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Neurotoxicity of Glia Activated by Gram-Positive Bacterial Products Depends on Nitric Oxide Production

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The present study examined the mechanism by which bacterial cell walls from two gram-positive meningeal pathogens, *Streptococcus pneumoniae* and the group B streptococcus, induced neuronal injury in primary cultures of rat brain cells. Cell walls from both organisms produced cellular injury to similar degrees in pure astrocyte cultures but not in pure neuronal cultures. Cell walls also induced nitric oxide production in cultures of astrocytes or microglia. When neurons were cultured together with astrocytes or microglia, the cell walls of both organisms became toxic to neurons. L-NAME, a nitric oxide synthase inhibitor, protected neurons from cell wall-induced toxicity in mixed cultures with glia, as did dexamethasone. In contrast, an excitatory amino acid antagonist (MK801) had no effect. Low concentrations of cell walls from either gram-positive pathogen added together with the excitatory amino acid glutamate resulted in synergistic neurotoxicity that was inhibited by L-NAME. The induction of nitric oxide production and neurotoxicity by cell walls was independent of the presence of serum, whereas endotoxin exhibited these effects only in the presence of serum. We conclude that gram-positive cell walls can cause toxicity in neurons by inducing the production of nitric oxide in astrocytes and microglia.

Gram-positive organisms cause many of the approximately 15,000 cases of bacterial meningitis in the United States each year. *Streptococcus pneumoniae* is the most common cause of bacterial meningitis in adults, while the group B streptococcus is a frequent pathogen in neonates (3). Brain injury frequently complicates bacterial meningitis, despite rapid identification of the disease and expedient administration of antibiotics. Depending on the population affected, up to 50% of survivors of meningitis have permanent neurologic damage ranging from deafness to motor deficits and global encephalopathy (11, 43).

Bacteria release cell wall components into the cerebrospinal fluid during meningitis during normal growth and spontaneous or antibiotic-induced autolysis. Major components of the cell walls of gram-positive bacteria are peptidoglycans and teichoic acids, while the biologically most active components of gram-negative organisms are their lipopolysaccharides (LPSs). Bacterial components induce a brisk inflammatory response when injected into the cerebrospinal space in animal models of meningitis (38). This inflammation induces the pathophysiologic changes that ultimately lead to brain injury. Whether direct cytotoxic effects of bacterial products on brain cells also contribute to brain injury during meningitis is unknown.

Most studies characterizing the response of brain cells to bacterial products have centered on the effect of LPS of gram-negative organisms (1, 33, 42). Relatively little is known about brain cell responses to gram-positive bacterial products. We have previously reported that glia, both astrocytes and microglia, were sensitive to cytotoxic effects of *S. pneumoniae* cell walls (19). Neurons, in contrast, were relatively resistant to the toxic effects.

The purpose of the present studies was to expand the findings obtained with *S. pneumoniae* to a second gram-positive meningeal pathogen, the group B streptococcus, and to determine if indirect mechanisms of neuronal injury result from the stimulation of glia by products of these gram-positive meningeal pathogens. Exploration of the effects of bacterial products on glial cells and the subsequent effects on neurons in vitro may allow the identification of mechanisms by which bacteria can cause neuronal damage during meningitis.

(This work was presented in part at the Infectious Diseases Society of America Meeting, San Francisco, Calif., September 1995 [20a].)

MATERIALS AND METHODS

All reagents were purchased from Sigma (St. Louis, Mo.), unless otherwise noted.

Tissue cultures. (i) Primary neuronal cultures. Neuronal cultures were prepared by methods previously described (19). Briefly, brains were removed from 16-day-old Sprague-Dawley rat embryos (Simenson, Gilroy, Calif.) and placed in modified Eagle's minimal essential medium–Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution (MEM-EBSS) (University of California, San Francisco, Cell Culture Facility). Dissociated cell suspensions were centrifuged and resuspended in MEM containing 2% Ultroser G (Gibco, Grand Island, N.Y.), 30 mM glucose, 2 mM glutamine, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 2× amino acids, and 4× vitamins. The purity of the neuronal cultures was ≥95%, as assessed by neuron-specific enolase staining.

(ii) Primary astrocyte and microglial cultures. Glial cell cultures were prepared by the method of McCarthy and deVellis (27). Whole brains were isolated from newborn Sprague-Dawley rat pups. The tissue was triturated and digested with 0.2% trypsin and 0.2% DNase. The suspension was centrifuged and resuspended in MEM with 10% fetal bovine serum and incubated at 5% CO_2 with 95% humidity at 37°C. These mixed glial cultures contained astrocytes, oligodendrocytes, and microglia.

For enriched microglial cultures, 2-week-old mixed cultures were placed in an orbital shaker at 180 rpm for 18 h at 37°C. The suspended cells were plated in culture flasks and further purified by repeated selection of the most firmly adherent cells. Final cultures consisted of >98% ameboid microglia, as stained by the microglia-specific epitope OX-42.

To obtain pure primary astrocyte cultures to serve as a feeder layer, medium was replaced in the flasks after aspiration of the supernatant after the 18-h shaking cycle (described above). Cultures were subjected to a second shaking cycle, and adherent cells were suspended, centrifuged, and plated on Fisher 96-well tissue culture plates (Fisher Scientific, Pittsburgh, Pa.). At confluence,

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cultures were pulsed with 20 μM cytosine arabinoside. Astrocyte cultures were ≥90% pure as assessed by glial fibrillary acidic protein staining.

(iii) Mixed neuron-glial cocultures. For mixed astrocyte-neuron cultures, neurons were plated on a feeder layer of primary rat astrocytes in Fisher 96-well culture plates. On day 4 in vitro, cultures were pulsed for 16 h with 5 μM cytosine arabinoside. Experiments were performed with 14-day-old cultures.

For neuronal cultures mixed with microglia, neurons were prepared as described above. Microglia were introduced into 10- to 14-day-old cultures in a 1:5 ratio of microglia to neurons. Experiments were performed after 24 to 48 h. All cells were incubated at 5% CO_2 with 95% humidity at 37°C.

(iv) Serum-containing media. To determine if neurotoxicity was serum dependent, experiments were performed with media containing 10 and ≤0.2% serum. Medium with 10% fetal bovine serum contains glutamate, which was neurotoxic in cultures. To circumvent this problem, MEM with 10% fetal bovine serum was placed in a flask containing an astrocyte monolayer. Astrocytes avidly take up the free glutamate, leaving a glutamate-depleted medium. After 24 h, the medium was centrifuged to sediment particulate material, passed through a 0.2-μm-pore-diameter filter, and stored at 4°C. Medium was used within 24 h for the experiments described below.

(v) Bacterial product conditioned glial supernatants. For supernatant toxicity experiments, conditioned supernatants were generated by exposure of either astrocytes or microglia to *S. pneumoniae* cell walls, group B streptococcal cell walls, or LPS for 48 h. Supernatants were stored at −70°C until use. Supernatants were then added to 14-day-old neuronal cell cultures (33% [vol/vol]). Toxicity was assessed as described below after 48 h.

Bacterial products. (i) *S. pneumoniae* and group B streptococcal cell walls. To generate gram-positive cell walls, rough strains of *S. pneumoniae* (R6; a kind gift from Alex Tomasz, Rockefeller University, New York, N.Y.) and group B streptococcus type III (a kind gift of Craig Rubens, University of Washington, Seattle) were grown in Todd-Hewitt broth to stationary phase and harvested in saline.

Cell walls were generated for both bacteria as previously described (19). Bacteria were disrupted by glass beads; boiled in 2% sodium dodecyl sulfate; incubated with DNase, RNase, and trypsin; and then extensively washed in distilled water, suspended in saline, and stored at −70°C. Ten to 30 μg of the cell wall preparation corresponds to approximately 10^8 CFU of *S. pneumoniae* and the group B streptococcus. In most experiments, 200 μg of cell walls per ml was used to challenge brain cell cultures. This dose is comparable to the maximally effective dose found in other in vitro studies of gram-positive cell walls (10, 15, 19).

(ii) LPS. Rough LPS from *Salmonella minnesota* Re 575 was diluted in phosphate-buffered saline and added to the culture medium. Brain cell cultures were exposed to 5 μg of endotoxin per ml for all experiments.

Trypan blue cytotoxicity assay. After 48 h of exposure to bacterial products, trypan blue (0.4%) was added to cell cultures. The live (trypan blue excluding) cells were enumerated in high-power fields at the center of each well, and the percentage of toxicity was calculated as follows: % viable cells = no. of live cells in sample/no. of live cells in control.

MTT assay. After 48 h of exposure to bacterial products, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay for cell viability, which is based on the reduction of MTT to tetrazolium blue crystals by viable cells, was performed as previously described (28). Acid isopropanol (0.04 N HCl) was added to the wells to solubilize the tetrazolium blue crystals. A_{540} was measured with a Multiscan MCC/340 enzyme-linked immunosorbent assay plate reader. Cytotoxicity was calculated as follows: % cytotoxicity = 1 − [(Abs sample − Abs lysed)/(Abs control − Abs lysed)], where Abs sample is the absorbance of the sample well, Abs lysed is the absorbance of wells with all cells lysed with paraquat (10 mM), and Abs control is the absorbance of wells with no treatment.

Nitrite assay. Quantification of nitrite was performed with the Griess reagent as described previously (16). Briefly, 40 μM NADPH and 14 mU of nitrate reductase in 50 μl of 20 mM TRIS (pH 7.6) were added to samples to reduce nitrate to nitrite. The reaction was terminated by the addition of distilled water. Griess reagent (equal volumes of 1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-1-naphthyl-ethylenediamine-HCl) was added. After 15 min, plates were read on a Spectramax 250 spectrophotometric plate reader at 546 nm. Concentrations were calculated by comparison of absorptions with a standard curve.

Presentation of data and statistics. All data are expressed as means ± standard deviations from experiments performed in quadruplicate wells. All experiments were repeated at least twice. All paired comparisons were assessed by Student's *t* test. Multiple group comparisons were assessed by Bonferroni *t* test.

RESULTS

Toxicity of *S. pneumoniae* and group B streptococcus. In previous studies, we found that *S. pneumoniae* cell walls induced dose-dependent toxicity in astrocyte and microglial cultures (19). To determine whether this is a unique feature of *S. pneumoniae* or can also be shown for the group B streptococcus, the second major gram-positive meningeal pathogen, we compared the toxicity of *S. pneumoniae* cell walls with that of group B streptococcal cell walls in primary astrocyte cell cultures. Toxicity was assessed by MTT assay. The toxicities of cell walls from both gram-positive organisms in astrocytes were comparable through the range of concentrations tested, although group B streptococcal cell walls were consistently slightly less potent than *S. pneumoniae* cell walls (Fig. 1).

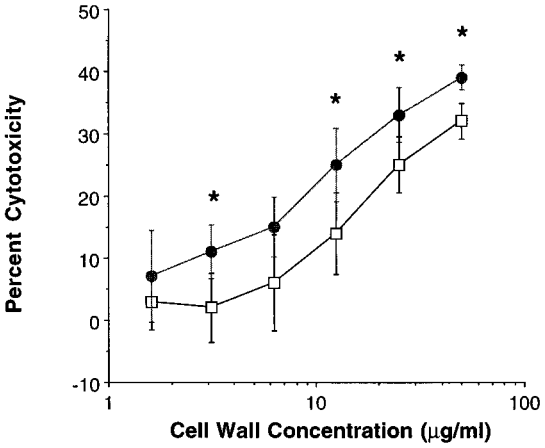

FIG. 1. Toxicity of gram-positive bacterial products in primary astrocytes. Primary rat astrocytes were exposed to either *S. pneumoniae* cell walls (●) or group B streptococcal cell walls (□) for 48 h. Toxicity was determined by the MTT assay. Data are shown as means ± standard deviations ($n = 4$ for all experiments). All paired comparisons between *S. pneumoniae* and group B streptococcal cell walls were by Student's *t* test. *, $P < 0.05$ by Student's *t* test analysis.

Mixed neuron-astrocyte cocultures. To examine whether bacterial products that show little toxicity to neurons in pure culture could induce neurotoxicity indirectly in the presence of astrocytes, mixed cultures of neurons on an astrocyte feeder layer were challenged with *S. pneumoniae* cell walls or group B streptococcal cell walls. Exposure to either *S. pneumoniae* or group B streptococcal cell walls resulted in extensive neurotoxicity after 48 h, with less than 50% of the neurons remaining viable at the end of this period.

Both nitric oxide (NO) and excitatory amino acids have been suggested to mediate glial-induced neurotoxicity in vitro (5, 7, 31, 32, 36). To test whether either of the neurotoxins contributed to the neurotoxicity in our system, we used an NO synthase inhibitor, L-NAME, and a glutamate receptor antagonist, MK801 in our system. The neurotoxicity of *S. pneumoniae* and group B streptococcal cell walls was to a great extent, but not completely, inhibited by 10 μM L-NAME (73% ± 6% and 79% ± 9% viable neurons; $P < 0.001$ for both groups compared with cultures with *S. pneumoniae* alone). The addition of 10 μg of MK801 per ml did not protect neurons from injury after exposure to *S. pneumoniae* or group B streptococcal cell walls (30% ± 12% viable neurons; *P* not significant compared with cultures with *S. pneumoniae* alone). Neither L-NAME nor MK801 had toxic effects on unstimulated neuron-astrocyte cultures (Fig. 2).

Mixed neuron-microglia cocultures. To test whether stimulation of microglia with gram-positive bacterial cell walls exhibited neurotoxic effects in neuronal coculture similar to those shown for astrocytes, mixed cultures of neurons and microglia in a ratio of 5:1 were challenged with bacterial products. Exposure of the neuronal cultures to unstimulated microglia or either *S. pneumoniae* or group B streptococcal cell walls without microglia was associated with only mild toxicity. In contrast, neuronal cultures exposed to cell walls from either gram-positive organism in the presence of microglia showed

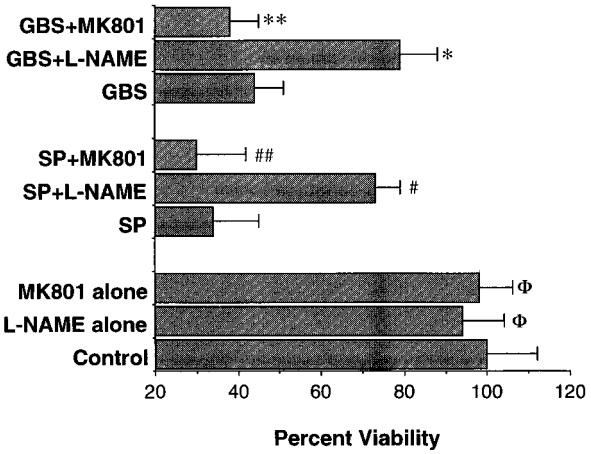

FIG. 2. Effect of L-NAME and MK801 on toxicity of gram-positive bacterial products in astrocyte-neuron cocultures. Astrocyte-neuron cocultures were challenged with either *S. pneumoniae* cell walls (SP [100 μg/ml]) or group B streptococcal cell walls (GBS [100 μg/ml]). Experiments were then repeated with either L-NAME (10 μM) or MK801 (10 μg/ml). At 48 h, viable neurons were counted after trypan blue exclusion. Controls included L-NAME alone and MK801 alone. Data are shown as means ± standard deviations ($n = 4$ for all experiments). Multiple comparisons analysis was by Bonferroni *t* test. **, P not significant compared with GBS alone; *, $P < 0.001$ compared with GBS alone; ##, P not significant compared with SP alone; #, $P < 0.001$ compared with SP alone; Φ, P not significant compared with control.

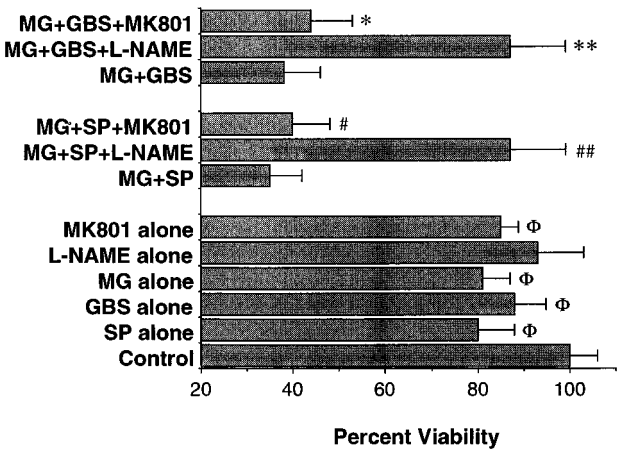

FIG. 3. Effect of L-NAME and MK801 on toxicity of gram-positive bacterial products in microglia-neuron cocultures. Microglia-neuron cocultures were challenged with either *S. pneumoniae* cell walls (SP [100 μg/ml]) or group B streptococcal cell walls (GBS [100 μg/ml]). Experiments were then repeated with either L-NAME (10 μM) or MK801 (10 μg/ml). At 48 h, viable neurons were counted after trypan blue exclusion. Controls included unstimulated microglia (MG) alone, L-NAME alone, and MK801 alone. Data are shown as means ± standard deviations ($n = 4$ for all experiments). Multiple comparisons analysis was by Bonferroni *t* test. *, P not significant compared with MG + GBS; **, $P < 0.001$ compared with MG + GBS; #, P not significant compared with MG + SP; ##, $P < 0.001$ compared with MG + SP; Φ, $P < 0.05$ compared with control.

marked neurotoxicity, with only 35% ± 7% of the neurons remaining viable after *S. pneumoniae* cell wall exposure and 38% ± 8% remaining viable after group B streptococcal cell wall exposure. Experiments performed in the presence of L-NAME showed complete inhibition of the toxic effect of *S. pneumoniae* cell walls and microglia (87% ± 12% viable neurons; $P < 0.001$ compared with cultures without L-NAME) and group B streptococcal cell walls (87% ± 10% viable neurons; $P < 0.001$ compared with cultures without L-NAME). MK801 showed no neuroprotective effect for either *S. pneumoniae* or group B streptococcal cell walls (40% ± 8% and 44% ± 9%; $P < 0.001$ compared with cultures with L-NAME) (Fig. 3).

Serum-dependent cytotoxicity of LPS. In initial experiments, in which we used LPS in parallel with the gram-positive cell wall preparations, no toxicity to neurons in either mixed astrocyte-neuron or microglia-neuron cultures was observed. Since LPS requires serum factors (e.g., LPS binding protein and CD14) to amplify and mediate its effects in many biologic systems (2), we sought to determine if serum is required to mediate the effect of LPS in our cell culture systems. We, therefore, performed experiments in the presence of 10% fetal bovine serum.

S. pneumoniae cell walls resulted in significant neurotoxicity both in media with low concentrations of serum (≤0.2%) and in 10% fetal bovine serum (neuronal viability, 44% ± 6% and 36% ± 8%, respectively). In contrast, LPS-induced neurotoxicity was dependent on the presence of serum in the medium. In low-serum conditions, LPS showed no toxicity (101% ± 5% viable neurons), while at 10% fetal bovine serum, LPS produced significant toxicity (63% ± 12% viable neurons; $P < 0.002$ compared with control cultures). As with gram-positive cell walls, LPS neurotoxicity was inhibited by L-NAME but not by MK801 (data not shown). A high concentration of serum alone was not toxic to neurons.

NO synthesis. Data from experiments with L-NAME (Fig. 2 and 3) suggested that NO plays a role in neurotoxicity in our system. We therefore examined whether microglia and astrocytes produced NO in response to challenge by *S. pneumoniae* and group B streptococcal cell walls. NO production was measured indirectly by generation of nitrite with the Griess reaction. NO production could be detected for all stimuli within 24 h. After 72 h, both microglia and astrocytes produced NO after challenge with either of the gram-positive cell walls in very-low-serum medium (≤0.2%) (Table 1). Addition of serum to gram-positive stimuli did not result in increased production of NO (data not shown). LPS elicited a brisk production of NO in microglia and astrocytes in medium with 10% serum; however, in low-serum medium, nitrite levels were ≤2 μM for both microglia and astrocytes at 72 h. Dexamethasone (1 μM) reduced the production of NO in both astrocytes and microglia independent of the stimulus used. Control wells of microglia and astrocytes with no stimuli produced <5 μM nitrite for all time points tested. The limit of detection of the Griess assay was 0.7 μM nitrite.

Toxicity of supernatants. The evidence for a role of NO, but not excitatory amino acids, in causing neuronal toxicity in our culture system suggests that the neurotoxic compound is not stable after storage. To test this, supernatants generated from pure microglial and astrocyte cultures after a 48-h challenge with bacterial products were assessed for neurotoxicity in pure neuronal cultures. As expected, there was no difference in neuronal viability after exposure to supernatants from cultures stimulated with *S. pneumoniae* cell walls (96% ± 13% of control cultures), group B streptococcal cell walls (89% ± 8% of control cultures), LPS (91% ± 10% of control cultures), and control (100% ± 12%).

Suppression of toxicity by dexamethasone. Dexamethasone suppresses inducible NO synthase, presumably through reduction of cytokine production (35, 36). Thus, it should reduce the production of NO and neurotoxicity in culture. A 2-h preincubation with dexamethasone (1 μM) markedly enhanced neuronal survival in experiments with both *S. pneumoniae* and group B streptococcal cell walls (86% ± 9% and 90% ± 7%, respectively) compared with cultures not preincubated with

TABLE 1. Nitrite concentration in supernatants of microglial and astrocyte cultures 72 h after exposure to bacterial products

Stimulus[a]	Nitrite concn (μM) in supernatant[b]			
	Microglia		Astrocytes	
	− dexamethasone	+ dexamethasone	− dexamethasone	+ dexamethasone
Control	4.24	2.39	1.84	2.21
S. pneumoniae cell walls (200 μg/ml)	10.91	4.19	12.33	2.33
Group B streptococcus cell walls (200 μg/ml)	10.15	4.75	13.33	1.77
LPS (5 μg/ml) in:				
10% serum	41.25	1.95	50.19	1.40
≤0.2% serum	3.22	ND[c]	3.67	ND

[a] All experiments with gram-positive stimuli were in medium with ≤0.2% serum.
[b] Concentration of dexamethasone was 1 μM.
[c] ND, not determined.

dexamethasone (44% ± 4% and 42% ± 12% viable neurons, respectively; $P < 0.001$ for both experiments compared with cultures with dexamethasone).

Synergy of glutamate and *S. pneumoniae* cell walls. Our previous studies have shown that glutamate is increased in the brain interstitial fluid during meningitis (14) and that treatment with glutamate antagonists reduces brain injury in experimental group B streptococcal meningitis (24). Recently published reports suggest that the neurotoxic effect of glutamate is mediated by NO (9, 18). Since bacterial products led to the production of NO in glia, we sought to evaluate whether excitatory amino acids and bacterial cell walls produce additive or synergistic neurotoxicity in neuron-glial cocultures. Minimally toxic concentrations of bacterial products and glutamate were added to mixed neuron-astrocyte cultures. The concentration of glutamate used (15 μM) resulted in mild toxicity (83% ± 8% viable neurons). *S. pneumoniae* cell walls (6 μg/ml) resulted in no significant cytotoxicity (94% ± 7% viable neurons) (Fig. 4). However, the same concentrations of glutamate and *S. pneumoniae* cell walls in combination in the mixed neuron-astrocyte cell cultures caused cytotoxicity to a much greater degree than the sum of their individual toxicities (54% ± 5% viable neurons; $P < 0.001$). This synergistic toxicity was greatly inhibited by the addition of L-NAME (87% ± 12% viable neurons; $P < 0.001$ compared with cultures without L-NAME). Results were similar for group B streptococcal cell walls (12 μg/ml) which had no significant toxicity (93% ± 9% viable neurons). The combination of 15 μM glutamate with group B streptococcal cell walls produced neuronal toxicity greater than the sum of the individual toxicities (47% ± 9% viable neurons; $P < 0.001$). Synergistic toxicity of group B streptococcal cell walls and glutamate was also markedly reduced by L-NAME (94% ± 10% viable neurons; $P < 0.001$).

DISCUSSION

The understanding of the molecular mechanisms of brain injury in bacterial meningitis is rudimentary. Pathophysiologic studies suggest that one important pathway leads to cerebral ischemia and subsequent neuronal injury (37, 41). However, some types of brain injury, such as neuronal loss in the dentate gyrus of the hippocampus and nonfocal cortical injury are difficult to explain on the basis of poor cerebral blood flow and may involve other neurotoxic mechanisms (20).

Both NO and the excitatory amino acid glutamate are thought to be involved in a variety of brain insults, such as ischemia, stroke, and degenerative and inflammatory brain diseases (8, 25). A number of reports support a potential role for NO and glutamate in bacterial meningitis. NO is present in the cerebrospinal fluid and mediates hyperemic blood flow in the early stages of bacterial meningitis (21), subarachnoid space inflammation, increased intracranial pressure (6), and anaerobic brain metabolism (40). Glutamate is present in the cerebrospinal fluid and brain interstitial fluid in bacterial meningitis (14), and treatment with an excitatory amino acid antagonist reduced brain injury in an infant rat model of group B streptococcal meningitis (24). In vitro, microglia stimulated by LPS release glutamate to levels two to three times the normal level (31). In mixed microglia-neuron cultures, glutamate antagonists reduced the neurotoxic effect of activated microglia (32).

We have previously shown that microglia and, to a lesser extent, astrocytes are sensitive to the cytotoxic effect of *S. pneumoniae* cell walls (19). Neurons, in contrast, were largely resistant to the direct cytotoxic effect of these toxins, suggest-

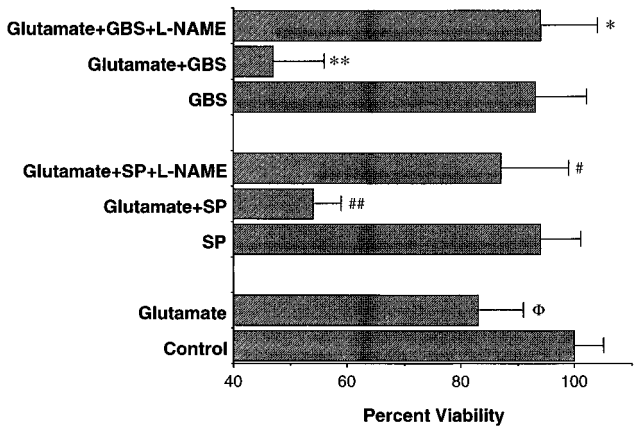

FIG. 4. Synergistic toxicity of gram-positive bacterial products and glutamate in astrocyte-neuron cocultures. Astrocyte-neuron cocultures were exposed to low concentrations of either *S. pneumoniae* cell walls (SP [6 μg/ml]) or group B streptococcal cell walls (GBS [12 μg/ml]) and glutamate (15 μM). Experiments were repeated in the presence of L-NAME (10 μM). At 48 h, viable neurons were counted after trypan blue exclusion. Controls included glutamate alone, SP alone, and GBS alone. Data are shown as means ± standard deviations ($n = 4$ for all experiments). Multiple comparisons analysis was by Bonferroni t test. *, P not significant compared with GBS alone; **, $P < 0.001$ compared with GBS alone; #, P not significant compared with SP alone; ##, $P < 0.001$ compared with SP alone; Φ, $P < 0.02$ compared with control.

ing that astrocytes and microglia are the cells that primarily respond to bacterial products. Rat glia produce NO after exposure to either proinflammatory cytokines or LPS (5, 12, 13, 35, 36). The aim of the present study was to determine the potential of gram-positive organisms to activate glia and thus induce neuronal injury by indirect mechanisms.

The current set of experiments document that glia respond to gram-positive bacterial products. After challenge with cell walls from either *S. pneumoniae* or the group B streptococcus, both astrocytes and microglia began to produce NO within 24 h. These findings are similar to those reported by others who found that rat astrocytes and rat and murine microglia produce NO after exposure to LPS or heat-killed *S. pneumoniae* (4, 5, 7, 12, 13, 36).

In keeping with our previous studies, there were only small differences in neuronal survival between cultures challenged with *S. pneumoniae* or group B streptococcal cell walls and controls in the absence of either microglia or astrocytes. However, in both mixed astrocyte-neuron or microglia-neuron cell cultures, the introduction of either *S. pneumoniae* or group B streptococcal cell walls resulted in significant neuronal cytotoxicity after 48 h. Toxicity was nearly completely inhibited by the NO synthase inhibitor L-NAME. This dramatic effect suggests that neuronal injury after challenge with gram-positive bacterial products is dependent on NO.

Dexamethasone improves neurologic outcome in pediatric bacterial meningitis and is commonly used as adjunctive therapy (22, 29). This beneficial effect is thought to result from the reduction of the inflammation in the central nervous system; however, the precise mechanism of action is unknown. Dexamethasone suppressed the production of NO in astrocytes and markedly reduced the associated neurotoxicity after challenge with LPS (35, 36). The mechanism of suppression of NO production is through the reduction of the synthesis of proinflammatory cytokines, which activate NO synthesis. In the present study, dexamethasone suppressed the production of NO in astrocytes and microglia after challenge with cell walls from both gram-positive pathogens and with LPS. Dexamethasone in cocultures stimulated with any of these bacterial products inhibited toxicity in neurons, again suggesting that neurotoxicity was NO dependent.

Glutamate seemed to play no independent role in mediating neuronal injury in either the neuron-astrocyte or neuron-microglial cell cultures, since the glutamate antagonist MK801 was not protective and no stable neurotoxin, such as glutamate, appeared to be present in the supernatants from cultures of astrocytes or microglia conditioned with *S. pneumoniae* cell walls, group B streptococcal cell walls, or LPS. These results are in contrast to those reported by other investigators, who found that LPS stimulated the release of glutamate from microglia and that glutamate was the major mediator of neurotoxicity in vitro (31, 32). While our findings do not exclude the possibility that glutamate is released from microglia activated by bacterial products, they argue against the release of glutamate in sufficient quantities to independently cause neurotoxicity. The reasons for the discrepancies are not clear but may be related to differences in the experimental systems (rat versus mouse, presence or absence of serum, and cerebellar versus cortical neurons).

Glutamate and NO may act in synergy within the same signaling pathways, and NO may potentiate neuronal sensitivity to glutamate. Some investigators have suggested that the cytotoxic effects of glutamate may be mediated through NO (9, 17). Given that the neurotoxicity of gram-positive bacterial products is NO dependent and that glutamate is present in brain interstitial fluid at increased concentrations in meningitis (14), we sought to determine if bacterial products and glutamate may act in synergy to produce brain injury. The toxicity caused by both stimuli in combination exceeded the sum of the toxicities of the individual stimuli. Again, toxicity was dependent on the presence of NO. Taken together, these results support the notion that in the brain interstitial milieu during bacterial meningitis, low levels of glutamate and gram-positive bacterial products may result in synergistic neurotoxicity.

In contrast to the neurotoxicity of gram-positive stimuli, which was serum independent, no nitrite was measured in cultures after LPS challenge in the absence of serum. In addition, LPS in the presence of a low concentration of serum had no neurotoxic effect in both coculture systems. The addition of serum to the culture system reinstated the production of NO as well as the neurotoxic effect of LPS in both microglia-neuron and astrocyte-neuron cultures. The biologic effect of LPS is mediated though the serum factors LPS binding protein and CD14 in some biological systems (2, 26, 34), and the serum requirement of the glial response to LPS likely reflects dependence on such serum factors for activation.

Our study shows that interaction of cell wall and glia may result in neuronal injury in vitro, but several open questions make it difficult to know whether this mechanism is relevant in vivo. First, while concentrations similar to those used in these studies induce inflammation in the cerebrospinal fluid of rabbits after intracisternal injection (38, 39), the concentrations of these bacterial cell wall products present in the cerebrospinal fluid or brain parenchyma during meningitis have not been determined. Second, while both rat astrocytes and microglia produce NO in neurotoxic concentrations in response to bacterial products, human microglia do not seem to synthesize such concentrations of NO (23, 30). Human astrocytes, however, are able to generate NO (23). Since astrocytes are the most numerous cell type in the brain, their activation by bacterial products could play a role in the development of brain injury in bacterial meningitis in humans. Nevertheless, it has not been formally documented that human or rat astrocytes produce NO in vivo during meningitis.

Another unresolved question is the precise nature of the cell wall components responsible for the biologic effects observed in this and other studies. Both in vitro and in vivo experiments have shown that heat-killed, unencapsulated organisms, whole cell walls, purified peptidoglycans of various sizes, and teichoic acid are all proinflammatory to generally similar degrees (4, 10, 15, 19, 38). In addition, lipoteichoic acid and peptidoglycan can induce tissue responses independently and synergistically (10). Thus, although subtle differences in the biological effects of the various components of bacterial cell walls may exist (38), it is clear that several chemically distinct cell wall structures can contribute to the induction of inflammation in response to the invading organism.

In summary, gram-positive cell walls from *S. pneumoniae* and the group B streptococcus induced the synthesis of NO from astrocytes and microglia. In coculture, these bacterial products caused significant neurotoxicity, which was greatly reduced by inhibitors of NO synthesis but not by glutamate antagonists. Neurotoxicity was also markedly inhibited by dexamethasone. Cell walls and glutamate toxicity were synergistic and could be greatly limited by NO inhibitors. Unlike LPS, which requires the presence of serum factors, cell walls from *S. pneumoniae* and group B streptococcus induced the synthesis of NO and neurotoxicity independent of serum. Gram-positive bacterial products thus activate glia and induce the production of NO in neurotoxic concentrations. This pathway may play a contributing role in the genesis of brain injury in bacterial meningitis.

ACKNOWLEDGMENT

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