*Parasite Immunology*, 1993: **15:** 415–421

# Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): downregulation of specific cytokine secretion (IL-9 and IL-10) correlates with poor mastocytosis and chronic survival of adult worms

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## SUMMARY

*Mice were infected either with* Trichinella spiralis *(day 0),* Heligmosomoides polygyrus *(day −14) or concurrently with both species and were killed in groups, together with naïve control mice, on 2 occasions (day 8 and 15 post infection with* T. spiralis, *corresponding to days 22 and 29 p.i. with* H. polygyrus*). The expulsion of* T. spiralis *was slowed significantly in concurrently infected mice and this was associated with a reduced mastocytosis and lower serum mucosal mast cell protease levels. Mesenteric lymph node (MLN) lymphocytes from all three experimental groups secreted IL-3 and IL-4 in copious amounts when stimulated in vitro by Concanavalin A (Con-A), but the secretion of high levels of IL-9 and IL-10 was essentially confined to mice infected with* T. spiralis *alone. It is suggested that adult* H. polygyrus *selectively modulate cytokine secretion by Th2 cells within the MLN during infection and that this is brought about as a direct consequence of the mechanism employed by* H. polygyrus *to depress mucosal inflammatory responses in order to facilitate its own survival.*

***Keywords*** Trichinella spiralis, Heligmosomoides polygyrus, *mice, IL-3, IL-4, IL-9, IL-10, mast cells, mastocytosis, immunoregulation, immunomodulatory factors, chronic GI infections, evasion of immunity*

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*Received*: 23 September 1992
*Accepted for publication*: 2 February 1993

## INTRODUCTION

The immunoregulatory circuits which control mucosal inflammatory responses are being increasingly well defined (Finkelman *et al.* 1991, Finkelman & Urban, 1992). A characteristic feature of such responses to gastrointestinal (GI) nematode infections is mucosal mastocytosis (MMC) which peaks soon after the elimination of worms (e.g., *Trichinella spiralis* and *Nippostrongylus brasiliensis*) and is believed to be one of several components of the overall response leading to worm expulsion (Rothwell 1989), although the exact role of mast cells in expulsion is still controversial (Moqbel & MacDonald 1990). Mastocytosis in response to GI nematode infections is orchestrated by activated T lymphocytes, which reside in the mesenteric lymph nodes (MLN) (Grencis, Riedlinger & Wakelin 1985) and which drive the response through the secretion of a number of cytokines (e.g., IL-3, IL-4, IL-9 and IL-10) all of which are required for an optimal response (Finkelman *et al.* 1991, Grencis, Hultner & Else 1991). Relevant cytokines are almost exclusively associated with the Th2 subset (Mosmann & Moore 1991, Else & Grencis 1991b, Urban *et al.* 1992, Finkelman & Urban, 1992).

In contrast to the typically acute infections generated by *T. spiralis* in mice, *Heligmosomoides polygyrus* gives rise to chronic infections which may last for many months (Robinson *et al.* 1989) and unlike the former species do not elicit a marked mastocytosis (Dehlawi, Wakelin & Behnke 1987). Moreover, the presence of *H. polygyrus* in concurrent infections with species which are normally expelled results in prolonged infections with the latter (Behnke, Wakelin & Wilson 1978) and a downregulation of mucosal mastocytosis (Dehlawi *et al.* 1987). Many studies have implicated adult *H. polygyrus* as the source of the immunodepressive effect (possibly through the

release of immunomodulatory factors, IMF, Behnke 1987) and data from adoptive transfer experiments suggest that the immunodepressive effect acts on the generation of T lymphocytes capable of promoting the development of MMC from precursor cells (Dehlawi & Wakelin 1988). The latter is also supported by the demonstration that tissue culture medium conditioned by spleen cells from mice infected with *H. polygyrus* does not support maturation of mast cells from bone marrow precursors *in vitro* (Reed, Dehlawi & Wakelin 1988). Therefore, the evasion strategy of *H. polygyrus* appears to be dependent primarily on preventing intestinal inflammatory responses from being generated, a conclusion consistent with data showing that the majority of worms are eliminated from the intestine if the inflammatory response is not prevented (Behnke, Cabaj & Wakelin 1992). Although downregulation of MMC may not be the only nor the crucial element in survival, mast cell responses can be easily quantified. An understanding of how such responses are downregulated by parasites may lead to a better understanding of immunoregulation of mastocytosis as well as of the mechanisms used by parasites to prevent the expression of potentially host-protective immunity.

In this paper we describe an experiment in which the relationship between worm expulsion, mucosal mastocytosis and the potential of MLN cells to secrete Th2 cytokines was re-examined, in order to unravel the basis of the low-intensity mastocytosis typically evident during infection with *H. polygyrus* and the downregulation of mastocytosis observed in concurrent infections with *T. spiralis*.

## MATERIALS AND METHODS

### Animals

Syngeneic NIH and outbred CFLP mice were either purchased from Harlan Olac Ltd., Bicester, Oxon, UK or were bred in the departmental animal house under conventional conditions. All animals were provided with food and water *ad libitum*.

### Parasite

The methods employed for maintenance, infection of mice and recovery of *Heligmosomoides polygyrus bakeri* (Behnke, Keymer & Lewis 1991) and *T. spiralis* at autopsy have all been described previously (Jenkins & Behnke 1977, Wakelin & Lloyd 1976).

### Experimental design

In order to simplify the timing of infections day 0 (d 0) is taken as the day on which *T. spiralis* was administered. Infections with *H. polygyrus* were given 14 days earlier (d − 14). The experiment comprised a total of 50 female NIH mice divided into eight groups. Four groups were infected with 250L3 of *H. polygyrus* on day −14. Two further groups were given 300 muscle larvae of *T. spiralis* on day 0 and two of the *H. polygyrus* infected mice were challenged with the same dose of *T. spiralis* on day 0. Two additional groups were left uninfected throughout. Eight days after infection with *T. spiralis*, one group of mice infected with *H. polygyrus* only ($n=6$), one group infected with *T. spiralis* only ($n=6$), one group infected with both species ($n=8$) and a naïve control group ($n=5$) were killed and autopsied. Each mouse was exanguinated (to provide serum for mucosal mast cell protease analysis), a section of the small intestine was fixed for mast cell counts, MLN were removed for quantification of cytokine secretion by T cells and the remaining intestine was incubated for worm counts. This procedure was repeated on day 15 for the remaining 4 groups.

### Mast cell counts and mucosal mast cell protease-1

A 2 cm length of small intestine taken 10 cm from the pyloric sphincter was fixed in Carnoy's fixative and processed using standard histological techniques. Sections cut at 5 μm were stained with Alcian Blue, counterstained with Safranin O and mounted in DPX using the method of Alizadeh & Wakelin (1982) with the following modifications. Sections were stained for 25–30 minutes in Mayer's haematoxylin, then for 20–25 minutes in phosphate buffered Safranin O before processing and mounting in DPX.

Mucosal mast cell protease (MMCP) was measured by the double antibody sandwich method as described by Huntley *et al.* (1987) and modified subsequently for murine studies (Tuohy *et al.* 1990). An affinity purified rabbit polyclonal MMCP-1 antiserum was used to coat microtitre plates overnight at 4°C. The plates were then washed and dilutions of test samples and standards were added and left for 90 min at room temperature. The plates were washed again and an affinity purified rabbit anti-MMCP-1 antibody conjugated with horse-radish peroxidase was added. The substrate hydrogen peroxide and O-phenylenediamine chromogen were used and the reaction was stopped with 25 μl of 2·5M $H_2SO_4$ before reading on a Dynatech reader at 490 nm. Concentrations of MMCP-1 in unknown samples were calculated from standard curves prepared from purified mouse MMCP-1 standards.

### Preparation of cell supernatants and measurement of cytokines

Briefly, mesenteric lymph node cell suspensions were prepared from infected and normal mice as described by Else & Grencis (1991b). Cells ($5 \times 10^6$/ml) were stimulated with Concanavalin A (Con-A) (Sigma, 5 μg/ml final concentration) and cell-free supernatants were recovered after 24 and 48 h incubation at 37°C and 5% $CO_2$, aliquoted and stored at −80°C prior to use. Cytokines present in supernatants were measured by sandwich ELISAs using methodology essentially as described previously (Else & Grencis 1991b, Else & Grencis 1993). The pairs of cytokine specific monoclonal antibodies (MoAbs) used were; IL-3, 8F8 & 43D11 (J.Abrams); IL-4, 1D11.2 & 24G.2 (AMS Biotechnology, Witney, Oxon, UK); IL-9, 229.4 & 1C10 (J.van Snick & C.Uyttenhove, Brussels, Belgium); IL-10, JES5-2A5 (AMS Biotechnology) & SXC-1 (T.Mosmann, Edmonton, Canada). The sensitivity of the assay was calculated as the mean optical density of 16 negative control wells plus three times the standard deviation. Any test wells with OD values above this sensitivity were considered to be positive for the cytokine in question. The cut off values were as follows: IL-3, 0·22; IL-4, 0·2; IL-9, 1·0; IL-10, 0·21 u/ml.

### Statistical analysis of results

Data are presented as group mean value ± standard error (SEM). Non-parametric statistical procedures were used to analyse the data sets, because normal distribution of data could not be assumed (Sokal & Rohlf 1969). In most cases statistical analysis of cytokine concentrations was carried out on values after conversion to u/ml. However, in the case of IL-3, IL-4 and IL-9 some samples had optical density (OD) readings in excess of the range covered by standards and since the relationship of OD to cytokine concentrations could not be assumed to be linear, these values were not converted to u/ml and are presented as exceeding the range of standards i.e., >62 for IL-3 and IL-4, and >1000 for IL-9. However, OD should still show a positive, albeit not necessarily linear, relationship with cytokine concentrations and therefore statistical analyses in these cases were carried out on OD values. When more than two groups required comparison at a single time point the Kruskal Wallis statistic $H$ was calculated to determine whether there was a treatment effect across experimental groups with the exception of naïve control mice. If significant, specific groups were compared to the group infected with *T. spiralis* only (or as stated) by the Mann-Whitney $U$ test. Probabilities were calculated from statistics tables and are presented as follows: *, $P=0{\cdot}05$; **, $0{\cdot}05>P\geqslant 0{\cdot}02$; ***, $0{\cdot}02>P\geqslant 0{\cdot}01$; ****, $0{\cdot}01>P\geqslant 0{\cdot}001$; *****, $P<0{\cdot}001$. However, where multiple analyses were undertaken, the cut off value for significance was lowered to $P=0{\cdot}01$ in order to avoid Type II errors.

## RESULTS

### Recovery of worms

The establishment of *H. polygyrus* was much as expected and 8 days p.i. with *T. spiralis* comparable worm burdens were recovered from *H. polygyrus* only (172·5 ± 12·3) and concurrently infected mice (158·0 ± 7·6). Worm burdens remained unchanged in the former group (178·5 ± 10·1) but by day 15 the concurrently infected mice had lost 44·5% of their worms (87·7 ± 19·5) much as described by Behnke, Cabaj & Wakelin (1992).

The establishment of *T. spiralis* was lower than expected but nevertheless 21·2% of the inoculated larvae were recovered on day 8 from the *T. spiralis* only group (63·7 ± 6·9) and 17·9% from the concurrently infected animals (53·7 ± 7·5, $P=$ ns). However, expulsion of *T. spiralis* was clearly delayed in concurrently infected mice ($P=0{\cdot}0013$) which still had a MWR of 7·6 ± 2·0 on day 15 (representing 14·2% of the established worms from day 8), whereas no worms at all were recovered from the *T. spiralis* only group.

### Mast cell counts

As expected from earlier studies *T. spiralis* generated a marked mastocytosis with mast cell numbers/20 vcus exceeding 700 on both day 8 and 15 p.i. (Figure 1). Throughout this period mast cells were not detected in control naïve mice and remained low in mice infected only with *H. polygyrus* (124·3 ± 32·7, 107·0 ± 13·5 respectively). Thus *H. polygyrus* elicited a relatively mild mastocytosis by comparison with *T. spiralis* and depressed significantly mastocytosis (24·6%) in concurrently infected animals ($P=0{\cdot}01$). Furthermore, whereas mast cell counts in *T. spiralis* only mice did not drop appreciably (4·8%, $P=$ ns) between days 8 and 15 p.i., there was a significant reduction in concurrently infected mice over the same period (50·3%, $P=0{\cdot}03$).

### Serum mucosal mast cell protease-1 levels

As with mast cells, there were pronounced differences in serum MMCP-1 concentrations between groups infected with *T. spiralis*, *H. polygyrus* or with both (day 8, $H=11{\cdot}86$, $P<0{\cdot}003$; day 15, $H=10{\cdot}82$, $P<0{\cdot}005$). MMCP-1 was lowest in naïve control mice (0·04 ± 0·02 μg/ml serum) and the greatest increase was observed in *T. spiralis* only mice with levels of 6·0 ± 0·3 μg/ml on day 8

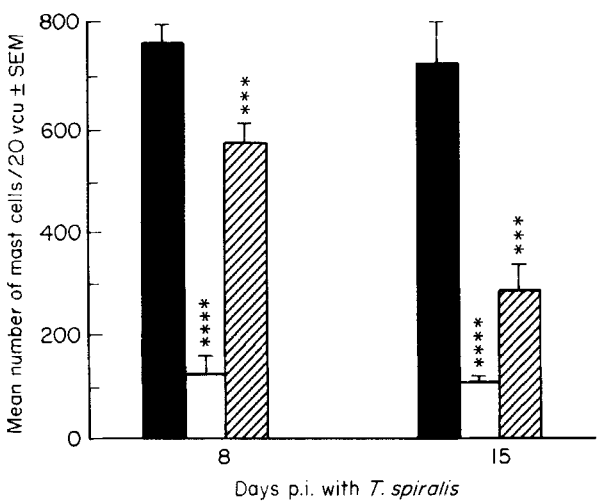


**Figure 1** The mucosal mast cell response of mice infected with *T. spiralis* alone, *H. polygyrus* alone and with both parasites concurrently. Groups of mice from all three experimental groups and from naïve control mice were killed for mast cell counts on days 8 and 15 p.i. with *T. spiralis* (days 22 and 29 p.i. with *H. polygyrus*). ■ *T. spiralis*; □ *H. polygyrus*; ▨ concurrent. No mast cells were detected in groups of naïve control mice killed on both occasions.

Statistical analysis.
Day 8, $H=11{\cdot}0167$ ($P=0{\cdot}004$)
Day 15, $H=7{\cdot}2$ ($P=0{\cdot}02$)

Groups were compared to the mice infected only with *T. spiralis* by the Mann-Whitney *U* test and the key to the symbols used is as following:
***, $0{\cdot}02>P\geqslant 0{\cdot}01$; ****, $0{\cdot}01>P\geqslant 0{\cdot}001$.

increasing to $8{\cdot}17\pm0{\cdot}6$ μg/ml on day 15. Mice infected with *H. polygyrus* alone or concurrently infected with *T. spiralis* had intermediate levels but the values were very similar for both groups (day 8, $3{\cdot}61\pm0{\cdot}2$, $4{\cdot}2\pm0{\cdot}2$; day 15 $5{\cdot}3\pm0{\cdot}4$, $5{\cdot}5\pm0{\cdot}4$, respectively). On both days these groups had significantly less MMCP-1 in the serum than the *T. spiralis* only group ($H=11{\cdot}86$, $P=0{\cdot}0027$; $H=10{\cdot}817$, $P=0{\cdot}0045$, respectively).

### Cytokines secreted by MLN cells

MLN cells of all infected groups irrespective of the combination of parasites to which the hosts had been exposed were capable to secreting large amounts of IL-3 and IL-4 following stimulation with Con-A *in vitro* (Table 1). Secretion of IL-3 and IL-4 peaked in *T. spiralis* mice on day 8, followed by a return to base levels by day 15. However, the MLN cells of mice harbouring *H. polygyrus*, whether alone or in combination with *T. spiralis*, continued to secrete both cytokines in large amounts until day 15 p.i. (29 days p.i. with *H. polygyrus*).

In marked contrast, on day 8 p.i. the MLN cells of mice infected with *H. polygyrus* alone secreted substantially less IL-9 and IL-10 ($P=0{\cdot}0028$, $0{\cdot}0051$ respectively) compared with mice infected with *T. spiralis*. Moreover, this depressed potential to secrete IL-9 and IL-10 was also evident in the concurrently infected group. Thus IL-9 and IL-10 concentrations in cell supernatants of concurrently infected mice were reduced by 46·9 and 55·7% respectively relative to *T. spiralis* only mice. We also measured

**Table 1** Concentration of cytokines in u/ml of supernatant from Con-A stimulated MLN cells, harvested from mice harbouring single or concurrent infections, 8 and 15 days after exposure to *Trichinella spiralis*

| | IL-3 | | IL-4 | | IL-9 | | IL-10 | |
|---|---|---|---|---|---|---|---|---|
| Group | Day 8 | Day 15 | Day 8 | Day 15 | Day 8 | Day 15 | Day 8 | Day 15 |
| *T. spiralis* only | >62 | 14·2±4·8 | 36·2±8·2 | 7·3±2·0 | >1000±27·6 | 78·3±1·1 | 7·9±0·1 | 0·65 |
| *H. polygyrus* only | >62**** | >62**** | 58·8±1·7 | >62**** | 144·8±12·3**** | 227·0±43·7** | 1·5±0·1**** | 1·5±0·1**** |
| *T. spiralis* + *H. polygyrus* | >62 | >62**** | 28·8±4·2 | >62**** | 530·6±52·8**** | 303·9±50·0** | 3·5±0·5*** | 1·24±0·7**** |
| Naïve control | 32·3±2·7 | 15·7±1·9 | 1·02±0·2 | 1·1±0·3 | 4·84±2·4 | 5·6±2·4 | 0·5±0·03 | 0·57±0·1 |
| Statistical analysis | | | | | | | | |
| *H* | 8·02 | 12·9 | 7·56 | 11·13 | 15·73 | 7·63 | 14·36 | 12·31 |
| *P* | 0·02 | 0·002 | 0·02 | 0·004 | 0·0004 | 0·02 | 0·0008 | 0·002 |

The three experimental groups (excluding the naïve control group) were compared by the Kruskal Wallis test to determine whether there was a significant difference in cytokine secretion among the three combinations of infections. Since many IL-3, and 4 values exceeded the range employed in standards, OD were used in statistical analyses. For IL-9 and IL-10 u/ml were used.
Additional groups were compared by the Mann Whitney *U* test to mice given *T. spiralis* only and have the following levels of significance: **, $0{\cdot}05>P\geqslant 0{\cdot}02$; ***, $0{\cdot}02>P\geqslant 0{\cdot}01$; ****, $0{\cdot}01>P\geqslant 0{\cdot}001$; *****, $P<0{\cdot}001$.

gamma interferon levels in cell supernatants (data not shown) and there were no statistically significant changes between days 8 and 15 p.i. nor between any of the groups on either day.

## DISCUSSION

The immunodepressive influence of adult *H. polygyrus* on mucosal immune responses was evident in this study in respect of three criteria, two of which have been previously reported. Firstly the expulsion of *T. spiralis* was retarded in concurrently infected mice with 14·2% of established worms still resident in the intestine 15 days p.i., at a time when control mice inoculated only with *T. spiralis* had expelled their entire worm burden (Behnke *et al.* 1978). Secondly, the slower expulsion of *T. spiralis* was accompanied by a reduced initial mastocytosis and a more rapid return to base levels in mice harbouring both parasites (Dehlawi *et al.* 1987). Thirdly, quantification of MMCP-1 levels confirmed that a significant depression of the mast cell component of the mucosal response to *T. spiralis* had taken place in concurrently infected mice. On these criteria our results concur with earlier studies indicating that *H. polygyrus* interferes with the immunoregulatory circuits controlling mucosal immune responses in order to favour its own survival. The question now arises as to how these effects are mediated.

In this study MLN were used as a convenient source of lymphocytes activated in response to intestinal infection, since these cells ultimately home to the gut mucosa and in response to further stimulation by antigen secrete the cytokines responsible for controlling local inflammatory events. When the potential of MLN cells to secrete cytokines was examined by stimulation with the polyclonal mitogen Con-A, we found that both parasites alone and in combination were associated with the presence of cells capable of producing large amounts of IL-3 and IL-4, both cytokines required for expression of mastocytosis *in vivo* (Finkelman *et al.* 1991). Indeed, levels of both IL-3 and IL-4 remained high or increased from day 8 to day 15 in those animals which experienced *H. polygyrus* alone or concurrently with *T. spiralis*.

However, other T cell derived cytokines have been shown to have effects upon the growth of mucosal mast cells *in vitro* including IL-9 (Hultner *et al.*, 1990) and IL-10 (Thompson-Snipes *et al.*, 1991). Whereas both cytokines were present in high levels in supernatants from MLN cells of mice infected with *T. spiralis* alone, there was a significant reduction in levels in concurrently infected mice, with lower levels of these cytokines from mice infected with *H. polygyrus* alone. The pattern of IL-9 and IL-10 secretion from the different groups correlated particularly well with the pattern of intestinal mastocytosis observed on day 8 p.i. although did not hold true for day 15 p.i. Based on our findings it is reasonable to suggest that the poor (*H. polygyrus* alone) or reduced (concurrrent infection) intestinal mastocytosis observed was related to the depressed secretion of IL-9 and IL-10. This is certainly consistent with the earlier reports which concluded that the suppression of mucosal mastocytosis by *H. polygyrus* was mediated by adult worm factors affecting the generation of T lymphocytes controlling mastocytosis (Dehlawi & Wakelin 1988), and which identified a lack of mast cell growth factors in culture medium conditioned by spleen cells from mice infected with *H. polygyrus* (Reed *et al.* 1988). However, worm derived immunomodulatory factors (IMF) would be expected to exert maximum effects locally in the mucosa in close proximity of the adult worms and to be short-lived (Behnke, Barnard & Wakelin 1992), and therefore, it does not follow that the effects observed on MLN cells, which are located at some distance from the gut mucosa, reflected their full scope of the interaction between *H. polygyrus* and host cells. Activated MLN T cells are known to show reduced homing to the intestinal mucosa of mice infected with *H. polygyrus* (Hagan & Wakelin 1982) and the activity of those cells which are retained in the mucosa is likely to be further affected by the higher local concentrations of IMF with possibly more wide ranging effects than those observed on MLN cells.

The control of intestinal mastocytosis is a complex and incompletely understood process, but is thought to involve a variety of cytokines, predominantly of T cell origin (IL-3, IL-4, IL-9 and IL-10, see above) although other growth factors, particularly stem cell factor also play a crucial role (Tsai *et al.* 1991, Grencis *et al.* 1993). The relative importance of each of these in regulating growth, proliferation, survival and maturation to a fully functional mucosal mast cell is unclear but it is known that some of these factors can act together synergistically and that the sequence to which mast cell precursors are exposed is important. IL-9 has been shown to enhance the survival of mucosal-like mast cells *in vitro* (Hultner *et al.* 1990) and IL-10 can function as a synergistic growth factor (Thompson-Snipes *et al.* 1991). Recently, more subtle effects of some cytokines have been demonstrated (Ghildyal *et al.* 1992). Bone marrow derived mucosal-like mast cells grown in IL-3 and subsequently exposed to IL-4 and IL-10 upregulated the expression of the gene encoding mouse mast cell protease 2 (MMCP-2, a protease specific for mucosal mast cells). The gene was only activated in the presence of IL-10, although a synergistic effect of IL-4 was apparent. Interestingly, the subsequent addition of IL-3 induced a downregulation of

the MMCP-2 gene and in this context it may be relevant that the levels of IL-3 and IL-4 remained high or increased between days 8 and 15 in mice harbouring *H. polygyrus* in contrast to those with *T. spiralis*. Therefore, in addition to downregulation of IL-9 and IL-10, upregulation of other cytokines may also have a role in the modulation of mast cell maturation and function during infection with *H. polygyrus*. Clearly the depression of intestinal mastocytosis during infection with *H. polygyrus* may operate at a number of distinct levels, with the same eventual outcome on the kinetics of cellular accumulation and survival in the mucosa.

The data presented here confirm the work of others indicating that the immune response generated against intestinal helminths is dominated by Th2 cells (Urban *et al.* 1992, Finkelman & Urban 1992, Grencis *et al.* 1991, Else & Grencis 1991a). We extend these observations by demonstrating a selective downregulation of certain Th cell derived cytokines (e.g., IL-9 and IL-10) and possibly upregulation of others (IL-3 and IL-4) by a helminth which normally causes chronic infections (*H. polygyrus*), leading to an alteration in intestinal mastocytosis and the expulsion kinetics of a second parasite (*T. spiralis*) concurrently resident in the host. Modulation of Th cell cytokine production has been shown to result in effective immune evasion in other parasite systems (see Alexander & Russell 1992, Else, Hultner & Grencis 1992). Our data suggest that subtle and precise interference by *H. polygyrus* with the generation of a potentially host-protective immune response at the level of cytokine secretion by Th cells (in this instance, within the Th2 subset), may be a potent mechanism for promoting its own survival. The nature of the modulatory influence on cytokine production by T-lymphocytes remains to be elucidated.

## ACKNOWLEDGEMENTS

We are grateful to Professors D.Wakelin and P.N.R. Usherwood for the provision of facilities for this study in the Department of Life Science at Nottingham University and to Dr G.F.J.Newlands for the provision of a MMCP-1 quantification kit. Drs Mosmann, Abrams, van Snick and Uyttenhove for kind gifts of cytokines and hybridoma cells. The work was supported by the MRC through project grant G8923735/T to J.M.B. We thank Mr D.Fox for supervision over the maintenance of our experimental animals and Mrs J.Brown for technical support.

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