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Responses of inbred mouse strains to infection with intestinal nematodes

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Abstract

Comparisons were made of the immune and inflammatory responses of four strains of inbred mice to infection with the intestinal nematodes *Trichinella spiralis* and *Nippostrongylus brasiliensis* to determine whether genetically determined 'high responsiveness' to infection, seen most clearly in intestinal responses, is independent of the parasite concerned and necessarily correlated with protection. The time course of infection was followed by counting adult worms at intervals after infection. Mucosal mast cells and Paneth cell numbers were determined as indices of the intestinal inflammatory response. Levels of IgG2a and IgG1 antibodies and of the cytokines IFN-γ and IL-5 released from *in vitro*-stimulated mesenteric node lymphocytes were measured to assess type 1 and type 2 responses. NIH and CBA mice were the most resistant to *T. spiralis* and *N. brasiliensis* respectively, resistance in each case being correlated with the most intense intestinal inflammatory responses. C57BL/10 (B10) and B10.BR were the least resistant to *T. spiralis*, but were as resistant as CBA to *N. brasiliensis*, despite their intestinal inflammatory responses to both parasites being much lower than the other two strains. Mice infected with *T. spiralis* made the expected switch from a type 1 (IFN-γ) to a type 2 (IL-5) response between days 2 and 8, and there were no significant differences in levels of these cytokines between the strains. In contrast, when infected with *N. brasiliensis*, CBA showed an IFN-γ response at day 4, all strains switching to IL-5 by day 8 and NIH mice releasing the greatest amount of IL-5. The results indicate that the 'high responder' phenotype to intestinal nematode infection is in part determined by host characteristics, but is also determined by the parasite concerned – seen most clearly by the differences between NIH and CBA when infected with *T. spiralis* and *N. brasiliensis*. The fact that 'low responder' B10 background mice were more resistant to *N. brasiliensis* than 'high responder' NIH implies that each parasite elicits a particular pattern of protective host responses, rather than parasites being differentially susceptible to the same response profile.

Introduction

Experimental work with intestinal nematodes in mice has provided information on many aspects of the immune response to parasitic infection. Among the more important of these are the roles of T helper subsets in the host response (Finkelman *et al.*, 1997; Artis & Grencis, 2001), the contribution of inflammatory changes to protective immunity and pathology (Lawrence *et al.*, 2001) and the influence of genetic factors in determining the outcome of infection (Wakelin, 1988). It has been clear for some time that particular strains of mice have characteristic patterns of response to intestinal worm

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infection. For example, NIH strain mice mount a rapid, acute inflammatory response (i.e. are high responders), whereas in mice of the C57BL/10 background (which are low responders) inflammation is much weaker (Alizadeh & Wakelin, 1983; Wakelin & Donachie, 1983). This suggests that the overall capacity of the intestine to respond to an infectious insult is a strain-dependent characteristic. However, the effectiveness of the intestinal response in controlling infection may vary depending upon the species of parasite concerned. For example whereas NIH strain mice control infections with *Trichinella spiralis* and *Trichuris muris* more rapidly than do CBA (Lee & Wakelin, 1982), both can sustain long-term primary infections with *Heligmosomoides polygyrus* (Robinson *et al.*, 1989; Wahid & Behnke, 1993).

An interesting issue arising from such studies is whether different parasites elicit similar intestinal responses but are differentially affected by particular components of these responses, or whether they elicit qualitatively different responses in a given host strain. One approach to answering this question is to carry out comparative studies with these parasites in a range of host strains. This should indicate whether the phenotypes 'high-responsiveness' or 'low responsiveness', which characterize the level of host response to one species, would be seen consistently when another species was used, regardless of the mechanisms involved in host protection. Although there have been several studies examining strain variation in responses to each parasite individually, there have been few adopting such a comparative approach.

In this paper we present the results of infections with *N. brasiliensis* and *T. spiralis* in four strains of inbred mice, whose patterns of response to *T. spiralis* have been described in some detail (Wakelin, 1980; Wassom *et al.*, 1983). These strains exhibit a range of responsiveness from NIH (high) to B10.BR (low). CBA mice have an intermediate phenotype, whilst C57BL/10 (B10), which are congenic with B10.BR, are intermediate to low-responders. In addition to monitoring worm recoveries as an index of resistance, we have measured specific parameters of immune and inflammatory responses. Levels of specific anti-parasite IgG2a and IgG1 antibodies and of the cytokines interferon-gamma (IFN-γ) and interleukin-5 (IL-5) were measured as indices of type 1 and type 2 responses respectively. Numbers of mucosal mast cells (MMC) and Paneth cells were used as a measure of the intestinal inflammatory response. Overall, the results show that genetic influences acted differentially in each host–parasite combination, supporting the view that parasites elicit different combination of host responses.

Materials and methods

Mice

Specific pathogen-free female NIH, CBA, C57 BL/10 and B10.BR mice were obtained from Harlan-Olac UK (Bicester, Oxon, UK) and maintained in the animal house of the School of Life and Environmental Sciences. Mice were used at approximately 8 weeks of age in groups of 4–6.

Parasites

The methods used for *T. spiralis* (London isolate, ISS 25) were as described by Wakelin & Lloyd (1976). Mice were infected orally with 300 larvae. The Moredun strain of *N. brasiliensis* was used as described by Ishikawa *et al.* (1998). Mice were infected subcutaneously with 550 or 1000 larvae. *Trichinella spiralis* enters the intestine immediately, whereas *N. brasiliensis* migrates through the lungs before reaching the intestine. For this reason, the timing for the initial examination of material was day 2 for *T. spiralis* and day 4 for *N. brasiliensis*.

Histology

Intestinal tissues were fixed in Carnoy's fixative for general histology and mucosal mast cell (MMC) counts, or in 10% buffered formalin for Paneth cell counts. The staining techniques were as described by Dehlawi & Wakelin (2002). Mucosal mast cells were counted in a total of 20 villus-crypt units (VCU) per mouse; Paneth cells were counted in 20 crypts per mouse.

Antibody and cytokine analysis

Serum samples were collected at *post mortem*. Levels of parasite-specific IgG1 and IgG2a were measured in ELISA against homogenate antigens prepared from muscle larvae of *T. spiralis* or adult worms of *N. brasiliensis* (Goyal *et al.*, 2002). Briefly, wells of 96-well plates were coated with antigen at $5\,\mu g\,ml^{-1}$ in carbonate/bicarbonate buffer. Serum samples were diluted 1:200 and bound antibody detected with alkaline-phosphatase-conjugated anti-mouse IgG1 or IgG2a (The Binding Site, Birmingham, UK).

Single-cell suspensions were prepared from the mesenteric lymph nodes (MLN) of infected and control mice, and cells (MLNC) cultured *in vitro* at 1×10^7 cells per well with $5\,\mu g\,ml^{-1}$ of the mitogen concanavalin A (ConA) (Sigma) or $25\,\mu g\,ml^{-1}$ of parasite antigen, as described by Hermanek *et al.* (1994). Cytokines were measured by sandwich ELISA, using pairs of cytokine-specific monoclonal antibodies (Pharmingen). For IFN-γ these were R46A2/biotinylated XMG1.2 and for IL-5 were TRFK-5/biotinylated TRFK-4. Recombinant IFN-γ and IL-5 (Pharmingen) were used as standards. Values are recorded in pg ml^{-1}. Because of the logistic difficulties in processing cells from several groups simultaneously, MLNC from individual mice were pooled and cytokine levels determined from duplicate samples. Analysis of responses to the two parasites was carried out on the same plates to ensure that values obtained were directly comparable.

Statistical analysis

Data were analysed using the non-parametric Mann-Whitney test. A value of $P < 0.05$ was considered statistically significant.

Results

Three experiments were carried out, one with *T. spiralis* and two with *N. brasiliensis*.

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Table 1. The numbers of *Trichinella spiralis* and *Nippostrongylus brasiliensis* recovered from four strains of inbred mice after infection with 300 larvae *per os* (*T. spiralis*) or 1000 larvae subcutaneously (*N. brasiliensis*).

	Mean (±S.E) no. of worms recovered			
	T. spiralis (exp. 1)		*N. brasiliensis* (exp. 2)	
Strain of mouse	Day 2	Day 8	Day 4	Day 8
NIH	130.8 ± 8.8	55.3 ± 10.0[a]	121.5 ± 20.9	39.2 ± 13.9
CBA	114.5 ± 4.1	110.0 ± 9.6	128.3 ± 18.9	0.0
C57 BL/10	148.8 ± 13.3	172.3 ± 8.3	173.3 ± 23.8	0.0
B10.BR	95.0 ± 4.3	171.5 ± 6.6	98.5 ± 6.8	0.0

[a] Day 8 mean significantly lower than day 4 ($P < 0.05$).

Trichinella spiralis

Eight mice of each strain were infected with 300 larvae on day 0. Four mice per group were killed on days 2 or 8, worms were recovered, sera and intestinal tissue collected, and MLNC taken for cytokine analysis.

Mean worm recoveries are shown in table 1. On day 2, 31–49% of the infection was recovered from NIH, CBA and B10 mice, although recovery was lower from B10.BR at this time. Similar numbers of worms were present in B10 and B10.BR on day 8, CBA had fewer worms, but not less than day 2, whereas NIH mice had lost a significant proportion of their worms ($P < 0.05$).

No IgG1 antibody was recorded and IgG2a levels were not significantly greater than uninfected controls on either day 2 or day 8 (data not shown). Cytokine data for cells stimulated with ConA are given in table 2. Moderate levels of IFN-γ were released from day 2 cells but no IFN-γ was released from day 8 cells. ConA-stimulated cells from uninfected mice and unstimulated cells from infected mice released no IFN-γ. No IL-5 was released from stimulated day 2 cells, but levels rose sharply by day 8. Unstimulated cells from infected B10 and NIH also released large amounts of IL-5 at this time (data not shown). ConA-stimulated cells from uninfected B10, B10.BR and CBA mice released no IL-5; NIH cells released small amounts (mean = 74 pg ml^{-1}). Overall, values for antigen-stimulated cells were similar to those obtained with ConA.

Mucosal mast cell and Paneth cell responses are shown in fig. 1. Counts of MMC/20 VCU were low in CBA, NIH and B10.BR mice on day 2 and no cells were recorded in B10. A sharp rise occurred by day 8, NIH mice having significantly more MMC than the other strains, and the B10BR significantly less. NIH mice had the largest number of Paneth cells on day 2, but there was a

Table 2. Levels of the cytokines interferon-gamma (IFN-γ) and interleukin-5 (IL-5) released from mesenteric lymph node cells taken from mice after infection with 300 *Trichinella spiralis* or 1000 *Nippostrongylus brasiliensis* and stimulated *in vitro* with ConA.

	Level of cytokines (pg ml^{-1})				
	T. spiralis		*N. brasiliensis*		
	IFN-γ	IL-5	IFN-γ	IL-5	
Strain of mouse	Day 2	Day 8	Day 4	Day 4	Day 8
NIH	199.8	1829.9	0.0	372.9	866.4
CBA	191.6	1646.5	237.1	149.5	30.8
C57Bl/10	271.7	1887.5	0.0	34.6	ND
B10.BR	222.1	2052.3	0.0	71.3	57.2

ND, not done.

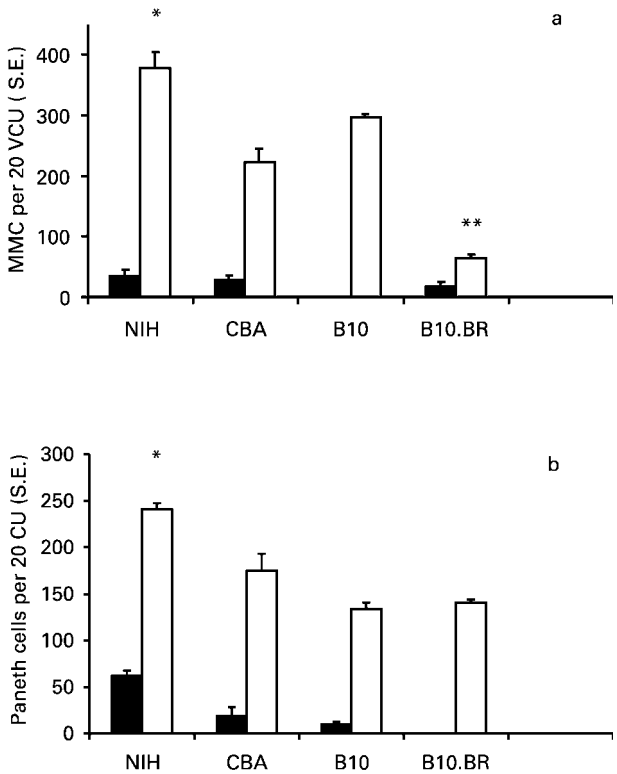

Fig. 1. Mucosal mast cell (MMC) (a) and Paneth cell (b) responses of NIH, CBA, C57BL/10 (B10) and B10.BR mice to infection with 300 larvae of *Trichinella spiralis*. Mice were infected on day 0 and tissues sampled on days 2 (■) and 8 (□). *NIH MMC and Paneth cell counts significantly greater than all other strains ($P < 0.05$). **B10.BR MMC counts significantly lower than all other strains ($P < 0.05$).

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significant increase between days 2 and 8 in all strains by day 8, NIH mice again having the largest number.

Nippostrongylus brasiliensis

Infections were carried out at two levels, 1000 larvae (experiment 2) and 550 larvae (experiment 3). The results of each were similar; those of experiment 2 are given in full. Six mice per group were killed on days 4 or 8, worms were recovered, serum and intestinal tissue collected, and MLNC taken for cytokine analysis.

Worm recoveries are given in table 1. B10 mice had the greatest number at day 4, although not significantly more than CBA and NIH. All strains except NIH had lost all of their worms by day 8, a pattern that was repeated in experiment 3.

No IgG1 responses were recorded on any day. Levels of IgG2a were low at day 4 but had increased significantly in NIH mice by day 8; B10 mice also showed an increase (fig. 2). Cytokine data from ConA stimulated cells are shown in table 2. Only day 4 cells from CBA mice released IFN-γ. No IFN-γ was released by unstimulated cells, by stimulated cells from uninfected mice or from day 8 cells. At day 4, both NIH and CBA mice released IL-5 when stimulated with ConA. Levels of IL-5 were maintained, although values were slightly lower, at day 8 except in NIH mice. As with *Trichinella spiralis* infections, unstimulated cells from infected NIH mice also released IL-5 at day 8. Values obtained with antigen-stimulated cells were irregular.

Mucosal inflammatory responses are shown in fig. 3. Increased MMC were present in CBA and NIH mice on day 4, and numbers in these strains rose sharply by day 8, being significantly greater in CBA. These strains also showed the greatest Paneth cell response.

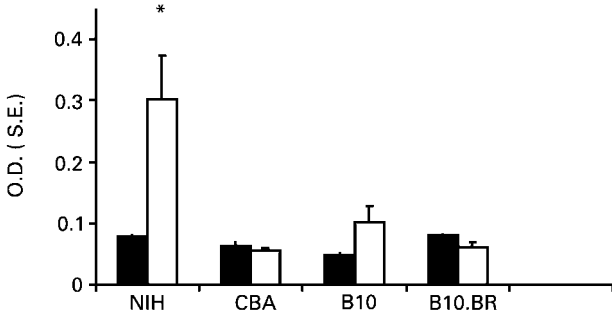

Fig. 2. Parasite-specific IgG2a responses of NIH, CBA, C57BL/10 (B10) and B10.BR mice after infection with 1000 larvae of *Nippostrongylus brasiliensis* on days 4 (■) and 8 (□). *NIH values significantly greater than all other strains ($P < 0.05$).

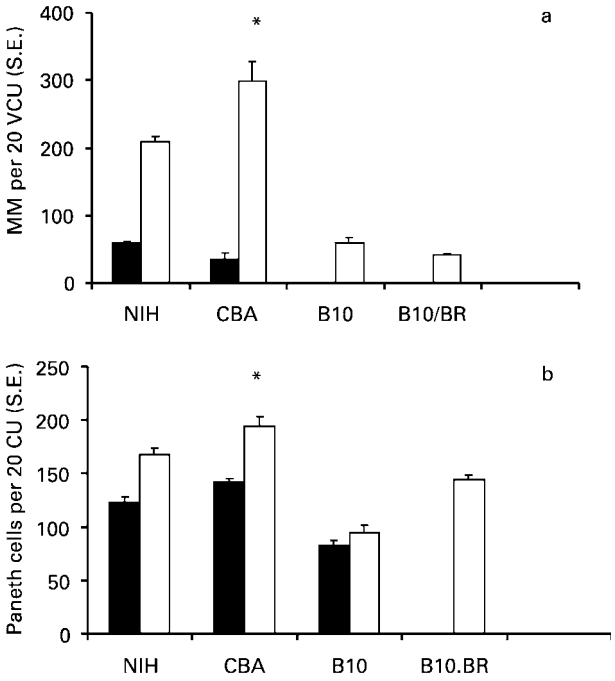

Fig. 3. Mucosal mast cell (MMC) (a) and Paneth cell (b) responses of NIH, CBA, C57BL/10 (B10) and B10.BR mice to infection with 1000 larvae of *Nippostrongylus brasiliensis*. Mice were infected on day 0 and tissues sampled on days 4 (■) and 8 (□). *CBA MMC and Paneth cell counts significantly greater than all other strains ($P < 0.05$).

Discussion

Recent studies have shown that there are significant functional differences in the responses of mice to infections with *N. brasiliensis* and *T. spiralis* (Urban *et al.*, 1998, 2000; Artis & Grencis, 2001). Whereas the Th2 lymphocyte signalling and transduction pathway involving STAT 6 is crucial for immunity to both, the type 2 cytokine IL-4 is of primary importance for immunity against *T. spiralis* and IL-13 for *N. brasiliensis*. Mucosal mast cells appear important only in expulsion of *T. spiralis* (Faulkner *et al.*, 1997). Although these, and similar, studies have provided valuable detail about the components required for protective host responses, few have made comparisons based on infections with each species in specific strains of mice. Such experiments should help to clarify whether different parasites elicit similar intestinal responses but are differentially affected by particular components of these responses, or whether they elicit qualitatively different responses in a given host strain.

The experiments described here were designed to provide details on particular aspects of the host's immune and inflammatory responses and relate these to specific combinations of parasite species and host strain. Although the host's protective response against both parasites is expressed against the adult worms in the intestine, it is important to remember that, in the case of *N. brasiliensis*, this phase is preceded by a systemic larval migration, providing an initial antigenic stimulus.

A number of important points emerge from the data:

1. NIH mice were the most resistant to *T. spiralis*, but least resistant to *N. brasiliensis.*
2. The intestinal inflammatory response to *T. spiralis* was greatest in NIH, that to *N. brasiliensis* greatest in CBA. B10 and B10.BR mice had the weakest intestinal inflammatory response to both parasites, but showed good resistance to *N. brasiliensis*.

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3. Systemic antibody responses were low in all cases. No IgG1 antibody was recorded; anti-*N. brasiliensis* IgG2a responses were highest in NIH.
4. The cytokine response to both *T. spiralis* and *N. brasiliensis* showed a switch to a predominant type 2 response later in infection; quantitatively this was greatest in cells taken from *T. spiralis*-infected mice. All strains made a strong IL-5 response to *T. spiralis*, but NIH were the only strain to respond strongly to *N. brasiliensis*.

The high responsiveness of NIH to *T. spiralis*, in comparison with other inbred strains, is well documented (Wakelin, 1988). No similar comparative studies involving NIH mice have been made with *N. brasiliensis*, but it is clear that loss of worms from this strain is much slower than in CBA, B10 and B10.BR. It is well known that NIH mice make strong intestinal responses to *T. spiralis* but the data presented here on MMC and Paneth cell numbers suggest that the strength of the response is, at least in part, the product of a particular host–parasite combination, as the inflammatory response of CBA to *N. brasiliensis* was greater than that of NIH, but that to *T. spiralis* was lower. Mucosal mast cell responses are to a large degree dependent on the activity of T helper 2 (Th2) cells (Madden *et al.*, 1991) although many other factors also are involved. Cells from *N. brasiliensis*-infected CBA mice released much less IL-5 than NIH, suggesting a reduced type 2 cytokine response, yet MMC numbers were significantly greater. This may show that CBA have a greater bone marrow-related capacity to produce MMC precursors, or produce more of the cytokines that are directly related to MMC production. Paneth cell responses are also T cell dependent (Kamal *et al.*, 2001), but less clearly type 2 dependent, and CBA mice again made the greatest response to *N. brasiliensis*. In contrast to these results, NIH mice made the greatest antibody response to *N. brasiliensis*, although the isotype involved was IgG2a, primarily (although not exclusively) a type 1 isotype. This is consistent with the results of Robinson *et al.* (1995), who also found that the early response to *T. spiralis* was IgG2a dominated.

In the absence of a clear understanding of the precise mechanisms that lead to worm expulsion, it is difficult to fully interpret the data presented here in terms of anti-parasite activity. The inflammatory and immunological components measured were chosen because they are easily monitored, and give an overall index of the host's response capacity, rather than because (with the exception of MMC) they are known definitely to be of key importance in protection. However, the data do imply that there are qualitative and quantitative differences between *T. spiralis* and *N. brasiliensis* in their capacity to elicit particular components of the host's intestinal responses, and that, at least in NIH and CBA mice, these differences override the host's genetically determined capacity to mount an intestinal response. An interesting observation is that the B10 background mice, though mounting a much-reduced intestinal inflammatory response compared with either NIH and CBA, nevertheless show a level of immunity to *N. brasiliensis* that is comparable with that in CBA, but are markedly less resistant than NIH to *T. spiralis*, again emphasizing the importance of parasite factors in the overall outcome of infection.

References

Alizadeh, H. & Wakelin, D. (1983) Genetic factors controlling the intestinal mast cell response in mice infected with *Trichinella spiralis*. *Clinical and Experimental Immunology* **49**, 331–337.

Artis, D. & Grencis, R.K. (2001) T helper cell cytokine responses during intestinal nematode infection: induction, regulation and effector function. pp. 331–371 *in* Kennedy, M.W. & Harnett, W. (*Eds*) *Parasitic nematodes. Molecular biology, biochemistry and immunology*. Wallingford, Oxon, CABI Publishing.

Dehlawi, M.S. & Wakelin, D. (2002) Parameters of intestinal inflammation in mice given graded infections of the nematode *Trichinella spiralis*. *Journal of Helminthology* **76**, 113–117.

Faulkner, H.F., Humphreys, N.H., Renauld, J.C., Van Snick, J. & Grencis, R.K. (1997) Interleukin-9 is involved in host-protective immunity to intestinal nematode infection. *European Journal of Immunology* **27**, 2536–2540.

Finkelman, F.D., Shea-Donohue, T., Goldhill, J., Sullivan, C.A., Morris, S.C., Madden, K.B., Gause, W.C. & Urban, J.F. (1997) Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. *Annual Reviews of Immunology* **15**, 505–534.

Goyal, P.K., Wheatcroft, J. & Wakelin, D. (2002) Tyvelose and protective responses to the intestinal stages of *Trichinella spiralis*. *Parasitology International* **51**, 91–98.

Hermanek, J.P., Goyal, P.K. & Wakelin, D. (1994) Lymphocyte, antibody and cytokine responses during concurrent infections between helminths that selectively promote T helper 1 or T helper 2 activity. *Parasite Immunology* **16**, 111–117.

Ishikawa, N., Goyal, P.K., Mahida, Y.R., Li, K-F. & Wakelin, D. (1998) Early cytokine responses during intestinal parasitic infections. *Immunology* **93**, 257–263.

Kamal, M., Wakelin, D., Oulette, A.J., Smith, A., Podolsky, D.K. & Mahida, Y.R. (2001) Mucosal T cells regulate Paneth and intermediate cell numbers in the small intestine of *Trichinella spiralis*-infected mice. *Clinical and Experimental Immunology* **126**, 117–125.

Lawrence, C.E., Kennedy, M.W. & Garside, P. (2001) Gut immunopathology in helminth infections – paradigm lost. pp. 373–397 *in* Kennedy, M.W. & Harnett, W. (*Eds*) *Parasitic nematodes. Molecular biology, biochemistry and immunology*. Wallingford, Oxon, CABI Publishing.

Lee, T.D.G. & Wakelin, D. (1982) The use of host strain variation to assess the significance of mucosal mast cells in the spontaneous cure response of mice to the nematode *Trichuris muris*. *International Archives of Allergy and Applied Immunology* **67**, 302–305.

Madden, K.B., Urban, J.F., Zitener, H.J., Schrader, J.W., Finkelman, F.D. & Katona, I. (1991) Antibodies to IL-3 and IL-4 suppress helminth-induced intestinal mastocytosis. *Journal of Immunology* **147**, 1387–1392.

Robinson, K., Bellaby, T. & Wakelin, D. (1995) Immune response profiles in vaccinated and non-vaccinated

https://doi.org/10.1079/JOH2003175 Published online by Cambridge University Press

high- and low-responder mice during infection with the intestinal nematode *Trichinella spiralis*. *Parasitology* **110**, 71–78.

Robinson, M., Wahid, F.N., Behnke, J.M. & Gilbert, F.S. (1989) Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): dose-dependent expulsion of adult worms. *Parasitology* **98**, 115–124.

Urban, J.F., Noben-Trauth, N., Donaldson, D.D., Madden, K.B., Morris, S.C. & Finkleman, F.D. (1998) IL-13, IL-4R alpha and Stat6 are required for the expulsion of the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*. *Immunity* **8**, 255–264.

Urban, J.F., Schopf, L., Morris, S.C., Orekhova, T., Madden, K.B., Betts, C.J., Gamble, H.R., Byrd, C., Donaldson, D., Else, K.J. & Finkleman, F.D. (2000) Stat 6 signaling promotes protective immunity against *Trichinella spiralis* through a mast cell- and T cell-dependent mechanism. *Journal of Immunology* **164**, 2046–2052.

Wahid, F.N. & Behnke, J.M. (1993) Immunological relationships during primary infection with *Heligmosomoides polygyrus*. Regulation of fast responder phenotype by H-2 and non-H-2 genes. *Parasitology* **107**, 343–350.

Wakelin, D. (1980) Genetic control of immunity to parasites. Infection with *Trichinella spiralis* in inbred and congenic mice showing rapid and slow responses to infection. *Parasite Immunology* **2**, 85–98.

Wakelin, D. (1988) Helminth infections. pp. 153–224 *in* Wakelin, D. & Blackwell, J.M. (*Eds*) *Genetics of resistance to bacterial and parasitic infection*. London, Taylor & Francis.

Wakelin, D. & Donachie, A.M. (1983) Genetic control of eosinophilia. Mouse strain variation in response to antigens of parasite origin. *Clinical and Experimental Immunology* **51**, 239–246.

Wakelin, D. & Lloyd, M. (1976) Immunity to primary and challenge infections of *Trichinella spiralis* in mice: a re-examination of conventional parameters. *Parasitology* **72**, 173–182.

Wassom, D.L., Brooks, B.O., Cypess, R.H. & David, C.S. (1983) A survey of susceptibility to infection with *Trichinella spiralis* of inbred mouse strains sharing common H-2 alleles but different genetic backgrounds. *Journal of Parasitology* **69**, 1033–1037.

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