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Meningeal and Perivascular Macrophages of the Central Nervous System Play a Protective Role During Bacterial Meningitis

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Meningeal (MM) and perivascular macrophages (PVM) constitute major populations of resident macrophages in the CNS that can be distinguished from microglial cells. So far, there is no direct evidence that demonstrates a possible role of MM and PVM in the CNS during normal or pathologic conditions. To elucidate the role of the MM and PVM during CNS inflammation, we have developed a strategy using a single intraventricular injection of mannosylated clodronate liposomes, which results in a complete and selective depletion of the PVM and MM from the CNS. Depletion of the MM and PVM during experimental pneumococcal meningitis resulted in increased illness, which correlated with higher bacteria counts in the cerebrospinal fluid and blood. This was associated with a decreased influx of leukocytes into the cerebrospinal fluid, which occurred despite an elevated production of relevant chemokines (e.g., macrophage-inflammatory protein-2) and a higher expression of vascular adhesion molecules (e.g., VCAM-1). In contrast, the higher bacterial counts correlated with elevated production of local and systemic inflammatory mediators (e.g., IL-6) indicating enhanced local leukocyte and systemic immune activation, and this may explain the worsening of the clinical signs. These findings show that the PVM and MM play a protective role during bacterial meningitis and suggest that a primary action of these macrophages is to facilitate the influx of leukocytes at the blood-brain barrier. More in general, we demonstrate for the first time that the PVM and MM play a crucial role during inflammation in the CNS. The Journal of Immunology, 2001, 167: 4644–4650.

Despite effective antibiotic treatment, bacterial meningitis is still an infection with a high mortality rate, particularly for very young and elderly patients (1). Furthermore, recovered patients may suffer from neurological damage, including cerebral infarction, recurrent seizures, hearing loss, learning disabilities, and/or mental retardation (2, 3). When the bacteria have entered the subarachnoidal space, endogenous cells of the CNS and infiltrated leukocytes are triggered to produce proinflammatory cytokines like TNF-α, IL-1β, and IL-6, which lead to an increased permeability of the blood-brain barrier (BBB)² (1). As a result of this enhanced permeability state, leukocytes, predominantly granulocytes, are recruited to the CNS. Activated granulocytes play a major role in bacterial clearance and also secrete a variety of inflammatory mediators (e.g., reactive oxygen species) that may not only kill bacteria, but also cause damage to the CNS (4–8). These mediators play a key role in the pathophysiology of bacterial meningitis and contribute to the unfavorable outcome of the disease due to intracranial complications such as decreased cerebral blood flow, raised intracranial pressure, and brain edema (6, 9).

The cerebrospinal fluid (CSF) lacks the presence of cell populations capable of initiating an effective immune response against invading pathogens. Therefore, important questions are which (and how) resident cell populations in the CNS initiate and regulate the inflammatory response during meningitis. Resident CNS macrophages, and in particular, the meningeal (MM) and perivascular (PVM) macrophages that line the meninges and surround local blood vessels (10), are prime candidates. The MM and PVM can be identified in the rat by the selective expression of the ED2 Ag (11). They express scavenger receptors and are highly phagocytic (12). Furthermore, under various inflammatory conditions the MM and PVM can express inflammatory mediators such as TNF-α, IL-1β, and cyclooxygenase-2 (13–15). Until now only one study has examined the role of the MM during bacterial meningitis (16). It was observed that reduction of the MM by intracisternal injection of clodronate liposomes did not affect inflammation in the subarachnoidal space. Unfortunately, the liposome treatment only resulted in a partial depletion of the MM, with no further information about: 1) the effect on the PVM and other CNS cell populations (e.g., microglial cells), 2) the effect on other parts of the CNS like the spinal cord and cerebrum, and 3) possible side effects on the periphery (e.g., Kupffer cells, splenic macrophages, and monocytes).

We have recently described a depletion method that results in a complete and selective depletion of the MM and PVM (17). Here we use this method to study the role of the MM and PVM in an
experimental bacterial meningitis model. Our results provide the first direct evidence for the role of MM and PVM in bacterial meningitis in particular, and in inflammation in the CNS in general.

Materials and Methods

Animals

Male Wistar rats were obtained from CPB-Harlan (Zeist, The Netherlands) and weighed 300–350 g at the time of the experiment. Animals were kept under routine laboratory conditions and were allowed free access to food and water. Microbiological status of the animals was according to Federation of European Laboratory Animal Science Associations recommendations. Approval of the Animal Ethics Committee of Vrije Universiteit was obtained for all animal experiments.

Preparation of liposomes

Multilamellar mannosylated liposomes were prepared as described before (18). Briefly, 178 mg of phosphatidylcholine and 27 mg of cholesterol were dissolved in 8 ml of chloroform, which was added to 9.25 mg of p-amino-phenyl-α-β-mannopyranoside (Sigma, St. Louis, MO) dissolved in 5 ml of methanol in a 500-ml round-bottom flask. This was dried in vacuo on a rotary evaporator to form a film. The molar ratio of phosphatidylcholine/cholesterol/mannoside was 7:2:1 (according to Ref. 19). The lipid film was dispersed in 10 ml of PBS (0.15 M NaCl in 10 mM phosphate buffer, pH 7.4) for the preparation of PBS-containing mannosylated liposomes. To enclose the clodronate, 2.5 g (a gift of Roche Diagnostics, Mannheim, Germany) of clodronate was dissolved in 10 ml of deionized water (adjusted to pH 7.3 with NaOH) in which the lipid film was dispersed, and the preparations were kept for 2 h at room temperature, sonicated for 3 min, washed, and resuspended in 10 ml of PBS. PBS-containing control liposomes and clodronate-containing liposomes were labeled with the fluorescent dye Dil (1,1-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; D282; Molecular Probes, Eugene, OR) as described by Claassen (20), with minor alterations. Dil was dissolved in absolute ethanol at a concentration of 2.5 mg/ml, sonicated for 5 min, and stored at 4°C. Dil was added to 1 ml of liposome suspension in a final concentration of 62.5 μg/ml and incubated for 30 min at 37°C. The suspension was washed three times with 2 ml of PBS (24,000 × g, 15 min) to remove free Dil, and the pellet was resuspended in 400 μl of PBS.

Stereotaxical injection of liposomes

The rats were deeply anesthetized with an i.m. injection of 1.0 ml/kg body weight of a mixture of 4 parts ketamine (1% solution of Ket; Aescos, Boxtel, The Netherlands) and 3 parts xylazine (2% solution of Rompun; Bayer, Brussels, Belgium) and mounted in a stereotaxic frame. Control or clodronate liposomes (50 μl) were slowly (over a total time period of 25 min) injected stereotactically with a blunt Hamilton syringe (Hamilton Bonaduz, Bonaduz, Switzerland) into the 4th ventricle. Coordinates (Bregma +11.6 and 5.5 mm dorsoventral) were derived from the atlas of Paxinos and Watson (21).

Bacterial meningitis model

Streptococcus pneumoniae type 6A (isolated from the CSF of a meningitis patient) were incubated in brain heart infusion broth at 37°C. After 16–17 h, 5 ml of this preincubation culture was mixed with 20 ml of fresh brain heart infusion medium and further incubated for 3–5 h at 37°C until OD620 = 1 was reached. Rats were mounted in a stereotaxic frame and injected intracisternally with −5 × 10^6 CFU of S. pneumoniae, 7 days post clodronate liposome (n = 11) or PBS liposome (n = 7) injection. Twelve hours post meningitis induction rats were clinically scored (0 = no clinical symptoms, 1 = red nose and eyes, 2 = reduced activity/apathy/supine recision, 3 = convulsions/respiratory problems) and sacrificed with an i.p. injection of 1 ml of Nembutal (Sanofi Sante Animale, Beneux B.V., Maassluis, The Netherlands). The inoculum (S. pneumoniae) was plated (1 × 10^10–1 × 10^5) on sheep blood agar-hemin menadione (BA-hm) plates and cultured overnight at 37°C to determine the amount of CFU injected.

Sample collection and processing

Blood was collected via a heart puncture for blood smears, and 1 × 10^10–1 × 10^7 eg of EDTA blood was plated on sheep BA-hm plates for identification and quantification of the bacteria. Serum was collected for measurement of TNF-α, IL-1β, IL-6 (National Institute for Biological Standards and Control, South Mimmns, Potter Bar, Hertfordshire, U.K.), and macrophage-inflammatory protein (MIP)-2 (BioSource International, Camarillo, CA) by ELISA. CSF was aspirated via a cisterna magna puncture, and 1 × 10^5–1 × 10^4 was plated on BA-hm plates, for the quantification of the bacteria. The leukocytes were counted using a hemocytometer, and cytocentrifuge preparations were made and stained with May-Grünewald Giemsa dye (Merck, Darmstadt, Germany), according to standard histological staining methods. CSF samples were then centrifuged, cells were stored in 10% DMSO/90% NBCS at −80°C, and the supernatant was stored at −80°C for determination of MIP-2 by ELISA.

The cerebellum, cerebrum, spinal cord (cranial-caudal), liver, cervical lymph nodes, and spleen were collected, snap-frozen, and stored at −80°C until histological evaluation.

Bacterial peritonitis model

Seven days post clodronate liposome (n = 6) and PBS liposome (n = 5) injection into the 4th ventricle as described above, the rats received an i.p. injection of 3 × 10^10 CFU/0.5 ml S. pneumoniae type 6A (culture conditions as described above). Four hours after this bacteria injection into the peritoneum, the rats were sacrificed by O2/CO2 exposure. The peritoneal cavity was washed with 20 ml of PBS.

Immunohistochemistry for light microscopy

Cryostat sections (8 μm) were cut serially, picked up on gelatin coated slides, and air dried overnight in a container with silica gel. Immunohistochemistry was applied after a 10-min fixation in dehydrated acetone to examine the presence of macrophages, neutrophils, and the expression of cerebral endothelial adhesion molecules. The murine mAbs in this study include the anti-rat macrophage markers ED1 and ED2. ED1 recognizes a lysosomal membrane-related Ag on both monocytes and macrophages, and ED2 recognizes an Ag on a subset of mature rat macrophages including the MM and PVM in the CNS (22). HIS48 recognizes an Ag on all granulocytes (23). 1A29 (a gift of T. Taniatami, Department of Immunology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) directed against ICAM-1 (24), 3A12 (a gift of W. F. Hickey, Dartmouth Medical School, Lebanon, NH) for platelet endothelium cell adhesion molecule (PECAM; Ref. 25), and 5F10 (a gift of R. Lobb, Biogen, Cambridge, MA), which recognizes VCAM-1 (26) on rat cerebral endothelial cells. Abs and conjugates were diluted in 0.1 M Tris buffer, pH 7.6, with 0.1% BSA (Organon Technika, Oss, The Netherlands) and used at optimal dilution of 2 μg/ml for ED1-biotin and ED2-biotin; 6 μg/ml for 5F10; 2.75 μg/ml for 3A12, 1/5 of the 1A29 supernatant, and 1 μg/ml for His48. Control slides included incubations in which the primary Ab was omitted. All incubations were conducted horizontally at room temperature. After incubation with the first Ab for 60 min, the slides were rinsed in Tris buffer, pH 7.6, with 0.1% BSA, incubated with conjugate/0.1% normal rat serum for 60 min, and washed again with Tris/BSA buffer. As conjugates we used streptavidin-alkaline phosphatase-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark) and alkaline phosphatase-conjugated rabbit anti-mouse IgG + IgM (DAKO). Alkaline phosphatase activity was demonstrated by incubation in naphth AS-Bl phosphate substrate (27) in 0.1 M Tris, pH 8.7, buffer for 10 min. Sections were rinsed in 0.1 M Tris, pH 7.6, light counterstained with hematoxylin, and mounted in VectaMount (Vector Laboratories, Burlingame, CA). The sections were examined with a microscope (Nikon Eclipse E800; Melville, NY), and the recordings were made with a Nikon DXM1200 camera.

Quantification of leukocytes

The number of leukocytes were counted with a light microscope using ×400 magnification. Two hundred total white blood cells were counted per CSF cytocentrifuge preparation and, subsequently, the percentage of neutrophils, eosinophils, macrophages, and lymphocytes of the total amount of white blood cells was counted.

Results

Pneumococcal meningitis model in a rat

Before investigating the role of the MM and PVM during meningitis, we evaluated the kinetics of the S. pneumoniae meningitis model in detail. We counted the number of bacteria and leukocytes in the CSF and blood of the animals after 4, 12, 24, and 48 h. We observed a decline in the number of bacteria in the CSF, 12 h post meningitis induction, followed by an increase after 24 and 48 h...
There were no detectable bacteria in the blood between 12 and 24 h, and they reappeared at 48 h post \textit{S. pneumoniae} injection into the cisterna magna (Fig. 1b). To see whether the decrease in bacteria correlated with the presence of granulocytes, which play a major role in bacterial clearance, we quantified the cells present in the CSF. We observed a maximal influx of granulocytes, mainly neutrophils (~75%), at 12 and 24 h post \textit{S. pneumoniae} inoculation, followed by a decrease after 48 h (Fig. 1c). The granulocytes were mainly situated within the subarachnoidal and Virchow-Robin space, and only a low percentage seemed to be located within the parenchyma of the brain (Fig. 1d). Based on these observations, we decided to investigate the effect of MM and PVM depletion 12 h post meningitis induction.

**MM and PVM depletion enhances clinical symptoms and bacterial numbers**

We have recently developed a liposome-based depletion method (17) that allows us to investigate the role of the MM and PVM during inflammation in the CNS. This method is based on a single intraventricular injection of clodronate liposomes resulting in a complete and selective depletion of the MM and PVM throughout the CNS. We induced the bacterial meningitis model 7 days post

**FIGURE 1.** \textit{S. pneumoniae} meningitis model in the rat. \textit{a} and \textit{b}, Number of CFU in the CSF and blood. \textit{c}, Number of infiltrated cells into the CSF. \textit{d}, Massive influx of granulocytes (HIS 48°, pink cells) in the cerebellum of the rat 12 h post meningitis induction (×100). The granulocytes are mainly located in the subarachnoidal space (large arrow), perivascular space (arrow head), and only a small percentage reside in the parenchyma (small arrow). Data are mean ± SE of four rats per time point.

**FIGURE 2.** Depletion of the MM and PVM from the CNS during meningitis. Staining of the MM and PVM with the mAb ED2 (pink cells). \textit{a}, Cerebellum of a control liposome-treated animal (×200). The small indent shows ED2° PVM near a blood vessel, at a higher magnification (×1000). \textit{b}, Cerebellum of a clodronate liposome-treated animal (×200). Again, Indent represents a higher magnification (×1000) of a blood vessel. Note the complete absence of PVM in \textit{b}.
depletion of the MM and PVM. At this time point, the liver and draining lymph nodes, which suffer from a mild and temporal macrophage depletion using this regime, are completely repopulated with macrophages (17). First, we tested whether this method could also be used to deplete the MM and PVM during meningitis induction; both the MM and PVM were still completely absent from all CNS areas tested (Fig. 2, a and b). We evaluated the animals for clinical symptoms 12 h post S. pneumoniae inoculation and observed that the depleted group was clearly more ill than the control group (Fig. 3a). In correlation with the more severe disease course \((p = 0.04)\), we observed profoundly higher bacterial counts in CSF (approximately \(\times 600\)) and blood (approximately \(\times 20\)) of the MM- and PVM-depleted animals (Fig. 3, b and c).

**MM and PVM depletion decreases leukocyte influx into the CSF**

Although the results described above were in agreement with a possible role of the MM and PVM in bacterial clearance, the dominant clearing cell is probably the granulocyte (2, 6). Therefore, it was important to investigate whether MM and PVM depletion was affecting the influx of granulocytes and other leukocytes into the CSF. We observed that the number of leukocytes that had extravasated was significantly decreased in comparison with the control group (Fig. 4a). Also no signs of increased cell death were observed. The leukocytes were mainly composed of granulocytes (~90%), and no difference in composition was noted between depleted animals and controls (Fig. 4b). To exclude the possibility that the decreased influx of granulocytes in the clodronate liposome-treated animals was caused by a direct effect of the clodronate liposomes on circulating neutrophils, a control experiment was performed. Seven days after the depletion of the MM and PVM, S. pneumoniae peritonitis was induced, and the number of immigrated cells in the peritoneal cavity was quantified 4 h post peritonitis induction. There was no significant difference between the control and depleted animals (Fig. 5a). Analysis of May-Grünewald Giemsa-stained peritoneal cell cytocientrifuge preparations showed that 4 h after i.p. bacteria injection the major infiltrating population is formed by granulocytes (~90%); this was the same for the control, MM-, and PVM-depleted animal groups (Fig. 5b). In other words, the intrinsic migratory capacity of leukocytes was not affected by the liposome treatment.

**FIGURE 3.** MM and PVM depletion enhances clinical symptoms and bacterial numbers. a. Clinical score of control \((n = 7)\) and depleted \((n = 11)\) animals 12 h post S. pneumoniae injection \((*, p < 0.05, \text{unpaired } t \text{test})\). The numbers of bacteria (CFU) are depicted in CSF (b) 12 h post meningitis induction \((**, p < 0.007, \text{Mann-Whitney } U \text{test})\).

**FIGURE 4.** MM and PVM depletion decreases leukocyte influx into the CSF. The amount of infiltrated leukocytes into the CSF, 12 h post meningitis induction, of control \((n = 7)\) and depleted \((n = 11)\) animals is shown in a \((*, p < 0.05, \text{unpaired } t \text{test})\). In both groups the leukocytes are predominantly composed of granulocytes (b).

**FIGURE 5.** MM and PVM depletion does not influence the intrinsic leukocyte migratory capacity. The number of immigrated cells, 4 h after S. pneumoniae peritonitis induction, was not significantly different between the control \((n = 5)\) and depleted group \((n = 6)\) \((p = 0.34, \text{unpaired } t \text{test})\). Infiltrated leukocytes were mainly granulocytes (b).
MM and PVM depletion enhances systemic proinflammatory cytokine production in meningitis

It is known that high levels of proinflammatory cytokines correlate with the pathophysiological symptoms and the clinical outcome in meningitis patients (reviewed in Ref. 1). Therefore, it was important to establish whether the enhanced numbers of bacteria in the circulation of the depleted animals were accompanied by higher levels of systemic proinflammatory cytokines. Significantly higher levels of IL-6 in the serum of the depleted group in comparison with the control group were observed (Fig. 6c). We could not observe detectable levels of TNF-α (<15 pg/ml) and IL-1β (<15 pg/ml) in the serum of either group.

MM and PVM depletion enhances MIP-2 production in the CSF during meningitis

To investigate whether the decrease in neutrophil influx after MM and PVM depletion was the possible consequence of a lowered chemokine expression in the CSF, we measured the expression levels of MIP-2. MIP-2 is both essential (28, 29) as well as sufficient (P. Zwijnenburg, unpublished observation) for the infiltration of granulocytes into the CSF during rodent bacterial meningitis. Moreover, MM and PVM appear to be the only endogenous CNS sources of MIP-2 in meningitis (28). Surprisingly, CSF MIP-2 levels of the depleted animals were much higher (530–2577 pg/ml) in comparison with the control meningitis group (34–448 pg/ml) (Fig. 6a). We could even detect elevated MIP-2 levels (7–22 pg/ml) in the serum of depleted animals as compared with controls, which displayed levels that were below detection (<10 pg/ml) (Fig. 6b). Thus, the decrease in leukocyte influx could not simply be explained by a decrease in MIP-2 expression.

MM and PVM depletion increases the expression of vascular adhesion molecules on the BBB during meningitis

An alternative explanation for the decreased influx of granulocytes into the CSF in MM- and PVM-depleted animals was a possible decrease in vascular adhesion molecule expression on the BBB. Therefore, we analyzed the vascular adhesion molecule expression on the cerebral endothelial cells, including VCAM-1 and ICAM-1, which are known to play a role in neutrophil extravasation (2, 30, 31) and PECAM. Although in both cases the bacterial infection resulted in an up-regulation of VCAM in comparison with noninfected animals (Fig. 7, a vs b and c), this was clearly enhanced in the MM- and PVM-depleted meningitis group. This enhancement was visible at both the level of staining intensity as well as the
number of VCAM-positive vessels. An up-regulation of ICAM-1 and PECAM was also observed, but this difference was clearly less profound than in the case of VCAM-1 (data not shown). This demonstrates that, despite a significantly enhanced expression of both relevant vascular adhesion molecules and chemokines, MM and PVM depletion suppresses leukocyte extravasation.

Discussion
The MM and PVM, as intrinsic components of the BBB, are considered to form a first line of defense when the endothelial integrity is circumvented (10). To elucidate the possible role of the MM and PVM during bacterial meningitis, we have used a previously developed strategy using a single intraventricular injection of clodronate liposomes, which results in a complete and selective depletion of the MM and PVM throughout the CNS (17).

Our present results show that depletion of MM and PVM during pneumococcal meningitis results in an aggravation of clinical symptoms (Fig. 3a) demonstrating for the first time that these macrophages play a protective role during bacterial meningitis. MM and PVM elimination resulted also in elevated numbers of bacteria, as well as enhanced levels of inflammatory mediators in both CSF (MIP-2) and blood (IL-6). Furthermore, a reduced influx of granulocytes and other leukocytes into the subarachnoid space was observed after MM and PVM depletion; this occurred despite elevated levels of the relevant chemokine (MIP-2) and a higher expression of vascular adhesion molecules (e.g., VCAM-1).

What does this reveal about the role of MM and PVM in the pathogenesis of bacterial meningitis? Based on the observation that depletion of MM and PVM, as intrinsic components of the BBB, reduces leukocyte influx (Fig. 4), it is concluded that MM and PVM somehow support leukocyte traffic across the BBB. We propose that, among the parameters studied, this role in leukocyte transmigration constitutes the primary action of MM and PVM and that this, together with our other observations, can explain their protective role during meningitis. Although our results indicate that MM and PVM play a supportive role in the infiltration of leukocytes across the BBB during bacterial meningitis, the underlying mechanism remains elusive. One obvious possibility was that in the early stages of meningitis MM and PVM are activated to produce mediators (cytokines/chemokines) that induce endothelial activation and leukocyte migration. We have investigated whether the reduced migration of leukocytes after MM and PVM depletion was accompanied by a reduced expression of relevant vascular adhesion molecules (e.g., VCAM-1, ICAM-1) or chemokines (e.g., MIP-2), and this was clearly not the case. Instead, enhanced VCAM-1 and MIP-2 levels were found. We believe that these findings may in fact be the consequence of a local and/or systemic immune response. An alternative explanation for the role of MM and PVM may be that they can affect the kinetics of the inflammatory response during meningitis. A thorough kinetic analysis may provide further insights.

Because granulocytes in the CSF are considered to eliminate the majority of bacteria during meningitis (2, 6, 32), a reduction in granulocytes resulted in increased bacterial numbers in the CSF (Fig. 3). The higher load of bacteria (and/or their degradation products) may turn potentiate leukocyte activation in the CSF, which will result in enhanced production of various effector molecules, including reactive oxygen species, that cause neuronal damage (9). These effector molecules, together with the elevated systemic cytokine response (e.g., IL-6; Fig. 6), can explain the observed worsening of the clinical signs (1).

It should be emphasized that our present results cannot demonstrate, or rule out, a possible direct role of MM and PVM in bacterial clearance. Of course, MM and PVM are actively phagocytic (12, 17), and the increase in bacteria in CSF after depletion of these cells is in line with a direct role in clearance. However, considering the concomitant reduction in granulocytes, which likewise represent the major scavengers during meningitis, we cannot presently decide what possible contribution MM and PVM have.

Taken together, these findings demonstrate that MM and PVM play a protective role during bacterial meningitis and suggest that this is, at least in part, due to their supportive role in the influx of leukocytes across the BBB. This also provides the first evidence for a role of MM and PVM during inflammation in the CNS.

References


