Experimental Pneumococcal Meningitis: Impaired Clearance of Bacteria from the Blood Due to Increased Apoptosis in the Spleen in Bcl-2-Deficient Mice

Andreas Wellmer, Matthias von Mering, Annette Spreer, Ricarda Diem, Helmut Eiffert, Christiane Noeske, Stefanie Bunkowski, Ralf Gold, and Roland Nau

Department of Neurology and Department of Bacteriology, University of Göttingen, D-37075 Göttingen, and Department of Neurology, University of Würzburg, D-97080 Würzburg, Germany

Received 8 August 2003/Returned for modification 15 December 2003/Accepted 1 March 2004

Necrotic and apoptotic neuronal cell death can be found in pneumococcal meningitis. We investigated the role of Bcl-2 as an antiapoptotic gene product in pneumococcal meningitis using Bcl-2 knockout (Bcl-2−/−) mice. By using a model of pneumococcal meningitis induced by intracerebral infection, Bcl-2-deficient mice and control littermates were assessed by clinical score and a tight rope test at 0, 12, 24, 32, and 36 h after infection. Then mice were sacrificed, the bacterial titers in blood, spleen, and cerebellar homogenates were determined, and the brain and spleen were evaluated histologically. The Bcl-2-deficient mice developed more severe clinical illness, and there were significant differences in the clinical score at 24, 32, and 36 h and in the tight rope test at 12 and 32 h. The bacterial titers in the blood were greater in Bcl-2-deficient mice than in the controls (7.46 ± 1.93 log CFU/ml versus 5.16 ± 0.96 log CFU/ml [mean ± standard deviation]; P < 0.01). Neuronal damage was most prominent in the hippocampal formation, but there were no significant differences between groups. In situ tailing revealed only a few apoptotic neurons in the brain. In the spleen, however, there were significantly more apoptotic leukocytes in Bcl-2-deficient mice than in controls (5.148 ± 3.406 leukocytes/mm² versus 1.070 ± 395 leukocytes/mm²; P < 0.005). Bcl-2 appears to counteract sepsis-induced apoptosis of splenic lymphocytes, thereby enhancing clearance of bacteria from the blood.

Streptococcus pneumoniae is one of the most important gram-positive bacterial species causing disease in humans, and the diseases that it causes include pneumonia, otitis media, and meningitis. In adults it is the most common cause of meningitis (7, 39). In spite of the continuing development of new antibiotics, the mortality rate for S. pneumoniae meningitis remains high (7). The overall mortality rate for bacterial meningitis has not changed over the last few decades in developed countries (7, 46, 39). Survivors of meningitis often suffer from severe neurological sequelae (3, 11). The pathological substrate for these long-term problems is neocortical and hippocampal neuronal damage, as observed at autopsy, by hippocampal magnetic resonance imaging volumetry, and in animal models (8, 10, 25, 33, 47, 44). Neuronal apoptosis and necrosis are caused by direct toxicity of bacterial compounds and the inflammatory reaction of the host (6, 21, 38, 40, 43, 47). They are mediated by free radicals, excitatory amino acids, and caspases (4, 5, 22, 25, 26, 34, 43).

The Bcl-2 protein family consists of pro- and antiapoptotic members that can homo- and heterodimerize with each other (23, 27, 35). An excess of proapoptotic dimers leads to cell death, whereas an excess of antiapoptotic molecules prevents cell death (23, 27). Bcl-2 itself is antiapoptotic. Bcl-2 knockout mice have a shortened life span and develop gray hair, polycystic kidneys, and premature lymphocyte apoptosis after initially normal lymphocyte development (19, 29, 31, 42). Also, apoptosis of neurons appears to depend on Bcl-2 family member concentrations. The absence of Bcl-2 results in degeneration of sympathetic, sensory, and motoneurons in the early postnatal period (29). Furthermore, increased susceptibility of aged rabbits to hippocampal apoptosis after aluminum treatment has been correlated with a short-lived early Bcl-2-response and a late intense Bax response, as opposed to the small Bax response and a maintained intense Bcl-2 response in young animals to this stimulus protecting against apoptosis (37). Moreover, Bcl-2 overexpression enhances survival of hippocampal neurons after oxidative stress, glutamate exposure, and hypoglycemia in rats (24). Neurological scores and infarct volume are increased in Bcl-2-deficient mice after 1 h of occlusion of the middle cerebral artery (13). Hypoxia of rat neocortical neuronal cultures leads to Bcl-2 expression and apoptosis that is aggravated when Bcl-2 is antagonized (1).

Therefore, we investigated the effect of an absence of Bcl-2 on the inflammatory changes, clinical course, and neuronal damage in experimental murine pneumococcal meningitis by using Bcl-2 knockout mice.

(This work was presented in part at the 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Canada, 2000.)
MATERIALS AND METHODS

Bacteria. A strain of S. pneumoniae serotype 3 (isolated from a patient with meningitis; a gift from M. G. Täuber, Department of Clinical Microbiology, University of Bern, Bern, Switzerland) was used. Bacteria were grown in tryptic soy broth for 18 h, harvested by centrifugation, washed and resuspended in 0.9% NaCl, divided into aliquots, and stored frozen at −70°C. Frozen aliquots were used for the experiments, and the concentration was adjusted to the required concentration, 10^7 CFU/25 μl of inoculum. The inoculum size was confirmed by plating 10-fold dilutions on blood agar.

Mice. Eleven Bcl-2-deficient mice and 13 control littermates with a C57BL/6 background (10 heterozygous and 3 homozygous) that were 40 days old and were kindly provided by B. Holtmann (Department of Neurology, University of Würzburg) were bred as described previously (29). Phenotypically, the Bcl-2-deficient mice had a slightly prolonged life span and exhibited less severe polyconvulsive kidneys than other Bcl-2-deficient mice described previously (31, 42). The Bcl-2-deficient mice were provided ad libitum. The experiments were approved by the Animal University Hospital of Goettingen and the District Government of Braunschweig, Lower Saxony.

Experimental meningitis. Mice were anesthetized with 100 mg of ketamin per kg and 10 mg of xylazine per kg. Then eight Bcl-2-deficient mice and nine control mice (Bcl-2−/− mice and six Bcl-2+/− mice) were infected by injection of 10^7 CFU in 25 μl of 0.9% saline into the right forebrain with a 27-gauge needle at a depth of 2 mm from the surface of the skull; this depth ensured that the bevel of the needle was partially placed in the subarachnoid space, which allowed a significant part of the inoculon to spread into the subarachnoid space (10). Twenty-five microliters of 0.9% saline was injected into uninjected controls (three Bcl-2−/− mice and four Bcl-2+/− mice) in the same way. After recovery from anesthesia the mice were clinically normal without apparent deficits. The mice were monitored by using clinical scores, a tight rope test, and weight before infection and 12, 24, 32, and 36 h after infection. The clinical scores were determined as follows: 0, healthy; 1, slightly lethargic; 2, moderately lethargic but able to walk; 3, severely lethargic and unable to walk; 4, dead (10, 44). At 36 h after infection the mice were sacrificed by decapitation, and blood was collected. Then the brain and spleen were removed from each mouse. Bacterial counts were determined for blood, spleen, and cerebellar homogenates. A part of the spleen and the brain were fixed in 4% paraformaldehyde and embedded in paraffin for hematoxylin-eosin staining and in situ tailing (47). For analysis of Bcl-2 expression in spleens of infected or uninfected mice, 10 6-week-old C57BL/6 mice (Charles River Laboratories, Sulzfeld, Germany) were used. The mice were kept in the Animal Care Facility of the University Hospital of Göttingen and by the Department of Neurology, University of Gottingen and by the Animal Care Committee of the Medical Faculty of the University of Göttingen and by the Department of Braunschweig, Lower Saxony.

Experimental meningitis. Mice were anesthetized with 100 mg of ketamin per kg and 10 mg of xylazine per kg. Then eight Bcl-2-deficient mice and nine control mice (Bcl-2−/− mice and six Bcl-2+/− mice) were infected by injection of 10^7 CFU in 25 μl of 0.9% saline into the right forebrain with a 27-gauge needle at a depth of 2 mm from the surface of the skull; this depth ensured that the bevel of the needle was partially placed in the subarachnoid space, which allowed a significant part of the inoculon to spread into the subarachnoid space (10). Twenty-five microliters of 0.9% saline was injected into uninjected controls (three Bcl-2−/− mice and four Bcl-2+/− mice) in the same way. After recovery from anesthesia the mice were clinically normal without apparent deficits. The mice were monitored by using clinical scores, a tight rope test, and weight before infection and 12, 24, 32, and 36 h after infection. The clinical scores were determined as follows: 0, healthy; 1, slightly lethargic; 2, moderately lethargic but able to walk; 3, severely lethargic and unable to walk; 4, dead (10, 44). At 36 h after infection the mice were sacrificed by decapitation, and blood was collected. Then the brain and spleen were removed from each mouse. Bacterial counts were determined for blood, spleen, and cerebellar homogenates. A part of the spleen and the brain were fixed in 4% paraformaldehyde and embedded in paraffin for hematoxylin-eosin staining and in situ tailing (47). For analysis of Bcl-2 expression in spleens of infected or uninfected mice, 10 6-week-old C57BL/6 mice (Charles River Laboratories, Sulzfeld, Germany) were used. The mice were kept in the Animal Care Facility of the University Hospital of Göttingen and by the Department of Neurology, University of Gottingen and by the Animal Care Committee of the Medical Faculty of the University of Göttingen and by the Department of Braunschweig, Lower Saxony.

Experimental meningitis. Mice were anesthetized with 100 mg of ketamin per kg and 10 mg of xylazine per kg. Then eight Bcl-2-deficient mice and nine control mice (Bcl-2−/− mice and six Bcl-2+/− mice) were infected by injection of 10^7 CFU in 25 μl of 0.9% saline into the right forebrain with a 27-gauge needle at a depth of 2 mm from the surface of the skull; this depth ensured that the bevel of the needle was partially placed in the subarachnoid space, which allowed a significant part of the inoculon to spread into the subarachnoid space (10). Twenty-five microliters of 0.9% saline was injected into uninjected controls (three Bcl-2−/− mice and four Bcl-2+/− mice) in the same way. After recovery from anesthesia the mice were clinically normal without apparent deficits. The mice were monitored by using clinical scores, a tight rope test, and weight before infection and 12, 24, 32, and 36 h after infection. The clinical scores were determined as follows: 0, healthy; 1, slightly lethargic; 2, moderately lethargic but able to walk; 3, severely lethargic and unable to walk; 4, dead (10, 44). At 36 h after infection the mice were sacrificed by decapitation, and blood was collected. Then the brain and spleen were removed from each mouse. Bacterial counts were determined for blood, spleen, and cerebellar homogenates. A part of the spleen and the brain were fixed in 4% paraformaldehyde and embedded in paraffin for hematoxylin-eosin staining and in situ tailing (47). For analysis of Bcl-2 expression in spleens of infected or uninfected mice, 10 6-week-old C57BL/6 mice (Charles River Laboratories, Sulzfeld, Germany) were used. The mice were kept in the Animal Care Facility of the University Hospital of Göttingen and by the Department of Neurology, University of Gottingen and by the Animal Care Committee of the Medical Faculty of the University of Göttingen and by the Department of Braunschweig, Lower Saxony.
RESULTS

At the beginning of the experiment all mice were healthy. Bcl-2-deficient mice and controls into which saline was injected intracerebrally did not develop signs of clinical disease within 36 h. Also, the performance in the tight rope test was not altered, and the results for the two groups were not significantly different. At zero time the median score in the tight rope test for the Bcl-2−/− mice was 3 and the minimum and maximum scores were 2 and 4, respectively, whereas for the Bcl-2+/− mice the median score was 2 and the minimum and maximum scores were 2 and 3, respectively (P = 0.4). At 36 h the median score for the Bcl-2−/− mice was 2 and the minimum and maximum scores were 2 and 4, respectively, whereas for the Bcl-2+/− mice the median score was 2 and the minimum and maximum scores were 2 and 2, respectively (P = 0.4). These mice did not show any neuronal damage other than that at the site of injection. The spleens of these mice contained few apoptotic lymphocytes, but the number of these cells was significantly greater in Bcl-2-deficient mice than in the controls. The spleens of the Bcl-2−/− mice contained 118 ± 42 apoptotic cells/mm², whereas the spleens of the control littermates contained 36 ± 23 apoptotic cells/mm² (P = 0.02) (Fig. 1A and 2E and F).

Bcl-2-deficient mice infected intracerebrally with pneumococci developed lethargy and other clinical signs of meningitis earlier than control mice developed such signs (Table 1). This was also reflected by the worse performance of the Bcl-2−/− mice in the tight rope test (Table 1). The bacterial titers in the blood were significantly higher in Bcl-2-deficient mice at 36 h than in the control littermates; the values for the Bcl-2−/− and control mice were 7.46 ± 1.93 and 5.16 ± 0.96 log CFU/ml, respectively (P = 0.0074) (Fig. 3). The bacterial titers in cerebellar and splenic homogenates were not significantly different; the values for the cerebellum for the Bcl-2−/− mice and the control littermates were 7.98 ± 2.25 and 7.95 ± 1.19 log CFU/ml, respectively (P = 0.97), and the values for the spleen were 5.30 ± 1.41 and 4.80 ± 1.06 log CFU/ml, respectively (P = 0.43). Severe secondary neuronal damage (other than the damage at the site of injection, where moderate direct injury occurred) was found predominantly in the hippocampal formation in hematoxylin-and eosin-stained brain sections (Fig. 2B and C). There were no significant differences when Bcl-2-deficient mice were compared with the control littermates for each individual region of the brain examined (data not shown). Also, the difference in the overall damage scores did not reach statistical significance; the overall neuronal damage scores (sums of scores for the four regions examined) for the Bcl-2−/− mice and the control littermates were 6.5 (25th and 75th percentiles, respectively, 5.0 and 8.0) and 5.0 (25th and 75th percentiles, respectively, 4.3 and 6.5), respectively (P = 0.20). In situ tailing revealed that the neuronal damage was mainly necrotic. Only a few animals had one or two apoptotic neurons per section as determined by in situ tailing (Fig. 2D).

Both groups developed histologically confirmed meningitis with high inflammation scores; the scores for the Bcl-2−/− mice and the control littermates were 16.2 (25th and 75th percentiles, respectively, 12.2 and 17.5) and 17 (25th and 75th percentiles, respectively, 16.0 and 17.0), respectively (P = 0.67) (Fig. 2A and 4A). Sections stained by in situ tailing showed that high proportions of the PML at the site of infection and in meningeal infiltrates were apoptotic. There were no significant differences between the groups, however; for the meningeal infiltrate the values for the Bcl-2−/− mice and the control littermates were 78 ± 37 and 69 ± 36 apoptotic cells/300 PML, respectively (P = 0.64) (Fig. 4B), and for the intraparenchymal infiltrate the values were 98 ± 24 and 81 ± 35 apoptotic cells/300 PML, respectively (P = 0.39) (Fig. 4C).

In the spleen 36 h after infection marked apoptosis of follicular lymphocytes was observed by in situ tailing. The number of apoptotic lymphocytes was higher in Bcl-2-deficient mice; the values for Bcl-2−/− mice and the control littermates were 5,148 ± 3,406 and 1,070 ± 395 apoptotic lymphocytes/mm², respectively (P = 0.0018) (Fig. 1B and 2G and H). Most apoptotic lymphocytes were located in the white pulp of the spleen. Double staining with CD3 and CD8 showed that cells were positive for in situ tailing in regions with T lymphocytes and cytotoxic T lymphocytes and also outside T-cell regions (Fig. 2I, K, and L). However, it was difficult to morphologically identify double-stained lymphocytes in these areas since the cytoplasm of apoptotic lymphocytes was shrunken, which impeded staining for lymphocyte markers (Fig. 2K and L).

In wild-type mice we observed expression of Bcl-2 in the
spleen by Western blotting. However, expression of Bcl-2 did not increase in mice infected intracerebrally with pneumococci compared to mice into which saline was injected (Fig. 5). Bcl-2-positive cells were found in spleen follicles by immunocytochemistry (Fig. 2M).

**DISCUSSION**

Bcl-2 knockout mice initially show normal lymphocyte development, but there is premature lymphocyte death (19, 29, 31, 42). In immunocompetent individuals lymphocyte death...
can be accelerated by stimuli, such as sepsis (14, 18). In our study Bcl-2-deficient mice developed clinically more-severe pneumococcal meningitis despite similar cerebellar bacterial titers, meningeal inflammation scores, and neuronal damage in the hippocampal formation, yet the bacterial titers in the blood were higher. This coincided with markedly enhanced lymphocyte apoptosis in the spleens of Bcl-2-deficient mice, which exceeded by far that in control mice (the means were 5,148 and 1,070 lymphocytes/mm², respectively). Bcl-2-deficient mice which received saline contained few apoptotic lymphocytes, yet the lymphocyte apoptosis was higher in uninfected Bcl-2-deficient mice than in the healthy wild-type littermates (the means were 118 and 36 lymphocytes/mm², respectively) (Fig. 1A and 2E and F). Lymphocyte apoptosis in the spleen appeared to affect all types of lymphocytes and, probably, also dendritic cells, although we were not able to show double staining for various lymphocyte markers with conventional immunohistochemistry, since cells that had undergone apoptotic cell death had lost the rim of cytoplasm.

Bcl-2−/− mice were less able to clear bacteria from the blood, which led to more severe sepsis. Also, in patients dying from sepsis and multiorgan dysfunction there is marked lymphocyte apoptosis in the spleen affecting B cells and CD4-positive T cells (14, 18). Hotchkiss et al. concluded that this acquired immunodeficiency may be partially responsible for the high mortality rate for sepsis. Accordingly, it was suggested

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Clinical score (25th percentile, 75th percentile)</th>
<th>Tight rope test (25th percentile, 75th percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bcl-2−/− mice (n = 8)</td>
<td>Controls (n = 9)</td>
</tr>
<tr>
<td>0</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td>12</td>
<td>0 (0, 0.5)</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td>24</td>
<td>0 (0, 1.5)</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td>32</td>
<td>2 (1.5, 2.5)</td>
<td>1 (0, 1)</td>
</tr>
<tr>
<td>36</td>
<td>3 (2.5, 3)</td>
<td>1 (1, 2)</td>
</tr>
</tbody>
</table>

*Time after infection. The data for time zero are preinfection data.

b P value as determined by the Mann-Whitney U test. NS, not significant.

FIG. 3. Bacterial titers in blood, cerebellar, and spleen homogenates of Bcl-2-deficient mice (open bars) (n = 8) and controls (solid bars) (n = 9) 36 h after intracerebral infection with 10⁸ CFU of S. pneumoniae in 25 µl. The bars indicate means, and the error bars indicate standard deviations. A number sign indicates that the P value is <0.01 (as determined by a t test).

FIG. 4. Meningeal inflammation in experimental S. pneumoniae meningitis 36 h after infection. Mice were infected intracerebrally with 10⁸ CFU of S. pneumoniae in 25 µl of saline. ○ and open bars, Bcl-2-deficient mice (n = 8); □ and solid bars, controls (wild type [WT]) (n = 9). (A) Meningeal inflammation score (median and 25th and 75th percentiles). (B) Apoptotic PML in the meningeal infiltrate. (C) Apoptotic PML in cerebral infiltrate. The bars indicate means, and the error bars indicate standard deviations. No statistically significant differences were observed.
that inhibition of lymphocyte apoptosis could be a new treatment option to improve survival in sepsis (35). The role of lymphocytes in sepsis is not completely clear. Certainly, B lymphocytes proliferate in bacterial infections and produce antibodies that may be important for opsonization of bacteria to improve their phagocytosis. Apoptotic lymphocytes are taken up by phagocytes themselves. When there is overwhelming infection with pneumococci (n = 5) or injection of normal saline (n = 5) as determined by Western blotting. ( ), mice with pneumococcal meningitis (n = 5); ( ), uninfected controls (n = 5).

We demonstrated that there was increased pneumococcal sepsis-induced lymphocyte apoptosis in the spleens of Bcl-2-deficient mice by in situ tailing. In pigs, bacterial endotoxins that cause septic shock and multiorgan failure induce apoptosis in the liver and spleen that correlates with a decrease in the Bcl-2 protein level (12). In our study, after intracerebral challenge with pneumococci wild-type mice did not show any change in the Bcl-2 protein concentration in the spleen, as estimated by Western blotting. Therefore, the presence of normal amounts of Bcl-2 may be sufficient to prevent the overwhelming lymphocyte apoptosis in gram-positive infections seen in Bcl-2-deficient mice. However, overexpression of Bcl-2 in transgenic mice led to a decrease in apoptosis of spleno- and thymocytes and to improved survival in sepsis (15, 16). Also, murine splenic T cells overexpressing Bcl-2 showed reduced apoptosis in vitro after stimulation with myelin oligodendrocyte glycoprotein (36). In conclusion, Bcl-2 is involved in prevention of infection-induced apoptosis of splenocytes and thymocytes. Bcl-2 probably acts as an inhibitor of activation of caspases involved in the mitochondrial pathway (41). Accordingly, caspase inhibitors were able to prevent lymphocyte apoptosis in the caecal ligation and puncture model of polymicrobial sepsis (17). In meningitis treated with antibiotics, PML in the subarachnoid space preferentially die by apoptosis and are impaired in Bcl-2-deficient mice. However, in Bcl-2 knockout mice with pneumococcal meningitis neuronal damage was not significantly enhanced. Apoptosis, however, accounted only for a small part of the neuronal damage in this model (10). Neuronal damage in meningitis can be a consequence of several alternative pathogenetic mechanisms (34). Infection-induced neuronal apoptosis could in part be caused by tumor necrosis factor alpha-induced caspase activation, which many researchers believe is not influenced by Bcl-2 (2, 17, 43, 45). The role of Bcl-2 in neuronal damage during bacterial meningitis needs to be investigated further by using a model of central nervous system infection with more pronounced apoptotic neuronal damage.

In conclusion, this study provided evidence concerning the role of Bcl-2 in preventing infection-induced apoptosis of lymphocytes in the spleen. A Bcl-2 deficiency leads to a more fulminating clinical course of pneumococcal meningitis after intracerebral infection, but it does not influence meningeval inflammation, PML apoptosis within the central nervous system, and necrotic neuronal damage. Future research could be targeted at direct application or stimulation of the synthesis of antiapoptotic members of the Bcl-2 protein family to prevent lymphocyte apoptosis in bacterial infections and thereby improve survival.

ACKNOWLEDGMENT

This project was supported by a grant from the Deutsche Forschungsgemeinschaft (Na165/4-3) to R.N.

REFERENCES


