Regulatory Role of Interleukin-10 in Experimental Group B Streptococcal Arthritis

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Intravenous inoculation of CD-1 mice with 10^7 CFU of type IV group B Streptococcus (GBS) results in a high incidence of diffuse septic arthritis, associated with high levels of systemic and local production of interleukin-1β (IL-1β) and IL-6. In this study, the role of the anti-inflammatory cytokine IL-10 in the evolution of GBS systemic infection and arthritis was evaluated. IL-10 production was evident in sera and joints of GBS-infected mice. Neutralization of endogenous IL-10 by administration of anti-IL-10 antibodies (1 mg/mouse) at the time of infection resulted in worsening of articular lesions and 60% mortality associated with early sustained production of IL-6, IL-1β, and tumor necrosis factor alpha (TNF-α). The effect of IL-10 supplementation was assessed by administering IL-10 (100, 200, or 400 ng/mouse) once a day for 5 days, starting 1 h after infection. Treatment with IL-10 had a beneficial effect on GBS arthritis, and there was a clear-cut dose dependence. The decrease in pathology was associated with a significant reduction in IL-6, IL-1β, and TNF-α production. Histological findings showed limited periarticular inflammation and a few-cell influx in the articular cavity of IL-10-treated mice, confirming clinical observations. In conclusion, this study provides further information concerning the role of IL-10 in regulating the immune response and inflammation and calls attention to the potential therapeutic use of IL-10 in GBS arthritis.

Despite advances in diagnosis and treatment, group B streptococci (GBS) are still a leading cause of perinatal morbidity and mortality and an emerging public health problem in non-pregnant adults (3, 8, 13, 16). Invasive neonatal GBS infection has either an early onset (usually the first 24 h after birth) or a late onset (7 days after birth). Common manifestations of GBS disease in neonates include pneumonia, septicemia, meningo- encephalitis, 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 age and serious underlying diseases in adults (26, 40–43). Articular lesions in GBS-infected mice are similar to those observed in human disease, making the mouse an excellent model for studying GBS arthritis (49, 50). In a mouse model of type IV GBS infection, we demonstrated that there is an altered cytokine profile in response to bacterial infection, with high-level systemic and local production of interleukin-6 (IL-6) and IL-1β and scant production of tumor necrosis factor-α (TNF-α) (51). In particular, a direct correlation was established between IL-6 and IL-1β concentrations in the joints and severity of arthritis. However, little is known about the production of immunomodulating cytokines, such as IL-10, during the development of GBS arthritis.

IL-10 is a pleiotropic cytokine and plays an important role in many immunological and inflammatory processes (10, 32, 34). IL-10 is mainly a product of T lymphocytes, B lymphocytes, and monocytes/macrophages. Murine IL-10 was initially identified as an important mediator of T helper 2 cell clones that suppressed the production of T helper 1 cell cytokines via its effects on the antigen-presenting cells (15). In addition to the inhibitory effects of IL-10 on T helper 1 cell cytokine production, IL-10 exerts various effects on different cell types, including macrophages (4, 14), B cells (18), cytotoxic T cells (7), mast cells (48), and thymocytes (31). Increasing evidence indicates that IL-10 possesses anti-inflammatory properties. IL-10 has been shown to inhibit the production of many proinflammatory cytokines, such as IL-1, IL-6, TNF-α, IL-8, granulocyte-mono-ocyte colony stimulating factor, granulocyte colony stimulating factor, macrophage inflammatory proteins 1α and 1β, and IL-12, by macrophages/monocytes or polymorphonuclear cells (4, 6, 11, 14, 28, 35). Furthermore, IL-10 has also been reported to down-regulate major histocompatibility complex class II expression (11, 21, 39) and to inhibit leukocyte chemotaxis (28). Interestingly, exogenous IL-10 has been found to exert suppressive effects in vivo, such as preventing endotoxin-induced lethality by inhibiting the overexpression of TNF (17) and delaying the onset of autoimmunity in NZB/W F1 mice (24). Thus, IL-10 appears to be an important immunoregulatory component during inflammatory and immune responses.

In the present study, the endogenous production of IL-10 during type IV GBS infection and its possible regulatory role in proinflammatory cytokine production were investigated. The effect of exogenous IL-10 supplementation on GBS-induced arthritis was also investigated.

MATERIALS AND METHODS

Mice. Outbred CD-1 mice of both sexes that were 8 weeks old were obtained from Charles River Breeding Laboratories (Calco, Italy).
Microorganism. The type IV GBS reference strain GBS 1/82 was used throughout this study. For experimental infection, the microorganisms were grown overnight at 37°C in Todd-Hewitt broth (Oxoid Ltd., Basingstoke, Hampshire, England) and then washed and diluted in RPMI 1640 medium (GIBCO Life Technologies, Milan, Italy). The inoculum size was estimated turbidimetrically, and viability counting was performed by plating preparations on tryptic soy agar–5% sheep blood agar (blood agar) and incubating the preparations 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 10⁷ CFU of GBS/mouse in a volume of 0.5 ml. Control mice were injected by the same route with 0.5 ml of RPMI 1640 medium.

Cytokines and antibodies. Murine recombinant IL-10 (6.7 × 10⁸ U/mg) was purchased from Sigma-Aldrich (Milan, Italy) and diluted according to the manufacturer’s recommendations in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). IL-10 (volume, 0.25 ml) was injected intraperitoneally (i.p.) at doses ranging from 100 to 400 ng/mouse 1 h after GBS infection and on days 1, 2, 3, and 4 after GBS infection. Control mice received PBS plus BSA according to the same protocol. Culture supernatants from the hybridoma line JESS-2A5 (American Type Culture Collection, Rockville, Md.) were used as a source of rat immunoglobulin G1 (IgG1) monoclonal antibodies (MAb) to murine IL-10. Antibody purification was performed by affinity chromatography with protein A-Sepharose CL-4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The protein concentration was determined by measuring the optical density at 280 nm. The neutralizing activity of anti-IL-10 MAb was assessed with a biosay by using MC9/9 cells (50% neutralizing dose, 3 μg/ml). Titration experiments were performed by using different doses of anti-IL-10 MAb (0.25, 0.5, and 1 mg/mouse) given i.p. at the time of infection. Since only the 1-mg/mouse dose resulted in total and prolonged in vivo neutralization of IL-10 activity, as assessed by enzyme-linked immunosorbent assays (ELISA), this dose was chosen for in vivo neutralization experiments. Control mice received 1 mg of normal rat IgG1 (Sigma) per mouse.

Clinical evaluation of arthritis and mortality. Mice injected with GBS and treated or not treated with IL-10 or MAb as described above were evaluated for mortality and arthritis. Mortality was recorded at 24-h intervals for 30 days. After challenge, mice were examined daily by two independent observers (L.T. and M.P.) for 30 days to evaluate the presence of joint inflammation, and scores for arthritis severity (macroscopic scores) were given as previously described (51, 54). Arthritis was defined as visible erythema or swelling of at least one joint. The clinical severity of arthritis was graded on a scale of 0 to 3 for each paw based on changes in erythema and swelling (0 = no change; 1 = mild swelling and erythema; 2 = moderate swelling and erythema; 3 = marked swelling, erythema, and/or ankylosis). Thus, a mouse could have a maximum score of 12. The arthritis index (mean ± standard deviation) was determined by dividing the total score (cumulative value for all paws) by the number of animals used in each experimental group.

Histological assessment. Groups of mice inoculated with GBS and treated or not treated with anti-IL-10 antibodies or IL-10 were examined 7 days after infection for histopathological features of arthritis. Arthritic hind paws (one paw per mouse) were removed aseptically, fixed in 10% (vol/vol) formalin for 24 h, and then decalcified in 5% (vol/vol) trichloroacetic acid for 7 days, dehydrated, embedded in paraffin, sectioned (thickness, 3 to 4 μm), and stained with hematoxylin and eosin. Samples were examined under blinded conditions. Tibia tarsal, tarsus metatarsal, and metatarsus phalangeal joints were examined, and a histologic score was assigned to each joint based on the extent 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. Arthritis severity was classified as mild (minimal infiltrate), moderate (presence of infiltrate, minimal exudate, integrity of joint architecture), or severe (presence of massive infiltrate or exudate, cartilage and bone erosion, and disrupted joint architecture).

FIG. 1. Serum and joint concentrations of IL-10 in mice infected with 10⁷ CFU of GBS/mouse and in control mice. Blood samples and supernatants from joint homogenates were collected at different times after infection and assayed for IL-10 by ELISA. The values are the means ± standard deviations from three separate experiments. Three mice per group were assayed at each time point. An asterisk indicates that the P value is <0.01 (GBS-infected mice versus uninfected controls), as determined by Student’s t test.

GBS growth in blood and joints. Blood and joint infections in GBS-infected mice treated or not treated with anti-IL-10 MAb were determined by evaluation of CFU at different times after inoculation. Blood samples were obtained by retroorbital sinus bleeding before sacrifice. Tenfold dilutions were made 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. All wrist and ankle joints from each mouse were removed, weighed, and homogenized in toto in 1 ml of sterile RPMI 1640 medium/100 mg of joints. After homogenization, joint samples were diluted and plated in triplicate on blood agar.

Sample preparation for cytokine assessment. Blood samples from GBS-infected mice that were treated or not treated with MAb or IL-10 and from uninfected controls were obtained by retroorbital sinus bleeding before sacrifice at different times after infection; sera were stored at −80°C until they were analyzed. Joint tissues were prepared as previously described (51). Briefly, all wrist and ankle joints from each mouse were removed and then homogenized in toto in 1 ml of lysis medium (RPMI 1640 containing [final concentrations] 2 mM...
phenylmethylsulfonyl fluoride, 1 μg of aprotonin per ml, 1 μg of leupeptin per ml, and 1 μg of pepstatin A per ml) per 100 mg of joints. The homogenized tissues were then centrifuged at 2,000 × g for 10 min, and the supernatants were sterilized by using a Millipore filter (pore size, 0.45 μm) and stored at −80°C until they were analyzed.

Cytokine determinations. IL-10, IL-6, IL-1β, and TNF-α concentrations in the biological samples were measured with commercial ELISA kits (Amersham Pharmacia Biotech Ltd., Amersham, United Kingdom) according to the manufacturer’s recommendations. Results were expressed in picograms per milliliter.

Statistical analysis. Differences in the arthritis index, number of CFU, and cytokine concentrations between the groups of mice were analyzed by Student’s t test. Differences between survival data for the groups were analyzed by the Mann-Whitney U test, and the incidence of arthritis and histologic data were analyzed by the χ² test. Each experiment was repeated two or three times. A P value of <0.05 was considered significant.

RESULTS

Detection of endogenous IL-10. IL-10 production was measured in sera and supernatants from the joints at different times after infection with 10⁷ CFU of GBS/mouse. As shown in Fig. 1, detectable levels of IL-10 were evident in the sera of infected mice as early as 4 h after challenge (50 ± 8 pg/ml of serum). A massive increase in IL-10 concentration was already evident at day 1; the peak value was on day 4 (947 ± 60 pg/ml of serum), and this was followed by progressive decreases on the subsequent days. In the joints, GBS infection resulted in significant enhancement (P < 0.01) of IL-10 production at all time points assessed.

Effect of IL-10 blockade on clinical course of arthritis. The clinical signs of joint swelling were observed as early as 24 h after injection of 10⁷ CFU of GBS in 30% of the mice. The incidence of arthritis increased to 85% by day 5, and the maximal prevalence was observed at day 7 after inoculation, when 90% of the mice manifested clinical signs of arthritis (Fig. 2B). Similarly, the arthritis index progressively increased, and the maximum value was obtained 10 days after GBS challenge (mean value ± standard deviation, 4.2 ± 0.5) (Fig. 2C); most of the animals had articular lesions in both the hind paws and the front paws. Forty percent of the mice died during the course of infection (Fig. 2A).

Neutralization of endogenous IL-10 was performed by administering anti-IL-10 MAb (1 mg/mouse) at the time of infection. This treatment affected both mortality and the severity of arthritis. In fact, only 40% of MAb-treated animals survived, compared to 60% of the infected control mice (P < 0.05) (Fig. 2A). Although the increase in the incidence of arthritis was not significant in MAb-treated mice compared with infected controls (Fig. 2B), differences were observed in the severity of articular lesions. The arthritis index reached a value of 5.5 ± 0.4, compared to the value of 4.2 ± 0.5 observed in the infected controls (Fig. 2C). Irrelevant antibodies did not affect either the mortality rates or the severity of arthritis.

Seven days after infection, mice were killed, and the most frequently affected paw (a hind paw) was removed for histologic examination. A microscopic analysis of hematoxylin- and eosin-stained sections was performed. In anti-IL-10-treated mice, 90% of the joints examined were classified as severely affected, with the presence of massive infiltrate and exudate, cartilage and bone erosion, and loss of joint integrity, and 10% were classified as moderately affected (data not shown).
contrast, only 54% of the joints of the untreated infected animals examined were classified as severely affected, and 38% were classified as moderately affected. Moreover, in this group, 8% of the joints were classified as mildly affected (data not shown). Thus, the clinical parameters of disease severity (clinical score) following anti-IL-10 MAb treatment were concordant with the enhanced histopathological severity.

GBS growth rates were also assessed in blood and joints of mice treated or not treated with anti-IL-10 MAb. No significant differences were observed between the experimental groups, regardless of the treatment used (data not shown).

**Effect of IL-10 blockade on cytokine production.** Since IL-6 and IL-1β have been shown to play a major role in the pathogenesis of GBS arthritis (51), the effect of IL-10 blockade on the levels of these cytokines was assayed. Systemic and local cytokine concentrations were measured in plasma and joint specimens. As shown in Fig. 3, early massive production of IL-6 was evident both systemically and at the joint level in anti-IL-10-treated mice. At 24 h after treatment, the IL-6 concentrations in serum and joint supernatants were 2,800 and 2,500 pg/ml, respectively, while 5 days was required to reach such values in untreated infected mice. Treatment with anti-IL-10 MAb also resulted in rapid, sustained local production of IL-1β. In fact, a high concentration of IL-1β (750 pg/ml of joint supernatant) was detected in the joints as early as 24 h after infection and anti-IL-10 MAb administration. Upon treatment with anti-IL-10 antibodies, a TNF-α concentration of 110 pg/ml was observed in the sera of mice 24 h after infection, while barely detectable levels were found in the sera of untreated infected animals. In the joints, IL-10 blockade resulted in significant increases in the TNF-α concentrations compared with those in untreated infected mice on days 1 and 5 after infection; baseline levels were detected in both groups on day 10. Irrelevant antibodies did not affect cytokine production (data not shown).

The efficacy of anti-IL-10 MAb treatment was assessed by measuring IL-10 levels in serum and joints 1, 3, and 5 days after antibody injection. Total elimination of endogenous IL-10 production was observed at all time points and in all samples examined (data not shown).

**Effect of IL-10 administration on mortality and arthritis.** Since endogenous IL-10 neutralization revealed a role for this cytokine in GBS infection, the effect of exogenous IL-10 supplementation on mortality and arthritis was investigated. Mice were injected i.p. with three doses of IL-10 (100, 200, and 400 ng/mouse) once a day for 5 days, starting 1 h after infection. The data in panel A are the cumulative results of three separate experiments, each with 10 animals per experimental group. For panels B and C, the values are the means ± standard deviations from three separate experiments, each with 10 animals per experimental group. *P* = 0.044 (mice treated with 400 ng of IL-10 versus PBS-treated mice) according to the Mann-Whitney *U* test. An asterisk indicates that the *P* value is <0.01 and a dagger indicates that the *P* value is <0.05 (mice treated with 400 or 200 ng of IL-10 versus vehicle-treated mice), as determined by Student’s *t* test.

Days after the end of IL-10 treatment, significant differences were still evident between treated and control mice; the incidence of articular lesions for the treated mice was 50%, compared with 90% for the control mice, and the arthritis indices were 1.8 ± 0.1 and 4.2 ± 0.4 for the treated mice and the control mice, respectively. In contrast, treatment with 200 ng of IL-10 per mouse did not significantly modify the incidence of arthritis, although significant differences in the severity of arthritis were observed as long as IL-10 was administered; the 100-ng/mouse dose was totally ineffective.

Histological analysis confirmed the clinical observations. In fact, 70% of the joints from mice treated with 400 ng of IL-10 per mouse were classified as mildly affected and none was classified as severely affected, while 55% of the joints from infected untreated controls were classified as severely affected and only 7.5% were classified as mildly affected (data not shown). No differences in histopathologic severity were observed between mice treated with 200 ng of IL-10 per mouse and uninfected untreated controls.

**Effect of IL-10 administration on cytokine production.** The animals infected with GBS and treated with IL-10 (400 or 200...
ng/mouse) or PBS plus BSA were monitored for systemic and local production of IL-6, IL-1β, and TNF-α. As shown in Fig. 5, a strong decrease in IL-6 and IL-1β serum concentrations was observed in the infected animals treated with the highest dose of IL-10 compared with infected untreated mice. The effect was limited to the period of IL-10 administration. IL-6 production was also affected by treatment with 200 ng of IL-10 per mouse, although not as dramatically as when the 400-ng/mouse dose was used. Down-regulation of the production of IL-6, IL-1β, and TNF-α following IL-10 treatment was also observed at the joint level. Again, a clear dose-dependent effect was evident.

**DISCUSSION**

The murine model of GBS infection has proven to be beneficial in the elucidation of bacterial and host factors responsible for the development of GBS arthritis (38, 49–52). Our previous studies pointed out a major role for proinflammatory cytokines, such as IL-6 and IL-1β, in the pathogenesis of GBS arthritis (51). In this study, we found that the anti-inflammatory cytokine IL-10 has a relevant influence on the degree of inflammation and severity of arthritis in GBS-infected mice.

GBS challenge induced rapid production of endogenous IL-10, similar to that observed by Cusumano et al. in a neonatal mouse model of type III GBS infection (9) or during *Pseudomonas aeruginosa* (20) or *Haemophilus influenzae* (12) experimental infections. Endogenous IL-10 plays an important role in GBS arthritis, since blockade of IL-10 resulted in 100% of the animals having articular lesions and in an increase in the severity of arthritis. Similar aggravation of the disease was achieved for collagen-induced arthritis (27), as well as in a mouse model of streptococcal cell wall arthritis after administration of neutralizing anti-IL-10 antibodies (30). It has been shown that addition of anti-IL-10 neutralizing antibody to synovial tissue cells isolated from patients with rheumatoid arthritis results in a substantial increase in IL-1β and TNF-α levels (30, 34), suggesting that IL-10 spontaneously produced in rheumatoid arthritis is an important immunomodulatory component of the cytokine network in this type of arthritis. In the present study, early sustained local production of IL-6, IL-1β, and TNF-α was observed as soon as 24 h after IL-10 blockade. This enhancement of proinflammatory cytokines is likely responsible for the worsening of articular lesions. In fact, TNF-α and IL-1β are known to contribute directly to tissue damage through induction of the release of tissue-damaging enzymes from synovial cells and articular chondrocytes and through activation of osteoclasts (2, 47, 53). Moreover, IL-6 participates together with IL-1 in catabolism of connective tissue components at sites of inflammation (25, 36) and activates osteoclasts, with a consequent increase in joint damage (19). The early exacerbated inflammatory response caused by IL-10 neutralization could also account for the increase in mortality rates observed in the anti-IL-10-treated mice. Interestingly, high levels of TNF-α were observed in the serum of anti-IL-10 treated mice, but not in untreated infected animals. Elevated concentrations of this cytokine have been reported to be correlated with disease severity and mortality in other animal models of GBS infection (45, 46). Thus, these results strongly suggest a regulatory role for endogenous IL-10 during the early stage of infection.

Addition of exogenous IL-10 increased the effect of endogenous IL-10, resulting in enhancement of survival and amelio-
ration of GBS arthritis. Systemic administration of recombinant IL-10 has been reported to suppress the incidence, to delay the onset, and to reduce the severity of disease in mice and rats with collagen-induced arthritis (27, 37, 44, 55). In a model of experimental streptococcal cell wall arthritis, amelioration of articular lesions after IL-10 treatment was associated with a decrease in the local levels of TNF-α (30). In vitro studies with isolated synovial tissues from patients with rheumatoid arthritis demonstrated that addition of exogenous IL-10 was able to suppress TNF-α and IL-1β production (29, 33). In our experimental model, the amelioration of articular lesions observed upon IL-10 administration was accompanied by a reduction in the local levels of IL-6, IL-1β, and TNF-α in a dose-dependent manner. Interestingly, administration of the 400-ng/mouse dose efficiently down-regulated cytokine production even after the end of the treatment, while the 200-ng/mouse dose was active only during treatment, thus reflecting clinical observations.

Severity of GBS arthritis is dictated by different parameters, including the extent of inflammatory infiltrate and exudate in the joints. A number of animal models have shown that endogenous production or administration of IL-10 may be beneficial in reducing organ injury as a consequence of the reduction in the number of inflammatory cells accumulating in these organs (1, 5, 22, 23). In our model, histological analysis revealed the presence of elevated inflammatory cells accumulating in these organs (1, 5, 22, 23). In our model, histological analysis revealed the presence of elevated inflammatory cells accumulating in these organs (1, 5, 22, 23). In our model, histological analysis revealed the presence of elevated inflammatory cells accumulating in these organs (1, 5, 22, 23). In our model, histological analysis revealed the presence of elevated inflammatory cells accumulating in these organs (1, 5, 22, 23).

In summary, this study provided evidence for a protective effect of IL-10 in GBS arthritis. Endogenous IL-10 is important in the early stages of the infection, although the levels produced in response to bacterial challenge are not sufficient to block progression of the disease. The beneficial effects of exogenous IL-10 appear to be associated with the ability of IL-10 to suppress inflammatory cytokine production and leukocyte recruitment at the joint level. From these findings IL-10 might emerge as a candidate for treatment of GBS arthritis. Although an excessive inflammatory response can lead to detrimental outcomes, such as arthritis, inflammation is nonetheless an essential component of host defenses against infections. The doses and timing of IL-10 administration used in this study seem to be effective and to not produce side effects. However, further studies are needed to determine the conditions under which IL-10 treatment produces the maximum protective effect in humans with GBS infections.

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