The intensity and duration of primary *Heligmosomoides polygyrus* infection in TO mice modify acquired immunity to secondary challenge

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Abstract

The effect of dose and duration of immunizing infections of *Heligmosomoides polygyrus* on protection against homologous challenge was studied in female TO mice. Primary infections were terminated at various levels with pyrantel embonate (adult infections) or ivermectin (larval infections) and mice were then challenged with 500 infective larvae (L3). The level of protection to secondary challenge positively correlated with the intensity of the primary immunizing infection but truncation of larval infection produced significantly better protection than termination of the adult nematode infection. The duration of the primary larval infection (1–6 days) positively correlated with the level of protection to secondary challenge, antibody responses and the proportion of circulating eosinophils. Histological changes in the gastrointestinal tract, peripheral leucocytic changes and antibody responses of the mice to *H. polygyrus* adult somatic antigens indicate both a cellular and humoral basis of host immunity to secondary challenge. Although the TO mice are slow responders in that they harbour chronic infections, immunization by intramucosal killing of the larval stage produced strong protection against secondary challenge infection. The presence of dead immunogenic larval stages within the intestinal wall may well be an important factor, since it exposes the host to stage specific antigens at an appropriate location. The implications of the findings for the control of gastrointestinal nematode infections are also discussed.

Introduction

Heligmosomoides polygyrus infection in mice is an excellent model for chronic gastrointestinal helminthosis of other mammals (Bartlett & Ball, 1972; Monroy & Enriquez, 1992; Fakae *et al*., 1994, 1997). Several immunization procedures have been devised in order to render susceptible mice resistant to homologous challenge with this nematode. One commonly employed method of oral immunization has been to administer two or three divided doses of *H. polygyrus* third stage larvae (L3) followed by anthelmintic treatment to remove the adults (Behnke & Wakelin, 1977). Some strains of mice require several sensitizing infections while others may need only one previous infection, terminated at an appropriate stage to develop strong resistance to reinfection (Behnke & Wakelin, 1977; Prowse *et al*., 1979; Wahid & Behnke, 1992). The status of TO mice in this regard has not been previously investigated.

The purpose of these experiments was to investigate the effect of the intensity and the duration of a primary immunizing infection on the level of protection attainable in TO mice. Changes in the peripheral leucocyte population, gastrointestinal histology and humoral

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https://doi.org/10.1017/S0022149X00700940 Published online by Cambridge University Press

responses associated with homologous *H. polygyrus* challenge infection were also examined.

Materials and methods

Experimental animals and parasite

140 six to eight-week-old outbred female TO mice raised as specific pathogen free and weighing 20–25 g (A. Tuck & Sons, Essex) were used for the study. Professor D. Wakelin kindly supplied the third larval stage of the nematode, *H. polygyrus*. The origin and maintenance of this parasite have been described in detail by Fakae *et al*. (1994).

Experiment 1: *immunization by termination of primary adult* H. polygyrus *infection*

Forty mice were divided into five groups of eight mice each. They were either not infected (controls) or orally infected with either 50, 250 or 500 *H. polygyrus* L3. All mice were treated with pyrantel embonate (100 mg kg^{-1} body weight), 12 days after infection (DAI) and all (apart from the controls) challenged 8 days later with 500 *H. polygyrus* L3. All mice were killed 35 days after the start of the experiment. The percentage protection was calculated with reference to the total worm burden in the group that did not receive any immunizing infection

Experiment 2: immunization by termination of larval H. polygyrus *infection*

One hundred female TO mice (as above) were randomly selected and placed in ten groups of ten mice each. Except for the controls, which did not receive any immunizing infection, the mice were infected with either 50 or 500 *H. polygyrus* L3. Infections were terminated with ivermectin treatment (20 mg kg^{-1}) at either 1, 2, 3 or 6 DAI. Control mice were also treated. Challenge infections of 500 L3 were then given to all the mice, except the naive controls, approximately three weeks after the anthelmintic treatment (table 1), by which time the ivermectin would have been metabolized and eliminated by the host (Wahid *et al*., 1989).

Mice were humanely killed and bled from the heart 20 days after challenge infection. Blood smears were prepared from each mouse and stained with Giemsa for a differential leucocyte count. With a light microscope, 100 cells were randomly differentiated per animal.

The sera obtained from individual mice were preserved at –80°C and used in enzyme-linked immunosorbent assay (ELISA) with *H. polygyrus* somatic antigens as described by Fakae *et al*. (1994). Briefly, 96-flat-bottomed plates were treated with 5 μg ml^{-1} of parasite antigen. Test serum samples were diluted to 1:50 in diluent while goat anti-mouse IgG/peroxidase (Nordic Immunological Laboratories, The Netherlands) was used as the enzyme conjugate. Colour reaction was developed with 3,3',5,5' tetramethylbenzidine (Sigma T-5525). The optical density at 450 nm was read on a Titertek Multiscan (Labsystems Ltd).

The small intestines of mice from each group were recovered and examined grossly. Histological sections from naive and infected (immunized and unimmunized) mice were prepared, and stained by haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) reaction to determine the histological changes in the intestines (Bancroft & Stevens, 1977). The worms were extracted from the intestines of each of the remaining mice, sexed and counted. Protection attributable to the different immunization regimes was calculated from the total number of worms recovered from the infected control group. Worm lengths were determined by traces made of mounted worms captured by a video camera linked to a monitor.

Statistics

The results are presented as mean values ± standard error (SEM). Non-parametric statistical analysis was used to analyse data sets with small sample sizes (Fowler & Cohen, 1990). Two groups, as stated, were compared by the Mann-Whitney *U* test for significant differences. Where appropriate, data were compared by a one-way analysis of variance (ANOVA). Least squares linear regression analysis and the correlation coefficient, *r*, given as appropriate, tested the relationship (rectilinear) between two variables. Differences were considered significantly different at $P < 0.05$. Statistical procedures were carried out with Instat (GraphPAD Software Inc., USA).

Table 1. Experimental design for immunization of TO mice in experiment 2.

Groups	Immunizing infection (L3)	Day of treatment after infection	Challenge* infection
1 Immunized	50	1	+
2 "	500	1	+
3 "	50	2	+
4 "	500	2	+
5 "	50	3	+
6 "	500	3	+
7 "	50	6	+
8 "	500	6	+
9 Challenge control	0	6	+
10 Naive control	0	6	-

*Except for treatment control group, mice were challenged with 500 L3 of *Heligmosomoides polygyrus*, 21 days after treatment.

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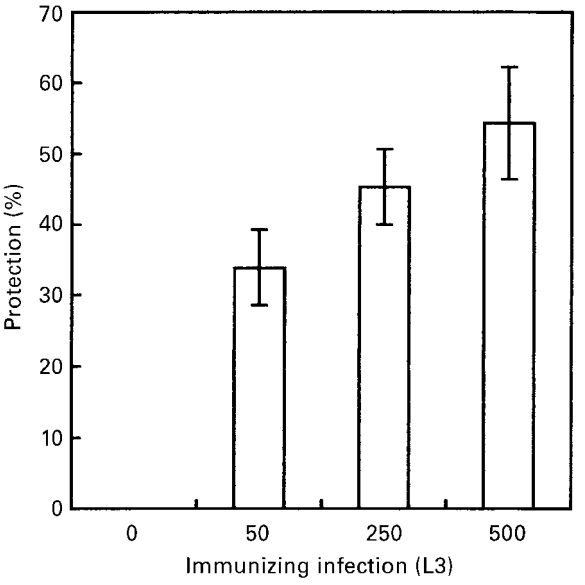

Fig. 1. The mean percentage protection (±SEM) in mice immunized by termination of an adult *Heligmosomoides. polygyrus* infection (experiment 1).

Results

Stimulation of immunity to H. polygyrus *by termination of adult infection*

The unstimulated control group harboured 360.5 (±12.9) worms. Protection against homologous challenge was directly related ($r=0.225$, $n=24$, $P<0.05$) to the immunizing dose of *H. polygyrus*, but at most reached just over 50% (fig. 1).

Stimulation of immunity to H. polygyrus *by termination of larval infection*

As shown in fig. 2, immunizing infections with 500 L3 and of 1, 2 or 3-day duration produced higher protection against a homologous challenge than those induced by 50 L3 of the same duration. However, only those of 3-day duration were statistically significant ($U=3.2$, $P<0.01$). There was no statistical difference between the protection obtained from infections of 6 day duration initiated with 50 or 500 L3 ($U=23$, $P=0.898$).

For logistic reasons, only worms pooled from the group immunized with 500 L3 and the control could be measured. Male and female worms from the immune animals were significantly shorter (fig. 3) than their counterparts from the uninfected control mice ($F_{1,38}=338.0$, $P<0.001$; $F_{1,38}=440.056$, $P<0.001$ respectively).

The proportion of eosinophils generally increased in the peripheral blood with the duration of the immunizing infection. This change was significantly higher in the mice immunized with 500 L3 than in those which had received 50 L3, especially in mice with a 6-day duration primary infection ($U=6.5$, $P=0.018$) (fig. 4a). Lymphocyte proportions in infected mice were markedly depressed (fig. 4b), the reverse was true for the neutrophils (fig. 4c), while monocyte results were rather inconclusive (fig. 4d).

The antibody titres in the groups immunized with 50 L3 were directly proportional to the duration of the immunizing infection ($r=0.984$, $n=32$, $P=0.0158$). The

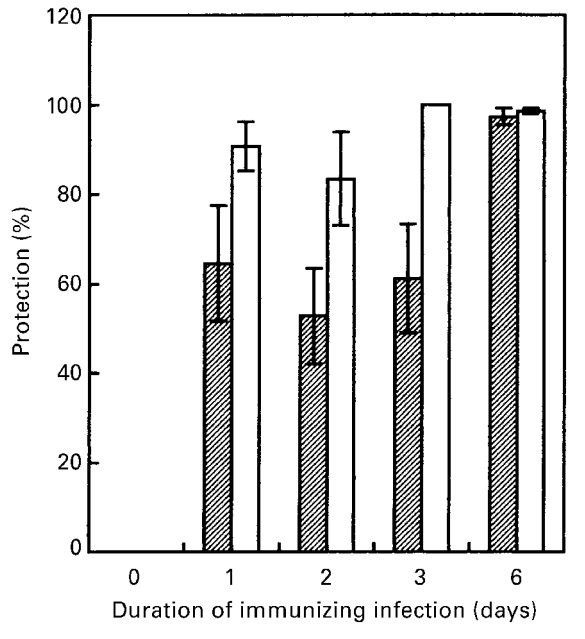

Fig. 2. The mean (±SEM) percentage protection obtained from mice immunized by termination of larval *Heligmosomoides polygyrus* infections (experiment 2). ▨, 50 L3; □, 500 L3.

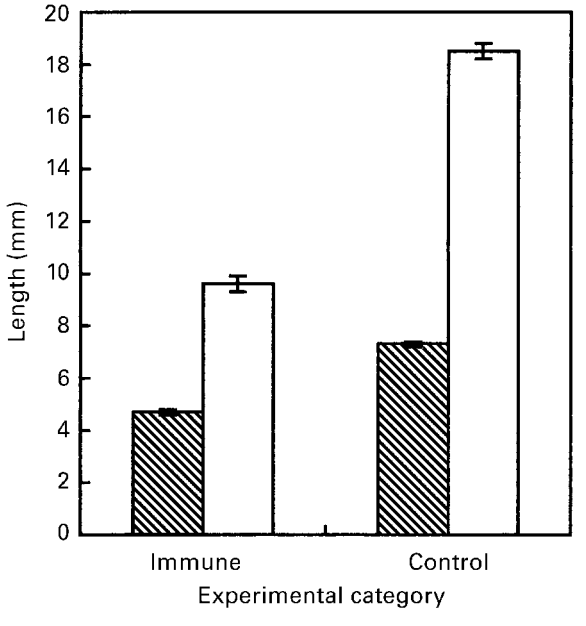

Fig. 3 . The mean (±SEM) lengths of male (▨) and female (□) *Heligmosomoides polygyrus* obtained from unimmunized controls and from mice immunized by termination of *H. polygyrus* larval infection (experiment 2).

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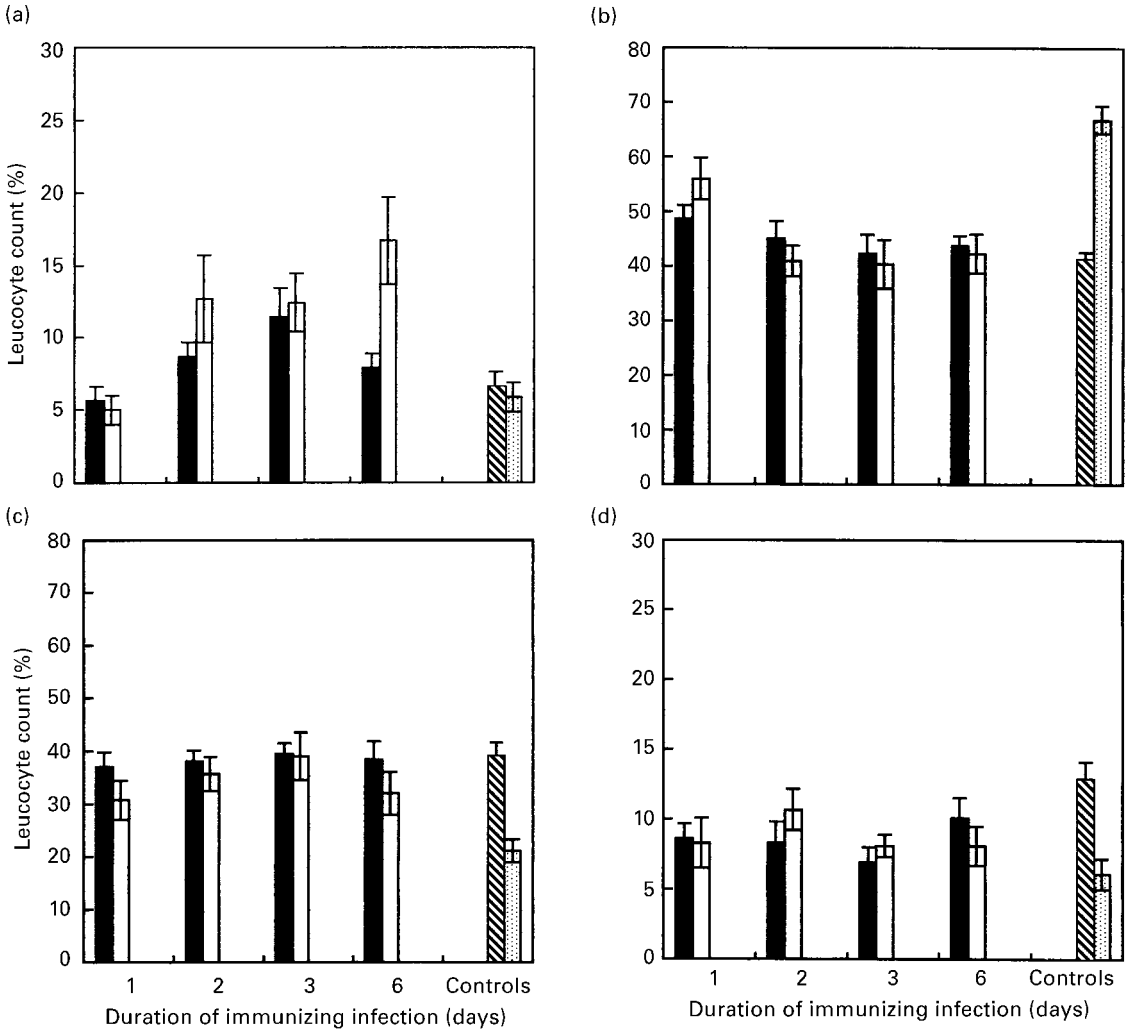

Fig. 4. The mean (±SEM) (a) eosinophil, (b) lymphocyte, (c) neutrophil and (d) monocyte counts per 100 leucocytes in the peripheral blood of control mice and those with varying doses and duration of *Heligmosomoides polygyrus* larval infection (experiment 2). ■, 50 L3; □, 500 L3; ▨, unimmunized; ⊞, uninfected.

antibody titres obtained by immunizing with 500 L3 also increased proportionately with the duration of infection ($r = 0.958$, $n = 32$, $P = 0.0416$) but did not exceed the level elicited by 6-day-old 50 L3 immunizing infection (fig. 5).

The small intestine of mice with primary *H. polygyrus* infections (challenge control group) were generally oedematous and translucent. There was hyperplasia of the epithelium, elongation of intestinal glands, distortion and hypertrophy of villi and about twice as many goblet cells as in the naive controls (fig. 6). In mice with secondary infections, the small intestine grossly showed many white parasitic nodules, which protruded from the serosal surface. There was much thickening of the *muscularis* with extensive formation of nodules associated with larvae killed within the intestinal wall. The intestinal glands of these mice were even larger than those found in the unimmunized group. There was also an intense cellular reaction involving infiltration of neutropolymorphonuclear cells, plasma cells and numerous eosinophils. PAS reaction revealed an increase in the number and size of the goblet cells as well as in the amount of mucus in the intestines of the immunized animals. These reactions, although present in the intestine of all immunized mice, were greater in the high dose groups and particularly in the group primed with 500 L3 for 6 days. The intestinal nodules contained areas of granulation and tissue necrosis with several remains of the larval parasite's cuticle (fig. 6).

Discussion

Significantly better protection was obtained against homologous *H. polygyrus* by terminating the primary infection at the larval, rather than at the adult stage, the level of protection being influenced by both the infective

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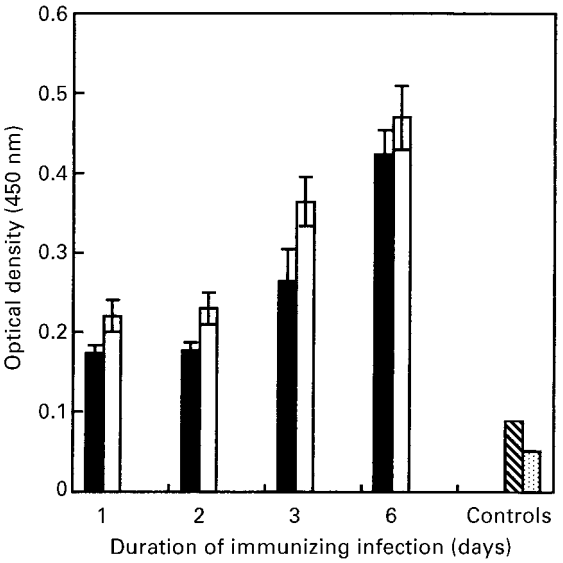

Fig. 5. The mean (±SEM) ELISA serum antibody activities in control mice and those with varying doses and duration of immunizing *Heligmosomoides polygyrus* larval infection (experiment 2). ■, 50 L3; □, 500 L3; ▨, unimmunized; ⊞, uninfected.

dose and the duration of the immunizing larval infection (compare figs 1 and 2). The histological changes in the gastrointestinal tract, the changes in the peripheral leucocyte population and the antibody responses reflect the immunological basis for resistance involving both the cellular and humoral components of host immunity.

Juvenile *H. polygyrus*, especially the fourth larval stage (L4), are highly immunogenic and provide the essential stimuli for the expression of host-protective immunity (Jacobson *et al*., 1982; Wahid & Behnke, 1992; Pleass & Bianco, 1994). Sensitization studies involving the transfer of adults via laparotomy directly into the duodenum did not stimulate resistance, confirming that stimulation of immunity is solely attributable to larval stages (Bartlett & Ball, 1974; Jacobson *et al*., 1982). In the present study even a larval infection with 50 L3 for one day protected TO mice better than a dose of 500 L3 terminated at the adult stage.

Killing the immunogenic larval stages with ivermectin while they are within the intestinal wall may well be an important factor, since it provides the host with the required specific antigens for continuous stimulation at the appropriate location (fig. 6). Dead larvae provide natural antigens exploited in the development of vaccines with irradiated larvae (Bain, 1999; Newton & Munn, 1999). The proportional increase in the protection with the size and duration of the immunizing larval infection observed here might be related to the amount of larval antigenic material to which the host was exposed. As demonstrated recently, the ensuing immunity is solid against non-specific immunosuppression from concurrent trypanosome infection (Fakae *et al*., 1999b).

As a corollary to this, it has been shown the adult stages of this parasite have an immunosuppressive effect (Jacobson *et al*., 1982; Pleass & Bianco, 1994). This effect has been thought to be due to secretion of immunomodulatory factors capable of depressing the expression of homologous immunity (Behnke *et al*., 1983).

Anti-parasite antibody, thymus-dependent lymphocytes and non-lymphoid cells are all thought to be involved in the loss of worms from immune animals (Wakelin, 1986). The positive correlation of antibody activity with the level of protection in this study supports a role for humoral immunity in protection against homologous *H. polygyrus* challenge infection in the TO mice. Earlier evidence of the role humoral immunity included the successful passive immunization of mice by transfer of immune serum (Bartlett & Ball, 1974; Behnke & Parish, 1979) and the identification of a specific protective anti-worm IgG (Pritchard *et al*., 1983; Williams & Behnke, 1983).

Investigations into the role of T-cell subsets and cytokines in the regulation of helminth infections suggest that CD4+ cells, especially the TH2 cells are important for host protection during *H. polygyrus* (Urban *et al*., 1991, 1996). Eosinophilia is a major and well-known host reaction to helminths, including *H. polygyrus* infections (Hurley & Vadas, 1983; Zhong & Dobson, 1996). Non-specific stimulation of eosinophilia in CBA/H mice, which are relatively poorly responsive to *H. polygyrus* enhanced their ability to expel, worms (Hurley & Vadas, 1983). Total leucocyte counts, which might have been more dependable, were not done in this study for logistic reasons. However the proportion of eosinophils correlated significantly with the level of protection, confirming and extending to the TO mice, that such changes in the peripheral blood could be directly related to immune reactions to *H. polygyrus* (Cypess, 1972; Zhong & Dobson, 1996). Moreover, antibody-mediated eosinophil killing of *Haemonchus contortus* exsheathed L3 *in vitro* has been demonstrated recently (Newton & Munn, 1999). The regulation of immune responses to *Heligmosomoides polygyrus* and other gastrointestinal helminths has been further described (Finkelman *et al*., 1991; Urban *et al*., 1996).

Although the process of expulsion of the challenge infection was not studied in the current experiments, the histological changes in the small intestine of the primed mice were extraordinary. The massive number of goblet cells, hyperplasia and accumulation of eosinophils and numerous stimulated lymphoid nodules suggested that a hostile environment awaited the incoming larva. Residual larvae were surrounded by inflammatory cells and probably killed in the inflammatory nodules, while those that survive return to the gut lumen as stunted pre-adults (fig. 4). Excessive mucus secretion and the thickening of intestinal epithelium, as seen in the histological sections, are physical obstacles which may also hinder the nematode's survival in the gut of immunized TO mice.

Finally, ruminants have been shown, both experimentally and under field conditions, to acquire resistance to gastrointestinal nematodes (McClure *et al*., 1992; Fakae *et al*., 1999a). This resistance may in part be responsible for the low-grade worm burdens characteristic of these infections in the subhumid savannah (Fakae, 1990). As it has been suggested that *H. polygyrus* infection in mice may form a useful laboratory model for other trichostrongylids of small ruminants (Monroy & Enriquez, 1992;

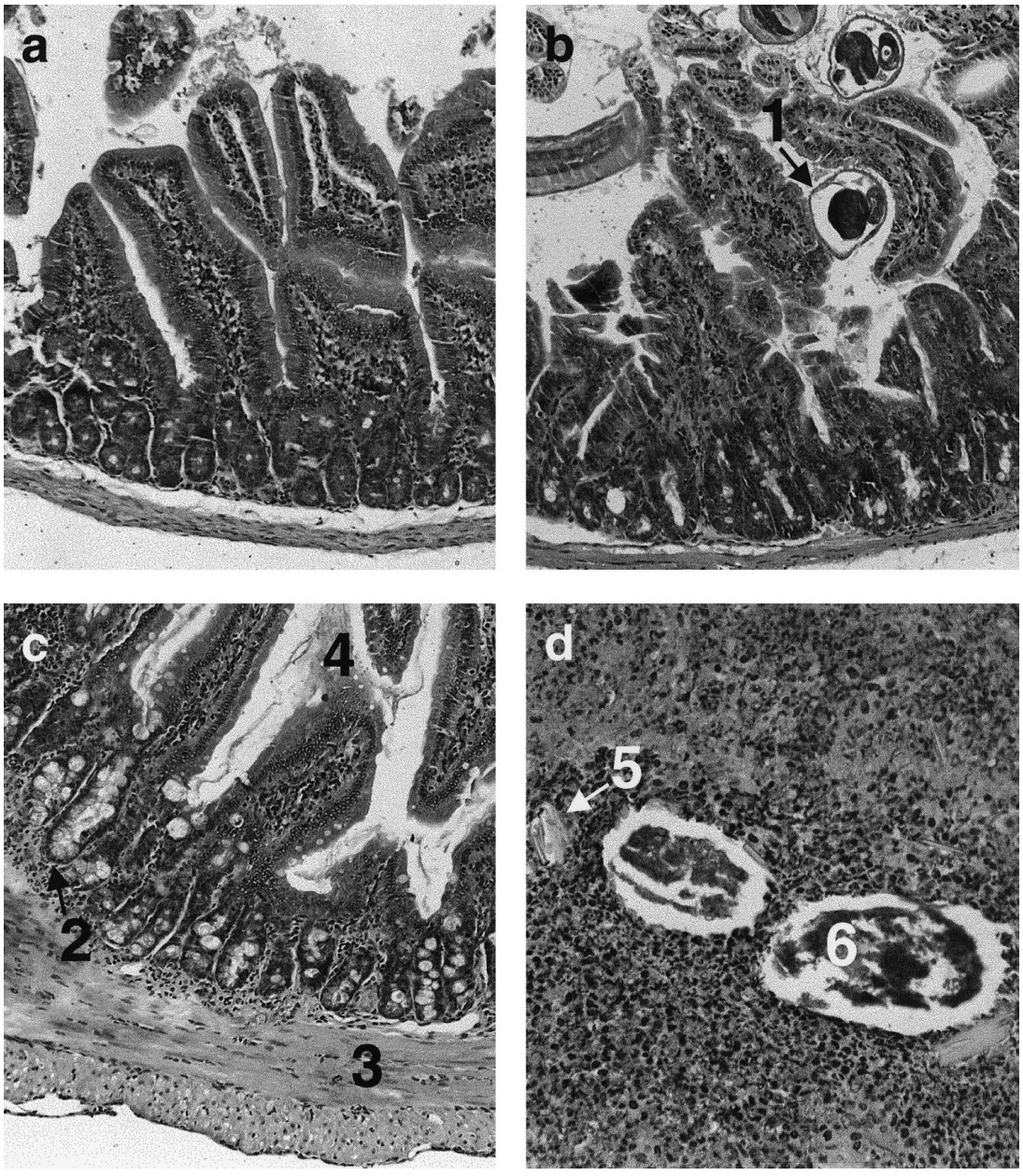

Fig. 6. Typical transverse sections of the small intestine of naive (a), unimmunized but infected with *Heligmosomoides polygyrus* (b) and immunized mice (c) stained with haematoxylin/eosin. (d) is a granulomata from an immunized mouse. (1, section of worm; 2, goblet cells and enlarged glands; 3, thickened *muscularis*; 4, secreted mucus; 5, remains of larval cuticle; 6, dead larva).

Fakae *et al.*, 1994), our observations may have some implications on planning control measures in these animals. If enhanced resistance through abbreviation of a primary larval nematode infection can be achieved after the first week of the grazing season in immunologically competent animals, it should be possible to combat to some extent the problem of rainy season parasitic gastro-enteritis of ruminants in the tropics. This possibility requires further study in the definitive hosts.

Acknowledgements

The authors would like to thank Professor D. Wakelin for the stock of *H. polygyrus* and Dr J.M. Behnke for critical reading of the manuscript. We gratefully acknowledge Dr G.R. Scott for advice on statistical analysis, Mr A.C. Roland for helping in the interpretation of histological sections, Mrs D. Bryce, Mr N. MacIntyre, Mr S.H. Wright and Mr H.R. Urquhart for their excellent technical support. The Association of Commonwealth Universities,

UK, supported B.B.F. This paper was, in part, an output of a project funded by the UK Department for International Development (DFID) for the benefit of developing countries. The views expressed are not necessarily those of the DFID.

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(Accepted 19 November 1999)