Severity of Group B Streptococcal Arthritis Is Correlated with β-Hemolysin Expression

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Septic arthritis is a clinical manifestation of group B streptococcal (GBS) infection in neonates and adults. To examine the potential role of GBS β-hemolysin in joint injury, mice were infected with 2 wild-type strains or with nonhemolytic (NH) or hyperhemolytic (HH) variants derived by transposon mutagenesis. Compared with mice infected with the parent strains, mice infected with the NH mutants had decreased mortality and bacterial proliferation. A reduced LD_{50} and a higher microbial load were obtained in mice infected with the HH mutants. Greater degrees of joint inflammation and damage were observed in the HH mutant–infected animals than in those infected with the parental strains. NH mutant–infected mice manifested only a mild and transient arthritis. Systemic and local levels of interleukin-6 mirrored the observed differences in virulence and severity of arthritis. These data support a direct correlation of GBS β-hemolysin expression with mortality and severity of articular lesions.

Group B streptococci (GBS) are a leading cause of life-threatening infection in neonates and young infants [1]. Invasive neonatal GBS infection has either an early (usually within 24 h of birth) or late (>7 days after birth) onset. Common manifestations of GBS disease in neonates are pneumonia, septicemia, meningitis, bacteremia, and bone or joint infections [1, 2]. Invasive infections caused by GBS have been increasingly recognized in adult populations [3, 4].

Septic arthritis has been well described as a clinical manifestation of late-onset GBS infection in neonates [1, 2]. In adults, GBS arthritis is often associated with advanced age and other risk factors, including cancer, diabetes mellitus, cardiovascular disease, chronic renal insufficiency, alcoholism, human immunodeficiency virus infection, neurologic disease, or cirrhosis [5–7]. Previously, we described an experimental mouse model of type IV GBS systemic infection with clinical features that closely resemble infection in humans, in particular the appearance of multifocal septic arthritis [8]. Subsequently, our studies demonstrated that GBS serotypes II, III, V, VI, and VII are also able to produce septic arthritis and that the incidence of articular lesions is influenced by the presence and amount of surface capsule, as well as by the amount of sialic acid in the capsular polysaccharide [9]. However, other bacterial factors also could contribute to the development and severity of articular lesions.

Most GBS clinical isolates demonstrate β-hemolysis when plated on sheep blood agar [10]. The GBS β-hemolysin is a potent membrane cytotoxin that is known to injure lung epithelial [11], lung endothelial [12], and brain endothelial cells [13] in vitro and thus is hypothesized to contribute to the pathogenesis of neonatal pneumonia and meningitis. Limited data exist, however, on the contribution of β-hemolysin to virulence in animal models of GBS disease. In neonatal rat models of pneumonia, mutants with a nonhemolytic (NH) phenotype were less virulent, and mutants with a hyperhemolytic (HH) phenotype more virulent, than the parental strains, suggesting involvement of the β-hemolysin in the initial pulmonary stages of early-onset neonatal disease [14, 15]. A potential role for the β-hemolysin in septicemia and in the localized complications, such as arthritis, that characterize late-onset neonatal and adult GBS infection has yet to be defined.

The aim of the present study was to perform a detailed investigation on the role of β-hemolysin in the development and severity of GBS septic arthritis in the murine model. Two type III GBS clinical isolates of differing hemolytic potential were selected for testing, along with isogenic transposon insertion mutants exhibiting an NH or HH phenotype relative to the parent strains. Because we recently documented a strong involvement of interleukin (IL)–6 and IL-1β but not tumor necrosis factor (TNF)–α in the pathogenesis of GBS arthritis [16] during type IV GBS infection, levels of these proinflammatory

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Table 1. Characterization of isogenic group B streptococcal strains.

Strain	Mutation	Reference	Hemolytic titer
COH-1	Wild type	[20]	1
COH1-20	Tn 916ΔE mutant	[11, 20]	0
IN-40	Tn 916ΔE mutant	[11, 20]	32
COH 31 r/s	Wild type	[19]	2–4
COH 31c12	Tn 916 mutant	[11, 21]	0
COH 31c35	Tn 916 mutant	[11, 21]	16

cytokines induced by type III strains of differing β-hemolysin expression were also analyzed.

Materials and Methods

Mice. Outbred CD1 mice of both sexes, 8 weeks old, were obtained from Charles River Breeding Laboratories (Calco, Milan, Italy).

Bacterial strains. Two serotype III GBS clinical isolates, together with their isogenic NH or HH mutants derived by transposon insertional mutagenesis [11], were used in this study. Strain COH1, from an infant with bacteremia [17, 18], is a highly encapsulated weakly hemolytic isolate, whereas COH31 r/s, from a diabetic adult with a colonized foot ulcer [19], is a weakly encapsulated isolate more hemolytic than COH1. The COH1-derived isogenic mutants were COH1-20 (NH) and IN-40 (HH) [11, 20], and the COH31 r/s–derived isogenic mutants were COH31c12 (NH) and COH31c35 (HH) [11, 21]. GBS strains were provided by C. E. Rubens (Children's Hospital and Medical Center, Seattle). All strains were tested for group B antigen expression by latex agglutination (Streptex; Glaxo-Wellcome, London), production of type-specific capsule by immunoblot assay, logarithmic growth rate in Todd-Hewitt (TH) broth (Oxoid, Basingstoke, UK) by optical density assay, production of CAMP factor on blood agar, and biochemical profile by use of a kit (API 20 Strep identification kit; BioMérieux, St. Louis) [11]. Mutants did not differ from their respective parent strains, except for slightly reduced CAMP factor expression by HH strains [11, 12]. The hemolytic activity (titer) of all GBS strains was defined elsewhere by Nizet et al. [11], by a modification of earlier methods [22, 23]. The assay conditions were standardized such that the weakly hemolytic wild-type strain COH1 possessed a hemolytic titer of 1. A detailed description of the GBS bacterial strains is shown in table 1. For experimental infection, all GBS strains were grown overnight at 37°C in TH broth and then washed, resuspended, and serially diluted in RPMI 1640 medium (GIBCO Life Technologies, Milan, Italy). The inoculum size was estimated turbidimetrically and corroborated by plate counts, as described elsewhere [8]. Presence of group B antigen, expected hemolytic phenotype, and transposon antibiotic marker were verified for each pure culture. Mice were inoculated intravenously (iv) in the tail vein with the desired number of microorganisms in a volume of 0.5 mL.

Virulence determination. To evaluate the overall virulence of the different GBS strains, groups of 20 CD1 mice were inoculated iv with 10^6–10^9 cfu/mouse, and mortality was recorded at 24-h intervals for 60 days. The LD_{50}, calculated by the method of Reed and Muench [24], represented the mean of 3 experiments.

Clinical evaluation of arthritis. Mice injected with 10^7 cfu of the different GBS strains were examined daily by 2 independent observers for 2 months, to evaluate signs of joint inflammation. Arthritis was defined as a visible erythema or swelling of $\geqslant 1$ joint. Time of onset, number of joints involved, duration of arthritis, and occurrence of ankylosis were recorded. To evaluate the intensity of arthritis, the following clinical scoring (arthritic index) was used for each limb: 0, no swelling or erythema; 1, mild swelling and erythema; 2, moderate swelling and erythema; and 3, marked swelling, erythema, and/or ankylosis. Thus, a mouse could have a maximum score of 12. The arthritic index was constructed by dividing the total score by the number of all animals used in each experimental group.

Histologic studies. To confirm clinical features of arthritis, groups of mice inoculated iv with 10^7 cfu of the different GBS strains/mouse were examined at selected intervals after infection for histopathologic features of arthritis. Joints were removed aseptically, fixed in 10% formalin (vol/vol) for 24 h, and then decalcified in 5% trichloroacetic acid (vol/vol) for 7 days, dehydrated, embedded in paraffin, sectioned at 5–7 μm, and stained with hematoxylin-eosin. Biopsy samples were examined under blinded conditions for the presence of synovial hypertrophy and cartilage and/or bone destruction.

GBS growth in blood, kidneys, and joints. Blood, kidney, and joint infections were assessed by quantitative culture at different times after inoculation with 10^7 cfu of the different GBS strains. Blood samples were obtained by retro-orbital sinus bleeding before death. Tenfold dilutions were made in RPMI 1640 medium, and 0.1 mL of each dilution was plated in triplicate on TH agar and incubated under anaerobic conditions for 24 h. The number of colony-forming units was determined, and the results were expressed per milliliter of blood. Kidneys were aseptically removed and homogenized with 3 mL of sterile RPMI 1640. Articular samples were removed, weighed, and homogenized in 1 mL of sterile RPMI 1640 medium/100 mg of joint weight. After homogenization, all tissue samples were diluted and plated in triplicate on TH agar, and results were expressed as the number of colony-forming units per whole organ or per milliliter of joint homogenate. GBS strains recovered from mice were always tested for phenotypic and biochemical characteristics as specified above, to verify the in vivo stability of the mutation.

Sample preparation for cytokine assessment. Because our previous study on type IV GBS has shown that maximal severity of arthritis and the highest cytokine levels are observed at day 10 after infection [16], blood and joint samples were collected at this time for cytokine assessment. Blood samples from mice injected with 10^7 cfu of each strain used in this study and from uninfected controls were obtained by retro-orbital sinus bleeding before death; serum samples were stored at −80°C until analysis. Joint tissues were prepared, as described elsewhere [16]. In brief, articular samples were removed and then homogenized in 1 mL of lysis medium (RPMI 1640 containing 2 mM phenylmethylsulfonyl fluoride and 1 mg/mL aprotinin, leupeptin, and pepstatin A)/100 mg joint weight. The homogenized tissues were then centrifuged at 2000 g for 10 min, and supernatants were sterilized by use of a filter (0.45 mm; Millipore, Bedford, MA) and stored at −80°C until analysis.

Cytokine determination. IL-6, IL-1β, and TNF-α levels in the serum and joints were measured with commercial mouse ELISA kits (Amersham Pharmacia Biotech, Amersham, UK) according

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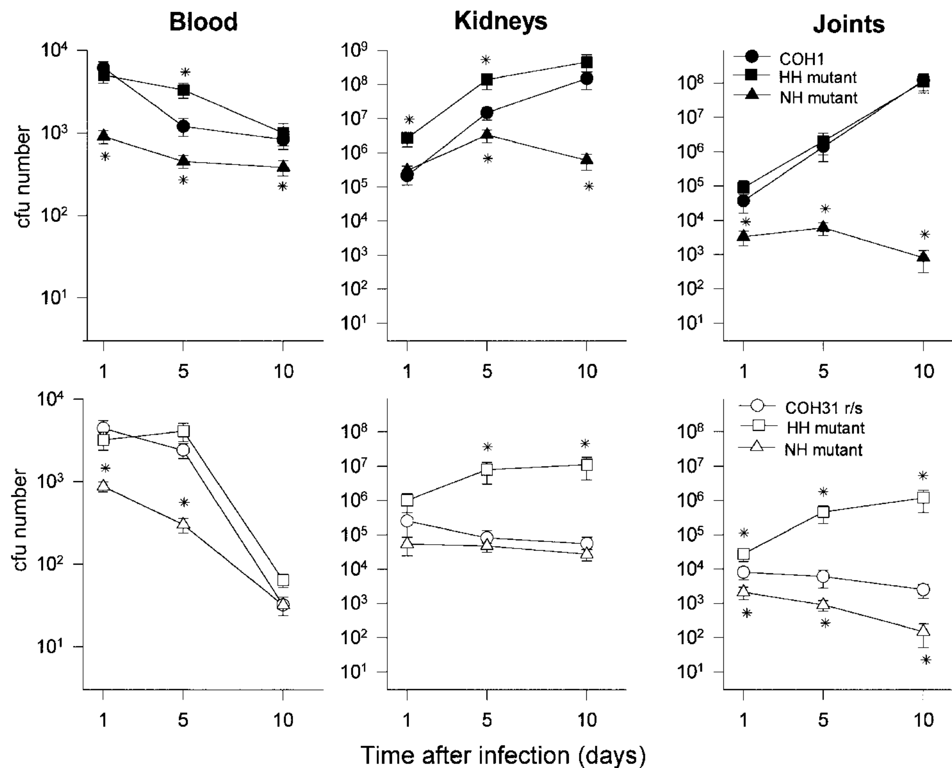

Figure 1. Growth kinetics of type III group B streptococcal strain COH1 (parental strain), IN-40 (hyperhemolytic [HH] mutant), COH1-20 (nonhemolytic [NH] mutant), COH31 r/s (parental strain), COH31c35 (HH mutant), or COH31c12 (NH mutant) in blood, kidneys, and joints of CD1 mice. Mice were intravenously injected with 10^7 cfu/mouse at time 0, and colony-forming units per milliliter of blood, per milliliter of both kidneys, or per milliliter of joint homogenate were determined. Results are mean ± SD of 3 separate experiments. Five mice/group were killed at each time point. *$P < .001$ (HH or NH mutants vs. parental strains, Student's t test).

to the manufacturer's recommendations. Results were expressed as picograms per milliliter of serum or of supernatants from joint homogenates. The detection limit of the assay was 7 pg/mL for IL-6, 3 pg/mL for IL-1β, and 10 pg/mL for TNF-α.

Statistical analysis. Differences in LD_{50}, quantitative bacterial culture, and cytokine concentrations between the groups of mice injected with the different GBS strains were analyzed by Student's t test. Differences in median survival times were evaluated by Mann-Whitney U test. Comparison of the incidence of arthritis was done by the χ^2 test, and differences in the arthritic index by Student's t test. Each experiment was repeated 3–5 times. $P < .05$ was considered significant.

Results

Effect of β-hemolysin expression on animal virulence. To assess the role of β-hemolysin expression on mortality, groups of CD1 mice were injected iv with different doses (10^6–10^9 cfu/mouse) of GBS clinical isolates or their isogenic hemolysin mutants. The LD_{50} and median survival time after iv inoculation with 10^8 or 10^9 cfu of each strain are shown in table 2. The LD_{50} of the weakly hemolytic but heavily encapsulated strain COH1 was 10-fold lower than that for the more hemolytic but weakly encapsulated strain COH31 r/s, confirming the well-documented importance of capsular polysaccharide in animal virulence. For the less virulent COH31 series, the LD_{50} was increased by 50% ($P < .01$) for the NH mutant and reduced >6-fold ($P < .001$) for the HH mutant, compared with the parent strain. For the highly virulent COH1 series, the LD_{50} was increased 20-fold ($P < .001$) for the NH mutant and decreased 13% for the HH mutant, but the latter did not reach statistical significance ($P = .149$). Median survival time was dramatically increased (from 4 to >60 days) for the NH mutant COH1-20 versus the parent strain COH1 and was markedly decreased (from >60 to 7 days) for the HH mutant COH31c35 versus the parent strain COH31 r/s (both $P < .001$).

Recovery of GBS from the blood, kidneys, and joints. Quantitative monitoring of bacteremia and GBS growth in the kidneys and joints was done 1, 5, and 10 days after iv injection of each wild-type strain or hemolysin mutant at 10^7 cfu/mouse (figure 1). The number of colony-forming units of the highly virulent strain COH1, its NH mutant, or its HH mutant in blood decreased slowly from days 1 to 10, although ~10^3 microorganisms/mL were still present at this time. In contrast, rapid and progressive growth of the parental strain and the HH

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Table 2. Virulence of type III group B streptococcal clinical isolates and their mutants in CD1 mice.

Strain	Description	LD_{50}	MST (no. dead/total)[a]	
			10^8 cfu/mouse	10^9 cfu/mouse
COH1	Wild type	1.48×10^7	4 (20/20)	1 (20/20)
COH1-20	NH mutant	2.81×10^8[b]	>60[b] (2/20)	1.5 (20/20)
IN-40	HH mutant	1.29×10^7	4 (20/20)	1 (20/20)
COH31 r/s	Wild type	1.94×10^8	>60 (6/20)	1 (20/20)
COH31c12	NH mutant	3.02×10^8[c]	>60 (1/20)	2 (20/20)
COH31c35	HH mutant	3.16×10^7[b]	7[b] (20/20)	1 (20/20)

NOTE. Mice were injected intravenously with 106–109 cfu of each strain at day 0. LD_{50} values represent means of 3 separate experiments (SDs, always <10%, were omitted). MST, median survival time in days; NH, nonhemolytic; HH, hyperhemolytic.

[a] At day 60.

[b] $P < .001$ (mutant vs. parental strains, Student's *t* test for LD_{50} and Mann-Whitney *U* test for MST).

[c] $P < .01$ (mutant vs. parental strains, Student's *t* test for LD_{50} and Mann-Whitney *U* test for MST).

mutant were observed in the kidneys and joints; the number of microorganisms increased to $>10^8$ on day 10 after infection. The NH mutant did not show similar proliferation. This disparity was most evident in the joints, where highly significant differences ($P < .001$) in colony-forming units recovered between the NH mutant and wild-type COH1 were observed at all time points tested.

When the less virulent GBS clinical isolate COH31 r/s and its isogenic hemolysin mutants were used, the number of microorganisms in the blood dropped from days 1 to 10, with clearance of all strains within 15 days (data not shown). Only the HH mutant showed progressive growth in the kidneys and joints, with ∼100-fold more than of parent COH31 r/s recovered at days 5 and 10 after infection ($P < .001$). No significant differences in organisms recovered from the kidneys were observed between strain COH31 r/s and its NH mutant; however, significantly fewer colony-forming units of the NH mutant were detected at all time points in the joints. All of the GBS strains and mutants recovered from mice maintained the original phenotypic and biochemical characteristics observed before in vivo inoculation.

Induction of septic arthritis. Parental strains and their NH and HH mutants were assessed for their ability to induce arthritis. Incidence and severity of articular lesions were evaluated after inoculation of each strain at 10^7 cfu/mouse, and a clinical scoring system was used to calculate arthritic index (figure 2). Parental strain COH1 and its HH mutant had a comparable incidence of articular lesions, except at day 5 after infection, when the percentage of articular lesions produced by the HH mutant was significantly ($P < .05$) higher. Furthermore, at days 5 and 10 after infection, the severity of arthritis was more pronounced in mice injected with the HH mutant, compared with the parental strain (mean ± SD: day 5, 2.0 ± 0.2 vs. 1.5 ± 0.1; day 10, 2.8 ± 0.3 vs. 2.3 ± 0.2). Lack of β-hemolysin expression in mutant COH1-20 resulted in a decreased incidence of arthritis, with all animals negative at day 20 after infection. Furthermore, the arthritic index for the NH mutant never exceeded a value of 0.8 ± 0.05.

The effect of overproduction or loss of β-hemolysin on development of arthritis was even more pronounced for COH31 r/s and its mutants. Compared with the parental strain, the incidence of arthritis was significantly higher ($P < .01$) in animals infected with the HH mutant and significantly lower ($P < .01$) in those infected with the NH mutant, beginning day 2 after infection. A corresponding pattern of marked differences among the 3 groups was evident when severity of arthritis was evaluated.

Histopathology. Histopathologic studies from the joints of mice injected with 10^7 cfu of each type III GBS strain and hemolysin mutant were done to confirm clinical signs of arthritis. An acute exudative synovitis with neutrophilic and monocytic infiltrates of the subsynovial and periarticular connective tissues was observed 2 days after infection with clinical isolate COH1, its HH mutant, and the HH mutant of isolate COH31 r/s (COH31c35). By day 10, articular cavities were filled with purulent exudate (figure 3*A,* 3*B,* 3*E*). Subsequently, arthritis progressed to joint destruction, loss of cartilage, bone erosion, and proliferation of granulation tissue (data not shown). In contrast, the joints of mice injected with strain COH31 r/s exhibited a reduced inflammatory process in the first days of infection, and, on day 10, the purulent infiltrate was limited to the subcutaneous and periarticular tissues (figure 3 *D*). In this group of mice, complete recovery was observed within 30 days after infection (data not shown). Finally, although at visual inspection the animals infected with strain COH1-20 and strain COH31c12 (NH mutants of the 2 clinical isolates) showed arthritic indices of ∼0.6 and ∼0.3, respectively, no synovitis or purulent infiltrate were observed at day 10 in the histologic samples (figure 3 *C,* 3*F*).

Cytokine levels in the serum and joints. To assess the inflammatory response of the different type III GBS strains to infection, samples from the blood and joints were taken on day 10, and cytokine levels were measured. As shown in figure 4, mice infected with HH mutants of both clinical isolates displayed significantly higher ($P < .001$) serum levels of IL-6 than did mice infected with the parental strains, whereas lack of β-hemolysin expression in NH mutants resulted in a significant decrease in IL-6 serum secretion, compared with mice infected with wild-type strains. Higher concentrations of the cytokine were also found in the joints of HH mutant–infected mice, compared with those infected with the parental strains. The effect of eliminating β-hemolysin production was also evident in the joints. IL-6 concentrations in joints of mice injected with the NH mutants COH1-20 and COH31c12 were 3- and 2-fold lower, respectively, than those detected in wild-type–injected mice. Although similar IL-1β concentrations were observed in serum of mice injected with HH mutants, NH mutants, or parental strains, significantly lower ($P < .001$) levels of IL-1β were detected in the joints of NH mutant–infected mice, com-

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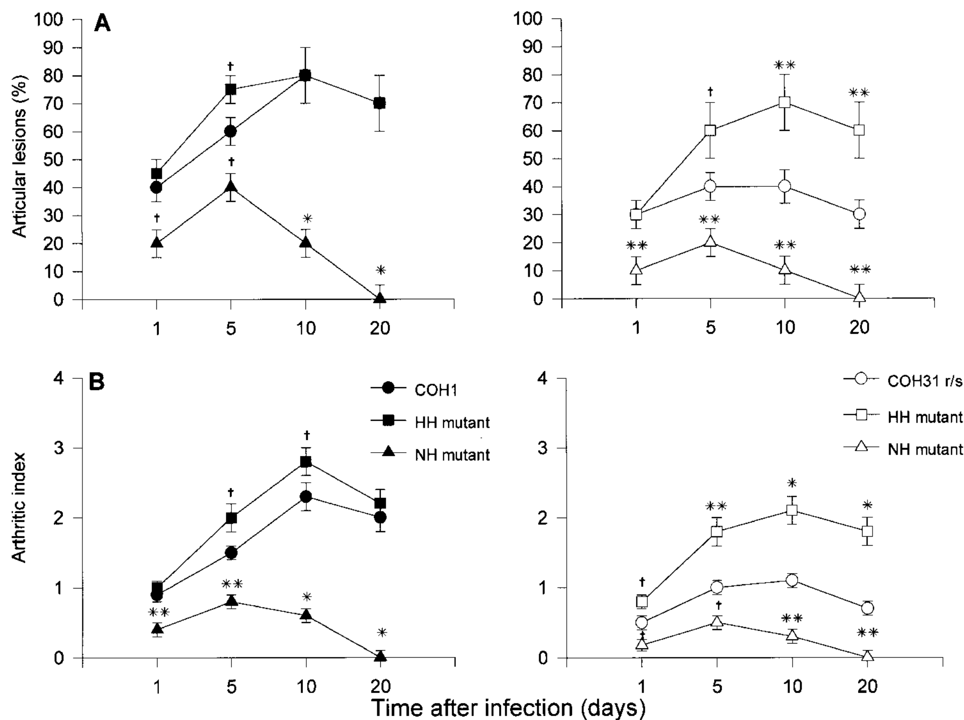

Figure 2. Incidence *(A)* and severity *(B)* of arthritis in CD1 mice injected with type III group B streptococcal strain COH1 (parental strain), IN-40 (hyperhemolytic [HH] mutant), COH1-20 (nonhemolytic [NH] mutant), COH31 r/s (parental strain), COH31c35 (HH mutant), or COH31c12 (NH mutant). Mice were injected intravenously with 10^7 cfu/mouse. Incidence and severity of arthritis were evaluated as described in Methods. Results are mean ± SD of 3 separate experiments with 20 mice each. *$P < .001$; **$P < .01$; †$P < .05$ (HH or NH mutants vs. parental strains, χ^2 for *A* and Student's *t* test for *B*).

pared with those infected with the parental strains. An effect of β-hemolysin overproduction leading to increased intra-articular IL-1β secretion was evident in mice infected with the HH mutant IN-40, compared with the parent strain COH1 ($P < .01$). No differences were observed in systemic and local TNF-α concentrations in mice injected with the different strains; the cytokine level never exceeded a value of 50 pg/mL in the serum and joints (data not shown).

Discussion

Although produced by most GBS clinical isolates, the GBS β-hemolysin has yet to be fully characterized. A major factor complicating analysis is that extracellular hemolytic activity is rapidly lost unless high-molecular-weight stabilizer molecules (albumin, starch, Tween 80) are present in the media [22, 25–27]. Nonetheless, the development of isogenic NH and HH transposon mutants of GBS wild-type strains has yielded powerful tools to explore the role of this β-hemolysin in various model systems of disease pathogenesis. Compared with the wild-type strains, HH mutants are more injurious, and NH mutants less injurious, to human lung epithelial cells [11], lung endothelial cells [12], and brain endothelial cells [13]. Hemolytic and cytolytic properties of hemolysin appear to involve pore formation in the target cell membrane [11, 23].

In the present study, involvement of the GBS β-hemolysin in animal virulence and in the pathogenesis of multifocal septic arthritis was demonstrated by use of isogenic NH and HH transposon mutants of 2 type III GBS clinical isolates. Previously, a role for β-hemolysin in virulence has been established only in GBS infection via the respiratory tract. For example, NH mutants were less virulent than the wild-type strains after intranasal inoculation of adult mice [15] or intrathoracic inoculation of infant rats [14]. However, previous studies on the effects of GBS β-hemolysin phenotype after establishment of systemic GBS infections yielded conflicting data. No difference in LD_{50} or altered pathophysiology was observed after subcutaneous or iv injection of NH mutants and parental strain in neonatal rats or piglets, respectively [21, 28]. In contrast, iv challenge of rabbits with the HH mutants resulted in significantly higher mortality and evidence of disseminated intravascular coagulation, compared with the parental strain or NH mutants [29]. Furthermore, crude β-hemolysin preparations from GBS cultures induce cardiotoxicity and hypotension after iv administration to rabbits or rats, a finding not observed with streptolysin S of group A streptococci [30].

Our results clearly demonstrate that loss or enhancement of

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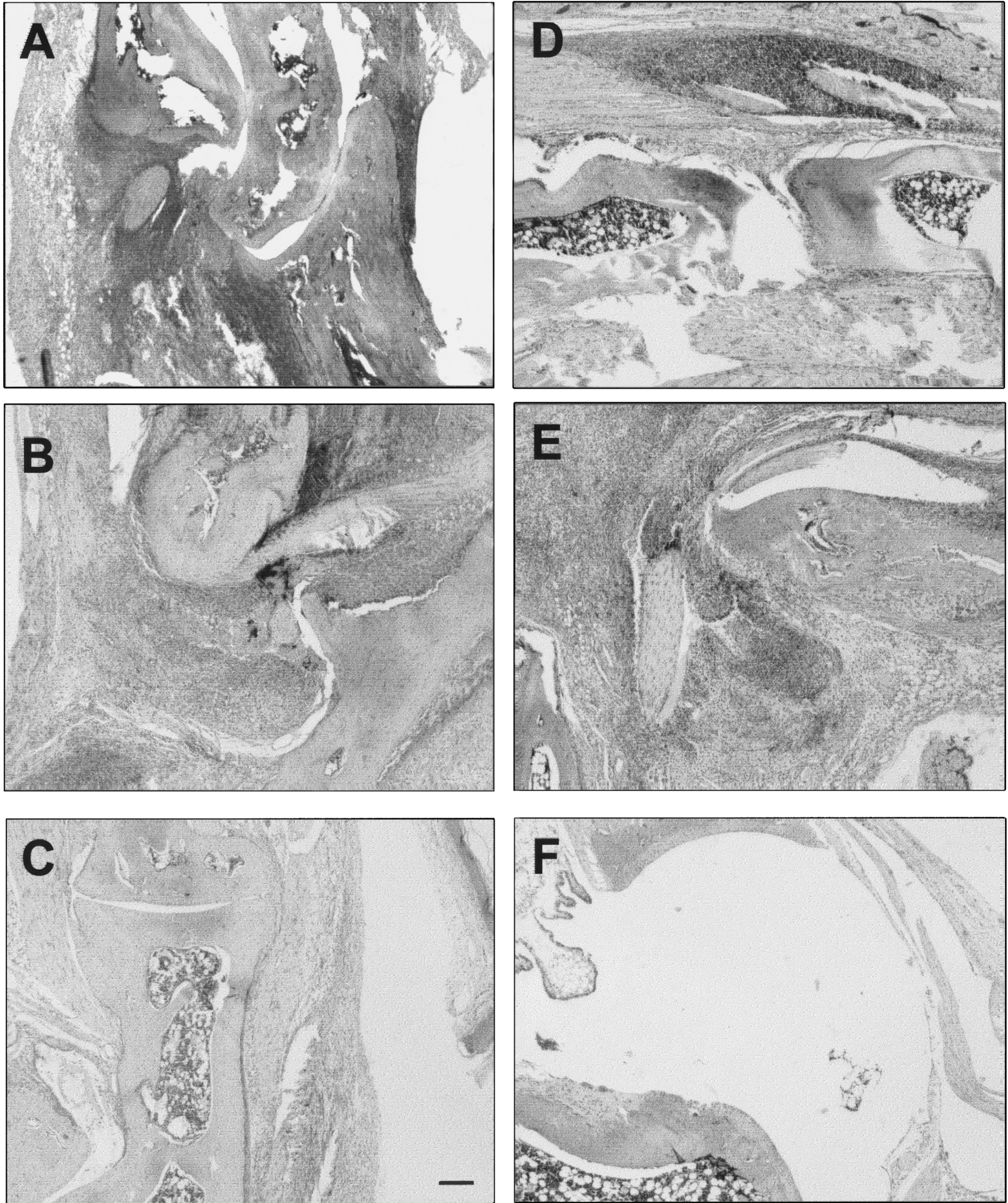

Figure 3. Histologic examination at day 10 of joints of mice injected with type III group B streptococcal strain: COH1 (parental strain; *A*); IN-40 (hyperhemolytic mutant; *B*); COH1-20 (nonhemolytic mutant; *C*); COH31r/s (parental strain; *D*); COH31c35 (hyperhemolytic mutant; *E*); and COH31c12 (nonhemolytic mutant; *F*). Mice were injected intravenously with 10^7 cfu/mouse at time 0. Samples from joints of forepaws were collected, processed, and stained (original magnification, ×4; bar, 100 μm).

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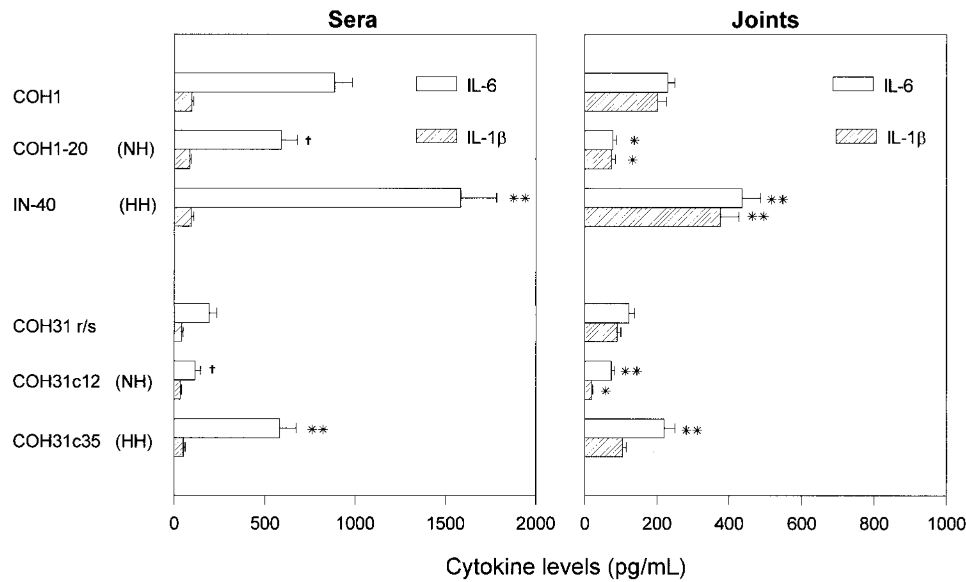

Figure 4. Interleukin (IL)–6 and IL-1β levels in sera or joints of mice at day 10 after inoculation with type III group B streptococcal parental strains or their hyperhemolytic (HH) or nonhemolytic (NH) mutants. Mice were injected intravenously with 10^7 cfu/mouse at time 0, and IL-6 and IL-1β concentrations in serum (picograms per milliliter) and joints (picograms per milliliter of supernatants from joint homogenate) were evaluated at day 10. Values represent mean ± SD of 3 separate experiments with 5 mice/group for each. *$P < .001$; **$P < .01$; †$P < .05$ (HH or NH mutants vs. parental strains, Student's *t* test).

β-hemolysin expression strongly influenced the virulence of GBS strains in adult mice. However, we found that the magnitude and direction of this effect are dependent on the background virulence of the parent strain. COH1 is a highly encapsulated, highly virulent GBS strain [17, 20]. In this background, the augmentation of a potential virulence factor in the isogenic HH mutant did not result in a further increase in pathogenicity, whereas the lack of β-hemolysin in the NH mutant impaired virulence significantly. In contrast, COH31 r/s is a weakly encapsulated and much less virulent parent strain [19]. In this background, the overproduction of β-hemolysin in the HH mutant led to a strong enhancement of virulence, whereas loss of hemolytic activity in the NH mutant resulted in only a modest, yet significant, increase in LD_{50}. Data on in vivo survival and proliferation of GBS strains demonstrated that clearance or persistence of bacteria correlated not only with the degree of encapsulation, as demonstrated elsewhere [9], but also with the level of β-hemolysin expression.

We have shown elsewhere that the appearance and severity of articular lesions are dependent on the viability and number of GBS injected [8] and on the presence and amount of surface capsule, as well as the presence of sialic acid in the capsular polysaccharide [9]. In this study, we for the first time demonstrate that the GBS β-hemolysin independently contributes to the appearance and severity of articular lesions. This represents the first evidence for this cytotoxin as a virulence factor in bone and joint infections characteristically observed in late-onset neonatal and adult GBS disease.

The GBS β-hemolysin, like other bacterial pore-forming toxins, possesses a broader range of host cell cytotoxicity and is therefore better classified as a cytotoxin [11–13, 26]. Several other gram-positive hemolysins, such as the α toxin of *Staphylococcus aureus* [31], streptolysin O [32] and S [33] of group A streptococci, and the plasmid pAD1-encoded hemolysin-bacteriocin of *Enterococcus faecalis* [34], are also known to injure mammalian epithelial cells and potentially contribute to virulence of the organism. The role of hemolysins produced by *S. aureus* in the pathogenesis of arthritis has been well documented [35, 36]. In a mouse model, *S. aureus* α toxin expression appeared to contribute to induction and severity of articular lesions [35], whereas *S. aureus* α and γ toxins jointly promoted progression of arthritis in subsequent studies [36].

In our experimental model, both the incidence and severity of arthritis appeared to be strongly influenced by GBS β-hemolysin expression. Inoculation of the HH mutant of the heavily encapsulated strain COH1 did not further enhance the already high incidence of arthritis observed with the parent strain but did result in a significantly greater severity of arthritis. Loss of β-hemolysin production in the NH mutant resulted in mild and transient arthritis. In the COH31 r/s series, both the incidence and severity of arthritis were increased with the HH mutant, reaching levels comparable to those seen with the more virulent parent strain COH1. Therefore, in this model of infection, overexpression of β-hemolysin compensated fully for the decreased virulence potential associated with poor encapsulation.

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In vitro studies making use of human epithelial and endothelial cell lines show a direct correlation of β-hemolysin expression with cellular injury [11–13]. Electron microscopy of injured cells revealed membrane disruptions, cellular swelling, loss of intracytoplasmatic density, and changes in organelles and nuclear chromatin [11, 13]. Our histologic studies were not able to directly confirm the destructive effect of β-hemolysin on joint tissues. However, even when similar numbers of organisms were recovered from the joints of mice infected with COH1 or its HH mutant, the severity of arthritis seen with the HH mutant was significantly greater than that observed with the parent strain. Thus, we can hypothesize a direct contribution of β-hemolysin to the worsening of arthritis.

The appearance of articular lesions is undoubtedly the byproduct of a multifactorial process. For instance, participation of proinflammatory cytokines in the pathogenesis of arthritis has been documented in both human and animal models [37–40]. In particular, IL-6 levels are persistently high in the synovial fluid of human patients with bacterial septic arthritis [41] and correlate with the severity of disease in murine *S. aureus* septic arthritis [36, 42]. In another study, the peptidoglycan component of the gram-positive cell wall has been shown to contribute to IL-6 induction and severity of experimental arthritis [43]. IL-6 is involved together with IL-1 in catabolism of connective tissue components at sites of inflammation [44, 45] and activates osteoclasts, with a consequent increase of joint damage during the arthritic process [46]. We demonstrated elsewhere that, in type IV GBS infection in mice, there is a direct correlation between severity of arthritis and high levels of IL-6 and IL-1β in the joints [16]. Here, we provide evidence that type III GBS infection also induces IL-6 and IL-1β secretion. In addition, we found a direct correlation of GBS β-hemolysin production with systemic and intra-articular IL-6 levels. HH mutants induced greater degrees of IL-6 release than did the parent strains, both systemically and in the joints. In contrast, the mild and transient arthritis observed in mice treated with NH mutants corresponded to the lowest IL-6 levels detected in the joints. Absence of β-hemolysin production also decreased IL-1β levels in the joints. As demonstrated elsewhere, in type IV GBS infection [16], TNF-α was not involved in the pathogenesis of arthritis induced by the type III strains.

In conclusion, our studies of experimental GBS arthritis in mice demonstrate that β-hemolysin expression contributes to the incidence and severity of joint lesions as well as overall mortality. These findings implicate the GBS β-hemolysin as a virulence factor in the pathogenesis of arthritis seen in late-onset neonatal and adult GBS infections.

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