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West Nile virus neuroinvasion and encephalitis induced by macrophage depletion in mice

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Summary. The encephalitic West Nile virus and its nonneuroinvasive variant, WN-25, were used to study the effect of macrophage depletion on viral invasion of the central nervous system. The in vivo elimination of macrophages was achieved by use of liposome-encapsulated drug dichloromethylene diphosphonate. Depletion of macrophages had an exacerbating effect on the course of the viral infection, exhibited by higher and extended viremia and accelerated development of encephalitis and death. Using a low dose of West Nile virus (5 PFU/mouse), an increase in mortality (from 50% to 100%) due to macrophage depletion was demonstrated. Furthermore, the attenuated noninvasive variant WN-25 showed high and prolonged viremia in the macrophage depleted mice ($\approx 5 \log 10$ PFU/ml versus 2 in control mice), that allowed the penetration of the virus into the central nervous system. The mortality rate caused by the attenuated virus in the macrophage-depleted mice was 70–75%, as compared to complete survival in the control inoculated mice. These results indicate a significant role of macrophages in the non-specific immediate defence system of the organism in case of viral infection.

Introduction

West Nile virus (WNV) is a mosquito-borne flavivirus of the *Flaviviridae*, which has been shown to be a major cause of endemic and epidemic infections, potentially causing encephalitis. In susceptible young adult mice, wild type WNV is both neurovirulent (i.e. able to infect the nervous system, replicate in some of its cells and injure them), and neuroinvasive (i.e., able to invade the sequestered CNS after replicating in the periphery) [2, 22]. WN-25 is an attenuated nonneuroinvasive mutant of WNV, which will cause encephalitis and death in weanling mice only when injected intracerebrally (i.c.), but not when introduced peripherally [6].

These two types of viruses have been shown to differ in their ability to proliferate in primary cultures and cell lines of murine macrophages [7, 11]. WNV, but not WN-25, infected cultures of mouse peritoneal macrophages, yielding a productive infection. As monocytes-macrophages have been implicated in the neuroinvasion of several viruses [13, 16], we studied the effect of macrophage depletion on CNS invasion by the two WN viruses. In vivo elimination of macrophages was achieved by use of the liposome encapsulated drug, dichloromethylene diphosphonate (Cl_2MDP). Macrophages ingest the liposomes, which are subsequently disrupted by phospholipase and Cl_2MDP is consequently released into the cell [17]. Cl_2MDP is believed to cause elimination of the macrophage due to its activity as a chelator of crucial metal ions or by a direct effect on ATP metabolism in the cell [18]. The effects of intravenously administered Cl_2MDP-liposomes on splenic and liver cell populations have been studied in great detail [19]. The selective depletion of macrophages in both organs has been confirmed using cytoplasmic and surface markers for detection as well as by functional studies (phagocytosis) and electron microscopy [20]. Furthermore, monocytes were depleted by Cl_2MDP- liposomes [8], but microglia were not [9]. Two types of liposomes were studied: PC liposomes – constituted of phosphatidylcholine and cholesterol; and PCMAN-liposomes containing a mannosylated ligand, which have been demonstrated to eliminate macrophages very efficiently during experimental allergic encephalomyelitis (EAE) [10].

The studies reported herein demonstrate the exacerbating effect of macrophage depletion on the course of infection caused by the two WN viruses. Macrophage depletion led to markedly higher viremia, even enabling infiltration of the noninvasive variant into the CNS, causing encephalitis and death.

Materials and methods

Mice

OF-1 mice from IFFA-Credo (France), were used at the age of 24 days (10–12 g body weight). In all studies, female mice of the same age and batch were compared. Mice were divided into groups following treatment and inoculation, and were observed for mortality until the end of the experiment (21 days).

Viruses

West Nile virus (WNV)

The original strain of virus was isolated from a human case of WNV infection [5]. The virus stock was prepared and assayed in Vero cells in our laboratory. The virus stock used for the experiments contained 3.0×10^8 PFU/ml.

Attenuated West Nile virus (WN-25)

Aedes aegypti cultures were infected with about one plaque-forming unit (PFU) per cell of WNV from mouse brain suspension. The infected culture was subcultured once or twice a week and the virus progeny was harvested. At passage 25, the virus was plaque-purified on

BHK cells (three times), regrown (three times) in C6/36 (Aedes albopictus cell line) and later on Vero cells. This strain was completely non-lethal when 2×10^5 PFU were injected intraperitoneally (i.p.); [12]. No infectious virus could be found in the brains of the WN-25 injected mice although all become immune [6]. The virus stock used contained 1.1×10^8 PFU/ml.

Virus inoculation

WNV was inoculated i.p. (0.2 ml) in doses of 50 or 5 PFU/mouse, and WN-25 was inoculated by the same route as 2×10^5 PFU/mouse. For virus titration, groups of mice were inoculated (0.03 ml) intracraneally (i.c.) with serial dilutions of the virus stock solution.

Isolation of WNV and WN-25 from the blood of infected mice

Liposome-treated and control mice were bled at various time points from the tail vein into serum separator tubes. Virus content in serum and brain are determined by titration of virus in Vero cells.

Tissue culture

The Vero cell line, originally derived from kidneys of normal African Green Monkeys, was grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS).

Titration of virus in tissue culture

For determination of WNV or WN-25 virus, the original plaque technique of Dulbecco and Vogt [4] was used. A dilution of virus was added to the Vero cell monolayers in Petri dishes and incubated at 37 °C for 1 h to permit viral adsorption. The monolayer is overlaid with MEM × 2 and tragacanth (Gum tragacanth Grade III G-1128, Sigma) containing 2% FBS and 2.4% $NaHCO_3$. The cultures were incubated (37 °C, 5% CO_2) for 72 h. Plaques were counted after staining the monolayer with neutral red (0.05%). All plaques were counted by an experienced investigator.

Liposomes

Multilamellar liposomes were prepared as described earlier [8, 19]. Briefly, to prepare the PC liposomes, 86 mg phosphatidylcholine (Lipoid KG, Ludwigshafen, Germany) and 8 mg cholesterol (molar ratio–6:1; Sigma Chemicals Co., St. Louis, MO) were dissolved in 10 ml chloroform in a 500 ml round-bottom flask, and dried in vacuo on a rotary evaporator to form a film. Subsequently the film was dispersed into liposomes after the addition of phosphate-buffered saline (PBS, 0.15 M NaCl in 10 mM phosphate buffer, pH 7.4). To enclose Cl_2MDP into PC liposomes, 2.5 g Cl_2MDP was added to the 10 ml PBS.

The preparations were kept for 2 h at room temperature (RT), sonicated for 3 min at 20 °C in a sonicator (50 Hz), and then kept at RT for another 2h. The liposomes were centrifuged at 100 000 × **g** for 30 min and finally resuspended in 4 ml PBS.

To prepare PCMAN liposomes, 70.9 mg phosphatidylcholine and 10.8 mg cholesterol were dissolved in 8 ml chloroform and added to 3.6 mg p-aminophenyl-D-mannopyranoside (Sigma Chemicals Co.), dissolved in 2 ml methanol and dried as described above. The dried lipid film was dissolved in chloroform and dried once again before the aqueous phase with or without Cl_2MDP was added. Liposomes were sonicated and centrifuged and resuspended in PBS as described above [8, 19].

Cl_2MDP was a gift of Boehringer Mannheim GmbH, Mannheim, Germany.

Liposome treatment

The liposome suspension (0.15 ml/mouse) was injected i.v. one day pre- and one day post-inoculation (p.i.) with the virus, and an additional injection was given i.p. on day 3 p.i. The efficacy of this protocol to eliminate spleen macrophages was verified by histological examination.

Statistical analysis

All experiments have been repeated at least twice. Data were analyzed by one-way ANOVA and the Student-Newman-Keul test for a-posteriori multiple comparisons.

Results

WNV strains characteristics

WN-25 strain is an attenuated non-neuroinvasive variant of the WNV. When the two virus strains were inoculated peripherally, their differential pathogenic traits were exhibited, as can be seen in Table 1. The comparison of their PFU/IPLD$_{50}$ values (IPLD$_{50}$-number of LD$_{50}$ per ml of stock solution when injected i.p.) demonstrates that less than 10 PFU of WNV inoculated i.p. will cause the death of mice, whereas 10^6 PFU of WN-25 will not induce any overt pathological effect. However, when injected i.c., the two viruses showed very similar characteristics (Table 1), with close PFU/ICLD$_{50}$ values and causing encephalitis with similar MDTD (mean day to death).

In previous studies we have found that the viruses WNV and WN-25 reacted differently with murine macrophages [7, 11]. WNV, when compared with WN-25, was shown to proliferate to higher levels in primary cultures and cell lines of murine macrophages. In order to evaluate the contribution of the virus-macrophage interaction to the neuroinvasive process, we studied the effect of selective elimination of macrophages.

Elimination of spleen macrophages

Recently, a new methodology was developed and characterized for in vivo elimination of macrophages using the liposome encapsulated drug Cl_2MDP

Table 1. Comparison of virulence of WNV and WN-25 in inoculated mice

Virus strain	Virus level (PFU/ml)[a]	ICLD50/ml	IPLD50/ml	Mean day to death (ic)[b]
WNV	3.0×10^8	1.3×10^8	7.0×10^7	5.5
WN-25	1.1×10^8	1.1×10^7	$< 3.2 \times 10$	6.0

[a] PFU titrations of the stock solutions were carried out on Vero cell monolayers

[b] Mean day to death (MDTD) was determined following i.c. inoculation of 10 PFU/mouse

[19]. This methodology selectively eliminated defined populations of macrophages (controlled by the route of administration of the liposomes), and minimized side effects on non-phagocytic cells [17]. Furthermore, the drug becomes effective only after the liposomes have been phagocytosed by the macrophages, whereas the free drug is immediately cleared from the organism. For comparison to the macrophage-depleted animals, the control mice should have normal healthy macrophages, therefore sham injections of PBS should be sufficient. Control treatments of PBS-containing liposomes could be evaluated, to minimize the possibility that the effects observed are not due to macrophage-depletion. However, it should be stressed that phagocytosis of empty liposomes, as other particulate compounds, may alter macrophage functions for some time, and therefore PBS buffer would be a more appropriate control (for a detailed discussion on control experiments, see [19]). We decided to study the effect of macrophage depletion by use of this methodology.

The macrophage-eliminating effect of the Cl_2MDP-liposomes, administrated according to the protocol described in Materials and methods, was evaluated by histochemical methods. One day after the last liposome administration the spleen was removed, cryostat sections were cut and checked for the presence of macrophages by staining for acid phosphatase (Fig. 1). Macrophages were completely eliminated from the red pulp, and the overall picture found was similar to that described previously for a single injection of Cl_2MDP-liposomes [3, 19, 21].

Effect of macrophage-depletion on wild-type WNV infection

As can be seen in Table 2, inoculation of mice with WNV (50 PFU/mouse) caused encephalitis and death of the infected mice. Administration of Cl_2MDP-liposomes accelerated the process, as demonstrated by the decrease in the mean time to death in the groups receiving the drug compared to the PBS-liposomes (groups III and V versus groups II and IV, respectively). Control mice receiving the different types of liposomes (without virus) did not show any overt effects of the treatments. An additional parameter that demonstrates the exacerbating effect of the depletion of macrophages was the level of viremia in the treated mice. As shown in Table 2, the virus level (determined on day 2 p.i.) in the blood of macrophage-depleted mice was much higher than in the control mice ($p < 0.001$).

The results described so far indicate that the elimination of macrophages permitted higher viremia and faster development of encephalitis and consequent death. However, the high rate of mortality of the untreated inoculated mice did not allow estimation of the effect on mortality of inoculated mice. Therefore we decided to test limiting conditions, namely using viral doses of 50 or 5 PFU/mouse. As can be seen in Table 3, the lower dose of virus inoculation did not cause the death of all the normal infected mice (group II), however it did kill all the macrophage-depleted mice (groups VI and VIII). Furthermore, the development of the disease was faster in the macrophage-depleted mice than in the sick normal mice (groups V–VIII versus groups I–IV).

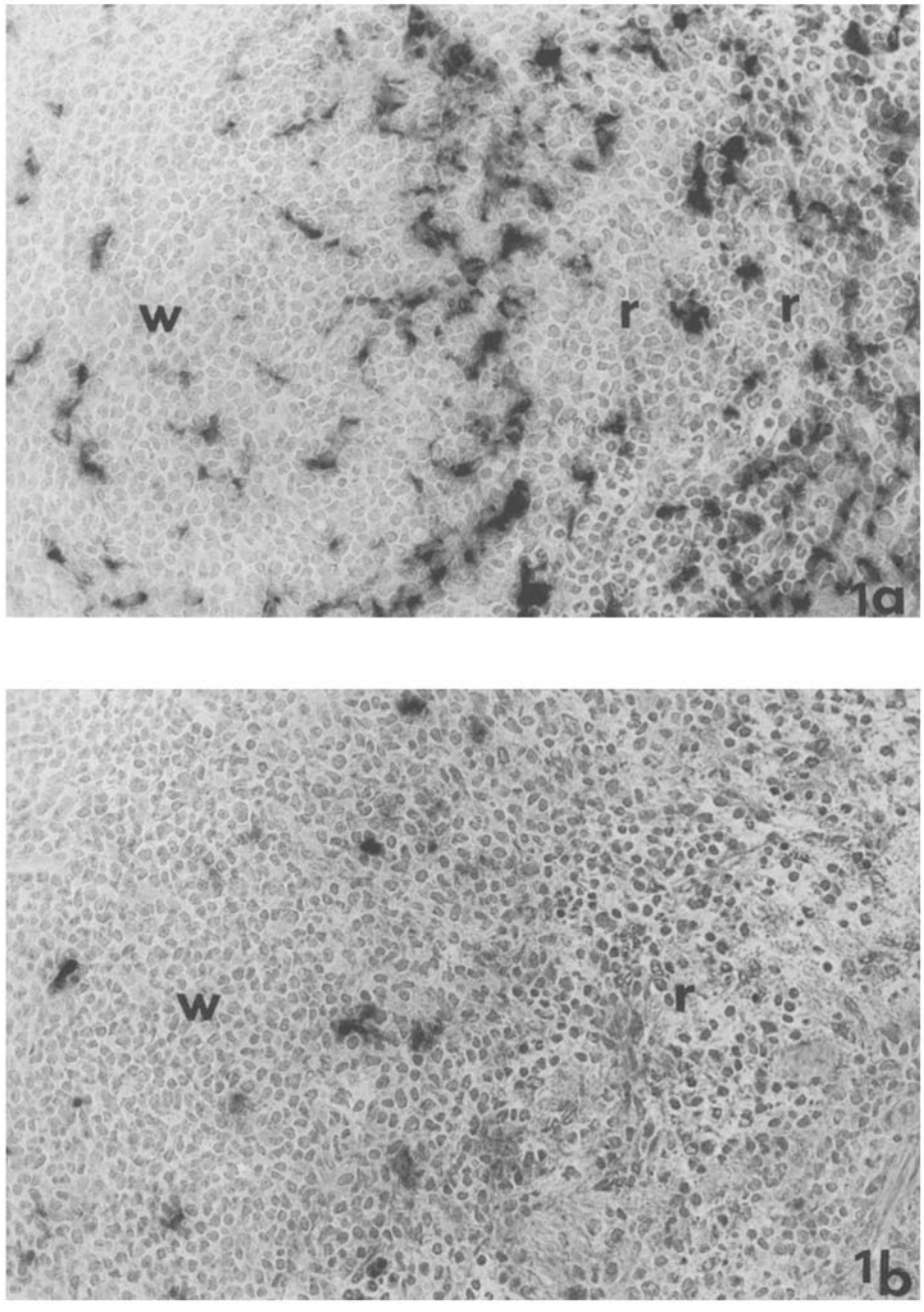

Fig. 1. Photographs of splenic tissue, stained for acid phosphatase activity, which is in the spleen, exclusively present in macrophages. **a** Normal spleen showing acid phosphatase positive macrophages in the red pulp (*r*) and marginal zone and to a lesser extent in the white pulp (*w*). **b** Spleen of a mouse after treatment with Cl_2MDP-liposomes. Note that the acid phosphatase positive cells have disappeared from the red pulp (*r*) and marginal zone. Only very few macrophages remain present in the white pulp (*w*)

Table 2. The effect of liposome administration on viremia and mortality of mice inoculated with West Nile virus

Group	Treatment[a]	Mortality dead/total	%	Mean days to death	Viremia[b] log 10 PFU/ml
I	PBS	10/12	83	8.8	1.9 ± 0.2
II	PC lip-PBS	11/12	91	9.3	2.0 ± 0.2
III	PC lip-Cl_2MDP	8/9	88	7.0	4.3 ± 0.2^b
IV	PCMAN lip-PBS	9/11	82	8.3	2.1 ± 0.1
V	PCMAN lip-Cl_2MDP	12/12	100	6.5	4.4 ± 0.15^b

Mice were inoculated i.p. with WNV-50 PFU/mouse (for viremia, N = 6). Viremia was determined on day 2 p.i.

[a] Liposome administration as described in Materials and methods

[b] $p < 0.001$ compared to groups I and IV

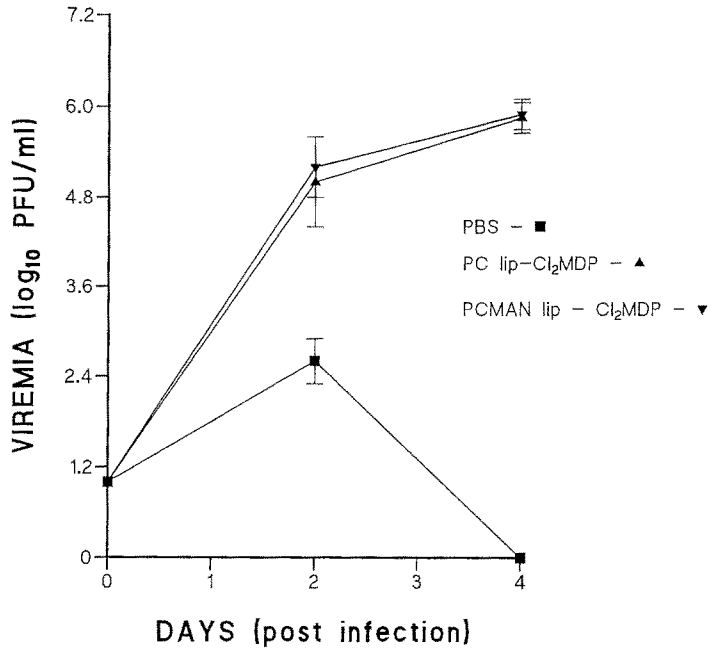

Fig. 2. The effect of macrophage depletion on the level of virus in the blood of mice inoculated with WNV. Mice were inoculated i.p. with 50 PFU/mouse (N = 6)

We have previously found that inoculation of mice with WNV resulted in a temporary viremia, peaking on day 2 p.i. and disappearing by day 4 [6]. Depletion of the macrophages by Cl_2MDP-liposomes promoted higher and longer viremia, as seen when the virus level in blood was determined on days 2 and 4 p.i. (Fig. 2).

Table 3. The effect of macrophage-depletion on mortality of mice inoculated with WNV

Group	Treatment[a]	Virus dose[b] (PFU/mouse)	Mortality D/T	%	Mean days to death
I	PBS	50	7/8	87	8.0
II	PBS	5	4/8	50	8.7
III	PC lip-PBS	50	7/8	87	8.1
IV	PC lip-PBS	5	5/8	62	8.8
V	PC lip-Cl_2MDP	50	10/10	100	6.5
VI	PC lip-Cl_2MDP	5	10/10	100	7.1
VII	PCMAN lip-Cl_2MDP	50	10/10	100	6.3
VIII	PCMAN lip-Cl_2MDP	5	10/10	100	6.9

[a] Liposome administration as described in Materials and methods
[b] Mice were inoculated i.p. with WNV
D/T Dead/total

Table 4. The effect of macrophage depletion on mice inoculated with the WN-25 attenuated virus

Treatment	Viremia[a] 2d p.i.	4d p.i.	Mortality D/T	%	Mean days to death
PBS	1.9 ± 0.2	< 0.6	0/6	0	–
PC lip-Cl_2MDP	5.3 ± 0.2[b]	4.8 ± 0.3[b]	7/10	70	7.7
PCMAN lip-Cl_2MDP	5.4 ± 0.3[b]	4.6 ± 0.4[b]	8/12	75	7.4

Mice were inoculated i.p. with 2×10^5 PFU/mouse (N = 6)
[a] Log 10 PFU/ml
[b] $p < 0.001$ compared to the PBS group

Effect of macrophage-depletion on attenuated WN-25 virus infection

Maintenance of a persistent high viremia for a few days was shown to induce the penetration of non-neuroinvasive viruses into the CNS [6]. Therefore we studied the effect of macrophage elimination on the viremia of the noninvasive variant of WN, the WN-25 virus. As can be seen in Table 4, in macrophage depleted mice the high viremia of WN-25 was maintained for several days, similar to the effect seen with WNV. The macrophage depletion permitted the invasion of the attenuated virus into the brain of the inoculated mice, causing encephalitis and death (Table 4). The MDTD in macrophage-depleted mice was found to be 7.5, 1.5 day longer than following i.c. inoculation of WN-25 (Table 1).

The neuroinvasion of the CNS by the attenuated virus was demonstrated by determination of the progeny virus in the brain of several of the macrophage-depleted dead mice and establishment of their neuroinvasive characteristics (Table 5). As can be seen, the virus in the brain proliferated to levels of 10^9–10^{10}

Table 5. Virulence of progeny virus isolated from brains of macrophage depleted mice inoculated with WN-25

Source of virus	Virus concentration (PFU/ml)[a]	ICLD50/ml	IPLD50/ml
Brain 1	9.2×10^8	1.1×10^8	< 32
Brain 2	5.3×10^8	7.3×10^7	< 32
Brain 3	2.3×10^8	1.7×10^8	< 32
Brain 4	4.3×10^8	4.9×10^7	< 32

[a] Virus concentration was determined in 20% brain homogenate

PFU/brain, similar to the levels measured after WN-25 i.c. inoculation (data not shown). The virus isolated from the brains of the dead mice did not invade the CNS when injected i.p., but killed mice when inoculated i.c. (Table 5), therefore we can conclude that the virus did not change its properties. These findings are of interest as in previous studies it was suggested that in immunosuppressed mice, i.e. during stress, WN-25-induced encephalitis was mediated by increased proliferation and selection of a neuroinvasive strain [1].

Discussion

The initial hypothesis that led to this study was that macrophages were involved in the pathway of viral neuroinvasion and therefore, the interaction of the neuroinvasive WNV with macrophages, which differed from the interaction of WN-25, would account for the neuroinvasive trait. However, depletion of macrophages exposed an additional neuroinvasive pathway, which did not depend on the genetic characteristics of the virus.

Macrophage depletion permitted the maintenance of high and long viremia, conditions that were previously shown to be sufficient to induce the penetration of noninvasive viruses into the CNS, when inoculated into SCID (severe combined immunodeficient) mice [6]. The effect of macrophage elimination on the development of encephalitis was shown in mice inoculated with either the neuroinvasive WNV or the noninvasive WN-25 virus. Comparison of the MDTD determined for the different treatments (Tables 1 and 2) indicated that penetration of WNV into the brain of macrophage-depleted mice occurred during the first 24 hours p.i.. Whereas the MDTD for WNV injected i.p. was 8–9 days and for i.c. inoculation 5–6 days, the value obtained for macrophage-depleted mice injected i.p. was 6–7 days. Therefore, the apparent interpretation would be that the high viremia (permitted by the macrophage depletion) caused immediate neuroinvasion, overriding the natural pathway for CNS penetration exhibited by the neuroinvasive WNV. The very efficient CNS penetration in macrophage-depleted mice was also seen with WN-25, where the MDTD after i.p. injection was 1–2 days longer than following i.c. injection. It should be noted that in SCID mice, WN-25 inoculation is followed by increasing viremia,

reaching values of 10^5 PFU/ml after 4–5 days, causing encephalitis and death 9–10 days p.i. [6]. Although macrophage depletion and lack of specific antibodies (as in SCID mice) resulted in high and prolonged viremia leading to encephalitis and death, macrophage elimination had a more pronounced and faster effect. (No significant difference was found between treatments with PC-liposomes and PCMAN-liposomes.)

Macrophages play a central role in the non-specific defence against viruses. Phagocytosis followed by digestion of intracellular virus particles, lysis of virus infected cells and interferon production are some examples of macrophages antiviral properties. In the WN system, the primary activity of the macrophages as scavengers is sufficient to control the early stages of viremia, preventing secondary effects like tissue invasion. Once this activity is eliminated, the development of the viral infection is exacerbated, permitting high viremia and consequently invasion of the CNS. These results support the hypothesis that the nature of interaction between virus and macrophages is an important determinant of the development of viremia [14]. Avoidance of uptake by macrophages would facilitate the maintenance of viremia, whereas inactivation of the virus by phagocytosis would terminate it. Inhibition of the phagocytic capacity of macrophages has been shown to amplify viremia and potentiate the severity of the viral infection [15, 23, 24].

The findings described in this report indicate a significant role for macrophages in the non-specific immediate defense system of the organism in case of a viral infection, controlling the development of viremia and limiting the severity of the following spread. Macrophage depletion was shown to inhibit early virus clearance, leading to high viremia and neuroinvasion of a noninvasive virus, causing fatal encephalitis.

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