HOST RESPONSE TO INFECTION

Role of interleukin-6 in determining the course of murine Tyzzer's disease

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Clostridium piliforme is an obligately intracellular bacterium that causes enterohepatic disease in many domestic and laboratory animal species. Susceptibility to infection is known to vary with the host immune status, species and strain, but little is known about specific immune mechanisms that regulate this disease. Subclinical infection was induced in weanling *C. piliforme*-susceptible DBA/2 or resistant C57BL/6 mice with either a toxic or a non-toxic *C. piliforme* isolate. Hepatic lesions and bacteria were evident in both mouse strains for 14 days after inoculation with the toxigenic bacterial isolate, but were never demonstrated following inoculation with the non-toxigenic isolate. All mice demonstrated increased interleukin-6 (IL-6) levels that were largely independent of host strain susceptibility to infection or virulence of the bacterial isolate. The severity of *C. piliforme*-induced hepatic lesions was increased by polyclonal anti-IL-6 treatment in both resistant (DBA/2) and susceptible (C57BL/6) mouse strains. These data indicate that IL-6 is important in mediating the course of murine *C. piliforme* infections but is not involved in determining host susceptibility to acute infection, nor is it influenced by the virulence of the *C. piliforme* isolate.

Introduction

Clostridium piliforme is a gram-negative obligately intracellular pathogen [1–6]. The bacterium induces an acute, often fatal enterohepatic disease in neonates and weanlings of a wide variety of laboratory and domestic animal species [3, 5–7]. Severity of infection varies widely between host species and strains, suggesting a role for host immunity in control of the infection. Additional evidence for host control of this infection is seen in recent reports of the isolation of subcutaneous *C. piliforme* in man [8, 9]. *C. piliforme* has not been reported previously in man and these patients were markedly immunocompromised. Although host immunity has an apparent role in regulating *C. piliforme* infection, specific factors that govern the severity of Tyzzer's disease are not well defined.

Murine Tyzzer's disease is often clinically silent but mouse strains differ in their susceptibility to both natural and experimental *C. piliforme* infections [7]. For example, DBA/2 mice often develop multiple

necrotic hepatic foci after experimental intravenous *C. piliforme* inoculation, whereas C57BL/6 mice develop few lesions and are considered resistant to *C. piliforme* [7]. The reasons for this differing susceptibility have not been thoroughly examined, but previous studies suggest that B lymphocytes, T lymphocytes and natural killer cells may be involved [4, 10, 11]. Recent investigations in this laboratory have demonstrated a role for neutrophils in mediating the course of experimental murine *C. piliforme* infection [10]. A significant rise in tumour necrosis factor- α (TNF- α) has also been demonstrated in *C. piliforme*-infected mice (R. A. Van Anandel, unpublished observations).

Interleukin-6 (IL-6) is known to regulate several aspects of gram-negative bacterial sepsis [12]. Specifically, IL-6 is important in neutrophil chemotaxis and activation [13] and is thought to modulate the immune response through downregulation of TNF- α activity [14]. IL-6 is known to alter the severity and persistence of infections produced by several pathogens such as *Listeria monocytogenes*, *Escherichia coli* and *Toxoplasma gondii* [15–18]. Given the role of IL-6 in mediating neutrophil and TNF- α responses to pathogens, the present study aimed to investigate the role of IL-6 in the regulation of murine *C. piliforme* infection.

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Materials and methods

Mice

Female DBA/2 and C57BL/6 mice were obtained at 24 days of age from Charles River Laboratories (Wilmington, MA, USA) and acclimatised for at least 4 days before use.

Bacteria

The toxic H1 isolate of *C. piliforme* was isolated from the livers of hamsters with clinical Tyzzer's disease and the non-toxic M1 isolate of *C. piliforme* was isolated from the livers of subclinically infected mice. Bacteria were subsequently maintained in tissue culture in murine liver cells (BNL, ATCC TIB 73), as described previously [19]. For animal inoculation, cultures containing bacteria in log-phase growth were washed twice with phosphate-buffered saline (PBS), scraped and resuspended in PBS. A sample of the harvested culture was removed and sonicated on ice to release intracellular bacteria for quantification with a haemocytometer. Mice were then inoculated intravenously with 1.0×10^5 unsonicated bacteria, as sonication has been shown to reduce *C. piliforme* infectivity (R. A. Van Andel, unpublished data). Control mice were inoculated intravenously with unsonicated BNL cells, as described previously [10].

Experimental design

To evaluate the role of IL-6 in subclinical *C. piliforme* infection, five groups of at least 15 susceptible (DBA/2) and 15 resistant (C57BL/6) juvenile (28-day-old) mice were challenged intravenously with 1.0×10^5 toxigenic (H1) or non-toxigenic (M1) *C. piliforme* organisms. Another five groups of at least 15 mice of each strain were inoculated with uninfected BNL cells as controls. Fifteen mice of each strain and inoculation group were killed at 0 (pre-inoculation), 1, 3, 7, 14 and 28 days after-inoculation. Livers were evaluated for lesions as described below. Cardiac blood samples from groups of five mice per inoculation group were collected, pooled, allowed to clot at room temperature, centrifuged at 5000 g for serum collection and stored at -20°C for evaluation by ELISA as described below.

In another series of studies, groups of 10 DBA/2 and 10 C57BL/6 juvenile mice were treated with either monoclonal or polyclonal anti-IL-6 antibody before inoculation with toxigenic *C. piliforme* H1, as previous studies in this laboratory have demonstrated that the H1 but not the M1 strain induces hepatic lesions and intracellular bacteria in mice [10]. Mice were killed at 3 days after inoculation and hepatic lesions and bacterial loads were evaluated as described below.

Evaluation of hepatic lesions and bacterial loads

At necropsy, livers were harvested for evaluation of gross and microscopic lesions and bacterial load as described previously [10]. Briefly, gross hepatic surface

necrotic foci were scored on a scale of 0–10 as follows: 0 = no necrotic foci; 1 = 1–10 necrotic foci per liver; 2 = 11–20 necrotic foci per liver, etc. Livers with >90 necrotic foci were given a score of 10. Sections of livers were fixed in formalin and processed for staining with either haematoxylin and eosin (H&E) for histopathological evaluation of lesions or with Steiner silver stain for estimation of bacterial load in 5- μm sections. Histological lesions were scored on a scale from 0 to 5 as follows: 0 = no lesions; 1 = 1–3 coagulative necrotic lesions per liver; 2 = 4–6 lesions; 3 = 7–10 lesions; 4 = >10 lesions; 5 = >10 lesions, more than half of which had caseous necrosis. Attempts in this laboratory to culture *C. piliforme* quantitatively from infected liver homogenates by plaque assays have been unsuccessful; however, the large size of *C. piliforme* (10–40 μm in length) allows visualisation of individual bacteria within hepatocytes. Therefore, bacterial load was assessed by counting organisms in 35 high-power silver-stained fields and scored on the following scale: 0 = no bacteria; 1 = 1–50 bacteria; 2 = 51–100 bacteria; 3 = 101–500 bacteria; 4 = 501–1000 bacteria; 5 = >1000 bacteria. Histological and bacterial scores were then doubled to provide a 1–10 scale for all lesions and bacterial counts.

Plaque assay

To determine the infectivity of bacteria from tissue culture, plaque assays were performed as described previously [20, 21]. Briefly, a sample of the bacteria harvested for mouse inoculation was diluted 1 in 500, 1 in 1000 and 1 in 2000 and inoculated on to confluent mouse fibroblasts cells (3T3, ATCC CCL 92) cultured in petri dishes. The cultures were overlaid with agarose 1% 3 h later. Then 24 h later, when motile bacteria were evident microscopically, plates were overlaid with a solution of agar 1% and neutral red 1.5% as described previously [10]. Plaques were counted visually after 24 h. Plaque assay results indicated that *C. piliforme* maintained 25–35% viability.

Cytokine ELISA

Serum samples from at least three groups of five animals at each time point were pooled and evaluated for IL-6 with a commercially available ELISA kit (Genzyme, Cambridge, MA, USA). Briefly, 50 μl of the pooled sera were diluted in 50 μl of buffer, loaded into anti-cytokine antibody-coated 96-well plates and assays were performed according to the manufacturer's instructions. To determine serum cytokine levels, the optical density of ELISA signal was compared to standards of known concentration with a plate reader (Bio-Tek Instruments, Winooksi, VT, USA). Each sample was examined in duplicate or triplicate.

Antibodies

To evaluate further the role of IL-6 in murine Tyzzer's disease, mice were treated with either monoclonal

(MAb) or polyclonal anti-IL-6 antibody to deplete IL-6 levels. Mice were then inoculated intravenously with *C. piliforme* H1 and the severity of disease and bacterial load were assessed at 3 days after-inoculation when hepatic *C. piliforme* lesions are most severe [10]. Rat IgG anti-mouse-IL-6 MAb (MP5-20F3, kindly provided by Dr J. Abrahms, DNAX, Palo Alto, CA, USA) was produced as ascites fluids grown in nu/nu mice (Frederick Cancer Research and Development Center, Frederick, MD, USA) and purified by ion-exchange chromatography (Bakerbond ABX column, J. B. Baker, Cambridge, MA, USA), as described previously [22]. To evaluate the effect of IL-6 depletion, test mice (17 DBA/2 and 15 C57BL/6) were given 0.25 mg of purified MAb intraperitoneally 2 h before intravenous bacterial inoculation, based upon published dosages and inoculation procedures [18, 23]. Control mice (15 DBA/2 and 16 C57BL/6) were given 0.25 mg of non-specific rat IgG 2 h before intravenous bacterial inoculation. MAb preparations had <0.1 ng of endotoxin/injected dose as determined by the E-toxate endotoxin quantitation kit (Sigma). Goat polyclonal anti-murine IL-6 was obtained from R&D Systems (Minneapolis, MN, USA; <10 ng of endotoxin/mg of polyclonal antibody). Experimental mice (10 DBA/2 and 8 C57BL/6) were inoculated with 10 µg of polyclonal antibody 4 h before bacterial inoculation as described previously [15]; control mice (6 DBA/2 and 8 C57BL/6) were given 0.25 ml (2.5 mg) of non-specific rat IgG intravenously 4 h before bacterial inoculation.

Statistical analysis

All data were evaluated for normal distribution by the Kolmogorov Smirnov test. When normally distributed, data were analysed by one-way ANOVA; when not normally distributed, analysis was performed by Kruskal-Wallis one-way ANOVA on ranks. All analyses were performed with SigmaStat (SSPS, San Diego, CA, USA) software. Results are reported as the mean and SEM; p values <0.05 were considered significant.

Results

IL-6 alterations induced by *C. piliforme*

To evaluate whether systemic IL-6 levels are altered by *C. piliforme* infection and if such alterations differed between *C. piliforme* isolates or between strains of mice, DBA/2 and C57BL/6 mice were inoculated intravenously with 10^5 bacterial cells of either H1 or M1 *C. piliforme*. Serum IL-6 levels were undetectable in mice before bacterial administration and in control mice after administration of uninfected BNL cells. However, both *C. piliforme* isolates induced a significant ($p < 0.05$) increase in serum IL-6 in both mouse strains which persisted from day 1 to day 14 after inoculation (Fig. 1). Serum IL-6 levels were not significantly different between the H1 and M1 strains,

Fig. 1. Serum levels of IL-6 after inoculation of mice with either (a) H1 or (b) M1 *C. piliforme* strain. Pooled serum from three groups of five *C. piliforme*-inoculated C57BL/6 (H1 or M1/C57; ■) and DBA/2 (H1 or M1/DBA; ○) or BNL-inoculated C57BL/6 (BNL/C57; ▲) and DBA/2 (BNL/DBA; *) mice was evaluated at 0, 1, 3, 7, 14 and 28 days by cytokine-specific ELISA ($p < 0.05$).

in spite of a lack of hepatic lesions after inoculation with the M1 strain. IL-6 levels returned to undetectable levels by 28 days after inoculation.

Effect of anti-IL-6 MAb administration on the development of *C. piliforme*-induced hepatic lesions

To evaluate further the role of IL-6 in control of *C. piliforme* and to evaluate the in-vivo activity of anti-IL-6 MAb, mice were treated with either anti-IL-6 MAb or rat IgG and were then inoculated with 10^5 H1 *C. piliforme* organisms. Administration of anti-IL-6 MAb before *C. piliforme* challenge resulted in serum IL-6 levels more than two-fold higher than in control *C. piliforme*-challenged mice (Table 1). This is consistent with reports from other laboratories [12, 24, 25], in which investigators noted a paradoxical increase in IL-6 after administration of anti-IL-6 MAb. In this study, MAb treatment did not significantly alter the *C. piliforme* load or severity of hepatic lesions in either strain of mice compared to control mice treated with normal rat IgG (Fig. 2).

Effect of polyclonal anti-IL-6 administration on the development of *C. piliforme*-induced hepatic lesions

Mice were treated with either polyclonal anti-IL-6 for IL-6 neutralisation or with rat IgG and were then

Table 1. Serum IL-6 levels in *C. piliforme*-infected mice treated with either monoclonal (MAb) or polyclonal (AAb) anti-IL-6 antibody

Mice	Mean (SEM) IL-6 levels (pg/ml)			
	MAb-treated	Control	PAb-treated	Control
DBA/2	137.0 (5.6)	68 (11)	7.7 (5.0)	57.2 (5.2)
C57BL/6	146.4 (7.8)	56.6 (3.8)	7.0 (2.8)	67.5 (6.3)

Numbers are the mean (SEM) of pooled serum from at least three groups of five *C. piliforme*-inoculated mice

Numbers in parentheses are SEM; $p < 0.05$.

Fig. 2. Gross, histological and bacterial load scores (a) in weanling DBA/2 mice treated with anti-IL-6 MAb ($n = 17$; ■) or IgG ($n = 15$; ■) and (b) in weanling C57BL/6 mice treated with anti-IL-6 MAb ($n = 15$; ■) or IgG ($n = 16$; ■). There was no significant difference between scores in treated and control mice ($p < 0.05$).

inoculated with 10^5 *C. piliforme* H1 organisms to elucidate the role of IL-6 in the control of *C. piliforme* infection. The serum IL-6 response of both mouse strains to *C. piliforme* challenge was markedly reduced by polyclonal anti-IL-6 administration compared with IgG-treated control mice (Table 1). In both strains of mice, the *C. piliforme* H1 hepatic load and severity of hepatic lesions was significantly increased by polyclonal antibody treatment compared with control mice treated with normal rat IgG (Fig. 3).

Fig. 3. Gross, histological and bacterial load scores (a) in weanling DBA/2 mice treated with polyclonal anti-IL-6 ($n = 10$; ■) or IgG ($n = 6$; ■) and (b) in juvenile C57BL/6 mice treated with polyclonal anti-IL-6 ($n = 8$; ■) or IgG ($n = 8$; ■). Scores were significantly higher in treated mice than in control mice ($p < 0.05$).

Discussion

The virulence factors of *C. piliforme* are poorly defined. It is known that the H1 strain produces a potent toxin not produced by the M1 strain [19], and that the H1 strain is more pathogenic than the M1 strain. As seen in this and previous studies [10, 26], the toxigenic H1 strain produced hepatic lesions with associated intracellular bacteria which decreased in severity after day 3, but persisted for up to 14 days after inoculation in both DBA/2 and C57BL/6 mice. Conversely, lesions were not induced by the non-toxigenic M1 isolate and no bacteria were visible in the limits of either DBA/2 or C57BL/6 mice after inoculation with strain M1.

In this study, IL-6 levels were similar in both strains of mice. A notable exception to this was evident on day 7 after inoculation with strain H1, at which time IL-6 levels were significantly higher in C57BL/6 than in DBA/2 mice. C57BL/6 mice are generally considered to be naturally resistant to *C. piliforme* infection relative to DBA/2 mice. As seen in this (Figs. 2 and 3) and previous studies in this laboratory [10] differences in strain susceptibility are evident by days 1 and 3 after inoculation. In the present study, at 1 and 3 days after inoculation serum IL-6 levels were not significantly different between C57BL/6 and DBA/2 mice, suggesting that IL-6 is not involved in mediating strain susceptibility in acute *C. piliforme* infection.

The similarity in IL-6 levels induced by the H1 and M1 strains was surprising, given that strain M1 induces no histologically detectable response, whereas strain H1 induces a pronounced neutrophil influx and tissue necrosis [10]. The H1 isolate also induces higher levels of TNF- α than does the M1 isolate (R. A. Van Andel, unpublished data). Given the role of IL-6 in neutrophil recruitment and TNF- α modulation [12, 14, 17], isolate H1 might be expected to induce dramatically higher IL-6 levels than isolate M1. However, cytokine secretion is not always proportional to the inflammatory response. For example, previous investigators have observed that monocyte production of IL-12 may be upregulated by phagocytosis of *Mycobacterium* and *Listeria* spp., whether or not the bacteria induce pronounced inflammation or lesions [27–29]. Previous PCR studies in this laboratory have documented that *C. piliforme* strain M1 established hepatic infection and that bacterial DNA persisted for several weeks, even though hepatic lesions and bacteria were not histologically evident (R. A. Van Andel, unpublished data). Thus, phagocytosis of *C. piliforme* M1 may upregulate IL-6 production in a manner similar to that seen with IL-12 in *Mycobacterium* and *Listeria* spp.

To further evaluate the role of IL-6 in *C. piliforme* infection, anti-IL-6 antibody was administered to DBA/2 and C57BL/6 mice. Studies were performed with both monoclonal and polyclonal anti-IL-6 antibodies, as other investigators have suggested that anti-IL-6 MAbs paradoxically increase serum IL-6 levels [12, 18, 24, 25, 30, 31]. Investigators suggest that this increase in IL-6 may be due to MAbs acting as 'chaperones,' shielding IL-6 from renal clearance [24]. However, it is unclear whether IL-6–MAb complexes enhance or hinder in-vivo IL-6 activity, leading to confusion in studies that used anti-IL-6 MAbs [18, 23, 24, 32]. Polyclonal antibodies are reported to deplete serum IL-6 [15], but to our knowledge, monoclonal and polyclonal anti-IL-6 antibodies have not been evaluated in the same study to determine whether MAbs enhance or impair IL-6 activity in vivo. Thus, in this study both MAbs and polyclonal antibodies were used to examine the role of IL-6 in *C. piliforme* infection.

Administration of anti-IL-6 MAb resulted in serum IL-6 levels more than two-fold higher in animals inoculated with *C. piliforme* than in control *C. piliforme*-inoculated animals (Table 1). Conversely, administration of polyclonal anti-IL-6 antibody to mice resulted in nearly undetectable serum IL-6 levels after *C. piliforme* challenge (Table 1). Mice treated with anti-IL-6 MAb before *C. piliforme* challenge developed hepatic lesions that were indistinguishable from those seen in IgG-treated, *C. piliforme*-challenged control mice (Fig. 2). *C. piliforme*-induced hepatic lesions in polyclonal anti-IL-6-treated mice were significantly worse than in control *C. piliforme*-challenged mice (Fig. 3). Data from the polyclonal study confirm that while IL-6 may not mediate strain susceptibility to acute *C. piliforme* infection, it does play a role in host resistance to Tyzzer's disease. The data from the MAb study confirm that IL-6–anti-IL-6 MAb complexes do not inactivate IL-6.

This study demonstrated that subclinical *C. piliforme* infections induce IL-6 perturbations, regardless of whether hepatic lesions or bacteria are ever apparent. This elevation is similar in both susceptible and resistant strains of mice and is prolonged, lasting at least 14 days. It is clear that IL-6 has a protective role in *C. piliforme* infection, but the degree of protection did not differ significantly between DBA/2 and C57BL/6 mice. These data suggest that IL-6 is important in host response to *C. piliforme* infection and is elevated even during subclinical infections, but is not involved in host strain susceptibility to acute Tyzzer's disease.

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