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Immunological relationships during primary infection with *Heligmosomoides polygyrus*: Th2 cytokines and primary response phenotype

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SUMMARY

The primary immune response to infection with *Heligmosomoides polygyrus* was studied in mice differing in response phenotype (fast–SWR, intermediate–NIH, slow–CBA). Marked IgG1 and IgE but not IgG2a antibody responses were detected in infected mice and the former were more intense in fast compared with slow responder strains. Mastocytosis, MMCP-1, and the secretion of cytokines by mesenteric lymph node cells, following stimulation *in vitro* by Con A, were also more intense initially in SWR mice. Secretion of IL-4 declined in all strains by the 4th week of infection, irrespective of response phenotype. IL-10 was only produced briefly by SWR mice. However, the temporal patterns of secretion of IL-3 and IL-9 clearly distinguished fast from slow responder phenotypes. Following initial intense secretion of IL-3, production declined in all strains but in the 5–6th weeks enhanced secretion was evident in SWR and NIH mice and was sustained until week 10 p.i. In contrast, CBA mice never recovered from the initial down-regulation in weeks 3–4 and secretion declined to background levels by week 6 p.i. despite the continued presence of adult worms. Temporal changes in the secretion of IL-9 were very similar: secretion declined in CBA mice by week 6 p.i., whilst SWR and NIH mice continued to secrete high amounts. We suggest that fast and slow responder mice differ not only in their initial responsiveness to parasite antigens but also in their ability to sustain a Th2 response to the parasite and we propose that the latter is in part determined by their different susceptibilities to parasite-mediated immunomodulation. Only the fast responder strains can sustain a Th2 response of sufficient intensity to facilitate expulsion of adult worms.

Key words: *Heligmosomoides polygyrus*, IL-3, IL-4, IL-9, IL-10, immunoregulation, Th2 lymphocytes, immunomodulatory factors, chronic GI infections, evasion of immunity.

INTRODUCTION

Intestinal immune responses to parasitic nematode infections are orchestrated by antigen sensitized T helper (Th) cells which undergo clonal expansion in the mesenteric lymph nodes (MLN) before migrating to the mucosa. After sequestration in the intestinal mucosa and following further exposure to antigen Th cells initiate the generation of inflammatory cells in the bone marrow and control their accumulation in the gut mucosa through cytokines which are released into their immediate environment and into the circulation (Moqbel & MacDonald, 1990). During such infections the Th cell subset responsible for these events is largely the Th2 subset which produces the cytokines that control the characteristic inflammatory changes often accompanying helminth infections (e.g. intestinal mastocytosis via release of IL-3, IL-4, IL-9 and IL-10; Finkelman *et al.* (1991); Madden *et al.* (1991); Urban *et al.* (1992)). There is also accumulating evidence supporting a role for Th2 cells in host protective immunity to intestinal helminths (Urban *et al.* 1991; Else, Hultner & Grencis, 1992).

The murine trichostrongylid gastrointestinal (GI) nematode *Heligmosomoides polygyrus* causes chronic infections in most mouse strains, in some cases lasting for over 30 weeks in duration (Robinson *et al.* 1989) and, in contrast to other GI nematodes such as *Trichinella spiralis* and *Nippostrongylus brasiliensis*, primary infections are not accompanied by intense mastocytosis (Dehlawi, Wakelin & Behnke, 1987), although other markers of the Th2 response are evident (e.g. IgE – Urban *et al.* (1991); eosinophilia – Cypess (1972); Ali, Behnke & Manger (1985); parasite specific IgG1 – Wahid & Behnke (1993*a*)). The low intensity mastocytosis and chronic survival of adult worms have both been attributed to the immunomodulatory activities of adult worms (Dobson & Cayzer, 1982; Behnke, 1987; Dehlawi & Wakelin, 1988), which have been shown to down-regulate the potential of MLN cells to secrete IL-9

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and IL-10 following stimulation by Concanavalin A (Con A) *in vitro* (Behnke *et al.* 1993). However, some strains of mice (e.g. SWR and SJL) limit primary infections within 8 weeks of infection (Wahid, Robinson & Behnke, 1989), considerably earlier than the majority of other strains and it has been proposed that these strains share genes which enable them to resist the immunomodulatory activities of adult worms (Wahid & Behnke, 1993*b*). However, worm loss from one of these strains (SJL) is not accompanied by prominent mastocytosis (Dehlawi & Wakelin, 1988). The possibility therefore exists that worm loss in SJL mice is mediated by Th1 cells or by an effector mechanism initiated by Th2 cytokines but not involving mast cells.

In this paper we report experiments in which we compared the potential of MLN cells to secrete Th2-associated cytokines, during primary infections in mouse strains representing the fast (SWR which like SJL mice lose worms by week 8), intermediate (NIH which generally tolerate infections for more than 8 weeks but lose worms by week 25) and slow (CBA which sustain chronic infections lasting > 30 weeks) responder phenotypes. Our experiments show that Th2 cells are activated in both fast and slow responder mouse strains but there is a profound difference between these strains in their ability to sustain the potential for secretion of the cytokines associated with inflammatory responses.

MATERIALS AND METHODS

Animals

Specific pathogen-free syngeneic SWR, NIH and CBA mice were purchased from Harlan Olac Ltd. Outbred CFLP mice 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) at autopsy have all been described previously (Jenkins & Behnke, 1977).

Experimental design

Two experiments were carried out comparing strains of mice representing slow (CBA) and fast (SWR) primary infection response phenotypes. NIH mice representing an intermediate responder strain were also included in the first experiment. In Exp. 1 mice were infected with 60 L3 and were killed in groups 2, 4, 6 and 10 weeks after infection (p.i.). Age-matched naive control mice were assayed in weeks 2, 4 and 10. Mesenteric lymph nodes (MLN) were used to provide cells for cytokine assays and the intestines were incubated for recovery of worms. MLN cell cultures were prepared from individual mice. At autopsy each mouse was exsanguinated to provide serum for assay of antibody and mast cell protease activities.

A second experiment was carried out employing only SWR and CBA mice but this time groups of mice were killed more frequently in the early stages of infection. Serum antibody and mucosal mast cell protease levels were measured, a section of the small intestine was fixed for mast cell counts, MLN were removed for quantification of cytokine secretion and the remaining intestine was incubated for worm counts. However, in this experiment MLN cells were pooled from all the nodes from mice within any particular experimental group.

Mast cell counts and mucosal mast cell protease-1 (MMCP-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 a thickness of 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 min in Mayer's haematoxylin, then for 20–25 min 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 horseradish peroxidase was added. The substrate hydrogen peroxide and *O*-phenylenediamine chromogen were used and the reaction was stopped with 25 μl of 2·5 M 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 antigens

Outbred CFLP mice were infected with 400 L_3 of *H. polygyrus* and were killed 14 days later. Adult worms were isolated by opening the small intestine and incubating sections harbouring worms in Hanks's saline at 37 °C in gauze bags held over small glass

beakers. When a sufficient number of worms had been collected, the parasites were washed 10 times in ice-cold sterile phosphate-buffered saline (PBS) and homogenized in a minimal volume of PBS using a glass tissue homogenizer held in an ice bath. The resulting suspension was centrifuged at 10000 g for 1 h at 4 °C to remove coarse particulate matter. The supernatant fraction was filtered (0·22 μm filter, Millipore), analysed for protein concentration using a method modified from Lowry *et al.* (1951), aliquoted and stored at −40 °C.

Measurement of parasite-specific serum antibody concentrations

Specific anti-worm antibodies were measured by a standard ELISA and the data are presented as optical density values. Briefly, ELISA microtitre plates were coated with 50 μl/well of worm antigen (5 μg/ml) and remaining protein binding sites were blocked by incubation for 1 h at 37 °C with 1–2 % BSA–PBS (100 μl/well). Alkaline phosphatase-conjugated sheep-anti-mouse IgG1 (Serotec) and goat anti-mouse IgG2a (Sera Labs, Crawley Down, Sussex, UK) were used to measure subclass-specific responses. After addition of the substrate (*P*-nitrophenylphosphatase) colour changes were read at 410 nm on a Dynatech MR700 Microplate Reader. Sera were assayed individually in triplicate after storage at −40 °C and a mean optical density (OD) value was obtained for each serum. Each plate included control hyperimmune serum (HIM) which was raised as described by Behnke & Parish (1979) and control serum from age- and sex-matched naive mice for quality control.

For measurements of parasite-specific IgE antibodies, microtitre plates were prepared as above and then incubated with test and control naive and hyperimmune sera (100 μl/well) for 3 days at 4 °C. The plates were then washed and, after addition at 100 μl/well of a rat anti-mouse IgE monoclonal antibody (MCA 419, [1 mg/ml] Serotec) diluted to 1 in 1000, were incubated at 37 °C for 1 h. Biotin-labelled goat anti-rat polyclonal serum (Jackson Immune Research Laboratories) was diluted 1 in 10^4 (v/v) in 2 % BSA–0·05 % Tween–PBS and added to washed plates at 100 μl/well and the plates were incubated for 1 h at 37 °C. The plates were washed again and streptavidin-conjugated horseradish peroxidase (Amersham), diluted 1 in 1000, was dispensed at 100 μl/well. The plates were incubated for 1 h at 37 °C and after further washing, the TMS (3,3′,5,5′-tetramethylbenzidine dihydrochloride, Amersham) and H_2O_2 (1 mg of TMS + 2 μl of 30 % H_2O_2 in 10 ml of citrate buffer, pH 5·0), were added at 100 μl/well. When a blue colour developed, the reactions were stopped by the addition of 25 μl/well of 2·5 M sulphuric acid. OD was read at 410 nm as described above.

Preparation of cell supernatants and measurement of cytokines

Briefly, MLN cell suspensions were prepared from infected and normal mice as described by Else & Grencis (1991). Cells (5×10^6/ml) were stimulated with Con A (Sigma, 5 μg/ml final concentration) and cell-free supernatants were recovered after incubation for 24 and 48 h at 37 °C and 5 % CO_2, aliquoted and stored at −80 °C prior to analysis. Cytokines present in supernatants were measured by sandwich ELISA using methodology essentially as described previously (Else & Grencis, 1991, 1993). The pairs of cytokine-specific monoclonal antibodies used were: IFN-γ, R4-6A2 (E. Havell) and XMG1.2 (T. Mosmann); IL-3, 8F8 and 43D11 (J. Abrams); IL-4, 1D11.2 and 24G.2 (AMS Biotechnology); IL-9, 229.4 and 1C10 (J. van Snick & C. Uyttenhove); IL-10, JES5-2A5 (AMS Biotechnology) and SXC-1 (T. Mosmann). 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, 0·24; IL-4, 0·2; IL-9, 1·1; IL-10, 0·22 units/ml.

Statistical analysis of results

For clarity, data are presented as group mean values ± standard error (S.E.M.). Non-parametric statistical procedures were used to analyse the data sets, because normal distribution of data could not be assumed. Analysis of cytokine, MMCP-1 and antibody concentrations was carried out on optical density (OD) values. In the case of cytokine and MMCP-1 levels this was necessary because some samples had OD readings in excess of the range covered by standards. Since the relationship of OD to actual concentration could be assumed to be positive but not necessarily linear, these values were not converted to exact units/ml and are presented as exceeding the range of standards, i.e. > 200 for IL-3 and IL-4, and > 1000 for IL-9. However, ODs should still show a positive, albeit not necessarily linear, relationship with cytokine concentrations and therefore statistical analyses by ranks were considered legitimate.

In order to avoid Type I errors arising from multiple comparisons within experiments, two *a priori* hypotheses were examined for each parameter measured during the course of infection. The first predicted that infected mice should show higher values for specific parameters than non-infected mice (infected > naive), a prediction which could only be tested legitimately on one day during the course of infection. For this purpose day 14 (Exp. 1) was selected for cytokine measurements, day 12 for

mast cell counts (Exp. 2) and day 42 p.i. for antibody levels (both experiments). A two-way ANOVA (Meddis, 1984) was employed specifying infection (infected *versus* non-infected) and strain (SWR *vs* NIH *vs* CBA [Exp. 1] or SWR versus CBA [Exp. 2]) as the two factors. The second hypothesis predicted that parameters should show the following relationship: SWR > NIH > CBA. In order to test this hypothesis individual values from infected mice were adjusted by subtraction of the mean value derived from the relevant naive control group and a one-way ANOVA (Meddis, 1984) was then employed to test the prediction that SWR > NIH > CBA. In some cases it was necessary to repeat the analysis late in the infection (e.g. day 70 p.i.) in order to test the hypothesis that the order of responsiveness (i.e. SWR > NIH > CBA) was maintained until later time-points in infection. In these cases the cut-off value for statistical significance was lowered accordingly.

For all *a priori* hypotheses examined the test statistic z is given as appropriate. In the event of a hypothesis being rejected (P = not significant [N.S.]), groups were compared by a general one-way ANOVA in order to determine whether there were any significant differences between the groups and the statistic H is given. In Exp. 2, where cells were pooled, levels of cytokines in the supernatants of cells from fast and slow responding strains were compared over time by a simple binary test. Where Exp. 1 had indicated that levels declined or increased, *a priori* hypotheses concerning relationships over time within strains were examined by the Spearman Rank Order Correlation Test and the statistic r_s is given.

Probabilities (P) of 0·05 or less were considered significant. However, multiple analyses could not be avoided in certain cases, and it was necessary to test *a posteriori* hypotheses in Exp. 1, and where these were undertaken, the cut-off value for significance was lowered to $P = 0{\cdot}025$ or 0·01 (depending on the number of tests) in order to avoid Type I errors.

RESULTS

Recovery of worms

The worm burdens recovered from infected mice in Exp. 1 are illustrated in Fig. 1A. The inoculum used showed 90·8% infectivity as assessed by the worm burden of NIH mice in week 2. However, the worm burdens of SWR and CBA mice were unusually low in week 2 (72·2 and 43·3% establishment) and there was an unexpected significant difference between the groups ($H = 10{\cdot}727$, $n = 6$ for all strains, $P = 0{\cdot}005$). However, in week 4, worm burdens were higher in CBA mice and similar to those in NIH mice ($P = 0{\cdot}3$, N.S.), suggesting that the low recovery of worms from CBA mice in week 2 may have been attributable to incomplete recovery of all worms from this strain.

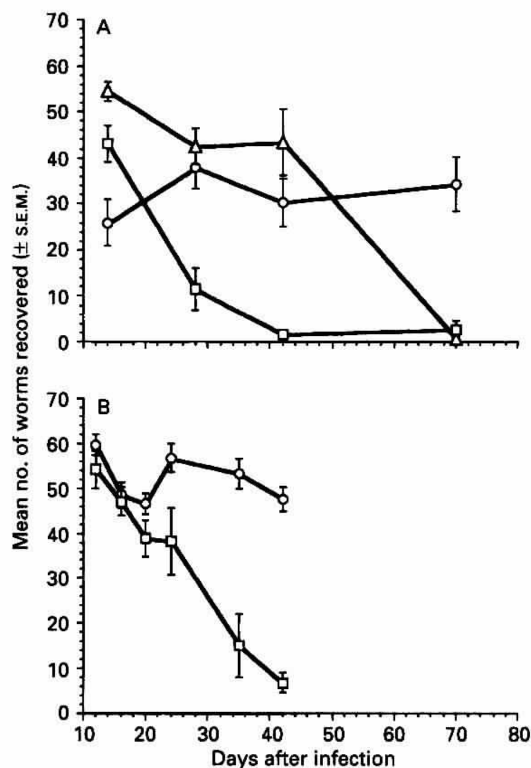

Fig. 1. The course of infection with *Heligmosomoides polygyrus* in fast responder SWR (□), intermediate responder NIH (△) and slow responder CBA (○) mice. For statistical analysis see text. (A) Exp. 1. For CBA, NIH and SWR mice $n = 6$ on all days except day 70 p.i. when for SWR mice $n = 7$. (B) Exp. 2. For CBA and SWR mice $n = 6$ on all days except day 12 when $n = 5$ for each strain, day 16 when $n = 5$ for SWR and day 42 when $n = 7$ for CBA and 5 for SWR.

As expected, SWR mice lost worms rapidly with 73·4% reduction in worm burdens by week 4 and 96·1% by week 6. NIH mice had stable worm burdens until week 6 but then lost 98·7% of their original worm burden by week 10. In contrast there was no loss of worms from CBA mice over the period of this experiment.

As is evident from Fig. 1B, the establishment of worms in Exp. 2 was similar in both strains (CBA, 99·3%; SWR 90·3%, $H = 1{\cdot}32$, P = N.S.). Again there was no change in the worm burdens of CBA mice during 6 weeks following infection: (for *a priori* hypothesis days 12–42 p.i., $r_s = -0{\cdot}176$, $n = 36$, P = N.S.), whereas in SWR mice loss of worms proceeded marginally slower than in Exp. 1 with 72% loss by week 5 and 87·5% loss by week 6: (for days 12–42 p.i., $r_s = -0{\cdot}766$, $n = 33$, $P < 0{\cdot}001$).

Serum parasite-specific antibody responses

Serum IgG1, IgG2a and IgE antibody to ES antigens of *H. polygyrus* were measured in both experiments on day 42 p.i. (Table 1). Specific IgG1 concentrations were higher in infected compared

Table 1. Parasite-specific serum IgG1, IgE and IgG2a antibodies to *Heligmosomoides polygyrus* 42 days after infection

Strain	Group	No. of mice	Mean optical density ± S.E.M. IgG1	IgG2a	IgE
			Experiment 1		
SWR	Infected	6	0·381 ± 0·041	0·055 ± 0·004*	0·633 ± 0·061
SWR	Naive	4	0·029 ± 0·006	0·094 ± 0·009	0·160 ± 0·027
NIH	Infected	6	0·412 ± 0·058	0·047 ± 0·004	0·398 ± 0·061
NIH	Naive	4	0·008 ± 0·002	0·061 ± 0·013	0·131 ± 0·017
CBA	Infected	6	0·122 ± 0·015	0·048 ± 0·003	0·351 ± 0·048
CBA	Naive	4	0·010 ± 0·008	0·083 ± 0·012	0·203 ± 0·024
			Experiment 2		
SWR	Infected	5	0·210 ± 0·024	0·364 ± 0·039	0·698 ± 0·094
SWR	Naive	4	0·026 ± 0·005	0·329 ± 0·053	0·140 ± 0·008
CBA	Infected	7	0·057 ± 0·010	0·266 ± 0·023†	0·414 ± 0·042†
CBA	Naive	4	0·018 ± 0·005	0·237 ± 0·022	0·214 ± 0·009

Statistical analysis For predictions	Infected > Naive z	P	SWR > NIH > CBA z	P
	Experiment 1			
IgG1	4·45	< 0·0001	2·76	< 0·005
IgG2a	−2·99	> 0·05	—	—
IgE	4·45	< 0·0001	3·03	< 0·0025
	Experiment 2			
IgG1	3·36	< 0·001	2·84	< 0·0025
IgG2a	0·74	> 0·05	—	—
IgE	3·63	< 0·001	2·76	< 0·005

* $n = 5$.
† $n = 6$.

with naive mice (Exp. 1, $z = 4{\cdot}45$, $P < 0{\cdot}0001$; Exp. 2, $z = 3{\cdot}36$, $P < 0{\cdot}001$), and the fast (SWR) and intermediate (NIH) responding strains had more antibody than slow responding CBA mice (Exp. 1, $z = 2{\cdot}76$, $P < 0{\cdot}005$; Exp. 2, $z = 2{\cdot}84$, $P < 0{\cdot}0025$). Similarly, there was more specific IgE in infected compared with naive mice (Exp. 1, $z = 4{\cdot}45$, $P < 0{\cdot}0001$; Exp. 2, $z = 3{\cdot}63$, $P < 0{\cdot}001$) and SWR produced a more intense response than the other strains (Exp. 1, $z = 3{\cdot}03$, $P < 0{\cdot}0025$; Exp. 2, $z = 2{\cdot}76$, $P < 0{\cdot}005$). However, the prediction that infection would increase IgG2a levels was not significant (Exp. 1, $z = -2{\cdot}99$, $P > 0{\cdot}05$; Exp. 2, $z = 0{\cdot}74$, $P > 0{\cdot}05$) although in Exp. 1 there was a significant difference between the groups ($H = 14{\cdot}582$, $P = 0{\cdot}012$) suggesting that IgG2a levels were lower among infected mice. In Exp. 2 there was no significant difference in IgG2a between the groups ($H = 4{\cdot}57$, $P = 0{\cdot}206$).

Mastocytosis and serum mast cell protease (MMCP-1) concentrations

In Exp. 1 serum MMCP-1 levels were measured 42 days p.i. and the results are shown in Fig. 2A. Infected groups had higher levels of MMCP-1 than naive controls ($z = 4{\cdot}58$, $P < 0{\cdot}0001$) and among the infected mice the prediction SWR > NIH > CBA was significant ($z = 2{\cdot}16$, $P < 0{\cdot}025$).

In Exp. 2 mastocytotis and serum MMCP-1 concentrations were quantified throughout infection and these results are shown in Fig. 2B and C. SWR mice produced a more rapid and intense mastocytosis than CBA mice, which became evident from day 8 onwards. No mast cells were observed in control naive mice on any of the days when samples were taken from these groups. Peak mastocytosis in infected animals was on day 12 p.i. when infected mice had significantly increased mast cell counts over naive controls ($z = 3{\cdot}22$, $P < 0{\cdot}001$) and SWR mice had significantly more mast cells than CBA mice ($z = 2{\cdot}31$, $P < 0{\cdot}025$). Mastocytosis then declined in both strains but this could only be tested statistically *a posteriori* (days 12–42 p.i. for SWR $r_s = -0{\cdot}862$, $n = 24$, $P < 0{\cdot}001$; for CBA $r_s = -0{\cdot}637$, $n = 24$, $P = 0{\cdot}001$). Declining mastocytosis was evident in SWR mice well before complete loss of worms and in CBA mice, despite the continued presence of worms. SWR mice had higher mast cell counts than CBA mice even 42 days p.i. ($z = 2{\cdot}31$, $P < 0{\cdot}025$) when mastocytosis had almost returned to control levels in the latter strain.

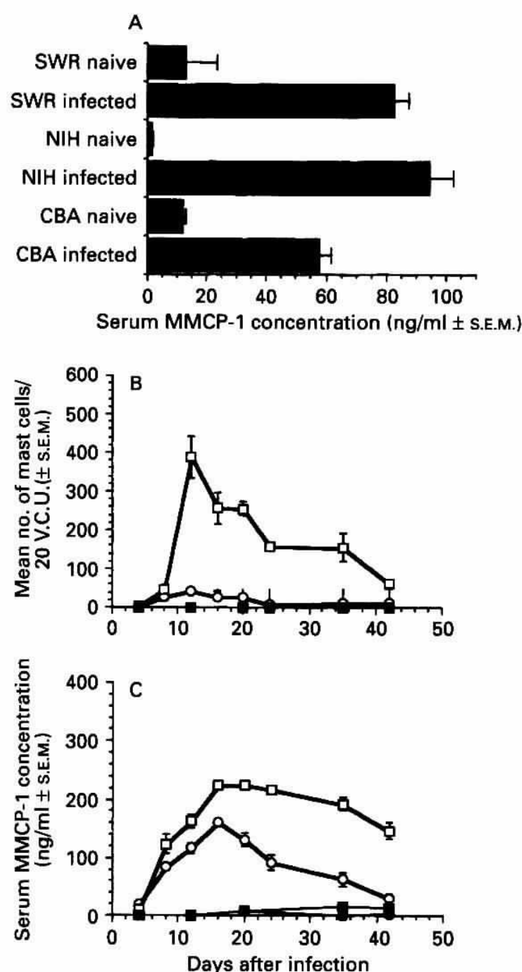

Fig. 2. Mucosal mast cells and serum MMCP-1 levels during the course of infection with *Heligmosomoides polygyrus* in fast and slow responder mouse strains. For statistical analysis see text. (A) Exp. 1. Serum MMCP-1 concentrations on day 42 p.i. For all infected groups $n = 6$, for naive control mice $n = 4$. (B) Exp. 2. Changes in mucosal mast cell numbers during the course of infection. For all groups $n = 4$. V.C.U., villus/crypt units. (C) Exp. 2. Changes in the serum concentrations of MMCP-1. For infected SWR and CBA mice on days 12–42 p.i. n was as in the legend to Fig. 1. In addition 5 infected SWR mice were compared on days 4 and 8 p.i. with 5 and 6 respectively, infected CBA mice. The control naive groups comprised 4 mice on each occasion except on day 35 p.i. when n for SWR mice was 3. (○) CBA infected; (●) CBA naive; (□) SWR infected; (■) SWR naive.

Serum MMCP-1 levels were elevated in infected compared with naive mice on day 12 p.i. ($z = 3{\cdot}56$, $P < 0{\cdot}001$) and throughout the period of observation (day 42 p.i., $z = 3{\cdot}21$, $P < 0{\cdot}001$). SWR mice had higher levels than CBA mice on day 12 ($z = 2{\cdot}2$, $P < 0{\cdot}025$) and on day 42 ($z = 2{\cdot}84$, $P < 0{\cdot}0025$). Peak serum MMCP-1 activity occurred on day 16 p.i. in both strains and declined afterwards (*a posteriori* analyses for days 16–42 p.i. for SWR $r_s = -0{\cdot}782$, $n = 27$, $P < 0{\cdot}001$; for CBA $r_s = -0{\cdot}897$, $n = 31$, $P < 0{\cdot}001$). Moreover, levels in infected CBA mice dropped more rapidly than those in SWR mice: in the period 20–42 days p.i. the percentage decline of MMCP-1 levels in CBA mice over naive controls was 76·8% and in SWR 39·2%.

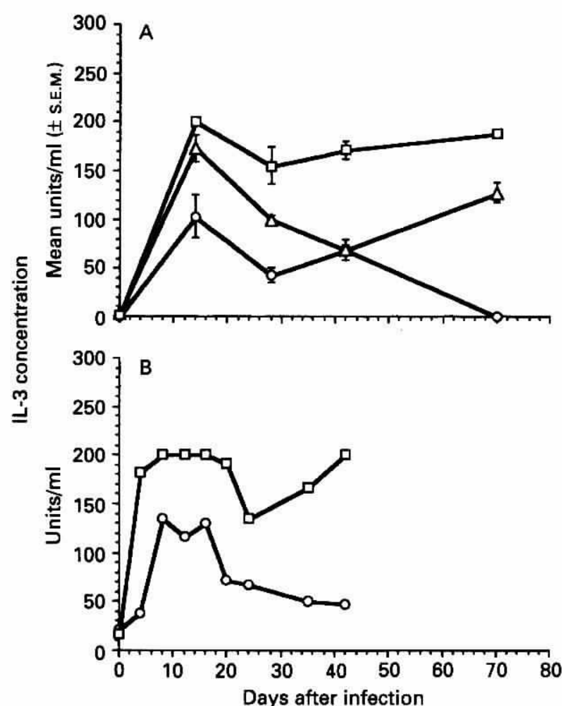

Fig. 3. Concentrations of IL-3 in culture supernatants, secreted by *in vitro* Con A-stimulated MLN cells from infected SWR (□), NIH (△) and CBA (○) during the course of infection with *Heligmosomoides polygyrus*. For n see legends to Figs 1 and 2. For statistical analysis see text. (A) Exp. 1. The levels secreted by cells from uninfected mice of each strain did not exceed 14·7 units on days 14 and 28. On day 70 cells from uninfected CBA cells gave 51 units/ml, SWR cells 99 units/ml while NIH cells gave 0·4 units/ml. (B) Exp. 2. The levels secreted by cells from uninfected CBA mice did not exceed 31 units/ml and SWR mice 18 units/ml.

Secretion of IL-3 by MLN cells

The results of IL-3 assays are shown in Fig. 3. In Exp. 1 MLN cells from all infected groups of mice showed evidence of enhanced IL-3 production *in vitro* during the first 6 weeks of infection as compared to naive controls (day 14 p.i., $z = 4{\cdot}57$, $P < 0{\cdot}0001$) and IL-3 secretion was greater in fast compared with slow responders (for SWR > NIH > CBA, $z = 3{\cdot}19$, $P < 0{\cdot}001$). The overall pattern of secretion was similar for NIH and SWR mice but differed in CBA mice in which secretion of IL-3 declined to naive control levels by day 70 p.i. (*a posteriori* hypothesis for days 14–70 p.i. $r_s = -0{\cdot}664$, $n = 22$, $P = 0{\cdot}001$). However, both infected NIH and SWR mice

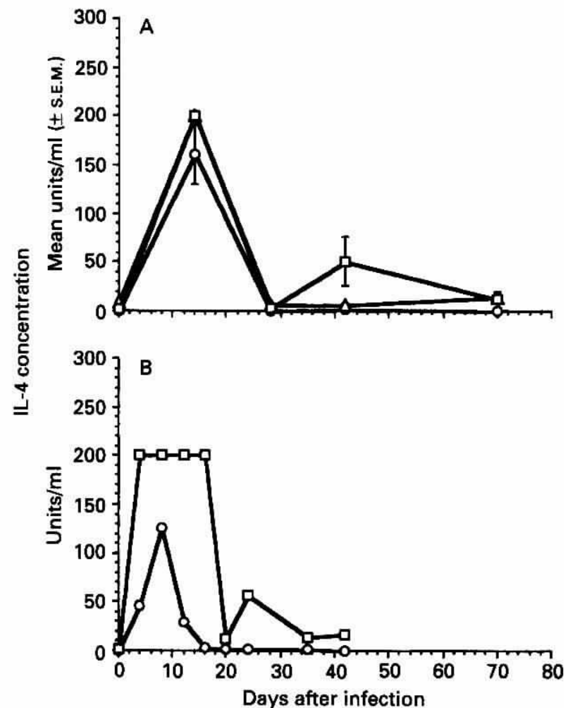

Fig. 4. Concentrations of IL-4 in culture supernatants, secreted by *in vitro* Con A-stimulated MLN cells from infected SWR (□), NIH (△) and CBA (○) during the course of infection with *Heligmosomoides polygyrus*. For *n* see legends to Figs 1 and 2. For statistical analysis see text. (A) Exp. 1. The levels secreted by cells from uninfected mice of each strain did not exceed 9 units/ml. (B) Exp. 2. The levels secreted by cells from uninfected mice of both strains did not exceed 2·5 units/ml.

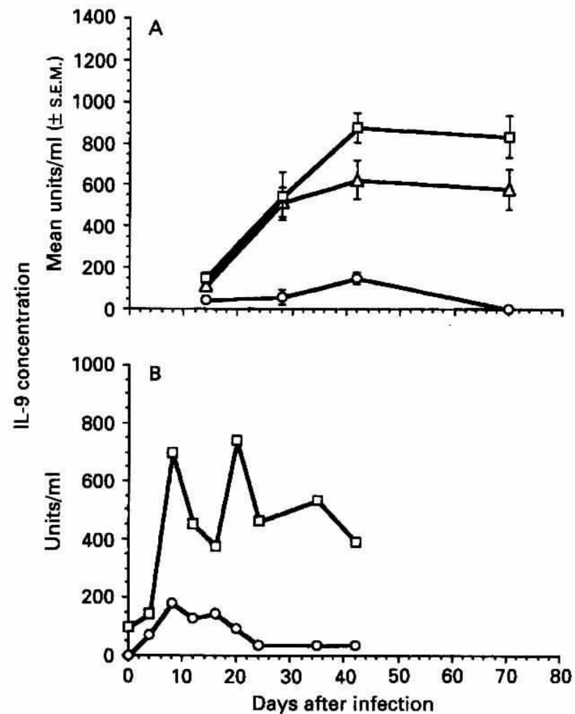

Fig. 5. Concentrations of IL-9 in culture supernatants, secreted by *in vitro* Con A-stimulated MLN cells from infected SWR (□), NIH (△) and CBA (○) during the course of infection with *Heligmosomoides polygyrus*. For *n* see legends to Figs 1 and 2. For statistical analysis see text. (A) Exp. 1. The levels secreted by cells from uninfected CBA mice did not exceed 5 units/ml. Values for cells from naive NIH mice were < 70 units/ml and those for SWR mice < 26 units/ml except on day 70 when 256·8 ± 82·2 units/ml were recorded. (B) Exp. 2. The levels secreted by cells from uninfected CBA mice did not exceed 6·5 units/ml whereas those from SWR mice were below 100 units/ml other than on day 35 when 111·3 units/ml were recorded and day 42 when 185·2 units/ml were recorded.

appeared to show a reduction in secretion in week 4 followed by enhanced secretion in week 10. This apparent recovery in the period 42–70 days p.i. was examined *a posteriori* by comparing the pooled values for IL-3 from days 28 and 42 p.i. against the values for day 70 p.i. in a general one-way ANOVA for each strain. Both showed a significant recovery in ability to secrete IL-3, although the higher probability for SWR has to be interpreted with caution because the test was carried out *a posteriori* (for SWR, $H = 4{\cdot}64$, $P < 0{\cdot}05$; for NIH, $H = 11{\cdot}38$, $P < 0{\cdot}01$).

In Exp. 2, mice were killed earlier (i.e. days 4, 8 and 12 days p.i.), and it is evident that both strains responded within 4 days of infection with elevated levels of IL-3 compared with controls (levels of IL-3 secreted by MLN cells from naive control mice never exceeded 31 units/ml). Although the response of SWR mice was more intense throughout infection (simple binary test for $n = 8$, $P < 0{\cdot}004$) and initially more rapid than that of CBA mice, lower secretion was apparent in weeks 3–4 before recovery and continued secretion at higher levels. As in Exp. 1, CBA initially responded well but did not recover their ability to secrete IL-3 once secretion declined in week 3 (*a priori* hypothesis for CBA days 8–42 p.i. $r_s = -0{\cdot}964$, $n = 7$, $P < 0{\cdot}001$).

Secretion of IL-4 by MLN cells

The levels of IL-4 in supernatants from naive mice of all strains in Exp. 1 were very low throughout the experimental period and never exceeded $8{\cdot}9 \pm 1{\cdot}06$ (NIH mice on day 14 p.i.). In contrast, supernatants taken from cells harvested from all infected mice 14 days p.i. contained significantly elevated levels of IL-4 ($z = 4{\cdot}41$, $P < 0{\cdot}0001$), although the data obtained in Exp. 1 did not yield a significant difference between strains (Fig. 4A, day 14 for specific prediction SWR > NIH > CBA, $z = 0{\cdot}43$, $P <$ N.S.). In Exp. 2 cells from SWR mice again secreted more IL-4 than those from CBA mice throughout infection (Fig. 4B; simple binary test for $n = 8$, $P = 0{\cdot}004$). However, when earlier time-points were examined, marked differences between SWR and

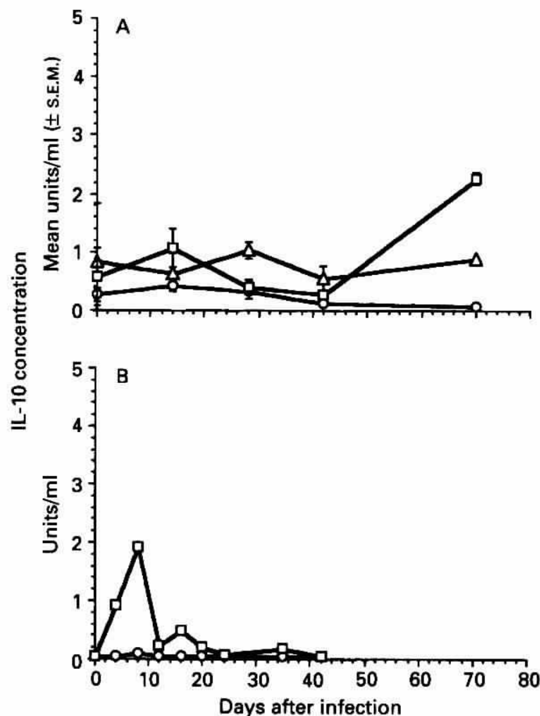

Fig. 6. Concentrations of IL-10 in culture supernatants, secreted by *in vitro* Con A-stimulated MLN cells from infected SWR (□), NIH (△) and CBA (○) during the course of infection with *Heligmosomoides polygyrus*. For *n* see legends to Figs 1 and 2. For statistical analysis see text. (A) Exp. 1. The levels secreted by cells from uninfected CBA mice did not exceed 0·6 units/ml, NIH mice 0·85 units/ml and SWR mice 0·97 units/ml. (B) Exp. 2. The levels secreted by cells from uninfected CBA and SWR mice did not exceed 0·08 units/ml at any time.

CBA mice became apparent, with SWR mice generating a more rapid response of greater magnitude than that evident in CBA mice, with high secretion evident as early as 4 days p.i. This initial period of vigorous IL-4 secretion was followed 20–42 days p.i. by a prominent loss of activity in both experiments, although the values for infected NIH and SWR mice were above the base levels of their respective naive control groups in weeks 6 (Exps 1 and 2) and 10 (Exp. 1).

Secretion of IL-9 by MLN cells

Infection with *H. polygyrus* induced enhanced secretion of IL-9 (Exp. 1, infected versus naive 14 days p.i. $z = 4{\cdot}25$, $P < 0{\cdot}0001$) and the potential to secrete IL-9 was greatest in the fast responder SWR strain and weakest in CBA mice in both experiments (Fig. 5A, day 14 p.i. for SWR > NIH > CBA, $z = 2{\cdot}43$, $P < 0{\cdot}01$; Fig. 5B, simple binary test $P = 0{\cdot}004$). In Exp. 2 SWR mice showed a first peak of activity on day 8 (699·2 units/ml), then a decline of 46·5 % to day 16 and a second peak of activity on day 20 (738·7 units/ml), before a second decline but overall there was no significant reduction from day 8 to day 42 p.i. (for *a priori* hypothesis $r_s = -0{\cdot}286$, $n = 7$, $P = 0{\cdot}535$). In contrast CBA mice showed significantly declining secretion of IL-9 from days 8 to 42 p.i. ($r_s = -0{\cdot}964$, $n = 7$, $P < 0{\cdot}001$). Overall the concentrations of IL-9 in the supernatants of cells from infected SWR mice were very much greater than those of CBA mice with the greatest disparity between the strains after week 2.

Secretion of IL-10 by MLN cells

The secretion of IL-10, is shown in Fig. 6. In Exp. 1, infected mice produced more IL-10 than naive animals on day 14 p.i. ($z = 2{\cdot}19$, $P < 0{\cdot}025$) and SWR mice produced significantly more than CBA mice (for SWR > NIH > CBA, $z = 2{\cdot}33$, $P = 0{\cdot}01$) but subsequently there was little evidence of IL-10 production above that of cells from control naive mice in any of the three mouse strains. The data from Exp. 2 support this conclusion with evidence of an early rise in IL-10 production by cells taken from infected SWR mice but not CBA (Fig. 6B). However, in this experiment, SWR mice secreted more IL-10 in the period 8–35 days p.i. (Fig. 6B, simple binary test for $n = 7$, $P = 0{\cdot}008$) although the levels were only marginally higher than naive SWR and infected CBA mice from day 20 p.i.

DISCUSSION

The data presented in this paper show that mice respond to a primary infection with *H. polygyrus* by activating lymphocytes within MLNs which have the potential to secrete cytokines characteristic of the Th2 subset, but emphasize that there is a marked disparity in the ability to secrete these cytokines between mice from strains representing slow and fast responder phenotypes. Overall, the data from both experiments presented a consistent and clear view of the changes which occurred. Firstly, it is apparent that SWR mice responded very rapidly to infection with *H. polygyrus* and already by days 4–9 p.i. their MLNs had the potential to produce IL-3 and IL-4 at considerably higher levels than comparable cell populations from CBA mice. These results concur with the recent demonstration by Svetic *et al.* (1993), employing the quantitative reverse transcriptase-polymerase chain reaction assay, of raised IL-3, IL-4 and IL-9 RNA within 4 days of infection with *H. polygyrus*. In our study, the levels of IL-3, IL-4 and IL-9 secreted by cells from SWR mice remained higher for longer than those of cells from CBA mice. The latter strain, nevertheless, showed some ability to respond in so far as secretion of IL-3, IL-4 and IL-9, but not IL-10, was elevated in comparison with naive control mice in the first 2 weeks following infection.

Evidence to support the involvement of Th2 cells was also found in the prominent serum parasite-

specific IgG1 and IgE responses of SWR mice and the lower intensity responses of CBA mice, and in the mastocytosis which was evident in both strains. SWR mice, in particular, responded with a prominent mucosal mast cell response, considerably more vigorous than that recorded in CBA mice and even in fast responder SJL mice during primary responses to *H. polygyrus* (Dehlawi *et al.* 1987). The levels of serum MMCP-1 also indicated that SWR mice produced a more prominent response than CBA mice. Further support for the importance of Th2-mediated responses in expulsion of *H. polygyrus* comes from studies in which *in vivo* administration of anti-IL-4 receptor monoclonal antibodies reversed host protective immunity generated by an abbreviated primary infection (Urban *et al.* 1991) and from the demonstration that administration of recombinant IL-4 to mice harbouring a chronic primary infection of *H. polygyrus* resulted in the expulsion of worms from the intestine (F. Finkelman, personal communication), presumably by enhancement of a protective Th2 response. IL-4 is essential for the induction of a variety of Th2-mediated responses *in vivo* following intestinal infections (Kopf *et al.* 1993) and initial activation of the IL-4 gene, is largely dependent on $CD4^+$ cells (Svetic *et al.* 1993). The high levels recorded in both SWR and CBA mice, in this study, show that Th2 cells were activated within 8 days of infection. Neither SWR nor CBA mice produced a significant IgG2a response and their T cells secreted low IFN-γ (data not shown), indicating that Th1 cell involvement was minimal.

The major differences between the fast and slow responder strains of mice were the kinetics and magnitude of the Th2 response: SWR mice mounted a rapid, intense and persistent response whilst CBA mice responded briefly and less vigorously. It is clear from the relative timing of events that the antigenic stimulus for activating the host response was provided by the invasive and tissue-resident larvae of the parasite, a finding which is consistent with earlier reports associating tissue stages with the induction of acquired immunity to *H. polygyrus* (Wahid & Behnke, 1992). Thus the onset of mastocytosis, and secretion of Th2 cytokines in SWR mice, occurred during the first 8 days of infection. In CBA mice these responses were less intense but probably also initiated by L3 and L4 stages. However, Svetic *et al.* (1993) have demonstrated that the early expression of genes for IL-3 and IL-9 is independent of $CD4^+$ cells but is subsequently driven by $CD4^+$ cells and it is possible that differences between the strains early during infection may have arisen as a result of variation in the contribution of these cytokines from non-Th2 sources, yet to be identified.

On the basis of these observations, coupled with the finding that SWR mice produced significant levels of IL-10 early after infection and CBA mice did not (IL-10 induces transcription of the gene for MMCP-1, Ghildyal *et al.* (1992)), it is tempting to suggest that the initiation of a vigorous Th2 response by SWR mice was directly related to their capacity to eliminate adult worms from the intestine. However, closer examination of our data suggests that the explanation may not be so simple. Firstly, mastocytosis began to decline in SWR mice from day 12 p.i. along with worm loss. Reports on other systems emphasize that mast cell numbers peak after and not preceding worm expulsion (Woodbury *et al.* 1984). Secondly, serum MMCP-1 levels did not show a difference between the strains consistent with the marked difference in mast cell counts and overall the serum concentrations were lower than would have been expected from mast cell counts. Hence, mast cell activation may not have been as efficient as in *T. spiralis* where MMCP-1 levels would be recorded in terms of μg/ml (> 8 μg/ml, Tuohy *et al.* 1990) and not ng/ml as in our study. Thirdly, the temporal pattern of cytokine secretion following infection in all strains showed a slight decline in the levels recorded in weeks 2–3 relative to earlier time-points. Following this initial period of instability in secretion, only the fast responder mice recovered the ability to secrete IL-3 and IL-9 and only these strains (SWR and NIH) lost worms promptly, but even here the earlier loss of worms in SWR was associated with more intense secretion and earlier recovery from down-regulation than in NIH mice. In the slow responder CBA mice, once the potential to secrete Th2 cytokines had begun to decline, it continued until virtually all 4 cytokines returned to background levels and this despite the continued presence of worms in the intestine. Neither strain continued to secrete IL-4 or IL-10 after week 3, irrespective of whether worm loss had taken place or not. It is pertinent that declining cytokine secretion and mast cell counts followed soon after the appearance of pre-adult worms in the intestine (from day 8 onward), an observation which is consistent with the known immunomodulatory activities of adult worms aimed at down-regulation of local mucosal immune responses (Behnke, 1987; Behnke, Barnard & Wakelin, 1992; Monroy & Enriquez, 1992). Recent data suggest that this modulation occurs at the level of T cell-derived cytokines, particularly IL-9 and IL-10 (Behnke *et al.* 1993) which contribute to the control of intestinal mastocytosis (Ghildyal *et al.* 1992).

In conclusion we propose that the difference between fast and slow responder mice which may account for their ability/inability to limit primary infections with *H. polygyrus* resides in two contrasting phenomena: first their ability to respond rapidly with Th2 cell activity to antigens provided by the L3 and L4 stages, possibly with a contribution of IL-3 and IL-9 from non-T cell sources and secondly, their relative sensitivity to parasite-

mediated modulation of cytokine secretion. *H. polygyrus* presumably secreted immunomodulatory factors in all hosts, but whilst SWR mice showed some susceptibility (depression of IL-3 and IL-9 in weeks 2–3, low IL-10 levels, low serum MMCP-1 concentrations) they managed to overcome the initial activity of freshly emerging adult worms and succeeded in mounting an intestinal response which removed most parasites from the gut lumen by week 6 p.i. In contrast, although CBA mice responded initially by generating a Th2-dominated response, the magnitude of the response as measured by *in vitro* cytokine production, never matched that of SWR with observed levels decreasing markedly as infection progressed. The precise mechanisms through which *H. polygyrus* modulates the immune system remain to be determined but, in common with other infections, interference appears to operate at the level of cytokine production. Our data clearly show that the capacity to expel the parasite from the host is associated with the potential to mount a strong and persistent Th2 response and that loss of this potential correlates with chronic infection and survival of the parasite.

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