Experimental model of infection with non-toxigenic strains of *Corynebacterium diphtheriae* and development of septic arthritis

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Corynebacterium diphtheriae is a well-known cause of localized respiratory tract infections. However, this micro-organism can also be associated with invasive infections, such as endocarditis, septic arthritis and osteomyelitis. Invasive infections are often caused by non-toxigenic strains. To set up an *in vivo* experimental model of *C. diphtheriae* infection, mice were infected intravenously with different doses (ranging from 1×10^7 to 5×10^8 bacteria per mouse) of three non-toxigenic strains, namely ISS-4749, ISS-4746 and ISS-3319. Similar mortality rates were observed with the three strains, with an LD₅₀ ranging from 9×10^7 to 1.2×10^8 . All strains were arthritogenic, although to different extents. ISS-4749 and ISS-4746 infection resulted in a maximum of 60 and 50 %, respectively, of animals with articular lesions, while in the ISS-3319-infected group only 25 % were positive. There were differences in systemic and joint cytokine production in the three experimental groups. ISS-4749- and ISS-4746-infected mice exhibited higher local levels of interleukin (IL)-6 and IL-1 β than ISS-3319-infected animals. At systemic levels, ISS-3319 was able to induce early and sustained production of interferon- γ (IFN- γ), but not IL-6. Conversely, infection with the other strains resulted in high IL-6, but not IFN- γ , production. In conclusion, an experimental model of *C. diphtheriae* infection was set up, with development of septic arthritis. This model could be useful in studies on the pathogenicity and characterization of virulence factors other than toxin production.

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INTRODUCTION

In recent decades it has become evident that *Corynebacterium diphtheriae* should not be considered only as the aetiological agent of diphtheria. The introduction of mass immunization against diphtheria in the 1950s in most European countries, as well in the USA and Canada, resulted in a very low incidence of this disease (Galazka, 2000) and circulation of toxigenic strains declined in all countries with a good vaccination coverage (Saragea *et al.*, 1979; von Hunolstein *et al.*, 2003). In contrast, non-toxigenic strains of *C. diphtheriae* have been increasingly documented as a cause of invasive disease, including endocarditis, bacteraemia, osteomyelitis and splenic abscesses (Alexander, 1984; Funke *et al.*, 1999; Poilane *et al.*, 1995; Belko *et al.*, 2000; Mattos-Guaraldi *et al.*, 2001). Skin lesions (cutaneous ulcers, bullous pemphigoid, scabies, open fractures) are most likely the portal of entry for infection. Septic arthritis, caused by

non-toxigenic *C. diphtheriae*, has also been described (Patey *et al.*, 1997; Guran *et al.*, 1979; Appelbaum & Dossett, 1982; Tiley *et al.*, 1993; Damade *et al.*, 1993; Barakett *et al.*, 1993). The first reported case was in France in 1979 (Guran *et al.*, 1979). In a study by Tiley *et al.* (1993), 50 % of patients manifested septic arthritis as a complication of endocarditis due to non-toxigenic *C. diphtheriae* var. *gravis*. These data were supported by a more recent study (Patey *et al.*, 1997), where joint involvement occurred in 27.5 % of patients infected with non-toxigenic strains. In this study, biotype *mitis* was the predominant one.

From the human cases of diphtheroid arthritis reported in the literature (Patey *et al.*, 1997; Guran *et al.*, 1979; Appelbaum & Dossett, 1982; Tiley *et al.*, 1993; Damade *et al.*, 1993; Barakett *et al.*, 1993), several observations can be made. First, infection may be present as an acute, purulent process or as chronic arthritis with plasmacytic or histiocytic inflammation. Second, patients generally have a pre-existing disease or condition that compromises their natural defences. Third, the disease occurs in both sexes, over a wide

Abbreviations: IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

age range. Usually, clinical examination of the affected joints reveals a tender, warm swelling with evidence of synovial effusion. Radiographic examinations show a widened joint space and no evidence of bony destruction or degenerative changes. A high number of inflammatory cells (mostly polymorphonuclear cells) and bacteria are present in joint aspirate.

For a better understanding of human *C. diphtheriae* disease, an experimental model of infection has been set up using CD1 mice injected intravenously with different non-toxicogenic strains of *C. diphtheriae*.

METHODS

Mice. Sex-matched, 8-week-old male or female outbred CD-1 mice were obtained from Charles River Breeding Laboratories.

Bacterial strains. Three strains of *C. diphtheriae* isolated from the throats of patients affected by severe pharyngitis/tonsillitis in the presence of fever were used in our study. The strains were identified at the Department of Infectious Diseases of Istituto Superiore di Sanità, Rome, Italy, and designated with laboratory numbers ISS-3319, ISS-4746, ISS-4749. All strains were non-toxicogenic as assessed by PCR analysis and penicillin-tolerant. The isolates were biotyped by API Coryne system profile (bioMérieux) and results were: ISS-3319, *C. diphtheriae* var. *mitis*; ISS-4746 and ISS-4749, *C. diphtheriae* var. *gravis*. Micro-organisms were grown overnight at 37 °C in Todd-Hewitt broth (Oxoid) under gentle agitation, washed, resuspended in PBS and adjusted spectrophotometrically at 600 nm at a concentration of 4×10^8 ml⁻¹ as checked by plating on tryptic soy agar/5% sheep blood agar (blood agar). Mice were infected intravenously via the tail vein with different doses of *C. diphtheriae* strains in a volume of 0.5 ml.

Clinical evaluation of arthritis and mortality. Mice injected with the different strains of *C. diphtheriae* were evaluated for signs of arthritis and for mortality. Mortality was recorded at 24 h intervals for 20 days. After challenge, mice were examined daily by two independent observers (L.T., M.P.) for 30 days to evaluate the presence of joint inflammation and scores for arthritis severity (macroscopic score) were given as previously described (Verdrengh & Tarkowski, 1997; Tissi *et al.*, 1999). Arthritis was defined as visible erythema and/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, mild swelling and/or 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 $\pm$ SD) was constructed by dividing the total score (cumulative value of all paws) by the number of animals in each experimental group.

Histological assessment. Mice were examined at different times after infection for histopathological features of arthritis. Arthritic paws (one per mouse) were removed aseptically, fixed in formalin 10% v/v for 24 h and then decalcified in trichloroacetic acid 5% v/v for 7 days, dehydrated, embedded in paraffin, sectioned at 3–4 μ m and stained with haematoxylin and eosin. Samples were examined under blinded conditions. Joints (three per paw) were examined for synovitis (defined as synovial membrane thickness of more than two cell layers), 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 (mild synovial hypertrophy, minimal infiltrate), moderate (moderate synovial hypertrophy, presence of infiltrate, minimal

exudate, integrity of joint architecture) and severe (marked synovial hypertrophy, presence of massive infiltrate/exudate, cartilage and bone erosion and disrupted joint architecture).

***C. diphtheriae* growth in the organs.** Blood, kidney, spleen and joint infections in *C. diphtheriae*-infected mice were determined by c.f.u. evaluation at different times after infection. Blood samples were obtained by retro-orbital sinus bleeding before mice were killed. Tenfold dilutions were prepared in RPMI 1640 medium (Gibco Life Technologies) 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 c.f.u. was determined and the results were expressed as the number of c.f.u. per ml blood. Kidneys and spleens were aseptically removed and homogenized with 3 ml sterile RPMI 1640. All wrist and ankle joints from each mouse were removed, weighed and homogenized *in toto* in RPMI 1640 medium (1 ml per 100 mg joint weight). After homogenization, all tissue samples were diluted and plated in triplicate on blood agar and the results were expressed as the number of c.f.u. per whole organ or per ml joint homogenate.

Sample preparation for cytokine assessment. Blood samples from *C. diphtheriae*-infected mice were obtained by retro-orbital sinus bleeding at different times after infection before the mice were killed. 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 toto* in 1 ml lysis medium (RPMI 1640 containing 2 mM PMSF and 1 μ g ml⁻¹ final concentrations of aprotinin, leupeptin and pepstatin A) per 100 mg joint weight. The homogenized tissues were then centrifuged at 2000 g for 10 min and supernatants were sterilized using a Millipore filter (0.45 μ m) and stored at –80 °C until analysed.

Cytokine assays. Interleukin (IL)-6, IL-1 β , tumour necrosis factor (TNF) α and interferon (IFN)- γ concentrations in the biological samples were measured with commercial ELISA kits purchased from R&D Systems according to the manufacturer's recommendations. Results were expressed as picograms per millilitre of serum or supernatant from joint homogenates. The detection limits of the assays were 7 pg ml⁻¹ for IL-6, 3 pg ml⁻¹ for IL-1 β , 5.1 pg ml⁻¹ for TNF α and 15 pg ml⁻¹ for IFN- γ .

Statistical analysis. Differences in the arthritis index, number of c.f.u. and cytokine concentrations between the groups of mice were analysed by Student's unpaired *t*-test. Differences in the incidence of arthritis were evaluated by the χ^2 test. Each experiment was repeated three times. *P* values less than 0.05 were considered significant.

RESULTS

Mortality rates and clinical evaluation of arthritis

The capacity of three different strains of *C. diphtheriae* to produce lethal infection was examined by intravenous inoculation of mice with different numbers of micro-organisms. Similar LD₅₀ values were observed in animals infected with the three strains, i.e. $9.1 \times 10^7 \pm 1.1 \times 10^7$ for strain ISS-4749, $9.8 \times 10^7 \pm 1.3 \times 10^7$ for strain ISS-4746 and $1.2 \times 10^8 \pm 1.1 \times 10^8$ for strain ISS-3319. With the highest infecting dose employed (5×10^8 c.f.u. per mouse), all the animals died within 3 days. By lowering the dose to 5×10^7 c.f.u. per mouse, mortality rates ranged from 10 to

20 %, while inoculation with 1×10^7 c.f.u. per mouse did not cause death.

The strains were also assessed for their ability to induce arthritis. They were all arthritogenic when injected at the dose of 5×10^7 c.f.u. per mouse (Fig. 1). Clinical signs of arthritis were evident starting 3 days after infection and the most frequently affected joints were ankle and wrist; the maximal incidence of articular lesions was reached in 6–8 days and then the number of affected animals gradually decreased, with complete remission of arthritis within 30 days. Differences were observed among the strains, in that ISS-4749 was the most arthritogenic, with 60 % of animals positive for articular lesions, while a maximum of 25 % incidence was observed in mice infected with ISS-3319. Moreover, 20 days after infection all ISS-3319-infected animals fully recovered, whereas 10–20 % of the animals infected with ISS-4746 or ISS-4749 were still positive.

Similarly, differences were also observed in terms of severity of arthritis. In fact, the arthritis index reached a maximum of 1.8 ± 0.4 in ISS-4749-, 1.1 ± 0.2 in ISS-4746- and 0.5 ± 0.1 in ISS-3319-infected animals.

Histopathological findings

Histopathological studies of the joints of mice infected with the different strains of *C. diphtheriae* were performed to

confirm clinical signs of arthritis. We have previously observed that paws that did not show macroscopic signs of arthritis also did not show any histological features of arthritis. Thus, we chose to examine histopathologically only paws showing clinical signs of arthritis. On day 3 after infection, three affected paws were examined in each experimental group. For each paw, three joints were assessed for histopathological score. In mice infected with ISS-4749, four of nine joints showed signs of inflammation, and in the mice infected with ISS-4746 or ISS-3319 the positive joints were three of nine. All the positive joints were classified as mildly affected (Fig. 2a), characterized by a mild synovial hypertrophy and presence of a scant polymorphonuclear-leukocyte-monocyte infiltrate in the subcutaneous tissues (Fig. 2b). At day 7 after infection, four affected paws were examined for each experimental group. Seven joints out of 12 in ISS-4749-infected mice and six joints out of 12 in ISS-4746- or ISS-3319-infected mice were positive for arthritis (Fig. 2a). In contrast to day 3, 25–40 % of joints were classified as moderately affected, with a moderate synovial hypertrophy, an inflammatory infiltrate present in the subcutaneous and periarticular tissues and a moderate cell influx in the articular cavities (Fig. 2c). No signs of cartilage destruction or bone erosion were present and joint integrity was maintained. The lack of cartilage and/or bone destruction prevented us from using a scoring system such as that employed by Josefsson *et al.* (2001) in which this parameter is relevant in attributing the histopathological score.

Recovery of *C. diphtheriae* strains from organs

Quantitative monitoring of *C. diphtheriae* bacteraemia and growth in the joints, kidneys and spleens was done 1, 3, 6, 9 and 15 days after injection of 5×10^7 c.f.u. per mouse of each strain. Numbers of c.f.u. in the blood and joints of mice infected with strain ISS-4749, ISS-4746 or ISS-3319 did not differ significantly at any of the time points assessed (Fig. 3). A consistent number of micro-organisms was detected in the blood at days 1 and 3 after infection, and all *C. diphtheriae* strains were cleared from the bloodstream within 6 days. In the joints, the number of micro-organisms reached a maximum between days 6 and 9 after infection and then gradually decreased, and no bacteria were recovered at day 20 (data not shown). Similar growth rates were observed in the kidneys and spleens of mice infected with strains ISS-4749 and ISS-4746. In contrast, significantly larger numbers of bacteria were recovered from the spleen and kidneys of *C. diphtheriae* strain ISS-3319-infected mice at days 3 and 6 after infection, respectively.

Cytokine production during *C. diphtheriae* infection

Cytokines appear to play an important role in inflammation and in the development of articular lesions in other experimental models of septic arthritis (Tissi *et al.*, 1999; Osiri *et al.*, 1998; Puliti *et al.*, 2000; Tarkowski *et al.*, 2002). To verify cytokine involvement in this model of *C.*

Fig. 1. Incidence (a) and severity (b) of arthritis in mice infected with 5×10^7 c.f.u. per mouse of the indicated non-toxicogenic strains of *C. diphtheriae* (●, ISS-4749; ■, ISS-4746; ▲, ISS-3319). Results are means $\pm$ SD of three separate experiments. *, $P < 0.05$ as determined by χ^2 test; †, $P < 0.05$ as determined by unpaired Student's *t*-test; *n*, 10 mice per group.

Fig. 2. Histopathological evaluation of arthritis in mice at day 3 and 7 after infection with the indicated non-toxicogenic strains of *C. diphtheriae*. (a) Percentage of joints mildly (open bars) or moderately (hatched bars) affected 3 and 7 days after infection with the indicated non-toxicogenic strains of *C. diphtheriae*. Arthritis was defined as mild or moderate on the basis of synovitis and extent of inflammation as detailed in Methods. (b) Representative histological features of joints on day 3 after infection. A few inflammatory cells are present in the subcutaneous tissues (arrowhead). (c) Representative histological features of joints on day 7 after infection. Moderate cell influx in the articular cavity (arrowhead). Bar, 400 µm. B, bone; M, muscle; star, joint space; Sc, subcutaneous tissues.

maximal concentration on day 6, and then progressively decreased. No differences in TNF α production were observed either in sera or joints, regardless of the strain used. Peak values were reached on day 9 after infection. IFN- γ production was not observed close to infection with strains ISS-4749 or ISS-4746. Barely detectable levels were evident at day 3 and on subsequent days a gradual increase of IFN- γ production was detected. In contrast, infection with strain ISS-3319 was characterized by a prompt and sustained systemic production of IFN- γ , which increased throughout the observation period, and by low levels of IL-6. In

Fig. 3. Bacterial growth in blood, joints, kidneys and spleen of mice at day 1, 3, 6, 9 and 15 after infection with 5×10^7 c.f.u. of the indicated non-toxicogenic *C. diphtheriae* strains (●, ISS-4749; ■, ISS-4746; ▲, ISS-3319). Data represent means $\pm$ SD of three separate experiments. *, $P < 0.05$ as determined by unpaired Student's *t*-test; *n*, 3 mice per group per time point.

diphtheriae-induced arthritis, systemic and local production of IL-6, IL-1 β , TNF α and IFN- γ was investigated at different time points after infection with the different strains. Similar kinetics of cytokine production were observed after infection with strain ISS-4749 or ISS-4746. In fact, sustained systemic levels of IL-6 were detected in mice soon after infection, followed by a gradual decrease on subsequent days (Fig. 4). Early IL-1 β serum production was also evident but, differently from IL-6, it continued to increase, reaching

Fig. 4. IL-6, IL-1 β , TNF α and IFN- γ levels in sera and joints of mice at day 1, 3, 6, 9 and 15 after infection with 5×10^7 c.f.u. of the indicated non-toxicogenic *C. diphtheriae* strains (●, ISS-4749; ■, ISS-4746; ▲, ISS-3319). Cytokine levels were determined by ELISA and expressed as the means $\pm$ SD of three separate experiments; values are pg ml⁻¹ for serum and pg (ml supernatant)⁻¹ for joint samples. *, $P < 0.05$ as determined by unpaired Student's t -test; n , 3 mice per group per time point.

the joints, IL-6 and IL-1 β production was evident, with concentrations reaching peak values at day 9 after infection with strains ISS-4749 and ISS-4746, but not with ISS-3319. Similar kinetics of TNF α production and barely detectable levels of IFN- γ were found in the joints upon infection with all the strains used.

DISCUSSION

Non-toxicogenic *C. diphtheriae* strains have been increasingly isolated from different types of infections, from cutaneous lesions and pharyngitis to bacteraemia and endocarditis

(von Hunolstein *et al.*, 2003; Funke *et al.*, 1999; Patey *et al.*, 1997; Mattos-Guaraldi & Formiga, 1998). Several studies have underlined that immunization against diphtheria toxin does not protect from the insurgence of non-toxicogenic *C. diphtheriae* diseases, supporting the hypothesis that diphtheria bacilli may possess other factors that enhance their virulence (Funke *et al.*, 1999; Wilson, 1995; Patey *et al.*, 1997). So far, there are no *in vivo* experimental models available for a better understanding of the pathogenetic mechanisms triggered by non-toxicogenic *C. diphtheriae*.

In the present study, we describe a mouse model of non-toxicogenic *C. diphtheriae* infection. Three strains were employed, two var. *gravis* and one var. *mitis*. All strains were poorly pathogenic in terms of acute lethality for mice, with LD₅₀ ranging from $(9.1 \pm 1.1) \times 10^7$ to $(1.2 \pm 1.1) \times 10^8$. The mortality rates recorded resembled those observed in humans, where fatality rates were 15–20% in patients with *C. diphtheriae* infection (Patey *et al.*, 1997; Tiley *et al.*, 1993). In our model, a long-lasting infection was observed in mice irrespective of the strain used. In fact, although bacteria were cleared from the bloodstream within 5 days, micro-organisms persisted in kidneys and spleens for more than 2 weeks after infection.

Interestingly, *C. diphtheriae* infection in mice was accompanied by the appearance of articular lesions in a relevant number of animals. Osteoarthritis has been described as a clinical feature of human diseases, since it has been reported in 27–50% of patients with *C. diphtheriae* systemic infection (Patey *et al.*, 1997; Tiley *et al.*, 1993). In humans, joint aspiration revealed the presence of elevated numbers of polymorphonuclear leukocytes and Gram-positive bacilli, further identified as *C. diphtheriae*. Radiographs reported a normal or widened joint space with no evidence of bony destruction or degenerative changes (Damade *et al.*, 1993; Barakett *et al.*, 1993; Afghani & Stutman, 1993). Similarly, in our model, bacteria were reisolated from the affected joints and histopathological examination revealed a mild-to-moderate arthritis, with presence of inflammatory cells but without bone or cartilage degradation.

Although similar amounts of bacteria were recovered from the joints of mice infected with the three non-toxicogenic strains of *C. diphtheriae*, differences were evident both in incidence and severity of arthritis. The appearance of articular lesions is undoubtedly the by-product of a multifactorial process, involving not only bacteria but also the host immune response. For instance, participation of pro-inflammatory cytokines (IL-6, IL-1 β and TNF α) in the pathogenesis of arthritis has been well documented in both human and animal models (Tissi *et al.*, 1999; Osiri *et al.*, 1998; Tarkowski *et al.*, 2002). In particular, IL-6 and TNF α levels are persistently high in the synovial fluid of patients with bacterial septic arthritis (Osiri *et al.*, 1998), and correlate with the severity of disease in murine *Staphylococcus aureus* septic arthritis (Nilsson *et al.*, 1999). In another study, the peptidoglycan component of the Gram-positive cell wall has been shown to contribute to proinflammatory

cytokine induction and severity of experimental arthritis (Fuseler *et al.*, 1997). Moreover, IL-6 is involved, together with IL-1, in the catabolism of connective tissue components at sites of inflammation (Ito *et al.*, 1992; Nietfeld *et al.*, 1990) and activates osteoclasts (Green *et al.*, 1994). A direct correlation between severity of arthritis and levels of IL-6, IL-1 β and TNF α in the joints has been demonstrated in a mouse model of group B *Streptococcus* septic arthritis (Tissi *et al.*, 1999). Here, we provide evidence that all the examined non-toxigenic strains of *C. diphtheriae* induce systemic and local IL-6, IL-1 β and TNF α secretion. In our opinion, in *C. diphtheriae*-induced septic arthritis these cytokines also actively contribute to articular damage. In fact, the more severe arthritis observed in mice infected with strains ISS-4749 or ISS-4746 is associated with the highest levels of IL-6 and IL-1 β , while strain ISS-3319, whose injection resulted in the lowest incidence and severity of arthritis, induced the lowest degree of IL-6 and IL-1 β local release. Surprisingly, high and early systemic levels of IFN- γ were found only in mice infected with strain ISS-3319. Thus, we might hypothesize that IFN- γ production counterbalances the proinflammatory response, leading to a more favourable outcome of the infection. Taken together, these observations indicate that the three strains of *C. diphtheriae* interact with the host in a different manner.

As with other pathogenic organisms, the *C. diphtheriae* adhesion process may involve bacterial surface-exposed structures (colonization and adhesion factors) able to recognize and bind to specific receptors of host cells (Mattos-Guaraldi *et al.*, 2000; de Oliveira Moreira *et al.*, 2003; Bertuccini *et al.*, 2004). The different adhesins (such as haemagglutinins, hydrophobins, exposed sugar residues and enzymes with *trans*-sialidase activity) may be involved in diverse biologically important host-pathogen interactions. The most striking difference among the strains examined in this study was the different cytokine pattern observed at both systemic and local levels. These data seem to suggest that differences exist in the cell surface structure of the bacilli, that, by engaging different cell receptors present on the host cells, may address a targeted cytokine production. Although such differences might appear to be related to the biotype, further *in vivo* and *in vitro* studies with other different strains of the two biotypes are needed to clarify this matter.

In conclusion, our mouse model, which shows similarities with human disease, offers outstanding potential for a better understanding of *C. diphtheriae* infection. The fact that different strains may induce a different cytokine pattern leads us to extend the study of surface structures as virulence factors and potential therapeutic targets.

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