Inhibition of Nitric Oxide Synthase Exacerbates Group B *Streptococcus* Sepsis and Arthritis in Mice

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The role of nitric oxide in group B *Streptococcus* (GBS) infection was evaluated by inhibiting its production with aminoguanidine (AG). AG-treated mice displayed higher mortality rates and more frequent and severe arthritis than controls. Worsening of arthritis correlated with a higher number of GBS cells in the joints and local interleukin-1β (IL-1β) production.

Group B streptococci (GBS) are a leading cause of life-threatening infection in neonates and young infants (1), and invasive infections caused by GBS have been increasingly recognized in adults (4, 7). Septic arthritis is one of the clinical manifestations of late-onset GBS infection in neonates (1) and is often associated with age and risk factors in adults (12, 16).

Our previous report described an experimental mouse model of type IV GBS systemic infection with clinical features that mimic the human situation, although a more frequent appearance of multifocal septic arthritis is observed (25). In this model, production of proinflammatory cytokines, in particular interleukin-6 (IL-6) and IL-1β, increased in response to GBS infection in sera and joints (26). A direct correlation between IL-6 and IL-1β concentrations in the joints and severity of arthritis was observed (26).

Nitric oxide (NO), produced in large amounts by inducible NO synthase (iNOS) (15), not only represents an important microbicidal agent in the host defense but also functions as a biological signaling and effector molecule in inflammation and immunity (2, 13). However, NO can contribute to tissue damage and has been implicated in the pathogenesis of tumors and infectious autoimmune and chronic degenerative diseases (3, 13, 23, 27). For example, NO inhibition leads to suppression of adjuvant and streptococcal cell wall-induced arthritis (14, 24) while it aggravates *Staphylococcus aureus*-induced septic arthritis (21).

The aim of this study was to evaluate the role of NO in the course of GBS-induced sepsis and arthritis by administration of aminoguanidine (AG), a potent and selective inhibitor of iNOS (5). Sex-matched, 8-week-old male or female outbred CD-1 mice were obtained from Charles River Breeding Laboratories (Calco, Italy). Type IV GBS, reference strain GBS 1/82, were grown overnight at 37°C in Todd-Hewitt broth (Oxoid Ltd., Basingstoke, England). A bacterial suspension was prepared as previously described (23). Mice were inoculated intravenously via the tail vein with 8 × 10⁶ GBS per mouse in a volume of 0.5 ml. Control mice were injected intravenously with 0.5 ml of RPMI 1640 medium (GIBCO, Life Technologies, Milan, Italy). AG hemisulfate (Sigma Chemical Co., St. Louis, Mo.) was administered in sterile drinking water (1%, wt/vol) throughout the study, starting 7 days before infection (22). In another set of experiments, L-arginine (Sigma) was administered in sterile drinking water (3%, wt/vol) along with AG. Mortality was recorded at 24-h intervals for 20 days. Mice were examined daily to evaluate joint inflammation. Arthritis was defined as visible erythema or swelling of at least one joint. Clinical scoring was used as follows for each limb: 0 = normal, 1 point = mild swelling and erythema, 2 points = moderate swelling and erythema, 3 points = marked swelling, erythema, and/or ankylosis (maximum score of 12). The arthritis index was constructed by dividing the total score by the number of animals used in each experimental group. Histological studies were performed as described elsewhere (25). Blood, kidney, and joint infections in mice infected with GBS, treated or not treated with AG, were determined by CFU evaluation as previously described (25). Peritoneal thioglycollate-induced macrophages were obtained as previously described and treated or not treated with AG (1 mM). Intracellular survival of GBS in macrophages was assessed as previously described (6). Blood and articular samples from mice injected with GBS, treated or not treated with AG, and from uninfected controls were obtained as previously described (26). IL-6 and IL-1β concentrations were measured with commercial enzyme-linked immunosorbent assay kits (R&D Systems, Inc., Minneapolis, Minn.), and urinary NO levels were measured by using a colorimetric NO₂⁻–NO₃⁻ commercial kit (R&D Systems) in accordance with the manufacturer’s recommendations.

The amount of nitrate in urine is a reliable indicator of the level of NO produced in all tissues of the mouse (9). A nitrate concentration of 140 ± 12 μM was detected in uninfected mice and was reduced to 55 ± 8 μM upon AG treatment. On day 5 after infection, a nitrate concentration of 270 ± 25 μM was detected in control mice, compared to 126 ± 12 μM in AG-treated mice (P < 0.01). Similar differences were observed on day 10 after infection (247 ± 36 μM in control mice versus 105...
Inhibition of NO production resulted in 50% mortality upon infection with $8 \times 10^6$ GBS/mouse, while only 7% of control mice died (Fig. 1A). AG-treated mice showed a significant increase in both the incidence and severity of arthritis (Fig. 1B and C). At day 10 after GBS infection, 80% of the AG-treated mice displayed articular lesions with an arthritis index of 2.6 ± 0.3, while the incidence of arthritis in control mice was 40% with an arthritis index of 1.0 ± 0.2. Addition of L-arginine reversed the effect of AG on mortality and the incidence and severity of arthritis. Histopathological analysis showed that 1 week after infection articular cavities of AG-treated mice were filled with purulent exudate, while in control mice the inflammatory infiltrate was limited to subcutaneous and periarticular tissues (Fig. 1D and E). The number of CFU in the bloodstream and kidneys of AG-treated mice was significantly higher than in those of controls at day 1 after infection; no differences were observed at days 5 and 10 (Fig. 2). The number of GBS recovered from the joints of AG-treated mice was always higher than in controls, although these differences were significant only at day 5 after infection. In vitro treatment of macrophages with AG resulted in reduced killing of GBS with respect to control macrophages. After 24 h, the number of GBS surviving in AG-treated cells was $1.6 \times 10^3 \pm 0.2 \times 10^3$ versus $8.1 \times 10^2 \pm 0.1 \times 10^2$ in untreated cells.

Levels of IL-6 in serum were significantly decreased in AG-treated animals during infection, whereas no significant differences were evident in systemic IL-1β levels (Fig. 3). Similar IL-6 concentrations were found at days 5 and 10 after infection in the joints of AG-treated mice, while a sustained increase in IL-6 levels was detected in articular samples from controls. On the contrary, AG treatment resulted in a significant increase in joint IL-1β concentrations at day 5 after infection.

There is evidence that GBS are able to induce NO production in murine macrophages (8) and that cell wall and β-hemolysin cooperate to substitute for the priming signal typically provided by gamma interferon (19). In contrast to what was observed in a rat model of group A streptococcal sepsis (20), our data account for NO as an essential protective mechanism during GBS infection. However, it must be considered that the group A streptococcal sepsis model is a high-lethality peritoneal sepsis, while our GBS model is a sublethal infection and that two different NOS inhibitors were used.

In our model, NO is likely to function both as a direct effector and as an immunoregulatory molecule. In the early phase of infection, AG-treated mice seemed unable to limit bacterial growth in the blood, in accordance with an in vitro
study showing a role for NO in controlling GBS growth (10) and our own observation. In the joints, this impaired antimicrobial function was evident at later time points. Evidence exists that GBS survive inside monocytes/macrophages, thus allowing dissemination to different body sites (6), including articular cavities (17), and that the number of microorganisms in the joints is a determinant of the severity of arthritis (25).

The higher number of GBS persisting in AG-treated macrophages could result in a greater bacterial load in the joints, thus accounting for the exacerbation of arthritis observed in AG-treated mice. Surprisingly, the worsening of articular lesions was not accompanied by a concomitant increase in IL-6 production, as previously observed (26). On the contrary, a significant decrease in both serum and local IL-6 levels was found upon iNOS inhibition. These findings are in agreement with previous reports showing a downregulating effect of NOS inhibitors on IL-6 during gram-positive infections (18, 21). However, it is noteworthy that IL-1β, which actively participates in GBS-induced joint injury (26), increases following AG treatment, at least at day 5 after infection, and this may be, together with the enhanced bacterial load, a potential cause of the worsening of articular lesions. Moreover, since it has been shown that NO inhibits leukocyte migration (11), its downregulation accounts for the augmentation of inflammatory cells locally recruited to the articular cavities of AG-treated mice. In conclusion, in contrast to models of autoimmune inflammatory arthritis (14, 24), in our experimental model of septic arthritis NO has a protective effect. Our study shows that treatment involving suppression of iNOS induction and NO production may not be suitable in the management of GBS infection.

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