The Polysaccharide Fucoidin Inhibits the Antibiotic-Induced Inflammatory Cascade in Experimental Pneumococcal Meningitis

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There is evidence that the treatment of bacterial meningitis with antibiotics liberates harmful bacterial products in the subarachnoid space (SAS). This enhances meningeal inflammation and in particular the recruitment of leukocytes into the cerebrospinal fluid (CSF), which has been shown to be more harmful than beneficial in this disease. In this study, we used a rabbit meningitis model based on intracisternal injection of live Streptococcus pneumoniae. Ampicillin (40 mg/kg of body weight given intravenously [i.v.] 16 h after induction of meningitis) caused a fivefold increase in CSF leukocytes over a 4-h period. Inhibition of leukocyte rolling by treatment with the polysaccharide fucoidin (10 mg/kg, i.v.) prevented the enhanced leukocyte extravasation into the SAS and attenuated the leakage of plasma proteins over the blood-brain barrier. These results suggest that certain polysaccharides that block leukocyte rolling have the potential to reduce leukocyte-dependent central nervous system damage in bacterial meningitis.

In bacterial meningitis, antibiotic-induced bacterial lysis liberates large amounts of harmful bacterial products in the cerebrospinal fluid (CSF), resulting in an increased number of active molecules capable of enhancing the inflammatory response (7). For example, in experimental pneumococcal meningitis in rabbits, Tuomanen et al. have demonstrated an increase in meningeal inflammation after ampicillin treatment, characterized by a massive influx of leukocytes and elevated levels of protein and lactate in the CSF (12, 13). Similar effects of antibiotic treatment have also been demonstrated in experimental Haemophilus influenzae meningitis (7, 10). These inflammatory changes, and in particular the accumulation of leukocytes, are believed to contribute to the central nervous system injury commonly associated with bacterial meningitis. In fact, there is evidence that the harmful effects of the leukocytes and their cytotoxic products outweigh their beneficial role in this disease, and the inhibition of leukocyte recruitment into the subarachnoid space has been shown to reduce mortality in experimental meningitis (10, 11, 13).

The process of inflammatory leukocyte recruitment involves several sequential steps: margination and rolling of leukocytes along the vascular endothelium, firm adhesion to the endothelial cells, and subsequent diapedesis through the vessel wall (3). Leukocyte rolling is mediated by adhesion molecules of the selectin family (3), while stationary leukocyte adhesion is critically dependent on leukocyte integrins such as CD11-CD18 and endothelial immunoglobulin gene-related adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) (3). Accordingly, the polysaccharide fucoidin, a homopolymer of sulfated L-fucose, blocks leukocyte rolling by binding to L- and P-selectins (5, 14) and monoclonal antibodies (MAbs) to CD11-CD18 (2) or ICAM-1 (3) block firm leukocyte adhesion. With regard to bacterial meningitis, we have recently shown that fucoidin treatment is an effective way to attenuate meningeal inflammation induced by pneumococcal cell wall fragments (4). In the present study, we tested whether fucoidin also has the capacity to inhibit the ampicillin-induced increase in CSF leukocytes in rabbits inoculated with live bacteria, as has previously been shown with anti-CD18 MAbs (10, 13).

MATERIALS AND METHODS

Bacterial strain. Streptococcus pneumoniae strain III, type III, a gift from Elaine Tuomanen, was cultured on blood-agar plates and suspended in pyrogen-free saline before injection.

Experimental meningitis model. A previously described meningitis model in female New Zealand White rabbits (2.0 to 3.5 kg) was used (6). Briefly, 0.25 ml of CSF was collected from the cisterna magna, into which an equal volume of live bacteria was then injected. Subsequent CSF samples (0.25 ml) were obtained at the time points indicated below. A total of 35 rabbits were injected intracisternally with live pneumococcal bacteria in a 0.25-ml saline suspension containing an inoculum of $10^6$ CFU/ml. Sixteen hours after bacterial inoculation, 30 animals received an intravenous (i.v.) injection of ampicillin (40 mg/kg of body weight) in an ear vein. Nineteen of these rabbits received ampicillin only and 11 rabbits were treated with fucoidin (10 mg/kg, i.v.) 5 min before the ampicillin dose and 2 (n = 11) and 4 (n = 4) h thereafter. Five rabbits served as controls, i.e., were inoculated with bacteria but did not receive any treatment. In all animals, aliquots of CSF were obtained just before pneumococcal inoculation and at 16 and 20 h. From the four animals receiving 3 doses of fucoidin and eight of the animals treated with ampicillin only, CSF was also obtained at 22 h. Immediately after collection, total and differential leukocyte counts of CSF were done. The remaining CSF was centrifuged (1,200 $\times$ g, 10 min), and the supernatants were stored at $-70^\circ$C until assayed for protein, lactate, and glucose (the 22-h CSF samples were not analyzed chemically). Fucoidin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in sterile phosphate-buffered saline (10 mg/ml at pH 7.3) and passed through a 0.2-μm-pore-size sterile filter prior to i.v. administration. Fucoidin has been found to block leukocyte rolling in a dose-dependent manner, without interfering with the process of firm leukocyte adhesion per se (5). Our choices of dose and regimen were based on previous studies, including our own (4). Ampicillin (Dokticillin; Astra, Södertälje, Sweden) was dissolved in sterile saline.

During each cisternal puncture, the animals were anesthetized intramuscularly with 0.25 ml of fluanxone/fentanyl (Hypnorm Vet; 10 mg of fluanxone and 0.2 mg of fentanyl per ml; Janssen Pharmaceutica) per kg and 0.25 ml of diazepam (Stesolid; 5 mg/ml; Dumex) per kg. The experiments were approved by an animal ethics committee at Södra Roslags Court House. All animals were euthanized within 24 h of bacterial inoculation.
FIG. 1. CSF leukocytes over time after intracisternal inoculation of rabbits with live pneumococci. Sixteen hours after bacterial injection, one group received a dose of ampicillin (40 mg/kg) resulting in a fivefold increase in CSF leukocytes at 20 h. This inflammatory increase was prevented when fucoidin (10 mg/kg) was given 5 min before and 2 h after ampicillin. Control animals, inoculated with pneumococci but given no treatment, did not show any significant change between the two time points. Values are means ± SE. ***P < 0.001 for ampicillin versus ampicillin plus fucoidin at 20 h.

Measurement of CSF leukocytes, glucose, lactate, and protein. Total and differential CSF leukocyte counts were carried out in a Bürker chamber after staining. The supernatants were assayed reflectometrically (Kodak 700XRc) for glucose, lactate, and protein with the Kodak Ektachem clinical chemistry slides GLU, LAC, and PROT (Eastman Kodak Company, Rochester, N.Y.). Statistical analysis. Results were analyzed by the Wilcoxon–Mann-Whitney test. Differences were considered significant at a P value of <0.05. Data are expressed as means ± standard errors (SE).

RESULTS

The 0-h mean CSF leukocyte count for all three groups together was 5 × 10⁶ ± 3 × 10⁶/liter (no differences between the groups). In the control group, which was inoculated with bacteria but given no treatment, the mean CSF leukocyte count did not change between 16 and 20 h (Fig. 1). When a dose of ampicillin was given at 16 h, the animals demonstrated a rapid increase in the influx of leukocytes into the CSF, i.e., a fivefold increase at 20 h (Fig. 1). This massive leukocyte accumulation was prevented if fucoidin was given 5 min before and 2 h after ampicillin (Fig. 1). In a subgroup of animals that was examined at 22 h (6 h after ampicillin treatment), the mean CSF leukocyte counts were 8,111 × 10⁶ ± 2,794 × 10⁶ cells/liter after ampicillin alone and 2,172 × 10⁶ ± 244 × 10⁶ cells/liter after ampicillin plus fucoidin, i.e., almost identical to the 20-h values.

The mean CSF protein level for all groups together at 0 h was 0.3 ± 0.08 g/liter (no differences between the groups), and all three groups inoculated with bacteria demonstrated a substantial increase in CSF protein at 16 h (Table 1). The mean CSF protein level in untreated controls did not change between 16 and 20 h. In the group receiving only ampicillin, the protein concentration increased slightly after 4 h, but not significantly (Table 1). The fucoidin-treated animals demonstrated a 50% reduction in mean protein concentrations in CSF between 16 and 20 h (Table 1), but this effect of fucoidin was not statistically significant.

The mean CSF lactate concentration at 0 h was 2.2 ± 0.2 mmol/liter for all groups, and the mean CSF glucose concentration was 4.2 ± 0.2 mmol/liter at 0 h (no differences between the groups). There were marked changes in lactate and glucose concentrations between 0 and 16 h in all three groups; however, the mean CSF lactate and glucose concentrations did not change significantly between 16 and 20 h (Table 1).

DISCUSSION

In a previous study (4), we showed that intravenous treatment with fucoidin, a homopolymer of sulfated fucose, almost completely inhibited leukocyte and protein accumulation in the CSF of rabbits injected intracisternally with pneumococcal cell wall products. Consistent with these results, Angstwurm et al. (1) recently demonstrated that fucoidin also has the effect of reducing the increase in brain water content, regional cerebral blood flow, and intracranial pressure in rats with meningoitis induced by pneumococcal cell wall components. In the present study, we show that fucoidin prevents CSF leukocyte recruitment and attenuates CSF protein accumulation when given i.v. after a dose of ampicillin to rabbits inoculated with live pneumococci.

Antibiotic-induced bacterial lysis has been shown to enhance the meningeal inflammation and cerebral circulatory pathology in both experimental and clinical studies, and treatment with anti-inflammatory drugs such as corticosteroids may reduce these indices of neurological damage (8, 10, 12, 13). Moreover, specific inhibition of leukocyte transmigration to the subarachnoid space through the blocking of firm leukocyte adhesion to vascular endothelium modulates the inflammation, reduces the increase in brain water content, and improves the survival rates of the animals; i.e., anti-CD18 MAbs have been documented to attenuate meningeal inflammation after treatment with antibiotics in both pneumococcal (13) and H. influenzae (10) experimental meningitis. Recently, the anti-inflammatory effects of anti-ICAM MAbs have also been demonstrated in experimental meningitis (15). Compared with these results, our present findings indicate that fucoidin is more effective in decreas-

| Treatment                        | Leukocytes (10⁶/liter) | Protein (g/liter) | Lactate (mmol/liter) | Glucose (mmol/liter) |
|----------------------------------|------------------------|-------------------|----------------------|----------------------|
|                                  | 16 h  20 h             | 16 h  20 h        | 16 h  20 h           | 16 h  20 h           |
| None (control group)             | 960 ± 326              | 662 ± 160         | 1.7 ± 0.7            | 1.8 ± 0.6            |
| Ampicillin                       | 1,456 ± 520            | 7,687 ± 2,022     | 2.1 ± 0.9            | 2.3 ± 0.6            |
| Ampicillin and fucoidin          | 1,994 ± 858            | 1,505 ± 444       | 3.1 ± 1.2            | 1.6 ± 0.3            |

* Fucoidin (10 mg/kg) and/or ampicillin (40 mg/kg) given i.v. at 16 h. Values are means ± SE.

* P < 0.001 versus ampicillin alone at 20 h.
ing the meningitic inflammatory changes: fucoidin completely inhibited the ampicillin-induced increase in leukocyte recruitment as measured 4 and 6 h after a dose of ampicillin, and it decreased protein levels 4 h after antibiotic administration. In the studies described above in which anti-CD18 treatment significantly reduced the enhancement of leukocyte and protein accumulation in rabbits with pneumococcal (13) or \textit{H. influenzae} (10) meningitis, both these indices of inflammation still increased after antibiotic treatment. Thus, in the former study (13), the median CSF leukocyte density did not increase 4 h after antibiotic treatment, but at 6 h, the median leukocyte count increased 10-fold. In the latter study (10), the mean CSF leukocyte and protein levels increased by at least 50% 2 h after a dose of antibiotics, and even more after 6 h. However, to make a strict comparison between fucoidin and MAb IB4, these drugs would have to be tested in the same animal model. One explanation of why fucoidin appears to be more effective than anti-CD18 therapy may be that firm leukocyte adhesion may involve beta-1-integrins in addition to the CD11-CD18 complex (3), while leukocyte rolling (inhibited by fucoidin but not anti-CD18 MAbs) is a precondition for the firm adhesion (5).

The other two indices of inflammation measured in this study, lactate and glucose levels, were markedly changed 16 h after bacterial inoculation but did not change significantly after treatment with ampicillin or ampicillin plus fucoidin. Although CSF lactic acidosis has been reported to be induced by a bolus dose of antibiotics in experimental meningitis (9, 10), our findings suggest that CSF lactate and glucose concentrations are not associated with the increase in CSF leukocytes after a dose of ampicillin.

Interference with the leukocyte endothelial adhesion process in bacterial meningitis has therapeutic potential in clinical situations in that it may be possible to inhibit the inflammatory burst elicited by the first dose of antibiotics. However, it must be noted in this context that the phenomenon of an inflammatory overload after a bolus dose of ampicillin in rabbits with experimental pneumococcal meningitis has been found to be dependent on the CSF bacterial concentration at the time of treatment (9). Thus, it has been shown that when the pneumococcal bacterial concentration reaches a threshold of about \(10^6\) CFU/ml there is a marked increase in CSF leukocytes, and a dose of ampicillin has no impact on the CSF leukocyte count under these conditions (9). However, the injection of ampicillin shortly before this threshold concentration is reached results in an inflammatory burst, while a delay in inflammation is seen if ampicillin is injected several hours earlier. Thus, it seems that the inflammatory increase induced by antibiotics occurs only during a limited phase in the course of experimental pneumococcal meningitis.

In conclusion, we have documented for the first time that the polysaccharide fucoidin effectively inhibits the accumulation of CSF leukocytes upon administration of antibiotics in experimental meningitis induced with live bacteria. As mentioned before, other therapeutic strategies are also possible, but in order to reduce CSF leukocyte recruitment it appears that the inhibition of leukocyte rolling (with, for example, fucoidin) is more efficient than the blocking of firm leukocyte adhesion with anti-CD18 or anti-ICAM-1 MAbs.

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