Journal of Helminthology (2002) **76,** 59–64 DOI: 10.1079/JOH200196

Effects of recombinant bovine interferon γ on *Strongyloides papillosus* infection in calves

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Abstract

The effects of interferon (IFN) γ on the course of infection with *Strongyloides papillosus* in calves were investigated. Calves (N = 7 each) were inoculated with recombinant bovine IFNγ or control solution daily from day 0 to day 15 following *S. papillosus* infection. Treatment with IFNγ induced an increase in faecal egg output in the peak stage of infection. The IFNγ-treated animals harboured more worms, especially more immature worms, in the small intestine than control animals at necropsy on day 17, with no decreases in intestinal mucosal mast cells. Both animal groups had similar small numbers of intestinal worms at necropsy on day 26. All control animals developed peripheral blood eosinophilia on day 7, while five of seven IFNγ-treated animals did not. Serum α1-acid glycoprotein concentrations increased on day 7 in both animal groups, with higher values in control animals than in IFNγ-treated animals. Control animals mounted a predominant IgG1 response to *S. papillosus* from day 10, while IFNγ-treated animals did from day 22. These data suggested that IFNγ inhibited some host protective responses to *S. papillosus* migrating larvae, resulting in an improvement of worm survival after a period when protective responses should be activated during the early stage of infection. The effects of IFNγ on intestinal worm expulsion should be confirmed by further experiments.

Introduction

Selective induction of T-helper (Th) 2 cytokines is promoted in rodents infected with gastrointestinal nematode parasites, such as *Trichuris*, *Heligmosomoides*, *Nippostrongylus* and *Strongyloides* spp. (Else *et al.*, 1992; Svetic *et al.*, 1993; Matsuda *et al.*, 1995; Abe *et al.*, 1998). Recent studies have demonstrated that interleukin (IL)-4 among Th2 cytokines plays a key role in protective immunity to gastrointestinal nematode infections (Urban *et al.*, 1991; Else *et al.*, 1994). In contrast, interferon (IFN) γ, a major Th1 cytokine, is considered to inhibit host immunity possibly by interfering with the induction of Th2 responses. Mouse strains susceptible to *Trichuris muris* mount a high production of IFNγ in the absence of Th2 responses following infection (Else *et al.*, 1992), and rapid worm expulsion is acquired in those mice by depletion of IFNγ (Else *et al.*, 1994). Among *T. muris* strains, a strain capable of inducing a high production of IFNγ accomplishes more persistent infections in mice than those inducing a low production of IFNγ (Koyama & Ito, 1996). Treatment with IFNγ, as well as an induction of endogenous IFN production, induced an increase and a persistence of egg production with delayed worm expulsion in mice infected with *Nippostrongylus brasiliensis* (Urban *et al.*, 1993). The administration of IL-12, which stimulates production of IFNγ and inhibits IL-4, also resulted in a prolonged infection of *N. brasiliensis* (Finkelman *et al.*, 1994).

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Strongyloides papillosus is a parasitic nematode of ruminants. The life cycle of the parasite, like that of *N. brasiliensis*, includes invasion by infective larvae (L3) into a host by skin penetration, a migratory phase in which some larvae sojourn in the lungs and an intestinal phase in which larvae develop into adult worms in the small intestine from 8 days post-infection (p.i.) and onwards (Turner *et al.*, 1960; Nwaorgu & Connan, 1980). When animals overcome a primary infection, most adult worms are expelled within several weeks p.i. and a strong resistance to reinfection is induced (Ratynska-Prill, 1980; Ooba *et al.*, 1996). We found a decreased expression of IFNγ-mRNA in mononuclear cells obtained from mesenteric lymph nodes of calves following *S. papillosus* infection (unpublished data). Bovine Th1/Th2 paradigm may be less restricted in nematode infections (Canals *et al.*, 1997), although it has been reported that some regulatory mechanisms of a cytokine network, such as immunoglobulin (Ig) isotype switching, are similarly characterized in cattle and mice (Estes, 1996).

To clarify the effects of IFNγ on *S. papillosus* infection, we investigated the course of infection in calves inoculated with recombinant bovine (rBo)-IFNγ. Our hypotheses were that Th2 mediated responses would be suppressed, and faecal egg output and intestinal worm burden would become higher by treatment with IFNγ in calves infected with *S. papillosus*.

Materials and methods

Animals, parasite and rBo-IFNγ

Male Holstein calves were obtained at 14 to 22 weeks of age from a farm where animals were born and kept housed indoors. Animals were kept in individual concrete-floored pens which were cleaned daily. Faecal examinations were carried out weekly to confirm the absence of parasite eggs and oocysts as described below. Animals were employed in experiments at 23 to 28 weeks of age. *Strongyloides papillosus* was maintained by serial passage in rabbits, and L3 were obtained by faecal culture as previously described (Nakamura *et al.*, 1994).

Baculo virus-derived rBo-IFNγ (expressed in *Bombyx mori* and purified by a series of chromatographies, >95% purity, 3.2×10^7 U mg^{-1} protein) was generously provided by the Chuo-Sanken Laboratory of Katakura Industries (Sayama, Japan). Aliquots of IFNγ solution were stored at −80°C until used. The solution was thawed at 37°C, diluted in 0.01 M phosphate buffered saline (PBS, pH 7.2) at an IFNγ activity of 8.0×10^5 U ml^{-1}, and passed through sterile 0.22 μm filters prior to use. The solution was free of detectable endotoxin using a *Limulus* assay. An elution buffer for purification (0.05 M Tris–0.5 M NaCl–0.5 M methyl glucoside, pH 8.0) was diluted in PBS and used as a control solution.

Experimental design

Fourteen calves were percutaneously infected with 100 000 L3 as previously described (Nakamura *et al.*, 1998). Animals were randomly allocated into two groups of seven each. Seven animals each were inoculated into the right abdominal cavity daily with 20 ml of IFNγ solution containing 1.6×10^7 U of rBo-IFNγ or control solution from day 0 (just before infection) to day 15 p.i. The dose and period of inoculation protocol was designed to introduce as much IFNγ as possible until the peak stage of infection (the third week p.i., Nwaorgu & Connan, 1980). Three and four animals in each group were killed on days 17 and 26 p.i., respectively, by exsanguination under an anaesthesia with sodium pentobarbital.

Faecal and blood examinations

Rectal faecal samples were collected on days 0, 7–11, 13, 15, 17, 19, 22, 24 and 26 p.i. to count eggs per gram of faeces by a centrifugation–flotation procedure (Cox & Todd, 1962). Animals were bled from the jugular vein twice a week. Blood samples (3 ml) were collected into tubes containing EDTA. Erythrocyte, leukocyte and platelet counts were monitored with a Coulter Counter (Coulter Electronics, Hialeah, Florida, USA). Eosinophils were counted using a haemocytometer. Blood samples (10 ml) were also collected into tubes without anticoagulant for serum collection. Serum was harvested 6 h later and stored at −80°C until used. Serum α1-acid glycoprotein (α1AG) concentration was determined using a radial immunodiffusion kit (Bovine α1AG Plate, Saikinkagaku Institute, Sendai, Japan). Serum anti-*S. papillosus* Ig isotype levels were determined by enzyme-linked immunosorbent assays as described below.

Antibody responses

Somatic extracts of *S. papillosus* L3 were prepared as previously described (Ooba *et al.*, 1996). Two additional calves were percutaneously infected with 100 000 L3 at every 4 weeks as previously described (Nakamura *et al.*, 1998). Serum samples were collected before initial infection and on day 10 after the third infection. Optimal concentrations of the antigen and horseradish peroxidase-conjugated rabbit anti-bovine IgM, IgA, IgG1 and IgG2 antibodies (Bethyl Laboratories, Montgomery, Texas, USA) for the assays were determined in box titrations with standard sera.

Microplates were coated with the antigen in 100 μl of 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. The plates were washed five times in PBS containing 0.05% Tween 20, and non-specific binding sites were blocked with 150 μl of PBS containing 5% skim milk for 1 h at 37°C. After washing, 100 μl of serum samples diluted 1:50 in PBS containing 0.15% Tween 20 were added and incubated for 1 h at 37°C. After washing, 100 μl of conjugated secondary antibodies were added and incubated for 1 h at 37°C. After washing, 100 μl of substrate solution (TMB Microwell Peroxidase Substrate System, Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, USA) was added and incubated for 20 min at room temperature. After adding 100 μl of 3 N phospholic acid, optical densities were read at a wavelength of 450 nm with a microplate reader.

https://doi.org/10.1079/JOH200196 Published online by Cambridge University Press

Intestinal mast cells and worm recovery

Tissue pieces were obtained from the upper part of the jejunum, fixed in Carnoy's solution and processed for staining with alcian blue (pH 0.3) and safranin-O (pH 1.0) (Madden *et al.*, 1991). A total of 50 villus crypts were scanned for counting mucosal mast cells. Alcian blue-positive and safranin-O-negative cells observed in the lamina propria and epithelium of a villus crypt were scored as mucosal mast cells.

The small intestine was slit longitudinally, cut into sections, incubated in water for 3 h at 37°C. The solution was adjusted to 19 l with water after the intestinal sections were rinsed and removed. For worm collection, 1900 ml of the solution was mixed with 100 ml of formaldehyde. Worms less than 3 mm (considered as immature worms) and above 3 mm in body length were differentially counted using a dissection microscope with a micrometer. Numbers counted were multiplied by 10 to calculate total worm burdens in the small intestine.

Statistical analysis

Data in each group were analysed for significant differences between day 0 and time points during the course of infection using paired *t*-tests. Data were also analysed for differences between animal groups at particular time points using *t*-tests. Time points selected were: day 7 p.i. for blood cell counts and α1AG concentrations, as transient eosinophilia was observed on day 7 in our previous study (Nakanishi *et al.*, 1993) and α1AG is an acute phase protein (Itoh *et al.*, 1990); on day 17 p.i., a sampling day in the peak stage of infection just after the last treatment with IFNγ, for faecal egg counts and antibody levels; and on day 17 or day 26 p.i., the day of necropsy, for intestinal mast cell and worm counts. Differences were considered significant at $P < 0.05$.

Results

Clinical findings

Animals appeared normal throughout the experiment except for one animal inoculated with IFNγ, which developed transient diarrhoea on day 9 p.i. No gross lesions were observed at the site of inoculation in any animals. Faecal egg output was established between day 9 and day 11 p.i. in all animals, and increased significantly from day 13 p.i. and onwards in both animal groups (fig. 1). Faecal egg counts on day 17 p.i. were significantly higher in IFNγ-treated animals than control animals. No significant changes were detected in erythrocyte, leukocyte and platelet counts throughout the experiments (data not shown). Transient peripheral blood eosinophilia developed on day 7 p.i. in all control animals and two of seven IFNγ-treated animals (fig. 2). Five IFNγ-treated animals did not show eosinophilia throughout the experiments. There was no significant difference in eosinophil counts between animal groups on day 7 p.i.

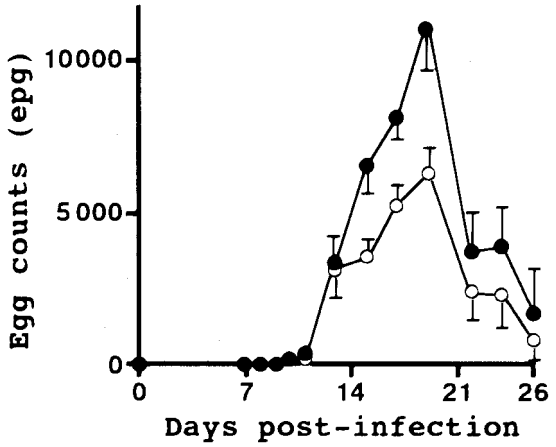

Fig. 1. Faecal egg output (eggs per gram) following a *Strongyloides papillosus* infection. Calves were inoculated with IFNγ (closed circles) or control (open circles) solution daily up to day 15 post-infection. Data show mean and standard error (N = 7 until day 17, N = 4 between days 19 and 26).

Serum α1AG and antibody responses

Serum α1AG concentrations increased transiently on day 7 p.i. in both animal groups, with significantly higher values in control animals than in IFNγ-treated animals (fig. 3). Control animals had elevated α1AG concentrations on day 10 p.i. in comparison to pre-infection levels.

Serum antibody responses are presented in fig. 4. Both animal groups mounted equivalent IgM and IgA

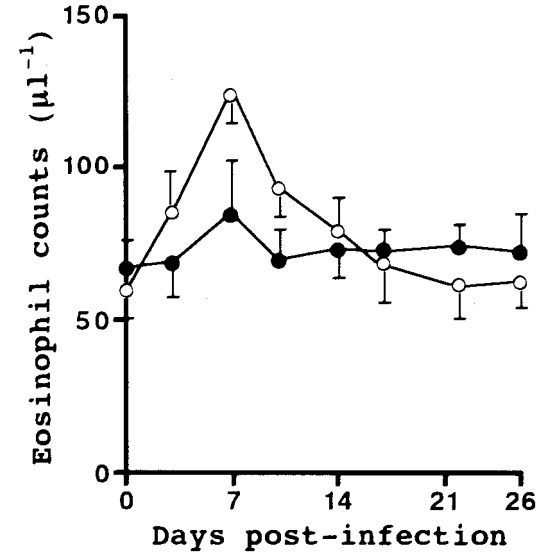

Fig. 2. Peripheral blood eosinophil counts following a *Strongyloides papillosus* infection. Calves were inoculated with IFNγ (closed circles) or control (open circles) solution daily up to day 15 post-infection. Data show mean and standard error (N = 7 until day 17, N = 4 on days 22 and 26).

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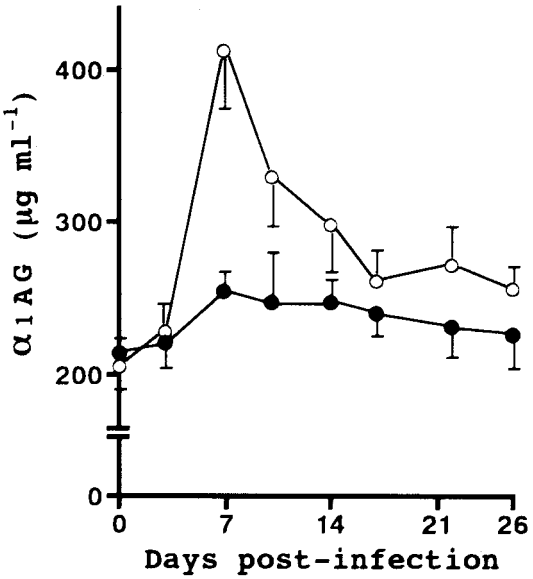

Fig. 3. Serum α1-acid glycoprotein (α1AG) concentrations following a *Strongyloides papillosus* infection. Calves were inoculated with IFNγ (closed circles) or control (open circles) solution daily up to day 15 post-infection. Data show mean and standard error (N = 7 until day 17, N = 4 on days 22 and 26).

antibody responses to *S. papillosus* from days 7 and 17 p.i., respectively. Serum IgG1 antibodies increased significantly from days 10 and 22 p.i. in control and IFNγ-treated animals, respectively. Serum IgG1 antibody levels on day 17 p.i. were significantly higher in control animals than in IFNγ-treated animals. Animals did not mount consistent IgG2 antibody responses throughout the experiments.

Intestinal mast cells and worm recovery

There was no significant differences in mucosal mast cell counts in the small intestine between animal groups on the days of necropsy (table 1). The IFNγ-treated animals harboured more worms in the small intestine than control animals on day 17 p.i. (table 1). In particular, the mean number of immature worms (length < 3 mm) from IFNγ-treated animals was three times greater than that in control animals. There was no significant difference in intestinal worm counts between animal groups on day 26 p.i.

Discussion

The daily treatment with IFNγ until day 15 p.i. induced approximately 1.5-fold increases in faecal egg output and intestinal worm burden in calves infected with *S. papillosus* during the middle stage of infection. However, IFNγ-treated animals showed a similar egg output during the early stage of patent infection in comparison with control animals.

A Th2 cytokine IL-5 is the main factor for the development, maturation and activation of eosinophils in mice infected with nematode parasites (Imamura & Takatsu, 1995). It has been reported in rodent strongyloidiasis that eosinophils play an important

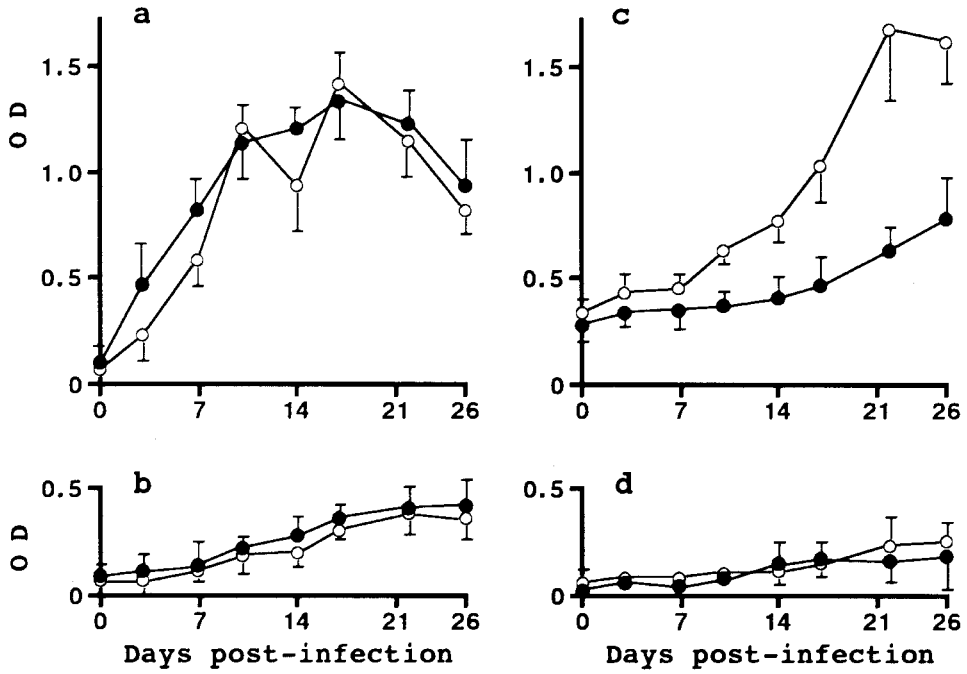

Fig. 4. Serum IgM (a), IgA (b), IgG1 (c) and IgG2 (d) levels following a *Strongyloides papillosus* infection. Calves were inoculated with IFNγ (closed circles) or control (open circles) solution daily up to day 15 post-infection. Data show mean and standard error (N = 7 until day 17, N = 4 on days 22 and 26) expressed in optical density (OD) units.

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Table 1. Mucosal mast cells and worm burdens in the small intestine of calves infected with *Strongyloides papillosus*.

Calf group	Number of muscosal mast cells per 50 villus crypts	Number of worms with body length	
		Less than 3 mm	Above 3 mm
Necropsied on day 17 post-infection (N = 3 each)			
IFNγ-treated	467±84[a]	8360±626[a]	22 010±1022[a]
Control	433±101[a]	2710±306[b]	15 570±671[b]
Necropsied on day 26 post-infection (N = 4 each)			
IFNγ-treated	603±87[a]	210±103[c]	2240±982[c]
Control	671±58[a]	110±78[c]	1990±668[c]

Values are expressed as mean±standard error. Means in columns with different superscripts (a–c) are significantly different ($P < 0.05$).

role in IL-5-dependent protection against migrating larvae of a secondary infection (Korenaga *et al.*, 1991). The function of eosinophils in the protective immunity to a primary infection of calves infected with *S. papillosus* remains unknown. Contrary to our expectation, there was no significant difference in peripheral eosinophil counts between animal groups in the early stages of infection.

Acute phase proteins such as α1AG are induced during an early course of inflammatory response associated with diseases and injuries (Itoh *et al.*, 1990). Some *S. papillosus* larvae are trapped in the lungs during the later period of the migratory phase, i.e. days 5–7 p.i., resulting in alveolar haemorrhages and interstitial infiltration (Turner *et al.*, 1960; Nwaorgu & Connan, 1980; Nakanishi *et al.*, 1993). In the present study, we determined serum α1AG as an index of inflammatory responses. Treatment with IFNγ suppressed the elevation of α1AG observed in the early stages of infection, suggesting that IFNγ reduced inflammatory responses associated with migrating larvae. Urban *et al.* (1993) reported that treatment with IFNγ reduced pulmonary inflammatory infiltrates in mice infected with *N. brasiliensis*. It was not surprising to find that serum α1AG concentrations decreased during the intestinal phase of infection even in control animals, mainly because of the absence of overt histological changes in the small intestine following an *S. papillosus* infection (Nakanishi *et al.*, 1993).

Specific IgG1 antibodies are predominantly induced among antibody responses of cattle to gastrointestinal nematode infections (Gasbarre *et al.*, 1993; Hilderson *et al.*, 1993). In rodent strongyloidiasis, IgG antibodies play an important role in protective immunity against migrating larvae (Murrell, 1981). In previous work using *S. papillosus*, we demonstrated that a systemic IgG production is induced against larval antigens and that partial protection is established against challenge infections by immunization with a larval somatic extract (Ooba *et al.*, 1996). The present results indicated that IgG1 and IgM responses were predominant antibody responses to *S. papillosus* infection in calves, and that treatment with IFNγ inhibited the establishment of an IgG1 response. *In vitro* studies with bovine B cells have shown that IL-4 upregulates the production of IgG1, IgM and IgE, while IFNγ not only enhances IgG2 and IgM production but also inhibits upregulation of IgG1 and IgE responses by IL-4 (Estes, 1996). The inhibition of the IgG1 response in IFNγ-treated animals following infection could have resulted from the suppression of Th2-like responses by IFNγ. Treatment with IFNγ did not enhance a specific IgG2 response, possibly because it is a minor response to *S. papillosus* infection. We did not investigate IgE responses.

Khan *et al.* (1993) demonstrated that worm expulsion from the intestine of mice with strongyloidiasis is dependent on intestinal mucosal mast cells, which are activated by IL-3 and IL-4 (Madden *et al.*, 1991). It was unlikely that the suppression of intestinal immune responses were responsible for the increased worm burden and egg output in IFNγ-treated animals during the peak stage of infection, since no reduction was observed in the number of intestinal mast cells. Instead, the following hypothesis can be generated. Protective responses to migrating larvae, such as acute phase and predominant antibody responses, became suppressed by IFNγ after a period when these responses should have been activated. The suppression was advantageous to larvae for their survival in tissues and successful migration to the small intestine. This resulted in increased numbers of intestinal worms, especially immature worms, in IFNγ-treated animals during the peak stage of infection. No differences were likely to occur in the number of larvae reaching the intestine between animal groups during the very early stage of infection before activation of those protective responses, as both animal groups showed similar rates of egg output up to day 13 p.i.

After the peak stage of infection, normal worm expulsion was observed even in IFNγ-treated animals. However, the animals had no treatment from day 16 p.i. and onwards in the present study. Further experiments including continuous treatment with IFNγ until the final stage of infection, should be undertaken to clarify the effects of IFNγ on intestinal immune responses involved in worm expulsion.

Acknowledgements

We are grateful to the Chuo-Sanken Laboratory of Katakura Industries for providing rBo-IFNγ. We are also grateful to Dr J.C. Williams of Louisiana State University for reviewing the manuscript. This study was supported by a Grant-in-Aid of Recombinant Cytokines Project

provided by the Ministry of Agriculture, Forestry and Fisheries, Japan (RCP1999-3120).

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(Accepted 26 July 2001)