Life-phase specific induction and expression of rapid expulsion in rats suckling *Trichinella spiralis*-infected dams

JUDITH A. APPLETON & D. D. MCGREGOR *James Baker Institute for Animal Health, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York, U.S.A.*

Summary. Rat dams infected with 1000 *Trichinella spiralis* muscle larvae, 4 weeks prior to breeding, provided their suckling offspring with immunity to challenge with 200 muscle larvae at 2 weeks of age. The immunity was expressed in the elimination of 75–99% of the challenge dose within 24 hr. The intestinal worm burden did not decline significantly after the initial expulsion. Infected dams continued to protect their offspring during three breeding cycles, for as long as 26 weeks after infection

Immunity was conferred upon pups by dams that had been selectively immunized with the parenteral phase of the parasite's life cycle. Immunization with a drug-terminated enteral infection was ineffective as was enteral immunization followed by the parenteral phase. Further analysis revealed that rapid expulsion by pups was dependent on the number of mature muscle larvae recovered from dams immunized with NBL. By comparison, the expulsive capacity of the same dams was not improved by increasing the numbers of NBL within the range tested.

INTRODUCTION

Adult rats convalescing from a primary infection with *Trichinella spiralis* are powerfully protected against reinfection with muscle-stage larvae. The immunity is conspicuously expressed in the phenomenon of 'rapid expulsion' whereby the host eliminates as many as 99% of larvae in an oral challenge inoculum within 24 hr (McCoy, 1940; Love, Ogilvie & McLaren, 1976; Castro *et al.*, 1976; Bell, McGregor & Despommier, 1979; Alizadeh & Wakelin, 1982). Mice may also express rapid expulsion of a challenge infection (Bell, McGregor & Adams, 1982; Wakelin & Lloyd, 1976; Wassom *et al.*, 1984). Immunity to *T. spiralis* is comprised of several phase-specific immune responses such as anti-fecundity, anti-adult worm and rapid expulsion (Bell *et al.*, 1979).

Immunity to *T. spiralis* manifest in rat dams is conferred on their suckling offspring (Culbertson, 1943; Appleton & McGregor, 1984). The immunity expressed in rat pups is kinetically similar to rapid expulsion in adult rats. The immunity in pups can be transferred passively by feeding the animals serum antibody from *T. spiralis*-immune donors (Appleton & McGregor, 1984). In this report, we document the specificity and endurance of that protection, and characterize the life phase responsible for inducing the mediator of maternally transferred immunity to the parasite.

MATERIALS AND METHODS

Rats
Both AO and PVG-AO congenic rats were used in
preliminary experiments, but subsequently, only AO
rats were used. Female rats were paired individually
with males for 2 weeks, then separated and left
undisturbed for 1 week prior to parturition and 1 week
post-partum.

Parasite
Trichinella spiralis was maintained in irradiated rats of
various strains (PVG, AO or congenic strains of PVG)
(Bell *et al.*, 1979). Infectious ML were harvested by 1%
pepsin-HCl digestion of muscle tissue (Crum, Des-
pommier & McGregor, 1977). Unless otherwise noted,
8–12 week-old rat dams were infected, 4 weeks prior to
breeding, with 1000 ML suspended in 1 ml 0·6%
nutrient broth-2% gelatin. Pups were challenged at 14
days of age with 200 ML in 0·5 ml nutrient broth-gela-
tin. Pups were separated from their dams for 2–4 hr
prior to challenge and returned 1–2 hr afterwards.
Dams were challenged with 500 or 640 ML approxi-
mately 1 week after their litters had been killed.

Assessment of intestinal worm burden
Litter and feed were removed from the cages of dams
the day before they were killed. Pups and dams were
killed by cervical dislocation 18–24 hr post-challenge,
unless noted otherwise. The intestines were removed
immediately, rinsed with saline to remove luminal
contents, and slit open. After 5 hr incubation in 0·85%
NaCl containing 200 IU penicillin and 200 μg strepto-
mycin per ml, the worms that migrated from intestinal
tissue were counted.

Assessment of muscle larvae burdens
Dams were challenged, killed 20–24 hr later, and their
small intestines removed for worm burden quantifica-
tion. Carcasses were skinned and eviscerated, leaving
the diaphragm with the carcass. After digestion over-
night in 1% pepsin/HCl, the larvae freed were concen-
trated and counted.

Life-phase restricted immunization of rat dams
The protocols outlined by Bell & McGregor (1979)
were used. Briefly, the procedures were as follows.

Parenteral immunization. Various numbers of NBL
harvested from 18 hr *in vitro* cultures of 6-day-old
adult worms were suspended in a volume of 2 ml

serum-free minimal essential medium and injected into
the lateral tail vein of rat dams. When delivered to
naive animals, 40–60% of the dose was recovered as
muscle larvae 8–10 weeks later.

Enteral immunization (TM regime). Dams were
infected orally with 1000 ML and fed 0·05% thiaben-
dazole-containing rat feed beginning 3 days later.
Methyridine (300 mg/kg) was injected subcutaneously
10 and 11 days after infection. This regime completely
inhibits the release of NBL (Bell *et al.*, 1979).

Mock complete infection. Enteral immunization was
followed by parenteral administration of NBL either 4
or 14 days after the last dose of methyridine (15 or 25
days after the oral infection).

Experimental design
The results shown in Figs 1, 2 and 3 were obtained by
immunizing dams with a complete, natural *T. spiralis*
infection, challenging the pups at 14–16 days of age
and assessing intestinal worm burdens 1, 2, 3, or 7 days
later. The experiment described in Table 1 involved
immunization of dams with the various life-phase
protocols described above. All dams were bred on the
same day—17 days after the injection of Groups D
and F with NBL. The experiment is described dia-
grammatically in Fig. 4a.
In order to analyse the dose response to NBL, dams
were injected with various numbers of NBL and bred 3
weeks later. The experiment is described diagrammati-
cally in Fig. 4b.

Measurement of antibody in pups' sera
Five pups per dam were challenged with muscle larvae
in order to assess rapid expulsion. If a dam delivered
more than five pups, she was allowed to rear them as
occasional infant mortality occurred. On the day of
challenge, surplus littermates were euthanized. In the
life-phase restricted immunization experiment de-
scribed above, the surplus littermates were bled and
blood samples collected from each group were pooled.
These sera were assayed in ELISA, using whole muscle
larvae as antigen. Briefly, larvae were digested free of
muscle (as described above) and washed with saline.
Worms were suspended in a solution of 2% gelatin in
PBS and 160 larvae were distributed to each well of a
polystyrene 'V'-bottomed 96-well plate. Larvae were
washed once with 0·1% gelatin in PBS then incubated
for 1 hr with 25 μl of various serum dilutions prepared
in 0·2% gelatin in PBS. Each serum dilution was tested

in triplicate wells. Larvae were washed three times with 0·1% gelatin-PBS and incubated for 1 hr with 25 μl of goat anti-rat IgG antibody conjugated to peroxidase (heavy and light chain-specific, Cappel Laboratories, Cochranville, PA) diluted in 10% normal goat serum in PBS. Unbound conjugate was washed away with five changes of 0·1% gelatin-PBS and the larvae were resuspended in 75 μl 0·1 M citrate buffer. Larvae were transferred to corresponding wells of a 96-well flat-bottomed polystyrene plate and 75 μl citrate buffer containing H_2O_2 and orthophenylenediamine were added to each well. Colour was developed for 45 min and then stopped by the addition of 50 μl 5 M H_2SO_4. Absorbances were measured at 495 nm on a Dynatech ELISA reader.

RESULTS

Kinetics of worm expulsion

The results of two experiments are described in Fig. 1 and 2. In the first experiment, pups suckling infected or uninfected dams were challenged at 14 days of age and killed 3 or 7 days later (Fig. 1). The majority of worms were expelled by the third day; few, if any, additional worms were expelled between Days 3 and 7. In the second experiment, pups were killed 1, 2 or 3 days after challenge (Fig. 2). Worms were expelled within 24 hr and no further expulsion took place on Days 2 or 3. In
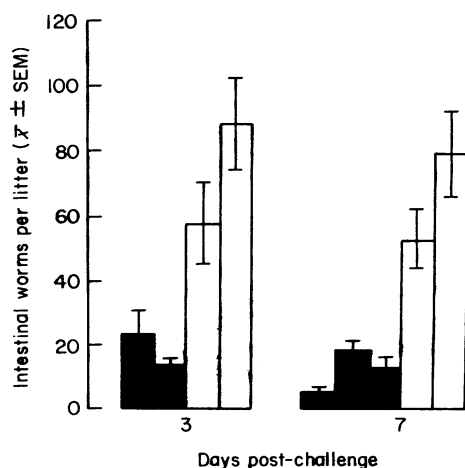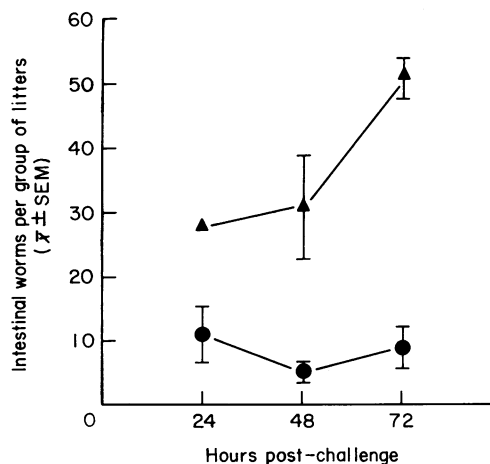

Figure 2. Kinetics of neonatal rapid expulsion of *T. spiralis*, 1, 2 and 3 days post-challenge. Each point represents the mean intestinal worm burden for 3 litters; (●), litters of infected dams; (▲), litters of uninfected dams.

this experiment, the pups suckling infected dams harbored significantly ($P < 0.05$, Student's *t*-test) fewer worms than those suckling uninfected dams, although the 'low take' in the non-immune pups is unexplained.

Persistence of maternal immunity to *T. spiralis*

A group of 25 infected and 25 uninfected rat dams were bred, and five litters from each group were challenged. The remaining rats were left with their pups until weaning and then bred again. A portion of the litters in this group were challenged and the remaining rats bred again. After the third breeding, only one uninfected dam whelped, presumably due to the declining reproductive efficiency of 8–10-month-old rats. The results are summarized in Fig. 3, where it can be seen that infected dams retained their capacity to protect pups for at least 26 weeks (the duration of the experiment), with the exception of one dam whose litter was challenged at 9 weeks. Immunity conferred on pups did not appear to decline during the course of the experiment.

Life-phase restricted immunization

Preliminary experiments indicated that the injection of dams with NBL produced an immunity in their pups comparable to that conferred by dams exposed to a complete infection (data not shown). Furthermore,
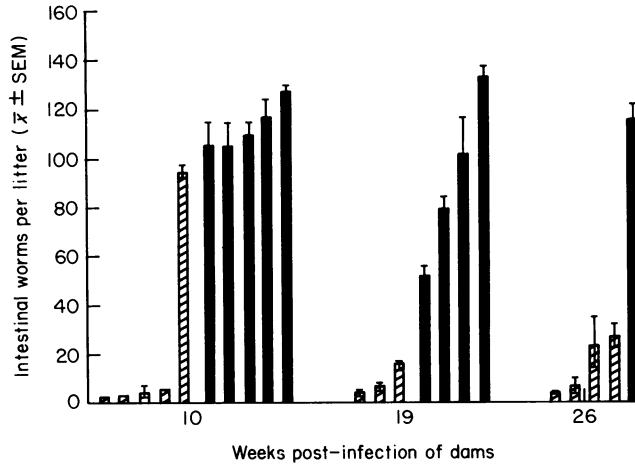

Figure 1. Kinetics of neonatal rapid expulsion of *T. spiralis*, 3 and 7 days post-challenge. Each bar represents the mean intestinal worms for a litter; (■), litters of infected dams; (□), litters of uninfected dams.

Figure 3. Persistence of maternal immunity to *T. spiralis*. Each bar represents the mean intestinal worm burden of one litter; (▨), litters of infected dams; (■), litters of uninfected dams.

dams who received the TM regime followed by NBL 14 days later were unable to protect their pups, although they were themselves immune. The inability of dams to protect their pups occurred in conjunction with a very low recoverable muscle larvae burden in TM-NBL recipients (data not shown). The experiment illustrated in Fig. 4a and shown in Table 1 was designed to substantiate these observations. Indeed, injection of dams with NBL was sufficient to induce

rapid expulsion in the dams and in their suckling pups. TM-NBL spaced by 4 or 14 days induced strong rapid expulsion in dams but afforded variable (TM-NBL 4) or no (TM-NBL 14) protection for pups. This corresponded to a very low mature muscle larvae burden in TM-NBL 14 dams and a variable burden in TM-NBL 4 dams, when compared with larvae burden in normal dams infected with an equal number of NBL.
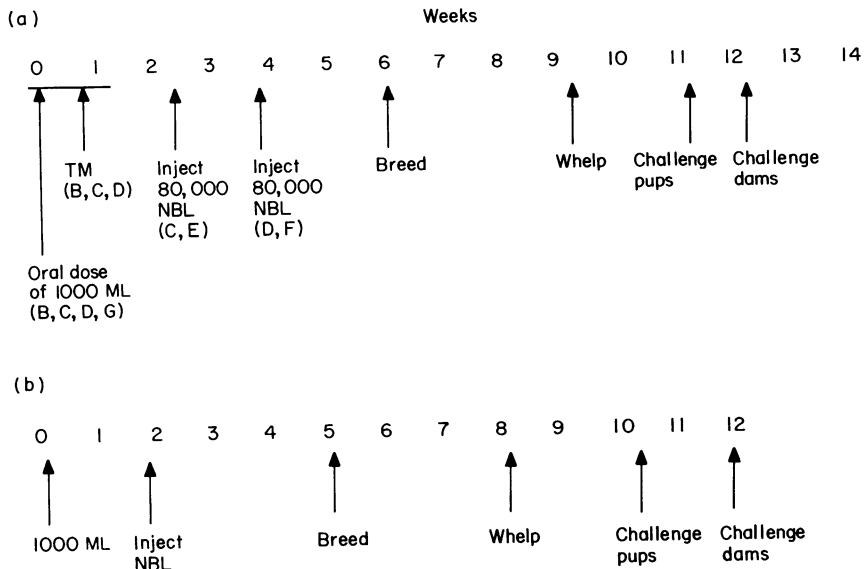
When tested for their ability to bind to whole muscle

Figure 4. Experimental design of *T. spiralis* life-phase restricted immunization schemes.

Table 1. Life-phase specificity of induction of neonatal rapid expulsion

Group*	Treatment of dam	Intestinal worm burden (% of dose recovered)		Muscle larvae burden in dam†
		Pups	Dam	
A.	None	61	50	ND‡
B.	TM	56	23	ND
C.	TM + NBL 4 days	21	10	27,900 ± 14,000
D.	TM + NBL 14 days	51	7	1200 ± 400
E.	NBL 4 days	7	11	49,200 ± 4300
F.	NBL 14 days	13	19	35,100 ± 1800
G.	Complete	3	2	92,700 ± 12,100

Statistical analyses (by the Studentized range test): pup intestinal worm burdens: C, E, F. G *vs* A—$P < 0.05$; B, D *vs* A—not significant; dam intestinal worm burdens: B, C, D, E, F, G *vs* A—$P < 0.05$; dam muscle larvae burdens: C or F *vs* E—$P > 0.05$; D *vs* F—$P < 0.05$ (Student's *t*-test).

* Groups were comprised of three to five dams with litters.
† Numbers rounded to the nearest hundred.
‡ ND, not done.

larvae in ELISA, sera from pups in the different treatment groups were clearly different (Fig. 5). Pups of TM or uninfected dams had negligible serum anti-*T. spiralis* antibody levels. Pups of complete, TM-NBL 4, and NBL 4 dams had high specific antibody levels; NBL 14 pups' sera had slightly lower but still substantial antibody levels. Considerably less antibody was present in sera of TM-NBL 14 pups. These results are roughly correlated with the immune status of the pups in the different treatment groups, as well as the muscle larvae burdens in the dams.

Dose response to NBL

In order to verify the dependency of neonatal rapid expulsion on the muscle larvae burden of dams, groups of females were injected with doubling dilutions of NBL. The results are shown in Fig. 6. Linear regression analysis of the data showed that intestinal worm numbers in pups after challenge were highly correlated ($r = -0.97$) with dam muscle larvae burden, while there was a weak correlation ($r = -0.64$) between dam intestinal worm numbers after challenge and muscle larvae burden. This suggests that the capacity to express rapid expulsion in suckling pups is dependent on the number of larvae in the dam's
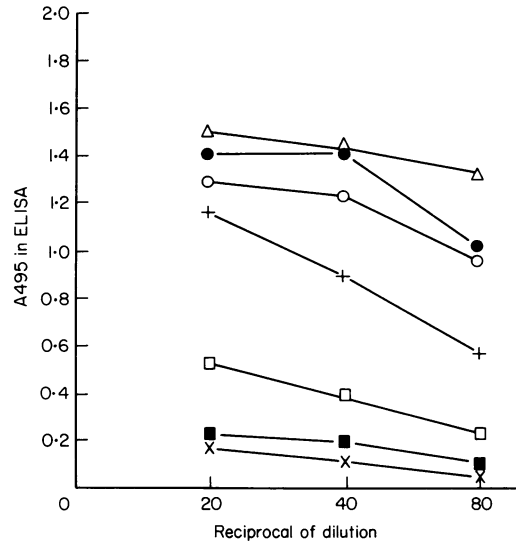

Figure 5. Titration of pup sera in ELISA. Pups born to dams immunized with life-phase restricted *T. spiralis* infections (Table 1) were bled at the time of challenge and sera from pups in each group were pooled. ELISA was performed as described using intact muscle larvae as antigen. Pups were born to dams immunized with: TM (×); TM-NBL 4 (○); TM-NBL 14 (□); NBL 4 (△); NBL 14 (+); complete infection (●); no infection (■).

muscles, whereas rapid expulsion in the dam is independent of larvae burden, at least over the dose range tested.

DISCUSSION

The experiments described here confirm earlier reports (Culbertson, 1943; Appleton & McGregor, 1984) that immunity to *T. spiralis* is conferred on suckling rats by their dams. Immunity is expressed in a phenomenon that has similar kinetics to rapid expulsion in infected adult rats (Lee & Ogilvie, 1982; Appleton & McGregor, 1984; Bell, Adams & Ogden, 1984). These experiments show that *T. spiralis*-immune dams protect their young several months after adult worms have been expelled from the intestine. The results suggest that immunity is maintained and possibly induced by encysted muscle larvae. This notion is supported by our finding that the immunity in protected pups is expressed mainly (perhaps exclusively) against the larval stage of the parasite's life cycle.

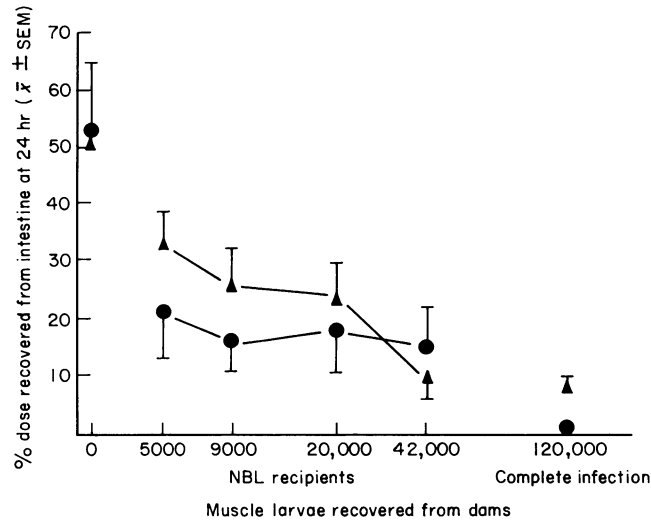Immunization of rat dams by life-phase restricted

Figure 6. Dose-response analysis of neonatal rapid expulsion induced by restricted immunization with parenteral *T. spiralis* larvae. Each point represents the mean ± standard error for *n* = 4 or 5 dams or litters per group, with the exception of the 0 NBL control group where *n* = 2; (●), dams; (▲), pups.

exposure to *T. spiralis* identified muscle larvae as the likely inducers of maternal immunity. Parallel analysis of the dam's own response to infection showed that the parenteral phase of the parasite's life cycle was able to induce a vigorous RE response.

Administration of NBL after enteral immunization produced the expected result in dams: a strong RE response. The enteral phase alone produced a more variable response among the group of rats tested. This confirms earlier results (Bell & McGregor, 1979) which indicated that the RE induced by the TM regime begins to wane 5–7 weeks after the infection is terminated. Although enterally immunized dams were variably protected, their pups were uniformly vulnerable. This disparity was also evident in pups reared by mothers immunized by the TM-NBL regime. Such pups were either variably protected (when NBL were administered 4 days after TM termination) or completely vulnerable (when NBL were administered 14 days after TM termination). The level of protection correlated with the number of ML recovered from the dams in each group. Thus, only 1200 ML matured in the TM-NBL 14 day group, while 27,900 ML matured in the TM-NBL 4 day group. It appears that on Day 25 after initiation of the enteral infection, the dam had mounted an immune response against NBL (or developing ML) such that only a small proportion of the NBL dose administered at this time was able to mature

in muscle. The variation observed in dams injected with NBL 4 days after a drug terminated enteral infection indicated that immunity to NBL was beginning to be expressed 15 days after oral infection. It is unclear how an immunity directed against NBL is induced by an enteral, drug-limited infection. However, the data reported here indicate that expression of rapid expulsion in neonates is dependent upon the establishment of larvae in the dam's muscles. Compelling evidence of this requirement was provided by the observed dose-response relationship between ML burden and the capacity of dams to immunize their pups.

The results of this investigation raise several questions concerning the immunity expressed in the intestine and mammary gland of *T. spiralis*-infected rats. It has been shown previously (Bell & McGregor, 1979) that the intestinal and parenteral phases of the infection act synergistically to both induce and prolong the expression of RE in the intestine. Thus, it is possible that, after priming the gut with the pre-adult to adult phases of the parasite, a short-lived RE response is induced which is later 'boosted' during the release of antigens by developing muscle larvae. Because the rats used in our experiments are not bred until after the immunizing infection is complete, one would expect the entero-mammary axis of lymphoblast migration described by others (Weisz-Carrington, Roux &

Lamm, 1977) to be inactive during the 'priming' intestinal immunization. In contrast, antigen release by muscle larvae may continue into gestation and would be expected to stimulate a strong systemic antibody response. The boosting dose of ML required for continued expression of immunity in the intestine may be considerably smaller than the 'immunizing' dose required for delivery of immunity to the mammary. A differential of this sort would explain the different immune responses manifest in pups and dams when the dams were immunized with the TM regime followed by NBL 14 days later.

The results reported here accord with our previously published data (Appleton & McGregor, 1984) which showed passive transfer of RE to suckling rats with orally or parenterally administered serum Ig. Absorption of such Ig with anti-IgG antibody prior to transfer abrogates the protection (J. A. Appleton and D. D. McGregor, unpublished observations). In this report, we have shown that sera from suckling pups contain high levels of muscle larvae-specific antibody only when dams carry large muscle larvae burdens. These factors, in turn, correlated well with expression of rapid expulsion. We attach significance to these findings and advance them as evidence in support of an hypothesis regarding the mechanism of maternally transferred immunity to *T. spiralis*. This hypothesis maintains that IgG antibodies to the parasite induced by encysted ML are transported from the blood into milk (some may also be transported in the milk after release by plasma cells resident in the mammary). Milk IgG is then rapidly and efficiently transported by epithelial cells lining the neonatal intestine (Morris & Morris, 1977), thereby providing a high level of specific antibody to *T. spiralis* at the very site where it would encounter incoming ML.

It is tempting to speculate that a similar mechanism underlies the expression of RE in adult rats, with the notable exception that the IgG antibodies which mediate expulsion would be delivered to the intestine *via* the blood. The intestinal phase of the infection may, in itself, encourage the diffusion of antibody to the reaction site. This notion accords with the observation that 'stimulation' of the intestine by the unrelated parasite *Heligmosomoides polygyrus* can promote the expression of RE in adult rats after parenteral immunization with larval antigens (Bell & McGregor, 1980). Because the neonate does not require prior intestinal stimulation in order to express RE, it provides a medium in which the specific, immune mediators of RE can be analysed in the absence of the

as yet unidentified intestinal change required for the full expression of RE in adult rats.

ACKNOWLEDGMENTS

REFERENCES

ALIZADEH H. & WAKELIN D. (1982) Comparison of rapid expulsion of *Trichinella spiralis* in mice and rats. *Int. J. Parasitol.* 12, 65.

APPLETON J.A. & McGREGOR D.D. (1984) Rapid expulsion of *Trichinella spiralis* in suckling rats. *Science*, 226, 70.

BELL R.G., ADAMS L.S. & OGDEN R.W. (1984) Intestinal mucus trapping in the rapid expulsion of *Trichinella spiralis* by rats: induction and expression analyzed by quantitative worm recovery. *Infec. Immun.* 45, 267.

BELL R.G. & McGREGOR D.D. (1979) *Trichinella spiralis*: role of different life cycle phases in induction, maintenance and expression of rapid expulsion in rats. *Exp. Parasitol.* 48, 51.

BELL R.G. & McGREGOR D.D. (1980) Rapid expulsion of *Trichinella spiralis*: coinduction by using antigenic extracts of larvae and intestinal stimulation with an unrelated parasite. *Infec. Immun.* 29, 194.

BELL R.G., McGREGOR D.D. & ADAMS L.S. (1982) *Trichinella spiralis:* characterization and strain distribution of rapid expulsion in inbred mice. *Exp. Parasitol.* 53, 301.

BELL R.G., McGREGOR D.D. & DESPOMMIER D.D. (1979) *Trichinella spiralis:* multiple, phase-specific anti-parasite responses mediate the intestinal component of protective immunity in the rat. *Exp. Parasitol.* 47, 147.

CASTRO G.A., BADIAL-ACEVES F., ADAMS P.R., COPELAND E.M. & DUDVICK S.J. (1976) Response of immunized, parenterally nourished rats to challenge infection with the nematode, *Trichinella spiralis*. *J. Nutrit.* 106, 1484.

CRUM E.D., DESPOMMIER D.D. & McGREGOR D.D. (1977) Immunity to *Trichinella spiralis*. I. Transfer of resistance by two classes of lymphocytes. *Immunology*, 33, 787.

CULBERTSON J.T. (1943) Natural transmission of immunity against *Trichinella spiralis* from mother rats to their offspring. *J. Parasitol.* 29, 114.

LEE G.B. & OGILVIE B.M. (1982) The intestinal mucus layer in *Trichinella spiralis*-infected rats. In: *Recent Advances in Mucosal Immunity* (eds W. Strober, L. A. Hanson and K. W. Sell), p. 319. Raven Press, New York.

LOVE R.J., OGILVIE B.M. & McLAREN D.J. (1976) The immune mechanism which expels the interstinal stage of *Trichinella spiralis* from rats. *Immunology*, 30, 7.

McCOY O.R. (1940) Rapid loss of *Trichinella* larvae fed to immune rats and its bearing on the mechanisms of immunity. *Am. J. Hyg.* 32, 105.

MORRIS B. & MORRIS R. (1977) The digestion and transmission of labeled immunoglobulin G by enterocytes of the proximal and distal regions of the small intestine of young rats. *J. Physiol.* **273,** 427.

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

WASSOM D.L., WAKELIN D., BROOKS B.O., KRCO C.J. &

DAVID C.S. (1984) Genetic control of immunity to *Trichinella spiralis* infections of mice. Hypothesis to explain the role of H-2 genes in primary and challenge infections. *Immunology,* **52,** 625.

WEISZ-CARRINGTON P., ROUX M.E. & LAMM M.E. (1977) Plasma cells and epithelial immunoglobulins in the mouse mammary gland during pregnancy and lactation. *J. Immunol.* **119,** 1306.