Intestinal infection of BALB/c mice with *Yersinia enterocolitica* O9 causes major modifications in phenotype and functions of spleen cells

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***Yersinia enterocolitica* serotype O9 may cause a persistent intestinal infection with few or no symptoms in humans and in BALB/c mice. The present study demonstrated profound alterations in the immune status of BALB/c mice infected with *Y. enterocolitica* O9. Infected mice developed splenomegaly and phenotypic analysis of spleen cells revealed increases in CD3⁺ total T cells, CD4⁺ helper T cells, CD8⁺ cytotoxic T cells and CD11b⁺ phagocytic cells. Spleen cells from infected mice exhibited impaired responses to mitogens and suppressed the proliferation of normal splenocytes in response to mitogens. Suppression of responses to concanavalin A and heat-killed yersiniae was associated with increased production of gamma interferon and reactive nitrogen intermediates. *Y. enterocolitica*-infected mice resisted challenge with a lethal dose of the intracellular pathogen *Listeria monocytogenes*. These findings suggest that infection of mice with *Y. enterocolitica* O9 induces gamma-interferon-secreting cells that promote macrophage activation, mediating resistance to infection with *L. monocytogenes*, and macrophage production of reactive nitrogen intermediates, which results in *in vitro* inhibition of lymphocyte response to mitogens.**

Keywords: *Yersinia enterocolitica*, gamma interferon, lymphocytes, macrophage activation

INTRODUCTION

Yersinia enterocolitica is a common cause of intestinal infections in humans (Cover & Aber, 1989; Bottone, 1997). Extraintestinal and systemic infections are most often caused by strains belonging to so-called high-virulence serotypes, such as serotype O8 (Heesemann *et al.*, 1993), although low-virulence serotypes, such as O3 and O9, may occasionally cause bacteraemia in patients with iron overload or other underlying diseases (Rabson *et al.*, 1975; Bouza *et al.*, 1980). The presence of a 70 kb plasmid (pYV) that carries a number of virulence genes is required for *Y. enterocolitica* strains to become virulent (Cornelis *et al.*, 1998).

Non-septic sequelae of human yersiniosis include reactive arthritis, erythema nodosum, Reiter's syndrome,

autoimmune thyroiditis and other immunopathological complications (Winblad, 1973; Bech *et al.*, 1974; Toivanen *et al.*, 1985). Alterations in the immune networks following infection with a number of micro-organisms are likely to play a role in the genesis of immunopathological diseases (Schwartz, 1993). The effects of experimental yersiniosis on immunity parameters, such as the size of spleen cell subsets or the production of immune cytokines, have been documented in mice infected with serotype O8 strains (Autenrieth *et al.*, 1994; Bohn *et al.*, 1998). In comparison, low-virulence serotypes have received little attention, although they possess immunomodulatory properties (Ruiz-Bravo *et al.*, 1985, 1996). In humans, enteric colonization with low-virulence serotypes may persist for periods of up to 14 weeks without symptoms (Morris *et al.*, 1991); however, there is no information available about the possible effects of the intestinal carriage of these strains on the immune condition of the hosts. In this study, we used a murine intestinal infection model (Ruiz-Bravo *et al.*, 1999) to examine the immune status

Abbreviations: Con A, concanavalin A; HKY, heat-killed yersiniae; IFN- γ , gamma interferon.

of mice infected with a low-virulence serotype of *Y. enterocolitica*.

METHODS

Mice. Six- to eight-week-old female BALB/c mice were provided by the Unit of Animal Experimentation, University of Granada (Granada, Spain). They were maintained under pathogen-free conditions.

Experimental infection with *Y. enterocolitica*. Strain IP383 of *Y. enterocolitica* was used in all experiments. It is a serotype O9 strain that carries the virulence plasmid pYV (Mazigh *et al.*, 1983). Bacteria were grown on Tryptic soy agar (Difco) at 25 °C for 24 h, harvested, washed and resuspended in sterile water to obtain 10^9 bacteria ml^{-1} . Mice were infected via the oral route, as described previously (Ruiz-Bravo *et al.*, 1999).

Phenotypic analysis of spleen cells. Spleens were homogenized in PBS containing 1% (w/v) BSA (Sigma). Splenocytes were sedimented by centrifugation, resuspended in red blood cell lysing buffer (Sigma) for 10 min, washed and resuspended in PBS containing BSA. Cell suspensions from individual mice were adjusted to 10^7 viable cells ml^{-1} . Aliquots (100 μl) were incubated with 5 μl fluorescein-isothiocyanate-conjugated monoclonal antibodies. The monoclonal antibodies used were anti-CD45R (clone RA3-6B2, which recognizes B lymphocytes), anti-CD3 (clone 29B, a pan T cell marker), anti-CD4 (clone H129.19, a marker for helper/inducer T cells and delayed hypersensitivity T cells), anti-CD8 (clone 53-6.7, a marker for cytotoxic T cells) and anti-CD25 (clone ATM-13, which recognizes the α -chain of the IL-2 receptor complex in activated lymphocytes), purchased from Sigma; and anti-CD11b (clone M1/70, which reacts with Mac-1 in macrophages and neutrophils), purchased from Pharmingen. After a 60 min incubation at 4 °C, cells were washed twice in PBS, fixed with 2% (v/v) paraformaldehyde and kept in the dark until analysed by flow cytometry. Cell samples were analysed by using a FACS-Vantage flow cytometer (Becton Dickinson Immunocytometry Systems), with FACScan LYSIS II software for data acquisition. Data for 10000 cells were acquired.

Spleen cell proliferation assays. Spleens were removed aseptically and homogenized in sterile Hanks' balanced salt solution (Sigma). After erythrocyte lysis, splenocytes were washed and suspended in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (100 U ml^{-1}), streptomycin (100 $\mu\text{g ml}^{-1}$) and amphotericin B (0.25 $\mu\text{g ml}^{-1}$). All supplements and medium were from Sigma. Cell suspensions were distributed (5×10^5 cells per well) into 96-well tissue culture clusters with flat-bottom wells (Costar). *Salmonella typhi* LPS (Sigma) was used at 2.5 $\mu\text{g ml}^{-1}$ as a B-cell mitogen, and concanavalin A (Con A; Sigma) was used at 1 $\mu\text{g ml}^{-1}$ as a T-cell mitogen. A preparation of heat-killed yersiniae (HKY) was obtained by heating cells of strain IP383 at 90 °C for 15 min and used in proliferation assays at 10^7 organisms ml^{-1} . After incubation at 37 °C in 5% CO_2 for 3 d, proliferation of spleen cells was measured by colorimetric reading of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction, as described by Mosmann (1983).

Suppressor cell assay. Cells from the spleens of three uninfected or infected mice were pooled and were referred to as cell suspensions of control and infected mice, respectively. Suppressor cell activity was evaluated by coculturing spleen cells from control mice (5×10^5 per well) with the cells tested for suppression (10^5 per well), i.e. spleen cells from control or infected mice. Cultures with Con A or HKY were performed

as outlined above. Lymphocyte proliferation was measured as described by Mosmann (1983).

Nitrite and gamma interferon assays. Spleen cells were cultured with LPS, Con A or HKY as described above. Supernatants were removed after 48 h for determination of nitrite levels, and after 72 h for quantification of gamma interferon (IFN- γ). Supernatants were stored at -20 °C until assayed. The nitrite concentration of the supernatants was measured by a standard Griess reaction adapted to microplates (Green *et al.*, 1982); the absorbance at 550 nm was determined, with reference to a sodium nitrite standard curve. IFN- γ was quantified by enzyme immunoassay (Endogen); the cytokine concentration was interpolated from the appropriate recombinant IFN- γ standard curve.

Challenge with *Listeria monocytogenes*. A virulent isolate of *L. monocytogenes* was kindly provided by Dr De La Rosa (Hospital Virgende las Nieves, Granada, Spain). Bacteria were grown on blood agar at 37 °C for 24 h, harvested in sterile PBS, washed twice and 10^8 organisms were injected into a mouse tail vein. Mice were observed daily and deaths recorded.

Statistical analysis. The differences between infected and control groups were analysed by using Student's *t*-test. A *P* value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Effects of *Y. enterocolitica* infection on splenic cell phenotypes

Mice developed splenomegaly after experimental infection with *Y. enterocolitica* O9 via the oral route. At 10 days after infection, the absolute number of nucleated cells in spleens from infected mice was significantly increased (1.75-fold, $P < 0.025$) compared with that in spleens from uninfected mice (Fig. 1). It has been

Fig. 1. Effect of *Y. enterocolitica* infection on spleen cell composition in BALB/c mice. Splenocytes were recovered from uninfected mice (open bars) and from 10-d-infected mice (solid bars) and analysed by flow cytometry. Results are means $\pm$ SD from three mice and are representative of two separate experiments. Asterisks indicate that the differences with control mice are significant.

Fig. 2. Effect of *Y. enterocolitica* infection on the mitogen-induced responses of mouse splenocytes. Spleen cells (5×10^5 per well) from control (open bars) and from 10-d-infected mice (solid bars) were stimulated *in vitro* with LPS, Con A or HKY. (a) Spleen cell proliferation was assessed by colorimetric reading of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction. Background values (wells with cells but no mitogen) were subtracted from all measures. Data are expressed as the mean $\pm$ SD of the mean for eight replicate wells from five mice. (b) Effect on IFN- γ production by splenocytes. IFN- γ levels were measured in supernatants by a specific immunoassay. (c) Effect on nitrite production by splenocytes. Nitrite levels were measured in supernatants by a standard Griess reaction. Results in (b) and (c) are means $\pm$ SD from three mice.

reported that viable yersiniae may be recovered from spleens of mice infected with low-virulence strains by the oral route (Bakour *et al.*, 1985). Therefore, an inflammatory reaction to bacterial components may be involved in splenomegaly development. However, in our assays, spleens from 10-d-infected mice were sterile, although recovery of yersiniae from faeces was always demonstrated (data not shown); this is in agreement with our previous description of the experimental infection of BALB/c mice with *Y. enterocolitica* O9 (Ruiz-Bravo *et al.*, 1999).

Flow cytometry analysis of spleen cells revealed that the absolute numbers of CD3⁺ total T cells, CD4⁺ helper T cells, CD8⁺ cytotoxic T cells and CD11b⁺ phagocytic cells were significantly increased (1.7-fold, $P < 0.05$; 1.4-fold, $P < 0.025$; 1.7-fold, $P < 0.04$; and 5.6-fold, $P < 0.04$, respectively) in 10-d-infected animals (Fig. 1). The number of activated lymphocytes expressing the high-affinity interleukin 2 receptor (CD25) was also significantly increased (3.2-fold, $P < 0.025$). The number of B cells remained unchanged. However, the mean percentage of B cells decreased from 39.8% in uninfected mice to 26.3% in infected mice. Percentages of total T cells and their helper and cytotoxic subsets were unaffected by infection, while the macrophage/neutrophil population expanded from 1.8% to 5.1%. It is interesting to note that C57BL/6 mice infected with *Y. enterocolitica* belonging to the high-virulence serotype O8 develop splenomegaly, whereas BALB/c mice do not develop splenomegaly (Autenrieth *et al.*, 1994). In the present work, total cells, CD4⁺ T cells and CD8⁺ T cells were found to be increased in spleens from BALB/c mice infected with *Y. enterocolitica* O9, as in *Y. enterocolitica* O8-infected C57BL/6 mice (Autenrieth *et al.*, 1994).

Effects of *Y. enterocolitica* infection on the splenocyte responses

We examined whether changes in spleen cell populations of infected mice were associated with modifications in the cell functions. The proliferative responses of spleen cells from 10-d-infected mice to LPS and Con A were decreased by 32.6% ($P < 0.025$) and 52% ($P < 0.001$), respectively, with regard to those of uninfected controls (Fig. 2a). HKY induced marked proliferation of spleen cells from control mice, but in 10-d-infected mice this response was abolished.

To investigate the possible presence of suppressor cells in spleens from *Yersinia*-infected mice, spleen cells from control and infected mice were placed in co-culture with spleen cells from control mice at a ratio of 1:5. The addition of spleen cells from infected mice reduced the response to Con A by 36% ($P < 0.002$) and the response to HKY by 80% ($P < 0.0001$) (data not shown).

IFN- γ is a cytokine able to stimulate the macrophage production of reactive nitrogen intermediates (Drapier *et al.*, 1988; Grazzini *et al.*, 1992), leading to inhibitory effects on T cell proliferation (MacMicking *et al.*, 1997). Thus, we investigated whether the suppression of lymphoproliferative responses of spleen cells from *Yersinia*-infected mice was associated with an increased production of IFN- γ . The results are shown in Fig. 2(b). Infection did not significantly affect the production of IFN- γ by unstimulated (medium) or by LPS-stimulated cultures. However, Con A-stimulated cells from infected mice produced significantly higher levels of IFN- γ than those from uninfected controls (mean increase of 2-fold, $P < 0.03$). In HKY-stimulated cultures, the production of IFN- γ by cells from infected mice was also increased (3.4-fold, $P < 0.03$).

We also examined the nitric oxide synthesis in

Fig. 3. Resistance of *Y. enterocolitica*-infected mice to the intracellular pathogen *L. monocytogenes*. Control mice (solid line) and 10-d-infected mice (broken line) were challenged via the intravenous route with *L. monocytogenes* (10^3 organisms per mouse). Day 0 is the day of challenge with *L. monocytogenes*. The set of results presented is from one of three similar experiments.

splenocyte cultures, by determining the nitrite concentrations in the culture supernatants (Fig. 2c). Con A-stimulated and HKY-stimulated cultures of spleen cells from *Yersinia*-infected mice contained significantly higher levels of nitrite than those from uninfected controls (mean increases of 2.1- and 3.5-fold respectively, with *P* values < 0.04 for both stimuli).

Infection of C57BL/6 mice with *Y. enterocolitica* O8 results in IFN- γ production by NK cells and CD4⁺ helper T cells, whereas this response is late and low in BALB/c mice (Autenrieth *et al.*, 1994; Bohn & Autenrieth, 1996). Previous studies showed that C57BL/6 mice are resistant to *Y. enterocolitica* O8, while BALB/c mice are susceptible (Hancock *et al.*, 1986). Our results suggest that low-virulence yersiniae are able to induce in BALB/c mice a response resembling that induced by high-virulence yersiniae in C57BL/6 mice, but not in BALB/c mice.

Our data have also shown that splenocytes from *Y. enterocolitica* O9-infected BALB/c mice exhibited impaired responses to mitogens and significantly suppressed the ability of normal splenocytes to respond to mitogens. The marked splenomegaly and the increase in the number of interleukin 2 receptor-positive cells in the spleen suggest the presence of large numbers of *in vivo* activated lymphocytes which may be refractory to the *in vitro* overstimulation with mitogens. The inhibition of proliferative responses to Con A and HKY was associated with an increased production of IFN- γ and reactive nitrogen intermediates. It has been documented that IFN- γ is the most potent endogenous activator of the NO synthase gene in macrophages, and NO suppresses the proliferative response of lymphocytes (Albina *et al.*, 1991; Candolfi *et al.*, 1995).

Resistance of *Y. enterocolitica*-infected mice to challenge with *L. monocytogenes*

Production of reactive nitrogen intermediates by IFN- γ -activated macrophages correlates with resistance to microbial growth (MacMicking *et al.*, 1997). To de-

termine whether increased production of IFN- γ and nitrite by splenocytes from *Yersinia*-infected mice in response to *in vitro* stimuli correlated with an increased resistance of mice against unrelated pathogens, we challenged animals with a lethal inoculum of *L. monocytogenes*. This intracellular pathogen causes a systemic infection in mice, and both IFN- γ -producing T cells and activated macrophages are required to overcome infection (Kaufmann, 1993; Unanue, 1997). As shown in Fig. 3, *Yersinia*-infected mice survived challenge by the intravenous route with a dose of *L. monocytogenes* that was lethal for 100% of control mice.

These results confirmed the presence of profound immunological alterations in a murine model for human infection with low-virulence serotypes of *Y. enterocolitica*. It will be interesting to determine the biological significance of these alterations in immunopathological disorders related to yersiniosis. Several hypotheses have been proposed to explain mechanisms for immunopathological sequelae of some infectious diseases: formation of immune complexes, alterations in the idiopathic networks, molecular mimicry, microbial superantigens and perturbations of the immune system (Schwartz, 1993). Previous studies with high-virulence strains of *Y. enterocolitica* O8 have provided evidence for some of these mechanisms: the production of factors with superantigenic activity on human T cells was shown to have been involved in the occasional development of autoimmune spondyloarthropathies in susceptible individuals following clearance of yersiniae (Stuart *et al.*, 1995), and the presence of an envelope lipoprotein that is cross-reactive with the human thyrotropin receptor and activates human monocytes has been related to the induction of autoimmune hyperthyroidism (Zhang *et al.*, 1997). Our results suggest that immunomodulation by low-virulence serotypes may be another mechanism involved in the genesis of some immunopathological sequelae of natural infections with *Y. enterocolitica*. There is evidence showing the importance of the interaction between T cells and macrophages in the pathogenesis of rheumatoid arthritis, with a central role for IFN- γ . This cytokine stimulates the overproduction of proinflammatory cytokines (Sebbag *et al.*, 1997) and induces the arthritis-typical alterations in the profile of integrin expression by mononuclear phagocytes and synovial fibroblasts (Pirila & Heino, 1996). Also, NO produced by IFN- γ -activated macrophages has been implicated in the pathogenesis of arthritis and other inflammatory diseases (Miyasaka & Hirata, 1997).

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