Nitric Oxide-Dependent Regulation of Pro-inflammatory Cytokines in Group B Streptococcal Inflammation of Rat Lung

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Abstract. Group B Streptococcus (GBS) infection leading to sepsis and lung injury is a major cause of neonatal morbidity and mortality. Lung injury may result from overproduction of pro-inflammatory mediators (cytokines), caused by nitric oxide (NO). Our objective was to characterize the molecular signaling events involving the pro-inflammatory mediators interleukin-6 (IL-6) and macrophage inflammatory protein (MIP-2) in the presence of aminoguanidine (AG), an inducible nitric oxide synthase (iNOS) specific inhibitor, in lung tissue from GBS-treated young rats. Changes in iNOS mRNA, lactic acid, and rectal temperature were determined as markers of the inflammatory response. Expression and regulation of IL-6 and MIP-2 mRNA in lung tissue were studied by RT-PCR with densitometry analysis. GBS treatment of young rats induced the expression of pro-inflammatory mediators IL-6 (6-fold) and MIP-2 (3-fold) in lung tissue compared to controls. AG decreased IL-6 and MIP-2 expression. Addition of L-arginine (L-arg) reversed the AG effect on IL-6 and MIP-2 expression. These data suggest a role for the nitric oxide pathway in the overproduction of pro-inflammatory mediators IL-6 and MIP-2 during GBS-induced lung inflammation. This pathway may be responsible for the initiation of lung injury. (received 10 August 2002; accepted 17 November 2002)

Keywords: Group B streptococcus, neonatal sepsis, lung injury, aminoguanidine, arginine, interleukin-6, macrophage inflammatory protein

Introduction

Group B Streptococcus is a leading cause of neonatal morbidity and mortality with long term sequelae despite antibiotic treatment [1,2]. Systemic inflammation is associated with increased synthesis and combined action of pro-inflammatory mediators such as interleukin-6 (IL-6), macrophage inflammatory protein-2 (MIP-2), and inducible nitric oxide synthase (iNOS) [3-10].

Nitric oxide (NO) is a biological signaling and effector molecule that regulates the expression of other pro-inflammatory mediators involved in the early response to GBS [3,4,7-10]. NO is derived from the conversion of L-arginine to L-citrulline by three isoforms of NOS. During the inflammatory process, iNOS, which is induced by bacterial products and cytokines, plays an important role in the early response [7-9]. Recent data suggest that the iNOS pathway is involved in the synthesis of pyrogenic cytokines during the inflammatory response [3,6-10]. One of these cytokines, IL-6 is implicated in the pyrogenic response as well as in the catabolic response, in particular glycolysis, provoked by severe bacterial infection [6-14].

Another cytokine that plays a significant role in rat lung inflammatory response is macrophage inflammatory protein-2 (MIP-2). MIP-2, expressed in rat lung tissue (analogous to IL-8 in humans), belongs to the C-X-C chemokine family and binds to IL-8 receptor [4,15,16]. Recently, IL-8 upregulation during systemic inflammation has been suggested to be NO-dependent [4]. The possible involvement of the nitric oxide pathway in the regulation of cytokines such as IL-6 and MIP-2

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during GBS inflammation of the lung has not been described.

The role of NO during disease states is assessed by the effect of specific NOS inhibitors on the NO pathway [3,7]. Leib et al [7] reported that inhibition of NOS produces beneficial results on neuronal injury. However, the effect of NOS inhibition on GBS-induced lung inflammation has not been examined. To determine the effect of nitric oxide on pro-inflammatory mediators during systemic GBS inflammation, we characterized the in vivo effect of aminoguanidine (AG), an iNOS specific inhibitor, on IL-6 and MIP-2 regulation during GBS-induced inflammation. We hypothesize that lung injury results from overproduction of pro-inflammatory mediators (cytokines) caused by nitric oxide (NO). In this study, we used a young rat model of systemic GBS inflammation to determine whether GBS-induced infection leads to induction of two pro-inflammatory cytokines, IL-6 and MIP-2, in lung tissue. The results may help to determine if the NO pathway is involved in their regulation.

Methods

**Experimental animals and treatment protocol.** This study was approved by the Wayne State University Animal Investigation Committee and was performed in accordance with NIH guidelines for use of animals in research. Lung tissue was obtained from 3-wk-old male Sprague Dawley rats that weighed 100 to 125 g. The rats were assigned to 1 of 5 treatment groups (4-6 rats/group), as follows: Group 1, controls (saline treated, ip); Group 2, aminoguanidine (AG, 100 mg/kg, ip); Group 3, heat-inactivated GBS (109 cfu/ml, ip); Group 4, aminoguanidine (AG, 100 mg/kg, ip) plus heat-inactivated GBS (109 cfu/ml, ip); and Group 5, L-arginine (L-arg, 300 mg/kg, ip) plus aminoguanidine (AG, 100 mg/kg, ip) plus heat-inactivated GBS (109 cfu/ml, ip).

**Lung tissue collection and RNA preparation.** At 18 hr post-treatment, total lung tissue RNA was prepared by homogenizing lung tissue (100 mg) in TRIzol solution (GIBCO BRL/Life Technologies, Inc., Baltimore, MD), following the manufacturer’s protocol. The quality and quantity of each RNA preparation was assessed by spectrophotometry and formaldehyde agarose gel electrophoresis [15].

**Serum lactic acid analysis.** Systemic inflammation in rats was assessed by measuring the serum level of lactic acid using an automated clinical analyzer.

**Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.** One µg of total lung tissue RNA was reverse-transcribed for 60 min at 42°C and amplified by a PCR method, according to the manufacturer’s protocol (BioSource International, Inc., Camarillo, CA). Amplification was done in 50 µl of reaction buffer containing 20 mM TRIS (pH 8.0), 50 mM KCl, 2.0 mM MgCl2, 0.2 mM each of dNTPs (dTTP, dGTP, dATP), 0.5 mM each of primers (IL-6, MIP-2, TIMP-2, iNOS and ß-actin) for 27-35 cycles (each cycle was 95°C, 1 min; 55-60°C, 45 sec; 72°C, 1 min), terminating at 72°C for 10 min. All primers spanned an intron so that genomic DNA contamination would not interfere with the analyses. Control (-RNA) and (-RT) samples were included in all PCR assays. The amplified cDNA fragments were subjected to 6% acrylamide/bis gel electrophoresis and autoradiography. After scanning and densitometry, the data were expressed relative to ß-actin, which served as the internal standard control gene.

**Statistical analysis.** Data are expressed as mean ± SD and analyzed by t-test; the criterion for statistical significance is p <0.05. The gel shown in Fig. 1B is representative of 4 separate experiments.

**Results and Discussion**

To assess the GBS-induced systemic inflammatory response in young rats, we measured the changes in serum lactic acid, rectal temperature, and lung expression of iNOS mRNA.

**Serum lactic acid and rectal temperature.** Microbial infection and inflammation are frequently associated with increased glucose catabolism, a persistent state of lactate production, and metabolic acidosis. The serum lactate levels in GBS-treated rats increased
compared to controls (3.9 ± 0.7 vs 1.4 ± 0.2 mmol/L, p < 0.04) and pretreatment with AG partially prevented this increase (2.6 ± 0.2 mmol/L, Fig. 1, panel A). In GBS-treated young rats, the rectal temperatures were slightly elevated (>38°C) compared to controls (37°C). Fig. 1B shows a representative RT-PCR autoradiogram that confirms the NO-responsiveness to GBS and AG treatment by studying iNOS mRNA expression in the lung tissue. Pretreatment with AG prevented the induction of the iNOS gene by GBS; however, the inhibitory effect of AG was reversed in the presence of L-arg.

**IL-6 mRNA production.** During bacterial infection, IL-6 production is regulated primarily by transcription, so we studied the changes in IL-6 mRNA expression by GBS and AG, using RT-PCR and quantitative densitometry. Fig. 2A shows that GBS treatment significantly (p < 0.005) induced changes in IL-6 mRNA expression (6-fold) at 18 hr, compared to controls. AG pretreatment suppressed IL-6 mRNA induction by GBS, and addition of L-arg, a NO donor, neutralized the AG effect, suggesting involvement of the NO pathway during GBS infection in lung tissue.

**MIP-2 mRNA production.** MIP-2 mRNA regulation by GBS and AG followed a pattern similar to IL-6 expression. Treatment with GBS increased the MIP-2 mRNA level in lung tissue (3-fold compared to controls) and addition of AG suppressed the MIP-2 mRNA induction close to the control level. Addition of L-arg reversed the suppressive effect of AG at 18 hr post-GBS (Fig. 2B). These data suggest
a role of the NO pathway in MIP-2 gene regulation during GBS infection.

**Cytokines, chemokines, oxidants, and metalloproteases play roles in inflammatory lung injury.**

In the present study, we examined the potential involvement of mediators IL-6 and MIP-2 in GBS-induced inflammation in rat lung and the role of NO in cytokine expression. To date, the correlation between induction of both the IL-6 and MIP-2 genes during in vivo GBS-induced inflammation of the lung tissue has not been studied. MIP-2 is a member of the C-X-C chemokine family (alpha-family) of inflammatory and immunoregulatory cytokines [11, 15, 16]. MIP-2 exhibits potent neutrophilic chemo tactic activity and is considered (together with CINC) a key mediator during inflammatory cell recruitment in response to lung injury and infection. Our data indicate that MIP-2 plays a significant role in GBS-induced inflammatory response in rat lung and that its expression in lung is mediated, at least in part, by increased production of NO.

One of the common responses to infection or inflammation is a febrile response. Various members of the cytokine family that participate in the inflammatory cascade are involved in the generation of fever. IL-6 is a cytokine associated with the febrile response. Recent studies have suggested that a NOS mechanism is involved in the synthesis of pyrogenic cytokines (in particular, IL-6) and in the attenuated febrile response by pretreatment with AG [3, 6, 7]. Our data support these studies. In this study, we confirmed that AG inhibits iNOS, known to be responsible for excess production of NO, and suppresses pyrogenic cytokines, such as IL-6.

IL-6 has been implicated in the catabolic effects (glucose metabolism, lactate production) that accompany inflammation [6, 11, 14]. Our data show

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**Fig. 2.** The effect of aminoguanidine (AG) on IL-6 and MIP-2 mRNA expression in lung tissue. Rats were treated as follows: (Group 1) saline; (Group 2) AG; (Group 3) GBS; (Group 4) AG + GBS; and (Group 5) L-Arginine + AG + GBS. At 18 hr, total lung RNA was extracted and changes in MIP-2 and IL-6 mRNA expression were analyzed by RT-PCR. Results are presented as relative changes in densitometric units (DU), compared to the controls (± SD). * p < 0.05.
that AG significantly prevents the production of lactic acid and IL-6 mRNA accumulation in GBS-treated rats, suggesting in vivo anti-inflammatory properties of this component.

TIMP-2 is released from a variety of lung cells and is one of the resident protease inhibitors thought to be important in regulation of tissue matrix metalloproteinases (MMP). TIMP-2 is a member of a family of enzymes that contribute to normal and pathological tissue remodeling. TIMP-2 also participates in the migration of normal and malignant cells through the body and acts as a regulatory molecule by processing matrix proteins, cytokines, growth factors and adhesion molecules [12]. Expression of the TIMP-2 gene was unaffected by any treatments, suggesting that the NO pathway does not have a significant role in these events [4].

We have demonstrated that GBS induces the major pro-inflammatory mediators in lung tissue, IL-6 and MIP-2, and that AG inhibits GBS-induction of MIP-2 and IL-6. In vivo addition of a NO-donor, L-arginine, reverses these effects, which suggests that the pro-inflammatory response in lung tissue is in part NO-dependent.

Presence of other iNOS isoforms in lung tissue may be responsible for NO effects, eg, the specific suppression of iNOS by AG. Studies are underway in our laboratory to determine whether different inhibitors of nitric oxide production may be therapeutic options for pediatric and neonatal infections. We are exploring the effects of an exogenously administered iNOS inhibitor on endotoxin-stimulated lung epithelium from newborn and adult lung.

Cytokines and chemokines are attractive targets for therapies to reduce the extent of inflammatory injury [13]. At present, the most notable approach to adjunctive therapy is the use of corticosteroids. Alternatives to corticosteroids for controlling the cytokine network have been explored experimentally (3-6,13). In addition to standard antimicrobial therapy for specific bacterial infections, the inhibition of GBS-induced pro-inflammatory mediators by an iNOS-specific inhibitor may suppress an inappropriate inflammatory response. Aminoguanidine may be a good candidate for potential adjunctive therapy during GBS infection.

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References


