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Neutrophils Promote Mononuclear Cell Infiltration During Viral-Induced Encephalitis

Jihhao Zhou,* Stephen A. Stohlman,*‡ David R. Hinton,* and Norman W. Marten*‡*

Neutrophils are the first infiltrating cell population to appear within the CNS during infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV). To determine whether neutrophils play a role in limiting acute JHMV infection, mice were depleted of neutrophils. Infection of neutropenic animals resulted in increased levels of virus replication and mortality compared with control mice. Furthermore, neutropenia resulted in significantly reduced mononuclear leukocyte infiltration possibly due to reduced loss of blood brain barrier integrity during acute JHMV infection. These data suggest that infiltrating neutrophils are crucial for limiting virus replication during acute JHMV infection, contribute to the loss of blood brain barrier integrity and play a role in shaping adaptive immunity within the CNS. The Journal of Immunology, 2003, 170: 3331–3336.

The CNS has numerous mechanisms to minimize destructive inflammatory responses, including the blood brain barrier (BBB), an absence of a lymphatic drainage and low MHC expression (1). Although these mechanisms limit the potential for immune mediated pathology, they can be overcome as in cases of encephalitis induced by either pathogens or autoimmune reactions (1, 2). Neutrophils are potent mediators of acute inflammation for several reasons including their ability to rapidly release preformed antimicrobial and proinflammatory molecules as well as the sheer number of circulating neutrophils (3). Rapid neutrophil recruitment is induced via chemokines, which also increase adhesion molecule expression (4). Interactions between neutrophil and endothelial adhesion molecules promote disassembly of tight junctions, permitting neutrophils to extravasate and enter damaged or infected tissues. Neutrophils also secrete preformed matrix metalloproteinases (MMPs) (5), which degrade extracellular matrix proteins including those constituting the basal lamina of the BBB, facilitating tissue entry (6).

Infection of the CNS with the neurotropic JHM strain of mouse hepatitis virus (JHMV) induces extensive inflammatory cell infiltration and has proven to be a useful model for studying both virus replication, inflammatory infiltrates also contain a variety of other potential immune effectors (9). The mechanism(s) by which these infiltrates cross the BBB and migrate into the parenchyma in response to inflammatory signals are still not completely understood. In this study, lethal JHMV-induced encephalitis was used to identify the accumulation and composition of CNS infiltrates. Neutrophils were the first, and remained the most abundant inflammatory cells entering the infected CNS. In addition, neutrophils possess much of the cellular machinery required to mediate transmigration of the BBB (5, 10, 11) and their infiltration correlated with a rapid decline in BBB integrity. Infection of neutropenic mice resulted in increased virus replication and mortality, suggesting either a direct or indirect role for neutrophils in limiting viral replication. Inflammatory cell infiltration was almost completely eliminated and loss of BBB integrity was significantly inhibited following infection of neutropenic mice, indicating that neutrophils play a critical role(s) in regulating subsequent CNS inflammatory events.

Materials and Methods

Animals and virus

Six-week-old male BALB/c (H-2b) mice were purchased from the National Cancer Institute (Frederick, MD) and were certified mouse hepatitis virus seronegative. Mice were infected intracerebrally with 100 PFU of the neurotropic DM variant of JHMV in 30 μl of Dulbecco’s PBS (12). Neutrophils were depleted by i.p. administration of 200 μg of anti-Ly-6G (RB6-8C5) mAb at days −1, +1, and +3 postinfection (p.i.). CD8 T cells were depleted by injection with 200 μg of an isotype matched anti-CD8 mAb (2.43).

CNS virus titers

Virus titers were determined from brain homogenates as described previously (12). Briefly, half-brains from three or more mice per time point were individually homogenized in 4 ml of Dulbecco’s PBS (pH 7.4) using Tenbroeck tissue homogenizers. Homogenates were clarified by centrifugation at 800 × g for 7 min at 4°C. Supernatants were stored at −70°C until assayed for infectious virus by plaque assay on DBT cell monolayers as described previously (12).

Isolation and analysis of CNS inflammatory cells

Infiltrating cells were isolated from the CNS of perfused JHMV-infected mice as described previously (13). Briefly, following perfusion with PBS, brains were removed, washed twice with RPMI 1640 medium supplemented with 25 mM HEPES (pH 7.2) and 1% fetal bovine serum, and homogenized in Tenbroeck tissue homogenizers. Cells were suspended in 30% Percoll (Pharmacia, Piscataway, NJ) and concentrated onto a 1-ml cushion of 70% Percoll by centrifugation at 1300 g for 10 min at 4°C. Cells were collected from the interphase and washed twice before analysis.

Abbreviations used in this paper: BBB, blood brain barrier; MMP, matrix metalloproteinase; JHMV, JHM strain of mouse hepatitis virus; p.i., postinfection; MIP, macrophage inflammatory protein; CXCL, CXC chemokine ligand; CCL, CC chemokine ligand.
A3-1) (Serotech, Raleigh, NC) and analyzed using a FACStar flow cytometer and CellQuest software (BD Biosciences, Mountain View, CA).

**Histology**

For histopathologic analysis, brains were removed and bisected in the mid-coronal plane. For paraffin fixed preparations, tissues were fixed for 3 h in Clark’s solution (75% ethanol, 25% glacial acetic acid) and embedded in paraffin. To assess for the presence of inflammatory cells, sections were stained with H&E. Distribution of JHMV Ag was examined in paraffin sections using anti-JHMV mAb J.3.3 as the primary Ab and horse antimouse mAb as a secondary Ab (14). Distribution of neutrophils was examined in frozen sections using anti-Ly-6G mAb (RB6-8C5; BD Pharmingen) as the primary Ab and goat anti-rat as a secondary Ab. Briefly, overnight incubation with primary mAb was followed by incubation with the biotinylated secondary Ab. Visualization was achieved via an avidin-biotin peroxidase complex using 3,3’-diaminobenzidine as chromogen (Vectastain-ABC kit; Vector Laboratory, Burlingame, CA). Control sections were incubated with isotype-matched primary mAb. All slides were scored in a blinded fashion.

**Assessment of BBB integrity**

One hour before sacrifice, mice were injected via the tail vein with 200 μl of 2% Evans blue dye (Sigma-Aldrich, St. Louis, MO) in PBS. Following perfusion with 50 ml of PBS, brains were removed and homogenized in 4 ml of PBS. Supernatants were collected following centrifugation at 1000 x g for 5 min. Quantitative analysis of BBB integrity was based on the method described by Kondo et al. (15). An equal volume of 10% (w/v) TCA was added to supernatants, and incubated at 4°C for 18 h. Supernatants were centrifuged following centrifugation at 1000 x g for 30 min. Dye content within supernatants was determined spectrophotometrically at 610 nm and compared with a dilution series from an Evans blue standard. Data are presented as milligrams of Evans blue per brain.

**Zymography**

Zymography was performed as described previously (16). Cells isolated from the CNS were disrupted by suspension in lysis buffer (1.0% Triton X-100, 300 mM NaCl, 50 mM Tris, pH 7.4). Lysates prepared from 2 x 10^6 cells were separated on a 10% acrylamide gel containing 1% gelatin (Bio-Rad, Hercules, CA) in the absence of reducing agents. Following electrophoresis, gels were washed at room temperature for 20 min in washing buffer (Bio-Rad). Gels were placed in developing buffer (Bio-Rad) for 20 min at room temperature before overnight incubation at 37°C. Gels were then stained with 0.25% Coomassie brilliant blue R-250 and destained with a mixture of 10% acetic acid and 10% methanol until bands became evident. Band intensities were quantitated using a Fluor-S imaging system (Bio-Rad).

**Statistical analysis**

Results generated from three or more samples per time point are represented as the mean ± SD and were analyzed using the Student’s t test.

**Results**

**Rapid and sustained neutrophil response to CNS viral infection**

Neutrophils, identified by flow cytometry as CD11b^+Ly-6G^high (16, 17), have been detected in the CNS of JHMV-infected mice following lethal and sublethal infections (16) as well as following adoptive transfer of activated T cells into immunodeficient recipients infected with the sublethal JHMV variant (17). However, the anti-Ly-6G mAb cross-reacts with the Ly-6C Ag expressed by activated inflammatory cells (18). Flow cytometric analysis of cells isolated from the CNS of JHMV-infected mice revealed a single population of Ly-6G^high/CD45^high/CD11b^+ cells, consistent with neutrophils (Fig. 1A). To confirm the identity of the CD11b^+/Ly-6G^high population, inflammatory cells were isolated from the CNS at day 4 p.i. and the CD11b^+/Ly-6G^high population sorted by FACS. Microscopic analysis demonstrated that the CD11b^+/Ly-6G^high population was comprised of >98% cells exhibiting morphology consistent with neutrophils (Fig. 1B). Infiltration of neutrophils into CNS parenchyma was also confirmed by both morphology (Fig. 1C) and immunohistochemistry (Fig. 1D). Anti-Ly-6G mAb weakly reacted with some CD8^+ cells derived from the CNS; however, the level of CD8^+ T cell expression was distinctly lower compared with the Ly-6G^high population (data not shown). No other Ly-6G^+ cells were detected within the CNS (data not shown).

**Kinetic analysis revealed that neutrophils** (CD11b^+/Ly-6G^high) appeared in the CNS as early as 24 h p.i. (Fig. 1E). Accumulation of neutrophils within the CNS peaked at day 4 p.i. and declined before death at days 6–8 p.i. (Fig. 1E). CNS entry of neutrophils was a consequence of active viral replication since neither injections of PBS nor UV-inactivated virus induced neutrophil infiltration (data not shown). CNS infiltration by other inflammatory cell populations was minimal until day 4 p.i. (Fig. 1E). NK cell and macrophage infiltration within the CNS peaked at day 4 p.i., whereas infiltration of CD4^+ and CD8^+ T cells and B cells did not peak until day 6 p.i. Therefore, throughout early stages of a lethal JHMV infection of the CNS, neutrophils constituted a major component of the acute inflammatory response.

**Elevated virus replication and reduced survival in neutropenic mice**

The rapid and sustained infiltration of neutrophils into the CNS following JHMV infection raised the possibility that neutrophils...
may be involved in limiting viral replication. This hypothesis was investigated by comparing virus titers in the CNS of infected neutropenic and wild type control mice. Levels of virus replication were nearly identical in both groups at day 1 p.i. (\(\sim 1 \times 10^3\) PFU/g; Fig. 2A). By contrast, at days 3 and 4 p.i., virus replication within the CNS of neutropenic mice was \(\sim 10\)-fold higher than in wild type mice (\(p < 0.05\)). Increased virus replication within the CNS of neutropenic mice correlated with a greatly increased prevalence of viral Ag detected within the CNS of infected neutropenic mice compared with those of wild type controls at day 4 p.i. (Fig. 2B). Consistent with the increased viral load in the CNS of neutropenic mice, lethal JHMV infection resulted in an increased rate of mortality compared with wild type mice (Fig. 2C). These data suggest neutrophils contribute to early control of viral replication in the CNS and prolong survival in response to a lethal viral challenge.

**Neutrophils regulate CNS mononuclear cell infiltration**

To further examine the role of neutrophils in limiting JHMV replication, infected neutropenic and control mice were compared via histological analysis. Only scattered inflammatory cells were detected in the parenchyma of infected neutropenic mice at day 4 p.i. (Fig. 3A). By contrast, both diffuse parenchymal inflammatory infiltrates and perivascular cuffs were found in infected control mice (Fig. 3A). The apparent absence of CNS inflammation in infected neutropenic mice was confirmed by flow cytometric analysis of CNS derived cells which revealed that accumulation of total CNS infiltrating inflammatory cells (CD45<sup>hi</sup>) was reduced by \(\sim 90\%\) at day 4 p.i. (Fig. 3B).

Analysis of the composition of the CNS infiltrating population via flow cytometry revealed a > 90% reduction in neutrophils in infected neutropenic mice as compared with wild type controls as expected (Fig. 4A). However, NK cell, macrophage and lymphocyte infiltration were also all reduced to near background levels in brains of virus-infected neutropenic mice compared with those of infected control mice at day 4 p.i. (Fig. 4A). To exclude the possibility that reduced CNS infiltration in neutropenic mice resulted from cross-reactive depletion of Ly-6C<sup>+</sup> cells (18), peripheral compartments in neutropenic and control mice were compared. Analysis of splenocytes confirmed neutrophil depletion in anti-Ly-6G mAb treated mice at day 4 p.i. whereas CD4<sup>+</sup> T cell, NK cell, B cell and macrophage populations all remained intact (Fig. 4B). By contrast, CD8<sup>+</sup> T cells were reduced by \(\geq 95\%\), consistent with the \(\sim 15\%\) reduction in total cell numbers recovered compared with control mice. The depletion of CD8<sup>+</sup> T cells following administration of anti-Ly-6G mAb in vivo has been reported previously (19), despite minimal cross reactivity detected by flow cytometry (Fig. 4C). However, CD8<sup>+</sup> T cells constituted <2% of the CNS inflammatory cellular response at day 4 p.i. (Fig. 4A). Furthermore, accumulation of virus specific CD8<sup>+</sup> T cells within the CNS before day 5 p.i. is negligible (20). Therefore, the absence of CD8<sup>+</sup> T cells was unlikely to have a significant influence on the total CNS inflammatory response at this early stage of infection. To confirm this hypothesis, the CNS inflammatory response in CD8<sup>+</sup> T cell depleted mice was examined at day 4 p.i. The absence of CD8<sup>+</sup> T cells had no effect on the CNS infiltration of other inflammatory cells (data not shown), nor any effect on virus replication compared with control mice at day 4 p.i. (4.9 \(\pm 0.10\) vs 4.9 \(\pm 0.02\) log<sub>10</sub> PFU/g, respectively).

**Neutrophils influence loss of BBB integrity**

Inflammatory responses involving neutrophils are frequently associated with increased vascular permeability (3). Therefore, the reduction in the CNS mononuclear response to JHMV infection in neutropenic mice may be associated with a reduced ability of mononuclear cells to cross the BBB. JHMV-infected mice were examined to test whether neutropenia altered BBB permeability. Kinetic analysis of JHMV- and sham-infected wild type mice revealed only minimal loss of BBB integrity 24 and 48 h p.i. (Fig. 5A). However, by day 4 p.i., JHMV-infected wild type mice exhibited reduced BBB integrity compared with controls. Virus-induced disruption of the BBB further increased at day 6 p.i., the last day examined. Comparisons of BBB integrity between JHMV-infected neutropenic and wild type control mice were made at day 4 p.i. as most neutropenic mice were moribund at later time points. Loss of BBB integrity was significantly reduced (\(p < 0.05\)) following infection of neutropenic mice compared with control mice (Fig. 5B), consistent with a role of neutrophils in mediating the loss of BBB integrity. MMP-9, which contributes to vascular basement membrane breakdown (6), increases during JHMV infection and is associated with infiltrating neutrophils (16). To determine whether MMP-9 expression was induced in the absence of neutrophils, MMP-9 activity was compared in infected neutropenic and control mice. MMP-9 activity was readily detected in extracts of CNS derived cells prepared from infected control but not neutropenic mice (Fig. 5C). The presence of functional MMP-9 enzyme associated with neutrophils suggests that MMP-9, possibly in conjunction with other neutrophil derived factors, contributes to the loss of BBB integrity during the infection. Taken together, these data suggest the following: 1) neutrophils represent the initial cellular response to neurotropic viral infection, 2) neutrophils contribute to the early control of viral replication, 3) neutrophils play a key role in promoting infiltration of mononuclear inflammatory cell populations responding to a CNS infection, possibly through their ability to compromise the integrity of the BBB.

**Discussion**

Circulating neutrophils provide a large pool of inflammatory cells, which can be rapidly mobilized to respond to proinflammatory...
chemotactic signals induced within injured/infected tissue. Although neutrophils are usually considered as important participants to combat extracellular bacterial infections, they are also prominent components of inflammatory responses induced by numerous viral infections (21–24). During herpes simplex virus infection, neutrophils have been shown to play a clear role in limiting virus replication following dermal, ocular and mucosal infections, however, no distinct antiviral mechanism(s) have been elucidated for neutrophils in any of these tissues (19, 25–28). In the case of lethal JHMV-induced encephalitis, neutrophils entered the CNS at least 24 h before other infiltrating mononuclear cells and remained the dominant inflammatory cell population throughout infection. Furthermore, neutrophilia was associated with elevated virus replication in the CNS and greatly reduced infiltration of all inflammatory cells in parallel with reduced loss of BBB integrity.

Neutrophil infiltration peaked rapidly and began to decline in conjunction with the accumulation of lymphocytic infiltrates (Fig. 1E). Murine neutrophils respond to chemotactic signals from macrophage inflammatory protein (MIP)-2, KC (murine CXC chemokine ligand (CXCL)1), and MIP-1α (CC chemokine ligand (CCL)3) (29). MIP-2 is rapidly up-regulated in response to CNS infection by JHMV (16, 30) and expression (16, 30) peaks coincident with neutrophil infiltration at day 4 p.i. (Fig. 1F). By contrast, expression of MIP-1α is delayed, peaking at day 6 p.i. (16) whereas KC mRNA expression has not been detected during JHMV infection (T. Lane, personal communication). These data suggest that MIP-2 is likely the major neutrophil chemokine in this model. Interestingly, MIP-2 expression is down-regulated by IFN-γ (31), and deficiencies in IFN-γ signaling are associated with

FIGURE 3. Depletion of neutrophils disrupts the inflammatory process. A, CNS inflammation in infected neutropenic and wild type control mice at day 4 p.i. (H&E). Arrows indicate inflammatory cells. B, CD45<sup>high</sup> inflammatory cells isolated from pooled brains of 5 or 6 infected neutropenic and control mice were examined by flow cytometry at day 4 p.i. Cell number = frequency × mean cell yield/brain. The data shown are representative of three experiments.

FIGURE 4. Neutropenia inhibits CNS inflammation. A, Brain inflammatory cells and B, Splenocytes from three to four infected neutropenic and control mice were stained with mAb specific for the indicated individual cell types and examined by flow cytometry at day 4 p.i. Cell number = frequency × mean cell yield/brain. C, Splenocytes of infected mice at day 4 p.i. were stained with anti-CD8 and anti-Ly-6G mAb and analyzed by flow cytometry. The data shown are representative of two or more experiments.

FIGURE 5. Neutropenia reduces BBB permeability. A, Kinetic analysis of BBB integrity was performed in JHMV- and sham-infected immunocompetent mice via the presence of extravascular Evans blue dye injected 1 h before sacrifice. Data are the average mass of dye recovered per brain from three to four mice per time point. Error bars represent SD. B, Virus-infected control, neutropenic and sham-infected mice were injected with Evans blue dye 1 h before sacrifice at day 4 p.i. and analyzed as described in A. Error bars represent SD. C, Lysates of inflammatory cells isolated from brains of virus-infected neutropenic and control mice at day 4 p.i. assayed for gelatinase activity via zymography. The data are representative of two or three experiments. Error bars represent standard deviations from the mean. *p < 0.05.
increased neutrophil infiltration (32). Thus, the accumulation of IFN-γ secreting T cells within the CNS may limit neutrophil infiltration during JHMV infection through inhibition of MIP-2 expression.

Inflammatory cell migration across the BBB is often associated with the loss of vascular integrity (33). Surprisingly, neutropenia greatly reduced infiltration of all inflammatory cells in addition to reducing loss of BBB integrity. Our data were unable to distinguish whether loss of BBB integrity was mediated directly by neutrophils or via subsequent infiltration of mononuclear inflammatory cells. However, several properties set neutrophils apart from mononuclear cells as initiators of CNS inflammation and BBB disruption. Neutrophils granules contain azurocidin, glutamate as well as MMPs, all of which can compromise BBB integrity. These granules are rapidly released following β2-integrin engagement (34), supporting a role for neutrophils as rapid mediators of BBB disruption and extracellular matrix degradation. Azurocidin mediates Ca2⁺ dependent cytoskeletal rearrangement of endothelial cells resulting in the disassembly of interendothelial cell junctions (10). Collard et al. (11) reported that glutamate regulates vascular endothelial barrier function most likely via its action on endothelial-expressed glutamate receptors. MMP-9, a protease specific for the type IV collagens and laminins, which are structural components of the BBB basal lamina, has been implicated in the BBB breakdown during cerebral ischemia (35). Furthermore, in contrast to mononuclear leukocytes, neutrophils do not express the MMP-9 inhibitor, tissue inhibitor of MMP-1 (6), suggesting that local degradative potential of neutrophil derived MMP-9 is higher than that secreted by activated mononuclear inflammatory cells. These data support the concept that neutrophils are crucial for the BBB disruption and subsequent mononuclear inflammatory infiltration during acute JHMV infection.

Neutrophils rapidly infiltrated into the CNS following JHMV infection, likely in response to MIP-2 and played an unexpected role in controlling CNS virus replication. Fewer neutrophils are recruited into the CNS following sublethal JHMV compared with a lethal infection (16). These differences in neutrophil infiltration may reflect differences in viral replication and/or tropism (16) in conjunction with an increased proportion of virus specific IFN-γ secreting mononuclear cells. Although BBB integrity is not as severely compromised during sublethal JHMV infection, preliminary experiments show both increased virus replication and dramatically decreased mononuclear cell infiltration during sublethal JHMV infection of neutropenic mice (data not shown). These data support the observations during lethal infection suggesting that neutrophils play a distinct role in controlling JHMV infection. Neutrophils can secrete TNF-α (36) and IFN-γ (37), suggesting a capacity to mediate direct anti-viral activity (26, 38). However, there is little evidence for early production of IFN-γ in response to JHMV infection of the CNS (39). In addition, no anti-viral effects of TNF-α have been detected during JHMV infection (40). Furthermore, neutrophils do not secrete the potent anti-viral IFN-αβ cytokines (36). Rather, the data presented here would suggest that in the case of JHMV-induced encephalitis, neutrophils may contribute indirectly to control of virus replication. Infiltrating neutrophils contributed to BBB integrity loss, possibly via MMP-9 secretion, as well as through other mechanisms. Neutrophils also secrete a variety of proinflammatory chemokines (e.g., CXCL1, CCL3, CCL4, CXCL10, CXCL9, and CCL2) (41) and granule-derived proteins with chemotactic activity (e.g., cathepsin G, azurocidin, and α-defensins) (42), which recruit mononuclear leukocytes, including virus specific CD4⁺ and CD8⁺ T cells. Therefore, neutrophils most likely contribute to control of viral replication by promoting CNS access to mononuclear anti-viral immune effectors.

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