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Tyrosine Kinase Inhibition Reduces Inflammation in the Acute Stage of Experimental Pneumococcal Meningitis

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Bacterial meningitis is an acute inflammatory disease of the central nervous system with a mortality rate of up to 30%. Excessive stimulation of the host immune system by bacterial surface components contributes to this devastating outcome. In vitro studies have shown that protein tyrosine kinase inhibitors are highly effective in preventing the release of proinflammatory cytokines induced by pneumococcal cell walls in microglia. In a well-established rat model, intracisternal injection of purified pneumococcal cell walls induced meningitis characterized by increases in the regional cerebral blood flow and intracranial pressure, an influx of leukocytes, and high concentrations of tumor necrosis factor alpha (TNF-α) in the cerebrospinal fluid. Compared with the values at the beginning of the experiment, intraperitoneal injection of tyrphostin AG 126 reduced the increases in regional cerebral blood flow (at 6 h, 127% ± 14% versus 222% ± 51% of the baseline value; $P < 0.05$) and intracranial pressure (at 6 h, 0.8 ± 2.4 versus 5.4 ± 2.0 mm of Hg; $P < 0.05$), the influx of leukocytes (at 6 h, 1,336 ± 737 versus 4,350 ± 2,182 leukocytes/μl; $P < 0.05$), and the TNF-α concentration (at 6 h, 261 ± 188 versus 873 ± 135 pg/μl; $P < 0.05$). These results demonstrate that inhibition of AG 126-sensitive tyrosine kinase pathways may provide new approaches for preventing excessive inflammation and reducing the increases in blood flow and intracranial pressure in the acute phase of bacterial meningitis.

Streptococcus pneumoniae is the major pathogen that causes bacterial meningitis in children and adults (24). Surprisingly devastating, even in the context of effective antibiotics, is the poor outcome of the disease, which has a mortality rate of up to 34% and results in permanent sequelae in up to 50% of the survivors (9).

Two major mechanisms that cause damage in the central nervous system have been proposed: (i) direct toxic effects of pneumococci (5) and (ii) excessive stimulation of the host immune system by the bacterial surface (2, 28). An important stimulus is the multilayered network of peptidoglycan that makes up the pneumococcal cell wall (PCW) (27). Purified PCW induces meningeal inflammation which is comparable to the acute inflammation caused by living bacteria, including the clinical hallmarks of the disease, such as an influx of leukocytes and increases in the intracranial pressure (ICP) and regional cerebral blood flow (rCBF) (2, 28, 31). The immunostimulatory effect of cell wall components is clinically important (34) because antibiotic lysis of bacteria induces the release of these components (11). Moreover, the concentration of the cell wall components in the cerebrospinal fluid (CSF) correlates significantly with the outcome of the disease (23).

It has been shown that heat-killed pneumococci, soluble peptidoglycan, and PCW induce signaling through Toll-like receptor 2 (33, 35). The downstream signaling involves activation of mitogen-activated protein kinases (MAPK) erk 1/2 ($p44/42^{MAPK}$) and p38, which has been demonstrated in astrocytes (25), microglia (14), and primary cerebral microvascular endothelial cells (33). Inhibition of the MAPK pathway in vitro reduces the production of tumor necrosis factor alpha (TNF-α) and nitric oxide induced by PCW (25). Specific inhibition of a protein tyrosine kinase that also controls MAPK erk 1/2 by tyrphostin AG 126 attenuates the release of proinflammatory cytokines in mouse microglial cells and decreases the number of invading leukocytes in the CSF (14). AG 126 is a selective protein tyrosine kinase inhibitor that interferes with substrate binding rather than with ATP binding by the corresponding kinase.

Tyrosine kinase inhibitors block experimental autoimmune encephalomyelitis by reducing lymphocyte entry into the central nervous system (7, 8). In a sepsis model AG 126 protects mice against endotoxin toxicity, probably by blocking TNF-α and nitric oxide production (18).

In a well-established rat model of meningitis (19, 31), we induced meningeal inflammation with PCW, a stimulus that obviates any interference by bacterial metabolism and mimics the inflammatory burst caused by bacterial lysis. We tested the effect of AG 126, a protein tyrosine kinase inhibitor, on the detrimental hallmarks of early meningitis, including leukocyte influx, TNF-α production, an increase in the blood flow, and an increase in the ICP.

MATERIALS AND METHODS

Animal model. We used a well-characterized model of the acute phase of bacterial meningitis (19, 31). In brief, male Wistar rats (280 to 330 g) were anesthetized with thiopental (Trapanal; Byk Gulden, Konstanz, Germany) by injecting 100 mg/kg intraperitoneally, followed by 15 mg/kg if required, for the whole experiment, and they were mechanically ventilated (AP-10; K. Efferberger, Pfaffing, Germany). The terminal expiratory partial CO_2 pressure

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(pCO_2), the arterial blood pressure (determined with a femoral catheter), the ICP (determined with a catheter in the cisterna magna and Statham P10 EZ pressure transducers [Spectramed, Oxnard, Calif.]), and the rCBF (determined by laser-Doppler flowmetry parietally through thinned bone with a PeriFlux 4001 Master [Perimed, Järfälla, Sweden]) were measured continuously. After a stable rCBF baseline was recorded (30 min), 75 μl of CSF was withdrawn and replaced by an equal volume of sample.

The ICP data were expressed as the difference between the value obtained and the value at the beginning of the experiment (in millimeters of Hg), and rCBF data were expressed as a percentage of the baseline value.

At the beginning of each experiment and then at 2-h intervals, blood samples were taken to determine arterial pO_2, pCO_2, and pH (COMPACT 1; AVL List, Graz, Austria). Leukocytes in the blood and CSF were counted before and after the experiment.

PCW preparation. PCW were purified as described previously (28, 33), with minor modifications. Unencapsulated *S. pneumoniae* R6 was cultured in chemically defined medium, heat killed, and mechanically disintegrated by using 0.1-mm beads. The suspension was digested with DNase (Promega, Mannheim, Germany) and RNase (U.S. Biochemical Corp., Cleveland, Ohio), treated with trypsin (with 10 mM $CaCl_2$; Sigma-Aldrich Chemie Gmbh), and after centrifugation (23,000 × *g*, 20 min) resuspended in 2% sodium dodecyl sulfate (Serva, Heidelberg, Germany); this was followed by extensive washing. The purified cell walls were resuspended in phosphate-buffered saline at an optical density at 620 nm of 1 (equivalent to 10^8 CFU/ml), and stored at −20°C. The composition of PCW has been described previously (15).

Experimental groups. Control animals were inoculated intracisternally with pyrogen-free saline ($n = 7$), and animals with meningitis were inoculated with PCW ($n = 7$). Tyrphostin AG 126 (Calbiochem-Novabiochem Corp., San Diego, Calif.), dissolved in 200 μl of dimethyl sulfoxide, was inoculated intraperitoneally 2 h before induction of meningitis (7.5 mg/kg [$n = 7$] or 15 mg/kg [$n = 9$]) or 1 h after induction of meningitis (15 mg/kg [$n = 6$] or 30 mg/kg [$n = 4$]). In untreated controls 200 μl of dimethyl sulfoxide was injected intraperitoneally ($n = 7$).

Statistical analysis. All data were expressed as means ± standard deviations. The means of independent groups were compared by one-way analysis of variance and the Student-Newman-Keuls multiple-comparison test and by the Student *t* test for two groups. A *P* value of <0.05 was considered significant.

RESULTS

Tyrphostin AG 126 prevented the PCW-induced increase in rCBF. Intracisternal injection of PCW resulted in a slow increase in rCBF over 6 h (Fig. 1A), as previously described (19, 31). Pretreatment with AG 126 reduced the increase in the rCBF in a dose-dependent manner compared to the increase in animals with untreated meningitis at 3 h (with 7.5 mg of Ag 126 per kg, 133% ± 18% versus 161% ± 22% [$P < 0.05$]; with 15 mg/kg, 120% ± 15% [$P < 0.05$]) and at 6 h (with 7.5 mg/kg, 153% ± 36% versus 222% ± 51% [$P < 0.05$]; with 15 mg/kg, 127% ± 14% [$P < 0.05$]; linear correlation, $r^2 = 0.5260$ [$P < 0.001$]) (Fig. 1A). Administration of AG 126 1 h after challenge with PCW was also effective when 15 mg/kg was used (at 3 h, 130% ± 12% [$P < 0.05$]; at 6 h, 150% ± 18% [$P < 0.05$]) and when 30 mg/kg was used (Fig. 1A). AG 126 treatment alone did not alter rCBF in control animals (Fig. 1B).

Tyrphostin AG 126 prevented the PCW-induced increase in ICP. Inflammatory hyperemia contributes to ICP. Therefore, we asked whether a reduction in the cerebral blood flow resulted in attenuation of ICP in AG 126-treated animals. Pretreatment with 7.5 mg of AG 126 per kg, as well as with 15 mg of AG 126 per kg, reduced the ICP significantly compared to the ICP in untreated rats with meningitis at 3 h (with 7.5 mg of AG 126 per kg, 0.7 ± 1.6 versus 2.0 ± 1.0 mm of Hg [difference not significant]; with 15 mg of AG 126 per kg, 0.6 ± 1.0 mm of Hg [$P < 0.05$]) (Fig. 2A) and at 6 h (with 7.5 mg of AG 126 per kg, 1.9 ± 2.1 versus 5.4 ± 2.0 mm of Hg [$P < 0.05$]; with 15 mg/kg, 0.8 ± 2.4 mm of Hg [$P < 0.05$]; $r^2 = 0.4394$ [$P < 0.01$])

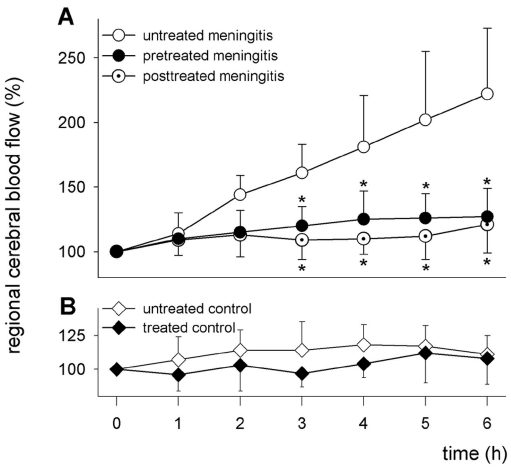

FIG. 1. Effect of tyrphostin AG 126 on rCBF in experimental meningitis. (A) Meningitis induced a slow increase in rCBF, which was significantly reduced by treatment with 15 mg of AG 126 per kg 2 h before induction of meningitis (●) or by treatment with 30 mg of AG 126 per kg 1 h after induction of meningitis (⊙). An asterisk indicates that the *P* value is <0.05 (as determined by analysis of variance and the Student-Newman-Keuls post hoc test). (B) rCBF was stable over time and did not change in control animals. The values are means ± standard deviations.

(Fig. 2B). Posttreatment with 30 mg/kg prevented the increase in ICP (at 3 h, 0.6 ± 1.0 mm of Hg [$P < 0.05$]; at 6 h, 1.1 ± 1.9 mm of Hg [$P < 0.05$]), whereas posttreatment with 15 mg/kg did not prevent the increase (at 6 h, 6.9 ± 4.6 mm of Hg [difference not significant]). In control animals and AG 126-treated controls there was no change in ICP over time (data not shown).

Tyrphostin AG 126 reduced the PCW-induced invasion of the CSF by leukocytes. Invasion by leukocytes is a hallmark of bacterial meningitis. AG 126 reduced the invasion of the subarachnoidal space by leukocytes in a dose-dependent manner (with 7.5 mg of AG 126 per kg, 1,984 ± 1,066 versus 4,350 ± 2,182 leukocytes/μl [$P < 0.05$]; with 15 mg of AG 126 per kg, 1,336 ± 737 leukocytes/μl [$P < 0.05$]; linear correlation, $r^2 = 0.4182$ [$P < 0.05$]) (Fig. 2C). The 30-mg/kg AG 126 treatment inhibited leukocyte invasion of the CSF even 1 h after induction of meningitis (1,011 ± 936 leukocytes/μl [$P < 0.05$]), whereas the 15-mg/kg posttreatment was not effective (4,439 ± 1,024 leukocytes/μl [difference not significant]).

Tyrphostin AG 126 reduced the TNF-α concentrations in the CSF. TNF-α is thought to be one of the key proinflammatory cytokines in meningitis (1, 17). Pretreatment of animals with meningitis with AG 126 reduced the concentration of TNF-α in the CSF significantly compared to the concentration in untreated animals with meningitis (with 15 mg of AG 126 per kg, 261 ± 188 versus 873 ± 135 pg/ml [$P < 0.05$]) (Fig. 2D). Posttreatment with 15 mg/kg was not effective, but posttreatment with 30 mg/kg decreased the concentration of TNF-α significantly (188 ± 97 pg/ml [$P < 0.05$]). The TNF-α levels were not different in controls and AG 126-treated controls (18 ± 3 and 28 ± 10 pg/ml).

AG 126 treatment had no effect on physiological parameters. Body temperature, mean arterial blood pressure, and arterial pH, pO_2, and pCO_2 were measured every hour, and

TABLE 1. Physiological parameters for the experimental groups[a]

Animals	Mean arterial blood pressure (mm of Hg)				pH				pCO_2 (mm of Hg)				pO_2 (mm of Hg)				White blood cell count (cells/μl)	
	Zero time	2 h	4 h	6 h	Zero time	2 h	4 h	6 h	Zero time	2 h	4 h	6 h	Zero time	2 h	4 h	6 h	Zero time	6 h
Control rats																		
Untreated	101 ± 10	102 ± 11	100 ± 9	97 ± 10	7.41 ± 0.03	7.40 ± 0.03	7.40 ± 0.02	7.40 ± 0.01	36 ± 5	33 ± 6	34 ± 8	33 ± 7	118 ± 16	115 ± 12	118 ± 15	114 ± 21	7,000 ± 1,600	8,300 ± 2,600
Pretreated with AG 126 (15 mg/kg)	105 ± 8	103 ± 14	96 ± 8	96 ± 11	7.42 ± 0.02	7.41 ± 0.01	7.39 ± 0.01	7.39 ± 0.01	40 ± 5	40 ± 4	38 ± 8	38 ± 7	133 ± 21	140 ± 25	126 ± 21	147 ± 30	7,100 ± 2,300	7,600 ± 1,500
Rats with meningitis																		
Untreated	105 ± 9	105 ± 5	102 ± 10	98 ± 7	7.40 ± 0.02	9.41 ± 0.03	7.39 ± 0.02	7.39 ± 0.01	37 ± 4	35 ± 6	36 ± 5	35 ± 5	125 ± 24	127 ± 22	143 ± 20	135 ± 15	5,100 ± 1,100	6,100 ± 1,700
Pretreated with AG 126 at:																		
7.5 mg/kg	103 ± 10	103 ± 8	101 ± 10	96 ± 11	7.41 ± 0.02	7.91 ± 0.02	7.40 ± 0.03	7.39 ± 0.03	37 ± 4	38 ± 7	35 ± 5	36 ± 3	127 ± 25	136 ± 19	138 ± 23	130 ± 16	5,800 ± 1,100	6,800 ± 3,800
15 mg/kg	97 ± 9	99 ± 10	95 ± 11	93 ± 11	7.40 ± 0.02	7.40 ± 0.02	7.38 ± 0.01	7.40 ± 0.03	36 ± 5	33 ± 5	34 ± 4	32 ± 4	119 ± 19	127 ± 21	131 ± 22	133 ± 21	6,800 ± 1,300	8,300 ± 1,800
15 mg/kg	109 ± 8	107 ± 9	106 ± 11	102 ± 12	7.41 ± 0.02	7.41 ± 0.02	7.41 ± 0.02	7.41 ± 0.02	38 ± 8	35 ± 7	37 ± 6	35 ± 4	113 ± 14	144 ± 18	132 ± 20	126 ± 21	5,800 ± 700	7,900 ± 2,600
30 mg/kg	104 ± 10	102 ± 7	99 ± 13	100 ± 11	7.42 ± 0.03	7.41 ± 0.03	7.40 ± 0.03	7.41 ± 0.04	39 ± 5	40 ± 5	39 ± 6	37 ± 9	119 ± 18	106 ± 19	109 ± 13	118 ± 14	6,500 ± 1,800	7,300 ± 1,000

[a] The data are means ± standard deviations. The values for the experimental groups were not significantly different (as determined by analysis of variance) and were within normal ranges.

FIG. 2. Tyrphostin AG 126 reduced ICP in a dose-dependent manner 3 h (A) and 6 h (B) after induction of meningitis. The CSF white cell count (C) and the TNF-α concentration (D) were also significantly reduced at 6 h compared to the values obtained for untreated animals with meningitis. The values are means ± standard deviations. An asterisk indicates that the P value is <0.05 (as determined by analysis of variance).

the values were comparable for all experimental groups (Table 1). Blood leukocytes were counted at the beginning and the end of the experiment, and there were no differences among the groups.

DISCUSSION

Adjunctive therapy in pneumococcal meningitis targets the overwhelming immune response due to the release of highly inflammatory components of lysed bacteria. Recently, pretreatment with dexamethasone has been proven to decrease the mortality and morbidity from pneumococcal meningitis (9). Further improvement must be based on a detailed understanding of the triggers of this immune response, as well as specifically targeted intervention in the inflammatory cascade. Most potential pharmacologic agents that have been identified recently do not cross the blood-brain barrier and therefore require intracisternal administration. The ability of AG 126 to cross this barrier makes it an attractive potential therapeutic agent.

Here we show that specific inhibition of a protein tyrosine kinase, most likely upstream of $p42/44^{MAPK}$, reduced the increases in the rCBF and ICP, as well as the influx of leukocytes

and the TNF-α concentration in the CSF in the acute phase of experimental meningitis.

AG 126 prevented an increase in rCBF, a measure of local vasodilatation, in a dose-dependent manner. Furthermore, treatment of the animals 1 h after induction of meningitis still inhibited the increase in rCBF. This is consistent with significantly improved hemodynamic parameters seen in tyrphostin-treated animals with multiorgan failure caused by *Escherichia coli* (26).

Clinically, increased ICP in the acute phase of bacterial meningitis is enormously important and may be attributed to at least three mechanisms (21): (i) vasogenic edema due to extravasation of plasma compounds and hyperemia; (ii) cytotoxic edema caused by toxins released from activated leukocytes (13), glia (20), endothelial cells (12), and bacteria; and (iii) an increase in CSF outflow resistance (22). Decreased ICP in tyrphostin-treated animals may result from attenuated hyperemia, as well as reduced leukocyte recruitment and TNF-α concentrations in the CSF.

Leukocyte influx into the CSF, the hallmark of bacterial meningitis, was significantly reduced in AG 126-treated animals compared to that in untreated animals. Reducing the leukocyte influx is believed to have potential therapeutic benefits because high numbers of leukocytes correlate with an unfavorable outcome (3), and blocking leukocyte invasion attenuates brain edema, an increase in the ICP, hyperemia, and neuronal damage and improves survival in experimental meningitis (4, 29, 32). In a model of zymosan-induced peritonitis AG 126 also inhibited the influx of polymorphonuclear leukocytes in a dose-dependent manner (10). Additionally, protein tyrosine phosphorylation mediates TNF-α-induced endothelial neutrophil adhesion in vitro (16). Autoimmune encephalomyelitis is suppressed by tyrosine kinase inhibitors (6, 7), most likely by inhibition of invasion of the brain parenchyma by leukocytes (8).

TNF-α is thought to be one of the key cytokines in bacterial meningitis, as the level of TNF-α is elevated in the CSF of meningitis patients (30) and in experimental models (17), and it dramatically accelerates experimental pneumococcal meningitis (1). Production of TNF-α requires $p42/44^{MAPK}$ activation, as shown in macrophages (18), astrocytes (25), and microglia (14). AG 126-treated mice survive a lipopolysaccharide-induced lethal shock, and the protection is correlated with decreased TNF-α production (18). Accordingly, AG 126 treatment reduced TNF-α levels in the CSF of animals with meningitis in the present study.

In conclusion, our study showed that an AG 126-sensitive protein tyrosine kinase is important in the early phase of pneumococcal meningitis. AG 126 effectively inhibited blood flow and an increase in the ICP and reduced the leukocyte influx and TNF-α concentration in the CSF. These results suggest that inhibition of selective tyrosine kinase phosphorylation may provide new approaches for decreasing the excessive inflammatory response caused by antibiotic lysis of bacteria and the release of highly inflammatory PCW.

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