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Role of macrophages in experimental group B streptococcal arthritis

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Summary

Septic arthritis is a clinical manifestation of group B *Streptococcus* (GBS) infection in both neonates and adults. Because macrophages are known to participate in tissue injury, the role of this cell population in GBS-induced arthritis was investigated. Mice were rendered monocytopenic by administration of etoposide, a drug that selectively depletes the monocyte/macrophage population and then injected with GBS (1×10^7 colony-forming units per mouse). Appearance of arthritis, mortality, GBS growth in the organs, and local and systemic cytokine production were examined. Etoposide-treated mice had a significantly less severe arthritis than control animals. Histopathological analysis of the joints confirmed clinical observations. Decreased joint levels of the proinflammatory cytokines interleukin 1 (IL-1) beta and IL-6 accompanied the less severe development of arthritis in monocytopenic mice. In contrast, mortality was increased in the etoposide-treated mice compared with controls. Monocytopenic mice exhibited elevated bacterial load in the blood and kidneys at all time points examined. These results indicate that lack of macrophages leads to less severe joint lesions, but also results in impaired clearance of bacteria, and consequent enhancement of mortality rates.

Introduction

Group B streptococci (GBS) are a leading cause of life-threatening infections in neonates and young infants (Baker and Edwards, 1995). Invasive neonatal GBS infection has 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, septicaemia, meningitis, bacteraemia, and bone or joint infections (Baker and Edwards, 1995). Invasive disease caused by GBS has also been recognized in adults (Cooper and Morganelli, 1998; Gardam *et al*., 1998). Septic arthritis is one of the clinical manifestations of late-onset GBS infection in neonates (Baker and Edwards, 1995) and requires prolonged antibiotic treatment to ensure an uncomplicated outcome. In adults, GBS septic arthritis is often associated with age and risk factors such as diabetes mellitus, cancer, cardiovascular disease, chronic renal insufficiency, alcoholism, intravenous drug abuse, human immunodeficiency virus infections, neurological disease, and cirrhosis (Jackson *et al*., 1995; Straus *et al*., 1997; Schattner and Vosti, 1998a,b).

We previously described a murine model of haematogenously GBS-induced sepsis and arthritis (Tissi *et al*., 1990; 1999). Mice given a single intravenous dose of GBS develop clinical signs of arthritis within 48 h. Appearance and severity of GBS arthritis are the byproduct of a multifactorial process. Viability and number of microorganisms injected and bacterial factors (i.e. presence and amount of capsule, amount of sialic acid in the capsular polysaccharide, β-haemolysin production) have been shown to influence the development of articular lesions (Tissi *et al*., 1998; Puliti *et al*., 2000a). Nevertheless, in the pathogenesis of GBS arthritis a crucial role is played by inflammatory cells (granulocytes and monocytes) that reach the joints (Puliti *et al*., 2000b; 2002) and by the production of proinflammatory cytokines, including interleukin 6 (IL-6), IL-1β and tumour necrosis factor-α (TNF-α) (Tissi *et al*., 1999).

Cells from the monocyte/macrophage lineage are, together with granulocytes, the earliest type of leucocytes entering tissue in response to invading pathogens. Once localized at a site of infection, monocytes/macrophages exert a wide range of effector and regulatory functions. In fact, they phagocytose infectious organisms and control the immune response via antigen presentation of phagocytosed cell constituents at their surface and through secretion of numerous mediators (for review see Nathan,

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1987; Moonis *et al*., 1992). The final outcome of these macrophage-mediated actions is often tissue destruction (Bellingan, 1999; Marton and Kiss, 2000). In septic arthritis, there appears to be a connection between macrophage accumulation and joint destruction (Goldengerg and Reed, 1985; Bremmel *et al*., 1992). In fact, invading macrophages and granulocytes produce proteolytic enzymes that contribute to cartilage and bone destruction observed in bacterial arthritis (Peters *et al*., 1996; Verdrengh and Tarkowski, 2000).

In the present study, mice rendered monocytopenic by etoposide administration were used to evaluate the role of macrophages in the pathogenesis of GBS-induced sepsis and arthritis.

Results

Effect of monocytopenia on GBS-induced mortality and arthritis

To analyse the impact of monocyte depletion on the course of systemic GBS infection, mice were intravenously (i.v.) inoculated with 1×10^7 colony-forming units (CFU) per mouse, after pretreatment with etoposide for 3 days. As shown in Fig. 1A, monocytopenic mice had a higher, although not statistically significant ($P = 0.170$), mortality rate than controls, starting 6 days after inoculation. Twelve days after infection, 14 mice of 30 in the monocytopenic group had died, versus nine of 30 in the control group. Differences in the appearance and severity of arthritis were also noted upon etoposide treatment. In fact, as early as 3 days after bacterial inoculation, the frequency of arthritis was significantly lower in etoposide-treated animals than in controls (Fig. 1B). On day 10, only 50% of etoposide-treated mice manifested clinical signs of arthritis versus 100% of controls ($P < 0.01$). The severity of arthritis was significantly less pronounced in monocytopenic mice, as etoposide treatment dramatically reduced the arthritis index, that never exceeded 0.9 ± 0.3 compared with 5.4 ± 0.6 observed in controls at day 10 after infection (Fig. 1C).

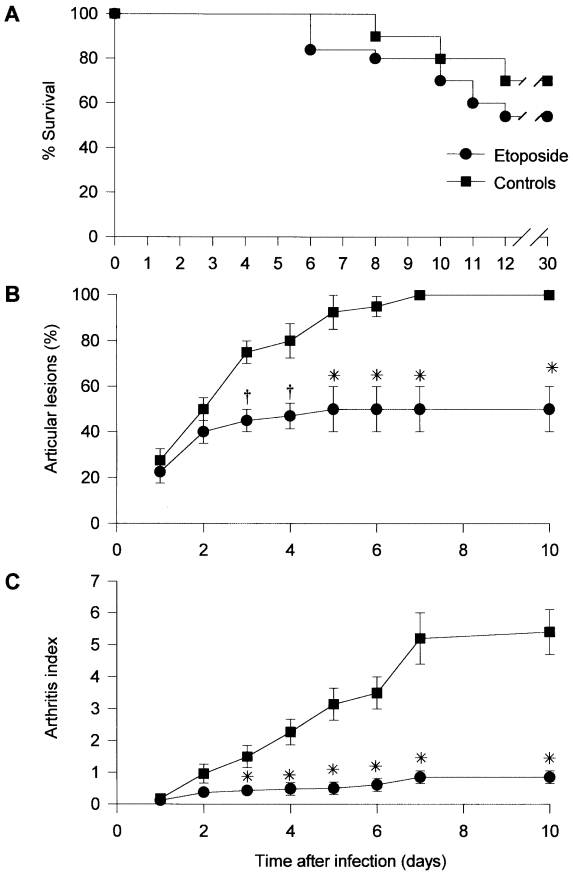

Fig. 1. Effect of etoposide treatment on survival (A), incidence (B) and severity (C) of arthritis in mice infected with GBS. Etoposide (12.5 μg/kg) was administered once a day starting 3 days before infection with 10^7 CFU per mouse of GBS. The data in A are the cumulative results of three separate experiments, each with 10 animals per experimental group. For B and C, the values are the means ± SD of three separate experiments, each with 10 animals per experimental group. * = $P < 0.01$ and † = $P < 0.05$ (etoposide-treated versus control mice).

Histopathology

Keeping in mind the clinical outcome of arthritis, histopathological analysis of joints from GBS-infected mice treated or not with etoposide was performed 7 days after infection. The left front and hind paws were examined in each mouse regardless of the macroscopic appearance of arthritis. Thirty per cent of monocytopenic mice showed a total absence of synovitis or erosions. The majority of the remaining mice showed signs of mild arthritis (Fig. 2). Only 10% of these mice displayed moderate arthritis with erosivity. In contrast, all control mice had histological signs of arthritis. In particular, 55% had severe arthritis, with extensive cartilage and/or bone destruction; only 7.5% of the animals displayed mild arthritis.

Effect of etoposide administration on recovery of GBS from the blood, kidneys and joints

In vivo GBS growth was assessed 1, 5 and 10 days after infection by quantitative monitoring of bacteraemia and bacterial growth in the kidneys and joints of mice treated or not with etoposide. As shown in Fig. 3, etoposide-treated animals had increased bacterial growth in the blood throughout the observation period. Higher growth of microorganisms was also observed in the kidneys of monocytopenic mice with respect to controls from day 5 after infection, with a significant difference in the number

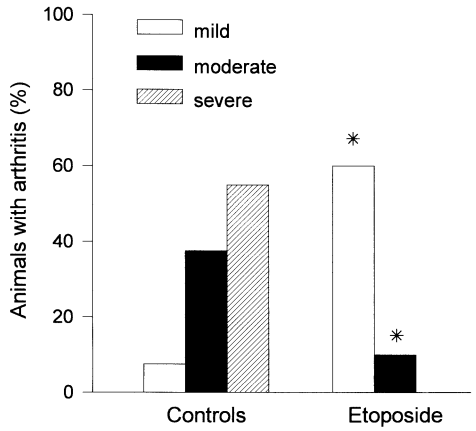

Fig. 2. Histopathological evaluation of arthritis in joints of mice infected with GBS and treated or not with etoposide. Etoposide (12.5 μg/kg) was administered once a day starting 3 days before infection with 10^7 CFU per mouse of GBS. Histopathological analysis was performed on day 7 after infection. Arthritis was defined mild, moderate or severe as detailed in *Experimental procedures*. Cumulative results of two experiments, each with five animals per experimental group, are represented. *$P < 0.01$ (etoposide-treated versus control mice).

of GBS recovered ($P < 0.05$). In contrast, consistent, although not significant, lower GBS growth was observed in the joints of etoposide-treated mice than in those of the controls at all time points assessed.

Effect of etoposide on cytokine production

Cytokines, such as IL-6, IL-1β and TNF-α have been shown to participate in the pathogenesis of GBS arthritis (Tissi *et al.*, 1999). Because monocytes/macrophages are known to produce these cytokines, the impact of etoposide treatment on their production was assessed. Animals infected with GBS and treated or not with the drug were monitored for systemic and local production of IL-6, IL-1β and TNF-α. A strong decrease in all cytokine concentrations was observed in the joints of etoposide-treated mice, with respect to controls (Fig. 4). Downregulation of IL-6 and IL-1β production following etoposide treatment was also observed in the serum at all the time points assessed, whereas a similar effect on TNF-α serum levels was found only 1–2 h after GBS infection (18.5 ± 5.2 pg ml^{-1} in etoposide-treated mice versus 30.1 ± 6.7 pg ml^{-1} in controls).

To further confirm the inhibitory effect of etoposide treatment on cytokine production, spleen cells from mice treated 3 days with the drug and from naive animals were cultured in the presence of concanavalin A (Con A) or heat-inactivated GBS (HI-GBS). Supernatants were assayed for the presence of IL-6, IL-1β, and TNF-α. As shown in Table 1, *in vivo* etoposide treatment significantly inhibited cytokine production by spleen cells, in particular when HI-GBS was used as a stimulating agent. In addition, *in vitro* treatment of naive spleen cells with different

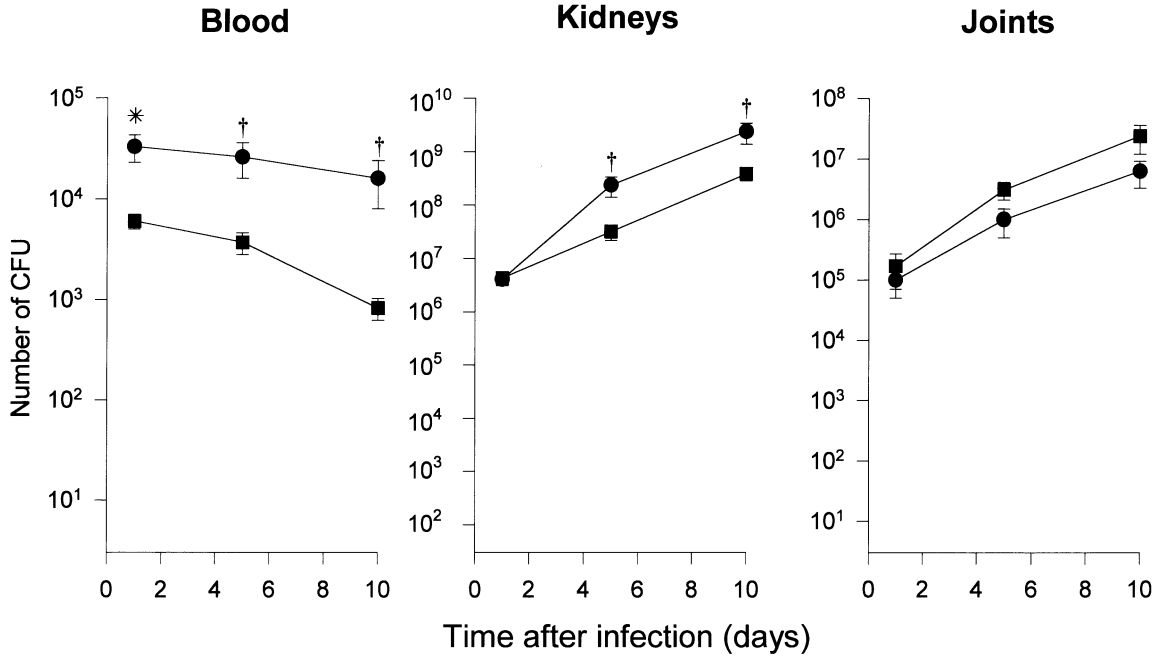

Fig. 3. Effect of etoposide treatment on bacterial growth in blood, kidneys and joints from mice infected with GBS. Etoposide (12.5 μg/kg) was administered once a day starting 3 days before infection with 10^7 CFU per mouse of GBS. Data represent the mean ± SD of three separate experiments. Three mice per group were sacrificed at each time point. Results are expressed as number of CFU per ml of blood, per whole organ or per ml of joint homogenate. *$P < 0.01$ and †$P < 0.05$ (etoposide-treated versus control mice).

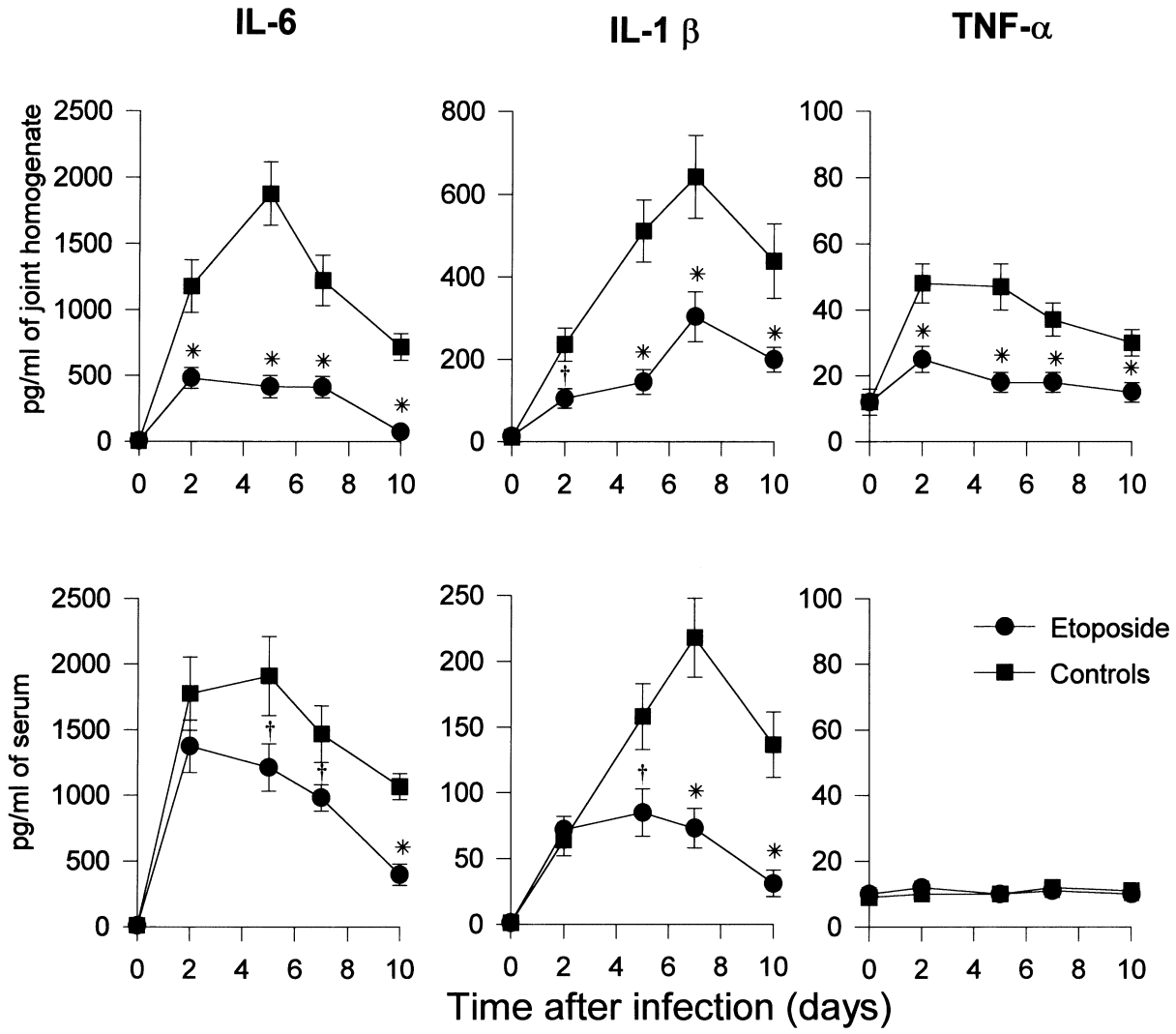

Fig. 4. Effect of etoposide treatment on cytokine production in sera and joints of mice infected with GBS. Etoposide (12.5 μg/kg) was administered once a day starting 3 days before infection with 10^7 CFU per mouse of GBS. Blood samples and supernatants from joint homogenates were collected at the indicated times after infection and assayed for IL-6, IL-1β, and TNF-α by ELISA. Values are the mean ± SD of three separate experiments. Three mice per group were sacrificed at each time point. *$P < 0.01$ and †$P < 0.05$ (etoposide-treated versus control mice).

doses of etoposide resulted in dose-dependent suppression of cytokine production (Table 2).

Impact of etoposide on peripheral blood monocyte, lymphocyte and granulocyte counts during the course of GBS infection

Mice treated for 3 days with etoposide before infection with GBS (day 0) showed a significant decrease in the number of circulating monocytes with respect to controls; no differences were observed in the number of lymphocytes or granulocytes (Table 3). During the course of GBS infection, the number of monocytes progressively increased in controls ($65 \pm 9.0 \times 10^3$ on day 0 compared with $323 \pm 51 \times 10^3$ on day 7), whereas in etoposide-treated animals the number of monocytes further decreased (from $9.0 \pm 1.1 \times 10^3$ on day 0–$0.8 \pm 0.1 \times 10^3$ on day 7). During infection, an increase in the number of granulocytes was observed both in control and etoposide-treated animals, although in the latter group this enhancement was less pronounced. No differences in the number of lymphocytes were found between the experimental groups, regardless of the treatment or the time point assessed.

Discussion

Macrophages participate in innate cellular immunity and initiate many host defence responses. Activated macrophages kill microorganisms by phagocytosis and generation

Table 1. Cytokine production by spleen mononuclear cells from etoposide-treated mice.[a]

Mice	*In vitro* stimulation	IL-6 (pg ml^{-1})	IL-1β (pg ml^{-1})	TNF-α (pg ml^{-1})
Etoposide treated	None	<7	4.5 ± 1.0	<10
	Con A	360 ± 72*	12.5 ± 3.2	32.5 ± 5.0
	HI-GBS	<7*	12 ± 2.9*	31.3 ± 7.1*
Controls	None	<7	7.2 ± 2.0	<10
	Con A	861 ± 92	15.5 ± 2.4	37.2 ± 6.1
	HI-GBS	777 ± 65	23.5 ± 4.1	217 ± 28

a. Spleens were explanted from mice treated and untreated with etoposide (12.5 mg kg^{-1}) for 3 days.
Cells were stimulated with Con A (5 μg ml^{-1}) or HI-GBS (10 CFU per cell) for 48 h. Data are the mean ± SD of three separate experiments. *$P < 0.01$ (etoposide-treated mice versus controls).

Table 2. *In vitro* cytokine production by spleen mononuclear cells from non-infected mice.

	IL-6 (pg ml^{-1})		IL-1β (pg ml^{-1})		TNF-α (pg ml^{-1})	
Etoposide (μM)	Con A	HI-GBS	Con A	Hi-GBS	Con A	HI-GBS
0	958 ± 88	790 ± 56	14.9 ± 1.2	24.0 ± 4.0	40.1 ± 5.3	232 ± 30
1	850 ± 91	766 ± 41	15.0 ± 2.2	21.1 ± 4.9	35.3 ± 3.5	211 ± 40
10	406 ± 55*	110 ± 22*	12.3 ± 3.1	7.6 ± 2.5*	25.1 ± 4.2*	58.4 ± 11*
100	<7*	<7*	<3*	<3*	<10*	<10*

Cells were incubated with the indicated concentrations of etoposide and stimulated with Con A (5 μg ml^{-1}) or HI-GBS (10 CFU/cell) for 48 h. Data are expressed as the mean ± SD of three separate experiments. *$P < 0.01$ (etoposide-treated cells versus untreated cells).

of reactive oxygen species, become more efficient antigen-presenting cells and produce mediators, such as cytokines and growth factors, that trigger local inflammation (Nathan, 1987; Moonis *et al*., 1992; Bellingan, 1999). However, these macrophage-mediated actions can result in host damage with tissue destruction (Bellingan, 1999; Marton and Kiss, 2000). The host defence system must tightly balance the protective role of macrophages against their potential role of causing injury.

The detrimental role of macrophages in aseptic chronic joint diseases has been firmly established (Van den Berg and van Lent, 1996). Synovial macrophages play a critical role in pathogenesis of joint erosion (Yanni *et al*., 1994), and macrophage-derived cytokines, such as TNF-α and IL-1 dominate the cytokine profile in inflammatory synovitis (Eigler *et al*., 1997; Jorgensen and Gay, 1998). In septic arthritis, the inflammatory process starts as an acute synovitis with neutrophil invasion (Goldengerg and Reed, 1985). These cells are followed by monocytes from the peripheral blood pool, that differentiate locally into macrophages, and contribute to the destructive process of inflammation by secreting proteolytic enzymes and cytokines (Goldengerg and Reed, 1985; Bremmel *et al*., 1992). In the present study, we evaluated the role of cells from the monocyte-macrophage lineage in the pathogenesis of GBS sepsis and arthritis.

Mice rendered monocytopenic and inoculated with GBS developed a less severe arthritis than controls, both clinically and histopathologically. A strong decrease of proinflammatory cytokine concentrations was observed in these mice, particularly at joint level. Interestingly, etoposide treatment resulted in decreased production of IL-6,

Table 3. Number of circulating monocytes, lymphocytes and granulocytes in mice treated and untreated with etoposide and infected with GBS.[a]

		Days after GBS infection		
Mice	Cells	0	3	7
Etoposide treated	Monocytes	$9.0 \times 10^3 \pm 1.1 \times 10^3$*	$3.3 \times 10^3 \pm 0.6 \times 10^3$*	$0.8 \times 10^3 \pm 0.1 \times 10^3$*
	Lymphocytes	$1.6 \times 10^6 \pm 0.2 \times 10^6$	$1.3 \times 10^6 \pm 0.1 \times 10^6$	$1.6 \times 10^6 \pm 0.3 \times 10^6$
	Granulocytes	$1.0 \times 10^6 \pm 0.1 \times 10^6$	$3.2 \times 10^6 \pm 0.5 \times 10^6$	$4.7 \times 10^6 \pm 0.7 \times 10^6$*
Controls	Monocytes	$65 \times 10^3 \pm 9.0 \times 10^3$	$169 \times 10^3 \pm 30 \times 10^3$	$323 \times 10^3 \pm 51 \times 10^3$
	Lymphocytes	$1.9 \times 10^6 \pm 0.3 \times 10^6$	$1.8 \times 10^6 \pm 0.2 \times 10^6$	$1.6 \times 10^6 \pm 0.4 \times 10^6$
	Granulocytes	$1.2 \times 10^6 \pm 0.3 \times 10^6$	$4.1 \times 10^6 \pm 0.8 \times 10^6$	$10.8 \times 10^6 \pm 2.0 \times 10^6$

a. Etoposide (12.5 μg/kg) was administered once a day starting 3 days before infection with 10^7 CFU per mouse of GBS. Differential leucocyte counts (number × mm^3) were performed at the indicated times after infection as described in *Experimental procedures*. Each value represents the mean ± SD of three separate experiments. Three mice per group were sacrificed at each time point. *$P < 0.01$ (etoposide-treated versus controls).

IL-1β and TNF-α not only *in vivo*, but also in *ex vivo* and *in vitro* experiments. In our opinion, this diminished cytokine production is likely the key factor behind the downregulation of arthritis. IL-6, IL-1β and TNF-α are known to exert pro-inflammatory activities in both septic (Bremmel *et al*., 1992; Tissi *et al*., 1999) and aseptic arthritides (Arend and Dayer, 1990; Nietfeld *et al*., 1990; Ridderstad *et al*., 1991; Green *et al*., 1994). TNF-α directly influences the migration of monocytes and lymphocytes into the synovium through its effect on endothelium expression of intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin (Bevilacqua *et al*., 1994), and indirectly through induction of chemokines such as IL-8 and monocyte chemotactic protein 1 (Cutolo *et al*., 1993). Moreover, 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 by activation of osteoclasts (Thomas *et al*., 1987; Arend and Dayer, 1995; Van de Loo *et al*., 1995). Similar detrimental properties are shown by IL-6, which participates together with IL-1 in catabolism of connective tissue components at sites of inflammation (Nietfeld *et al*., 1990; Ito *et al*., 1992), and activates osteoclasts, with a consequent increase in joint damage (Green *et al*., 1994). Based on these findings, it may be hypothesized that the amelioration of arthritis in etoposide-treated mice is attributable not only to deletion of peripheral blood monocytes and consequent minor cell influx in the joints, but also to downregulation of cytokine production and, likely, tissue-destructive enzymes.

The number of GBS recovered from the joints of etoposide-treated mice was lower then that of controls. It has been demonstrated that GBS, after *in vitro* phagocytosis, survive and persist intracellularly in macrophages up to 24–48 h (Cornacchione *et al*., 1998). Macrophages may carry GBS to different body sites, such as joints, thus allowing dissemination of infection. Keeping this in mind, it is conceivable that lack of monocytes may result in a smaller number of bacteria reaching the joints. Thus, we cannot exclude that the lower bacterial load in the joints of monocytopenic mice might concur, together with a lower cytokine production and number of inflammatory cells locally recruited, to a less severe development of arthritis.

Although monocytopenic mice displayed less severe arthritis, they were more susceptible than controls to GBS infection. In fact, the higher number of bacteria in the blood and in the kidneys, as a consequence of the lack of phagocytosing monocytes, could be the reason for enhanced mortality observed in etoposide-treated mice. However, the less pronounced increase in the number of peripheral granulocytes observed during GBS infection in these mice compared with controls may also have contributed to the development of septicaemia.

In conclusion, our results indicate that monocytes/macrophages have a relevant role in the development of GBS-induced arthritis and sepsis. On one hand, they participate in tissue destruction in the joints, while on the other hand they contribute to a more efficient elimination of bacteria from the blood stream. A combination therapy of etoposide together with antibiotics might be expected to lead to a favourable outcome of GBS septic arthritis.

Experimental procedures

Mice

Sex-matched, 8-week-old male or female outbred CD mice were obtained from Charles River Breeding Laboratories (Calco, Italy).

Microorganism

Type IV GBS, reference strain GBS 1/82, was used throughout the study. For experimental infection, the microorganisms were grown overnight at 37°C in Todd–Hewitt broth (Oxoid, Basingstoke, Hampshire, UK) and then washed and diluted in RPMI 1640 medium (Gibco, Life Technologies, Milan, Italy). The inoculum size was estimated turbidimetrically and viability counts were performed by plating on tryptic soy agar – 5% sheep blood agar (blood agar), and overnight incubation under anaerobic conditions at 37°C. A bacterial suspension was prepared in RPMI 1640 medium. Mice were inoculated i.v. via the tail vein with 1×10^7 GBS per mouse in a volume of 0.5 ml. Control mice were injected in the same way with 0.5 ml of RPMI 1640 medium.

Induction of monocytopenia

Etoposide (Sigma-Aldrich, Milan, Italy) is a drug that leads to selective decrease in peripheral blood monocytes in mice (Van't Wout *et al*., 1989). Etoposide inhibits the function of DNA topoisomerase II, thus interrupting the late S-G2 phase of the cell cycle (Smith *et al*., 1994). Etoposide was diluted 1 : 10 in phosphate-buffered saline (PBS) from a stock solution of 20 mg ml^{-1}, and injected subcutaneously at the dose of 12.5 mg kg^{-1} into mice once a day during the course of the experiment, starting 3 days before GBS i.v. inoculation. The dose and time of etoposide administration were chosen based on those established in previous studies (Calame *et al*., 1994; Verdrengh and Tarkowski, 2000). Induction and maintenance of monocytopenia were assessed in each experiment by haematological analysis as detailed below.

Haematological analysis

Mice were bled from retro-orbital sinus into heparinized tubes 3 days before, and 0, 3 and 7 days after bacterial inoculation. Leucocyte counts were performed in a haemocytometer (QBC VetAutoread, IDEXX Laboratories, Westbrook, ME). Fifty microlitres of the heparinized blood was analysed in a FACScan cytometer (Becton Dickinson, San Jose, CA) to determine the percentage of lymphocytes, granulocytes and monocytes. The following antibodies have been employed: PE-Ly-6G/Ly-6C (for

granulocytes detection), FITC-anti-mouse CD3e monoclonal antibody (for lymphocytes detection), both purchased from BD Biosciences Pharmingen, San Diego, CA, and FITC rat anti-mouse F4/80 (for monocyte detection), purchased from Serotec, Oxford, UK. The antibodies were used following manufacturers' recommendations. The absolute number of different leucocyte subsets were then calculated from total leucocyte counts.

Clinical evaluation of arthritis and mortality

Mice injected with GBS and treated or not with etoposide 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.) to evaluate the presence of joint inflammation; scores for arthritis severity (macroscopic score) were given as described (Tissi *et al.*, 1999). Arthritis was defined as visible erythema or swelling of at least one joint. Clinical severity of arthritis was graded on a scale of 0–3 for each paw, according to changes in erythema and swelling (0, no change; 1 point, mild swelling and erythema; 2 points, moderate swelling and erythema; 3 points, marked swelling, erythema and/or ankylosis). Thus, a mouse could have a maximum score of 12. The arthritis index (mean ± SD) was constructed by dividing the total score (cumulative value of all paws) by the number of animals used in each experimental group.

Histological assessment

Groups of mice inoculated with GBS and treated or not with etoposide were examined 7 days after infection for histopathological features of arthritis. Two standard pairs of limbs (left fore and hind) from each mouse were removed, fixed in formalin 10% v/v for 24 h, decalcified in a RDO® solution (active reagent, hydrochloric acid, APEX engineering products, Plainfield, IL) for 1–2 h, dehydrated, embedded in paraffin, sectioned at 3–4 μm and stained with haematoxylin and eosin. Samples were examined under blinded conditions with regard to synovial hypertrophy, defined as synovial membrane thickness of more than two cell layers (Bremmel *et al.*, 1992), presence of infiltrate/exudate, cartilage and bone destruction, and loss of joint architecture. Histological scoring was based upon the degree of synovial hypertrophy, extent of infiltrate/exudate, and degradation of cartilage and/or bone. Scores were 1 point for mild, 2 points for moderate and 3 points for severe synovial hypertrophy, presence of massive infiltrate/exudate and joint destruction.

GBS growth in blood, kidneys and joints

Blood, kidneys and joint infections in GBS-infected mice treated or not with etoposide were determined by CFU evaluation 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. The number of CFU was determined and the results were expressed as the number of CFU per ml of blood. Kidneys 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 1 ml/100 mg joint weight of sterile RPMI 1640 medium. After homogenization, all tissue samples were diluted and plated in triplicate on blood agar and the results were expressed as the number of CFU per whole organ or per ml of joint homogenate.

Sample preparation for cytokine assessment

Blood samples from GBS-infected mice, treated or not with etoposide and from uninfected controls were obtained by retro orbital sinus bleeding before sacrifice at different times after infection; sera were stored at –80°C until analysed. Joint tissues were prepared as previously described (Tissi *et al.*, 1999). Briefly, all wrist and ankle joints from each mouse were removed and then homogenized in 1 ml/100 mg joint weight of lysis medium (RPMI 1640 containing 2 mM phenylmethylsulphonyl fluoride (PMSF) and 1 μg ml^{-1} of aprotinin, leupeptin and pepstatin A, final concentration). The homogenized tissues were then centrifuged at 2000 *g* for 10 min and supernatants were sterilized using a Millipore filter (0.45 μm) and stored at –80°C until analysed.

Spleens were obtained from mice treated with etoposide once a day for 3 days and from untreated controls. Three animals were pooled and a single-cell suspension was prepared as previously described (Derrico and Goodrum, 1996). Briefly, cells were washed and resuspended in RPMI 1640 complete medium (10% fetal calf serum, 50 μM mercaptoethanol, 2 mM L-glutamine, and 50 μg ml^{-1} gentamicin) to a density of 5×10^6 cells per ml. Spleen cells were stimulated with 5 μg ml^{-1} of Con A (Sigma) or HI-GBS (10 CFU per splenocyte) for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. After culturing, supernatants were removed and stored at –80°C until assayed. In another set of experiment, spleen cells from naive mice were *in vitro* treated with etoposide at concentrations ranging from 1 to 100 μM and stimulated with Con A or HI-GBS as described above. After culturing, supernatants were removed and stored at –80°C until assayed.

Cytokine determination

IL-6, IL-1β and TNF-α concentrations in the biological samples or in spleen cell supernatants were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits (Amersham Pharmacia Biotech, Amersham, UK) according to the manufacturer's recommendations. Results were expressed as pg ml^{-1} of serum or supernatants from joint homogenates or from spleen cultures. The detection limits of the assays were 7 pg ml^{-1} for IL-6, 3 pg ml^{-1} for IL-1β and 10 pg ml^{-1} for TNF-α.

Statistical analysis

Differences in the arthritis index, number of CFU, cytokine concentrations and leucocyte counts between the groups of mice were analysed by Student's *t*-test. Differences between the groups with respect to survival data were analysed by Mann–Whitney *U*-test, and incidence of arthritis and histologic data by the c^2 test. Each experiment was repeated three times. A *P*-value < 0.05 was considered significant.

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