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Granulomatous inflammation during *Heligmosomoides polygyrus* primary infections in FVB mice

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Abstract

Host responses to primary infections with *Heligmosomoides polygyrus* were studied in fast responding FVB mice (H-2^q). Pathological changes in the intestinal mucosa, mesenteric lymph nodes and spleen were examined. Features of the fast response were typical: low effectiveness of infection and limiting of parasite survival and egg production, with worm expulsion occurring about 60 days post-infection. The intestinal inflammatory response involved infiltration by different cells into the intestinal mucosa and granulomata formation. As is typical for intestinal nematode infection enteropathy, decreased villus:crypt ratio and hyperplasia of goblet and Paneth cells were also present. Reactions of the intestinal mucosa, mesenteric lymph nodes and spleen increased over time post-infection and after worm expulsion. Enteropathy may help worm expulsion by creating an unfavourable environment for *H. polygyrus*. The implications of these findings and the potential role of intestinal intraepithelial lymphocytes in the pathogenesis of generated lesions are discussed.

Introduction

Heligmosomoides polygyrus is a trichostrongyloid nematode occurring in the murine small intestine. The life cycle of this parasite is strictly enteric. Following oral inoculation, third stage larvae (L3) embedded in the *muscularis externa* develop and moult twice (tissue phase of the infection). After 7–8 days, preadult worms emerge in the intestinal lumen (intestinal phase of the infection), where they reside as adults (Wahid & Behnke, 1992).

The nematode, particularly its larval stage, acts as a stimulus for intestinal inflammatory reactions, but adult worms are highly immunosuppressive (Monroy & Enriquez, 1992; Telford *et al.*, 1998). Primary infections tend to be chronic, in some cases lasting for over 30 weeks (Lawrence & Pritchard, 1994; Wahid *et al.*, 1994) although challenge infections are readily expelled (Else & Finkelman, 1998). Data on protective responses and worm expulsion are therefore usually based on reactions seen during secondary infections. However, different mouse strains show varying ability to expel the nematode during primary infections, associated with the phenotype of the major histocompatibility complex (MHC). Fast responders (SJL: H-2^s, SWR: H-2^q) limit the infection after about 8 weeks (Enriquez *et al.*, 1988; Wahid *et al.*, 1994), intermediate responders (BALB/c, DBA, both H-2^d) take 10–20 weeks, whilst in low responders (CBA, C3H, both H-2^k) it takes up to 35 weeks for expulsion to occur (Monroy & Enriquez, 1992; Lawrence & Pritchard, 1994; Wahid *et al.*, 1994; Urban *et al.*, 1995).

The intestinal pathology in fast (Webster) and low (C3H) responders was described by Liu (1965a,b) and Jones & Rubin (1974). On primary exposure they observed an inflammatory reaction provoked by developing larvae, their excretory/secretory products and sheaths left behind after moulting. Lesions were similar in fast and low responders, although less intensive in the

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latter. In contrast to other intestinal nematodes, primary infections with *H. polygyrus* are not accompanied by prominent mastocytosis (Wahid *et al.*, 1994). In fact, mastocytosis is suppressed by immunomodulatory activities of adult worms (Monroy & Enriquez, 1992; Wahid *et al.*, 1994), which were also shown to down-regulate the potential of lymphocytes from mesenteric lymph nodes (MLNs) to secrete cytokines following *in vitro* stimulation (Behnke *et al.*, 1993). It is also postulated that, as other intestinal parasites, *H. polygyrus* induces increases in goblet and Paneth cell numbers (Monroy & Enriquez, 1992; Fakae *et al.*, 2000).

On reinfection (Liu, 1965b; Prowse *et al.*, 1978) and challenge infection (Jones & Rubin, 1974) sites of larval development become the foci of intense reactions resulting in prominent granulomatous lesions, particularly in fast responders. Such lesions have never been observed during primary infections.

Heligmosomoides polygyrus has been extensively studied as a model for chronic helminthiasis (Monroy & Enriquez, 1992), but less is known about mechanisms involved in limiting primary infections in fast responders. The status of the FVB mouse strain has never been investigated, however their MHC haplotype (H-2^q) suggests that the responses should be fast. We found in our experiments that the fast reaction of the FVB strain involves development of granulomatous lesions in the intestine, which is not typical for primary exposure.

In the present paper we investigated changes in intestinal pathology and inflammatory reaction that may influence fast worm expulsion during *H. polygyrus* primary infection in FVB mice. Enteropathy parameters, namely villus:crypt ratios, numbers of goblet (GC) and Paneth cells over time post-infection and after worm expulsion and histomorphology of granuloma formation are described.

Materials and methods

Experimental protocol

A total of 132 specific pathogen-free, 6–8 weeks old, female FVB mice were purchased from the Breeding Department, Medical Research Centre, Polish Academy of Sciences, housed under 12 h light:dark, with access to food (standard mouse diet) and water *ad libitum*.

The kinetics of worm expulsion were examined in 60 mice orally inoculated with 120 *H. polygyrus* L3 larvae in 0.2 ml of distilled water, using a ball-tipped feeding tube. Larvae were maintained as previously described by Bryant (1973) from infected C57 mice, a kind gift from the Parasitology Department, Institute of Zoology, Faculty of Biology, Warsaw University. At 10, 16, 20, 25, 30, 35, 40, 45, 50 and 60 days post-infection (d.p.i.) free adult worms were collected from the intestine, washed in PBS and counted. Faecal egg counts were assessed by the McMaster technique.

Another 60 mice were divided into six groups, each of ten mice and inoculated as previously. They were killed by cervical dislocation on 2, 5, 10, 16, 50 and 100 d.p.i. The proximal part of the small intestine (10 cm distal to the pylorus), MLNs and spleen were recovered, washed in warm PBS and fixed in 10% phosphate-buffered formalin for histological analysis. For flow cytometry analysis, the intestine was placed in cold PBS. Twelve mice were treated as controls. They were given 0.2 ml of water by the same method as the inoculum was administrated to the infected group and they were killed at the same intervals, two mice each time.

Histological analysis

Intestinal samples cut into 1.0 cm segments, MLNs and spleens were embedded in paraffin wax, cut into 5 μm sections and stained with haematoxylin and eosin (HE), Alcian blue and periodic acid-Schiff (PAS), and immunohistochemically with biotin-conjugated anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (MoAb), Pharmingen. Villus:crypt ratios were calculated from 100 measurements of villus height and crypt depth in HE and PCNA stained sections respectively. Paneth cells were counted in 100 crypts in HE stained sections. Goblet cells were examined in HE and PAS/Alcian blue stained sections and enumerated in the latter in 1 mm^2 area units, with ten counts being made. Analyses were performed using the LUCIA 4.21 screen measurement. Intestinal, MLN and spleen inflammatory reactions were estimated in HE stained sections.

Flow cytometry

Using curved scissors, granulomatous lesions were cut off from the wall of the proximal part of the intestine and dissociated in RPMI-1640 (Gibco) containing 2 mM L-glutamine (Gibco), 50 μM 2-mercaptoethanol and 1.25 ml per 100 ml antibiotic/antimycotic solution (Sigma). Twenty granulomata were isolated from each mouse. After washing three times, cells were resuspended in PBS containing 2.5% foetal calf serum (FCS) and counted. Cells in concentrations of 2×10^3 cells μl^{-1} were incubated with PE-conjugated anti-CD103 MoAb (M290, Pharmingen) and FITC-conjugated anti-TCR$\alpha\beta$ MoAb (H57-597, Pharmingen) or anti-TCR$\gamma\delta$ MoAb (GL3, Pharmingen) for 30 min at 4°C. Cells were then washed twice in PBS, fixed in 1% paraformaldehyde and analysed on a FACStrak (Becton Dickinson).

Statistical analysis

Data are expressed as mean values ± S.E.M and were analysed using the non-parametric Mann-Whitney test. A value greater than $P \leq 0{\cdot}05$ was considered non-significant.

Results

Worm expulsion

The worm burden recovered from infected mice and faecal egg counts are shown in fig. 1. The inoculum used showed 72.7% infectivity as assessed by the worm burden on days 10–30 p.i. From 16 d.p.i. most adult nematodes were found in the jejunum. As was expected, worm loss was rapid, with a 35% reduction by day 35 and 99% by day 60. Faecal egg output was highest on days 16–25,

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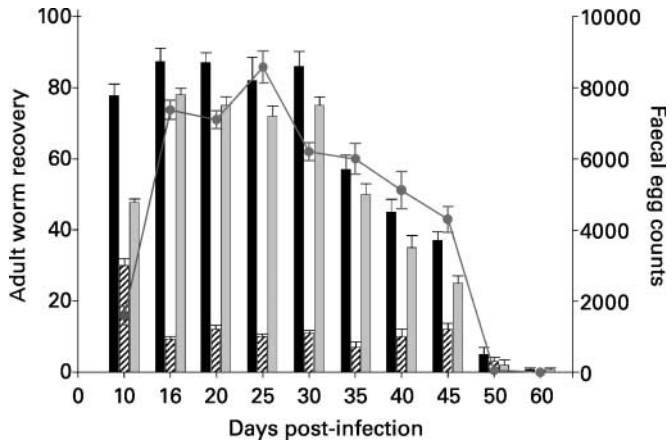

Fig. 1. Worm burdens of *Heligmosomoides polygyrus* in the small intestine (■), duodenum (▨) and jejunum (▩) and faecal egg counts (-●-) in FVB mice up to 60 days post-infection.

decreased on days 30–45 and then rapidly declined to zero on day 50.

Intestinal pathology

Infected mice expressed severe enteropathy compared with uninfected controls. Villus:crypt ratios decreased during the tissue phase of infection, (2 and 5 d.p.i) and after worm expulsion (fig. 2). There was a cellular reaction (fig. 3) involving infiltration of mononuclear cells, plasma cells and mast cells even after worm expulsion. The inflammatory infiltration was most intense at 16 d.p.i. Mast cells were present during the infection and after worm expulsion, although mastocytosis was not prominent. A large number of plasma cells was present in the early tissue phase (2 d.p.i), intestinal phase (16 d.p.i) and after worm expulsion. The reactions in Peyer's patches were strong and on day 2 p.i. numerous macrophages phagocytosing lymphocytes were observed (fig. 4). Subsequently, a strong lymphocyte proliferation took place until worm expulsion occurred. On day 100 p.i. there was no difference compared with controls. The number of Paneth cells in infected mice increased from 2 d.p.i. to 50 d.p.i. with a peak occurring on day 10 p.i. (fig. 5).

On day 10 p.i. granulomatous lesions formed in the sites of larval development (fig. 6). Granulomata changed in size and morphology over time post-infection. Necrosis was observed in the centre of the granulomata in the latter phase of infection and fibrosis was predominant after worm expulsion. Percentages of cell types forming granulomata are shown in table 1.

The PAS reaction revealed an increase in the number and size of goblet cells as well as in the amount of mucus in the intestine only on 10–16 d.p.i. (figs 7, 8).

Mesenteric lymph nodes and spleens were distinctively enlarged in infected mice even at 2 d.p.i. During the infection and after worm expulsion an increase in lymphocyte number and the number and size of nodules were observed. The reactions were observed in the cortex, paracortex and medulla in MLNs and in the periarteriolar lymphoid sheath, germinal centres and mantle zone in the spleen.

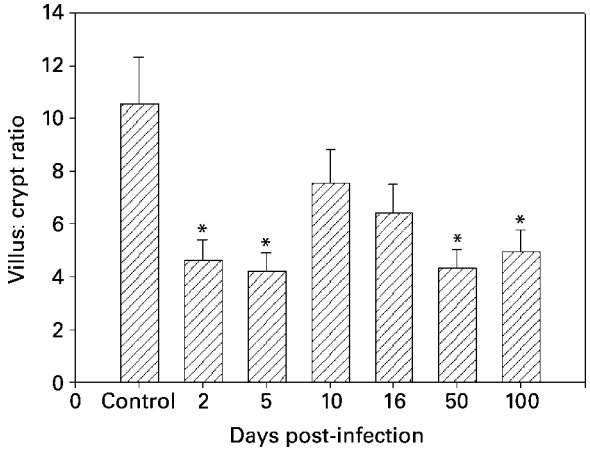

Fig. 2. Mean (±SEM) villus:crypt ratios in FVB mice up to 100 days post-infection with *Heligmosomoides polygyrus*. *Significantly different from controls ($P \leq 0.05$)

Discussion

Intestinal helminthiases are accompanied by severe enteropathy caused by direct damage from the attachment, migration/burrowing and feeding activities of worms but also by the host's immune responses to the parasites. Pathological changes promote limited worm survival or fecundity by creating an unfavourable environment for parasites, which are then forced from their preferred niches. Increased permeability caused by local inflammatory responses results in an increased exposure to components of the systemic response (Wakelin, 1978). On the other hand, these reactions may be more harmful than beneficial for the host (Garside *et al.*, 2000). *Heligmosomoides polygyrus* primary infections usually cause limited enteropathy (Garside *et al.*, 2000), because the nematode downregulates intestinal changes and can suppress the expulsion of other nematodes (Behnke *et al.*, 1978, 1993). Expulsion may also occur in

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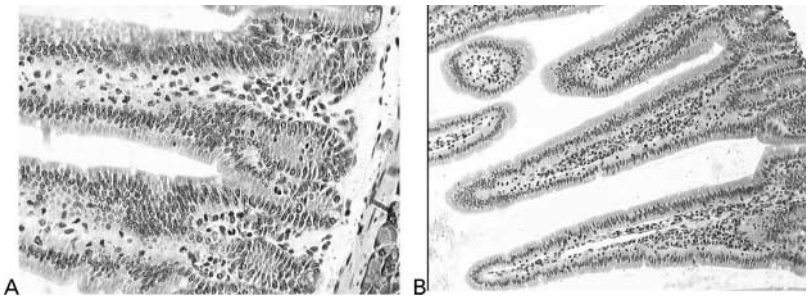

Fig. 3. Sections of the duodenum of FVB mice: (A) uninfected (HE, 40×); (B) 10 days after infection with *Heligmosomoides polygyrus*, showing infiltration of mononuclear cells (HE, 20×).

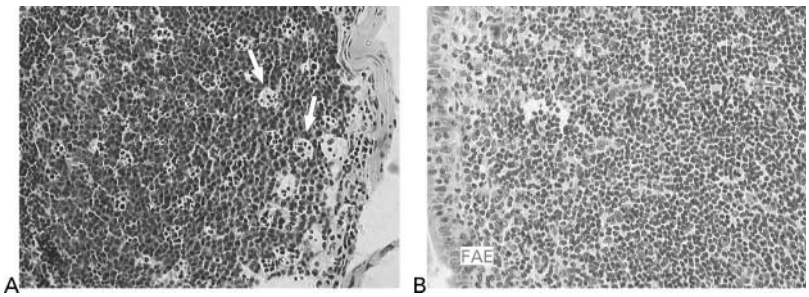

Fig. 4. Sections of Peyer's patches from FVB mice infected with *Heligmosomoides polygyrus*. A. Macrophages (arrowed) phagocyting lymphocytes, 2 d.p.i (HE, 40×); B. Stimulation with *H. polygyrus* infection, 5 d.p.i, FAE – follicle associated epithelium, (HE, 40×).

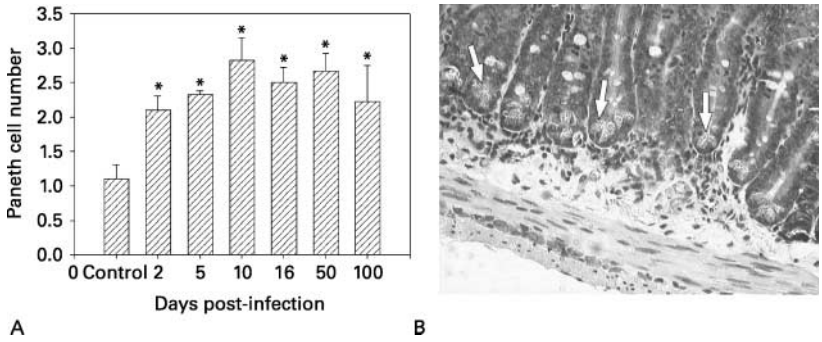

Fig. 5. A. Paneth cell numbers per crypt in FVB mice up to 100 days post-infection with *Heligmosomoides polygyrus*. B. Paneth cells (arrowed) in small intestine of FVB mice 10 days post-infection with *H. polygyrus* (HE, 40×).

mice in which intestinal inflammation has been previously induced non-specifically by other pathogens (Doligalska & Mijal, 2000).

In experimental animal models, helminthiasis-induced enteropathy is characterized by villus atrophy, crypt hypertrophy, goblet cell hyperplasia and infiltration of the mucosa by a variety of inflammatory cells (Garside *et al.*, 1992). It has been shown in infections with *Trichinella spiralis* (Garside *et al.*, 1992; Bożić *et al.*, 2000) and *Nippostrongylus brasiliensis* (Miller *et al.*, 1981), that changes in mucosal architecture are T lymphocyte dependent.

Sequence enteropathy was observed during *H. polygyrus* primary infection in FVB mice as well as granulomatous lesions, which are unusual in the case of primary exposure. Changes in the mucosal architecture and inflammation occurred in the early intestinal phase of the infection, changed over time after infection and persisted after worm expulsion. Some of these changes seem to be related to epithelial stem cell activity. A decrease in the villus:crypt ratios is the result of villus atrophy and crypt hypertrophy. This occurred during the tissue phase of infection, at day 16 p.i., when adult worms were present in the intestine and after worm expulsion, hence these changes cannot arise just as a consequence of direct damage from the presence of nematodes in the intestine. In the case of *H. polygyrus* infection, the villus length determines habitat selection by this nematode species (Bansemir & Sukhdeo, 1996) since worms attach to the intestinal mucosa and

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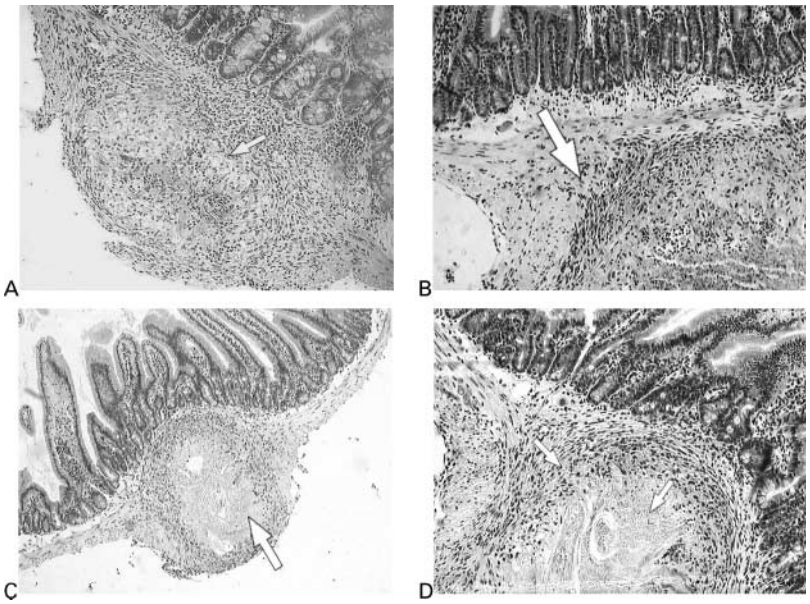

Fig. 6. Granulomata (arrowed) in the intestine of FVB mice infected with *Heligmosomoides polygyrus*: A, 10 d.p.i. (40×); B, 50 d.p.i. (20×); C, 100 d.p.i. (10×); D, 100 d.p.i. showing fibrosis (arrow) in the periphery and necrosis (arrow) in the centre of lesions (20×). All sections stained with HE.

Table 1. Percentage (±SEM) of cell types forming granulomata in the intestinal wall in FVB mice up to 100 days post-infection with *Heligmosomoides polygyrus*.

	Days post-infection			
Cell type	10	16	50	100
Macrophages	72.79 ± 5.26	75.84 ± 3.86	66.51 ± 5.37	53.21 ± 9.3
Eosinophils	14.85 ± 1.6	13.31 ± 2.42	16.89 ± 3.03	12.85 ± 1.35
Lymphocytes	7.11 ± 1.3	5.9 ± 1.42	9 ± 2.43	10.15 ± 2.5
$CD103^+$ cells	22.37 ± 2.84	35.00 ± 4.11	Not tested	Not tested
Neutrophils	2.5 ± 0.67	1.5 ± 0.33	0 ± 0	0 ± 0
Mast cells	2.25 ± 0.41	2.79 ± 0.62	1.8 ± 0.05	4.8 ± 0.15
Fibroblasts	0.5 ± 0.16	0.66 ± 0.17	5.8 ± 0.87	18.99 ± 1.01

maintain their position by coiling around single or several villi, with long villi being preferred. It has also been found that worms move away from short villi regions and any treatment that shortens the villus length causes a forward migration (Bansemir & Sukhdeo, 1996). The longest villi are usually found in the duodenum, which is known to be favoured by *H. polygyrus*. Worm distribution during the early intestinal phase of infection (on day 16 p.i. most of them were located in the jejunum) suggests that the duodenum in FVB mice was not favourable for them. Villus atrophy is a feature of several helminthiases and is intraepithelial (IEL) T cell dependent (Garside *et al.*, 1992). We found that the number of these cells bearing CD103 (integrin $\alpha E\beta 7$) (Poupon & Cerf-Bensussan, 1999) increased dramatically during the intestinal phase of *H. polygyrus* infection in FVB mice (Cywińska *et al.*, 2001) and they probably act in the same way as in *T. spiralis* infections.

Another important non-specific factor in host protection against intestinal nematodes is goblet cell hyperplasia. These exocrine cells of the intestinal epithelium are specialized in mucin exocytosis. Following mucus secretion, mucins hydrate and form a viscous gel which acts as a barrier between the intestinal contents and gut mucosa (Khan *et al.*, 2001) and mediate the response by enveloping worms and interrupting attachment (Nawa *et al.*, 1994). Goblet cell (GC) hyperplasia and increases in mucus secretion were prominent during infections with *T. spiralis* (Garside *et al.*, 1992; Bożić *et al.*, 2000; Khan *et al.*, 2001) and *Trichuris muris*, with a correlation between peak mucin production and worm expulsion for *T. muris* (Else & Finkelman, 1998). Although goblet cell hyperlasia is known to be mediated by the CD4+ Th2 immune response (Else & Finkelman, 1998; Garside *et al.*, 2000), it is also postulated that secreted mucin proteins form the basis of an innate mucosal response independent of specific IFN-γ, TNF and IL-4 cytokines (Garside *et al.*, 2000). An increase in goblet cell number was concurrent with a significant rise

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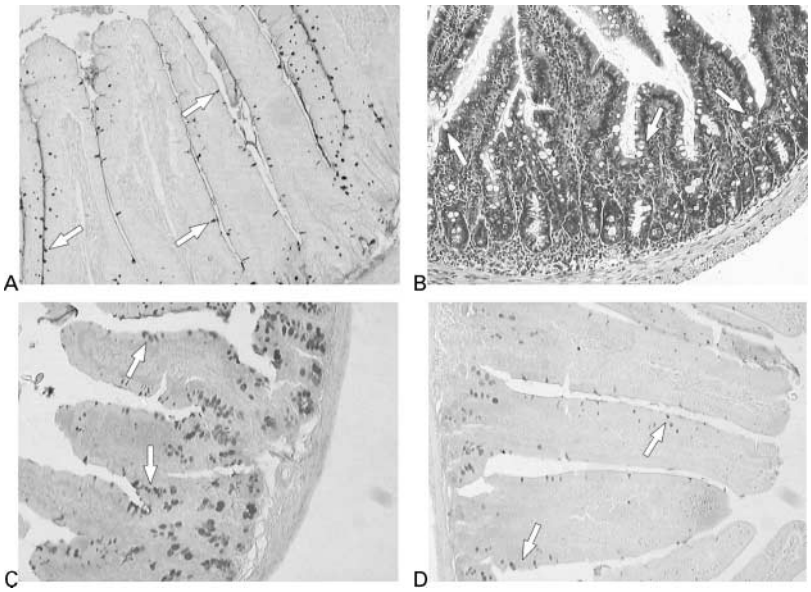

Fig. 7. Goblet cells (arrowed) in the duodenum of uninfected (A) FVB mice and 10 days (B), 16 days (C) and 50 days (D) post-infection with *Heligmosomoides polygyrus*. All sections 20×, stained with HE (B) and AB/PAS (A, C, D).

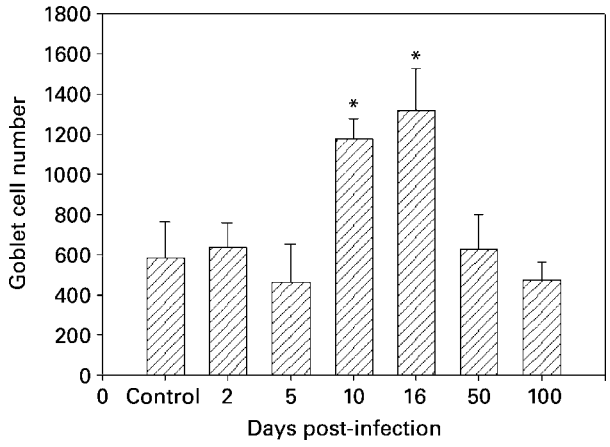

Fig. 8. Mean number (± SEM) of goblet cells up to 100 days post-infection with *Heligmosomoides polygyrus*. * Significantly different from controls ($P \leq 0.05$).

in TCR$\gamma\delta$ i-IEL during *T. spiralis* infections (Bożić *et al.*, 2000). These cells regulate epithelial homeostasis by the production of a keratinocyte growth factor (KGF), which enhances the growth and functioning of epithelial cells (Boismenu & Havran, 1994; Boismenu *et al.*, 1996) and specifically stimulates goblet cell differentation (Housley *et al.*, 1994). These facts, together with other findings (Bożić *et al.*, 2000), clearly demonstrated the critical role for TCR$\gamma\delta$ i-IELs in the generation of mucosal GC hyperplasia and host protective immunity following *T. spiralis* infections (Bożić *et al.*, 2000).

In the present experiments, GC hyperplasia was significant during the early (10, 16 d.pi.) intestinal phase of the *H. polygyrus* infection and was associated with an increase in TCR$\gamma\delta$ i-IEL number in Peyer's patches (Cywińska *et al.*, 2001). These lymphocytes seem to generate GC hyperlasia non-specifically, as in the case of *T. spiralis* infections. Excessive mucus secretion is a physical obstacle which probably hinders nematode attachment and survival. However, *H. polygyrus* expulsion was not accompanied by GC hyperplasia and this reaction is Th2 dependent, it might be suppressed by the immunosuppressive activity of adult worms in the latter phase of infection.

Another interesting feature of *H. polygyrus* associated enteropathy is the increase in the number of Paneth cells during both phases of infection but not after worm expulsion. In fast responding FVB mice the pattern of Paneth cell response differed from that observed in the intermediate responder NIH mice (Kamal *et al.*, 2002). In the latter, hyperplasia of the Paneth cells accompanies an increased expression of Th2 cytokines during larval development in the intestinal mucosa. When immunosuppressive adult worms occurred in the intestine, the number of Paneth cells decreased due to downregulation of the expression of Th2 cytokines (Kamal *et al.*, 2002), although these authors do suggest that Paneth cell hyperplasia may also be regulated by thymic-independent T cells derived from cryptopatches (Kamal *et al.*, 2001, 2002). It is therefore possible that these T cells regulate Paneth cell hyperplasia during the intestinal phase of *H. polygyrus* infection and immunomodulatory factors secreted by adult worms do not interfere with the functions of this unique T cells population.

A very unusual feature of *H. polygyrus* primary infection in FVB mice was the development of granulomatous lesions. During challenge infections in immunized mice, residual larvae are surrounded by inflammatory cells and often killed in inflammatory nodules (Fakae *et al.*, 2000). The protection was better when primary infections were terminated at the larval not adult stage and killed immunogenic larvae act as a

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continuous source of specific antigen stimulation in the gut (Fakae *et al.*, 2000). Similar processes may occur when larvae are killed in granulomata during the challenge infection (Fakae *et al.*, 2000). Granulomata formed around dead larvae or the remaining cuticular sheaths are an active source of stimulation. This can explain why intestinal inflammatory reactions increased when changes in Peyer's patches were absent and why features of chronic inflammation were found in the MLNs and spleen of infected mice after worm expulsion, even after 100 d.p.i. Cells found in the granulomata produce several mediators regulating inflammatory reactions and other cellular activities. In the case of *Schistosoma mansoni* infection, neurokines physiologically present in the intestine, namely P substance, somatostatin and VIP, are produced in granulomata. They can regulate the Th1/Th2 balance by suppression (somatostatin) or enhancement (P substance) of IFNγ production (Weinstock, 1999). However it still remains unclear why such lesions occur during primary infections. It may be true that TCR$\gamma\delta$ cells are involved in this process. They are also postulated to stimulate other cells for granuloma formation. However, in *S. mansoni* infections the absence of TCR$\gamma\delta$ cells had no impact on the granuloma morphology, in contrast to TCR$\alpha\beta$ cells (Lindberg *et al.*, 1999). However, the number of both TCR$\alpha\beta$ and TCR$\gamma\delta$ cells increased at the time of granuloma formation during *H. polygyrus* infection in FVB mice. Both types of cells were present in granulomata, although TCR$\alpha\beta$ positive cells prevailed.

The intestinal inflammation provoked by *H. polygyrus* infection in fast responder mice is continuous and persists even after worm expulsion. It changes over a time post-infection due to differing stimulations by immunogenic larvae, immunosuppressive adult worms and possibly due to differing antigen presentations. The reactions seem to be controlled not only by Th2 cells but also by cells forming granulomata and by other lymphocytes, probably distinct, thymus independent T cells. Intestinal mucosal lymphocytes especially CD103 (both TCR$\alpha\beta$ and TCR$\gamma\delta$) positive cells may play a role in generating the development of intestinal pathology during *H. polygyrus* infections in FVB mice.

It is not clear if inflammatory lesions are critical for worm expulsion but they exert a profound effect on the structure and function of the intestinal wall, create an unfavourable environment for worms and influence the host–parasite relationship.

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