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Pretreatment with Granulocyte Colony-Stimulating Factor Attenuates the Inflammatory Response but Not the Bacterial Load in Cerebrospinal Fluid during Experimental Pneumococcal Meningitis in Rabbits

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A possible immunomodulatory role of granulocyte colony-stimulating factor (G-CSF) was investigated in an experimental pneumococcal meningitis model in rabbits. Animals were pretreated with G-CSF (10 μg/kg subcutaneously twice a day) starting 48 h before in vivo and ex vivo experiments, causing a five- to six-fold increase in the peripheral leukocyte level. Meningitis was induced by intracisternal inoculation of ~4×10^5 CFU of *Streptococcus pneumoniae* type 3. Neutrophil pleocytosis and interleukin-8 (IL-8) levels were significantly attenuated in G-CSF-pretreated animals compared to untreated animals ($P < 0.05$). Furthermore, G-CSF pretreatment significantly delayed alterations in cerebrospinal fluid (CSF) tumor necrosis factor alpha and IL-1β levels, as well as protein and glucose levels ($P < 0.05$). No difference in CSF bacterial concentrations was found, whereas the blood bacterial concentration was significantly decreased in G-CSF-pretreated animals ($P < 0.05$). Ex vivo chemotaxis of neutrophils isolated from G-CSF-pretreated animals was significantly decreased compared to that of neutrophils from untreated animals ($P < 0.05$). In conclusion, G-CSF pretreatment attenuates meningeal inflammation and enhances systemic bacterial killing. Further preclinical studies are required to investigate whether this may affect the clinical course of meningitis and thus whether G-CSF treatment may have a beneficial role in pneumococcal meningitis.

The pathophysiology of pneumococcal meningitis has been studied intensively throughout the last decade in animal models (see reference 32 for a review). Pneumococci or pneumococcal cell wall fragments induce a local inflammatory response characterized by neutrophil influx into the brain or the cerebrospinal fluid (CSF) (42). Various types of anti-inflammatory treatments (e.g., antibodies to cytokines [33, 36], leukocyte-endothelial adhesion molecules [11, 35, 43], inhibitors of neutrophil activation products [22, 24], and inhibitors of neuro-excitatory amino acids [25]) reduce the development of increased intracranial pressure, brain edema, cerebral ischemia, or neural injury. It has been speculated that an influx of neutrophils is the primary cause of these changes.

Another approach to improve our understanding of the role of the neutrophils in the pathophysiology of bacterial meningitis is to increase the number of neutrophils in the peripheral blood over the course of the meningitis. Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein which stimulates proliferation and differentiation of hematopoietic progenitor cells and increases the total number of neutrophils in the blood (31; for a review, see reference 4).

G-CSF treatment has previously been demonstrated to improve survival in nonneutropenic models of systemic infections (27). However, conflicting results on survival have been obtained in various animal studies on local infections (12, 16, 28, 44). The explanation for this remains to be defined, but it could be due to the influence of G-CSF treatment on local host defense mechanisms. By using the rabbit meningitis model, it is possible to study the kinetics of the pleocytosis, since sequential CSF tappings can be performed.

The purpose of this study was to investigate the influence of pretreatment with G-CSF on the kinetics of local inflammation in an experimental pneumococcal meningitis model in rabbits. Our initial working hypothesis was that by increasing the number of neutrophils in peripheral blood, the rate of influx of neutrophils in the CSF would increase. However, our data showed that G-CSF-induced elevation of the peripheral leukocyte (WBC) level was associated with a consistent decrease in CSF pleocytosis, likely because of impaired chemotaxis by the G-CSF-induced neutrophils and/or impaired production of local cytokines.

MATERIALS AND METHODS

Bacterial strain. The bacterial strain used was a *Streptococcus pneumoniae* type 3 strain (68034). The frozen organisms were thawed and grown on 5% blood agar plates for 24 h, and the colonies were suspended in beef broth to an optical density of 0.35 at 540 nm and incubated for 1 h. The test organism was diluted in sterile beef broth to a final concentration of approximately 2×10^6 CFU/ml (1×10^6 to 6×10^6 CFU/ml; there was no significant difference between the G-CSF-treated group and the untreated control group), as confirmed by quantitative cultures, and 0.2 ml was used for intracisternal inoculation.

G-CSF treatment. Animals were pretreated with G-CSF (Neupogen; kindly provided by Amgen, Hellerup, Denmark) (10 μg/kg subcutaneously [s.c.] twice a day) starting 48 h before in vivo and ex vivo experiments. After bacterial inoculation, no further G-CSF was given.

Ex vivo experiments. All ex vivo studies were carried out with corresponding blood cells from G-CSF-treated and untreated animals in at least six independent experiments.

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(i) Separation of rabbit neutrophils and monocytes. Blood was drawn from rabbits sedated with fentanyl/fluanisone (Hypnorm; Janssen Pharmaceutica N.V., Beerse, Belgium) (0.1 ml/kg intravenously) from a central ear artery into 4% (wt/vol) citrated anticoagulated tubes, mixed 1/6 with 5% (wt/vol) dextran (Statens Serum Institut, Copenhagen, Denmark), and left to sediment. WBC-rich plasma was layered over Histopaque (1.082 g/ml; Sigma Chemical Co., St. Louis, Mo.) and centrifuged for 30 min at $600 \times g$. The mononuclear band was carefully removed, and then the plasma and the density medium above the neutrophil pellet were removed. Neutrophils and monocytes were washed in 0.2% (wt/vol) saline to remove erythrocytes and resuspended in minimal essential medium (MEM) (Statens Serum Institut) for neutrophil chemotaxis and monocyte cytokine production assays or in Krebs Ringer buffer (KRB) (Statens Serum Institut) for chemiluminescence assay. Means of 4×10^8 and 3×10^7 neutrophils and 1×10^7 and 1×10^8 monocytes were isolated from 50 ml of blood taken from G-CSF-treated and untreated rabbits, respectively.

(ii) Neutrophil chemotaxis. Chemotaxis was measured in triplicate by using a modified Boyden chamber assay as previously described (19). Briefly, the upper compartment, containing 10^6 cells/ml in a final volume of 500 μl suspended in MEM with 1% (vol/vol) human serum albumin (Statens Serum Institut), was separated from the lower compartment by a cellulose filter (3-μm pore size; Millipore, Bedford, Mass.). The lower compartment contained the following chemoattractant: 10^{-8} M fMLP (Sigma Chemical Co.), a 1:200 dilution of zymosan-activated human or rabbit serum (both prepared at our laboratory), or MEM as a control in a final volume of approximately 500 μl. The chambers were incubated at 37°C in CO_2 for 2.5 h. Cells migrating to the lower surface of the filter were stained with hematoxylin and counted automatically by a computer-assisted image analysis system (Image House, Copenhagen, Denmark) in five randomly chosen fields per filter.

(iii) Neutrophil chemiluminescence. A previously described luminol-enhanced chemiluminescence assay was used (20). Briefly, 5×10^5 neutrophils suspended in KRB with 7×10^{-5} M luminol (Sigma Chemical Co.) were tested in duplicate with the following stimulus: 2×10^{-6} M fMLP, 2 mg of zymosan per ml, or KRB as a control in a final volume of 1 ml. Peak chemiluminescence was recorded with an LKB 1251 instrument (Bio-Tec Instruments, Winooski, Vt.).

(iv) Cytokine production from mononuclear cells. Cytokine production from mononuclear cells was assayed as previously described (23). However, we used only freshly prepared mononuclear cells in this study. Briefly, 5×10^5 mononuclear cells were incubated in microtiter wells (Nunc, Roskilde, Denmark) at 37°C in ambient air with whole *S. pneumoniae* type 3 or lipopolysaccharide (LPS), each in a twofold dilution from 5×10^3 to 5×10^7 CFU or from 9.8 pg to 2.5 μg, respectively, in RPMI medium (Statens Serum Institut) in a final volume of 0.2 ml. After 24 h of incubation, the plates were stored at −80°C for subsequent cytokine analysis.

In vivo experiments. (i) Experimental meningitis model. The Danish animal experiment inspectorate approved the experimental protocols. A modification of the model originally described by Dacey and Sande (5) was used. New Zealand White rabbits, approximately 2.5 kg in weight, were anesthetized with midazolam (Dormicum; F. Hoffmann-La Roche AG, Basel, Switzerland) (0.5 mg/kg s.c.) and Hypnorm (0.35 ml/kg intramuscularly), and a dental acrylic helmet containing a half turnbuckle was attached to the skull. The next day the rabbits were reanesthetized with ethyl carbamate (Urethane; Fluka Kemi AG, Buchs, Switzerland) (1.75 g/kg s.c.) and pentobarbital (Nykomed, Roskilde, Denmark) (10 mg/kg) and immobilized in a stereotaxic frame. A 25-gauge spinal needle was introduced into cisterna magna for the inoculation of 0.2 ml of bacterial suspension and repetitive CSF sampling every second hour. The rate of removal of CSF did not exceed the rate of CSF formation, which is approximately 0.4 ml/h (38). Blood samples were taken from a central ear artery. After testing for bacterial concentration and WBC count, CSF and blood samples were centrifuged and supernatants were immediately stored at −80°C for subsequent analysis. Animals were euthanized 16 h after bacterial inoculation. After 16 h from the time of bacterial inoculation, the infected rabbits were starting to be clinically unstable because of sepsis and the continued anesthesia. At that time, the inflammatory response in the CSF was pronounced. The objective of the study was to monitor the kinetics of this response. For these reasons, the rabbits were sacrificed at this time with 50 mg of pentobarbital (Nykomed) per kg.

(ii) Bacterial concentration. Bacterial titers in blood and CSF were determined by plating undiluted sample and 10-fold serial dilutions on 5% blood agar plates (Statens Serum Institut). The lowest detectable bacterial concentration was 50 CFU/ml.

(iii) WBC, glucose, lactate, and protein levels. WBC and differential counts were determined on an automatic cell counter (Swelab, Årsta, Sweden). The lowest detectable WBC level was 100×10^3 cells/ml. Lactate and glucose contents were measured with commercial kits (Sigma Chemical Co.). Protein content was determined as described by Lowry et al. (26).

(iv) TNF-α. Tumor necrosis factor alpha (TNF-α) levels in CSF and blood were measured by bioassay as previously described (9, 13). Briefly, 5×10^4 cells (WEHI 164, subclone 13; kindly provided by T. Espevik) per well in RPMI 1640 with 0.25 μg of actinomycin D (A 9415; Sigma Chemical Co.) per ml were incubated with a standard (human TNF-α, T 6674; Sigma Chemical Co.) or 10-μl samples in triplicate on microtiter plates (167008; Nunc) for 18 to 24 h in ambient air at 37°C. MTT (M 2128; Sigma Chemical Co.) at 1 mg/ml was used to indicate cell kill; 20% (wt/vol) sodium dodecyl sulfate (L 4509; Sigma Chemical Co.) was dissolved in 50% (vol/vol) DMF (D 8654; Sigma Chemical Co.) and in water and used as the lysing buffer, and the optical density was measured on an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tec) at 570 nm. The coefficient of variation was less than 10%. The lowest detection level was 50 pg/ml.

(v) IL-1β. Interleukin-1β (IL-1β) levels in CSF were measured in a two-step assay which was a modification of the assay of Gearing et al. (10). In the first step, 2×10^5 cells (per well) of the murine T-cell line EL-4, subclone NOB 1, which produces IL-2 upon exposure to IL-1, in RPMI 1640 medium were incubated with a standard (human IL-1β; Sigma Chemical Co.) or 10-μl CSF samples in triplicate on microtiter plates (167008; Nunc) for 18 to 24 h in ambient air at 37°C. In the second step, 100 μl of the supernatants was carefully collected for subsequent analysis of IL-2 content with a commercial mouse IL-2 ELISA kit (Genzyme Diagnostics, Cambridge, Mass.), essentially as described by the manufacturer. The coefficient of variation was less than 20%. The lowest detection level was 30 pg/ml.

(vi) IL-8. IL-8 levels in CSF and blood were measured with a modification of a rabbit-specific ELISA as previously described (14, 21). Briefly, microtiter plates (Maxisorb 439454; Nunc) were coated overnight with WS-4 (a kind gift of K. Matsushima, Tokyo, Japan), a mouse monoclonal antibody to human and rabbit IL-8, at 0.5 μg/ml in 0.05 M carbonate buffer (pH 9.6) at 4°C. After the plates were washed with phosphate-buffered saline containing 0.05% (vol/vol) Tween 20 (buffer A), the unbound sites were blocked by adding 150 μl of 1% (vol/vol) bovine serum albumin in buffer A (buffer B) per well at 37°C for 1 h. After another wash, the standard (rabbit-IL-8; a kind gift of K. Matsushima) or samples diluted in buffer B were added in duplicate and incubated overnight at 4°C. Five washes were carried out between each of the subsequent steps. Goat anti-human IL-8 immunoglobulin G (R&D Systems Europe Ltd., Abingdon, United Kingdom) at 1 μg/ml in buffer A (buffer C) was added as the detection antibody and incubated at 37°C for 2 h. The specificity of goat anti-human IL-8 for rabbit IL-8 has previously been tested by Western blot analysis and found to be similar to that of guinea pig anti-rabbit IL-8 (unpublished observations). In the next step, alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin G (R&D Systems Europe Ltd.) diluted 1:3,000 in buffer C was added and incubated for an additional 1 h at 37°C. *o*-Phenylenediamine (Sigma Chemical Co.) at a concentration of 0.8 mg/ml in 0.05 M citric acid/sodium phosphate buffer containing a 1/1,000 dilution of 30% (vol/vol) hydrogen peroxide was added and allowed to react for 30 min at room temperature. The enzyme reaction was stopped by the addition of 100 μl of 4.5 N H_2SO_4, and the optical density at 490 nm was measured with the ELISA reader. The lowest detection level was 20 pg/ml. The coefficient of variation was less than 10%.

Statistical analysis. All results are provided as medians and ranges (minimum to maximum). Comparison between groups was performed by nonparametric tests (Mann-Whitney test for unpaired data and Wilcoxon signed rank test for paired data). $P < 0.05$ was considered significant.

RESULTS

Effect of G-CSF treatment on neutrophil pleocytosis. Intracisternal challenge with pneumococci resulted in neutrophil pleocytosis in untreated animals, with peak levels at 12 to 14 h after the bacteria were inoculated, whereas in G-CSF-treated animals, the CSF WBC response was significantly attenuated ($P < 0.05$) (Fig. 1). This was despite the fact that the blood WBC level (10^3 cells/μl) was increased approximately five- to sixfold in G-CSF-treated animals compared to untreated animals at the time of bacterial inoculation (28.7 [20.4 to 35.4] versus 5.4 [3.7 to 8.3], respectively; $P = 0.0001$) and throughout the study (data not shown).

CSF cytokine kinetics. Median CSF TNF-α levels (picograms per milliliter) were lower in G-CSF-treated than in nontreated animals from the start of meningeal inflammation (from approximately 8 to 10 h) and were significantly reduced at 12 h (175 [0 to 666] versus 1,848 [195 to 29,030], respectively; $P = 0.015$) (Fig. 2a). Median CSF IL-1β levels (picograms per milliliter) were also lower from the start of meningeal inflammation and were significantly reduced at 12 h (713 [155 to 1,524] versus 0 [0 to 120], respectively; $P = 0.004$) (Fig. 2b). Similar but more consistent trends over longer periods were observed for IL-8 levels in CSF, with a significant difference from 8 h throughout the study period ($P < 0.05$) (Fig. 2c).

The corresponding cytokine levels in plasma were low and not significantly different according to usage of G-CSF (for both groups, 0 to 1,000 pg/ml for TNF and <30 pg/ml for IL-8;

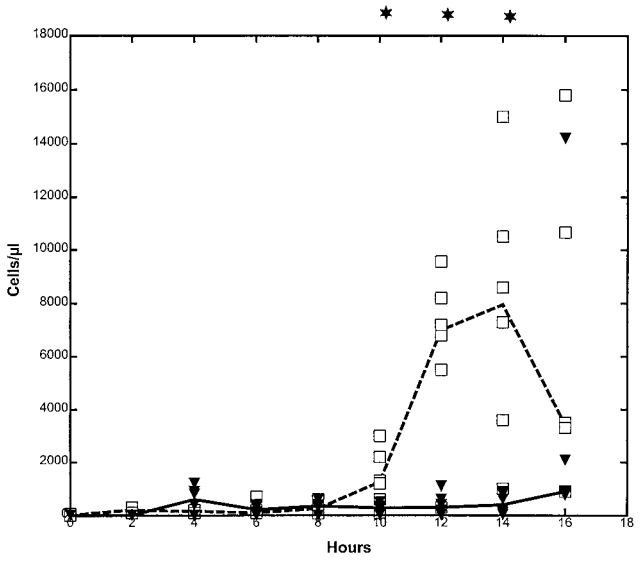

FIG. 1. Median CSF WBC levels in G-CSF-pretreated and untreated rabbits during experimental pneumococcal meningitis. G-CSF (10 μg/kg) was administered twice a day starting 48 h before intracisternal bacterial challenge (0 h). The CSF WBC level was significantly attenuated in the G-CSF-treated group (closed symbols [median, solid line]) compared to the untreated control group (open symbols [median, dashed line]) between 10 and 14 h ($n = 6$ for both groups). *, $P < 0.05$ (Mann-Whitney test).

$P > 0.05$), confirming that cytokines are locally produced in bacterial meningitis.

Cytokine production from rabbit monocytes ex vivo. To address whether the attenuated levels of proinflammatory cytokines in the CSF were due to impaired ability of the monocytes to produce cytokines, monocytes were isolated and stimulated with live pneumococci or LPS. However, no difference in release of cytokines was detected regardless of whether the animals were pretreated with G-CSF or not (for both groups the range of measurements were 0 to 18,817 pg/ml for TNF-α and 279 to 5,000 pg/ml for IL-1β; $P > 0.05$).

Rabbit neutrophil chemotaxis ex vivo. Another possible cause of the diminished pleocytosis is that the neutrophils in the peripheral blood had a reduced ability to migrate towards a chemotactic gradient. In fact, chemotaxis for various chemoattractants (fMLP and rabbit and human C5a) was reduced by more than 40% in neutrophils isolated from G-CSF-treated animals compared to neutrophils from untreated animals (Fig. 3) ($P < 0.05$).

Bacterial growth. (i) Bacterial concentration in CSF. During the 16-h study period, the bacterial concentration in the CSF increased exponentially by more than 2 $\log_{10}$ CFU/ml but without significant differences in concentration according to whether the rabbits were pretreated with G-CSF or not (Fig. 4a) ($P > 0.05$).

(ii) Bacterial concentration in blood. The rabbits started to become bacteremic 4 to 6 h after the bacteria were inoculated in the CSF. Thereafter, the growth curve was exponential for both groups (Fig. 4b). Although the times that the rabbits started to become bacteremic were similar regardless of pretreatment with G-CSF at all subsequent time points, G-CSF pretreatment was consistently associated with a decreased concentration of pneumococci in the blood ($P < 0.05$).

Luminol-enhanced chemoluminescence (percentage of the peak value in millivolts) was significantly enhanced in neutrophils isolated from G-CSF-pretreated rabbits compared to untreated rabbits when opsonized zymosan and fMLP were used as stimuli (36.3% [5.8 to 596.0%] and 38.5% [20.4 to 205.9%], respectively; Wilcoxon signed rank test, $P < 0.05$).

Indices of meningeal inflammation and blood-brain barrier damage. To assess the metabolic activity within the central nervous system, levels of glucose and lactate in CSF were measured. Levels of glucose in CSF (milligrams per deciliter) remained stable throughout the study period for rabbits pretreated with G-CSF, whereas they started to decrease from 12 h in the course of bacterial meningitis in untreated animals. The difference became statistically significant at 16 h after bacterial inoculation (99.7 [65 to 135] versus 43.0 [27 to 101]; $P < 0.05$). The lactate concentration in the CSF gradually increased from 10 h in both groups, without any consistent difference (data not shown). Finally, to obtain an impression of the damage to the blood-brain barrier, levels of protein in CSF were measured. The CSF protein concentration gradually increased late in the course of the meningitis, but this increase started significantly later in rabbits pretreated with G-CSF, with the difference being statistically significant at 10 and 12 h (Fig. 5) ($P < 0.05$).

DISCUSSION

In the present study, we found that pretreatment of rabbits with G-CSF attenuated the enhanced neutrophil pleocytosis in pneumococcal meningitis, despite a five- to sixfold increase in peripheral WBCs when the bacteria were inoculated. Two other studies have previously addressed the role of G-CSF in neutrophil recruitment to an inflamed site in vivo. A study by Price et al. found impaired neutrophil migration to skin chambers in normal humans with G-CSF-induced neutrocytosis (31), whereas Nelson et al. found an elevated number of neutrophils in brochioalveolar lavage of rats with *Klebsiella pneumoniae* pneumonia pretreated with G-CSF (28). Differences in the organs and bacteria studied, as well as differences in animal species used, may well explain these conflicting findings.

In the present study, we further investigated possible explanations for why G-CSF pretreatment results in an attenuated pleocytosis. Theoretically, these include (i) a reduction in CSF bacterial concentration, (ii) an inhibition of proinflammatory cytokine production within the central nervous system, (iii) a reduction in neutrophil chemotactic ability per se, and (iv) a modulation of adhesion molecules on neutrophils and vascular endothelium (and hence impaired rolling, adhesion, and diapedesis over the blood-brain barrier).

(i) In this study, no difference was found in the CSF bacterial concentration regardless of whether the rabbits were pretreated with G-CSF. Thus, diminished bacterial antigenic stimulation of host cells responsible for initiating and sustaining the inflammatory response within the central nervous system is an unlikely explanation for the observed attenuated pleocytosis in G-CSF-pretreated animals.

The fact that a lower number of neutrophils in the CSF does not affect the growth of bacteria in CSF in experimental models of meningitis is in accordance with several other studies in which the CSF WBC level was reduced either by nitrogen mustard-induced peripheral neutropenia (8, 41), blockage of integrins with anti-CD18 monoclonal antibodies (35, 43), or inhibition of selectins with fucoidin (unpublished observations). Moreover, an enhanced neutrophil pleocytosis in pneumococcal meningitis after treatment with fMLP intracisternally has previously been shown not to influence bacterial growth in the CSF (41). Previous studies on the effect of G-CSF on local bacterial or fungal killing have generated conflicting results

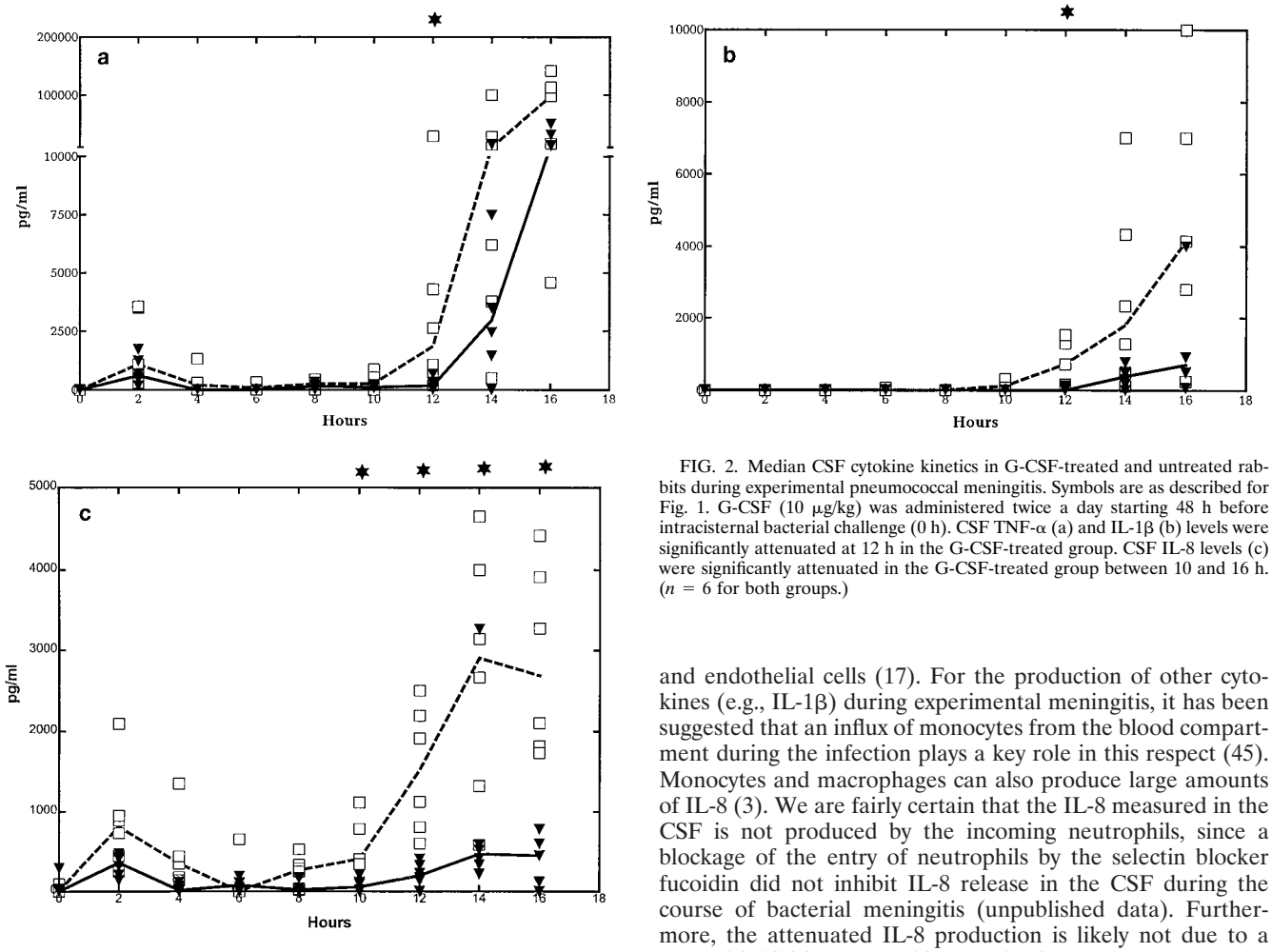

FIG. 2. Median CSF cytokine kinetics in G-CSF-treated and untreated rabbits during experimental pneumococcal meningitis. Symbols are as described for Fig. 1. G-CSF (10 μg/kg) was administered twice a day starting 48 h before intracisternal bacterial challenge (0 h). CSF TNF-α (a) and IL-1β (b) levels were significantly attenuated at 12 h in the G-CSF-treated group. CSF IL-8 levels (c) were significantly attenuated in the G-CSF-treated group between 10 and 16 h. (n = 6 for both groups.)

(12, 16, 28, 44), so caution should be used when findings from one model of infections are extrapolated to other models.

(ii) We found consistently decreased CSF IL-8 levels in the G-CSF-pretreated animals compared to the untreated animals from 8 h after the bacterial inoculation until the end of the study. In contrast, only a slight delay in CSF elevation of TNF-α and IL-1β in the G-CSF-pretreated animals compared to the untreated animals was observed. A reduction in levels of TNF-α and IL-1β in CSF after intracisternal treatment with monoclonal antibodies to TNF-α and IL-1β has previously been shown to reduce neutrophil pleocytosis in experimental pneumococcal and *Haemophilus influenzae*-induced meningitis (33, 36). IL-8 has been demonstrated to be an important chemokine for neutrophil influx in vivo (1) and in vitro (37) but has not previously been addressed in models of bacterial meningitis. In further support of a role of IL-8 as the responsible chemokine in attracting neutrophils into the CSF, we have recently shown that treatment with a monoclonal antibody to IL-8 attenuates neutrophil pleocytosis in pneumococcal meningitis (unpublished data). Thus, decreased production of IL-8, rather than TNF-α and IL-1β, appears to be the most likely explanation for the attenuated pleocytosis, if chemokines really do play a key role as chemoattractants in bacterial meningitis.

The cellular source of IL-8 detected in the CSF has not at present been identified. Various local cells within the CNS are able to produce IL-8, including microglia (7), astrocytes (2), and endothelial cells (17). For the production of other cytokines (e.g., IL-1β) during experimental meningitis, it has been suggested that an influx of monocytes from the blood compartment during the infection plays a key role in this respect (45). Monocytes and macrophages can also produce large amounts of IL-8 (3). We are fairly certain that the IL-8 measured in the CSF is not produced by the incoming neutrophils, since a blockage of the entry of neutrophils by the selectin blocker fucoidin did not inhibit IL-8 release in the CSF during the course of bacterial meningitis (unpublished data). Furthermore, the attenuated IL-8 production is likely not due to a general inhibition of cytokine production from monocyte-derived cells by G-CSF, since we found that G-CSF pretreatment starting 48 h before stimulation of rabbit monocytes with pneumococci or LPS ex vivo does not influence TNF-α or IL-1β production. This is in accordance with two human studies, where G-CSF pretreatment for longer than 24 h did not influence TNF-α or IL-1β production after stimulation of whole blood with LPS (6, 15). Further investigations are necessary to determine the relative importance of local cells versus incoming monocytes as producers of CSF IL-8 during bacterial meningitis.

(iii) In the present study, neutrophils obtained from rabbits which were pretreated with G-CSF had reduced chemotaxis towards three different chemotactic agents compared with neutrophils from untreated rabbits. This observation is in accordance with two studies of human neutrophils (18, 39). Thus, the diminished neutrophil pleocytosis in bacterial meningitis associated with G-CSF pretreatment may well be caused by an impaired mobility of the neutrophils towards a chemotactic gradient. The exact mechanism behind this remains to be defined.

(iv) An impaired ability of neutrophils to roll and adhere to the endothelium and/or impaired diapedesis over the blood-brain barrier is a potential cause of the attenuated pleocytosis observed in our study. Adhesion molecules (e.g., selectins, integrins, and immunoglobulins) are important for neutrophil adherence to the vascular endothelium and subsequent transmigration. Various up- and down-regulation of adhesion mol-

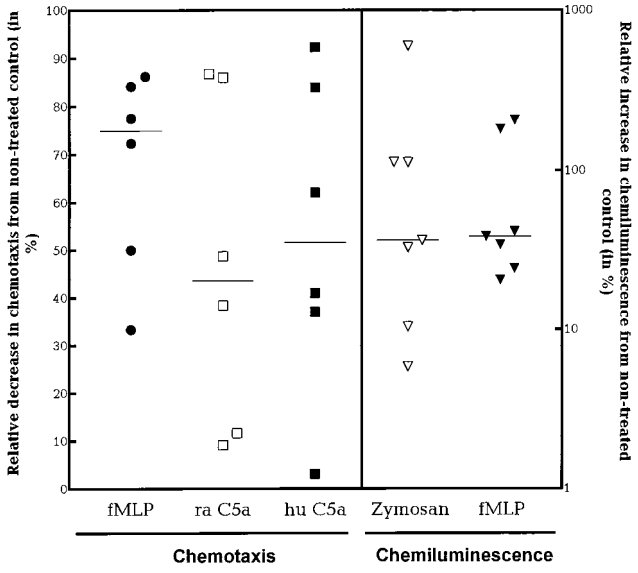

FIG. 3. Effect of G-CSF treatment on rabbit neutrophil function ex vivo. G-CSF (10 μg/kg) was administered twice a day, starting 48 h before isolation of neutrophils. Data are expressed as the relative decrease (chemotaxis) or increase (chemiluminescence) for G-CSF-treated rabbits compared to corresponding untreated rabbits in at least six independent experiments. Chemotaxis (number of migrated neutrophils) to fMLP and to rabbit (ra) and human (hu) C5a was significantly decreased in the G-CSF-treated animals compared to the untreated animals. Chemiluminescence (peak value in millivolts) with opsonized zymosan and fMLP was significantly increased in the G-CSF-treated animals compared to the untreated animals. ($P < 0.05$ [Wilcoxon signed rank test] for all experiments.)

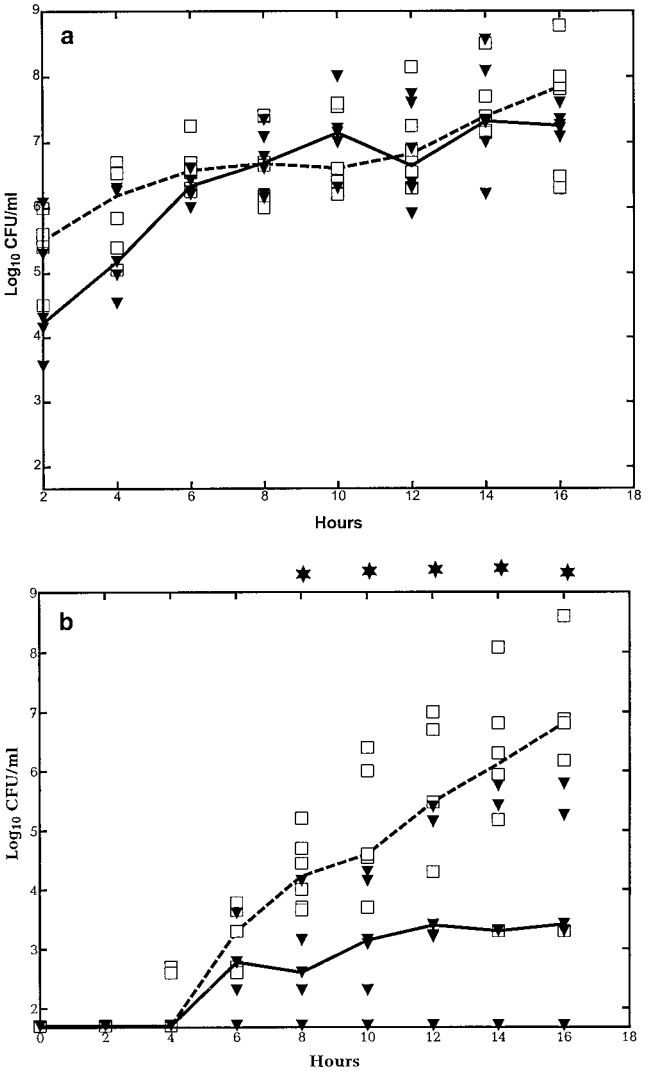

FIG. 4. Median bacterial concentration in CSF (a) and in blood (b) in G-CSF-treated and untreated rabbits during experimental pneumococcal meningitis. Symbols are as described for Fig. 1. G-CSF (10 μg/kg) was administered twice a day starting 48 h before intracisternal bacterial challenge (0 h). The bacterial concentration in blood was significantly reduced in the G-CSF-treated animals compared to the untreated animals between 8 and 16 h. ($n = 6$ for both groups.)

ecules occurs during G-CSF treatment (40). A down-regulation of the selectin leukocyte adhesion molecule-1 on neutrophils (30) and/or an increase in levels of soluble L- and E-selectins in serum occurs during G-CSF treatment (29). Intravenous administration of fucoidin, which binds to and blocks selectins (11) as well as peptides that mimic selectins (34), results in a decreased neutrophil pleocytosis in experimental meningitis. Thus, an altered expression or competitive binding of selectins may explain the decreased pleocytosis seen in our model after G-CSF administration.

Thus, a complex range of causes may explain the attenuated pleocytosis observed after G-CSF pretreatment in this experimental meningitis model. We have shown that the local production of proinflammatory cytokines and the ability of the neutrophils to move against a chemotactic gradient are impaired in animals which were pretreated with G-CSF. However, as discussed above, several other potential mechanisms may also be important and should be further studied.

Beside its influence on neutrophil pleocytosis and CSF cytokine levels, G-CSF pretreatment caused an attenuated enhancement of CSF protein levels and an attenuated decrease in CSF glucose levels. This supports the opinion that a reduction in meningeal inflammation may prevent disruption of the blood-brain barrier during bacterial meningitis (32). The influence of G-CSF pretreatment on brain damage or outcome in experimental pneumococcal meningitis was not studied in the present study. Further studies with experimental models suitable for this purpose should be performed.

Another important finding in this study was that G-CSF pretreatment limited the secondary bacteremia in experimental meningitis. We found in the present study that G-CSF-treated rabbits, all with high numbers of neutrophils in the blood, had significantly reduced blood bacterial concentrations compared to untreated rabbits. Human studies have previously found that G-CSF treatment increases neutrophil phagocytic and fungicidal activity and oxidative burst ex vivo, whereas little is known about the effect on bacterial killing (40). Here we found a significantly increased chemiluminescence activity of rabbit neutrophils after G-CSF pretreatment. Based on this, we assume that the observed increased bacterial killing in the blood after G-CSF pretreatment may be due to an increase in the total number of peripheral neutrophils and an increased ability of individual cells to respond.

Others have claimed that prevention of blood-brain barrier disruption by treatment with monoclonal antibodies to integrins alone or in combination with dexamethasone accounts for a delay in the secondary bacteremia in pneumococcal and *H. influenzae*-induced meningitis (35, 43). However, an increased number of peripheral neutrophils could be responsible for this decrease in secondary bacteremia, since rabbits treated with monoclonal antibodies to integrins or dexamethasone had

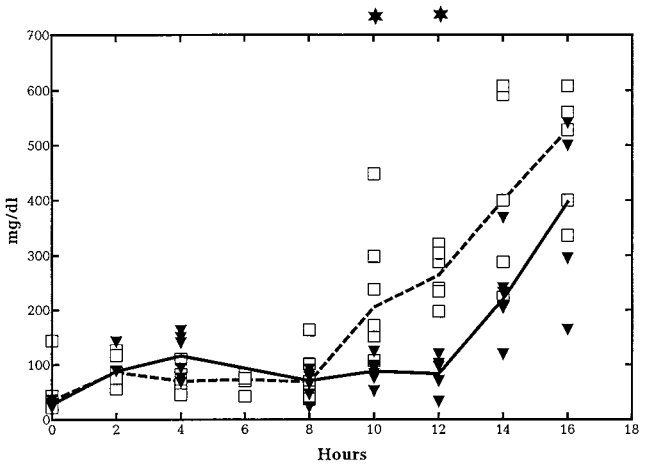

FIG. 5. Median levels of protein in CSF in G-CSF-treated and untreated rabbits during experimental pneumococcal meningitis. Symbols are as described for Fig. 1. G-CSF (10 μg/kg) was administered twice a day starting 48 h before intracisternal bacterial challenge (0 h). The levels of protein in CSF were significantly reduced in the G-CSF-treated animals compared to the untreated animals at 10 and 12 h and at 16 h, respectively. (n = 6 for both groups.)

significantly increased WBC counts in the blood (35, 43). Moreover, rabbits with neutropenia induced by nitrogen mustard had increased blood bacterial concentrations compared to untreated rabbits in pneumococcal meningitis, despite the fact that no difference in meningeal inflammation was seen between groups (8).

In conclusion, G-CSF pretreatment reduces meningeal inflammation and blood bacterial concentration in experimental pneumococcal meningitis. Whether this attenuated inflammatory response affects the clinical course of the meningitis should be further investigated in animal models before G-CSF treatment is used in patients with bacterial meningitis.

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