Glucuronoxylomannan, the Major Capsular Polysaccharide of *Cryptococcus neoformans*, Inhibits the Progression of Group B Streptococcal Arthritis

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Received 7 June 2004/Returned for modification 5 July 2004/Accepted 21 July 2004

Glucuronoxylomannan (GXM), the principal constituent of the *Cryptococcus neoformans* capsule, modulates the inflammatory response of human monocytes in vitro. Here we examine the efficacy of GXM as a novel anti-inflammatory compound for use against experimental septic arthritis. Arthritis was induced in mice by the intravenous injection of 8 × 10^6 CFU of type IV group B streptococcus (GBS). GXM was administered intravenously in different doses (50, 100, or 200 μg/mouse) 1 day before and 1 day after bacterial inoculation. GXM treatment markedly decreased the incidence and severity of articular lesions. Histological findings showed limited periarticular inflammation in the joints of GXM-treated mice, confirming the clinical observations. The amelioration of arthritis was associated with a significant reduction in the local production of interleukin-6 (IL-6), IL-1β, macrophage inflammatory protein 1α (MIP-1α), and MIP-2 and an increase in systemic IL-10 levels. Moreover, peritoneal macrophages derived from GXM-treated mice and stimulated in vitro with heat-inactivated GBS showed a similar pattern of cytokine production. The present study provides evidence for the modulation of the inflammatory response by GXM in vivo and suggests a potential therapeutic use for this compound in pathologies involving inflammatory processes.

Glucuronoxylomannan (GXM), the principal constituent of the capsular material of *Cryptococcus neoformans* (7) and exerts many immunoregulatory effects (40). Soluble GXM has numerous biological activities that may contribute to the pathogenesis of infection, and high levels of GXM have been found in sera from patients with cryptococcosis (4). After systemic administration, GXM is retained for an indefinite period in tissues containing mononuclear phagocytes (11, 18, 24), and macrophages serve as a long-term reservoir for GXM storage (13). It was recently demonstrated that GXM is continuously accumulated by macrophages for longer than 1 week in vitro and is retained inside these cells, altering their biological functions (23). The binding and internalization of GXM affect monocyte/macrophage effector and secretory functions. In particular, a secretion of interleukin-10 (IL-10) (41) and an inhibition of the production of proinflammatory cytokines, such as IL-1β and tumor necrosis factor alpha (TNF-α) (42), are observed after in vitro treatment of monocytes/macrophages with GXM. In addition, GXM inhibits the migration of neutrophils in response to IL-8 (19) and delays the translocation of leukocytes across the blood-brain barrier in an animal model of acute bacterial meningitis (20).

Despite advances in diagnosis and treatment, group B streptococci (GBS) are still a leading cause of perinatal morbidity and mortality and are also an emerging public health problem for nonpregnant adults (3, 8, 9, 33). Invasive neonatal GBS infections have either an early (usually the first 24 h after birth) or late (7 days after birth) onset. Common manifestations of GBS disease in neonates include pneumonia, septicemia, meningitis, bacteremia, and bone or joint infection (3). Septic arthritis is one of the clinical manifestations of GBS infection in neonates (3) and is often associated with aging and serious underlying diseases in adults (16, 30, 31, 33). Articular lesions in GBS-infected mice are similar to those observed for human disease, making the mouse an excellent model for studying GBS arthritis (35, 36). Mice inoculated with serotype IV GBS show clinical signs of arthritis that are characterized by an early onset and by evolution from an acute exudative synovitis to permanent lesions with irreversible joint damage and/or ankylosis (36). In this model, the production of proinflammatory cytokines, in particular IL-6 and IL-1β, increases in sera and joints in response to GBS infection, and a direct correlation was observed between IL-6 and IL-1β concentrations in the joints and the severity of arthritis (37). Additional evidence for the detrimental role of proinflammatory cytokines was obtained by use of the anti-inflammatory cytokine IL-10. The administration of exogenous IL-10 produced a decrease in pathology that was associated with a significant local reduction in IL-6, IL-1β, and TNF-α levels (28), while the administration of neutralizing anti-IL-10 antibodies resulted in a deleterious effect. Recently, a pivotal contribution of macrophages to GBS-induced arthritis was demonstrated with monocytic mice, which showed a less severe development of arthritis associated with a decrease in IL-1β and IL-6 production in joints (27).

Since GXM displays anti-inflammatory activity, we postulated the possibility that GXM may act as an anti-inflammatory agent in this model of GBS-induced septic arthritis. For the present study, we investigated the effect of this microbial compound on the course of GBS-induced arthritis.
MATERIALS AND METHODS

Mice. Eight-week-old female outbred CD-1 mice were obtained from Charles River Breeding Laboratories (Calco, Italy).

Microorganism. A type IV GBS reference strain, GBS 1/82, was used throughout the study. For experimental infections, the microorganism was grown overnight at 37°C in Todd-Hewitt broth (Oxoid Ltd., Basingstoke, Hampshire, England) and then was washed and diluted in RPMI 1640 medium (Gibco, Life Technologies, Milan, Italy). The inoculum size was estimated by observations of turbidity, and viability counts were performed by plating the cells on tryptic soy agar-5% sheep blood (blood agar) and then incubating them overnight under anaerobic conditions at 37°C. A bacterial suspension was prepared in RPMI 1640 medium. Mice were inoculated intravenously via the tail vein with 8 × 10⁶ GBS in a volume of 0.5 ml. Control mice were injected by the same route with 0.5 ml of RPMI 1640 medium.

Cryptococcal polysaccharide. GXM was isolated from the culture supernatant of a serotype A strain of C. neoformans (CN 6) that was grown in liquid synthetic medium (7) in a gyratory shaker for 4 days at 30°C. GXM was isolated by differential precipitation with ethanol and hexadecyltrimethyl ammonium bromide (Sigma, St. Louis, Mo.) (6). The isolation procedure was described previously in detail (14). GXM was diluted in saline and injected intravenously in a 0.3-ml volume at a dose of 50, 100, or 200 μg/mouse 1 day before and 1 day after GBS infection. Control mice received saline according to the same experimental schedule.

Clinical evaluation of arthritis and mortality. Mice injected with GBS and treated with GXM or saline as described above were evaluated for clinical signs of arthritis and for mortality. Mortality was recorded at 24-h intervals for 30 days. After disease challenge, the mice were examined daily under blinded conditions by two independent observers for 30 days to evaluate the presence of joint inflammation, and scores for arthritis severity (macroscopic scores) were given as previously described (28, 36, 37). Arthritis was defined as visible erythema and/or swelling of at least one joint. The clinical severity of arthritis was graded on a scale of 0 to 3 for each paw, according to changes in erythema and swelling (0 = no change; 1 = mild swelling and/or erythema; 2 = moderate swelling and erythema; 3 = marked swelling, erythema, and/or ankylosis). Thus, each mouse could have a maximum score of 12. The arthritis index (mean ± standard deviation [SD]) was constructed by dividing the total score (cumulative value for all paws) by the number of animals used for each experimental group.

Histological analysis. Groups of mice infected with GBS and treated with GXM or saline were examined 7 days after infection for histopathological features of arthritis. Arthritic hind paws (one per mouse) were removed aseptically, fixed in 10% (vol/vol) formalin for 24 h, decalcified in 5% (vol/vol) trichloroacetic acid for 7 days, dehydrated, embedded in paraffin, sectioned into 3- to 4-μm-thick sections, and stained with hematoxylin and eosin. The samples were examined under blinded conditions. Tibia-tarsal, tarsus-metatarsal, and metatarsus-thick sections, and stained with hematoxylin and eosin. The samples were examined under blinded conditions. Tibia-tarsal, tarsus-metatarsal, and metatarsus-phalangeal joints were examined, and the extents of infiltrate (presence of inflammatory cells in the subcutaneous and/or periarticular tissues), exudate (presence of inflammatory cells in the articular cavity), cartilage damage, bone erosion, and loss of joint architecture were evaluated. The arthritis severity was classified as mild (minimal infiltrate), moderate (presence of infiltrate, minimal exudate, and arthritis index <4), or severe (presence of massive infiltrate and/or exudate, cartilage and bone erosion, and disrupted joint architecture). GBS growth in blood, kidneys, and joints. Blood, kidney, and joint infections in GBS-infected mice treated with GXM or saline were determined by evaluation of CFU at different times after infection. Blood samples were obtained by retro-orbital sinus bleeding before the mice were sacrificed. Tenfold dilutions were prepared in RPMI 1640 medium, and 0.1 ml of each dilution was plated in triplicate on blood agar and incubated under anaerobic conditions for 24 h. The numbers of CFU were determined, and the results were expressed as numbers of CFU per milliliter of blood. Kidney samples were aseptically removed and homogenized with 3 ml of sterile RPMI 1640. All wrist and ankle joints from each mouse were removed, weighed, and homogenized in toto in sterile RPMI 1640 medium (1 ml/100 mg of joint weight). After homogenization, all tissue samples were diluted and plated in triplicate on blood agar, and the results were expressed as numbers of CFU per whole organ or per milliliter of joint homogenate.

Sample preparation for cytokine assessment. Blood samples from the different experimental groups were obtained by retro-orbital sinus bleeding at different times after infection before the mice were sacrificed. Sera were stored at −80°C until analysis. Joint tissues were prepared as previously described (37). Briefly, wrist and ankle joints from each mouse were removed and then homogenized in toto in 1 ml of lysis medium (RPMI 1640 containing 2 mM phenylmethysulfonyl fluoride and a 1-μM final concentration [each] of aprotinin, leupeptin, and pepstatin A)/100 mg of joint weight. The homogenized tissues were then centrifuged at 2,000 × g for 10 min, and the supernatant fluids were sterilized through a Millipore filter (0.45-μm pore size) and stored at −80°C until analysis.

RESULTS

Effect of GXM administration on clinical course of arthritis. Clinical signs of joint swelling were observed in 35% of the mice as early as 24 h after the injection of 8 × 10⁶ CFU of GBS. The incidence of arthritis increased to 80% by day 5, and 85% of the animals had articular lesions on day 10 after infection (Fig. 1A).
Similarly, the arthritis index progressively increased and reached its maximum value 10 days after the GBS challenge (mean value, 3.2 ± 0.3) (Fig. 1B); most of the animals had articular lesions in both the hind paws and front paws. Twenty percent of the mice died during the course of infection. GXM was administered 1 day before and 1 day after infection at a dose of 50, 100, or 200 µg/mouse. Treatment with 100 or 200 µg of GXM resulted in a smaller number of animals with articular lesions than that for controls; differences between GXM-treated mice and controls were significant starting 7 days after infection. Similarly, the arthritis indexes for GXM-treated mice were significantly lower than those for controls from day 5 on, reaching maximum values of 1.9 ± 0.4 and 2.0 ± 0.2 for mice given doses of 100 and 200 µg of GXM, respectively, compared to 3.2 ± 0.3 for controls. The dose of 50 µg had no effect on the incidence or severity of GBS arthritis (data not shown). The survival rates of GXM-treated mice did not differ from those of controls at any dose employed (data not shown).

Arthritic hind paws were removed for histologic examination on day 7 after infection. For controls, >50% of the examined joints were classified as severely affected (Fig. 2A), with the presence of a massive periarticular infiltrate and evidence of cartilage degradation (Fig. 2B). In contrast, most of the joints examined from GXM-treated mice were classified as moderately affected (Fig. 2A), with the inflammatory infiltrate limited to subcutaneous and muscular tissues (Fig. 2C). Only 18% (with 100 µg of GXM) and 14% (with 200 µg of GXM) of the joints were classified as severely affected.

GBS growth rates were assessed for blood, kidneys, and joints of mice that were treated or not treated with GXM. No significant differences were observed between the experimental groups, regardless of the treatment used (data not shown).

Effect of GXM administration on cytokine production. Plasma and joint specimens from mice that were treated with GXM (100 µg) or saline were collected on days 0, 2, 5, and 10 after GBS infection for assessments of cytokine (IL-6 and IL-1β) and chemokine (MIP-1α and MIP-2) production. A rapid increase in IL-6, IL-1β, MIP-1α, and MIP-2 production was observed for the joints of GBS-treated mice, with sustained levels still present 10 days after infection (Fig. 3). GXM treatment significantly affected these cytokine and chemokine levels. In fact, although similar concentrations of the inflammatory products were present 2 days after infection for the two experimental groups, a marked decrease was observed in the subsequent days for GXM-treated mice. The phenomenon was still evident at the end of the experimental period (Fig. 3). A down-regulation in the production of IL-6 and IL-1β following GXM treatment was also observed for systemic levels in the first week after infection (data not shown).

Since members of our laboratory recently pointed out a beneficial role of IL-10 in GBS arthritis (28), we also studied...
treated versus control mice, according to unpaired Student’s t
versely, a significant inhibition of IL-1 production. As shown in Fig. 4, the in vivo GXM treatment inactivated GBS (10 GBS/cell) for 6 or 24 h, and cell culture thioglycolate injection. The cells were stimulated with heat-mouse) or saline was administered the same day and 2 days after macrophages from GXM-treated or naive mice. GXM (100
production, we performed in vitro experiments with peritoneal two experimental groups.

The beneficial effect of GXM was confirmed by histopathological studies. A fivefold reduction in the number of lesions that were classified as severely affected was observed for GXM-treated mice. In addition, a limited cellular influx of inflammatory cells was present in the joints of treated animals, likely due to the decreased production of MIP-1α and MIP-2 at the joint level. These data are in agreement with previous studies showing the abilities of GXM to limit chemotaxis (19, 20) and to suppress the expression of a chemotactic receptor such as C5aR on inflammatory cells (22). The fact that no effect was observed with 50 μg of GXM and that similar therapeutic effects were observed with 100 and 200 μg of GXM are consistent with the results of earlier in vitro studies (23) and suggest a sharp dose-response curve. In vitro experiments showed that the loading of GXM in macrophages and neutrophils occurred at doses of 50 to 100 μg/ml; higher doses (200 μg/ml) did not augment GXM internalization, suggesting a cell saturation or threshold effect (23). Thus, it is conceivable that the in vivo inoculation of 50 μg of GXM did not result in any effects because this concentration was not sufficient to produce the monocyte/macrophage saturation that is required to affect their functions. Conversely, the inoculation of 100 or 200 μg of GXM induced maximal loading, resulting in no significant differences between the two doses.

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<tr>
<th>Tissue</th>
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<th>IL-10 production (pg/ml) on indicated day after infection</th>
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<tr>
<td></td>
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<tr>
<td>Serum</td>
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<td>GXM</td>
<td>25 ± 6</td>
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<td>Joints</td>
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<td>12 ± 3</td>
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a Mice were treated with GXM (100 μg/mouse) or saline 1 day before and 1 day after infection with 8 × 10⁶ GBS/mouse. Uninfected mice (day 0) received GXM or saline 1 day before infection. At the indicated times, three mice per experimental group were sacrificed. Values represent the means ± SD from three separate experiments.

³ P < 0.01 (for GXM-treated versus control mice, according to unpaired Student’s t test).
IL-1 cytokines are known to contribute directly to articular damage. The means of mice were injected intraperitoneally with 1 ml of 10% thioglycolate and then treated with GXM (100 μg/mouse) or saline the same day and 2 days after thioglycolate injection. Peritoneal macrophages were then recovered and stimulated as described in Materials and Methods. Data represent the means ± SD of three separate experiments. *P < 0.05; **P < 0.01 (for GXM-treated versus control mice, according to unpaired Student’s t test).

The clinical benefit mediated by GXM was accompanied by a marked reduction in local IL-6 and IL-1β production. Both cytokines are known to contribute directly to articular damage. IL-1β, together with TNF-α, induces the release of tissue-damaging enzymes from synovial cells and articular chondrocytes and activates osteoclasts (2, 38). IL-6 participates with IL-1 in the catabolism of connective tissue components at inflammation sites (15, 25) and activates osteoclasts, resulting in joint destruction (12). GXM treatment also resulted in a strong increase in the IL-10 concentration in the sera of GBS-challenged mice, although we failed to detect a GXM effect on IL-10 production in the joints. This may have been due to a prompt reutilization of locally produced IL-10 or, alternatively, to a lack of susceptibility of local cells such as synoviocytes, which are known to produce IL-10 (29), to GXM. In vitro experiments confirmed the in vivo results, since peritoneal macrophages from GXM-treated mice produced remarkably less IL-1β and IL-6 upon GBS stimulation in vitro, while increased IL-10 production was observed. It is noteworthy that the observed modification in local and systemic cytokine and chemokine production was not dependent on the number of microorganisms, since similar bacterial loads were present in the GXM-treated and untreated mice.

In this study, the anti-inflammatory effect of GXM was evident starting 2 days after infection. Keeping in mind that GXM accumulates in macrophages (23) and that this cell population reaches the joints 2 to 3 days after infection in bacterial arthritis (5, 10, 26), our results suggest that the beneficial effects observed at the local level are mediated by GXM-loaded macrophages. Indeed, GXM does not prevent the induction of inflammatory cytokines or chemokines, but rather limits their production, thus restraining pathological processes.

Cryptococcal lesions are often characterized by the absence of an inflammatory response, which is mainly attributable to the negative signals provided by GXM during cryptococcosis (40). Although inflammation is an essential component of the host defense against infections, nonetheless an excessive inflammatory response can lead to a detrimental outcome, such as arthritis. Here we demonstrated that the negative properties of GXM may become positive for other pathologies. In conclusion, this study provides evidence for a beneficial effect of GXM in septic arthritis. Its effect is likely due to the ability of GXM to modulate macrophage activities, thus limiting local proinflammatory cytokine and chemokine production and leukocyte recruitment. The advantages of this product, in contrast with others that have been proposed for arthritis therapy, such as cytokines or specific antibodies, are that GXM is readily produced in large amounts, has extended pharmacokinetics, and shows no overt signs of toxicity in vivo. The dose and timing of GXM administration used for this study seemed to be effective without producing side effects. Based on the detrimental role of macrophages (27, 39, 43) and proinflammatory cytokines and chemokines in septic and aseptic chronic disease (1, 17, 25, 32, 34, 37), our results raise the possibility of the therapeutic use of GXM to cure or prevent arthritic pathologies induced by inflammatory processes.

ACKNOWLEDGMENTS

This work was supported by the Ministero dell’Università e della Ricerca Scientifica, Italy, 2003-2004, Prot. 2003068044_005 and Prot. 2003068044_006, and by Public Health Service grant AI4209 from the National Institute for Allergy and Infectious Diseases.

We thank Maurizio Mazzanti for graphic support, Jo-Anne Rowe for dedicated editorial assistance, and Alessandro Braganti and Stefano Temporini for their excellent technical assistance in histological processing and animal care.

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Editor: J. T. Barbieri