Differential Effect of p47<sub>phox</sub> and gp91<sub>phox</sub> Deficiency on the Course of Pneumococcal Meningitis

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Received 5 December 2002/Returned for modification 21 January 2003/Accepted 3 March 2003

Bacterial meningitis is a severe inflammatory disease of the central nervous system and is characterized by massive infiltration of granulocytes into the cerebrospinal fluid (CSF). To assess the role of NADPH oxidase-derived reactive oxygen species (ROS) in pneumococcal meningitis, mice deficient in either the gp91 subunit (essential for functioning of the phagocyte enzyme) or the p47 subunit (essential for functioning of homologous enzymes in nonphagocytic cells) were intracisternally infected with live Streptococcus pneumoniae, and defined disease parameters were measured during the acute stage of infection. While none of the parameters measured (including CSF bacterial titers) were significantly different in gp91<sup>−/−</sup> and wild-type mice, the infection in p47<sup>−/−</sup> mice was associated with significantly increased inflammation of the subarachnoid and ventricular space, disruption of the blood-brain barrier, and the presence of interleukin-1β, tumor necrosis factor alpha, and matrix metalloproteinase 9 in the cortex. These changes were associated with <i>10</i>-fold-higher CSF bacterial titers in p47<sup>−/−</sup> mice than in wild-type mice (<i>P</i> < 0.001). In contrast to infection with live bacteria, the inflammatory response, including CSF leukocytosis, was significantly attenuated in p47<sup>−/−</sup> mice (but not gp91<sup>−/−</sup> mice) challenged with a fixed number of heat-inactivated pneumococci. Impairment of the host defense appeared to be responsible for the higher bacterial titers in p47<sup>−/−</sup> mice. Therefore, these results indicate that ROS generated by a gp91-independent NADPH oxidase(s) are important for establishing an adequate inflammatory response to pneumococcal CSF infection.
MATERIALS AND METHODS

Animals. gp91^+/− and p47^+/− mice, generated as described previously (11, 23), were bred under barrier isolation conditions at the animal care facility of Johann Wolfgang Goethe University and were sent to Switzerland for experiments. Animals were backcrossed onto the C57BL/6 background for at least five generations. Deletion of the NADPH oxidase subunits was verified by the absence of blood leukocyte superoxide production, as measured by lucigenin-enhanced chemiluminescence (3). C57BL/6 mice from Harlan (Horst, The Netherlands) were used as wild-type (WT) controls. All of the mice were used when they were 12 to 16 weeks old and weighed between 22 and 32 g, and they were sex matched for each set of experiments. Animal experiments were approved by the Animal Care and Experimentation Committee of the Canton of Berne, Switzerland, and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Induction of meningitis with live bacteria. To induce meningitis with live bacteria, a type 3 Streptococcus pneumoniae strain originally isolated from a patient with bacterial meningitis was used as the infecting organism (16). A total of 22 WT mice, 17 gp91^+− mice, and 20 p47^+− mice anesthetized by intraperitoneal injection with 40 mg of ketamine (Ketalar; Parke-Davis) per kg and 30 mg of xylazine (Xylasol; Graeub AG) per kg received a single injection of 10^10 CFU/ml of a preparation containing ~2 × 10^10 CFU of bacteria per ml. A total of 19 WT mice, 14 gp91^+− mice, and 11 p47^+− mice were mock infected with sterile saline and served as uninfected controls. Eighteen hours after infection, the animals were weighed, and CSF was obtained by puncture of the cisterna magna. Five-microliter portions were cultured quantitatively on blood agar plates. Animals were sacrificed with an overdose of pentobarbital (100 mg/kg given intraperitoneally) between 20 and 26 h after infection (i.e., when they became moribund), and they were perfused with ice-cold phosphate-buffered saline via the left cardiac ventricle. The rate of spontaneous death was <30% at this time, and no significant difference in the mortality rates of the different strains was observed.

Induction of sterile meningitis. To induce sterile meningitis, the nonencapsulated S. pneumoniae mutant R6 was used (31). R6 grown at 37°C in brain heart infusion broth to the mid-logarithmic phase was harvested by centrifugation, washed with sterile saline, and resuspended in saline at a concentration of ~5 × 10^10 CFU/ml. The bacteria were heat inactivated at 80°C for 20 min. Ten-microliter portions of heat-inactivated R6 pneumococci were intracisternally injected into WT mice, seven gp91^−/− mice, and six p47^−/− mice. Four WT animals injected with sterile saline served as controls. Animals were sacrificed 12 h after injection, following collection of the CSF. Each CSF sample was centrifuged for 10 min at 10,000 × g and 4°C, and the resulting pellet was used to determine white blood cell (WBC) counts after dilution in 0.4% Turk’s solution. The CSF supernatant was frozen at −80°C until cytokines were analyzed as described below.

Preparation of cortical homogenates. Animals were perfused with ice-cold phosphate-buffered saline, and cortical hemispheres were removed, separated from meninges, immediately frozen on dry ice, and stored at −80°C until analysis. Tissue was homogenized (1:9, wt/vol) in 20 mM HEPES buffer (pH 7.4) containing 1 mM EGTA, 0.1 mM dithiothreitol, 0.1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and a cocktail of additional protease inhibitors (complete mini; Roche Diagnostics, Rotkreuz, Switzerland). Homogenates were centrifuged at 9,000 × g, and aliquots of the supernatant were stored at −80°C. Protein concentrations were determined by the Pierce bicinchoninic acid assay (Pierce, Rockford, Ill.).

Determination of subarachnoid and ventricular inflammation. Since the limited amount of CSF available was used to measure bacterial titers in experiments with live bacteria, inflammation was semiquantitatively determined by histological evaluation of tissue sections. Twenty-micrometer-thick coronal sections of formaldehyde-fixed, paraffin-embedded brain tissue at the level of the hippocampus and third ventricle were stained with hematoxylin and cosin and investigated by light microscopy for five animals per group. Images were captured with OpenLab (Improvision, Coventry, United Kingdom) by using a digital camera (Hamamatsu, Photonics, Hamamatsu, Japan) and were scored for the extent of inflammatory infiltrate (~90% of which was neutrophilic granulocytes [30]) by using NIH Image. Subarachnoid inflammation was determined by measuring the area occupied by infiltrating cells in the subarachnoid space covering the area of (i) the retrospenial motor cortex or (ii) the perihiral cortex over a length of 850 μm by using the density slicing procedure (4). The data were expressed in square micrometers of infiltrate per micrometer of subarachnoid space and were transformed into scores as follows: 0 (no inflammation), 1 (~0 to 10 μm^2/μm), 2 (~10 to 20 μm^2/μm), 3 (~20 to 30 μm^2/μm), etc. (i.e., 1 point per 10 μm^2/μm). Ventricular inflammation was determined by expressing the area of infiltrate as a percentage of the area of the ventricle by using the outline tool and then was given one of the following scores: 0 (no infiltrate), 1 (>0 to 2.5%), 2 (>2.5 to 10%), 3 (>10 to 25%), or 4 (>25%). Finally, the scores for the three separate areas were added to obtain the total inflammation score. Noninfected animals (those for each strain) had a score of 0.

BBB integrity. To visualize differences in BBB integrity, animals received 0.5 ml of 2% (wt/vol) Evans blue intraperitoneally 45 min before sacrifice. Ten-micrometer-thick cryosections were examined for Evans blue extravasation by fluorescence microscopy essentially as described previously (12). To quantitatively assess changes in BBB permeability, extravasation of serum proteins into the cortex was assessed by measuring serum albumin levels with a mouse-specific enzyme-linked immunosorbent assay (ELISA) kit (Exocell Inc., Philadelphia, Pa.).

MMP-9. Matrix metalloproteinase 9 (MMP-9) levels were determined by gelatin zymography with minor modifications (16), essentially as described previously (14). MMP-9 levels were determined by densitometric analysis of the gelatin lysis zone at ~95 kDa by using NIH Image. Only the latent form of MMP-9 was detected. Purified human neutrophil MMP-9 (Calbiochem, Darmstadt, Germany) was used as the standard. Under these conditions, samples were within the linear range of the standard curve.

Cytokines. In animals infected with live bacteria, interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) were quantitatively determined by using mouse-specific ELISA kits (Quantikine M; R&D Systems Inc., Minneapolis, Minn.). Cortex homogenate supernatants were diluted 1:1 in assay buffer and analyzed in duplicate. The detection limit of the assay was <3.0 pg/ml for IL-1β and <5.1 pg/ml for TNF-α. In animals challenged with heat-inactivated R6 pneumococci, IL-12p70, TNF-α, gamma interferon, monocyte chemoattractant protein 1, IL-10, and IL-6 were simultaneously measured by flow cytometry by using a mouse inflammation cytometric bead array kit (BD Biosciences Pharmingen, San Diego, Calif.). CSF samples were prediluted 1:10 in assay diluent, while cortex homogenates were used undiluted. The detection limits ranged from ~2.5 pg/ml for gamma interferon to ~50 pg/ml for monocyte chemotactic protein 1.

Statistical analysis. Data were analyzed by nonparametric statistics for multiple comparisons (Kruskal-Wallis test followed by Dunn’s test) by using Prism 3.0 (GraphPad, San Diego, Calif.). Data were considered to be statistically different at a P value of <0.05. Data in the figures are expressed as medians with upper and lower quartiles and minimum and maximum values.

RESULTS

Clinical and histopathological findings after live infection. At 18 h after infection, WT and knockout mice infected with the type 3 pneumococci all exhibited significant weight loss (~5% compared to corresponding controls; P < 0.001) and signs of severe illness (lethargy, ruffled fur, tremors). There was no difference in the extent of weight loss among the different mouse strains. While the CSF bacterial titers were similar in the gp91^−/− and WT mice, there was a significant ~10-fold increase in the CSF bacterial titers in the p47^−/− mice compared to the titers in the WT animals (P < 0.001) (Fig. 1A). As is characteristic of bacterial meningitis, hematoxylin and eosin staining of coronal sections revealed marked granulocyte infiltration of the subarachnoid and ventricular space in infected WT animals (Fig. 2A and D). While the extent of inflammation in gp91^−/− animals (median inflammation score, 4; range, 3 to 6) was similar to that in WT animals (median score, 7; range, 3 to 8) (the difference was not significant) (Fig. 2B and E), granulocyte infiltration was markedly more intense in p47^−/− animals (median score, 10; range, 8 to 15) (P < 0.05 compared with infected WT mice; P < 0.01 compared with infected gp91^−/− mice) (Fig. 2C and F), which is in line with the elevated bacterial titers in these animals. The more intense inflammation was associated with especially extensive subarachnoid, ventricular, and intraparenchymal hemorrhages (Fig. 2C and F).
Effect of gp91 and p47 deficiency on other parameters. We next assessed the effect of NADPH oxidase deficiency on BBB disruption in mice infected with live bacteria. To visualize a defect in the integrity of the BBB, animals were injected with the albumin tracer Evans blue. Fluorescence microscopy revealed Evans blue extravasation primarily around penetrating and parenchymal blood vessels and in the subarachnoid space of infected WT animals (data not shown). The absence of Evans blue fluorescence in uninfected control animals indicated that the BBB was intact. Extravasation of Evans blue appeared to be more extensive in NADPH oxidase-deficient animals, especially p47\(^{-/-}\) animals (data not shown).

To quantitatively assess BBB disruption, the extravasation of serum albumin into the cortex was measured by a quantitative ELISA. As expected from the Evans blue data, infection led to significantly elevated cortical albumin levels in all of the mouse strains investigated compared to the levels in the correspond-

FIG. 1. Differential effects of gp91 and p47 deficiencies on CSF bacterial titers (A), BBB disruption (B), cortical MMP-9 levels (C), and IL-1\(\beta\) levels (D) in mice infected with live bacteria. (A) CSF bacterial titers at 18 h after infection were significantly higher in p47\(^{-/-}\) mice \((n = 11)\) but not in gp91\(^{-/-}\) mice \((n = 16)\) than in WT animals \((n = 16)\). (B) Infection led to significantly increased leakage of albumin into the cortex in all three strains tested. The data are expressed as percentages of the value for the corresponding uninfected control group (which was defined as 100\%). The basal levels in uninfected WT, gp91\(^{-/-}\), and p47\(^{-/-}\) mice were 10.6 \( \pm \) 3.7, 10.1 \( \pm \) 5.6, and 7.4 \( \pm \) 2.6 \( \mu \)g/mg of protein (means \( \pm \) standard deviations), respectively, and these values did not significantly differ from each other. (C) In all groups of infected animals, cortical MMP-9 levels were significantly increased compared to the levels in the corresponding uninfected controls. The basal levels in uninfected WT, gp91\(^{-/-}\), and p47\(^{-/-}\) mice were 17.1 \( \pm \) 31.9, 17.6 \( \pm \) 19.3, and 65.8 \( \pm \) 58.9 pg/mg of protein (means \( \pm \) standard deviations), respectively, and these values did not differ significantly from each other. (D) Increased cortical IL-1\(\beta\) levels in infected animals. The basal level was 1.1 \( \pm \) 0.1 \( \mu \)g/mg of protein (mean \( \pm \) standard deviation) in all backgrounds. The data indicate the median, upper and lower quartile, and minimum and maximum values for 10 or more WT infected animals, for six or more gp91\(^{-/-}\) infected animals, and for 11 or more p47\(^{-/-}\) infected animals unless indicated otherwise. Significant differences between the different strains are indicated as follows: one asterisk, \( P < 0.05\); two asterisks, \( P < 0.01\); three asterisks, \( P < 0.001\); ns, not significant.

Since MMP-9 has been thought to contribute to BBB disruption as a result of bacterial meningitis (18, 21), we investigated whether the increased disruption of the BBB in p47\(^{-/-}\) mice was associated with higher levels of this extracellular matrix-degrading enzyme. Approximately 24 h after infection, the MMP-9 levels (as measured by quantitative gelatin zymography) were significantly increased in all of the mouse strains compared to the levels in the corresponding uninfected controls \(( P < 0.01)\). In comparison, the levels of the constitutively expressed MMP-2 remained unchanged. As with accumulation of albumin, the meningitis-induced increase in the MMP-9 level was more than threefold greater in p47\(^{-/-}\) animals than in infected WT animals \(( P < 0.05)\), while the level in gp91\(^{-/-}\) animals was not significantly different from the level in WT control animals (Fig. 1C). In line with the notion that MMP-9 is directly involved in bacterial meningitis-mediated BBB disruption (21), there was a highly significant correlation between MMP-9 levels and the extent of albumin leakage into the cortex \(( P < 0.0001; r = 0.70)\) when data from all infected animals (i.e., WT and NADPH oxidase-deficient animals) were combined.

Compared to the responses of both WT and gp91\(^{-/-}\) infected animals, the histopathologically observed increase in the inflammatory response in p47\(^{-/-}\) animals (Fig. 2) was associated with significantly higher cortical levels of the proinflammatory cytokines IL-1\(\beta\) and TNF-\(\alpha\), which are known to be involved in BBB disruption due to bacterial meningitis (13, 25). The median level of IL-1\(\beta\) was about fivefold higher in p47\(^{-/-}\) animals (Fig. 1D) than in WT animals \(( P < 0.01)\), and significantly elevated TNF-\(\alpha\) levels due to infection were detected only in p47\(^{-/-}\) animals (median concentration, 1.74 pg/mg of protein; range, 0.97 to 14.71 pg/mg of protein) \(( P < 0.01)\) compared with the values for uninfected p47\(^{-/-}\) animals.

Effect of gp91 and p47 deficiency on the inflammatory response induced by sterile meningitis. Since the CSF bacterial titers were more than 10-fold higher in p47\(^{-/-}\) animals, a second set of experiments was conducted, in which meningitis was induced by inoculation of the CSF with heat-inactivated pneumococci. Twelve hours after intracisternal challenge with heat-inactivated R6 pneumococci, both WT and knockout mice exhibited symptoms similar to those observed in animals infected with live bacteria. However, in contrast to the response after infection with live bacteria, the inflammatory response induced by heat-inactivated bacteria was not more intense in p47\(^{-/-}\) mice than in WT or gp91\(^{-/-}\) mice. While the TNF-\(\alpha\) levels in the cortex were similar for the three strains of mice (data not shown), the CSF levels of TNF-\(\alpha\) and IL-6 were lower in p47\(^{-/-}\) animals than in WT animals (Table 1). Like the responses to live pneumococci, the response in gp91\(^{-/-}\) animals was similar to the response in WT animals. The lower
DISCUSSION

Using transgenic mice, we found that a deficiency in the NADPH oxidase subunit gp91 does not have a significant effect on CSF bacterial titers, BBB disruption, or the overall inflammatory response in animals infected with live pneumococci. In contrast to these results, infection was more severe in mice deficient in the p47 subunit. p47<sup>−/−</sup> animals had significantly higher CSF bacterial titers and exhibited more pronounced inflammation and increased BBB disruption than both WT and gp91<sup>−/−</sup> animals.

The results obtained with the gp91<sup>−/−</sup> animals are in line with the results of previous studies on the role of granulocytes in the pathogenesis of pneumococcal meningitis. Thus, bacterial growth and final titers in CSF were only marginally higher in rabbits rendered leukopenic than in normal rabbits (6, 30). Although pneumococci are sensitive to the bactericidal effects of ROS (24) and granulocytes from animals with pneumococcal meningitis are known to generate ROS (2), several factors can explain why these ROS are not able to effectively control pneumococcal growth in the CSF. Effective oxygen-dependent killing requires close proximity between bacteria and the location where ROS are generated (e.g., the phagolysosome). However, in CSF the levels of opsonizing complement and immunoglobulins necessary for efficient phagocytosis are low, and encapsulation of bacteria largely prevents phagocytosis by granulocytes. Furthermore, virulent pneumococci express high levels of manganese superoxide dismutase, which also contributes to protection from host-derived ROS (35). Overall, these findings, including the ones presented here, document that gp91<sup>−/−</sup>-dependent ROS formation does not contribute significantly to defense against pneumococcal CSF infection.

Compared to the results obtained with gp91<sup>−/−</sup> animals, levels of the two inflammatory cytokines in p47<sup>−/−</sup> animals were associated with lower WBC counts in the CSF.

**TABLE 1. CSF inflammatory responses in mice challenged with heat-inactivated pneumococci**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Conc of cytokines in CSF (pg/ml)</th>
<th>Conc of WBC in CSF (10&lt;sup&gt;6&lt;/sup&gt; cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNF-α</td>
<td>IL-6 (10&lt;sup&gt;3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>WT</td>
<td>1,121 ± 351</td>
<td>45.2 ± 20.0</td>
</tr>
<tr>
<td>gp91&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>741 ± 504</td>
<td>26.6 ± 10.4</td>
</tr>
<tr>
<td>p47&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>469 ± 379&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.0 ± 14.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> The data were obtained by a cytometric bead array analysis. The values are means ± standard deviations for eight WT animals, seven gp91<sup>−/−</sup> animals, and three p47<sup>−/−</sup> animals. All parameters were significantly elevated in WT animals challenged with heat-inactivated R6 pneumococci compared to the values obtained for saline-treated controls (P < 0.05, as determined by a Mann-Whitney U test).

<sup>b</sup> Significantly different (P < 0.01) than the value obtained for WT animals.

<sup>c</sup> Significantly different (P < 0.05) than the value obtained for WT animals as determined by an unpaired t test.
infection of p47−/− mice with live pneumococci was significantly more severe. A striking observation was the markedly more intense inflammatory response in p47−/− mice than in the other two strains of mice. The most likely explanation for this more intense inflammatory response is the increased bacterial titer in p47−/− mice compared to the bacterial titers in the two other strains. Our experiments with sterile inflammation provide a likely explanation for the higher CSF titers in p47−/− mice. In these animals, the inflammatory response (in particular, the WBC infiltration in the CSF) was greatly diminished, while the response in gp91−/− mice was similar to that in WT animals.

Extravasation of leukocytes into the CSF during bacterial meningitis is dependent on expression of intercellular adhesion molecule 1 (ICAM-1) on endothelial cells of the cerebral vasculature (1, 32). A recent study by Fan et al. showed that TNF-α-induced pulmonary ICAM-1 expression is greatly reduced in p47−/− mice compared to expression in WT mice (7). This effect was attributed to a defect in activation of nuclear factor κB, which is important for induction of ICAM-1 expression. Interestingly, inhibition of ICAM-1 expression appeared to be less pronounced in gp91−/− mice than in p47−/− mice. A possible explanation for this observation is that oxidants produced by cells that possess a p47-dependent but gp91-independent NADPH oxidase(s), such as vascular smooth muscle cells, are involved in control of endothelial ICAM-1 expression.

In addition to the differential effect of gp91 and p47 on the course of pneumococcal meningitis, our data are of interest with regard to the role of MMPs in the disruption of the BBB in bacterial meningitis. In our model, cortical MMP-9 levels correlated significantly with accumulation of albumin in the cortex, a marker for BBB disruption. A correlation between the level of MMP-9 in CSF and BBB disruption has also been observed for patients suffering from bacterial meningitis (18).

In a rat model of meningococcal meningitis, treatment with batimatstat, which inhibits MMP-9 and other MMPs, effectively diminished BBB disruption and the increase in intracranial pressure (21). Since MMPs such as MMP-9 can be activated by ROS (20, 26), one could anticipate that granulocyte-derived ROS contribute to BBB disruption via activation of latent MMPs. However, our data clearly show that NADPH oxidase-dependent formation of superoxide is not required for BBB disruption in pneumococcal meningitis.

The actual source of MMP-9 implicated in BBB disruption also remains unresolved. Since we measured MMP-9 levels in cortex homogenates that did not contain contaminating neutrophilic granulocytes (as judged by the absence of increased cortical myeloperoxidase activity [unpublished results]), our data suggest that at least some MMP-9 originates within the parenchyma. This hypothesis is supported by the fact that MMP-9 appears in the CSF before granulocytes can be detected in the CSF (15). It is known that astrocytes (33), microglia (9), and endothelial cells (10) all produce MMP-9 after stimulation with cytokines in vitro and may therefore be potential sources of MMP-9 in bacterial meningitis, in addition to infiltrating granulocytes. Indeed, exposure of rat brain slices to heat-inactivated R6 pneumococci resulted in increased secretion of MMP-9 in the absence of granulocytes (20).

In summary, in the present study we demonstrated that ROS formation by granulocytes cannot properly control CSF bacterial growth in pneumococcal meningitis. However, our results obtained with p47-deficient animals demonstrated that ROS formation by a gp91-independent NADPH oxidase(s) is important for establishing an adequate inflammatory response to pneumococcal CSF infection.

ACKNOWLEDGMENTS

This work was supported by Swiss National Science Foundation grants 32-66845.01 and 632-66057.01 and by Meningitis Research Foundation grant 14/00.

We especially thank Yoeng-Delphine Bifriere, Corinne Siegenthaler, and Jürg Kummer for excellent help with animal experiments. We are indebted to Ekatherina M. Vassina from the Institute of Pharmacology, University of Berne, for her help with the flow cytometry analysis.

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Vol. 71, 2003 PNEUMOCOCCAL MENINGITIS IN MICE 4091

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Editor: F. C. Fang