CONCISE COMMUNICATION

Antibodies to the Junctional Adhesion Molecule Cause Disruption of Endothelial Cells and Do Not Prevent Leukocyte Influx into the Meninges after Viral or Bacterial Infection

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A hallmark of infectious meningitis is the invasion of leukocytes into the subarachnoid space. In experimental meningitis triggered by tumor necrosis factor–α and interleukin-1β, the interaction of leukocytes with endothelial cells and the subsequent migration of the cells through the vessel wall can be inhibited by an antibody to the junctional adhesion molecule (JAM). In contrast to the cytokine-induced meningitis model, anti-JAM antibodies failed to prevent leukocyte influx into the central nervous system after infection of mice with *Listeria monocytogenes* or lymphocytic choriomeningitis virus. Furthermore, in bacterial meningitis, anti-JAM IgG antibodies, but not Fab fragments, caused disruption of the endothelium. Likewise complement-dependent antibody-mediated cytotoxicity was observed in cultured brain endothelial cells treated with anti-JAM IgG but not with its Fab fragment.

In bacterial meningitis, bacterial cell wall components and toxins lead to inflammation of the meninges, which frequently has neurologic sequelae, including impairment of motor and cognitive functions, seizures, and mental retardation [1]. Influx of leukocytes into the central nervous system (CNS) is initiated by cytokine-induced expression of adhesion molecules on endothelial cells and by the production of chemokines that attract leukocytes into the subarachnoid space [2, 3]. Intracisternal injection of tumor necrosis factor (TNF)–α, interleukin (IL)–1, macrophage inflammatory protein (MIP)–1, or MIP-2 leads to pleocytosis with accumulation of neutrophils in the cerebrospinal fluid (CSF) [4]. Previous experimental strategies, which aimed to prevent the recruitment of leukocytes into the CNS, have focused on the blockade of interactions of adhesion molecules on leukocytes and endothelial cells. In rabbits inoculated intrathecally with pneumococcal antigens, the administration of the polysaccharide fucoidin, which interferes with the function of L-selectin, was found to inhibit leukocyte rolling on endothelial cells. Leukocyte rolling is a precondition for firm leukocyte adhesion and subsequent transendothelial migration [5]. Intravenous injection of antibodies to CD11/CD18, which is expressed on leukocytes and promotes endothelial adhesion of the cells, inhibits the development of leukocytosis in rabbits infected intracisternally with *Streptococcus pneumoniae* [6]. Antibodies to the intracellular adhesion molecule 1 diminish the influx of leukocytes into the CSF of rats with pneumococcal cell wall–induced meningitis [7]. The difficulty of achieving complete blockade of leukocyte recruitment into the CNS probably results from a cooperative involvement of endothelial selectins. In mice with inactivation of the P- and E-selectin genes, only the double knockout mice showed a nearly complete inhibition of CNS leukocyte influx triggered by injection of inflammatory cytokines [8].

Recently a new target for therapeutic intervention has been suggested by the demonstration that an antibody to the endothelial cell-cell junctional adhesion molecule (JAM) blocks recruitment of neutrophils into the CNS induced by cytokines (IL-1β and TNF-α) or the chemokine monocyte chemoattractant protein (MCP)–3 [9]. JAM seemed especially promising as a target to limit the inflammatory response in the CNS, because the transmembrane protein is highly expressed in cells that form well-organized tight junctions, such as ependymal cells and the endothelium of the blood-brain barrier [10]. We investigated the effect of anti-JAM antibodies in a bacterial and a viral model of meningitis, and we discuss the results with respect to

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the reported results in the cytokine-triggered, self-limited form of meningitis.

Materials and Methods

Reagents. The monoclonal rat anti–mouse JAM IgG2b antibody BV-11 was purified from spent hybridoma medium by adsorption to protein G sepharose (Amersham-Parmacia, Zurich) and elution with 0.1 M glycine, pH 2.8. The antibody was further purified by anion-exchange chromatography (Resource Q; Amersham-Pharmacia) at pH 8.0 and was dialyzed in PBS. The antibody was maintained in sterile aliquots at –80°C. The endotoxin content was determined as 0.42 EU/mg Ig, using a commercial *Limulus* amoebocyte assay (KQCL test; BioWhittaker, Verviers, Belgium).

Fab fragments were obtained by papain digestion of BV-11 (1:200 [w/w]) and purified by cation-exchange chromatography (MonoS; Amersham-Pharmacia) at pH 6.0, followed by gel-filtration chromatography (Superdex 75; Amersham-Pharmacia). The endotoxin content was 0.2 EU/mg Fab. Antibodies and Fab fragment were diluted before injection with endotoxin-free Hanks' balanced salt solution (HBSS).

Listeria monocytogenes–*induced meningitis.* Female 12-week-old C57BL/6 mice purchased from RCC (Fullinsdorf, Switzerland) were pretreated intraperitoneally (ip) with 200 μg BV-11 IgG in 200 μL HBSS or with 200 μL HBSS as control. After 16 h the mice were infected intracranially with 600 cfu of *L. monocytogenes* in a 25-μL volume. Six, 24, and 48 h after infection, 2 mice each were anesthetized and perfused with 20 mL Ringer's solution (Braun, Sempach, Switzerland). For the 48-h time point, mice were again given 100 μg BV-11 antibodies at 24 h after infection. CSF was collected with an elongated Pasteur pipette, and CSF cells were counted, using a Neubauer hematocytometer. When using the BV-11 Fab fragment, 4 mice per time point and treatment group were infected intracranially with 100 cfu of *L. monocytogenes* in a 25-μL volume. After 12 and 24 h, mice were perfused, and CSF was collected. For the 12-h time point, 1000 μg BV-11 Fab was administered ip 30 min before infection. For the 24-h time point, 800 μg BV-11 Fab was given 4 h before infection, and 1000 μg BV-11 Fab was given 9 h after infection. Control mice received HBSS. The percentage of neutrophils, monocytes, and lymphocytes in CSF was measured on cytospins stained with Diff-Quik (Dade, Duedingen, Switzerland). In all cases, titers of *L. monocytogenes* in brain and liver were determined by plating various dilutions of organ homogenates on agar plates and counting colonies after 24 h of incubation at 37°C.

Lymphocytic choriomeningitis virus (LCMV)–induced meningitis. C57BL/6 mice were treated ip with 185 μg BV-11 IgG in 200 μL HBSS at days 0 and 3. Mice infected with LCMV for a total of 6 days were additionally given 100 μg BV-11 IgG in 200 μL HBSS on day 5. HBSS (200 μL) was injected as a control. All mice were infected intracranially with 30 pfu LCMV Armstrong (provided by R. Zinkernagel, University of Zurich) in a 25-μL volume immediately after the first antibody treatment (day 0). At 5 and 6 days after infection, 2 mice per time point and treatment group were perfused, and CSF was collected as described above.

Culture of murine endothelial cell line bEnd.4. The endothelial cell line bEnd.4 [11] was provided by K. Ballmer (Friedrich Miescher Institute, Basel, Switzerland). The cells were grown in Dulbecco's MEM (DMEM; Life Technologies, Basel) supplemented with 10% fetal calf serum (FCS; Life Technologies), 2 mM *N*-acetyl-L-alanyl-L-glutamine (Biochrom, Berlin), and 20 μg/mL gentamycin (Life Technologies).

Detection of BV-11 antigen expression. For flow cytometric analysis of JAM expression, bEnd.4 cells were trypsinized from culture flasks. After being washed in HBSS, 10^6 cells were resuspended in a volume of 100 μL flow cytometry buffer containing PBS (pH 7.4), 1% FCS, and 0.01% sodium azide and were incubated for 30 min on ice with either BV-11 IgG (rat IgG2b) or an isotype control (both 1 μg/10^6 cells), followed by incubation with a 1:20 diluted R-phycoerythrin (PE)–conjugated $F(ab')_2$ goat anti–rat IgG (Serotec; Inotech, Dottikon, Switzerland) for 30 min on ice. The cells were washed twice in flow cytometry buffer, and a total of 10^4 viable cells were analyzed on an Epics profile analyzer (Coulter, Miami).

BV-11 antibody–mediated cytotoxicity of endothelial cells. To determine complement-dependent cytotoxicity, bEnd.4 cells were cultured in 96-well microtiter F-plates (Falcon Plastics, Oxnard, CA) at a density of 2×10^4 cells/well in 100 μL DMEM containing 10% FCS, 2 mM *N*-acetyl-L-alanyl-L-glutamine, and 20 μg/mL gentamycin. After 24 h, medium was replaced with 100 μL cytotoxicity medium (serum-free DMEM and nutrient mixture Ham's F10 [Life Technologies] in a 1:1 ratio, containing 2 mM *N*-acetyl-L-alanyl-L-glutamine and 20 μg/mL gentamycin). The bEnd.4 monolayers were then treated for 1 h with the antibodies (25 μg/mL), followed by the addition of active or heat-inactivated (56°C for 30 min) Low-Tox-M rabbit complement (Cedarlane, Hornby, Ontario, Canada) at a 1:25 dilution (final assay volume, 150 μL). Cytotoxicity was determined after 3 h by using the AlamarBlue assay (Lucerna Chem, Lucerne, Switzerland), according to the manufacturer's instructions. Percent specific cytotoxicity was based on fluorescence units (FU) and was calculated as follows: specific cytotoxicity = [1 − (FU of antibody- and complement-treated cells − FU of cell-free cytotoxicity medium)/(FU of complement-treated cells − FU of cell-free cytotoxicity medium)] × 100.

Results

As described elsewhere [12], C57BL/6 mice inoculated intracerebrally with *L. monocytogenes* developed clinical signs of disease within 24 h and showed severe illness at 48 h. Pretreatment of mice with anti–JAM antibodies (200 μg ip) neither prevented nor delayed the development of bacterial meningitis. On the contrary, mice treated with anti-JAM antibody showed more-severe clinical signs (hunched posture, immobility, eye discharge) at 48 h than did saline-treated animals. When examined histologically at the 48-h time point, antibody-treated as well as control saline-treated animals showed severe meningeal inflammation, characterized by infiltrates in which neutrophils predominated. In animals treated with anti–JAM IgG, the blood vessels of the brain showed severe damage, including hemorrhages in the brain parenchyma and subarachnoid space (figure 1). These findings were not noted in saline-treated an-

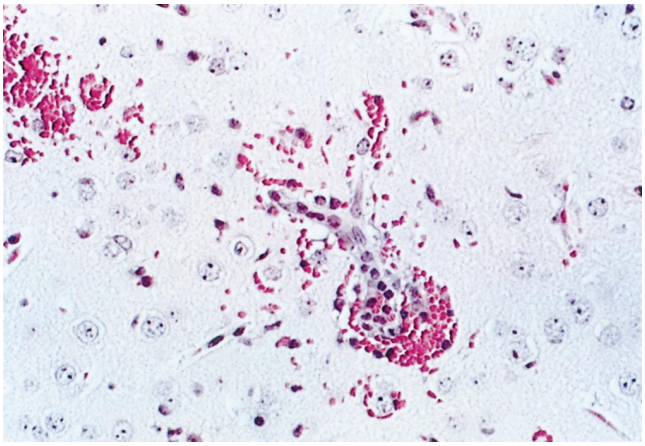

Figure 1. Leukocyte infiltration and central nervous system hemorrhages in mice with bacterial meningitis treated with BV-11 anti–junctional adhesion molecule (JAM) antibody. Mice were infected with *Listeria monocytogenes* and treated with BV-11 anti–JAM IgG. The photograph shows a brain tissue section stained with hematoxylin-eosin from day 2 after infection with *L. monocytogenes* (original magnification, ×40).

imals (data not shown). Numbers of inflammatory cells in the CSF did not differ between anti-JAM–treated and saline-treated mice at 6, 24, and 48 h after infection (figure 2*A*). At 24 h after infection, the CSF cells of both groups of animals consisted of ~90% granulocytes and ⩽10% macrophages, with only a few lymphocytes (data not shown). In addition, no difference in *Listeria* titers was found in brain and liver homogenates at any time point (data not shown). These experiments were repeated 4 times (2 mice per group and time point) with similar results. Treatment of uninfected control mice with anti-JAM antibody did not lead to histological changes in the brain or to CSF pleocytosis 48 h after treatment (data not shown). Thus, anti–JAM IgG antibodies induced hemorrhages in the brain parenchyma and subarachnoid space only in the presence of *L. monocytogenes* infection.

In contrast to bacterial meningitis, no disruption of the blood vessel wall was observed in LCMV-induced meningitis treated with anti-JAM antibodies (data not shown). Signs of disease at day 6 (weakness, hunched posture, or scruffy fur), as well as the extent of infiltrates of monocytes and lymphocytes in the meninges at days 5 and 6, did not differ from those among HBSS-treated control mice (data not shown). Histological findings correlated with the accumulation of inflammatory cells in the CSF. No difference in CSF counts was observed between BV-11 IgG–treated and control mice after infection with LCMV (figure 2*B*).

Activation of complement has been well described in bacterial infections, including meningitis [13]. The finding that anti–JAM IgG antibodies caused endothelial damage in bacterial, but not viral, meningitis led us to suspect that complement-dependent cytotoxicity was mediated by binding of the antibody to the endothelial junction protein. To test this hypothesis, the brain-derived endothelial cell line bEnd.4 was investigated for expression of JAM and susceptibility to cytotoxicity induced by anti–JAM IgG. As shown in figure 3*A*,

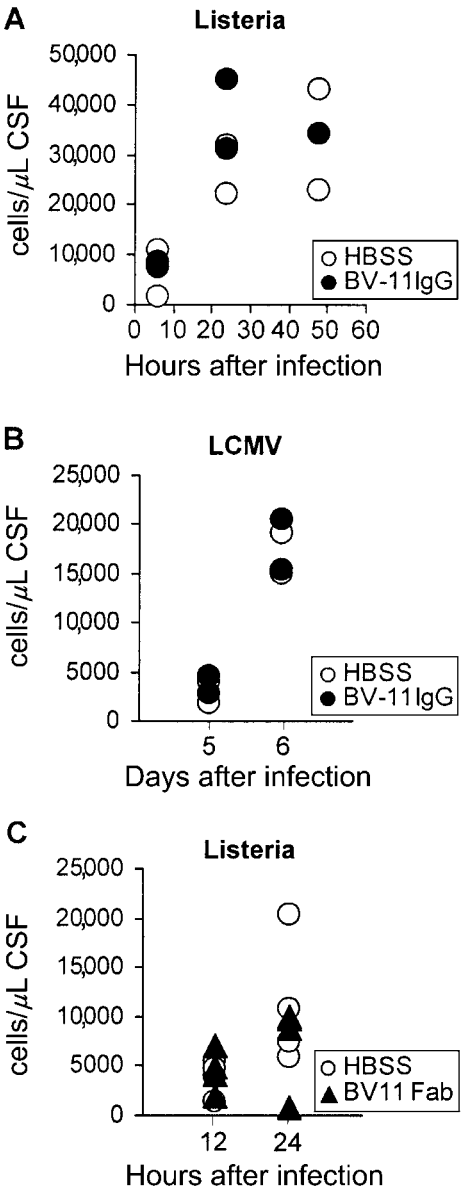

Figure 2. Pleocytosis in the cerebrospinal fluid of mice infected with *Listeria monocytogenes* or lymphocytic choriomeningitis virus (LCMV) and treated with anti–junctional adhesion molecule (JAM) antibodies. C57BL/6 mice were infected with *L. monocytogenes (A* and *C)* or LCMV *(B)* and were treated with BV11 anti–JAM IgG *(A* and *B)* or anti–JAM Fab *(C)* or HBSS intraperitoneally (see Materials and Methods); 2 *(A* and *B)* or 4 *(C)* mice per time point or treatment mode were analyzed. Inflammatory cells in the cerebrospinal fluid were counted at the indicated time points.

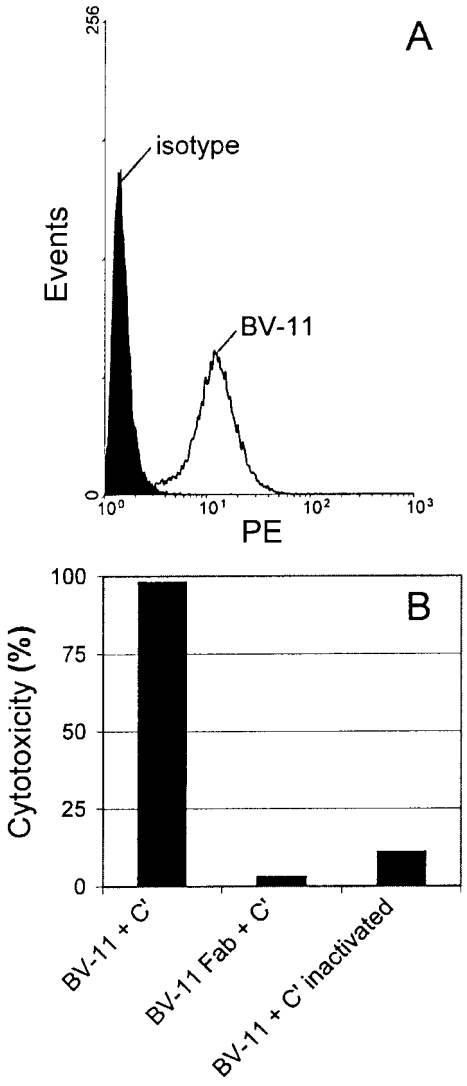

Figure 3. Brain endothelial cells express junctional adhesion molecule (JAM) and are susceptible to complement-dependent cytotoxicity induced by anti–JAM IgG. *A,* Flow cytometric analysis of bEnd.4 cells stained with BV-11 anti-JAM IgG or an isotype control. *B,* Cytotoxicity of bEnd.4 cells treated with BV-11 anti-JAM IgG in the presence of rabbit complement. No such effect was seen when either the Fab fragment of the anti-JAM IgG antibody was used or the complement was heat inactivated (see Materials and Methods).

JAM is expressed at high levels on the surface of bEnd.4 cells. Treatment of these cells with BV-11 anti–JAM IgG resulted in almost complete cellular cytotoxicity, the effect being dependent on the presence of active complement and of the Fc part that harbors the complement-binding domain of the IgG molecule (figure 3*B*).

When the non–complement-binding Fab fragments of the anti–JAM IgG molecule were injected before and after infection with *L. monocytogenes* no intracerebral bleeding was observed for as long as 48 h after infection, although higher doses (total, 1800 μg) of antibody were administered than in the experiments where intact anti–JAM IgG had been used (data not shown). However, the progression of *L. monocytogenes*–induced disease in anti-JAM Fab-treated mice was similar to that in control mice. Histological analysis did not reveal a protective effect of the anti-JAM Fab antibody on recruitment of leukocytes into the CNS (data not shown). No differences were found between anti-JAM Fab-treated mice and controls in the degree of CSF pleocytosis at 12 and 24 h after infection (figure 2*C*). This experiment was repeated 3 times (4 animals per group and time point) with similar results.

Discussion

This study fails to demonstrate that antibodies to the endothelial tight junction protein JAM protect against leukocyte accumulation in the meninges or improve the outcome of meningitis in mice infected with either *L. monocytogenes* or LCMV. The anti-JAM antibody did not prevent neutrophils, monocytes, or lymphocytes from transmigrating through blood vessel walls in the CNS. These data differ considerably from those of previous experiments showing that anti-JAM antibodies attenuate experimental meningitis induced by cerebroventricular injection of cytokines (TNF-α and IL-1β) or the chemokine MCP-3. In the latter model of disease, the antibody reduced the accumulation of leukocytes in the CSF, meninges, and brain parenchyma. The contradictory results in the 2 meningitis models suggest that the mechanisms underlying leukocyte traffic through the endothelial layer in infectious meningitis are different from those in cytokine-induced meningitis. Recently it has been shown that treatment of endothelial cells with the combination of TNF-α and IFN-γ, 2 cytokines known to be expressed in infectious meningitis and to be capable of modulating transmigration of leukocytes across endothelial cells, causes disappearance of JAM from intercellular junctions without decreasing the total amount of JAM on the cell surface [14]. By this mechanism the "glue-like effect" of JAM to stabilize intercellular junctions may be lost in infectious meningitis but not in the experimental cytokine-induced meningitis model used by Del Maschio et al. [9]. Therefore, it may be crucial to use infectious models of meningitis for the development of therapeutic tools to prevent brain inflammation. Our data clearly show that the strategy of using the tight junction protein JAM as a target for therapeutic intervention in bacterial meningitis in humans might be dangerous, because bacteria-induced activation of the complement system may initiate antibody-mediated complement-dependent cytotoxicity, thereby leading to the destruction of the vessel wall and to CNS bleeding. Moreover, the removal of the complement-activating Fc part of the anti-JAM antibody prevented CNS bleeding but did not show any beneficial effect in either bacterial or viral meningitis.

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