (MOI) of 3 PFU per cell. Cell cultures were washed twice in PBS to remove serum components, and 0.25 ml of appropriately diluted virus stock solution was added to each well. Plates were incubated at 37°C for 2 h to allow virus adsorption. Uninfected cells received 0.25 ml of serum-free medium. After this 2-h incubation, 0.75 ml of 1% FCS-containing medium was added to each well.

(i) **Cytolytic effect.** The number of brain macrophages was estimated at different times after infection with Theiler's virus. As shown in Table 1, no significant difference was found between infected and uninfected cultures. The decrease in cell number from day 1 to day 4 represents the death of brain macrophages cultured in 1% FCS-containing medium without stimulating cytokines. Therefore, we did not observe any significant cytolytic effect of Theiler's virus on cultures of brain macrophages.

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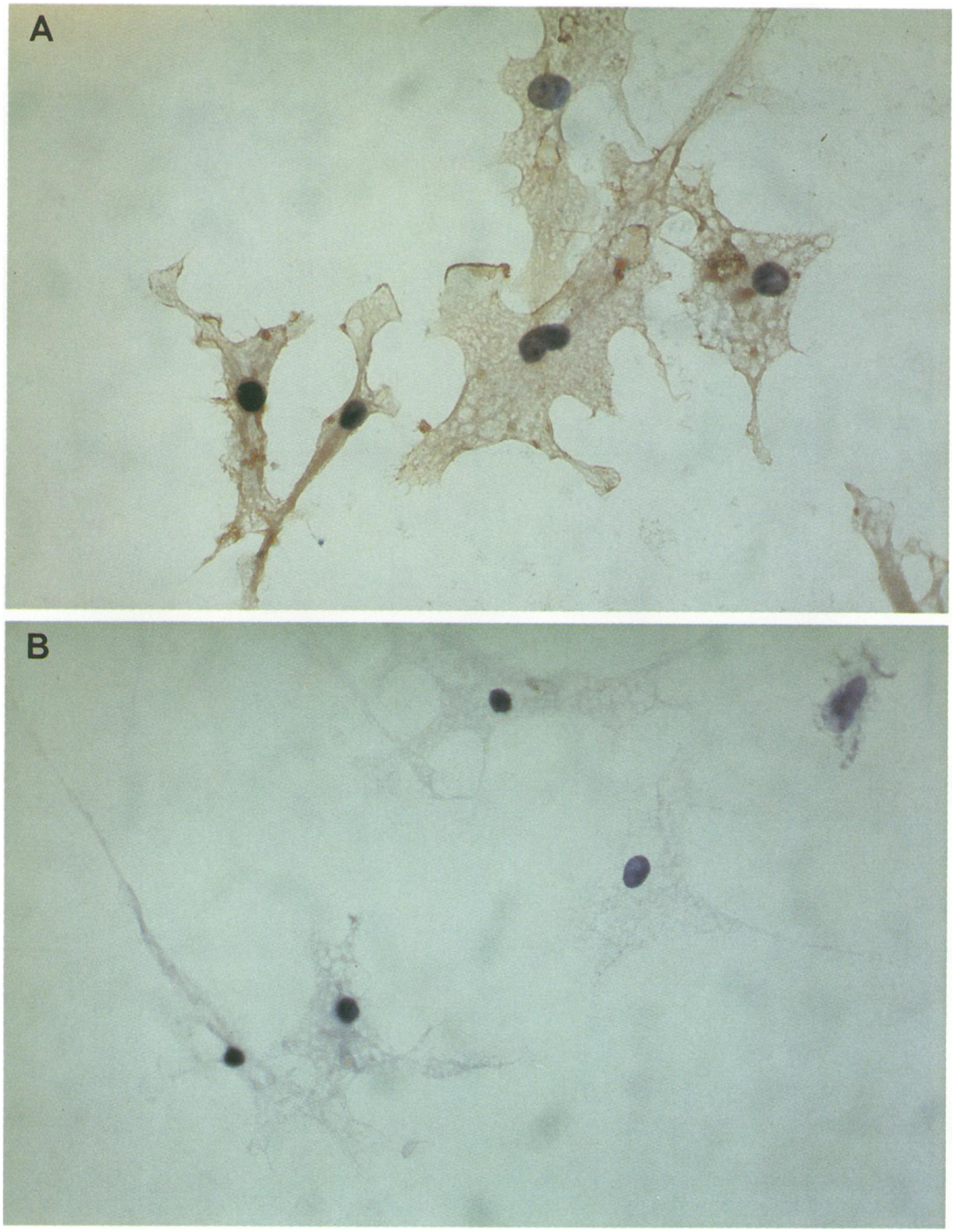

FIG. 1. Photomicrographs of brain macrophages cultured during 3 days at low density (1.5×10^4 cells per cm^2). (A) Immunostaining with antibody F4/80; (B) immunostaining with anti-glial fibrillary acidic protein antiserum. After detection with peroxidase, cells were counterstained with Mayer's hematoxylin. Magnification, ×491.

(ii) Proportion of brain macrophages infected in vitro. The presence of viral RNA in brain macrophages was estimated using a highly sensitive in situ hybridization assay. Cells cultured on 12-mm-diameter glass slides were fixed with PFA (4% in PBS) for 15 min at room temperature and kept in 70% ethanol at 4°C before hybridization. Riboprobes were prepared according to Wilkinson et al. (43) as modified by Sassoon et al. (35). Viral cDNA was cloned in a Bluescript vector and transcribed with high efficiency from the T3 or T7 bacterial promoter to make antisense cRNAs. The insert contained 280 bp of cDNA corresponding to the 5′ end of the Theiler's virus genome. Radioactive labeling of viral riboprobe and details concerning the in situ hybridization technique have been described previously (32). The amount of radioactivity bound to the cells was determined by counting the average number of autoradiographic grains per cell per minute of exposure for 200 cells taken at random on the different slides. Following hybridization, slides were ex-

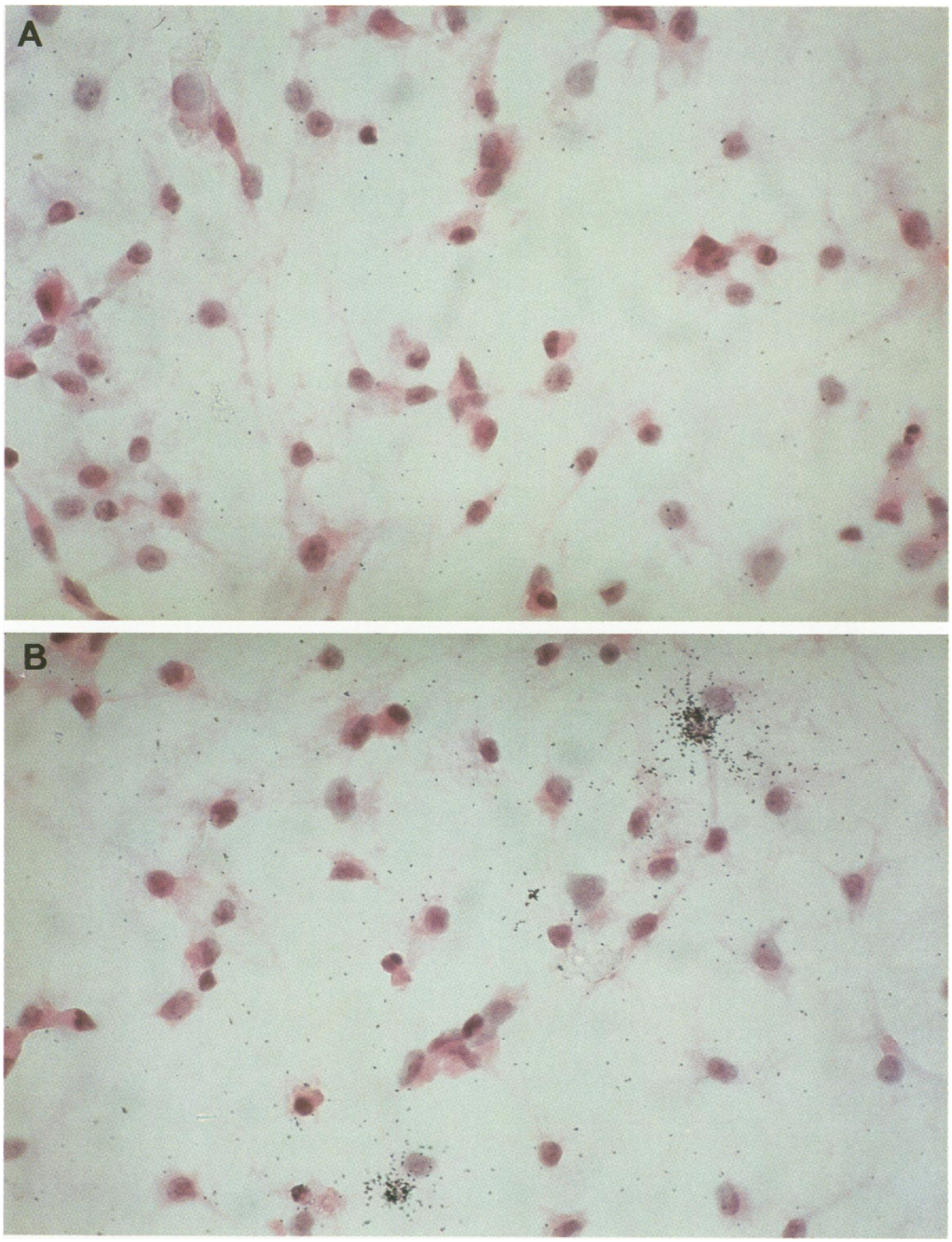

FIG. 2. Detection of Theiler's virus mRNA by in situ hybridization on brain macrophages 24 h postinfection. (A) Uninfected cultures; (B) infected cultures. Cells were counterstained with Harris's hematoxylin and eosin. Magnification, ×491.

posed for 1, 2, or 3 days. Figure 2 shows representative microscope fields from uninfected (Fig. 2A) and infected (Fig. 2B) brain macrophage cultures. An average of 1,000 cells was examined for each slide at different times after infection. The percentage of infected cells was of the order of 1.5 to 6.5%. This percentage did not vary much between 1 and 4 days postinfection.

(iii) Viral replication. Viral replication was estimated with in situ hybridization by counting autoradiographic grains over randomly chosen cells at different times postinfection. As shown in Table 2, 2 h postinfection, the average number

TABLE 1. Estimation of the number of brain macrophages in uninfected cultures and cultures infected with Theiler's virus

Time (h) postinfection	No. of brain macrophages/field[a] (mean ± SD)	
	Uninfected cultures	Infected cultures
24	113.2 ± 21.1	82.0 ± 10.2
72	78.4 ± 14.0	82.8 ± 8.2
96	50.2 ± 10.2	48.0 ± 5.5

[a] At least six different fields were counted on each slide after hematoxylin-eosin staining.

TABLE 2. Assessment of viral replication in brain macrophages

Time (h) postinfection[a]	Determination							
	Brain macrophages				BHK cells			
	% of positive cells[b]	Avg no. of grains/cell/min of exposure[c]	Avg no. of grains/positive cell[b]	Total no. of grains in positive cells[d]	% of positive cells	Avg no. of grains/cell/min of exposure	Avg no. of grains/positive cell	Total no. of grains in positive cells
2	1.5	0.0024	73.1	219	1.5	0.0007	48.7	146
7	5	0.0096	240.2	2,402	32	0.0450	173.0	11,075
24	6.5	0.0166	192.3	2,500	—[e]	—	—	—

[a] Cells were infected at an MOI of 3 PFU per cell in the presence of 1% FCS.
[b] As estimated by the presence of viral RNA (grains) after in situ hybridization. Cells are scored as positive when exhibiting more than 30 autoradiographic grains.
[c] As calculated for 200 cells randomly chosen on each slide. The background (0.0040 for brain macrophages and 0.0030 for BHK cells) has been subtracted.
[d] As obtained by adding the number of grains counted on positive cells.
[e] —, all cells were dead.

of grains per brain macrophage per minute of exposure, which directly reflects the number of viral RNA copies, was 0.0024. Five hours later, this number increased to 0.0096, and 5% of the cells were considered as positive. This increase indicates that viral RNA replication occurred in brain macrophages. In comparison, at the same time postinfection, 32% of BHK cells, cells permissive for Theiler's virus replication, were infected. There was no significant difference between the average number of grains per positive cell in brain macrophages (240.2) and BHK cells (173.0). These results show that Theiler's virus, although it infected a smaller percentage of brain macrophages than BHK cells, behaved in the same way in both types of cells in terms of viral RNA replication 7 h postinfection.

(iv) Viral titers in brain macrophage cultures. Brain macrophages grown on glass slides were infected with Theiler's virus at an MOI of 3 PFU per cell. After virus adsorption at 37°C for 2 h in 0.2 ml of serum-free Dulbecco's modified Eagle's medium, 0.8 ml of 1% FCS-containing medium was added to each well. At different times after infection, the culture supernatants were sampled and the virus was titrated by conventional plaque titration on BHK cells (26). Table 3 shows the kinetics of virus production. The titer remained rather constant over 6 days of infection. Because this level of infectivity is of the same order as for the inoculum, it was important to study the rate of inactivation of Theiler's virus in the culture medium. For this purpose, the inoculum was incubated in medium without cultured cells and titrated in parallel. As shown in Table 3, the infectivity of the virus alone decreased steadily over 6 days of incubation, reflecting virus inactivation in the medium. Therefore, the constant infectivity titer observed in the supernatant of infected brain macrophages reflects a continuous production of infectious virus.

(v) Ia expression on brain macrophages. Brain macrophages in culture were also tested for Ia expression. By using a rat monoclonal anti-$Ia^{b,d,q}$ antibody (M5/114.15.2; American Type Culture Collection), an average of 2.2% of cells were stained in pure brain macrophage cultures (45 of 2,000). After 3 days of infection with Theiler's virus at an MOI of 3 PFU per cell, the percentage of Ia^+ cells significantly increased to an average of 4% (81 of 2,000).

In this report, we show that pure primary cultures of brain macrophages can be infected with the DA strain of Theiler's virus. There is no major cytolytic effect in these cultures, at an MOI of 3 PFU per cell, even 6 days after infection. This result could be due to the small number of brain macrophages infected by Theiler's virus in the cultures. In fact, no more than 5 to 7% of the brain macrophages in culture contained viral RNA, as detected by in situ hybridization assay. Since Theiler's virus infects a small subset of brain macrophages in vitro, this result could indicate that only a minor population of those cells express a receptor for the virus. Whether the few infected cells die after infection cannot be established, since only 5% of the cells are infected and the standard error on cell counts is greater than 5% (Table 1). Viral replication was assessed by comparing the average number of grains in positive cells at the time of virus adsorption (2 h) with the average number of grains in positive cells at the end of one viral cycle (7 h) and at 24 h postinfection. The results show that viral RNA replication takes place in brain macrophages. The fact that Theiler's virus RNA replicated in a similar way in brain macrophages and BHK cells 7 h postinfection may indicate that brain

TABLE 3. Theiler's virus titers in supernatants of brain macrophage cultures and in control virus suspension

Time postinfection	Mean virus titer (PFU/ml)[a]			
	Brain macrophages[b]		Virus alone[c]	
	Expt 1	Expt 2	Expt 1	Expt 2
2 h	1.3×10^5	2×10^5	7.8×10^4	1.3×10^5
7 h	6.5×10^4	6.6×10^4	1.0×10^4	1.5×10^4
24 h	6.7×10^4	5.6×10^4	ND	3.0×10^3
3 days	ND	8.9×10^4	1.2×10^3	1.3×10^3
6 days	2.2×10^4	1.7×10^4	ND	6.6×10^1

[a] As determined by plaque assay in BHK cell monolayers (dilution of supernatants was tested in triplicate). ND, not determined.
[b] Brain macrophage cultures were infected at an MOI of 3 PFU per cell.
[c] As a control, 1.1×10^5 PFU of virus per ml was incubated in medium alone and sampled at various time intervals.

macrophages represent the minority of infected cells which contain several thousand viral genomes in the white matter of the spinal cord after intracerebral inoculation of Theiler's virus (8). If brain macrophages die after infection, the constant percentage of infected brain macrophages found in culture (≈5%) could be due to the infection of newly formed susceptible cells. Indeed, it has been shown that brain macrophages divide in culture, although at a slow rate (17). Thus, the small number of self-renewing brain macrophages susceptible to infection, compensating for the death of infected macrophages, may explain the constant number of infected cells found in the cultures. Alternatively, brain macrophages may survive the infection, and the constant percentage of infected cells may represent cells infected from the beginning.

Brain macrophages are able to produce continuously new virus after infection. Theiler's virus capsid antigens and viral RNA have been found to persist in macrophages within the CNS (1, 11, 34). Our results are in agreement with these observations. It is still unclear whether these brain macrophages represent the invasion and proliferation of blood-derived monocytes or an expansion of macrophages normally present in the brain tissue.

Macrophages are the reservoir for numerous viruses which persist in the CNS, in particular visna virus, which is also responsible for a demyelinating disease (19, 29, 30), and human immunodeficiency virus (18, 24).

Classical immune reactions as well as autoimmune responses can be envisioned when one considers the mechanisms of Theiler's virus-induced inflammation and demyelination. Brain macrophages can function as antigen-presenting cells. The expression of Ia antigen on infected brain macrophages significantly increased after viral infection. Brain macrophages could present processed Theiler's virus antigenic determinants in the context of major histocompatibility complex class II molecules, resulting in the activation of $CD4^+$ T-cell subsets, which in turn could provide helper function for major histocompatibility complex class I-restricted T cells as well as for virus-specific antibody-producing cells.

Furthermore, infected brain macrophages could release IL-1 (22, 33), which activates $CD4^+$ T cells to produce IL-2 and enhances the expression of IL-2 receptors on T cells. IL-1 also activates B cells and NK cells and stimulates its own production (15, 21). IL-2 can in turn activate and induce the proliferation of $CD8^+$ T cells. Specific T cells can release lymphokines which lead to the recruitment, accumulation, and activation of additional brain macrophages in the CNS tissue. Brain macrophages can also produce tumor necrosis factor alpha, which induces oligodendrocyte necrosis and demyelination (6, 17, 36), prostaglandin E (16), and other mediators such as leukotrienes and phospholipases, proteinases that have been shown to have high myelinolytic activity (7, 42). The amount of tumor necrosis factor alpha (37) and of IL-1 (40) in the cerebrospinal fluid and sera of patients with MS has been correlated with the intensity of the disease. Moreover, brain macrophages have been shown to exhibit natural cytotoxicity against oligodendrocytes in vitro (28). The degradation of myelin could be due to nonspecific effects of the chronically stimulated brain macrophages or to the factors which these cells secrete.

Autoimmune demyelination may occur after presentation of myelin antigens to T lymphocytes through the generation of myelin debris. This stimulation of T cells might be due to immune cross-reactions between virus and host components (31).

Taken together, our results support the importance of the infection of brain macrophages in the course of inflammation and demyelination observed in disease affecting the CNS and related to viral persistence.

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