HOST RESPONSE TO INFECTION

Prolonged perturbations of tumour necrosis factor- α and interferon- γ in mice inoculated with *Clostridium piliforme*

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Clostridium piliforme is an obligate intracellular bacterium that causes enterohepatic disease in many animal species. *C. piliforme* infections are commonly subclinical in laboratory rats and mice, and little is known about host regulation of disease or of the effects of *C. piliforme* infections on investigations that use subclinically infected animals. To assess host regulation of subclinical *C. piliforme* infections and the effects of those infections on laboratory mice, the expression of the pro-inflammatory cytokines tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) was evaluated at 0, 1, 3, 7, 14 and 28 days after inoculation with *C. piliforme*. 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* strain. Hepatic lesions and bacteria were demonstrated histologically in both mouse strains for 14 days after inoculation with the toxigenic bacterial strain, but were never demonstrated histologically following inoculation with the non-toxigenic strain. Hepatic TNF- α and IFN- γ mRNA and serum protein levels were similarly elevated in both mouse strains 1 day after inoculation with both *C. piliforme* strains, as evaluated by reverse transcription PCR and enzyme-linked immunosorbent assays, respectively. Elevation of IFN- γ persisted for 14 days after inoculation; TNF- α remained elevated at 28 days after inoculation.

Introduction

Clostridium piliforme, an obligately intracellular bacterium which strains gram-negative, is the causative agent of Tyzzer's disease. The bacterium may induce acute and often fatal enterohepatic disease in neonates and weanlings of many domestic and laboratory animal species [1–6]. The severity of disease is governed by several poorly defined bacterial and host factors [7–11]. Bacterial factors that influence disease are not well characterised, but it is known that some *C. piliforme* isolates produce toxins, whereas others do not [9]. The precise role of toxins in the pathogenesis of *C. piliforme* infection is unknown, but toxic isolates are generally more virulent than non-toxic isolates [9]. Previous studies in this laboratory indicated that toxigenic H1 *C. piliforme* isolates induced hepatic

lesions in two strains of mice, while no lesions were seen following inoculation with the non-toxigenic M1 isolate [12].

Although Tyzzer's disease is severe in many animal species, infections are often subclinical. In mice, Tyzzer's disease is often clinically asymptomatic, yet mouse strains differ in susceptibility to both natural and experimental *C. piliforme* infections [4]. For example, DBA/2 mice often develop multiple necrotic hepatic foci following experimental intravenous *C. piliforme* inoculation, whereas C57BL/6 mice develop few lesions and are considered resistant to *C. piliforme* [4]. The reasons for this differing susceptibility have not been examined thoroughly, but previous studies suggest a role for B lymphocytes, T lymphocytes and natural killer cells in mediating strain susceptibility [4, 8, 13]. Recent reports of subcutaneous *C. piliforme* infection in CD4⁺ T-lymphocyte-deficient human patients [14, 15] provide further anecdotal support for the role of host immunity in mediating susceptibility to *C. piliforme*. Although these data suggest an important role for cell-mediated immunity in the control of

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Tyzzer's disease, there has been no systematic evaluation of lymphocyte-mediated factors that control susceptibility to Tyzzer's disease. Similarly, although it has been reported that subclinical Tyzzer's disease alters murine hepatic metabolism of pharmacological agents [16], long-term systemic sequelae to murine *C. piliforme* infection have not been examined.

Molecular characterisation of a number of cytokines and their genes provides methods to evaluate host responses to infectious agents. Both local and systemic cytokine responses are now known to be important in determining murine susceptibility to endotoxin and various intracellular pathogens [17–23]. Tumour necrosis factor- α (TNF- α) is one of the earliest cytokines released in inflammatory responses and is crucial in determining host susceptibility to infection with a number of intracellular pathogens [18, 21, 24]. Interferon- γ (INF- γ) mediates several functions associated with local inflammatory processes, is important in initiating cell-mediated (Th1) responses in bacterial infections and acts synergically with TNF- α in response to intracellular bacterial infections [21]. In the present study, the roles of TNF- α and INF- γ in mediating murine strain susceptibility to acute *C. piliforme* infection were evaluated. The potential long-term sequelae to this acute bacterial infection were also evaluated by examining TNF- α and INF- γ expression for 28 days after inoculation.

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 allowed to acclimatise for 4 days before use.

Bacteria

The toxic H1 *C. piliforme* strain was isolated from the livers of hamsters with clinical Tyzzer's disease; the non-toxic M1 *C. piliforme* strain was isolated from livers of subclinically infected mice. These isolates were then propagated in tissue culture as described previously [9]. For animal inoculation, bacteria were grown in murine liver cells (BNL; ATCC TIB 73). Cultures with bacteria in log-phase growth were washed twice with phosphate-buffered saline (PBS) and harvested. To determine infective units, a sample of the culture was sonicated to release intracellular organisms and bacteria were counted with a haemocytometer. Mice were subsequently inoculated intravenously with 1.0×10^5 unsonicated *C. piliforme*-infected BNL cells, as sonication has been shown to reduce *C. piliforme* infectivity (Van Anandel, unpublished data). Control mice were inoculated intravenously with unsonicated BNL cells.

Experimental design

Five groups of 10 susceptible (DBA/2) and resistant (C57BL/6) juvenile (28-day-old) mice were inoculated intravenously with 1.0×10^5 toxigenic (H1) *C. piliforme* organisms in BNL cells, 1.0×10^5 non-toxigenic (M1) *C. piliforme* organisms in BNL cells, or uninfected BNL cells (control animals). Ten mice of each strain and inoculation group were killed at 0 (pre-inoculation), 1, 3, 7, 14 and 28 days after inoculation. At necropsy, livers were evaluated for gross and histological lesions and bacterial load, as described previously [13]. Additional liver sections were snap-frozen in liquid nitrogen before storage at -80°C for subsequent PCR analysis. Cardiac blood samples were collected from groups of five mice per inoculation group, pooled, allowed to clot at room temperature, centrifuged at 5000 *g* and the sera were pooled and stored at -20°C for subsequent ELISA evaluation.

Endotoxin analysis

The endotoxin content of bacteria and tissue culture cells was measured with the *Limulus* amoebocyte lysate endotoxin quantitation kit (E-toxate, Sigma).

Plaque assay

The infectivity of bacteria in BNL cells was determined by plaque assays, as described previously [13, 25, 26]. Plaque assay results indicated that *C. piliforme* maintained 25–35% viability after preparation and inoculation.

Bacterial PCR

Livers removed from *C. piliforme*-infected mice at necropsy were examined for the presence of *C. piliforme* by PCR. Briefly, DNA was isolated from 25 mg of hepatic tissue with a Quiagen column (Quiagen, Chatsworth, CA, USA). Nucleic acid samples were then amplified in 0.5-ml tubes with 16S rRNA gene sequences that are conserved among all *C. piliforme* isolates evaluated to date (Riley, unpublished data). The PCR mixture contained 25 mM MgCl₂; buffer containing 50 mM KCl, 10 mM Tris-HCl and Triton X-100 0.1%; 500 μM of each dNTP; *Taq* DNA polymerase (Perkin-Elmer, Alameda, CA, USA) 2.5 U and 10 μM of each primer. Sequences for these primers are 5'-ACCATTGACAGCCTACGTAA-3' and 5'-GTCTCGCTTCACTTTGTTGTA-3'. Samples were denatured at 94°C for 1 min followed by 40 cycles of 94°C for 2 s, 53°C for 5 s and 72°C for 30 s. A 7-min final extension at 72°C was performed subsequently. Products were examined by electrophoresis on a Nusieve agarose 2% gel (FMC Bioproducts, Rockland, ME, USA) and visualised by ethidium bromide staining.

Reverse transcription for preparation of cDNA

Mice were killed 0, 1, 3, 7, 14 and 28 days after bacterial inoculation. Hepatic RNA was isolated as described previously [18]. Briefly, 10–50 mg of hepatic tissue were pulverised under liquid nitrogen and resuspended in Ultraspec solution (Biotecx, Houston, TX, USA). RNA was extracted with chloroform; the purity and concentration of RNA samples were determined by 260/280 nm spectrophotometry. Reverse transcription was performed with 1 μ g of total RNA, in 25 mM MgCl₂; buffer containing 50 mM KCl, 10 mM Tris-HCl and Triton X-100 0.1%; 0.5 mM of each dNTP; 10 μ M oligo dT primers and Superscript Reverse Transcriptase (Gibco BRL, Gaithersburg, MD, USA) 200 U.

Cytokine-specific PCR

Cytokine PCR was performed with 1 μ g of cDNA with primers specific for murine β -actin, TNF- α or IFN- γ . β -Actin and TNF- α primers were synthesised by Integrated DNA Technologies (IDT, Coralville, IA, USA) from previously described sequences [24, 27, 28]. IFN- γ primers were synthesised with sequences kindly provided by Steven Kunkel (University of Michigan, USA). The cytokine primer sequences were as follows: β -actin: 5'-GTGGGCCGCTCTAGG CACCAAGGT-3' and 5'-CTGGATGGCTACGTACAT GGC-3'; TNF- α : 5'-CCTGTAGCCACGTCGTAGC-3' and 5'-TTGACCTCAGCGCTGAGTTG-3'; and IFN- γ : 5'-GAGTCTCTTCTTGGATATCT-3' and 5'-CAGTCA GCCGCTTGGCTGTC-3'. PCR was performed in a 50- μ l mixture as described above for bacterial PCR. Samples were denatured at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 2 min, with a 7-min final extension at 72°C. Products were examined by electrophoresis on a Nusieve agarose 2% gel and visualised with ultraviolet light after staining with ethidium bromide.

Cytokine ELISA

Serum samples were examined for the presence of TNF- α and IFN- γ with commercially available ELISA kits (Genzyme, Cambridge, MA, USA). Briefly, 50 μ l of the pooled sera were diluted in 50 μ l 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 intensity of the ELISA signal was compared to standards of known concentration with a plate reader (Bio-Tek Instruments, Winooksi, VT, USA). Each sample was run in duplicate or triplicate.

Statistical analysis

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 of <0.05 were considered significant.

Results

C. piliforme-induced alterations in IFN- γ

Hepatic IFN- γ mRNA was detected at day 1 after inoculation with both bacterial isolates and remained elevated until 14 days after inoculation in both C57BL/6 and DBA/2 mice (Figs. 1 and 2). No hepatic IFN- γ mRNA was evident before inoculation with *C. piliforme* or at any time point in BNL cell-inoculated control mice. Serum ELISA values for IFN- γ were low in uninoculated C57BL/6 and DBA/2 mice (Fig. 3). Inoculation with either H1 or M1 *C. piliforme* caused elevated serum IFN- γ levels for 1–14 days after

Fig. 1. Results of RT-PCR for hepatic IFN- γ (A) and β -actin (B) in C57BL/6 mice at 0, 1, 3, 7, 14 and 28 days after inoculation with toxigenic (H1) or non-toxicogenic (M1) *C. piliforme*. M, mol. wt marker; B, control mice 3 days after inoculation with uninfected BNL cells; 0, day 0 of infection (uninoculated mice).

Fig. 2. Results of RT-PCR for hepatic IFN- γ (A) and β -actin (B) in DBA/2 mice at 0, 1, 3, 7, 14 and 28 days after inoculation with toxigenic (H1) or non-toxicogenic (M1) *C. piliforme*. M, mol. wt marker; B, control mice 3 days after inoculation with uninfected BNL cells; 0, day 0 of infection (uninoculated mice).

Fig. 3. Serum levels of IFN- γ following inoculation with either *C. piliforme* or uninfected BNL cells. (a) C57BL/6 and DBA/2 mice inoculated with toxigenic H1 *C. piliforme* (H1/C57, ■, and H1/DBA, ○, respectively) or BNL cells (BNL/C57, ▲, and BNL/DBA, ×, respectively). (b) Mice inoculated with non-toxicogenic M1 *C. piliforme* (M1/C57, ■, and M1/DBA, ○) or BNL cells (BNL/C57, ▲, and BNL/DBA, ×). Pooled sera from three groups of five treated and control mice were evaluated at 0, 1, 3, 7, 14 and 28 days by cytokine-specific ELISA. Bars represent SEM. $p < 0.05$.

inoculation. In both strains of mice, strain H1 induced significantly ($p < 0.01$) higher levels of IFN- γ than strain M1. There were no consistent significant differences in IFN- γ levels between the mouse strains. By 28 days after inoculation, serum IFN- γ had returned to pre-inoculation levels in all mice. Serum IFN- γ levels were unaltered by inoculation of control animals with uninfected BNL cells.

C. piliforme-induced alteration in TNF- α

No hepatic TNF- α mRNA was detected by RT-PCR before bacterial inoculation or in BNL-inoculated control animals at any time (Figs. 4 and 5). Hepatic TNF- α mRNA was elevated by day 1 and remained elevated at day 28 after inoculation with both bacterial strains and in both strains of mice. Serum TNF- α was undetectable by ELISA in either mouse strain before inoculation. Inoculation of both DBA/2 and C57BL/6 mice with either *C. piliforme* strain induced significant elevations in serum TNF- α as compared with control mice (Fig. 6). Toxigenic H1 *C. piliforme* induced higher serum TNF- α levels than did non-toxicogenic M1. Serum TNF- α remained elevated at 28 days after inoculation with strain H1 whereas in the mice inoculated with strain M1 the levels of serum TNF- α

Fig. 4. Results of RT-PCR for hepatic TNF- α (A) and β -actin (B) in C57BL/6 mice at 0, 1, 3, 7, 14 and 28 days after inoculation with toxigenic (H1) or non-toxicogenic (M1) *C. piliforme*. M, mol. wt marker; B, control mice 3 days after inoculation with uninfected BNL cells; 0, day 0 of infection (uninoculated mice).

Fig. 5. Results of RT-PCR for hepatic TNF- α (A) and β -actin (B) in DBA/2 mice at 0, 1, 3, 7, 14 and 28 days after inoculation with toxigenic (H1) or non-toxicogenic (M1) *C. piliforme*. M, mol. wt marker; B, control mice 3 days after inoculation with uninfected BNL cells; 0, day 0 of infection (uninoculated mice).

were significantly lower at 28 days than at day 14 after inoculation.

Persistence of C. piliforme DNA in hepatic tissues

Hepatic tissues were examined for the presence of *C. piliforme* with bacteria-specific PCR primers. No bacterial DNA PCR signal was detected in uninoculated or BNL-inoculated animals. Bacterial DNA was amplified from mice inoculated with either of the *C. piliforme* strains from day 1 to day 28 after inoculation. Consistent with previous findings in this laboratory [13], no lesions or bacteria were ever evident histologically in mice inoculated with the M1 strain. After inoculation with strain H1, a few mice had mild lesions at 7 days, but lesions were not evident after day 14.

Fig. 6. Serum levels of TNF- α following inoculation with either *C. piliforme* or uninfected BNL cells. (a) C57/BL6 and DBA/2 mice inoculated with toxigenic H1 *C. piliforme* (H1/C57, $\blacksquare$ —, and H1/DBA, $\circ$ —, respectively) or BNL cells (BNL/C57, $\blacktriangle$ —, and BNL/DBA, $\times$ —, respectively). (b) Mice inoculated with non-toxicogenic M1 *C. piliforme* (M1/C57, $\blacksquare$ —, and M1/DBA $\circ$ —) or BNL cells (BNL/C57, $\blacktriangle$ —, and BNL/DBA, $\times$ —). Pooled sera from three groups of five treated and control mice were evaluated at 0, 1, 3, 7, 14 and 28 days by cytokine-specific ELISA. Bars represent SEM. $p < 0.05$.

Endotoxin status of *C. piliforme*

Unlike most members of the clostridial genus, *C. piliforme* stains weakly gram-negative [10]. However, to our knowledge there are no previous reports evaluating the *Limulus* amoebocyte lysate assay response of *C. piliforme*. Thus, toxic H1 and non-toxic M1 *C. piliforme* strains and uninoculated BNL cells were evaluated for evidence of endotoxin by the *Limulus* amoebocyte lysate (LAL, E-toxate) assay. Both H1 and M1 *C. piliforme* strains gave positive results in this assay, there were no differences in LAL assay reaction between the H1 and M1 strains. No evidence of LAL reactivity was seen in the BNL cells.

Discussion

Serological evidence suggests that subclinical murine *C. piliforme* infection may be widespread in both animals and man [2, 15, 29, 30]. However, host factors regulating disease expression have not been examined thoroughly. Furthermore, although subclinical *C. piliforme* infection is not uncommon in various laboratory rodent populations, the potential effect of subclinical *C. piliforme* has not been evaluated systematically. This present study documents several important findings regarding subclinical murine Tyzzer's disease. First,

perturbations of IFN- γ and TNF- α were similar in susceptible and resistant mouse strains, suggesting that these cytokines are integral to the host response to *C. piliforme*, but do not mediate strain susceptibility to this infection. Secondly, significant IFN- γ and TNF- α disturbances were seen in the absence of clinical disease, and in the case of strain M1, with no histological evidence of infection. Thirdly, perturbations in cytokines, particularly TNF- α , persisted well after the resolution of histologically detected lesions. TNF- α remained elevated 28 days after inoculation in both mouse strains, irrespective of the severity of hepatic lesions.

An interesting finding in this study was the persistence of *C. piliforme* DNA in hepatic tissues. Bacterial DNA was evident with both bacterial strains from days 1 to 28 after inoculation. The presence of bacterial DNA was not correlated with histological evidence of infection. No lesions or bacteria were ever evident histologically in mice inoculated with strain M1; most lesions induced by strain H1 had resolved by 7 days after inoculation and no lesions were evident after day 14. Investigators have debated whether murine *C. piliforme* infection is latent or transient [10, 29]. This question is complicated by the fact that plaque assays from *C. piliforme*-infected tissue are insensitive for determining infection or quantifying bacterial load [12, 13]. PCR is highly sensitive and capable of detecting minute quantities of DNA. Thus, it is possible that these data suggest the presence of persistent DNA products rather than persistent infection. However, studies with other gram-negative and gram-positive bacteria indicate that the host cytokine response declines rapidly as pathogenic bacteria lose viability [31–34], making it unlikely that small quantities of non-viable *C. piliforme* DNA would induce systemic cytokine alterations such as those reported here 28 days after inoculation. Thus, persistence of *C. piliforme* DNA and prolonged disturbances in IFN- γ and TNF- α suggests that hepatic *C. piliforme* infection may be sustained. Further studies will be required to document such persistent *C. piliforme* infection.

The taxonomic classification of *C. piliforme* was changed recently from *Bacillus piliformis* to *Clostridium piliforme* based upon 16S ribosomal RNA analysis [35]. Unlike most members of the genus *Clostridium*, *C. piliforme* is considered gram-negative based upon staining characteristics [9]. However, to our knowledge, production of endotoxin by *C. piliforme* has not been evaluated previously. In this study, strains H1 and M1 of *C. piliforme* were positive in the *Limulus* amoebocyte lysate assay, suggesting production of endotoxin. Endotoxin is known to induce both TNF- α and IFN- γ , raising the question as to the contribution of endotoxin present in the initial inoculum to the cytokine perturbations seen in this study. Although endotoxin released from cultured bacteria may contribute to initial cytokine perturbations

associated with experimental *C. piliforme* infection, it is unlikely to account for the sustained cytokine expression seen in the present study. Various reports document that cytokine levels drop rapidly (within 12 h) following a single dose exposure to endotoxin and diminish significantly within 24 h of a single dose of *Escherichia coli* [32, 36]. Furthermore, transient IFN- γ perturbations are reportedly seen following intravenous inoculation with 10^7 but not 10^3 *E. coli* [32]. IFN- γ levels in the present study were elevated at 14 days after inoculation with strain M1, even with no histological evidence of infection. Further study is required to evaluate bacterial virulence mechanisms, but it is unlikely that endotoxin alone from so few *C. piliforme* organisms was sufficient to account for the cytokine perturbations seen in this study.

Cytokine perturbations may have diverse deleterious effects on research investigations that utilise subclinically infected animals. For example, it has been documented previously that elevated serum TNF- α levels in subclinical bacterial infections alter immune responses and prolong survival of mice during subsequent experimental infection with agents such as *Listeria monocytogenes* and *Candida albicans* [19, 37]. Elevations in IFN- γ are known to increase murine resistance to experimental infection with *Brucella abortus*, *L. monocytogenes* and *Mycobacterium tuberculosis* [18, 20, 34, 37]. Elevated cytokine levels are also known to decrease growth of experimentally transplanted tumours in mice [38]. Data from the present study suggest that experimental animals with subclinical *C. piliforme* infections may be unsuitable for many research projects.

In conclusion, it has been demonstrated that prolonged perturbations in TNF- α and IFN- γ are induced by subclinical murine *C. piliforme* infection. Elevations were similar in both DBA/2 and C57BL/6 mice, suggesting that TNF- α and IFN- γ responses are not directly involved in mediating strain susceptibility to *C. piliforme*. Notably, the duration of cytokine elevations was largely independent of bacterial strain or presence of hepatic lesions. While TNF- α does not appear to mediate host susceptibility directly, it remained elevated for several weeks after inoculation. Thus, subclinical murine *C. piliforme* infections may have a dramatic impact on many biomedical investigations even in the absence of histologically identifiable lesions or bacteria.

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References

- Hook RR, Riley LK, Franklin CL, Besch-Williford CL. Seroanalysis of Tyzzer's disease in horses: implications that multiple strains can infect Equidae. *Equine Vet J* 1995; **27**: 8–12.
- Motzel SL, Riley LK. *Bacillus piliformis* flagellar antigens for serodiagnosis of Tyzzer's disease. *J Clin Microbiol* 1991; **29**: 2566–2570.
- Veazey RS, Paulsen DB, Schaeffer DO. Encephalitis in gerbils due to naturally occurring infection with *Bacillus piliformis* (Tyzzer's disease). *Lab Anim Sci* 1992; **42**: 516–518.
- Waggie KS, Hansen CT, Ganaway JR, Spencer TS. A study of mouse strains susceptibility to *Bacillus piliformis* (Tyzzer's disease): the association of B-cell function and resistance. *Lab Anim Sci* 1981; **31**: 139–142.
- Waggie KS, Thornburg LP, Grove KJ, Wagner JE. Lesions of experimentally induced Tyzzer's disease in Syrian hamsters, guinea pigs, mice and rats. *Lab Anim* 1987; **21**: 155–160.
- Young JK, Baker DC, Burney DP. Naturally occurring Tyzzer's disease in a puppy. *Vet Pathol* 1995; **32**: 63–65.
- Fries AS. Studies on Tyzzer's disease: acquired immunity against infection and activation of infection by immunosuppressive treatment. *Lab Anim* 1979; **13**: 143–147.
- Livingston RS, Franklin CL, Besch-Williford CL, Hook RR, Riley LK. A novel presentation of *Clostridium piliforme* infection (Tyzzer's disease) in nude mice. *Lab Anim Sci* 1996; **46**: 21–25.
- Riley LK, Caffrey CJ, Musille VS, Meyer JK. Cytotoxicity of *Bacillus piliformis*. *J Med Microbiol* 1992; **37**: 77–80.
- Riley LK, Franklin CL, Hook RR, Besch-Williford CL. Tyzzer's disease: an update of current information. In: Charles River Laboratories Technical Bulletin, Fall 1994.
- Waggie KS, Ganaway JR, Wagner JE, Spencer TH. Experimentally induced Tyzzer's disease in Mongolian gerbils (*Meriones unguiculatus*). *Lab Anim Sci* 1984; **34**: 53–57.
- Van Andel RA, Hook RR, Franklin CL, Besch-Williford CL, Riley LK. Interleukin-12 has a role in mediating resistance of murine strains to Tyzzer's disease. *Infect Immun* 1998; **66**: 4942–4946.
- Van Andel RA, Hook RR, Franklin CL, Besch-Williford CL, van Rooijen N, Riley LK. The effect of neutrophil, natural killer cell, and macrophage depletion on murine *Clostridium piliforme* infection. *Infect Immun* 1997; **65**: 2725–2731.
- Hilyard EJ, Hadfield TL, D'Nicola ME, Smith K, Skelton HG. Detection of Tyzzer's bacillus in humans from paraffin-embedded tissue using polymerase chain reaction. In: Abstracts of the 96th General Meeting of the American Society for Microbiology, New Orleans, LA. Washington, DC, American Society for Microbiology. 1996: C-420, p. 72.
- Smith KJ, Skelton HG, Hilyard EJ et al. *Bacillus piliformis* infection (Tyzzer's disease) in a patient infected with HIV-1: Confirmation with 16S ribosomal RNA sequence analysis. *J Am Acad Dermatol* 1996; **34**: 343–348.
- Fries AS, Ladefoged O. The influence of *Bacillus piliformis* (Tyzzer) infection on the reliability of pharmacokinetic experiments in mice. *Lab Anim* 1979; **13**: 257–261.
- Cain TK, Rank RG. Local Th1-like responses are induced by intravaginal infection of mice with the mouse pneumonitis biovar of *Chlamydia trachomatis*. *Infect Immun* 1995; **63**: 1784–1789.
- Ilzawa Y, Wagner RD, Czuprynski CJ. Analysis of cytokine mRNA expression in *Listeria*-resistant C57BL/6 and *Listeria*-susceptible A/J mice during *Listeria monocytogenes* infection. *Infect Immun* 1993; **61**: 3739–3744.
- Louie A, Baltch AL, Smith RP et al. Tumour necrosis factor alpha has a protective role in a murine model of systematic candidiasis. *Infect Immun* 1994; **62**: 2761–2772.
- Sarmiento AM, Appelberg R. Relationship between virulence of *Mycobacterium avium* strains and induction of tumor necrosis factor alpha production in infected mice and in vitro-cultured mouse macrophages. *Infect Immun* 1995; **63**: 3759–3764.
- Wagner RD, Czuprynski CJ. Cytokine mRNA expression in livers of mice infected with *Listeria monocytogenes*. *J Leukoc Biol* 1993; **53**: 525–531.
- Urban BC, Ferguson DJP, Pain A et al. *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature* 1999; **400**: 73–77.
- Wang H, Bloom O, Zhang M et al. HGM-1 as a late mediator of endotoxin lethality in mice. *Science* 1999; **285**: 248–251.
- Lukacs NW, Strieter RM, Chensue SW, Widmer M, Kunkel SL. TNF- α mediates recruitment of neutrophils and eosinophils during airway inflammation. *J Immunol* 1995; **154**: 5411–5417.
- Kawamura S, Taguchi F, Fujiwara K. Plaque formation by

- Tyzzler's organism in primary monolayer culture of mouse hepatocytes. *Microbiol Immunol* 1983; **27**: 415–424.
26. Wike DA, Tallent G, Peacock MG, Ormsbee RA. Studies of the rickettsial plaque assay technique. *Infect Immun* 1972; **5**: 715–722.
 27. Alonso S, Minty A, Bourlet Y, Buckingham M. Comparison of three actin-coding sequences in the mouse; evolutionary relationships between the actin genes of warm-blooded vertebrates. *J Mol Evol* 1986; **23**: 11–22.
 28. Novaković S, Buldough I. In vitro TNF- α production and in vivo alteration of TNF- α RNA in mouse peritoneal macrophages after treatment with different bacterial derived agents. *Cancer Lett* 1994; **81**: 99–109.
 29. Fries AS. Antibodies to *Bacillus piliformis* (Tyzzler's disease) in sera from man and other species. In: 7th ICLAS Symposium, Utrecht. New York, Gustav Fischer Verlag. 1980: 245–252.
 30. Gibson SV, Waggle KS, Wagner JE, Ganaway JR. Diagnosis of subclinical *Bacillus piliformis* infection in a barrier-maintained mouse production colony. *Lab Anim Sci* 1987; **37**: 786–788.
 31. Cusumano V, Mancuso G, Genovese F *et al.* Role of gamma interferon in a neonatal mouse model of group B streptococcal disease. *Infect Immun* 1996; **64**: 2941–2944.
 32. Kohler J, Heumann D, Garrotta G *et al.* IFN- γ involvement in the severity of gram-negative infections in mice. *J Immunol* 1993; **151**: 916–921.
 33. Walley KR, Lukacs NW, Standiford TJ, Strieter RM, Kunkel SL. Balance of inflammatory cytokines related to severity and mortality of murine sepsis. *Infect Immun* 1996; **64**: 4733–4738.
 34. Zhan Y, Kelso A, Cheers C. Cytokine production in the murine response to brucella infection of immunization with antigenic extracts. *Immunology* 1993; **80**: 458–464.
 35. Duncan AJ, Carman RJ, Olsen GJ, Wilson KH. Assignment of the agent of Tyzzler's disease to *Clostridium piliforme* comb. nov. on the basis of 16S rRNA sequence analysis. *Int J Sys Bacteriol* 1993; **43**: 314–318.
 36. Heinzel FP, Rerko RM, Ling P, Hakimi J, Schoenhaut DS. Interleukin 12 is produced in vivo during endotoxemia and stimulates synthesis of gamma interferon. *Infect Immun* 1994; **62**: 4244–4249.
 37. Ehlers S, Mielke MEA, Blankenstein T, Hahn H. Kinetic analysis of cytokine gene expression in the livers of naïve and immune mice infected with *Listeria monocytogenes*. The immediate early phase in innate resistance and acquired immunity. *J Immunol* 1992; **149**: 3016–3022.
 38. Molloy RG, Mannick JA, Rodrick ML. Cytokines, sepsis and immunomodulation. *Br J Surg* 1993; **80**: 289–297.