Requirements for in vivo IFN- induction by live microfilariae of the parasitic nematode, Brugia malayi

Requirements for *in vivo* IFN-γ induction by live microfilariae of the parasitic nematode, *Brugia malayi*

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SUMMARY

Lymphatic filariasis caused by the parasitic nematode, *Brugia malayi*, is a chronic human disease immunologically characterized by stimulation of Th2 cells and reduced antigen-specific T cell responses. Single stage intra-peritoneal infections with infective larvae (L3) or adult nematodes induce Th2 cells, while the microfilarial stage (Mf) stimulates IFN-γ and Mf-specific IgG1, IgG2a, IgG2b, IgG3 and IgM, but not IgE. To investigate whether IFN-γ is elicited by live Mf in their natural site of infection, mice were infected intravenously. Intravenous infection had a striking effect on the response to Mf and high levels of IgE were induced even in the presence of IFN-γ. Indeed IgE levels to Mf increased markedly with the number of immunizations, higher doses of Mf and prolonged exposure to Mf suggesting that under conditions of chronic antigen exposure, typical of human disease, Mf will stimulate high levels of IgE. The ability of Mf-induced IFN-γ to modulate or regulate a pre-existing Th2 response, was investigated by infecting mice initially with adult male worms to induce a Th2 response, followed 14 days later by infection with Mf. Although Mf stimulated IFN-γ in the presence of male adults, the antibody isotypes elicited did not reflect IFN-γ induction and IgG1and IgE dominated the response. Although it cannot be discounted that IFN-γ induction by Mf may act locally as an inflammatory mediator or modulator of Th2 cells, these data suggest that Mf-stimulated IFN-γ does not have a profound effect overall on progression of the Th2-dominated immune response to filarial infection.

Key words: *Brugia malayi*, filariasis, IFN-γ, microfilariae, antibody, isotype.

INTRODUCTION

Human lymphatic filariasis caused by the filarial nematodes, *Wuchereria bancrofti* and *Brugia malayi*, induces prominent Th2 cell responses in infected individuals. In a mouse model of infection we have shown that both infective-stage larvae (L3) and adult worms generate Th2 cells and that the female adult worm, in particular, is a profound IL-4 stimulant (Lawrence *et al*. 1994). Interestingly, single-stage infections with microfilariae (Mf) of the parasite induce significant levels of the inflammatory cytokine IFN-γ. In single-sex adult female infections Mf are continually produced, however, IFN-γ is only induced in the absence of host IL-4 (Lawrence *et al*. 1995). These data imply that in filarial disease ontogeny a Th2 response is rapidly established to the invasive L3 and later to the adult worm. In the environment of this already Th2-polarized response, the subsequent appearance of Mf in the bloodstream, at about 60 days p.i., may not allow modulation by IFN-γ. However, it is well known that in human filarial disease, individuals with active infection have a high IgG4:IgE ratio, while individuals with chronic pathology who are generally Mf negative have a low IgG4:IgE ratio (Kurniawan *et al*. 1993). The immunoglobulin class switch to both IgG4 and IgE in humans is regulated by IL-4 and IFN-γ can inhibit IgE but not IgG4 switching (Ishizaka *et al*. 1990). Thus it is an interesting possibility that modulation of the immune response by Mf-induced IFN-γ may be involved in the differential regulation of IgG4 and IgE in active and chronic filarial infection.

Microfilariae are the major cause of pathology in onchocerciasis. Mf have also been strongly implicated in the hypo-responsiveness to parasite antigens of T cells taken from filarial patients, in both lymphatic filariasis and onchocerciasis. Therefore a greater understanding of the responses elicited by Mf is essential. At present it is known that live Mf inoculation i.p. induces high levels of IFN-γ in the first 21 days p.i., and thereafter, there appears to be an upregulation of Th2 cell responses (Pearlman *et al*. 1993*a*; Lawrence *et al*. 1994). In addition multiple immunizations with Mf extract induce IL-5 and IgE, in combination with IFN-γ production

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(Pearlman *et al*. 1993*c*). However, it is not clear from previous work whether it is the chronicity of infection with live Mf that leads to Th2 cell upregulation. Indeed an alternative explanation is that exposure of the host to somatic Mf antigens (ag) in Mf extract may lead to Th2 responses and the late Th2 response seen in live Mf infections maybe related to responses against antigen release from dead or dying Mf. Therefore, to clarify the immune response elicited by Mf during natural infection, we analysed a variety of conditions which may affect the outcome of the immune response to the live Mf. We investigated the influence of (i) tissue location of live Mf, (ii) exposure to dead Mf, (iii) chronicity of exposure in terms of dose, length of exposure and the number of times live Mf were seen and (iv) prior exposure to a Th2-driving parasite stage. We showed that although Mf drive profound IFN-γ responses initially in infection, in the context of natural infection, Mf may not modulate the overwhelming Th2 response. Indeed, chronic exposure to live intact Mf may potentiate Th2 cell responses.

MATERIALS AND METHODS

Parasites

Brugia malayi-infected jirds were obtained from TRS Labs (Athens, GA). Male and female worms and Mf were isolated from the peritoneal cavity as previously described (Lawrence *et al*. 1994). Killed parasites were prepared by repeated freeze/thawing until the worms were motionless.

Mice

Six-week-old male BALB/c or CBA/Ca mice were used in all experiments. Mf were injected either in the peritoneal cavity or intravenously in the tail vein of mice using a 27-gauge needle. In i.v. experiments the number of Mf were counted in 20–50 μl of blood at varying time intervals p.i. In some experiments 20 adult male worms were surgically implanted into the peritoneal cavity. At autopsy sera were collected and the spleens removed for use in cellular assays. In some experiments tracheo-bronchial lymph nodes were also used in cellular assays.

Antigen

Somatic extracts of Mf or adult *B. malayi* worms of mixed sex were prepared by homogenization in PBS as described previously (Lawrence *et al*. 1994).

Antigen-specific immunoglobulin isotypes

Measurement of parasite-specific immunoglobulin isotype levels has been described previously (Lawrence *et al*. 1994). Briefly, plates were coated with 1 μg/ml of *B. malayi* extract diluted in carbonate buffer (pH 9·6). The plates were blocked with 10% fetal calf serum (FCS). Serum samples were diluted 1 in 100 in PBS/0·05% Tween 20 and incubated for 2 h at 37 °C. Peroxidase-conjugated goat anti-IgG1 (50 ng/ml; Southern Biotechnology Associates Inc. 1070-05), anti-IgG2a (100 ng/ml; SBA 1080-05), anti-IgG2b (100 ng/ml; SBA 1090-05) or anti-IgG3 (400 ng/ml; SBA 1100-05) were diluted in PBS/0·05% Tween 20 to the concentration indicated and incubated for 45 min at 37 °C. After each incubation the plate was washed 4 times in PBS/0·05% Tween 20. Finally, ABTS substrate (KPL Biotechnology) was added. The level of total serum IgE was measured by capture ELISA as previously described (Lawrence *et al*. 1994).

Proliferation assays

Splenocytes or tracheo-bronchial lymph node (TBLN) cells for proliferation assays were cultured in RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (4 mM) plus 10% FCS. Spleens and TBLN were teased apart and erythrocytes lysed in 0·84% NH_4Cl. Whole spleen or TBLN cells from individual mice were incubated at 2×10^6 cells/ml in 100 μl triplicate cultures and proliferation was assessed by the addition of 1 μCi of [^{3}H]thymidine for the last 6 h of a 72 h incubation. TBLN cells from control mice were pooled to provide enough cells for 2×10^6 cells/ml, infected mice were generally treated individually; however, in some cases cells from 2 individual mice were pooled. The optimal concentration of *B. malayi* extract was 10 μg/ml. Supernatants from proliferation assays were collected at 66 h in culture for use in cytokine assays.

Cytokine assays

All cytokine assays have been previously described (Lawrence *et al*. 1994). IL-2 and IL-4 were measured using the NK cell line (Swain *et al*. 1981). Proliferation of NK cells at 10^4 cells/well in the presence of 11B11 anti-IL-4 antibody (ATCC) at 2 μg/ml was used to measure IL-2, while IL-4 was measured in the presence of 1 μg/ml S4B6 anti-IL-2 (AMS Biotechnology). The assay detected $> 0{\cdot}25$ U/ml of IL-2 and > 1 U/ml of IL-4.

IFN-γ was measured by ELISA on plates coated with 50 μl of R46A2 (ATCC) anti-IFN-γ (2 μg/ml) diluted in 0·06 M carbonate buffer. The plate was blocked for 1 h at 37 °C with 200 μl of 10% FCS in carbonate buffer. Samples diluted 1 to 2 in PBS/0·05% Tween 20 were incubated for 4 h at 37 °C. Polyclonal biotinylated rabbit anti-IFN-γ (1 μg/ml) was incubated for 1 h at 37 °C followed by streptavidin peroxidase (Sigma) 1 μg/ml for 30 min at

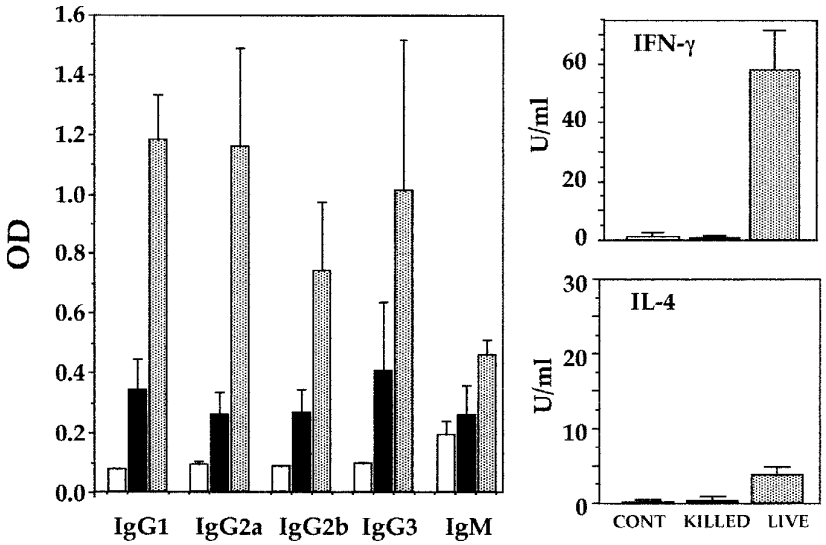

Fig. 1. Mf-specific immunoglobulin responses and splenocyte cytokine responses in BALB/c mice infected i.p. with $2{\cdot}5 \times 10^5$ live Mf or $2{\cdot}5 \times 10^5$ freeze-killed Mf. Sera and splenocytes were taken at 14 days p.i. from control mice (□), mice infected with killed Mf (■) and mice infected with live Mf (▨). Immunoglobulin levels were measured by ELISA using plates coated with Mf extract. Splenocytes were recovered from individual mice and restimulated *in vitro* with 10 μg/ml Mf extract for 66 h. Supernatants were tested for IL-4 and IFN-γ by bioassay and ELISA respectively. Results are expressed as the mean ± S.D. of 4 individual mice.

37 °C. After each incubation the plate was washed 3 times in PBS/0·05% Tween, finally ABTS substrate (KPL Biotechnology) was added. The ELISA detected IFN-γ at concentrations > 1 U/ml.

IL-5 was also measured by ELISA. Antibodies TRFK 5 and TRFK 4 (AMS Biotechnology) were used, respectively, as capture (2 μg/ml) and detection (2 μg/ml) antibodies for IL-5. These antibodies detected IL-5 at a concentration of 0·1 ng/ml. Other aspects of the ELISA were the same as for the IFN-γ ELISA. In each lymphokine assay a dilution series of recombinant IL-2, IL-4, IL-5 or IFN-γ (Genzyme) was run in order to compute a standard curve for each lymphokine in Units/ml or ng/ml. From the resulting calibration, test sample dilutions that fell within the linear part of the curve were converted to Units/ml or ng/ml.

Statistical analysis

Statistical differences between groups were compared using the Mann–Whitney U-test where $P < 0{\cdot}05$ was considered significant.

RESULTS

Induction of IFN-γ by live microfilariae but not by dead microfilariae or microfilarial extract

We have previously shown that an i.p. infection of live Mf primarily induces IFN-γ-driven Th1 induction with the production of Mf-specific antibody of the isotypes IgG1, IgG2a, IgG2b, IgG3 and IgM but not IgE (Lawrence *et al.* 1994). In addition, we and others have observed that 21 days post-Mf infection there is a rise in the Th2 cytokines IL-4 and IL-5 in response to Mf (Pearlman *et al.* 1993*a*; Lawrence *et al.* 1994). As Mf survive for a limited period of time in BALB/c mice the change in Th response could be due to dead/dying nematodes. To investigate whether dead Mf can elicit a qualitatively different response from live Mf we compared the response of BALB/c mice inoculated with live parasites to those inoculated with killed parasites. In addition, analysis of the response to dead Mf enabled investigation of the role of excretory/secretory (E–S) antigens in response induction, as dead parasites are intact but do not secrete E–S proteins.

Both live and killed Mf induced parasite-specific IgM, IgG1, IgG2a, IgG2b and IgG3 responses by 14 days p.i. (Fig. 1). Neither live nor dead Mf injected i.p. induced IgE production. Live parasites were more efficient at inducing all classes of IgG antibody. Although killed parasites induced antibody production there was little cytokine response by splenocytes restimulated with extract *in vitro* at this time-point p.i. In contrast, as we have previously observed (Lawrence *et al.* 1994), live parasites induced high levels of IFN-γ (56 U/ml) but low levels of IL-4 (3·5 U/ml) at 14 days p.i. (Fig. 1). (In a repeat experiment similar results were obtained.) The qualitative similarity of antibody isotype profiles, between groups of mice infected with live and killed Mf indicates that dead Mf *per se* do not stimulate a Th2 response although we cannot eliminate the possibility that dying Mf may stimulate the late Th2 response. At days 14 and days 21 p.i., no killed Mf could be recovered from the peritoneal cavity of mice while live Mf could be readily recovered. Thus, although the results could indicate

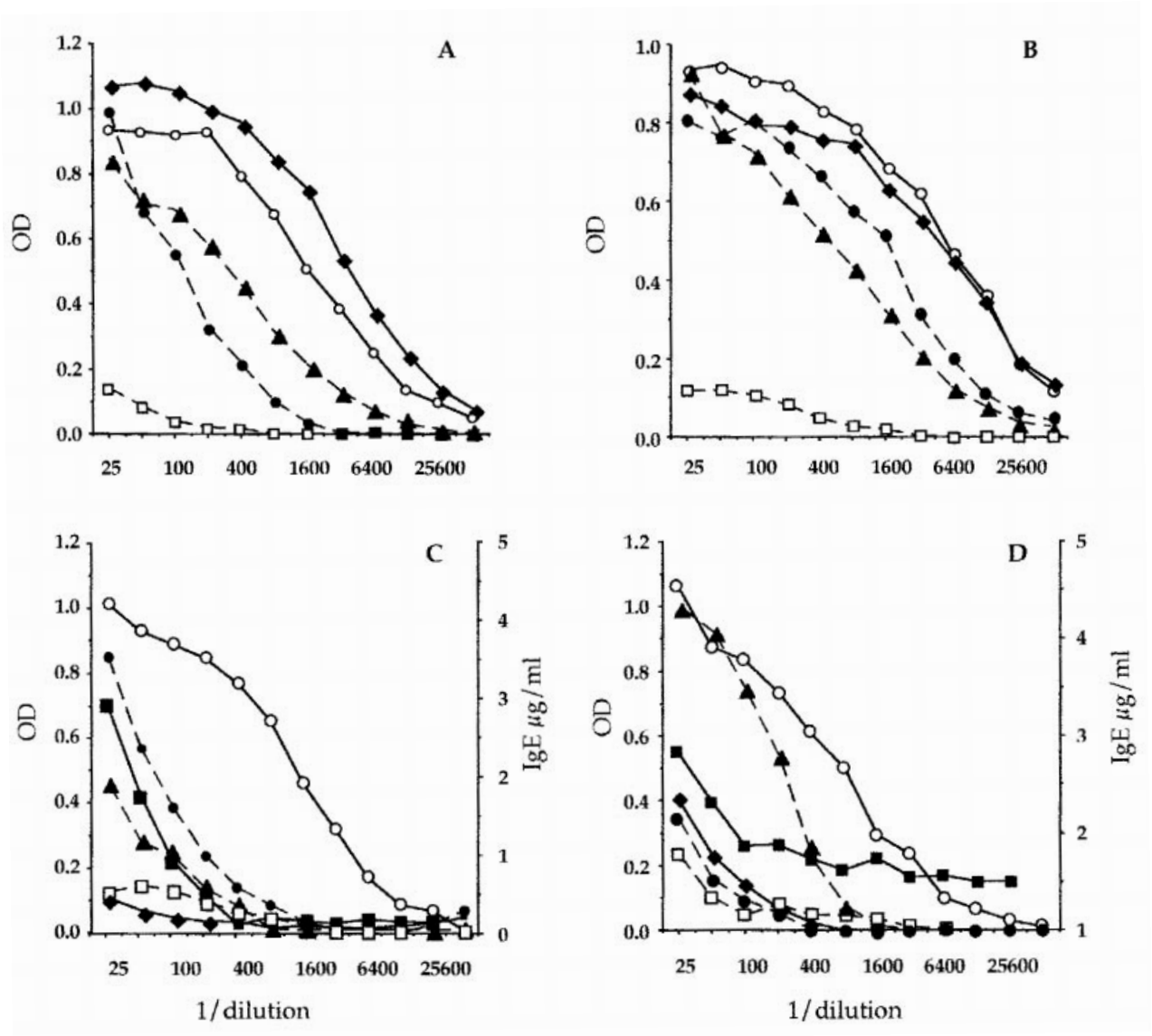

Fig. 2. Immunoglobulin isotype responses in BALB/c (A) and CBA/Ca (B) mice multiply inoculated with live Mf i.v. and BALB/c (C) and CBA/Ca (D) mice multiply immunized with Mf extract s.c. BALB/c (A) and CBA/Ca (B) mice were given 3 doses of $2{\cdot}5 \times 10^5$ live Mf over 56 days and the serum isotype responses examined 14 days after the final injection. A second group of BALB/c (C) and CBA/Ca (D) mice were given $3 \times 200\ \mu$l doses of 12 μg/ml Mf extract over 56 days and the serum Ig isotype responses were examined 14 days after the final injection. Mf-specific IgG1 (○), IgG2a (◆), IgG2b (▲), IgG3 (●), IgM (□) and total IgE (■) were measured by ELISA and the results are expressed as the mean of 4 individual mice in a serum dilution series.

that live Mf are more immuno-stimulatory as a result of E–S products released, a more likely explanation is that dead and live Mf are processed differently by the immune system and dead Mf have been more rapidly removed.

Previous studies have suggested that multiple Mf extract immunizations primarily induce a Th2-type response (Pearlman *et al*. 1993*a*). To investigate whether the Th2 responses resulted from multiple exposure to antigens not normally expressed in live infection or whether multiple inoculation of live Mf infection could also induce Th2 we multiply immunized mice with either live Mf or with Mf extract. As BALB/c mice are known to be particularly prone to Th2 cell upregulation we included the CBA/Ca strain of mouse which, in contrast to BALB/c, rapidly eliminate live Mf infection (Fanning & Kazura, 1983). BALB/c and CBA/Ca mice were injected i.v. with 250000 live Mf 3 times over a 56-day period and the immune response was examined 14 days after the final immunization. In comparison another group of BALB/c and CBA/Ca mice were immunized s.c. 3 times over a 56-day period with 200 μl of Mf extract (12 μg/ml) and the immune response was analysed 14 days after the final immunization. Both BALB/c mice (Fig. 2A) and CBA/Ca mice (Fig. 2B) multiply injected with live Mf i.v. stimulate high levels of all IgG isotypes. Interestingly, very high levels of IgE were induced in both groups of mice, $> 10\ \mu$g/ml and $5{\cdot}5\ \mu$g/ml respectively. This may have been a result either of the intravenous infection route or the chronic

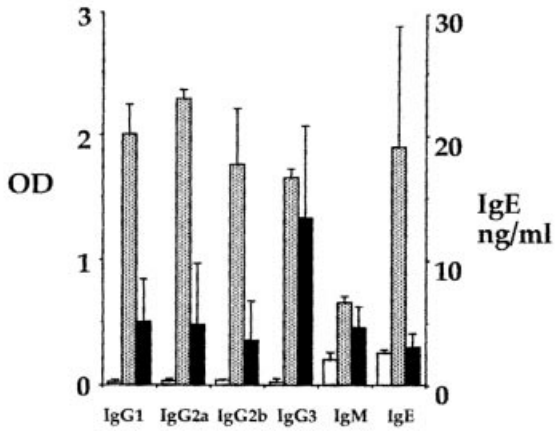

Fig. 3. Influence of route of infection on immunoglobulin responses to Mf. Serum immunoglobulin responses were measured in groups of 4 control uninfected BALB/c mice (□), mice injected with $2{\cdot}5 \times 10^5$ Mf i.v. (▨), and mice infected with $2{\cdot}5 \times 10^5$ Mf i.p. (■). Total IgE was measured by ELISA, Mf-specific immunoglobulins were measured by ELISA for the other isotypes. Results are expressed as the mean ± S.D. of 4 individual mice.

exposure to antigen caused by multiple immunizations. BALB/c mice multiply immunized with Mf extract (Fig. 2C) also produce high levels of IgE (3·0 μg/ml) but, in contrast to animals given live infections, these mice produced high levels of only the IgG1 isotype of IgG. IgG2b and IgG3 were produced at very low levels and IgG2a was virtually absent (Fig. 2C) indicating that Mf extract may induce a more Th2 phenotype than live infection. Similarly, CBA mice multiply stimulated with Mf extract produced high levels of parasite-specific IgG1 and relatively high levels of IgG2b but low levels of other IgG isotypes (Fig. 2D). Levels of IgG2a and IgG3 are good 'historical' indicators of IFN-γ activity and indeed the more 'Th2' nature of the response to Mf extract was also reflected in the cytokine response of *in vitro*-stimulated splenocytes from both CBA and BALB/c mice which produced no IFN-γ but did secrete IL-4, IL-5 and IL-10 (data not shown). Interestingly, splenocytes from CBA mice multiply infected with live Mf produced IFN-γ and IL-10, but no IL-4 or IL-5 upon restimulation, while splenocytes from BALB/c mice produced a similar profile to those immunized with Mf extract (data not shown).

Mf injected intravenously stimulate IgE production

The route of administration of an antigen is known to influence the type of immune response to that antigen. The natural environment for Mf is the bloodstream so we investigated whether the immune response to a single i.v. immunization of Mf was similar to that of a single i.p. infection.

Figure 3 shows that Mf injected i.v. into BALB/c mice stimulate significantly higher levels of specific IgG1, IgG2a, and IgG2b ($P > 0{\cdot}05$) than mice given Mf i.p. at day 50 p.i. The levels of IgM and IgG3 were similar between the 2 infection groups. Interestingly, the level of IgE stimulated by an i.v. infection of Mf is very high in comparison to an i.p. infection in which IgE production is rarely observed. Proliferative responses of splenocytes stimulated with specific antigen were similar in both infection groups.

Both dose and length of exposure to Mf can alter the response seen

Antigen dose can bias the Th1/Th2 balance of an immune response (Parish & Liew, 1972). Our earlier investigations have shown that although live Mf infections i.p. stimulate strong IFN-γ responses over a 28-day time-period, by day 28 both IL-4 and some IL-5 are also produced by splenocytes (Lawrence *et al*. 1994). In human infections Mf are continually circulating in the bloodstream over many years resulting in a chronic exposure to Mf antigen. We were therefore interested in modeling the effect of chronic exposure to Mf in the mouse. We approached this in 2 different ways. In the first experiment we infected 5 BALB/c mice with a single live i.v. infection of $2{\cdot}5 \times 10^5$ Mf and compared the immune response to 5 BALB/c mice that were given 3 infections of $2{\cdot}5 \times 10^5$ Mf i.v. on days 0, 20 and 40 p.i. In the second experiment we injected BALB/c mice with either a single high dose of live Mf i.v. ($2{\cdot}5 \times 10^5$ Mf) or a single low dose of Mf ($2{\cdot}5 \times 10^4$). In order to investigate the effect of length of exposure on the immune response, 4 mice per group were analysed on days 20, 40, 60, and 80.

The major findings from these 2 experiments were analogous. In the first experiment, Mf-specific IgM and Mf-specific IgG of each isotype were produced at similar levels irrespective of the number of inoculations (data not shown). By day 36 post-initial infection, although multiply immunized mice had received only 2 infections, IgE production was notably higher than mice receiving a single inoculum ($P > 0{\cdot}05$). By day 60 post-initial infection IgE had dramatically increased to 3 μg/ml in the chronically infected animals (Fig. 4A). In the second experiment, only mice given the higher dose of Mf produced IgE and these mice began IgE production between days 40 and 60 p.i. (Fig. 4B). Mf-specific IgM and all IgG isotypes were at high levels over the whole course of infection (data not shown). Taken together these results show that high dose or chronic and prolonged exposure to Mf leads to IgE production, however, other isotypes of antibody are not down-regulated.

Local immune responses vary from splenic responses

It has been observed in many host–parasite systems that responses in the local secondary lymphoid

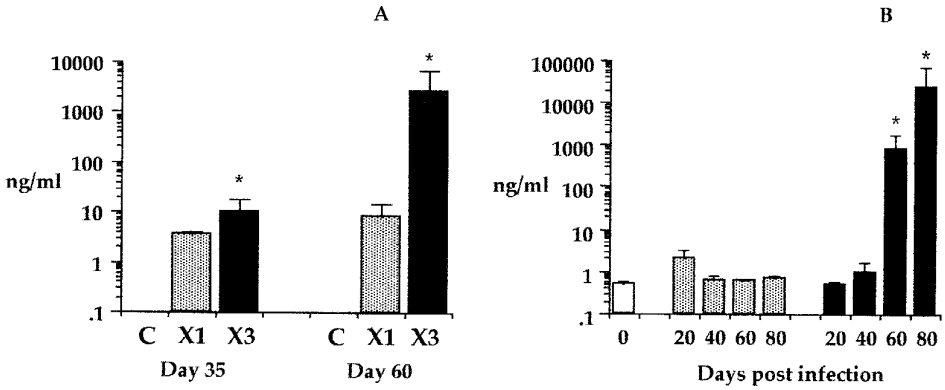

Fig. 4. Effect of chronicity of exposure to Mf on total IgE levels. (A) Five BALB/c mice were infected $\times 1$ with $2{\cdot}5 \times 10^5$ Mf i.v. on day 0 (▨), 5 mice were infected $\times 3$ with $2{\cdot}5 \times 10^5$ Mf i.v. on days 0, 20 and 40 post-initial infection (■) and 5 mice were not infected (□). (B) Five BALB/c mice were infected with a low dose of Mf ($2{\cdot}5 \times 10^4$) i.v. (▨), 5 mice were infected with a high dose of Mf ($2{\cdot}5 \times 10^5$) i.v. (■) and 5 mice were not infected (□). Total IgE was measured by ELISA and the result expressed as mean ± S.D. of individual mice. * Represents statistical significance at the 95% level using the Mann–Whitney U-test in (A) between mice infected $\times 1$ and mice infected $\times 3$ and in (B) between mice given high dose or low dose Mf at a particular time-point.

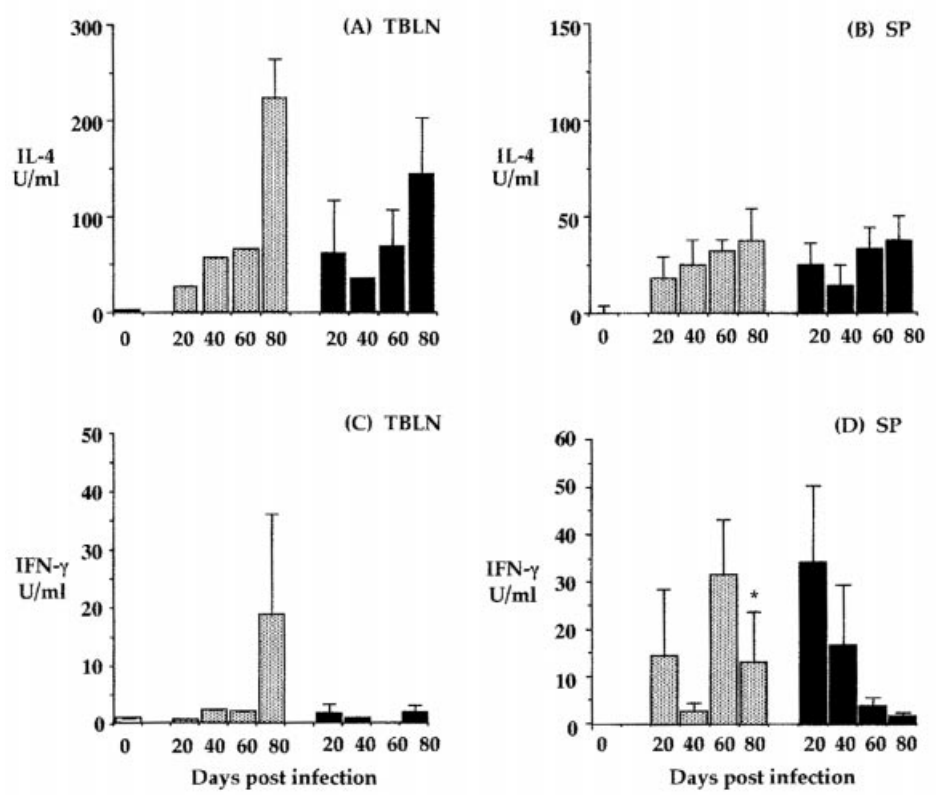

Fig. 5. Effect of chronicity of exposure to Mf on cytokine levels. Five BALB/c mice were infected with a low dose of Mf ($2{\cdot}5 \times 10^4$) i.v. (▨), 5 mice were infected with a high dose of Mf ($2{\cdot}5 \times 10^5$) i.v. (■) and 5 mice were not infected (□). Cytokine levels in tracheo-bronchial lymph nodes (A and C) and spleen (B and D) were assayed. Splenocytes and tracheo-bronchial lymph node cells were recovered from individual mice and restimulated at 2×10^6 cells/ml *in vitro* with 10 μg/ml Mf extract for 66 h. Supernatants were assayed for IL-4 by bioassay (A and B) and IFN-γ by ELISA (B and D). Results are expressed as the mean ± S.D. of 5 individual mice. * Represents statistical significance at the 95% level using the Mann–Whitney U-test between mice given high dose and low dose Mf at a particular time-point.

organs draining the site of infection reflect a more Th1/Th2 skewed response than those seen in the major lymphoid organ, the spleen (Kelly *et al*. 1991; Tonkonogy & Swain, 1993). As the majority of Mf are known to reside in the lung post-intravenous infection (Grove, Davis & Warren, 1979) we examined responses in the local draining lymph nodes of the lung, the tracheo-bronchial lymph

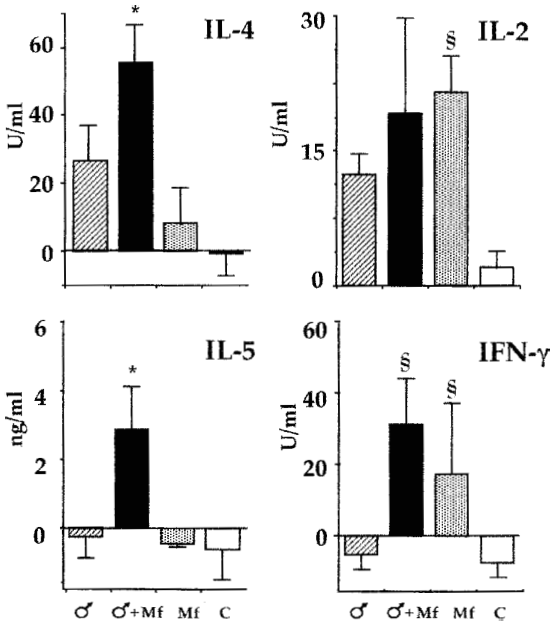

Fig. 6. Influence of Mf on pre-existing Th2 cytokine responses. Cytokine responses in splenocytes taken from 4 BALB/c mice infected i.p. with 20 adult male nematodes (▨), 4 mice infected with 20 adult male nematodes followed by $2{\cdot}5 \times 10^5$ Mf i.p. 14 days later (■), 4 mice infected with $2{\cdot}5 \times 10^5$ Mf i.p. at 14 days only (▩) and 4 control uninfected mice (□). Splenocytes were recovered 28 days post-initial adult infection and restimulated at 5×10^6 cells/ml *in vitro* with 10 μg/ml *Brugia* extract for 66 h. Supernatants were assayed for IL-4 and IL-2 by bioassay, and, IL-5 and IFN-γ by ELISA. Results are expressed as the mean ± S.D. of 4 individual mice. * Represents statistical significance at the 95% level using the Mann–Whitney U-test where one group is significantly greater than all other groups. § Represents statistical significance at the 95% level when 1 group is greater than the control group and the male-implanted group.

nodes, in addition to the splenocyte responses of these mice. The cytokine responses of mice which received $2{\cdot}5 \times 10^5$ Mf or $2{\cdot}5 \times 10^4$ Mf i.v. were analysed at day 20, 40, 60 and 80 p.i. Interestingly the IL-4 response at the local draining lymph node increased with time p.i. at both Mf doses and at day 80 was significantly higher than at earlier time-points (Fig. 5A). The IL-4 response by splenocytes was similar at both Mf doses and varied little over the infection time-course (Fig. 5B). IFN-γ production by the tracheo-bronchial nodes was generally absent and only seen at the lower dose of Mf at day 80 (Fig. 5C). IFN-γ production by splenocytes was reduced at the higher dose of Mf and interestingly decreased in a time-dependent manner at this dose. Chronicity of Mf exposure therefore appears to skew the Mf-specific response away from IFN-γ-mediated responses and, as shown in other parasite systems, the up-regulation of Th2 cell responses is more apparent in the local draining lymph node.

Male worms followed by Mf induce IL-4 and IFN-γ but only the IL-4-dependent immunoglobulin isotypes, IgG1 and IgE

Both infective larvae and adult worms predispose the immune response in a Th2 direction (Lawrence *et al*. 1994, 1995), but as Mf have the capacity to induce IFN-γ this may modulate the response during disease ontogeny. To test this hypothesis, we implanted mice i.p. with 20 adult male worms which induced an IL-4 response (Lawrence *et al*. 1994) and 14 days later challenged 1 group of male-implanted mice with $2{\cdot}5 \times 10^5$ Mf i.p. (We chose to challenge with Mf by the i.p. route as very high IFN-γ production is obtained via this route. Thus if IFN-γ can regulate the Th2 response we considered that we would be able to most easily assess its effect in this system.) At 28 days post-initial adult implantation we measured serum antibody responses and the cytokine response of spleen cells.

Adult male worm infections alone induced 26 U/ml of IL-4 and no IFN-γ, while single-stage infections with Mf alone induced 18 U/ml IFN-γ but little IL-4 production. Surprisingly, however, splenocytes from mice that received adult male worms followed by Mf made substantial amounts of both IL-4 and IFN-γ when stimulated with *Brugia* extract *in vitro* (Fig. 6). In fact the mice that received males and Mf produced higher levels of IL-4, IFN-γ and measurable amounts of IL-5 in comparison to the control groups presumably because shared antigens present on both Mf and adult male nematodes acted effectively as an antigenic boost. At the antibody level, however, the down-regulating effect of IL-4 exerted itself (Fig. 7). Mice that received either adult male worms or adult males plus Mf produced IgG1 and IgE. The IgG2a, IgG2b and IgG3 seen in response to Mf alone was prevented from developing in the presence of a prior adult male worm infection. Thus despite the ability of IFN-γ to down-regulate both IgG1and IgE production in the mouse, IFN-γ induction by Mf did not influence the levels of these antibody isotypes in this model system.

DISCUSSION

Potent IFN-γ production is a characteristic response to single-stage infection with the microfilarial stage of *B. malayi*. However, active infection with adult nematodes that produce Mf is associated with strong Th2 responses and down-regulation of Th1 and IFN-γ responses (Lawrence *et al*. 1994). This study was designed to investigate the circumstances under which live Mf induce IFN-γ and whether, in this long-term chronic disease, IFN-γ-induction by live Mf could modulate the effects of the profound Th2 induction by infective larvae and adult-stage nematodes.

We have shown that live Mf potently induce

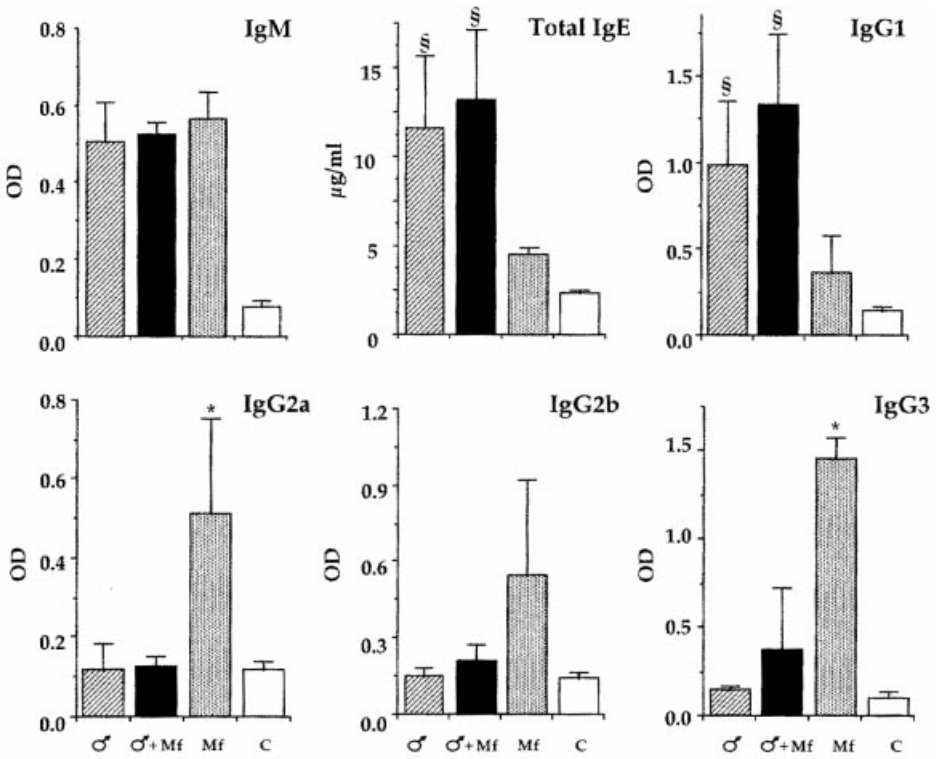

Fig. 7. Influence of Mf on pre-existing Th2-driven antibody response. Immunoglobulin isotype responses were measured in sera from 4 BALB/c mice infected i.p. with 20 adult male nematodes (▨), 4 mice infected with 20 adult male nematodes followed by $2{\cdot}5 \times 10^5$ Mf i.p. 14 days later (■), 4 mice infected with $2{\cdot}5 \times 10^5$ Mf i.p. at 14 days only (▩) and 4 control uninfected mice (□). Sera were taken 28 days post-initial adult infection and total IgE was measured by ELISA, for all the other isotypes *Brugia*-specific isotypes were measured by ELISA. Results are expressed as the mean ± S.D. of 4 individual mice. * Represents statistical significance at the 95% level using the Mann–Whitney U-test where 1 group is significantly greater than all other groups. § Represents statistical significance at the 95% level when one group is greater than the control group and the Mf injected group.

IFN-γ and IFN-γ-mediated antibody responses upon both single and multiple infections. Although no splenocyte IFN-γ production was seen in response to killed Mf at the time-point studied, the antibody isotype profile was similar (albeit weaker) to that seen against live infection and IFN-γ-driven isotypes, such as IgG2a and IgG3, were present. Repeated immunizations with Mf extract, however, did not stimulate IFN-γ or IFN-γ-mediated antibody responses, suggesting that the Th1-inducing antigen may be an integral component of intact Mf. Indeed our preliminary experiments suggest that exsheathed Mf stimulate higher levels of IL-4 and lower levels of IFN-γ than their intact counterparts implying that IFN-γ production may be in response to a protein or glycoprotein intimately associated with the microfilarial sheath (unpublished observations). Interestingly, recent work showing that Mf have gram-negative bacteria of the genus *Wolbachia* (Taylor *et al*. 1999), suggests that IFN-γ production may be a by-product of endotoxin release from these bacteria. However, if this is the case, Mf extract would be expected to possess similar levels of endotoxin and, in addition, IFN-γ production is not seen in human infections unless Mf are absent (Ravichandran *et al*. 1997).

A number of studies have addressed the question of whether route of infection affects the quality of the immune response engendered. Many of these studies suggest that antigens administered via mucosal surfaces such as the oral route or via the respiratory system generate anergic responses (Garside & Mowat, 1997). In addition there is some evidence that antigens administered intravenously are also capable of inducing a tolerogenic response usually via massive Th2 production at the expense of a Th1 response. Our previous work has studied the immune response to Mf that have been injected into the peritoneal cavity in order that we could make a comparison between the immune response to adult nematodes and infective larvae that were implanted by the same route. The natural site of infection of Mf, however, is in the bloodstream and our experiments have compared both i.p. and i.v. sites of Mf infection. Interestingly, although the profile of IgG isotypes observed is similar, all IgG isotypes are significantly more elevated in mice given an i.v. infection. Moreover, i.v. Mf infections generated a notable IgE response. Thus although Mf i.v. stimulates IFN-γ there is enough IL-4 or IL-13 induced via this route to allow the switch to IgE. We have not observed IgE responses in any of several i.p.

infection experiments with Mf. The differences may relate to differences in draining lymphatic organs (spleen versus lymph node) or relate to dose as Mf present in the bloodstream may be more accessible to lymphatic organs than those in the peritoneal cavity.

Lymphatic filariasis is a chronic long-term infection in humans in which adult nematodes can survive for more than 10 years. Although in mice it is difficult to replicate the effects of this long-term infection, we have shown that higher doses of Mf, higher numbers of boosts and longer lengths of infection all induce elevated IgE production, an IL-4/IL-13 dependent isotype. Experiments in malaria-infected mice have also shown that the higher the number of infections the greater the level of IgE production (Helmby *et al*. 1996). Indeed, in another parasitic nematode model high doses of parasites have been shown to generate Th2 responses and consequent resistance to infection while doses below a particular threshold generated Th1 responses and susceptibility (Bancroft, Else & Grencis, 1994). The continual production of Mf, which can live for 100–300 days, by adult female nematodes during natural infection is likely to ensure that the immune response is constantly boosted and bombarded by high levels of antigen and this may also lead to the development of anergy. The consensus of opinion from work in single antigen systems is that very low doses of antigen priming promote Th2 responses, intermediate to high doses promote Th1 responses and very high doses promote Th2 polarization (Hosken *et al*. 1995). In a chronic infection such as lymphatic filariasis in which parasites can survive for a period of years the system is likely to be similar to one in which very high doses of antigen are used. In this study the down-regulation of IFN-γ at a high Mf dose is clearly seen in the spleen while higher levels of IL-4 production in response to Mf is apparent in the local draining lymph node. Thus although subtle effects of IFN-γ secretion in the local microenvironment of the worms cannot be discounted overall it appears that live Mf induce local IL-4 production particularly at high infection doses.

With a chronic disease of this nature the potential for modulation or reversibility of a biased immune response is of vital importance. In our model we have been unable to show that Mf can modulate the antibody response to adult nematodes despite high levels of IFN-γ production even in adult worm presence. It is possible, however, that IFN-γ production may play a role in modulation of IgG1 or IgE by influencing the class switch of B cells that are not yet committed. For instance, Mf share many antigens with other developmental stages that precede them in the life-cycle (Maizels & Selkirk, 1988) and responses to these particular antigens will be severely biased prior to Mf appearance. The effect of IFN-γ production in response to Mf-specific antigens is, however, difficult to assess and may play a role in regulation of the Th2 response. In other *Brugia*–mouse models it has been seen that the dominant Th2 response to *Brugia* sp. can regulate the Th1-driven pathological effects of an unrelated concurrent *Plasmodium* infection (Yan *et al*. 1997) or the Th1 response to an unrelated antigen PPD (Pearlman *et al*. 1993*b*). In both mouse and human filarial infections, filarial-specific Th1 cells are known to be antigen-primed but appear to be immunologically silenced as treatment of cells *in vitro* with anti-IL-10 can restore antigen-specific Th1 responses in both hosts (Mahanty *et al*. 1997; Osborne & Devaney, 1999). In mice, committed Th2 responses are notoriously difficult to reverse (Murphy *et al*. 1996) and it remains to be seen whether commitment to the Th2 lineage in human immune responses is reversible *in vivo*. The strong Th2 bias seen in filarial infection, particularly in long-term active microfilaraemic infections, suggests, however, that the Mf stage of the parasite, although a potent inducer of IFN-γ, does not act as a natural regulator of the Th2 immune response and may indeed contribute to the Th2 bias itself.

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