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# Efficacy of Oral Vaccination against the Murine Intestinal Parasite *Trichuris muris* Is Dependent upon Host Genetics

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**Oral vaccinations with *Trichuris muris* adult worm homogenate antigen with cholera toxin as the adjuvant were successful in both high-responder BALB/c and low-responder C57BL/10 mice, resulting in high levels of protection against subsequent infection, but were ineffective in the low-responder B10.BR mice. Subcutaneous vaccination with antigen in Freund's complete adjuvant resulted in protection of all of these strains but was most effective in high-responder BALB/c and least effective in B10.BR mice. Oral vaccination resulted in a *T. muris*-specific intestinal immunoglobulin A response only in the two protected strains. High levels of serum immunoglobulin G1 antibody were induced by Freund's complete adjuvant vaccination in all cases. A relationship between vaccine efficacy, expulsion phenotype, and induced T-helper subset-associated cytokines (interleukin-5 and gamma interferon) was noted. It was concluded that effective vaccination against *T. muris* requires the induction of Th2 responses and that this can be achieved by both oral and parenteral administration of antigens.**

An effective antiparasite vaccine should induce high levels of protective immunity in a large proportion of a genetically heterogeneous host population (18). To determine an effective mode of vaccination, it is therefore important to examine individuals with diverse responses to vaccination so that differences in the underlying immune mechanisms might be identified and nonresponsiveness might be corrected.

Expression of protective immunity to the murine intestinal nematode *Trichuris muris* is T-cell dependent and strongly influenced by host genetics (26). Genetic factors determine the rate of expulsion of worms from the large intestine, making it possible to classify different mouse strains as having high-responder (HR) low-responder (LR), or nonresponder (NR) expulsion phenotypes (9). Expulsion phenotype is closely correlated with T-helper-cell subset responses; i.e., HR mice elicit a Th2-type response, while NR mice activate the Th1 subset (6). The *T. muris*-mouse host-parasite relationship therefore provides an excellent model for analyzing the conditions necessary for using vaccine strategies to induce effective T-cell-dependent immunity in hosts of widely divergent response potential.

Most studies on vaccination against intestinal nematode infections have concentrated on inducing high levels of systemic immunity. This is despite current thought that host protection against enteric diseases should be elicited at mucosal surfaces where the infection occurs (17) and that mucosal immune responses, especially secretory antibody production, are best stimulated by local exposure to antigen (20).

The present study was undertaken to investigate the possibility that protective immune responses against *T. muris* infection could be induced by oral and parenteral routes. Comparisons of anti-worm immunity and of antigen-specific immune responses were made in vaccinated HR (BALB/c) and LR (C57BL/10 and B10.BR) mice. Parameters measured included expulsion of challenge infection, serum and intestinal luminal antibody isotype responses, and levels of mesenteric lymph node cell (MLNC) interleukin-5 (IL-5) and gamma interferon (IFN-γ) secretion as markers of T-helper subset activity.

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## MATERIALS AND METHODS

**Mice.** Female C57BL/10, BALB/c, and B10.BR mice were purchased from Harlan-Olac (Bicester, Oxon, United Kingdom). Outbred CFLP mice were bred in the Department of Life Science, University of Nottingham. Mice were used at 6 to 8 weeks of age.

**Parasite.** *T. muris* was maintained as described previously (25) in immunosuppressed CFLP mice by oral infections with 400 eggs.

**Antigen.** Homogenate antigen preparations from freshly isolated *T. muris* adult worms (designated H) were made and filtered through a sterile 0.2-μm-pore-size filter before dialysis, freeze-drying, and storage at −40°C (14).

**Preparation of material for immunization.** A 2-mg/ml solution of H in phosphate-buffered saline (PBS) was emulsified at a ratio of 1:1 in Freund's complete adjuvant (FCA; Sigma, Poole, Dorset, United Kingdom) to give a final concentration of 100 μg of H per 0.1 ml. Antigen solutions were mixed with a 500-μg/ml solution of cholera toxin (CT; Sigma) in PBS and diluted by 50% in 0.35 M sodium bicarbonate (Sigma) to provide 150 μg of H and 5 μg of CT per 0.1-ml dose.

**Experimental protocol.** Groups of six mice were immunized subcutaneously (s.c.) with 100 μg of H in FCA (H+FCA) on days −27 and −17 or orally with 150 μg of antigen and 5 μg of CT (H+CT) on days −27, −17, and −7 before oral infection with 400 *T. muris* eggs on day 0.

BALB/c and C57BL/10 mice were killed on day 15, and B10.BR mice were killed on day 21 post-*T. muris* infection. These days were chosen by reference to published data (9) as suitable for detection of vaccine-induced reduction in worm burden. At these times, worms were recovered from the large intestine and counted, serum samples were taken, intestinal lavages were done, and mesenteric lymph nodes were removed.

**Collection of intestinal lavage fluid.** Following removal of the small intestine, 3 ml of a 0.1-mg/ml concentration of soybean trypsin-chymotrypsin inhibitor (Sigma) in 50 mM EDTA (Sigma) was flushed through (10). The recovered samples were kept on ice before vortexing and spinning at 650 × *g* for 10 min at 4°C. Thirty microliters of 100 mM phenylmethylsulfonyl fluoride was added to the supernatants, which were then centrifuged for a further 20 min at 27,000 × *g*. Twenty microliters of phenylmethylsulfonyl fluoride and 20 μl of 1% sodium azide (Sigma) were added, and the supernatants were left to stand for 15 min before 100 μl of fetal calf serum (GIBCO Ltd., Paisley, United Kingdom) was added. Samples were aliquoted and stored at −40°C until required.

**Antibody enzyme-linked immunosorbent assay (ELISA).** Levels of specific immunoglobulin G1 (IgG1), IgG2a, and IgA in samples of serum diluted to 1/50 and in undiluted intestinal lavage fluid were determined by a method described previously (2).

**Preparation of cell culture supernatants for cytokine assay.** As described previously (23), 200-μl aliquots of pooled MLNC suspensions from groups of six mice at $5 \times 10^6$ cells per ml in RPMI 1640 medium (GIBCO), supplemented

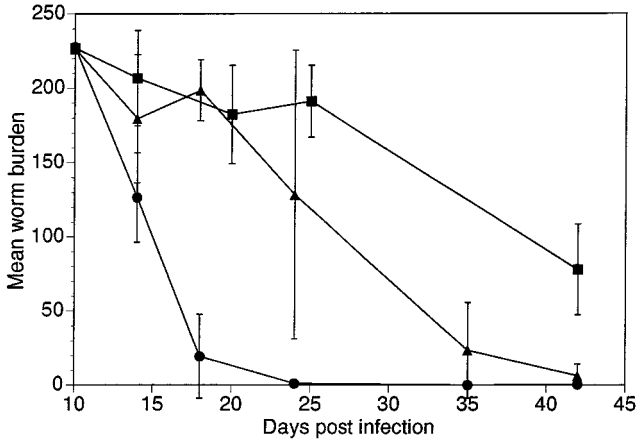


FIG. 1. Mean cecal *T. muris* worm burdens of BALB/c (●), C57BL/10 (▲), and B10.BR (■) mice throughout a time course of infection. Mice were infected on day 0 with 400 eggs, and at several points during the infection, groups of six mice were killed and their worm burdens were determined. Error bars represent standard deviations.

with 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and $7.5 \times 10^{-5}$ M monothioglycerol (BDH Chemicals Ltd., Poole, Dorset, United Kingdom), were cultured in 96-well flat-bottom tissue culture trays (Nunc, Life Technologies Ltd., Paisley, United Kingdom). Fifty micrograms of H per ml was added. After 48 h of incubation, supernatants were removed and centrifuged at 10,000 × *g* in a microcentrifuge for 1 min to pellet any cells. The sterile samples were aliquoted and stored at −80°C until required.

**Assays for the cytokines IL-5 and IFN-γ.** IL-5 and IFN-γ levels in duplicate samples of the pooled cell supernatants were measured by sandwich ELISA (6). The monoclonal antibodies TRFK-5 and biotinylated TRFK-4 (Pharmingen, San Diego, Calif.) were used in IL-5 assays. R46A2 and biotinylated XMG1.2 antibodies (Pharmingen) were used in IFN-γ assays. Although IL-4 is, functionally, a more important Th subset marker than IL-5, our own data and those of others (8) have shown that IL-4 levels are low in *T. muris*-infected mice, even in HR strains, and that IL-5 measurements are sufficient markers of the Th2 response.

**Statistics.** Comparisons between groups were made by the Mann-Whitney U test. A *P* value of greater than 0.05 was considered nonsignificant. It should be noted that since MLNC from the groups were pooled, statistical analysis of the cytokine data could not be carried out.

## RESULTS

**Expulsion phenotypes of three inbred strains of mice.** The BALB/c, C57BL/10, and B10.BR strains of mice have been designated previously as HR, LR, and NR to *T. muris*, the phenotype being determined by the rate of expulsion of infection (5, 9). To confirm these classifications before vaccination experiments were carried out, mice of each of these strains were infected with 400 *T. muris* eggs. Groups of six mice were killed at regular intervals during the course of infection, and cecal worm burdens were determined (Fig. 1). The HR BALB/c strain expelled all worms before day 25 postinfection, whereas the C57BL/10 and B10.BR mice retained their infections longer. In experiments described previously, the B10.BR strain was found to retain almost all of its infection load after day 35 (9); however, the data presented here show that some 50% of the worms were lost between days 25 and 42. The LR C57BL/10 mice expelled their infection later than the BALB/c mice but earlier than the B10.BR mice.

**Oral and s.c. vaccination of three murine strains against *T. muris*.** Groups of six BALB/c, C57BL/10, and B10.BR mice were given oral and s.c. vaccinations before infection with 400 *T. muris* eggs. On days 15 (BALB/c and C57BL/10) and 21 (B10.BR) postinfection, the cecal worm burdens were compared with those of nonvaccinated animals infected on the same day (Fig. 2). Vaccinations with H+FCA s.c. resulted in significant reductions in worm burdens of all three strains

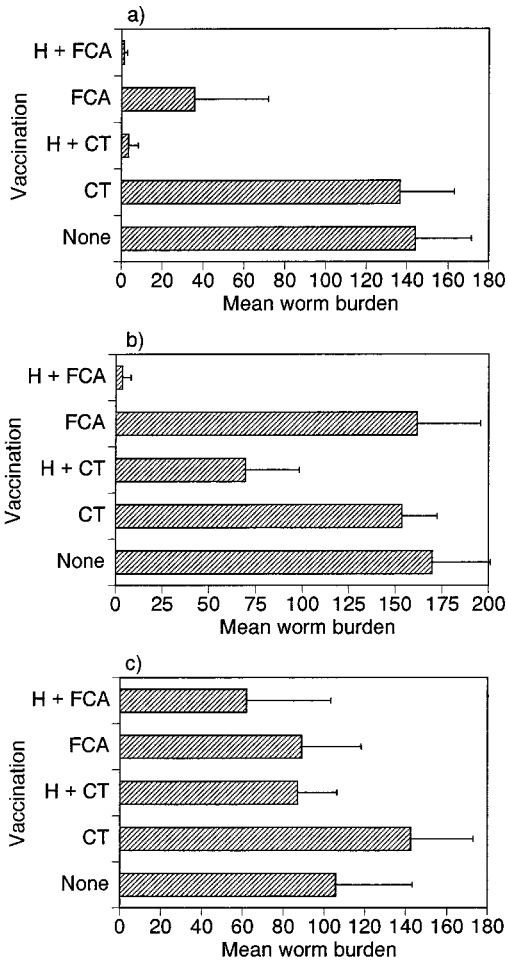


FIG. 2. Mean worm burdens of groups of BALB/c (a), C57BL/10 (b), and B10.BR (c) mice previously vaccinated s.c. with H+FCA, FCA in the absence of antigen as adjuvant control, or orally with H+CT or CT without antigen on days 15 (BALB/c and C57BL/10) and 21 (B10.BR) postinfection with 400 *T. muris* eggs. Error bars represent standard deviations.

(BALB/c, 99.2% protection [$P = 0.001$]; C57BL/10, 97.8% protection [$P = 0.001$]; B10.BR, 41.4% protection [$P = 0.05$]). It has been noted previously (unpublished data) that the FCA control injection may result in nonspecific worm expulsion mechanisms in HR but not LR mice. Significant worm loss as a result of administration of FCA alone was noted in the case of the BALB/c strain but not for the C57BL/10 or B10.BR groups. Oral H+CT vaccination significantly protected the BALB/c (97.6% [$P = 0.001$]) and C57BL/10 (59.0% [$P = 0.001$]) mice but not the B10.BR (17.8% [$P > 0.05$]) mice. CT control injection had no effect on the worm burdens of any of the mouse strains.

**Immune responses to oral and s.c. vaccinations.** Groups of six mice were vaccinated as they were in the experiment described previously, but on the day that infections should have been administered, i.e., 7 days after the last CT dose and 17 days after the last FCA boost, these mice were killed, and sera, intestinal lavage fluids, and MLNC were collected. The serum and lavage samples were tested by indirect ELISA for *T. muris*-specific antibody of the IgG1 and IgG2a isotypes (indicative of Th2 and Th1 subset activation, respectively) and also for IgA antibody in the case of the intestinal lavages. MLNC from

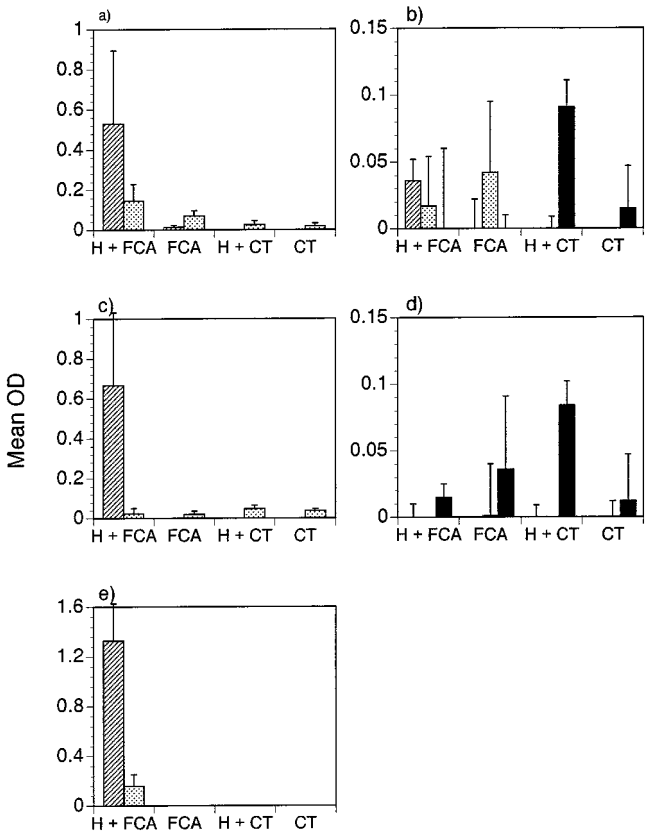


FIG. 3. *T. muris*-specific IgG1 (▨), IgG2a (▤), and IgA (■) antibody levels detected in serum (a, c, and e) and intestinal lavage (b and d) samples taken from groups of six BALB/c (a and b), C57BL/10 (c and d), and B10.BR (e) mice vaccinated s.c. with H+FCA or FCA alone or orally with H+CT or CT alone 17 (H+FCA and FCA) and 7 (H+CT and CT) days previously. No *T. muris*-specific antibody was detected in intestinal lavage fluids of the B10.BR groups; therefore, these data were omitted. Error bars represent standard deviations.

vaccinated and infected mice were used for cytokine measurement.

H+FCA vaccination resulted in high levels of IgG1 and low IgG2a serum antibody in each of the strains (Fig. 3). Oral H+CT vaccination did not result in significant serum antibody production, but high-level intestinal IgA responses were produced by the BALB/c and C57BL/10 strains. Production of high levels of intestinal IgA therefore correlated with protection induced by oral immunization. No significant antibody levels were detected in either serum or intestinal fluids of the adjuvant control groups.

MLNC from vaccinated, vaccinated and infected, infected, and naive mice of the three strains were stimulated in vitro with H for 48 h before culture supernatant collection. These supernatants were tested for the T-helper-associated cytokines IL-5 (Th2) and IFN-γ (Th1) (Table 1).

MLNC of BALB/c mice, successfully vaccinated with H+FCA, released IL-5 but not IFN-γ. When cells were tested 15 days after infection, the levels of these cytokines remained approximately the same. Injection with FCA alone did not induce cells capable of producing these cytokines upon restimulation, but when the mice were infected, MLNC were then able to secrete IL-5. When BALB/c mice were given H+CT by oral vaccination, levels of IL-5 production by MLNC were lower than those when FCA was used. Fifteen days after infection, the MLNC of these mice produced increased levels

TABLE 1. IL-5 and IFN-γ secreted by MLNC of vaccinated and infected mice stimulated in vitro with H[a]

| Treatment[b] | Cytokine (U/ml) secreted by: | | | | | |
|---|---|---|---|---|---|---|
| | BALB/c mice | | C57BL/10 mice | | B10.BR mice | |
| | IL-5 | IFN-γ | IL-5 | IFN-γ | IL-5 | IFN-γ |
| H+FCA (V) | 120 | 0 | 320 | 0 | 4 | 0 |
| H+FCA (VI) | 160 | 0 | >500 | 0 | 15 | 170 |
| FCA (V) | 4 | 0 | 120 | 0 | 0 | 0 |
| FCA (VI) | 190 | 20 | 170 | 240 | 4 | 70 |
| H+CT (V) | 85 | 18 | 290 | 18 | 0 | 0 |
| H+CT (VI) | 160 | 12 | 0 | 230 | 0 | >300 |
| CT (V) | 0 | 0 | 130 | 0 | 0 | 0 |
| CT (VI) | 90 | 42 | 130 | 260 | 0 | 16 |
| Infection | 110 | 60 | 320 | >300 | 0 | 8 |
| None | 0 | 0 | 8 | 0 | 4 | 2 |

[a] BALB/c, C57BL/10, and B10.BR mice were vaccinated with H+FCA s.c. or with H+CT orally before infection with 400 eggs. Groups receiving only vaccinations were killed on the day that the others were infected. Infected BALB/c and C57 BL/10 mice were killed on day 15 postinfection, whereas B10.BR mice were killed on day 21. Pooled MLNC at $5 \times 10^6$ cells per ml were cultured with 50 μg of H per ml for 48 h. Supernatants were tested for IL-5 and IFN-γ (units per milliliter) by sandwich ELISA.

[b] V, vaccination; VI, vaccination and infection.

of IL-5. CT in the absence of antigen did not induce detectable levels of these cytokines.

Cells from C57BL/10 mice vaccinated with H+FCA, like the BALB/c strain, produced high levels of IL-5 but no IFN-γ. After infection (day 15), IL-5 levels were enhanced. FCA alone resulted in IL-5 production, and IFN-γ secretion was induced upon infection. Oral vaccination with H+CT induced IL-5 and IFN-γ secretion, but after infection, the MLNC produced high levels of IFN-γ in the absence of IL-5.

MLNC of B10.BR mice that had been vaccinated with H+FCA secreted low levels of IL-5, while cells taken at day 21 from vaccinated and infected mice produced high IFN-γ and low IL-5 levels. Exposure of mice to FCA alone did not induce MLNC capable of secreting these cytokines, and neither did the unprotective oral vaccine. After infection, MLNC of such orally vaccinated mice secreted high levels of IFN-γ but no IL-5.

The values reported here were taken after vaccination-induced worm loss should have taken place. The cytokine profile of cells taken from similarly treated groups of BALB/c, C57BL/10, and B10.BR mice on day 7 postinfection (i.e., when antiworm responses were ongoing) showed trends similar to the day 15 or 21 results (data not shown).

## DISCUSSION

The responses of mice to *T. muris* range from resistance, associated with effective Th2-type immune responses, to susceptibility resulting in chronicity, associated with ineffective Th1 subset activation (6). These responses are governed by both major histocompatibility complex-linked and background genes (9). Three inbred strains, which respond differently to infection, were compared in this study to determine whether mucosal immunization was more effective than systemic administration of antigens in inducing protection against this intestinal parasite. The B10.BR strain, reported previously to maintain a chronic *T. muris* infection and to be unable to expel worms from the intestine (5), has apparently undergone a slight shift in phenotype and has, in our recent experiments, showed a degree of worm expulsion between days 25 and 42. It could not therefore be described as a true NR strain but, of the

three strains, exhibited the slowest response and did appear to mount a Th1-type response upon infection as described for NR mice (6).

Expression of protective immunity to intestinal infections is often associated with considerable pathology, avoidance of which in vaccinated individuals is therefore a high priority. It has been shown previously that immunity to *T. muris* in HR and LR mouse strains can be induced by exposure to abbreviated infections (5), in which pathology is minimized. This suggests that if appropriate conditions for presenting antigens directly to mucosal surfaces could be found, it should be possible to induce immunity without enteropathology. The mucosal immune response to most orally administered soluble antigens is usually weak, requiring large and frequent doses, which may then result in systemic tolerance (21). A combination of antigen with CT has facilitated induction of high-level mucosal immune responses in several viral, bacterial, and parasitological systems (1, 4, 13, 19) and is thought to act through enhancement of antigen presentation, promotion of immunoglobulin class switching to IgA, and stimulation of cytokine release (12). This approach has been used previously to generate protective immunity against *Trichinella spiralis* (22) with limited results, since only HR mice were protected. A LR strain, unprotected by a range of vaccine formulations (24), was also unaffected by oral CT vaccination.

The results of the present study agree with those of other workers (20) in that the oral mode of vaccination was more efficient in raising a mucosal antibody response. This mucosal response, however, was apparently not as potent as the systemic response to s.c. vaccination in inducing protection against infection. High serum IgG1 levels were detected in all three H+FCA-vaccinated mouse strains, while intestinal IgG1 and IgG2a were detected only in BALB/c mice. Oral vaccinations resulted in mucosal IgA production when protection was demonstrated, i.e., in BALB/c and C57BL/10 mice, but not in the case of the unprotected B10.BR mice; no systemic humoral responses were detected. It is said that oral vaccination should be more relevant for intestinal infections (17), but s.c. FCA vaccination induced protective immunity in all three strains of mice.

The route of vaccine administration has been shown to govern the type of immune response elicited (28), and it is thought that antigen-presenting cells (APCs) at the immunization site have an important role in determining the T-helper-cell subset activated (27). For example, murine splenic APCs have been found previously to be capable of stimulating the proliferation of Th1 and Th2 cell clones, whereas APCs resident in other tissues were capable only of stimulating Th1 clones (16). It is thought that Peyer's patches may contain specialized APCs which selectively promote Th2 cell subset responses (28). Adjuvants have also been shown to determine the type of immune response elicited by vaccination in terms of antibody isotype and T-helper subset activation (3). Modulation of T-helper cytokine responses by vaccination against *T. muris* in the B10.BR mouse strain has been proved possible previously (7). These mice, whose inability to expel a *T. muris* infection is due to the induction of a Th1 response (6), produced a Th2 type response following s.c. vaccination with *T. muris* antigen in Freund's incomplete adjuvant and were able to expel worms.

Classically, FCA should induce Th1-type responses characterized by IFN-γ secretion and IgG2a antibody production (11), and oral vaccination with CT should induce Th2 responses (IL-5, IgG1, and IgA production) (15). If each form of vaccination did elicit such responses against *T. muris*, then vaccination with CT should be highly protective while vaccination with FCA should not, inducing an inappropriate immune response profile for protection. This was not found to be the case. As in *T. spiralis* vaccination studies (22), the main serum IgG antibody isotype detected in FCA-vaccinated mice was IgG1 in each of the strains, even in the B10.BR strain, which then produced a dominant IgG2a response upon subsequent infection with *T. muris* (data not shown). The cytokine data confirm that FCA vaccination did not modulate the response to *T. muris* antigen in favor of Th1 subset activation, and in fact, the reverse was true.

In the case of the HR BALB/c strain, both types of vaccination resulted in high IL-5 and low or no IFN-γ secretion by MLNC, and upon subsequent infection, this pattern remained the same. This cytokine response has been shown to promote worm expulsion (6), and both types of immunization were indeed protective. In the case of the B10.BR strain, however, the protective H+FCA vaccination resulted in low IL-5 secretion in the absence of IFN-γ (as found by other workers [7]). Upon infection, high levels of IFN-γ were detected but IL-5 secretion also continued. The nonprotective oral vaccination did not result in secretion of either of these cytokines, and no intestinal antibody response was detected. Upon infection, high levels of IFN-γ were measured in the absence of IL-5. This type of vaccine therefore failed to induce a relevant T-helper response in these mice, whereas FCA vaccination resulted in a low-level IL-5 secretion, indicating some Th2 subset activation. The data obtained from the C57BL/10 groups was interesting. The protective H+FCA vaccination resulted in cytokine profiles similar to those of the BALB/c strain. High levels of IL-5 were detected in the absence of IFN-γ, and this was unaffected by infection. The oral vaccine induced similar results, but when the mice were subsequently infected, the pattern of cytokine production changed. On day 7 postinfection, the concentrations of IL-5 were reduced by more than half (110 U/ml), while IFN-γ secretion increased to 60 U/ml (data not shown). By day 15 postinfection, no IL-5 was detected but IFN-γ production was high. This cytokine profile would normally indicate an inappropriate immune response for worm expulsion to take place. A possible explanation for the unexpected efficacy of this vaccine may be that the reduction in worm burden on day 15 compared with that of untreated or control groups was a measure of an earlier expulsion event when there was a higher level of Th2 activity.

A relationship between expulsion phenotype and vaccine efficacy was noted in that the highest levels of protection were observed in HR mice, while the lowest levels were obtained in the lowest-responder strain. MLNC of HR mice produced high levels of IL-5 and low levels of IFN-γ as a result of both vaccination (s.c. and oral) and infection. Cells from the B10.BR mice, however, produced low levels of IL-5 following s.c. vaccination and high levels of IFN-γ upon infection. Failure to achieve protection by oral vaccination was correlated with production of no IL-5 or IFN-γ and no detectable intestinal IgA. From these data, it seems clear that vaccination must induce a degree of Th2 cell subset activation if it is to provide protection against *T. muris*. By doing so, it is possible to correct for the genetically determined infection-induced immune responses that are polarized towards ineffective Th1-type responses.

## ACKNOWLEDGMENT

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