*Journal of Helminthology* (1977) 51, 167—176

# *Nematospiroides dubius:* stimulation of acquired immunity in inbred strains of mice

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**ABSTRACT**

The development of immunity to *Nematospiroides dubius* was studied in three strains of inbred mice (BALB/c, C3H and NIH). Although a primary infection in NIH mice persisted for two months without evidence of a reduction in worm numbers, female mice of this strain readily developed resistance to reinfection. The degree of resistance was enhanced when an immunizing infection of 600 larvae was administered as 6 separate doses of 100 larvae given between days 0 and 11, and the worms removed by anthelmintic treatment given on days 15, 21, 28 and 35. Immunity in mice immunized in this way was manifest both as a reduction in worm recoveries on days 9–14 after challenge and also as an expulsion of established worms from the intestine. BALB/c mice were initially less resistant, but expelled most of the worms which became established; C3H mice showed no evidence of expulsion. The finding that inbred NIH and BALB/c mice acquire resistance to *N. dubius* offers possibilities for the systematic analysis of lymphoid cell activity in initiating and expressing immunity to this parasite.

Studies of immunity to nematode parasites have largely concentrated on species such as *Nippostrongylus brasiliensis*, *Trichinella spiralis* and *Trichuris muris*, in which the larval or adult stages are expelled from the host before the third week of a primary infection (Ogilvie and Love, 1974; Larsh and Race, 1975; Wakelin, 1975; Wakelin and Lloyd, 1976), and in consequence there is now much information about the role of components of the host immune response in initiating and effecting protective immunity against these species. In marked contrast relatively little is known of the immune response to the murine trichostrongyle *Nematospiroides dubius*, which survives for many months after a primary infection and in which the adult worms appear not to be adversely affected in animals resistant to reinfection (Liu, 1966; Bartlett and Ball, 1974). Analysis of immunity in this host-parasite system has been hampered by difficulties in stimulating effective levels of resistance to challenge and by failure to stimulate immunity in inbred strains of mice. Although the development of acquired immunity to *N. dubius* has been studied by several authors, the experimental protocols used to bring about immunity have usually involved several immunizing infections, each separately terminated by anthelmintic treatment (Baker, 1954; Van Zandt, 1961; Panter, 1969a, b; Cypess and Zidian, 1975). The use of such multiple infections interspersed with periods of non-stimulation (i.e. after removal of the parasites by anthelmintic treatment) complicates the interpretation of data from subsequent challenge experiments and prolongs the period of experimental investigation. Failure to readily stimulate immunity in inbred mice has prevented the use of cell transfer techniques in analysing immunity to *N. dubius*; such techniques are of course effective only when syngeneic animals are available (Kelly, Love and Dineen, 1974).

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The present paper reports the results of experiments in which several immunizing schedules were compared in previously used strains of inbred mice (BALB/c and C3H) as well as in the relatively unstudied NIH strain, which has recently been shown to reject *Trichinella spiralis* and *Trichuris muris* more rapidly than any other strain of inbred mouse (Wakelin, 1975; Wakelin and Lloyd, 1976).

## MATERIALS AND METHODS

Inbred NIH, BALB/c and C3H strain mice were bred in the Wellcome Laboratories for Experimental Parasitology and were used at 6–8 weeks of age. When necessary, additional NIH mice were purchased from Anglia Laboratory Animals.

The strain of *N. dubius* was obtained in 1975 from the Wellcome Research Laboratories, Beckenham where it has been maintained since 1956. This strain of the parasite originated in the United States of America and is thought to correspond to *Heligmosomoides polygyrus bakeri* as described by Durette-Desset, Kinsella and Forrester (1972). The parasite was maintained at the Wellcome Laboratories for Experimental Parasitology as a stock infection in outbred CFLP mice. Infective third stage larvae were cultured as described by Bryant (1973). Mice were infected by the oral administration of larvae in 0·2 ml of suspension. The procedures used to recover and count worms have already been described (Jenkins and Behnke, 1977).

The drug used to remove worms from infected mice was pyrantel embonate (*Strongid-P* paste, Pfizer) given to the mice as an aqueous suspension by the oral route. A dose rate of 200 mg/kg bodyweight was found to be adequate to remove all adult worms from the intestinal lumen.

## RESULTS

*Survival of primary* Nematospiroides dubius *infections in NIH mice*

There is no published data concerning the pattern of primary *N. dubius* infections in NIH mice. Several initial experiments were therefore carried out in which male or female NIH mice were infected with *N. dubius* and the course of infection followed over a two month period in order to ascertain whether the parasite gave rise to chronic infections, as has been described for other strains of mice (Liu, 1966; Bartlett and Ball, 1974).

The results of two such experiments are shown in Fig. 1. In the first experiment male NIH mice were given 350 larvae of *N. dubius* and in the second female NIH mice were infected with 400 larvae. Groups of six mice were then killed for worm counts at intervals over the next two months. It can be seen that there was no appreciable change in the number of worms recovered over the duration of the experiments, confirming that in this strain of mice, as in others, *N. dubius* survives for at least two months after infection and that there is no host-response effective against the adult worms during this time.

*Attempts to stimulate immunity in inbred mice by single and divided primary infections*

Preliminary experiments in outbred CFLP mice indicated that mice which received a divided primary infection, i.e. one in which a given total number of larvae was administered in several proportionally smaller infections over a period of 9–14 days, showed an enhanced secondary response against subsequent challenge infection. Since this method of immunization was superior to immunization by a comparable number of larvae given as a single infection, an attempt was made to determine whether inbred BALB/c and C3H mice

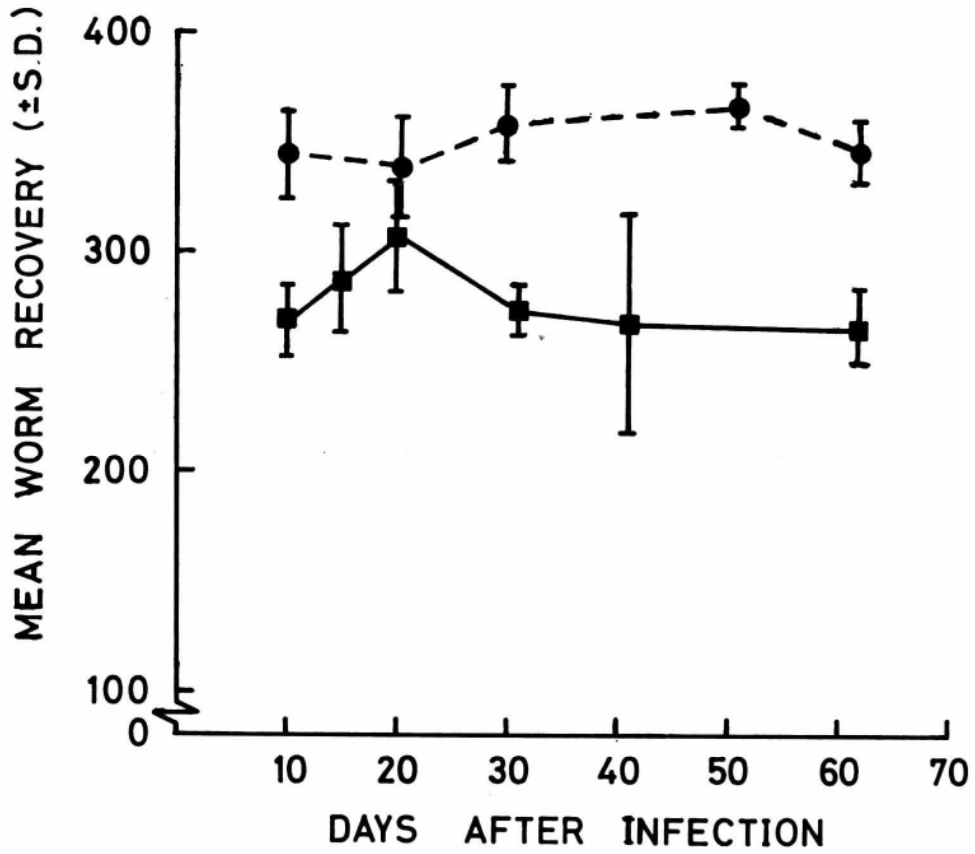


FIG. 1. The survival of *Nematospiroides dubius* in male (■) and female (●) NIH mice. The figure was constructed from two separate experiments in the first of which male mice were infected with 350 larvae of *N. dubius* and in the second female mice were infected with 400 larvae of *N. dubius*.

could also be protected from reinfection in this way. The experimental design and the results of this experiment are presented in Table 1. Although numerically there was some reduction in the worm recoveries of all the previously infected groups, there was no statistical evidence for a more effective response in the mice given divided primary infections.

NIH mice are known to respond more rapidly than either BALB/c or C3H mice to infection with other nematode parasites and therefore an experiment was carried out to determine whether this strain of mice could be made resistant to reinfection by a divided primary infection with *N. dubius*. Groups of six female NIH mice were given various combinations of either single or divided primary infections over a period of nine days. Anthelmintic was given on days 20, 21 and 22, the challenge infection on day 29 and the mice were killed for worm counts 14 days after the challenge infection.

The results of this experiment are presented in Table 2. Only the groups given a single infection of 100 or 250 larvae failed to develop substantial immunity; all the remaining groups had significantly fewer worms at autopsy. These results indicate that the inbred NIH strain of mice can be made resistant to reinfection by exposure to a divided primary infection consisting of a total of no more than 100 larvae of *N. dubius*.

**TABLE 1**

Recovery of *Nematospiroides dubius* after a challenge infection of 150 larvae given to male BALB/c and C3H mice, previously immunized either by a single or a divided primary infection.

| Strain of mice | No. of infections ×[1] no. of larvae in each dose | No. mice | Mean worm recovery | ± S.D. |
|---|---|---|---|---|
| BALB/c | None | 6 | 122·0 | 4·0 |
| | 1 × 500 | 4 | 117·8 | 14·0 |
| | 6 × 80 | 6 | 92·5 | 16·7 |
| | 11 × 80 | 3 | 92·0 | 5·7 |
| C3H | None | 6 | 131·5 | 13·1 |
| | 1 × 500 | 5 | 115·4 | 12·1 |
| | 6 × 80 | 8 | 119·9 | 8·3 |
| | 11 × 80 | 5 | 119·2 | 17·0 |

[1] For each group, the total dose was administered over a period of 10 days at the number and level of infections shown. Anthelmintic was given on days 20, 21 and 22, the challenge infection on day 29 and the mice were killed 15 days after the challenge infection.

*The pattern of worm loss in inbred mice immunized by divided primary infections*

The experimental schedule by which mice were immunized with a divided primary infection was standardized as follows. Mice were given 100 larvae on days 0, 2, 4, 7, 9 and 11; anthelmintic was given on days 15, 21, 28 and 35. The challenge infection was given either on day 42 at the earliest or on a subsequent occasion depending on the experimental circumstances.

**TABLE 2**

Recovery of *Nematospiroides dubius* after a challenge infection of 150 larvae given to female NIH mice, previously immunized either by a single or a divided primary infection.

| No. of infections ×[1] no. of larvae in each dose | No. mice | Mean worm recovery | ± S.D. |
|---|---|---|---|
| None | 6 | 133·3 | 11·3 |
| 1 × 100 | 6 | 128·7 | 12·8 |
| 10 × 10 | 6 | 97·0* | 8·8 |
| 1 × 250 | 6 | 117·3 | 22·8 |
| 10 × 25 | 6 | 86·0* | 16·0 |
| 1 × 500 | 6 | 83·5* | 13·7 |
| 5 × 100 | 6 | 67·5* | 16·0 |
| 10 × 50 | 5 | 64·4* | 7·6 |

* Significantly different from control group $P < 0{\cdot}005$.

[1] For each group, the total dose was administered over a period of 9 days at the number and level of infections shown. Anthelmintic was given on days 20, 21 and 22, the challenge infection on day 29 and the mice were killed 14 days after the challenge infection.

Table 3 shows the results of three experiments in which the survival of *N. dubius* was compared in NIH and C3H mice. In experiments 1 and 3, the challenge infection was given on day 43 in relation to the first dose of the divided immunizing infection. In experiment 2, the interval was increased and the mice were not challenged until day 71, in order to allow possible non-specific intestinal disturbances to return to normal.

**TABLE 3**

Recovery of *Nematospiroides dubius* at various times after a challenge infection of 100 larvae given to female NIH and C3H mice, immunized by a divided primary infection.

| | | | DAYS AFTER CHALLENGE | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 9 | | 14 | | 38, 41 or 44 | |
| | Group | | Mean worm recovery | ± S.D. | Mean worm recovery | ± S.D. | Mean worm recovery | ± S.D. |
| Expt. 1 | NIH | Control | 95·5 | 9·5 | 96·3 | 7·2 | 100·4 | 5·6 |
| | | Immune | 15·0 | 7·1 | 13·3 | 11·3 | 2·4 | 2·0 |
| | C3H | Control | 87·6 | 9·3 | 101·8 | 5·7 | 95·2 | 8·4 |
| | | Immune | 47·5 | 13·3 | 66·6 | 6·9 | 59·2 | 34·6 |
| Expt. 2 | NIH | Control | 84·2 | 11·6 | not done | | 103·0 | 5·9 |
| | | Immune | 14·8 | 9·9 | not done | | 0·7 | 0·5 |
| | C3H | Control | 79·0 | 11·9 | not done | | 99·1 | 11·0 |
| | | Immune | 39·4 | 19·6 | not done | | 86·2 | 12·9 |
| Expt. 3 | NIH | Control | not done | | not done | | 104·3 | 14·6 |
| | | Immune | not done | | not done | | 4·7 | 1·5 |
| | C3H | Control | not done | | not done | | 109·7 | 12·6 |
| | | Immune | not done | | not done | | 66·0 | 27·4 |

It can be seen from these results that NIH mice developed a marked resistance to re-infection, which was reflected in reduced worm recoveries on day 9 and also in the elimination of the surviving worms by day 38–44. All the immunized NIH groups killed five or more weeks after challenge infection had fewer worms than comparable groups killed earlier in the experiments; C3H mice were much less successful in resisting the challenge infection. Although on day 9, the worm recoveries from immunized C3H mice were significantly lower than controls, there was no evidence of worm loss such as was shown to occur in NIH mice. In experiment 1 the mean worm recovery from immunized C3H mice was consistently lower than that from control mice although the variation between individual mice gave rise to high standard deviations by day 38. In the second experiment, substantially more worms were recovered on day 41, indicating that the lower recoveries on day 9 may have resulted from a partial inhibition of larval development with the consequent failure to recover all the worms at autopsy.

Table 4 shows the results of two experiments in which the survival of a challenge infection was followed in BALB/c mice immunized in the standardized way described above. The pattern of survival of *N. dubius* in these mice was distinct from either that described for NIH or for C3H mice. BALB/c mice showed partial reduction in worm recovery on days 9–10, but the worm burdens were never quite as low as those found in NIH mice. Subsequently the established worms were almost completely eliminated by the fifth week. Thus, although in BALB/c mice fewer worms were initially inhibited or destroyed than in NIH mice, both strains of mice were then able to eliminate the established worms; C3H mice did not show evidence of such a rejection.

*Recovery of worms from the mouse intestine during the standardized immunization procedure*

Two experiments were carried out in which female NIH mice were killed and their worms were recovered at various times after the initiation of the immunizing procedure. The pooled results from these experiments are presented in Fig. 2. Each of the six immunizing infections was known to be 90–95% infective, so the low worm recoveries during the first week represent only those larvae which were sufficiently close to the intestinal mucosa to escape from the host tissue during incubation. Over the course of the following week the worm

**TABLE 4**

The survival of a challenge *Nematospiroides dubius* infection in female BALB/c mice immunized by a divided primary infection.

| Day After challenge infection | | Mean no. of worms recovered ± S.D. after challenge with 120 larvae | | | |
|---|---|---|---|---|---|
| Expt. 1 | Expt. 2 | Expt. 1 | | Expt. 2 | |
| | | Control group | Immunized group | Control group | Immunized group |
| 9 | 10 | 111·2 ± 8·4 | 50·7 ± 15·8 | Not done | 36·4 ± 10·8 |
| – | 17 | Not done | | Not done | 17·8 ± 9·7 |
| 20 | 23 | 119·0 ± 9·5 | 63·2 ± 24·7 | Not done | 12·5 ± 10·2 |
| 34 | 33 | 114·7 ± 19·9 | 6·0 ± 2·3 | 109·3 ± 3·6 | 4·5 ± 1·8 |

numbers increased as the worms given in the first infections completed their development and returned to the gut lumen. Most of the recovered worms were late fourth stage larvae and adult worms, with only a few younger larvae representing unestablished worms from the last doses of infective material. Treatment with anthelmintic on day 15 removed about 70% of worms from the intestinal lumen. During the course of the next week worm numbers increased again until day 21, but by this time the mice were already showing some degree of resistance in that relatively greater numbers of larval worms were recovered indicating some inhibition of development. The anthelmintic given on day 21 removed a further 77% of worms from the intestinal lumen and the last two doses of anthelmintic on days 28 and 35 ensured that no more worms persisted in the intestinal lumen beyond the fifth week of infection.

## DISCUSSION

Unlike the rat trichostrongyle *N. brasiliensis*, the murine trichostrongyle *N. dubius* gives rise to a chronic primary infection. Whereas there is a great deal of information about the mechanism of worm expulsion in *N. brasiliensis* infection, relatively little is known as to why a similar mechanism is not initiated, or fails to become effective, in primary *N. dubius* infection in the mouse. This situation could be remedied if an inbred strain of mice, which readily develops resistance to the parasite, was available and if immunization could be achieved after a relatively simple immunizing procedure. This would not only enable the clarification of the processes involved in worm loss in immune animals, but would also allow a systematic analysis of the role of different cell populations during immunization by facilitating cell transfer to syngeneic recipient mice, an approach which has yielded valuable information in other host-parasite systems (Ogilvie and Jones, 1968; Love, 1975a, b; Wakelin, 1975; Wakelin and Lloyd, 1976).

It is evident from the results reported in this paper that the inbred NIH mouse can readily be immunized and therefore meets the requirements set out above. As in other mouse strains, there is no effective response against the primary infection and consequently the worms survive for several months. However, in marked contrast to other inbred strains, NIH mice develop strong immunity following a single immunizing infection with 500 larvae of *N. dubius*. Furthermore, the level of resistance to challenge infection can be enhanced by administering the initial infection not as a single dose but in several proportionally smaller doses over an 11 day period.

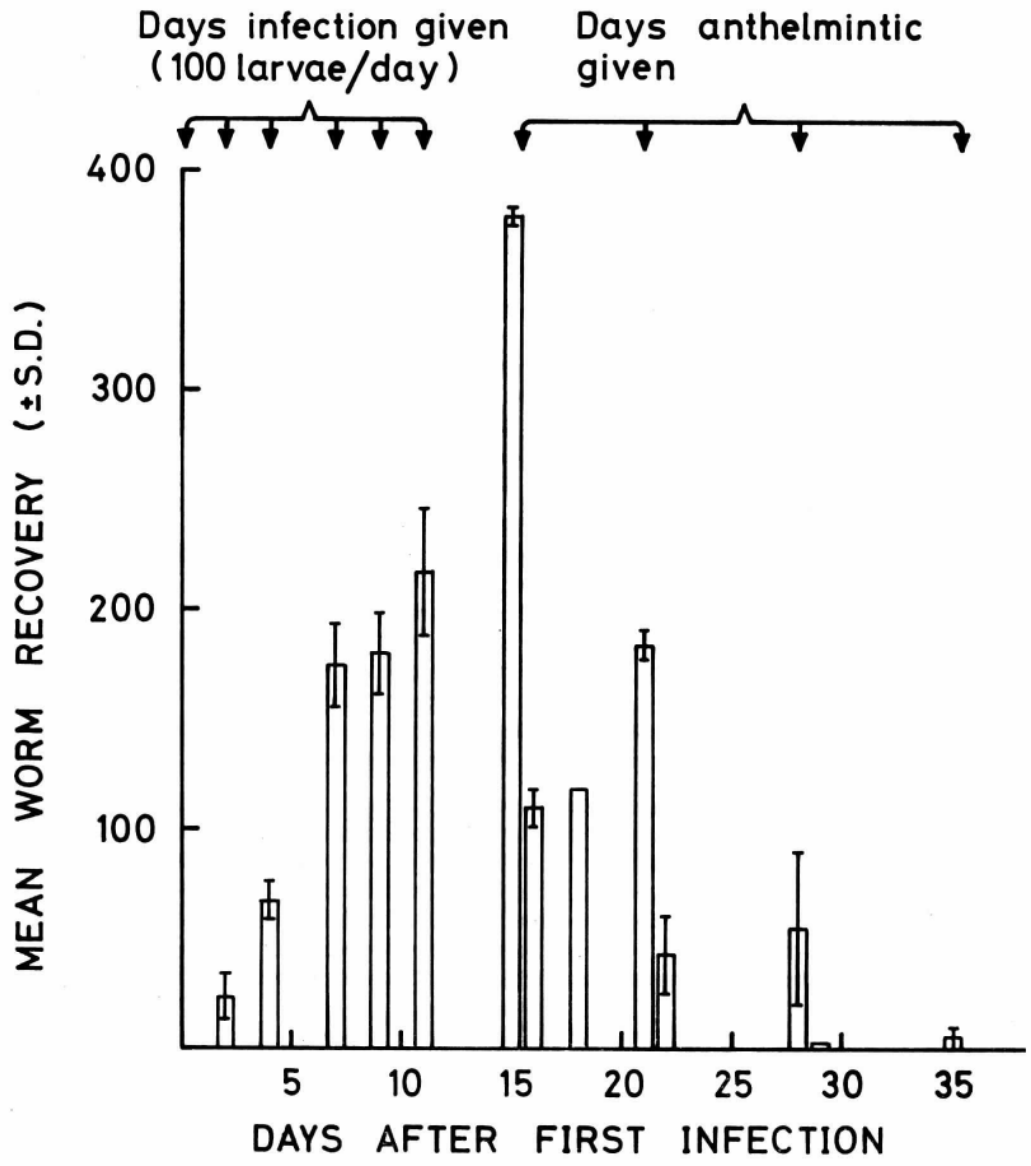


FIG. 2. Recovery of *Nematospiroides dubius* during the standardized immunization procedure. Groups of mice were killed and their worms were recovered at various times after the initiation of the immunization procedure.

A number of such schedules was examined and a standardized divided primary infection regimen was selected for further analysis. Although by day 11 mice had been given a total of 600 larvae, only a proportion of these worms, corresponding to the first two or three doses given on days 0, 2 and 4 were actually in the lumen of the intestine at this time. The tissue phase of *N. dubius* is known to be 6 to 8 days in duration (Bartlett and Ball, 1974). Treatment with anthelmintic on day 15 removed a proportion of the total worm burden, i.e. worms in the gut lumen and the remaining worms persisted until day 21 by which time most were already in the lumen and consequently susceptible to the action of the next anthelmintic treatment. The net result of this method of immunization was a build-up of worm numbers in different stages of development in the intestinal wall and later in the intestinal lumen. Treatment with the anthelmintic ensured that large numbers of worms were never present in the intestinal lumen, thus making it possible for the animals to be exposed to a potentially lethal level of infection.

Although at present the reason why a divided primary infection evokes greater immunity than a corresponding single dose is not known, there is evidence that the larvae are highly immunogenic (Van Zandt, 1961; Panter, 1969b; Bartlett and Ball, 1972). In a divided infection, such as was used in the present work, the host would be exposed to larval stages for a considerably longer period of time than in animals given a single infection and hence the degree of immunity may be related to the duration of the presence of the immunogenic phase as well as to the intensity of infection.

When the standardized immunizing schedule was compared in different strains of inbred mice, it became clear that each of the three strains behaved in a characteristic way. Thus *N. dubius* was only partially inhibited in development in immunized C3H mice killed on day 9, since larger numbers were recovered from the intestinal lumen when mice were killed on later occasions. There was no evidence of worm loss after day 9. BALB/c and NIH mice both resisted challenge infection, but on day 9 the worm recoveries from NIH mice were always lower than in BALB/c, reflecting a greater capacity to induce inhibition of larval development or destruction of tissue-phase worms. Subsequently, both strains expelled the established worms, few persisting beyond the fifth week of infection.

The results reported in this paper clearly indicate that at least two inbred strains of mice develop resistance to *N. dubius* when exposed to a divided immunizing infection. This method of immunization is superior to the multiple immunizing infections used by previous workers, in which mice were alternatively given infections and anthelmintic treatment until immunity was evoked. It is suggested that these two findings, i.e. that inbred mice can be immunized and that a divided primary infection enhances the development of immunity, offer possibilities for the systematic analysis of lymphoid cell activity in initiating and expressing immunity to *N. dubius*.

**ACKNOWLEDGEMENTS**

Some of the experiments described in this paper were carried out in the Zoology Department of Nottingham University and we would therefore like to thank Professor P. N. R. Usherwood for providing facilities for the work. Jack Keys, Roy Gilder and David Reffin maintained our experimental animals. This work was supported by Ministry of Overseas Development Grant R2993.

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*Accepted* 24 *March*, 1977