INFECTION AND IMMUNITY, Feb. 1994, p. 733–737
0019-9567/94/$04.00+0

Vol. 62, No. 2

Immunization against Experimental Coccidiosis Produces Contrasting Results in Inbred Mice of Differing Susceptibility to Infection

M. ELAINE ROSE,[1]* PATRICIA HESKETH,[1] AND D. WAKELIN[2]

Department of Immunology and Pathology, Institute for Animal Health, Compton, Nr Newbury, Berkshire RG16 ONN,[1] and Department of Life Science, University of Nottingham, University Park, Nottingham NG7 2RD,[2] United Kingdom

Received 7 September 1993/Returned for modification 15 October 1993/Accepted 15 November 1993

Pretreatment of inbred mice with intravenous and/or intraperitoneal injection of an antigen prepared from sporozoites of *Eimeria vermiformis* modulated the course of infection with the parasite in a manner that depended on the resistance-susceptibility phenotype of the host. Mice with a resistant background (BALB) produced more oocysts and those with a susceptible background (C57BL) produced fewer oocysts than their respective controls. The optimum conditions for producing these effects were established, and evidence is presented which suggests that the phenomenon might also apply in the target host, the chicken.

Coccidiosis is an economically important disease of domestic livestock, especially poultry, caused by protozoan parasites of the genus *Eimeria*. In the poultry industry, coccidiosis is controlled mainly by prophylactic medication, although live vaccines are used for a small and specialized proportion of flocks. Vaccines in current use consist of live parasites whose pathogenic effects are mitigated either by controlling the number of organisms administered or by attenuating their virulence (22). Both systems have disadvantages, including drug resistance for the first and high cost and the problems of handling living, potentially unstable material for the second. Consequently, there is interest in the development of vaccines consisting of nonliving parasite-derived material, including recombinant forms (2, 10). Success to date has been limited, with the achievement of only partial and variable protection (reviewed in reference 2). From the results of experiments carried out with inbred lines of chickens, it is clear that both MHC-linked (8) and non-MHC-linked (9) genes influence the effects of vaccination, the latter perhaps being of greater importance.

We have found that infection of inbred mice with *Eimeria vermiformis* provides a particularly useful model for investigating the immunology of coccidiosis. It has enabled us to show that $CD4^+$ lymphocytes are effective in controlling primary infections, without the essential participation of an inflammatory response, and that gamma interferon (IFN-γ) plays an important role in this process (reviewed in reference 25). Some of this work has exploited the marked strain variation in susceptibility to infection with *Eimeria* spp. found between inbred strains of mice (5, 13, 17, 23). In primary infections of the BALB mouse, the replication of *E. vermiformis* is curtailed by a prompt immune response, i.e., this strain shows a resistant phenotype, whereas infection runs a prolonged course in the susceptible C57BL mouse (16). This difference in phenotype arises from temporal rather than qualitative (20) variation in the immune response to primary infection, and both strains are completely resistant to challenge infection. We have now extended our use of this model to investigate the potential of nonviable material prepared from *E. vermiformis* to immunize against homologous infection in the two strains of mice. Our results show that treatment with sporozoite-derived material produces contrasting effects in BALB and C57BL mice, and there are indications, from preliminary experiments, that a similar variation may occur in chickens.

Animals, parasites, and antigens. BALB/c, BALB/B, C57BL/6 (B6), and C57BL/10 (B10) female mice were obtained from Harlan Olac Ltd. (Shaw's Farm, Bicester, Oxford) and kept as described previously (17). BALB/B and B10 mice are $H\text{-}2^b$ compatible. Chickens of the N and Brown Leghorn (BL) lines were bred at Houghton and produced at the Compton Laboratories of the Institute for Animal Health. They were used, unsexed, at 3 weeks of age and kept as described before (15). The origin of *E. vermiformis*, the methods for its propagation, and the measurement of infection by estimation of the passage of oocysts in daily collections of the total fecal output of individual mice throughout patency have been described (17). *Eimeria tenella* was the H.P.R.S. strain, handled in accordance with the standard methods of the laboratory (12). Antigens were prepared from sporulated oocysts (14) or from purified (18) sporozoites suspended (5×10^8 ml) in 0.1 mM phenylmethylsulfonyl fluoride for disintegration by ultrasound (Soniprep 150 MSE; eight 20-s bursts) and three cycles of freeze-thawing in liquid nitrogen. The sporulated oocyst antigen (diluted 1:100, equivalent to 5×10^3 oocysts) was used in an enzyme-linked immunosorbent assay (19) to measure antibodies in the serum of mice.

Design of experiments. After "immunization" (see below), animals were challenged orally with an inoculum of 10^3 oocysts of *E. vermiformis* (mice) or 10^2 oocysts of *E. tenella* (chickens). The ensuing infections were measured (17) by fecal oocyst output. Control groups consisted of animals that were untreated or injected with phosphate-buffered saline (PBS) before challenge. Blood samples were obtained from the tails of mice for estimation of serum antibodies to *E. vermiformis* immediately before and 14 days after challenge. The numbers of animals in each group are given in the figure legends and the table, and the results were analyzed by Student's *t* test.

Experiments with mice. Initial attempts to influence parasite replication in BALB/B, B6, and NIH mice by a course of subcutaneously (s.c.) injected antigen of either type, sporulated oocyst or sporozoite, given in various adjuvants gave

* Corresponding author. Phone: (635) 578411. Fax: (635) 578844.

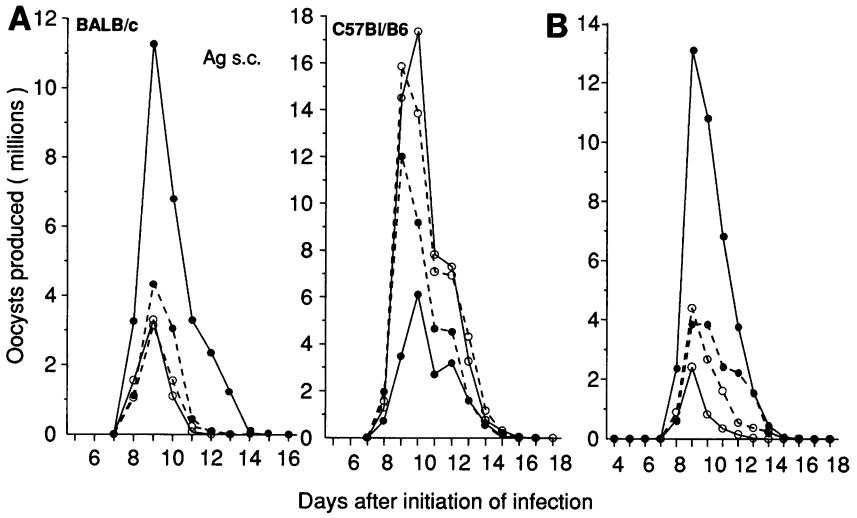

FIG. 1. Effect of antigen treatment on the course of infection with *E. vermiformis* in BALB (resistant) and C57BL (susceptible) mice. (A) BALB/c and C57BL/6 mice treated with antigen (●) or mock-treated with PBS (○) by the s.c. (– – –) or i.v./i.p. (——) route. (B) B10 (●) and BALB/B (○) mice treated with antigen (– – –) or mock-treated with PBS (——). Treatment with antigen consisted of three injections, each equivalent to 4×10^6 sporozoites (in 0.2 ml), at intervals of 7 days. The infective inoculum of 10^3 oocysts of *E. vermiformis* was given 3 days after the third injection of antigen or PBS. Values plotted are means for groups of seven or eight mice. Within strains, total oocyst production differed significantly ($P < 0.05$) between i.v./i.p. antigen-injected groups on the one hand and between s.c. antigen-injected (A) and PBS-injected (A) or untreated (B) groups on the other.

uniformly negative results (unpublished observations), and this stimulated us to investigate other protocols. Schedules in which three doses of sporozoite antigen were injected, either twice intravenously (i.v.) and once intraperitoneally (i.p.) (i.v./i.p. route) or thrice s.c., at intervals of 7 days were used in the first instance. Each injection of antigen (0.2 ml) was equivalent to 4×10^6 sporozoites, and the challenge inoculum of oocysts was given 3 days after the last antigen injection. A number of experiments of this type, involving three different preparations of antigen, yielded consistent results. Whereas s.c. injections of antigen had no effect on oocyst production, this was significantly affected by the i.v./i.p. route in a strain-dependent manner. The number of oocysts was increased in resistant BALB/c mice (six experiments) but reduced in susceptible B6 mice (four experiments); the difference varied with the antigen preparation but ranged from three- to sixfold, and there was a greater tendency for the duration of patency to be altered in BALB/c than in B6 mice.

The results of representative experiments are illustrated in Fig. 1A, which also shows the difference in parasite replication in untreated mice of the two strains. The differences between the strains in the effect of treatment on the replication of the parasite was not reflected in their antibody responses (data not shown). There was some variation with different preparations of antigen, and B6 mice tended to have lower levels of antibody than BALB/c mice, but this was not always statistically significant. The antibody response to s.c. injection was less than that to i.v./i.p. injection; values were increased by subsequent infection with the parasite in all mice.

The modulating effects of antigen injection were not affected by MHC haplotype, as shown by the results (Fig. 1B) obtained when BALB/B and B10 mice were treated by the i.v./i.p. route before challenge with sporulated oocysts of *E. vermiformis*. BALB/B mice were rendered more and B10 mice were rendered less susceptible than the corresponding controls, similar to BALB/c and B6 mice.

Thus, i.v./i.p. antigen treatment reversed the strain susceptibility to infection normally seen in mice of the BALB and C57BL backgrounds. This effect was defined further, as described below.

Concentration of antigen. The effect of antigen concentration on the enhancement of oocyst production in BALB/c mice was examined in two experiments with different preparations of antigen in which the protocol used was two i.v. injections followed by one i.p. injection, with a challenge inoculum of oocysts given 3 days later. In both experiments, oocyst production varied directly with the antigen concentration (Table 1). The exacerbation of infection by antigen treatment was also evident as an extension of the patency period. Serum antibodies were produced as before, and variations in the concentration of antigen within the range tested had little or no effect on

TABLE 1. Enhancement of replication of *E. vermiformis* in BALB/c mice by injection of sporozoite antigen is concentration dependent[a]

Antigen prepn	Antigen injected/dose (sporozoite equivalent)	Results of challenge inoculum[b]	
		Mean no. (10^6) of oocysts produced ± SEM	Patency (days)
A89	4×10^6	22.7 ± 5.8*	6.7 ± 0.3*
	2×10^6	12.2 ± 1.3†	5.0 ± 0†
	1×10^6	9.9 ± 1.1†	4.8 ± 0.3†
	0 (PBS)	4.2 ± 0.7§	4.0 ± 0†
A91	4×10^6	20.7 ± 3.4*	6.7 ± 0.2*
	2×10^6	11.4 ± 1.8†	5.9 ± 0.5†
	1×10^6	9.9 ± 1.0†	5.4 ± 0.2†
	0 (PBS)	3.3 ± 0.5§	4.6 ± 0.2§

[a] All mice received three injections of 0.2 ml each, two i.v. followed by one i.p., at weekly intervals. The challenge inoculum was 10^3 sporulated oocysts of *E. vermiformis* given 3 days after last injection of antigen.

[b] Values are means for groups of five to seven mice. Within columns and experiments, different superscripts indicate that the values differ significantly from each other ($P < 0.05$).

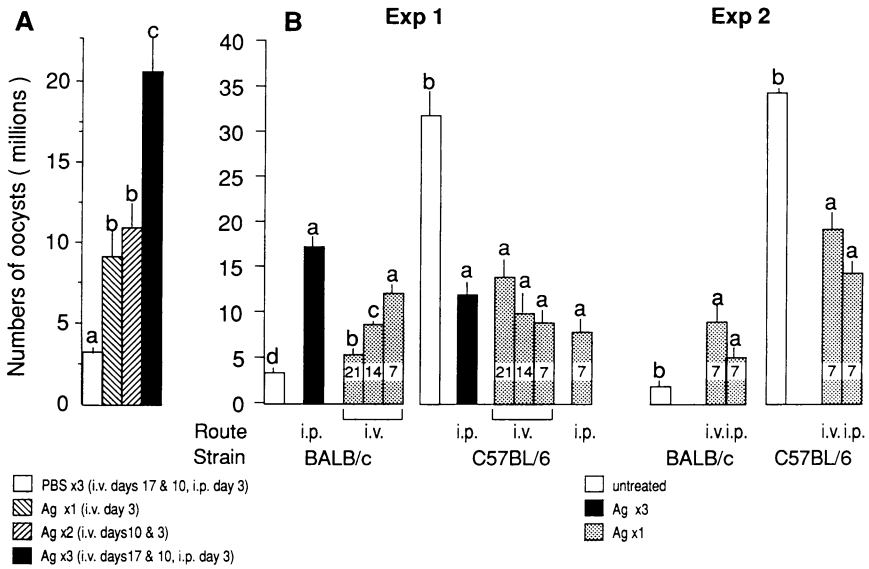

FIG. 2. Effect of different schedules of antigen treatment on subsequent resistance to challenge with *E. vermiformis* in BALB/c and C57BL/6 mice. (A) Variation in the number of antigen injections (BALB/c mice). (B) Variation in timing of a single injection. Each injection of antigen (Ag) was the equivalent of 4×10^6 sporozoites in 0.2 ml. Days of treatment (shown within the columns in panel B) refer to the number of days before oral challenge with 10^3 sporulated oocysts. When the antigen was administered in three doses, injections were given on days 21, 14, and 7 before challenge. The values are mean number of oocysts per mouse ± standard error of the mean for groups of six or seven mice. Different letters indicate that values (within an experiment and strain) differ significantly from each other ($P < 0.05$).

this response either before or after challenge (data not shown). Antigen used at a concentration equivalent to 4×10^6 sporozoites per dose produced between five- and sixfold enhancement of infection in BALB/c mice, and this concentration was used in most of the subsequent experiments.

Variation in the schedule of antigen treatment. Different schedules of antigen injection were examined in an attempt to expedite and facilitate the priming of mice for altered resistance or susceptibility. The results obtained with one antigen preparation are given in Fig. 2. For BALB/c mice (Fig. 2A), the schedule was based on the standard i.v./i.p. protocol, as above, but with omissions; for BALB/c and B6 mice (Fig. 2B), a single dose of antigen was given either i.v. or i.p. on different days. The greatest enhancement of oocyst production in BALB/c mice was produced in response to three injections of antigen either as the standard treatment (i.v./i.p.) or i.p., but even a single injection was effective and was even more effective when given 1 week, rather than 2 or 3 weeks, before challenge (Fig. 2B, experiment 1). Experiments with B6 mice yielded similar but reverse results, showing that a single injection of antigen given either i.v. or i.p. 7 days before challenge could reduce oocyst production (Fig. 2B). Indeed, in this strain, a single injection was as effective as a course of three injections (Fig. 2B, experiment 1). In general, the effects on patency reflected those on the total number of oocysts produced (data not shown). The levels of circulating antibodies (measured immediately before challenge) reflected the schedule of immunization, i.e., the highest levels were found in mice given multiple injections, followed by those given the single dose 14 days previously (data not shown). Their relationship to oocyst production was direct for BALB/c mice and inverse for B6 mice. Subsequent infection had a boosting effect on antibody levels.

The results of these experiments show that identical treatment of two inbred mouse strains with a parasite-derived antigen had contrasting effects on the outcome of a subsequent infection with the homologous organism. Infection in the normally resistant BALB mouse was exacerbated, whereas the comparatively susceptible C57BL mouse was substantially protected. The reversal in host phenotype thus produced was striking in some experiments; the number of oocysts passed by treated BALB mice approached that found in untreated C57BL mice, and vice versa (Fig. 1). The effect, like that on resistance or susceptibility in untreated mice (4), was independent of H-2 haplotype and was quickly elicited by comparatively small amounts of this crude whole-sporozoite antigen by the i.v. and/or i.p. route. S.c. administration of this and sporulated oocyst antigen, even when given with adjuvants, had previously been found to have no effect on subsequent challenge infections. The effects on infection could not be correlated with the serum antibody responses elicited by treatment, although the levels immediately before challenge tended to be higher in BALB/c than in B6 mice; they were similarly boosted by infection.

The susceptibility of mice to infection with *Leishmania major* can be altered by immunization with nonviable parasite material, but the effects differ markedly from those reported here for *E. vermiformis*. With *L. major*, the route of immunization determines the outcome, not the resistance phenotype of the host. In both susceptible (BALB background) and resistant (C57BL and CBA) strains of mice, protection is afforded by i.v. or i.p. immunization with *Leishmania* antigen, whereas treatment given s.c. exacerbates disease and can even produce a long-lasting blockage of the prophylactic effect of i.v. injection (7). Extensive work on this system has subsequently revealed that the preferential induction of a Th-2 cell response promotes disease, both after s.c. immunization and in infection in untreated susceptible, as opposed to resistant, strains of mice (6, 11, 21), and that this effect may be determined by the nature of the antigen-presenting cell (3). Whether i.v./i.p. immunization with *Eimeria* sporozoite antigen induces an inappropriate T-cell response in BALB mice, as opposed to an

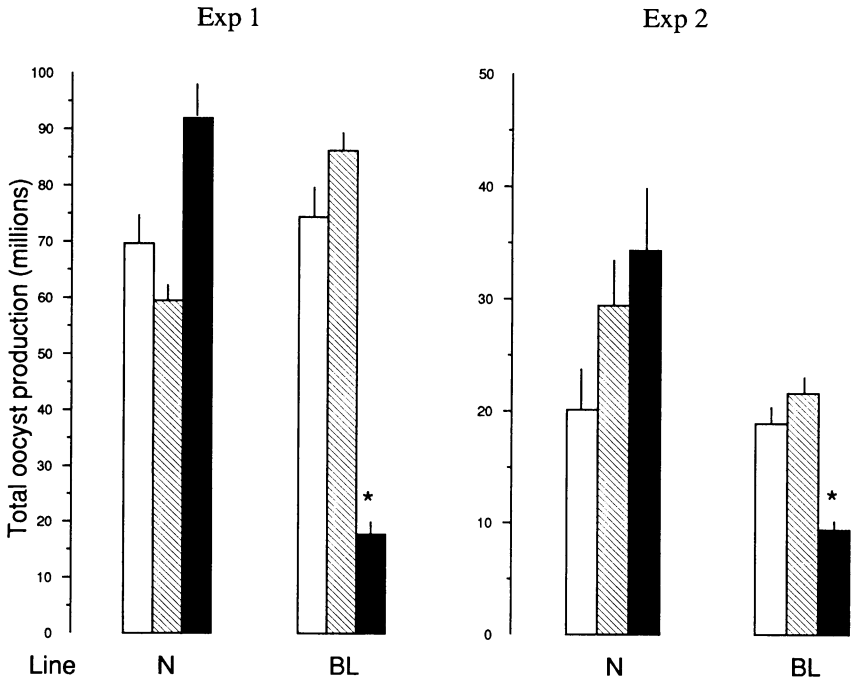

FIG. 3. Effect of treatment with *E. tenella* sporozoite antigen on subsequent infection with *E. tenella* in inbred chickens. Three doses of antigen, each equivalent to 4×10^6 sporozoites (in 0.2 ml), were given either i.v. (solid bars) or i.p. (hatched bars) before the chickens were orally challenged with 10^2 sporulated oocysts of *E. tenella*; all intervals were 7 days. Open bars, untreated chickens. The values are means ± standard error of the mean for groups of 8 to 10 chickens. *, value differs significantly ($P < 0.05$) from those for the other groups in the experiment; no other differences were significant (comparisons between lines within an experiment).

appropriate one in C57BL mice, must be determined. The differential susceptibility of the untreated strains to infection, however, does not appear to be due to a mechanism of this kind, since the cytokine responses of their mesenteric lymph node cells to restimulation in vitro differ more in kinetics than in kind (26).

In attempts to vaccinate against nematodes, for example, *Trichinella spiralis* (24), the results differ yet again. The outcome of s.c. injection of antigen does depend on the response phenotype of the mouse to the parasite in question, but resistant strains are easily immunized (i.e., expulsion of worms is accelerated), whereas the kinetics of challenge infections in low-responder strains are unaltered. It is likely, in this case, that immunization is ineffective in susceptible strains because of their relative inability to translate immune responsiveness into the intestinal inflammatory responses required for worm expulsion rather than because they fail to respond immunologically to the antigens given.

Experiments with inbred chickens. To determine whether the different effects of antigen treatment on subsequent challenge infection described above for mice might also be apparent in the target host, two experiments were carried out in inbred chickens. Two lines, N and BL, selected for resistance and susceptibility, respectively, to *E. tenella* from the results of Bumstead and Millard (1), were treated with a sporozoite antigen prepared from this parasite. The schedule was based on that found to be most effective in mice and consisted of three doses, each equivalent to 4×10^6 sporozoites, given either i.v. or i.p. before the chickens were challenged with 10^2 sporulated oocysts of *E. tenella*; all intervals were 1 week. The results (Fig. 3) show that i.p. treatment with antigen had no effect on the replication of the parasite in either line, whereas i.v. treatment significantly depressed oocyst production in the BL line (reportedly susceptible). The number of oocysts was increased by i.v. treatment in the reportedly resistant N line, but because of variations in the values obtained for individual birds, this was not statistically significant. Within experiments, untreated birds of the two lines passed similar numbers of oocysts.

The abilities of various recombinant antigens to induce T-cell responses and protection against challenge infections are known to differ in different lines of inbred chickens (8, 9), but to our knowledge, there have been no reports of induction of greater susceptibility by antigen treatment or even of reversal of comparative host susceptibility phenotype. Indeed, in the investigations referred to above, T-cell response to antigen treatment and the development of partial protection to challenge were greater in lines of birds that were normally comparatively resistant to infection (8, 9). Our experiments with inbred birds were limited and complicated by the fact that, in our hands, the lines did not show the contrasting susceptibility to infection with *E. tenella* that had been the basis for their original selection (1). Nevertheless, the results reflected, at least to some extent, those obtained with the mice: the reputedly susceptible BL line was protected by antigen treatment, and the reputedly resistant line N was not (the increased mean number of oocysts passed by treated birds of this line indicates enhanced susceptibility, but the interbird variation was such that the difference was not statistically significant). In contrast to the mice, i.p. injections of antigen had no effect on subsequent challenge infections. This may be related to the different anatomy of mammals and birds, including the absence of mesenteric lymph nodes in the latter.

Our results have shown interesting differences in the effects

of treatment with *Eimeria* antigens on subsequent challenge infections and may have important theoretical and practical implications for the immunology of coccidial infections. It is encouraging that we were able, consistently and unequivocally, to protect hosts with a susceptible phenotype, but disturbing that identical treatment resulted in an exacerbation of infection in resistant hosts. Further work will be directed towards understanding the mechanisms involved.

We thank Angela Stebbings for technical assistance, the staff of the animal houses at the Houghton and Compton laboratories for care of the animals and collection of fecal samples, and Sam Cook for typing the manuscript.

This work was supported in part by grant 031118 from the Wellcome Trust.

REFERENCES

1. **Bumstead, N., and B. Millard.** 1987. Genetics of resistance to coccidiosis: responses of inbred chicken lines to infection by *Eimeria tenella* and *Eimeria maxima*. Br. Poultry Sci. **28:**705–715.
2. **Danforth, H. D., P. C. Augustine, and M. C. Jenkins.** 1993. A review of progress in coccidial vaccine development, p. 49–60. *In* J. R. Barta and M. A. Fernando (ed.), Proceedings of the VIth International Coccidiosis Conference. University of Guelph, Ontario, Canada.
3. **Doherty, T. M., and R. L. Coffman.** 1993. Leishmania antigens presented by GM-CSF-derived macrophages protect susceptible mice against challenge with *Leishmania major*. J. Immunol. **150:** 5476–5483.
4. **Joysey, H. S., D. Wakelin, and M. E. Rose.** 1988. Resistance to infection with *Eimeria vermiformis* in mouse radiation chimeras is determined by donor bone marrow cells. Infect. Immun. **56:**1399–1401.
5. **Klesius, P. H., and S. E. Hinds.** 1979. Strain-dependent differences in murine susceptibility to coccidia. Infect. Immun. **26:**1111–1115.
6. **Liew, F. Y.** 1989. Functional heterogeneity of $CD4^+$ T cells in leishmaniasis. Immunol. Today **10:**40–45.
7. **Liew, F. Y., C. Hale, and J. G. Howard.** 1985. Prophylactic immunization against experimental leishmaniasis. VI. Subcutaneous immunization prevents the induction of protective immunity against fatal *Leishmania major* infection. J. Immunol. **135:**2095–2101.
8. **Lillehoj, H. S., M. C. Jenkins, and L. D. Bacon.** 1990. Effects of major histocompatibility genes and antigen delivery on induction of protective mucosal immunity to *E. acervulina* following immunization with a recombinant merozoite antigen. Immunology **71:**127–132.
9. **Lillehoj, H. S., M. C. Jenkins, L. D. Bacon, R. H. Fetterer, and W. E. Briles.** 1988. *Eimeria acervulina*: evaluation of the cellular and antibody responses to the recombinant coccidial antigens in B-congenic chickens. Exp. Parasitol. **67:**148–158.
10. **Lillehoj, H., and J. M. Trout.** 1993. Coccidia: a review of recent advances on immunity and vaccine development. Avian Pathol. **22:**3–31.
11. **Locksley, R. M., and P. Scott.** 1991. Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function, p. 1758–1761. *In* C. Ash and R. B. Gallagher (ed.), Immunoparasitology today. Elsevier Trends Journals, Cambridge, U.K.
12. **Long, P. L., L. P. Joyner, B. J. Millard, and C. C. Norton.** 1976. A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. Folia Vet. Lat. **6:**201–217.
13. **Mahrt, J. L., and Y. Shi.** 1988. Murine major histocompatibility complex and immune responses to *Eimeria falciformis*. Infect. Immun. **56:**270–271.
14. **Rose, M. E.** 1977. *Eimeria tenella*: skin hypersensitivity to injected antigen in the fowl. Exp. Parasitol. **42:**129–141.
15. **Rose, M. E., and P. Hesketh.** 1987. *Eimeria tenella*: effects of immunity on sporozoites within the lumen of the small intestine. Exp. Parasitol. **63:**337–345.
16. **Rose, M. E., and B. J. Millard.** 1985. *Eimeria vermiformis*: host strains and the developmental cycle. Exp. Parasitol. **60:**163–172.
17. **Rose, M. E., D. G. Owen, and P. Hesketh.** 1984. Susceptibility to coccidiosis: effect of strain of mouse on reproduction of *Eimeria vermiformis*. Parasitology **88:**45–54.
18. **Rose, M. E., A. L. Smith, and D. Wakelin.** 1991. Gamma interferon-mediated inhibition of *Eimeria vermiformis* growth in cultured fibroblasts and epithelial cells. Infect. Immun. **59:**580–586.
19. **Rose, M. E., D. Wakelin, H. S. Joysey, and P. Hesketh.** 1988. Immunity to coccidiosis: adoptive transfer in NIH mice challenged with *Eimeria vermiformis*. Parasite Immunol. **10:**59–69.
20. **Rose, M. E., D. Wakelin, and P. Hesketh.** 1990. *Eimeria vermiformis*: differences in the course of primary infection can be correlated with lymphocyte responsiveness in the BALB/c and C57BL/6 mouse, *Mus musculus*. Exp. Parasitol. **71:**276–283.
21. **Scott, P.** 1989. The role of TH1 and TH2 cells in experimental cutaneous leishmaniasis. Exp. Parasitol. **68:**369–372.
22. **Shirley, M. W.** 1993. Live vaccines for the control of coccidiosis, p. 61–72. *In* J. R. Barta and M. A. Fernando (ed.), Proceedings of the VIth International Coccidiosis Conference. University of Guelph, Ontario, Canada.
23. **Stockdale, P. H. G., M. J. Stockdale, M. D. Rickard, and G. F. Mitchell.** 1985. Mouse strain variation and effects of oocyst dose in infection of mice with *Eimeria falciformis*, a coccidian parasite of the large intestine. Int. J. Parasitol. **15:**447–452.
24. **Wakelin, D., L. A. Mitchell, A. M. Donachie, and R. K. Grencis.** 1986. Genetic control of immunity to *Trichinella spiralis* in mice. Response of rapid- and slow-responder strains to immunization with parasite antigens. Parasite Immunol. **8:**159–170.
25. **Wakelin, D., and M. E. Rose.** 1990. Immunity to coccidiosis, p. 281–306. *In* P. L. Long (ed.), Coccidiosis of man and domestic animals. CRC Press, Boca Raton, Fla.
26. **Wakelin, D., M. E. Rose, P. Hesketh, K. J. Else, and R. Grencis.** 1993. Immunity to coccidiosis: genetic influences on lymphokine and cytokine responses to infection with *Eimeria vermiformis* in inbred mice. Parasite Immunol. **15:**11–19.