

STIMULI FOR ACQUIRED RESISTANCE TO *HELIGMOSOMOIDES* *POLYGYRUS* FROM INTESTINAL TISSUE RESIDENT L3 AND L4 LARVAE

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Abstract—WAHID F. N. and BEHNKE J. M. 1992. Stimuli for acquired resistance to *Heligmosomoides polygyrus* from intestinal tissue resident L3 and L4 larvae. *International Journal for Parasitology* 22: 699–710. L3 and L4 stages of *H. polygyrus* were prevented from developing further and were probably killed within 24 h of treatment with ivermectin although total parasite burdens, particularly when treatment was given 4–6 days after infection, declined over a longer period lasting several days. Strong resistance to challenge infection was expressed by infected mice dosed with ivermectin during the tissue phase of larval development. Even immunizing infections as brief as 12–36 h (when only L3 larvae would have been present in the mucosa) elicited strong acquired immunity. When infections were terminated 4–6 days after infection, acquired resistance was 95–100%. The stronger resistance of mice exposed to both L3 and L4 stages was associated with the recognition of low molecular weight polypeptides in adult worm homogenate and there was a highly significant correlation between percentage protection and anti-L4/anti-adult worm serum IgG1 antibodies.

INDEX KEY WORDS: *Heligmosomoides polygyrus* (*Nematospiroides dubius*); nematode; abbreviated infections; adult worm homogenate (AH) antigens; arrested development; enzyme-linked immunosorbent assay (ELISA); excretory/secretory (ES) antigens; immunomodulatory factors (IMF); ivermectin; mouse; strains; responder status; third-stage larvae (L3); fourth-stage larvae (L4).

INTRODUCTION

THE longevity of parasitic nematodes in vertebrate hosts is still an enigmatic phenomenon for which there is no conclusive explanation. Both tissue-dwelling species such as the filariae and intestinal species such as hookworms have the capacity to cause chronic infections in man and his domestic animals (reviewed by Behnke, 1987; Behnke, Barnard & Wakelin, in press; Nutman, 1989) and yet their evasive strategies, whilst anticipated on evolutionary considerations *vis-à-vis* the arms race (Behnke & Barnard, 1990), are hardly understood. The possibility that complex immunomodulatory strategies have evolved to downregulate potentially protective host responses has been explored extensively and a variety of mechanisms have been considered (Barriga, 1984; Behnke, 1987; Nutman, Kumaraswami & Ottesen, 1987).

The murine trichostrongylid *Heligmosomoides polygyrus*, a widely investigated model species in this context, is a natural parasite of mice but in several

respects the parasitic life cycle of this worm is unusual. The L3 larvae penetrate through the mucosa and come to lie in the *muscularis externa* where development involving two moults takes place (Bryant, 1973). After 7–8 days the preadults emerge into the intestinal lumen where they reside as adults for up to 10 months post-infection (p.i.) depending on strain of mouse (Ehrenford, 1954; Robinson, Wahid, Behnke & Gilbert, 1989). On primary exposure to the parasite, there is no significant loss of worms during this tissue phase, despite the inflammatory reaction provoked by the developing stages, their excretory/secretory (ES) products and the sheaths left behind after moulting (Liu, 1965; Sukhdeo, O'Grady & Hsu, 1984).

On secondary exposure, these sites of development in the gut tissues become the foci of intense reactions resulting in prominent granulomatous lesions (Prowse, Mitchell, Ey & Jenkin, 1979). Larvae developing in immune mice are usually stunted (Bartlett & Ball, 1974; Sitepu, Dobson & Brindley, 1985), take longer to complete their tissue phase of development (Ey, 1988a), may become arrested (Behnke & Parish, 1979a) and are less fecund as adults (Enriquez,

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Scarpino, Cypess & Wassom, 1988; Kerboeuf, 1985; Sitepu *et al.*, 1985). These manifestations of acquired resistance are all well documented. However, there is still controversy over the stage which finally succumbs to host immunity. Initially it was believed that the intense foci of inflammation surrounding developing larvae killed the parasites *in situ* (Bartlett & Ball, 1974; Chaicumpa, Prowse, Ey & Jenkin, 1977), but it was subsequently shown that numerous worms survived for a very long time in such lesions, possibly returning to the gut lumen to be expelled as preadults (Behnke & Parish, 1979b; Cypess, Lucia, Dunsford & Enriquez, 1988).

There is little argument that the tissue larval stages of *H. polygyrus* provide essential stimuli for the expression of host-protective immunity (Van Zandt, 1961; Jacobson, Brooks & Cypess, 1982), although the relative contribution of L3 and L4 stages growing *in vivo* in the normal site of development has not been evaluated. Moreover, this is complicated by the opposing effect of adult worm immunomodulation which not only prevents host immunity affecting adult worm survival (Jacobson *et al.*, 1982; Robinson, Behnke & Williams, 1988) but also downregulates acquired immunity elicited by juvenile stages (Behnke, Hannah & Pritchard, 1983; Cayzer & Dobson, 1983; Enriquez, Cypess & Wassom, 1988; Pritchard & Behnke, 1985). This has been clearly demonstrated in experiments where infections terminated before the adult phase of infection were shown to generate considerably more potent immunity than those in which the infection was allowed to progress to patency (Behnke & Robinson, 1985; Kerboeuf & Jolivet, 1984).

Although protective immunity is most effectively acquired when exposure is limited to tissue larvae, the exact stages of *H. polygyrus* responsible for stimuli eliciting acquired immunity still remain to be elucidated. Following on from our earlier work, the studies of Enriquez, Cypess & Wassom (1988) and the recent work of Larrick, Semprevivo, Maloney & Tritschler (1991), we report here that infections terminated during the L4 stage provide by far the most immunogenic signals, but unexpectedly L3 larvae are also shown to be capable of eliciting potent host-protective immunity by themselves.

MATERIALS AND METHODS

Mice. The mice used in this study were purchased from Harlan Olac Ltd or were bred in the departmental animal house. All animals were housed under conventional animal house conditions with access to food and water *ad libitum*.

Parasite. The parasite used in this work was originally obtained in 1975 from the Wellcome Research Laboratories (Beckenham, Kent) since when it has been passaged regularly

in CFLP mice. Our strain corresponds to *H. polygyrus bakeri* as reported by Durette-Desset, Kinsella & Forrester (1972) and Behnke, Keymer & Lewis (1991). The methods used for maintenance and infection have been reported previously (Jenkins & Behnke, 1977). Adult parasites were recovered by a 6 h incubation, at 37°C, of small intestines suspended in a gauze, in 50 ml beakers containing Hanks's saline as described by Jenkins & Behnke (1977). Larval stages of the parasite were recovered by separate digestion of intestinal tissues from individual mice in a mixture of pepsin/hydrochloric acid (5 g pepsin (BDH), 5 ml conc. HCl and 500 ml distilled water), in conical flasks maintained in a waterbath at 37°C for 1.5 h. Following incubation the contents were poured through a stainless steel mesh (size 38 µm, Endecott Sieves) held in a separating funnel. The funnel was then rinsed with PBS five times into a beaker and the fluid containing worms was transferred to a 10 cm Petri dish for counting under a dissecting microscope.

Measurement of worms. Worms were fixed in 2.5% formalin and were drawn to scale using a camera lucida arrangement attached to a microscope. The drawings were then measured using a digitizer pad and an IBM computer with a program for conversion of lengths traced into discrete units (courtesy of Dr R. Ramsey). A maximum of 20 females were measured from the pooled worms from each experimental group. When the total recovery was lower all available female worms were measured.

Preparation of antigens. Outbred CFLP mice were infected with 400 L3 of *H. polygyrus* and were killed 6 or 14 days later to provide L4 and adult worms, respectively. L4 stages were isolated by gently scraping off the mucosa and then incubating anterior sections of the small intestine in Hanks's saline at 37°C in gauze bags held over small glass beakers. Adult worms were isolated by opening the small intestine and incubating tissues with gut contents in the same manner. When sufficient numbers of worms had collected, the parasites were washed 10 times in ice cold sterile PBS and were homogenized in a minimal volume of PBS using a glass tissue homogenizer held in an ice bath. The resulting suspension was centrifuged at 10,000g for 1 h at 4°C to remove coarse particulate matter. The supernatant was filtered (0.22 µm filter, Millipore), analysed for protein concentration using a method modified from Lowry, Rosenborough, Farr & Randall (1951), aliquoted and stored at -40°C.

Anthelmintic. Ivermectin (IV) was available as a commercial preparation (Ivomec) which contained 1% w/v of the anthelmintic (MSD, AGVET). A dose of 20 mg kg⁻¹ was obtained by appropriate dilution in distilled water and the resulting fine suspension was injected in a volume of 0.2-0.3 ml within minutes of preparation (Wahid, Behnke & Conway, 1989). Pyrantel embonate (Strongid-P paste, Pfizer) was administered orally to some mice in experiments 7 and 10 at a dose of 100 mg kg⁻¹, which is known to be adequate for removing all adult worms from the intestinal lumen (Behnke & Wakelin, 1977).

Measurement of antibody responses. Specific anti-worm antibodies were measured by a standard ELISA. Briefly, ELISA microtitre plates were coated with 50 µl of worm antigen (5 mg ml⁻¹). Alkaline phosphatase conjugated sheep-

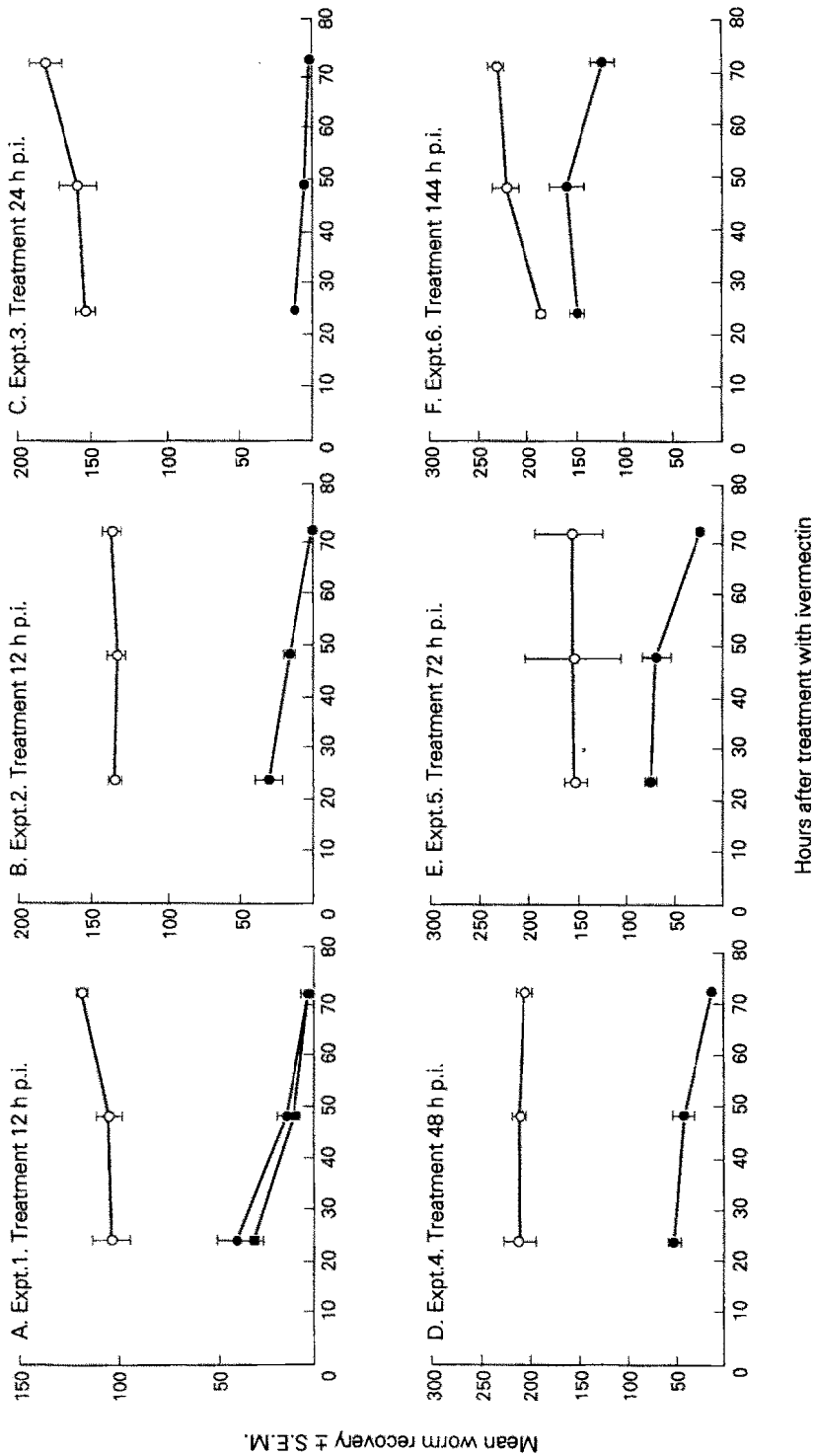


FIG. 1. Experiments 1-6. Survival of *H. polygyrus* during the 3-day period following treatment with ivermectin. A. In Experiment 1, mice were treated orally (■), or subcutaneously (●) with 20 mg kg⁻¹ at 12 h following infection with 150 L3. Control group not treated with ivermectin (○). Mice were killed in groups of three at the times shown for recovery of worms by digestion of the entire small intestine. B-F. Experiments 2-6 in which ivermectin was administered at the time p.i. shown. Female NIH mice were used in all experiments.

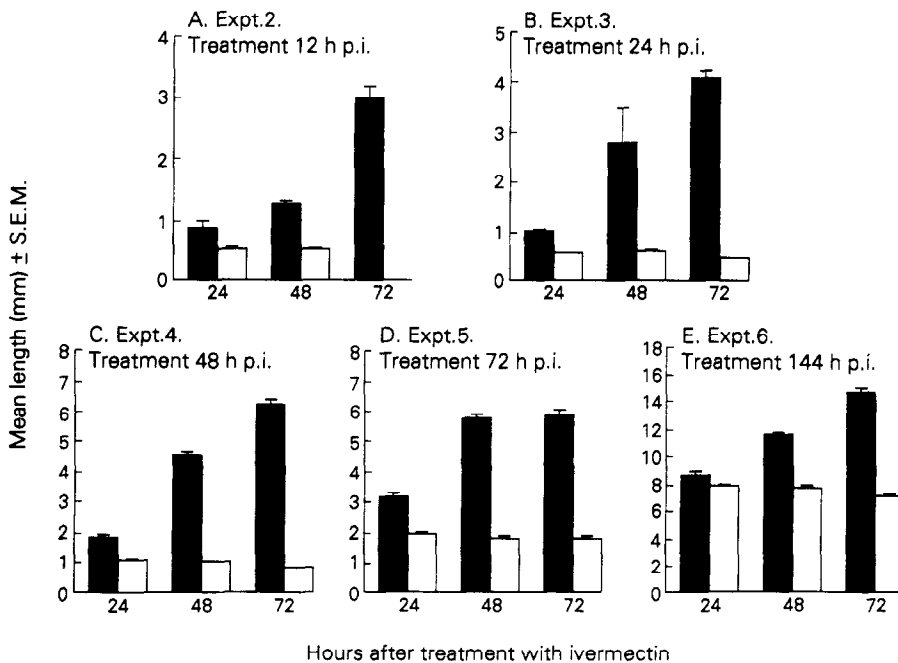


FIG. 2. Experiments 2–6. Growth of female *H. polygyrus* during the 3-day period following treatment with ivermectin. Experimental details as in legend to Fig. 1. Closed bars represent control, untreated groups. Open bars represent groups treated with ivermectin at the times shown. When available 20 female worms were measured at each time point.

anti-mouse IgG (whole molecule, Sigma) or IgG1 (Serotec) were used to measure total or 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 after storage at -40°C . Each plate included control hyperimmune serum (HIM) which was raised as described by Behnke & Parish (1979b) and naive serum and these were used to calculate the relative response index (RRI). Sera were assayed in triplicate and the mean optical density (O.D.) for individual sera was expressed as a percentage of the O.D. obtained with the hyperimmune reference serum, after subtraction of the value for naive control serum from each value. RRIs measured on individual sera on different occasions showed little variation and the results were consistently reproducible. The same stocks of aliquoted HIM and control naive serum were used throughout the study. The spectrum of antigens recognized by antibodies in the sera of infected mice was examined by the Western blotting technique essentially as described by Carr & Pritchard (1987). PBS-soluble adult worm antigen was electrophoretically transferred to nitrocellulose membranes (Schleicher & Scuell) following separation on 5–20% gradient SDS-PAGE. Strips of membrane were blocked with 5% skimmed milk in PBS-Tween 20 (0.05%) and then incubated overnight at 4°C with appropriate test sera (1:100) using 5% skimmed milk in PBS-Tween 20. After washing in PBS-Tween 20 the strips were incubated with ^{125}I -sheep anti-mouse polyvalent immuno-

globulins (18.5 kBq/strip, Amersham) for 2 h at room temperature. Following washing in PBS-Tween 20 (3×20 min) the strips were air dried, fixed to the interior of an X-ray cassette and exposed to an X-ray film (Fuji) at -80°C in the presence of an intensifying screen. The molecular weights were estimated with reference to the migration of standard molecular weight markers.

Statistical analysis of results. The results are presented as group mean values \pm standard error (S.E.M.). Non-parametric statistical procedures were used to analyse the data sets, because of small sample sizes (Sokal & Rohlf, 1969). 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 significant treatment effect. If significant, specific groups were compared to the control group (or as stated) by the Mann-Whitney U test. Correlations between variables were tested by the Spearman Rank Correlation Test and the statistic r_s is given, as appropriate. Probabilities were calculated from statistics tables and are presented as follows: * $P = 0.05$; ** $0.05 > P \geq 0.02$; *** $0.02 > P \geq 0.01$; **** $0.01 > P \geq 0.001$; ***** $P < 0.001$.

RESULTS

The effect of ivermectin (IV) on tissue resident stages of Heligmosomoides polygyrus

A prerequisite for the main objective of this project was certainty that IV would kill or at least prevent

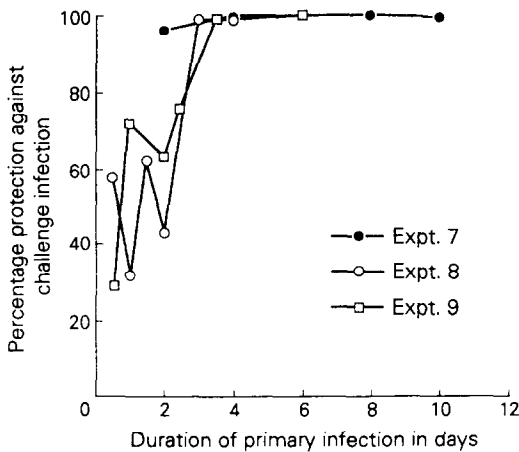


FIG. 3. Experiments 7-9. Acquired immunity to *Heligmosomoides polygyrus*, elicited by primary infections ranging from 12 h to 10 days in duration, in female NIH mice. Details are given in Table 1.

further development of worms, within hours of treatment, otherwise we could not be certain that elicited immunity would be attributable to stimuli from parasite stages resident in host tissues prior to or at the time of treatment. Therefore, it was essential to assess the speed of larvicidal activity of IV after injection, a feature not examined in earlier studies (Wahid *et al.*, 1989).

The results of six experiments, each examining a different period of exposure, are illustrated in Fig. 1. It is evident that in all cases stable worm populations were recovered from control mice over the 3-day period of observation. Furthermore, the numbers of worms recovered in relation to the inocula administered show that recovery by pepsin digestion was efficient and that few worms were missed. Additional, infected but not treated, control groups were killed 3 weeks after each infection and the worms were isolated by the standard Baerman procedure at a time when all the worms could be accurately recovered. These control groups yielded 162, 182, 214, 155 and 199 worms in Experiments 2-6, respectively, thereby confirming that the recovery of worms in the early stages of infection by the digestion technique was accurate and efficient.

By comparison with control groups relatively few worms were recovered from mice treated with IV. When IV was given 12 h after infection the reduction in worm recovery 24 h later (i.e. 1.5 days after infection) was 77.2%. By 48 h it was 87.5% and by 72 h, 99.5%. When older infections were treated the yield of parasites was greater. For example when the 6-day infection was treated (Fig. 1F, Experiment 6 144h), the reduction 24 h later was only 19.7%.

However, it is evident that while worms in control

mice continued developing normally in each case, those in IV-treated mice showed no further growth as adjudged by their size 24-72 h after treatment (Fig. 2). Furthermore careful examination of these specimens showed that in no case did worms from mice treated with IV 12, 24 or 48 h after infection moult to the L4 stage (Fig. 2A-C) and none treated 72 and 144 h p.i. developed into juvenile worms. In all cases the mean length of worms from control groups was longer than from IV-treated groups 24 h after administration of the drug.

One further control was used to confirm that worms in animals treated with IV died rapidly after treatment. In Experiment 6 in which the reduction of worm burden 24 h after treatment was relatively small, additional groups of mice were treated as follows. The intestine was scraped gently and the tissues were then incubated without enzyme in a standard Baerman procedure. The recovery of worms from control mice treated in this way 24, 48 and 72 h after treatment with saline was 118, 183 and 186. Mice dosed with IV yielded 41, 14 and three worms showing that the parasites in treated mice were far less mobile, since this recovery technique depends on the parasites migrating out of the tissues. After incubation the tissues were digested in enzyme and the yields were as follows, control mice 65, 44 and 41, respectively (indicating that the majority had migrated out earlier), whilst those of treated mice were 105, 125 and 128 (indicating that the majority has stayed *in situ*).

Acquired immunity elicited during infections of varying duration, abbreviated during the tissue phase of development

Four experiments, in which ivermectin was administered to groups of mice at varying time points following infection, were carried out to determine the minimal period of infection required to elicit acquired immunity. Experiments 7-10 involved female NIH mice, a primary infection with 250 larvae, followed by ivermectin treatment of relevant groups, a period of rest to allow ivermectin to be metabolized and eliminated by the host, followed by a challenge infection. The exact protocol for each experiment, together with details of minor variations in detail between the experiments and the statistical analysis, are presented in Table 1. For greater ease of interpretation the data from Experiments 7-9 are also expressed in terms of percentage protection in Fig. 3.

In Experiment 7 ivermectin was given 48 and 96 h and 6, 8 and 10 days after infection. Acquired immunity exceeded 95% in all cases with the weakest (96.1%) in the group treated 48 h after exposure. The second experiment in this series (Experiment 8) examined earlier periods of treatment. Again

TABLE 1—ACQUIRED IMMUNITY TO *Heligmosomoides polygyrus* STIMULATED BY PRIMARY INFECTIONS OF VARYING DURATION

Primary infection‡	Treatment hours (h) or days (d) after infection§	Number of worms recovered \pm S.E.M. (n)†			
		Experiment 7	Experiment 8	Experiment 9	Experiment 10
No	None	135 \pm 5.6 (5)	90.7 \pm 2.6 (7)	98.4 \pm 4.2 (5)	79.0 \pm 3.2 (6)
No	10 d	143.4 \pm 17.1 (5)	—	—	—
No	6 d	—	—	94.0 \pm 10.0 (6)	—
Yes	12 h	—	38.3 \pm 15.5 (6) ****	69.6 \pm 16.0 (14)	12.9 \pm 7.5 (14) ****
Yes	24 h	—	61.9 \pm 13.7 (7) **	27.3 \pm 15.3 (11) **	6.9 \pm 5.2 (10) ****
Yes	36 h	—	34.3 \pm 14.5 (6) ****	—	—
Yes	48 h	5.2 \pm 2.3 (6) ****	51.8 \pm 13.7 (5) ****	35.9 \pm 12.0 (8) ****	6.1 \pm 6.0 (10) ****
Yes	60 h	—	—	23.3 \pm 13.7 (8) ****	—
Yes	72 h	—	1.0 \pm 0.6 (6) ****	—	—
Yes	84 h	—	—	1.0 \pm 0.9 (8) ****	0.2 \pm 0.2 (6) ****
Yes	96 h	0 (5) ****	0.8 \pm 0.5 (6) ****	—	—
Yes	6 d	0 (6) ****	—	0 (12) ****	0 (6) ****
Yes	8 d	0.2 \pm 0.2 (6) ****	—	—	—
Yes	10 d	0.5 \pm 0.3 (6) ****	—	—	—
Yes¶	35 d	58.2 \pm 20.8 (6) ****	—	31.7 \pm 11.7 (7) ****	—

† The mice in Experiments 7–9 were female NIH, those in Experiment 10 were female SWR. Challenge infections comprised 150 L3 (Experiment 7), 100 L3 (Experiments 8 and 9) or 120 L3 (Experiment 10) and were given 38, 34, 35 and 35 days after the primary infection, respectively.

‡ The primary infection consisted of 250 L3 in each experiment. Additional groups of mice were killed on day 21 after primary infection to monitor the level of establishment and the numbers of worms recovered were as follows: Experiment 7, 242.5 \pm 16.4 ($n=4$); Experiment 8, 214.7 \pm 8.7 ($n=3$); Experiment 9, 171.0 \pm 9.5 ($n=4$) and Experiment 10, 178.0 \pm 3.4 ($n=4$).

§ Groups of mice were treated at the hours or days shown after primary infection. In each case additional groups of two to three mice (not shown), infected with the primary infection and then treated at the times shown, were killed on day 21 p.i. to confirm the efficacy of ivermectin. Of 62 mice examined across the four experiments only one mouse in Experiment 8 (treated with ivermectin 48 h after infection) carried 126 worms. The remaining mice were all without worms.

|| Control groups for persistence of ivermectin.

¶ These two groups were not treated with ivermectin but were given pyrantel a few days (Experiment 7, day 35; Experiment 9, day 33) before challenge to eliminate adult worms.

Statistical analysis of results

Experiment 7, $H = 39.442$ ($P < 0.001$); Experiment 8, $H = 29.070$ ($P < 0.001$); Experiment 9, $H = 43.196$ ($P > 0.0001$); Experiment 10, $H = 25.259$ ($P = 0.00012$).

Additional comparisons were made using the Mann-Whitney U test and control groups (no primary infection and no anthelmintic treatment) in each case.

**0.05 $> P \geq 0.025$.

****0.01 $> P \geq 0.001$.

treatment with ivermectin after 72 h of infection generated solid resistance to challenge. Earlier treatment produced acquired immunity but variable in degree ranging among individual mice from 0 to 100%. Groups treated 12, 24, 36 and 48 h after infection had variable worm burdens following challenge with curiously fewer worms in the groups treated 12 and 36 h compared with those treated 24 and 48 h after infection, respectively. However, in each case there was a significant reduction in worms relative to controls and we attribute the lack of a consistent pattern to small sample sizes at a time when events are delicately poised *vis-à-vis* appropriate stimulation for acquired immunity. Because of this variation in the 12–48 h period, a third experiment was carried out in which larger sample sizes were employed particularly in groups treated 12 and 24 h after infection (Experiment 9). This time treatment 12 h after infection did not generate significant protection from challenge but treatment at 24 h gave a 72% reduction in worm burden and comparable reduction in groups treated 60 and 72 h after exposure. Overall it can be seen from these data that infections terminated up to 60 h post-infection (p.i.) were surprisingly effective at eliciting acquired immunity in NIH mice, despite some variation among groups and between experiments. In contrast all mice treated with IV after 60 h p.i. showed > 95% protection.

A fourth experiment along the same lines was carried out in SWR mice which expel worms from the fifth week of a primary infection (Table 1, Experiment 10). Accordingly the period to challenge was reduced to 4 weeks on this occasion, to ensure that comparisons could be made before the control mice also began to reject parasites. In this case a 12 h period of exposure was sufficient to elicit 83.7% protection to challenge.

IgG and IgG1 serum antibody responses to infection

Antibody responses were measured in Experiment 9 using adult worm homogenate antigen in ELISA and the results are presented in Fig. 4. Sera taken at autopsy, 5 weeks after challenge, were also analysed for responses to L4 antigens and these results are given in Fig. 5. It is evident that the longer the infection was allowed to progress the more intense an antibody response was elicited. Following challenge the differences between the groups were further exaggerated in both total IgG and IgG1 subclass antibody. Moreover, there was an almost perfect correlation between the intensity of the IgG1 antibody response to L4 and adult worm antigens and the percentage protection at autopsy 5 weeks after challenge infection.

Recognition of parasite antigens

The pattern of recognition of antigens was studied

using the Western blotting technique (data not shown). Four antigens, 123, 62, 46 and 34 kDa were strongly recognized by the control hyperimmune serum. None were seen by control naive serum. Sera from mice exposed to abbreviated infections followed by challenge recognized all four antigens, although the intensity of recognition varied. In general sera from mice exposed to longer periods of primary exposure exhibited stronger recognition of these antigens. Challenge control sera recognized only the 123 and 34 kDa antigens.

An additional three antigens of approximate molecular weight 16, 18 and 20 kDa were recognized by sera from the experimental groups, particularly by mice from group H which had been exposed to a 6-day immunizing infection and showed solid resistance to challenge. These three bands were weakly recognized by control hyperimmune serum and there was also weak reactivity in the challenge control group.

DISCUSSION

Although *H. polygyrus* is considerably more resistant to ivermectin than other parasitic nematodes, doses of 10–20 mg kg⁻¹ are totally effective in eradicating the worms (Wahid *et al.*, 1989). However, before using IV to abbreviate infections at crucial stages of larval development, it was necessary to determine precisely how rapidly the drug exerted its effect on parasites, an aspect not considered in earlier publications. Our data (Fig. 1) show that the recovery of early developmental stages is reduced by over 70% in the 24 h period following treatment. The remaining worms recovered by digestion may have been alive at autopsy, but measurements showed that there was no growth and development after treatment (Fig. 2) and we are therefore confident that mice treated 12–48 h after infection did not experience L4 stages at all. Treatment at 72 and 144 h resulted in a greater proportion of the worm burden being recovered by digestion. These L4 were considerably larger than the earlier stages but again did not grow from the time of treatment (Fig. 2D,E) and were considered to be immobilized and/or dead. Their slower loss was likely attributed to a greater size and the associated difficulties in their complete removal from the *muscularis externa* by the host response, rather than to resistance to IV. We conclude that ivermectin at the dose employed immobilized the worms rapidly, within 24 h, enabling their destruction by a local inflammatory and granulomatous reaction and consequently all the antigenic components of these stages were made available to the host's immune system.

Previous attempts to induce immunity to *H. polygyrus* using infective larvae have had mixed fortunes. Vaccination with dead exsheathed larvae given by

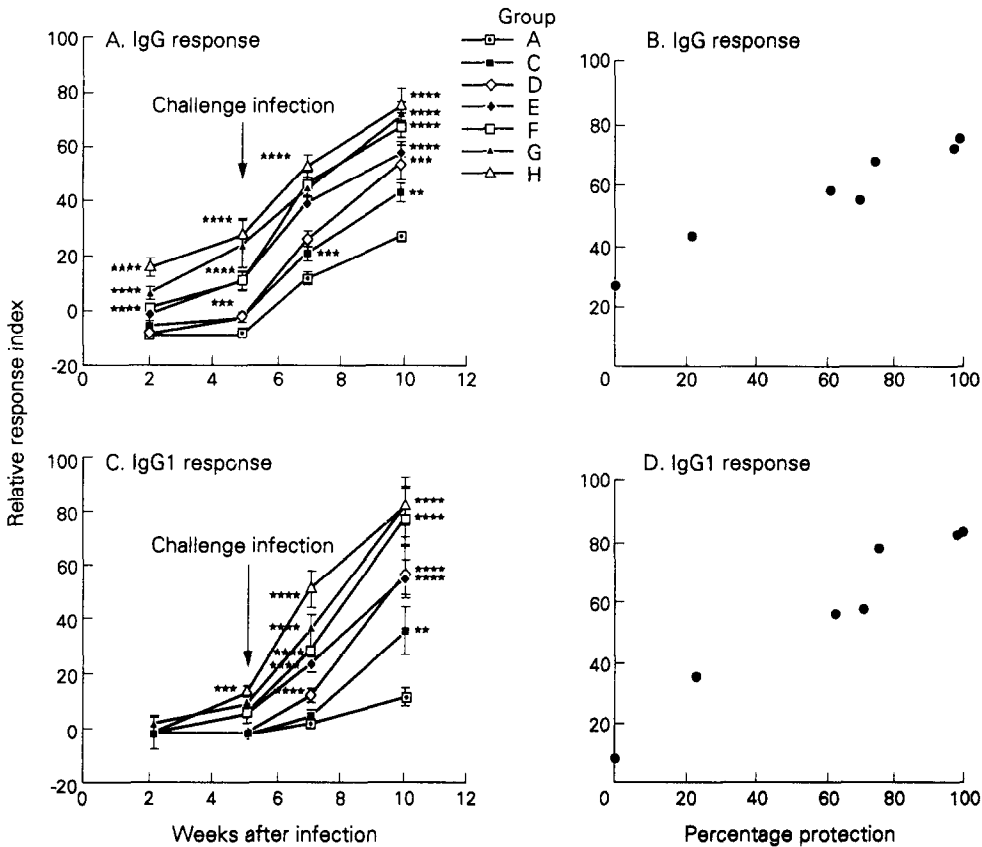


FIG. 4. Experiment 9. IgG and IgG1 antibody responses to adult *Heligmosomoides polygyrus* during the course of primary and challenge infections and the relationship between the intensity of antibody activity and protective immunity. Each group comprised female NIH mice and were treated as follows. A: Challenge infection only. C-H: Primary infection terminated at 12, 24, 48, 60, 84 h and on day 6, respectively with ivermectin, then challenge infection. 4A. IgG response; 4B. IgG response in relation to protection; 4C. IgG1 response; 4D. IgG1 response in relation to protection. *Statistical analysis of results:* A. Week 2, $H = 35.6053$ ($P < 0.0001$); week 5, $H = 34.5333$ ($P < 0.0001$); week 7, $H = 33.8198$ ($P < 0.0001$); week 10, $H = 28.82970$ ($P = 0.0001$). B. $r_s = 0.9643$ ($P = 0.0182$). C. Week 2, $H = 3.6522$ ($P = \text{NS}$); week 5, $H = 30.774$ ($P < 0.0001$); week 7, $H = 35.6092$ ($P < 0.0001$); week 10, $H = 23.9949$ ($P = 0.0005$). D. $r_s = 1.0000$ ($P < 0.002$). Additional comparisons were made using the Mann-Whitney U test and group A as the group for comparison in all cases. The key to symbols used is as follows: ** $0.05 > P \geq 0.025$; *** $0.025 > P \geq 0.01$; **** $0.01 > P \geq 0.001$.

intravenous (Ey, 1988a), intraperitoneal, intramuscular or subcutaneous (Chaicumpa *et al.*, 1977) injection failed to elicit resistance. Likewise subcutaneous injection of live ensheathed larvae (Lueker, Rubin & Andersen, 1968) or an extract of ensheathed larvae (Van Zandt, 1962) proved ineffectual. In contrast potent immunity was elicited by vast doses (4000) of live exsheathed larvae given subcutaneously (and therefore into an abnormal tissue site where development would have been transient, Lueker *et al.*, 1968; Rubin, Lueker, Flom & Andersen, 1971) and, although this protocol was refined later with

smaller doses being equally effective in outbred mice (Hosier, Sackman & Idell, 1974), protection in inbred C57BL and AKR mice never exceeded 30% (Lueker & Hepler, 1975). Acquired immunity was also induced successfully by intravenous injection of live L3 larvae, a proportion of which succeeded in migrating to the mucosa and developed normally. Anthelmintic treatment was given 10 days after the last of one to three injections, allowing in the latter cases some adult worms temporary residence before challenge. After one such inoculation protection was $< 50\%$. However, more recently Larrick *et al.* (1991) showed

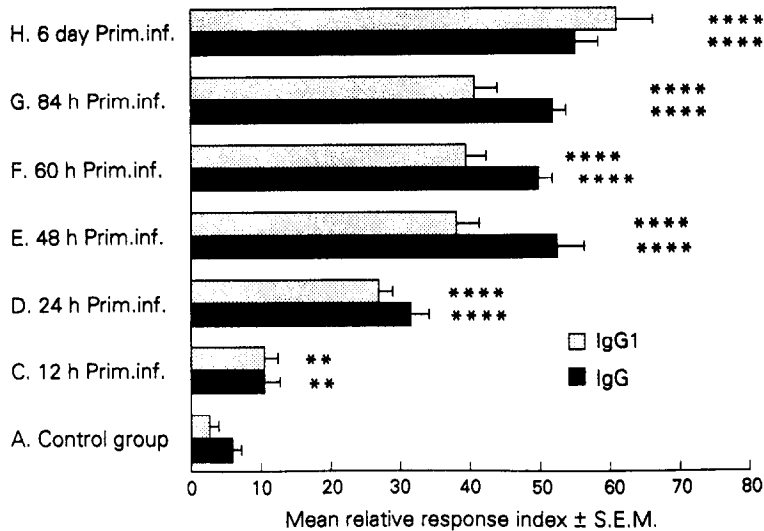


FIG. 5. Experiment 9. IgG and IgG1 antibody responses to L4 larvae of *Heligmosomoides polygyrus* 5 weeks after challenge infection and the relationship between the intensity of antibody activity and protective immunity. Groups as in Fig. 4. *Statistical analysis of results*: IgG response in week 5, $H = 38.8579$ ($P < 0.0001$). IgG1 response in week 5, $H = 41.4152$ ($P < 0.0001$). Additional comparisons were made using the Mann-Whitney U test and group A as the comparative group in all cases. The key to symbols used is as follows: ** $0.05 > P \geq 0.025$; **** $0.01 > P \geq 0.001$. Intensity of the IgG response vs percentage protection in week 5: $r_s = 0.7857$ ($P = 0.0543$). Intensity of the IgG1 response vs percentage protection in week 5: $r_s = 0.9643$ ($P = 0.0182$).

that subcutaneous injections of L3 (isolated on day 2) and L4 induced acquired immunity to *H. polygyrus* in BALB/c mice. Our results confirm and extend their findings, showing for the first time that L3 stages *in situ* in the intestinal mucosa provide key antigenic stimuli for acquired resistance in responder mouse strains.

During the first 12–24 h period of infection, mice would have experienced little else apart from the invasive L3 stages, in the mucosa and submucosa (Sukhdeo *et al.*, 1984). The worms do not reach the *muscularis externa* until 1–3 days after infection and the moult to the L4 occurs 90–94 h p.i., although signs of development of L4 features become apparent from 60 h (Bryant, 1973). Clearly, on the basis of our results, these early invasive stages are potentially immunogenic even during the first 36 h period of infection.

It is perhaps pertinent that mice vaccinated with L3 larvae developed intense antibody responses to a common somatic antigen of L3 and adult worms and to cuticular antigens of invasive larvae but did not show anti-L3-ES antibody activity (Ey, 1988a), and whilst we did not test sera for anti-L3-ES activity, it is probable that our immunizing protocol was superior in this respect. Indeed immunization with repeated doses of L3-ES products eventually induced detectable

resistance to challenge, but overall percentage protection was still poorer than in our experiments with 24 h periods of exposure to infection (Ey, 1988a). Furthermore, whereas vaccination with live 2-day p.i. L3 generated 60% protection (Larrick *et al.*, 1991), our SJL mice showed 83.7 and 91.2% protection when primary exposure was restricted by IV treatment 12 and 24 h after infection, respectively. Exsheathed larvae secrete essentially a single dominant molecule of 23 kDa during the first 10 h of culture and in relatively small amounts (< 0.2 ng per larva per day). It is not until later that additional factors appear in ES products (Ey, 1988b). If the same holds true for worms *in vivo*, this 23 kDa antigen is likely to be the source of host-protective immunity. All the other L3 antigens were presumably made available to mice when dead larvae were used without success in immunization (Ey, 1988a).

The strongest resistance to challenge was acquired when primary infections were terminated 4–6 days after infection at a time corresponding almost exactly to the L4 phase of infection, the preadult moult occurring 144–166 h p.i. (Bryant, 1973). We are therefore in total agreement with Larrick *et al.* (1991) that L4 stages of *H. polygyrus* are potentially

immunogenic, but on the basis of our experiments we cannot separate the contribution of L4 stages from that provided by the earlier L3. Larrick *et al.* (1991) showed that L4 stages of *H. polygyrus* induce protective immunity in isolation from L3 stages, although their experimental procedure involved subcutaneous rather than mucosal presentation of the antigens. Taken altogether, our studies establish that both larval stages release highly immunogenic antigens which elicit potent acquired immunity. This extraordinary immunogenicity of larval stages has to be contrasted with the prolonged survival of adult worms on primary exposure when patent infections last for more than 20 weeks in NIH mice (Robinson *et al.*, 1989). Although immunizing infections in which larvae were allowed to progress to the patent adult stage were only briefly considered here (Experiments 7 and 9), it is evident from these that once adult worms developed, acquired immunity was significantly weakened confirming earlier reports (Behnke & Robinson, 1985; Dobson, Sitepu & Brindley, 1985; Enriquez, Cypess & Wassom, 1988; Kerboeuf & Jolivet, 1984). The immunodepressive properties of adult parasites are believed to inhibit the acquisition of acquired resistance (Behnke *et al.*, 1983; Pritchard & Behnke, 1985) and exposure, limited to larval stages free from this influence, generated potent acquired resistance.

The greater resistance of mice experiencing a 6-day immunization was associated with a more intense antibody response to L4 and adult worm antigens (both IgG and IgG1). Indeed there was an almost perfect correlation between the intensity of the IgG1 antibody response and the percentage protection 5 weeks after challenge infection, supporting the role of IgG1 antibodies in mediating resistance to *H. polygyrus* (see Pritchard, Williams, Behnke & Lee, 1983; Williams & Behnke, 1983). The intensity of the antibody response to adult worm antigens was surprising since the mice would not have experienced antigens unique to adult worms during the priming infections. A significantly more intense secondary response to adult worms was reported by Jacobson *et al.* (1982), but these authors used a 16-day priming infection, during which mice would have experienced adult worm antigens. Several antigens in homogenates of adult and larval stages share identical molecular weights (e.g. molecule(s) at 65 kDa) suggesting that there may be common epitopes on the different development stages of the worm (Pritchard, Maizels, Behnke & Appleby, 1984; Pritchard & Carr, 1987). However, stage-specific antigens have also been recognized (Adams, Monroy, East & Dobson, 1987; Adams, East, Monroy, Washington & Dobson, 1987) including a 16 kDa molecule on the cuticular surface of L4 (Pritchard *et al.*, 1984). In the present study mice

would have been sensitized only to L3 and L4 antigens depending on the duration of the priming infection and indeed, 2 weeks after exposure as well as on the day of challenge, groups experiencing a 12 and 24 h immunizing infection had antibody titres only marginally higher than naive mice. Then depending on the intensity of the secondary response, adult stages would have been experienced transiently and it was at this stage that anti-adult worm antibody titres rose significantly above control groups (Fig. 4).

The strong recognition of low molecular weight adult worm antigens may be particularly significant. Antigens of 17, 18 and 20 kDa were immunoprecipitated by purified IgG1 from hyperimmune sera, demonstrably effective in passive transfer (Pritchard *et al.*, 1983). In the present study these antigens were also recognized by the control hyperimmune serum, as expected, but there was considerably more reactivity in sera from mice sensitized by 4-6 day abbreviated infections, despite the fact that their overall reactivity against adult worm homogenate was less intense than that of HIM. Thus exposure to L4 stages, which possess antigens with comparable molecular weights although not necessarily the same molecules (Pritchard *et al.*, 1984), led to preferential stimulation of a response against these antigens, indicating that some are indeed likely to be identical or possess common epitopes (Pritchard & Carr, 1987).

Monroy, Dobson & Adams (1989) have suggested that the low molecular weight antigens (< 20 kDa) of adult worms represent the elusive immunomodulatory factors (IMF) of the parasite. However, if adult and L4 stages share low molecular weight factors, the potent immunogenicity of the larval stages in comparison to the relatively poor immunogenicity of adult worms (Jacobson *et al.*, 1982) poses an intriguing paradox. One explanation may be that larval immunogens enter the circulation eliciting immunity whilst their IMFs keep cellular effectors at bay, protecting the larvae locally in their sites of development. In support of the latter hypothesis, 6-day old L4 stages have been found to secrete < 14 kDa IMF (Losson, Lloyd & Soulsby, 1985). Furthermore, larval IMFs may explain the resilience of tissue stages in the inflamed intestines of sensitized mice where development is slowed (Ey, 1988a) and despite the vigorous granulomatous reactions around them (Jones & Rubin, 1974), many worms survive to return to the gut lumen as stunted preadults (Ey, 1988a) from which the majority are lost soon after arrival. Perhaps experience of dying L4 stages and their IMFs enables the host to resist larval and adult worm immunomodulation on secondary exposure, facilitating a sufficiently intense local inflammatory response to damage the worms before their evasive strategies

become fully effective, with consequent slower growth and loss from the gut lumen on completion of development.

Finally, our experiments have demonstrated unequivocally that the early developmental stage of *H. polygyrus*, the parasitic L3, provides important stimuli for acquired resistance in responder strains of mice. Acquired resistance was enhanced when mice also experienced L4 stages and therefore protective immunity to *H. polygyrus* may be evoked by responses to antigen sets from both L3 and L4 stages. The crucial issues to tackle next will be to confirm the identification of the L3 antigens involved (*vis-à-vis* Ey's 23 kDa antigen) and to determine whether the expression of acquired immunity in mice which have experienced only L3 stages is mediated by similar effector mechanisms to those known to act against L4 and preadult parasites or whether a novel combination is involved.

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