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The pattern of peripheral blood leucocyte changes in mice infected with *Nematospiroides dubius*

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ABSTRACT

Experiments were carried out to define the haematological changes taking place during the first six weeks of a primary infection with *Nematospiroides dubius*. The general pattern of changes was observed to comprise a rapid increase in circulating leucocytes (4 to 5-fold increase) which consisted of a neutropl a, lymphocytosis, monocytosis and an eosinophilia. However, in strong responder NIH mice leucocyte counts returned to normal more rapidly than in other strains (by day 28). In contrast, in weak responder C_{57}BL/10 mice the leucocyte counts whilst falling significantly relative to day 7 did not return to normal within the experimental period. Mice infected with irradiated larvae did not experience as high a leucocytosis as did mice given an identical number of normal larvae. The peak lymphocytosis, neutrophilia and monocytosis were all lower. The removal of adult worms from infected animals by treatment with pyrantel on days 9, 11, 13 and 16, also significantly altered the pattern of leucocytosis. The neutrophilia which was evident on day 7 returned rapidly to normal, whereas in mice which had retained their worms a peak neutrophilia was observed on day 14. These haematological changes were discussed and related to the failure of host-protective immunity to operate effectively during the early stages of a primary infection with *N. dubius*.

INTRODUCTION

Pronounced qualitative and quantitative changes in the haematological picture constitute some of the most frequent and easily observed manifestations of infection. However, meaningful conclusions are often complicated by variation in cell counts between different animals and by significant daily changes in individual animals (OGILVIE *et al.*, 1978). Nevertheless such studies coupled with histological analysis of the type and number of cells arriving at the site of infection have contributed significantly to our understanding of the mechanisms of resistance to parasitic diseases (OGILVIE *et al.*, 1980; MOQBEL, 1980; ROTH & LEVY, 1980; BOYER *et al.*, 1971).

Information on the haematological changes in mice infected with *Nematospiroides dubius* is scarce. Only three authors have tackled this aspect of the host-parasite relationship of *N. dubius* and only one of these has studied the primary infection alone. Thus, BAKER (1962) found that infection with *N. dubius* resulted in a marked leucocytosis commencing as early as three days after infection and peaking on days 7 to 9, during the period of emergence of the larvae from the mucosal walls. This leucocytosis was composed of a relative neutrophilia and lymphocytosis. There was a slight rise in the monocyte numbers but no significant eosinophilia was reported. In most animals the total counts returned to normal by day 25, although some animals had a persisting leucocytosis.

CYPESS (1972) and PROWSE *et al.*, (1978) were more concerned with the haematology of repeatedly infected mice. However, their data does contain some information which is pertinent to the present study. Both authors monitored haematological changes during the first two weeks of a primary infection, after which the mice received a second inoculum of larvae and they confirmed Baker's work in that both reported a

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leucocytosis composed mainly of lymphocytes and neutrophils. However, PROWSE *et al.*, (1978) recorded in addition a significant monocytosis corresponding to the time of peak leucocytosis, five to eight days after infection.

It is clear from this review that *N. dubius* does elicit major changes in the haematology of infected mice during the first two weeks of infection, and it is most intriguing that these changes do not reflect effective resistance in operation against the parasite, since during a primary infection, worms complete development and survive for some 8 to 10 months (EHRENFORD, 1954). It would be expected that events during the early stages of infection play a crucial role in determining the outcome of infection. Adult *N. dubius* are known to depress the expression of homologous immunity in the host (BEHNKE *et al.*, 1983; CAYZER & DOBSON, 1983) and furthermore *N. dubius* causes significant non-specific immunodepression (NSID) which is maximal at 14 days after infection, coinciding with the period when leucocytosis has just peaked (ALI & BEHNKE, 1983, 1984). Infection protocols have now been described whereby appropriately manipulated single primary infections can stimulate very high levels of resistance to challenge infection (irradiated infection larvae—HAGAN *et al.*, 1981; 9-day abbreviated infection—Robinson & Behnke, unpublished observation). It was considered that a haematological comparison of mice which were subjected to immunogenic regimes versus normal infection (which is poorly immunogenic) might shed some light on the significance of the pronounced changes in haematology which follow a primary infection and might help to clarify their relevance to host protective immunity to *N. dubius*.

MATERIALS AND METHODS

Animals

Randomly bred CFLP mice and inbred NIH and C_{57}BL/10 mice were bred and maintained under conventional animal house conditions.

Nematospiroides dubius

The *N. dubius* used in the present study was obtained in 1975 from the Wellcome Research Laboratories (Beckenham, Kent) and has been maintained since in CFLP mice. The maintenance of the parasite and the methods used for infection of animals and recovery of worms have already been described (BEHNKE & WAKELIN, 1977; JENKINS & BEHNKE, 1977). The infective third-stage larvae of *N. dubius* were exposed to gamma radiation from a Cobalt 60 source in the Chemistry Department of Nottingham University. The methods used for irradiation have been published (BEHNKE *et al.*, 1980).

Anthelmintic

Pyrantel embonate (Strongid-P paste, Pfizer) was used to remove adult *N. dubius* from the intestines of infected mice. A dose of 100 mg/kg body-weight was given orally as an aqueous suspension. This dose level is known to be more than 95% effective in removing adult worms from the intestinal lumen (BEHNKE & PARISH, 1979).

White blood cell counts

The animals used in all the experiments reported here were part of a larger study in which the lymph nodes and spleens were also studied. These results will be reported elsewhere (ALI & BEHNKE, 1985). The mice were killed with chloroform, weighed and were bled by cardiac puncture. One ml of blood was transferred into small autoanalyser

pots containing a few EDTA crystals as an anticoagulent. These samples were kept at 4°C until processed.

Total white blood cells (WBC) counts were carried out by using a Coulter counter (Coulter Electronics Ltd., England). 40 μl of blood was discharged into 10 ml of isotonic saline solution (Isotone II, Coulter Electronics) and then three drops of zap-oglobin were added immediately before the readings were taken.

Thin blood films for WBC differential counts were prepared by conventional methods and were stained in May Grunwald and Giemsa. Differential counts were carried out by counting 100 cells/slide.

Eosinophils were counted using Discombe's. 20 μl of blood was delivered and mixed with 180 μl of Discombe's. The cells were kept for a few minutes at 4°C and were then counted using a haemocytometer.

Statistical analysis of results

The results are expressed as the group mean $\pm$S.E. Where S.E.s on the figs. overlap only one S.E. is shown or alternatively the S.E. bar of one group is shown slightly to one side of the others. Statistical significance was determined by the non-parametric Wilcoxon test (SOKAL & ROHLF, 1969). A value of $p > 0{\cdot}05$ was considered to be significant.

RESULTS

Comparison of the haematological response in different strains of mice

Three separate experiments were carried out each of which considered the haematological response of NIH, C_{57}BL/10 or CFLP mice to infection with *N. dubius*. Each experiment comprised 65 mice of which 30 were infected and 35 remained as uninfected controls. The infection levels used were 400 larvae for NIH and CFLP mice. C_{57}BL/10 mice, however, were given 250 larvae since worm burdens in excess of 250 cause heavy mortality in experimental groups of this strain. Five mice from each group were killed at weekly intervals and the values for control animals were averaged out over the entire six-week period. These are presented in Figs. 1 and 2 as a line representing the mean with S.E. bars either side.

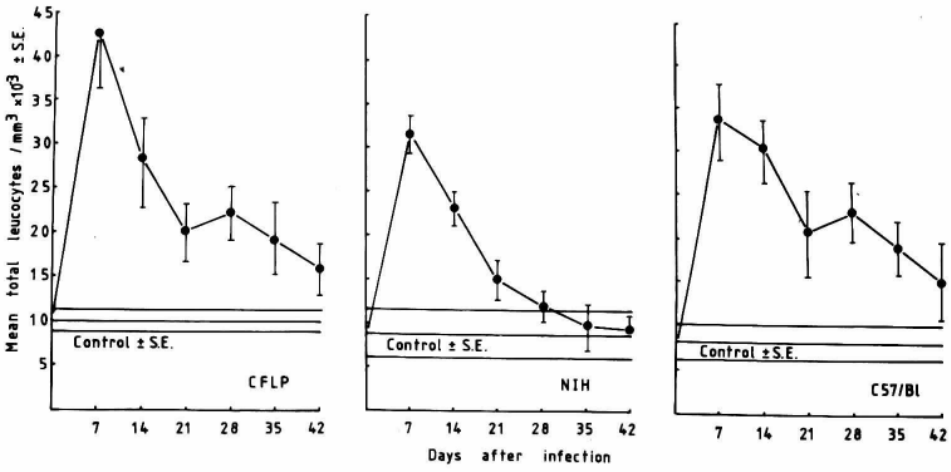

FIG. 1. Mean number of leucocytes/mm^3 of blood in CFLP, NIH and C_{57}BL/10 mice following a primary infection with *N. dubius*. The number of worms recovered from CFLP, NIH and C_{57}BL/10 mice was 370·4 $\pm$17·6, 422·6$\pm$13·8, 233·9$\pm$11·4 respectively.

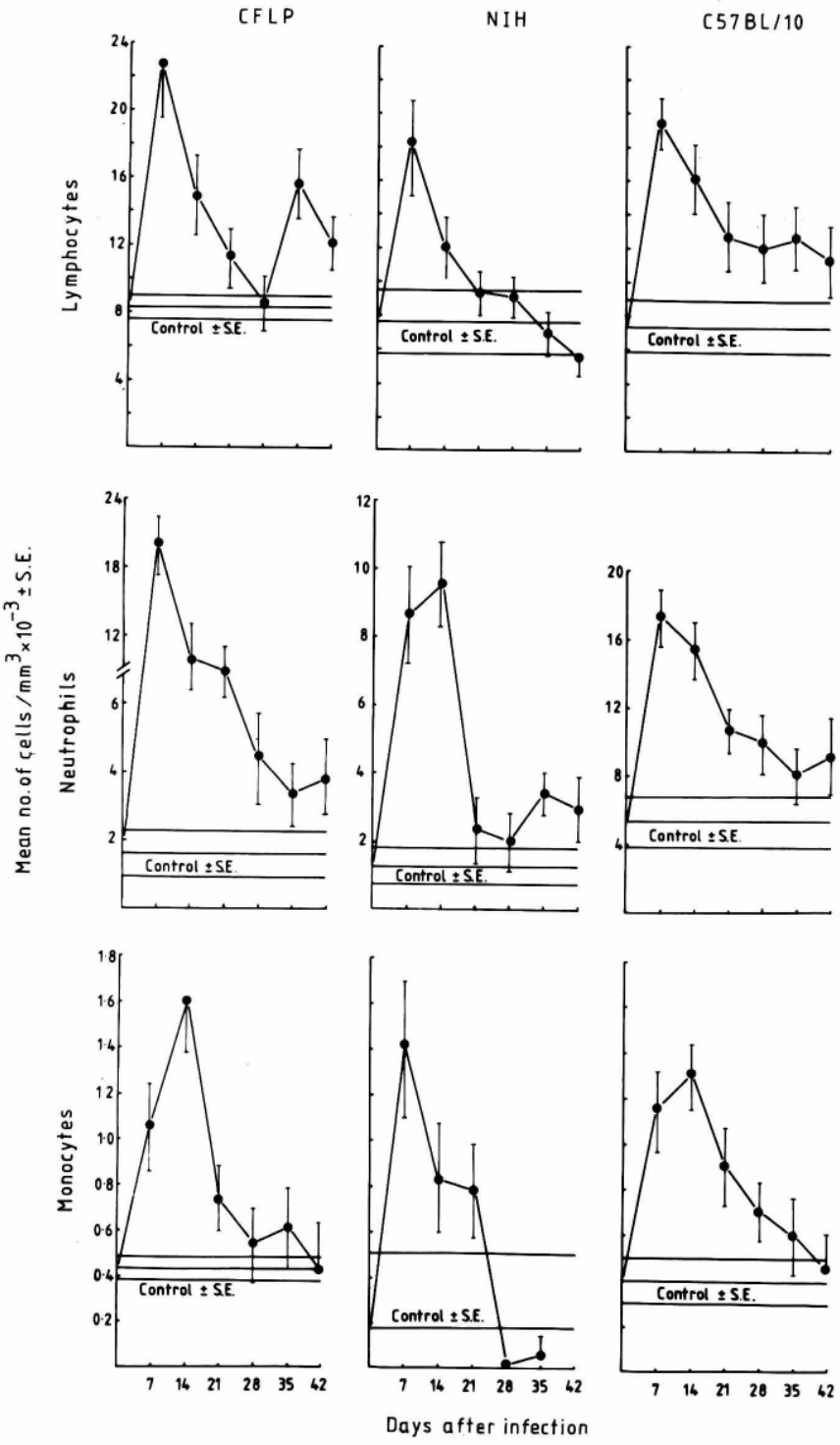

FIG. 2. The mean number of lymphocytes, neutrophils and monocytes/mm^3 of blood in different strains of mice following a primary infection with *N. dubius*.

The general pattern of the haematological changes following infection with *N. dubius* can be seen (Figs. 1 and 2) to be similar to that described by BAKER (1962). Thus all strains responded by a rapid increase in circulating leucocytes which comprised a neutrophilia, lymphocytosis and a monocytosis. However, interesting differences were observed between the strains.

It is clear from both Figs. that in NIH mice leucocyte counts returned to normal more rapidly than in other strains. By day 28 following infection, lymphocyte, monocyte and neutrophil counts were within the normal range for NIH mice. In contrast, the leucocyte counts in $C_{57}BL/10$ mice, whilst falling significantly relative to day 7, did not return to normal within the experimental period. Lymphocyte and neutrophil counts in particular remained high in this strain. CFLP mice fell somewhere between these two extremes in that there was a rapid drop in circulating leucocytes on days 7 to 21 but thereafter the return to normal values was slower than in NIH mice.

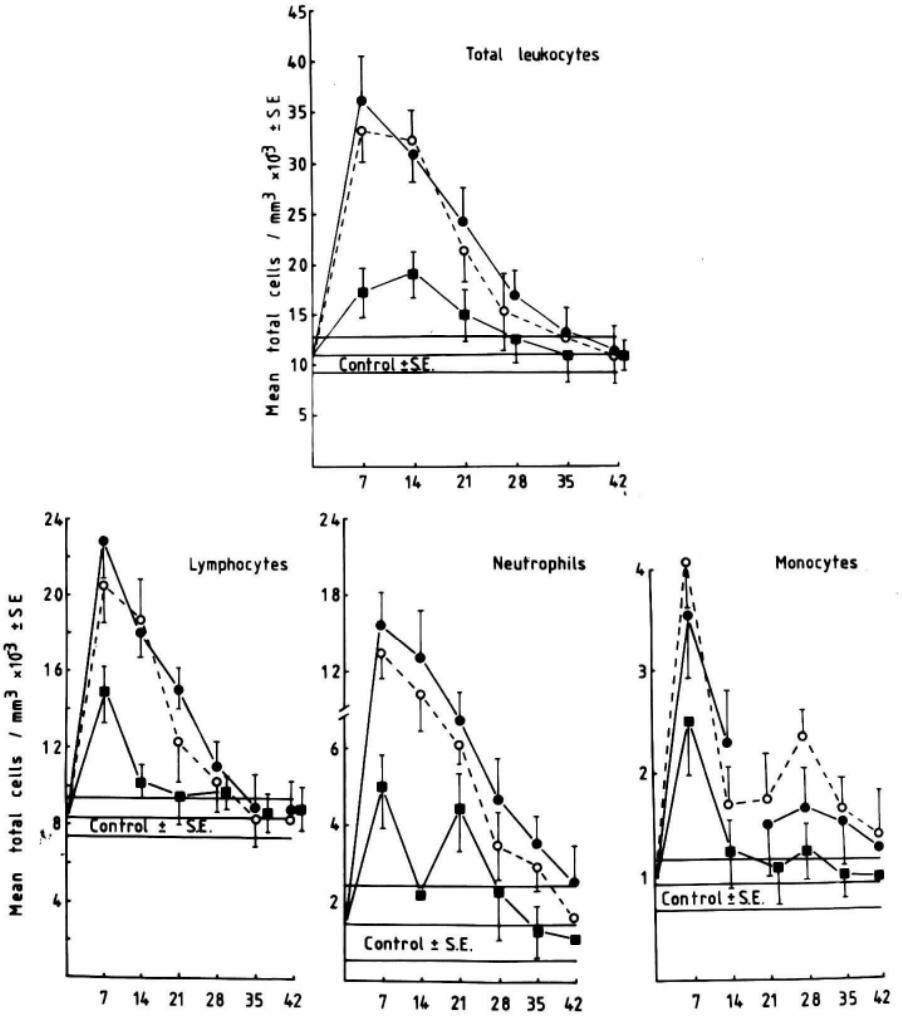

FIG. 3. Mean number of leucocytes/mm³ of blood in CFLP mice given a single infection with 400 (●), 275 (○) or 65 (■) larvae of *N. dubius*. The number of worms recovered was 368 ± 12·8, 272·3 ± 9·8 and 63·6 ± 5·9 respectively.

Comparison of different infection levels

An experiment was carried out in CFLP mice, in which three groups of 30 animals were infected with 400, 275 or 65 larvae of *N. dubius*. A further group of 20 uninfected mice was included as the control group. Five mice from each group were killed and autopsied at weekly intervals as described above. The results are presented in Fig. 3.

Both of the groups given the higher levels of infection (400 and 275) responded as in the previous experiment, but there was little to suggest that mice given 400 larvae behaved any differently from those given 275 larvae. The values were only marginally higher in the group given 400 larvae. However, the mice given 65 larvae had significantly lower counts than the other two groups; but nevertheless there was still a significantly elevated level of lymphocytes, neutrophils and monocytes in the circulation on day 7 when compared to the control animals.

The effect of truncated infections and infection with irradiated larvae

Finally two experiments were carried out in which the mice were prevented from experiencing adult worms. In the first of these, irradiated larvae were used. Thus two groups of 36 mice each were infected either with normal or irradiated (25 krad) larvae. In the second experiment two groups of 30 mice were infected with normal larvae, but one group was treated with pyrantel on days 9, 11, 13 and 16 after infection. A further group of 20 mice was left without infection but was treated with anthelmintic and a final group of 20 untreated mice served as the control to this second experiment. For ease of interpretation comparable date from both experiments are set alongside one another. The results are summarized in Fig. 4.

The figure shows that mice infected with irradiated larvae did not experience as high a leucocytosis as did mice given an identical number of normal larvae. The peak lymphocytosis, neutrophilia and monocytosis were all significantly depressed in relation to the mice given normal larvae. Nevertheless, the group infected with irradiated larvae did experience a significant leucocytosis when compared to the control mice.

The anthelmintic drug, pyrantel, had no effect at all on the haematology of treated uninfected mice, but the removal of adult worms from infected animals by treatment with pyrantel did significantly alter the pattern of leucocytosis. In mice from which the adult worms were removed, the neutrophilia which was evident on day 7 returned rapidly to normal, whereas in mice which had retained their worms a peak neutrophilia was observed on day 14.

As a final component to this last experiment the eosinophils were measured. The data is presented in Fig. 5 and it shows that a primary infection with *N. dubius* does indeed cause a transient eosinophilia. The counts were, however, very variable and although pyrantel-treated mice had lower counts than mice from which the worms had been removed, the difference was only significant on day 28.

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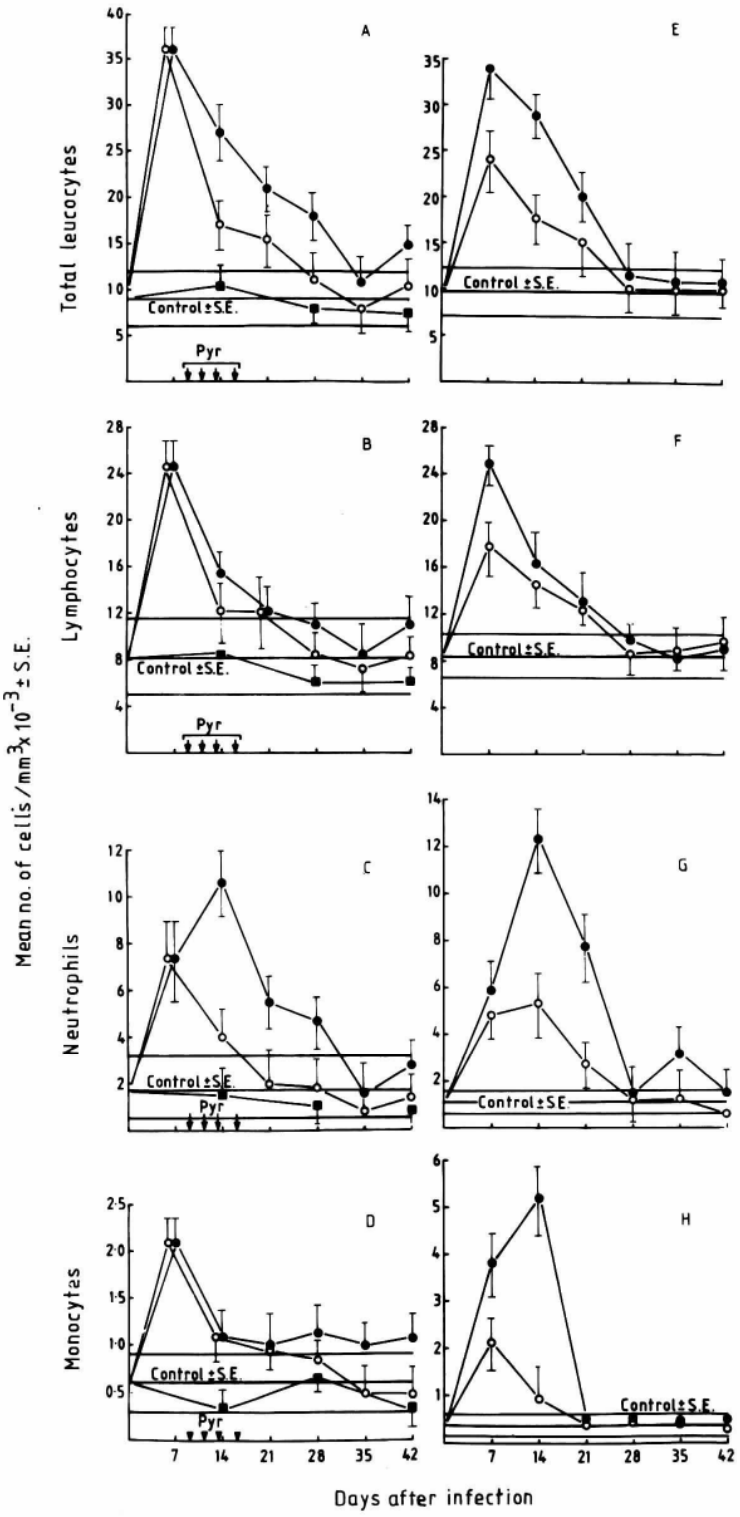
FIG. 4. A, B, C and D. Mean number of leucocytes/mm^3 of blood in CFLP mice given a single infection with 500 larvae of *N. dubius* followed by treatment with anthelmintic drug.
Key to symbols used

● Infected control group (MWR = 459·0 ± 14·1). ○ Infected group treated with anthelmintic (Pyr) on days indicated by arrows (MRW = 1·8 ± 0·08). ■ Group treated with anthelmintic alone.

FIG. 4. E, F, G and H. Mean number of leucocytes/mm^3 of blood in CFLP mice given a single infection with 500 irradiated larvae of *N. dubius*.
Key to symbols used

● Normal larvae (MWR = 462·6 ± 6·0). ○ Irradiated larvae (MWR = 8·4 ± 1·5).


A
E
B
F
C
G
D
H
Total leucocytes
Lymphocytes
Neutrophils
Monocytes
Control ±S.E.
Pyr
Mean no. of cells/mm^3 x 10^{-3} ± S.E.
Days after infection

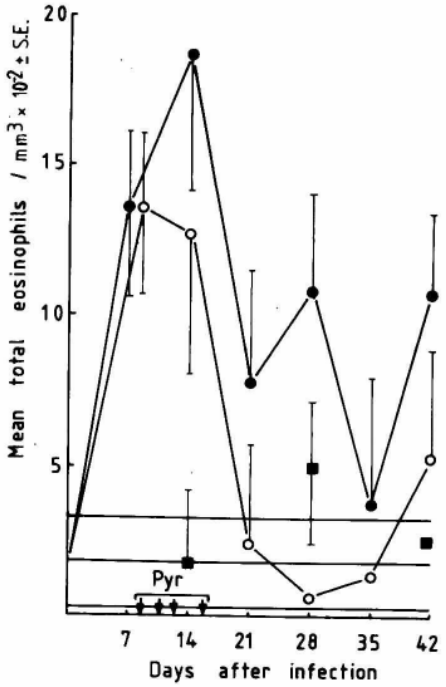


FIG. 5. Mean number of eosinophils/mm³ of blood in CFLP mice given 500 larvae of *N. dubius* followed by treatment with anthelmintic drug (Pyr) on the days indicated by arrows. ●, normal infected group; ○, infected group treated with anthelmintic; ■, group given anthelmintic alone.

DISCUSSION

Haematological changes represent a facet of the host's over-all response to infection and have therefore been widely studied and reported in the literature (OGILVIE *et al.*, 1978, 1980; MOQBEL, 1980; RUITENBERG *et al.*, 1977). In gastrointestinal infections this aspect has been well documented, firstly from a description point of view (PRZYJALKOWSKI *et al.*, 1980) and secondly in an attempt to pinpoint the cells that may be important in host protective immunity (OGILVIE *et al.*, 1980; WAKELIN & DONACHIE, 1983). Circulating WBC numbers increase two to three times above the resting level in *Trichinella spiralis*, *Nippostrongylus brasiliensis*, and *Strongyloides ratti* (see YARINSKY, 1962; OGILVIE *et al.*, 1978; MOQBEL, 1980) but, in *N. dubius*, BAKER (1962) found a peak leucocytosis of 53,000 cells/mm³, over seven times the resting level in control mice (7,000 cells/mm³). In CFLP mice, used in the present work, the resting level of WBC was about 10,000 cells/mm³, somewhat higher than in Baker's experiments and the peak leucocytosis observed on day 7 was represented by values ranging from 35,000 to 45,000 cells/mm³. Our work has confirmed that infection with *Nematospiroides dubius* elicits a rapid increase in all the categories of white blood cells in the circulation and the cell type which showed the maximum relative increase was the neutrophil, which in some experiments increased tenfold.

In rats infected with *Nippostrongylus brasiliensis* WBC counts returned to normal by about day 16 of infection when the parasites had been eliminated from the intestine (OGILVIE *et al.*, 1978). Within this period lymphocytes, large mononuculear cells and eosinophils were found to increase in a biphasic manner but basophils peaked only on

day 14. It was suggested that this last cell type may have a significant but as yet undetermined role in worm expulsion. Although in the present experiments WBC were counted at weekly intervals, this was based on preliminary experiments which gave no indication that more than a single but prolonged peak of leucocytosis was taking place. The increase in WBC and especially the early neutrophilia was associated with the damage caused by developing larvae in the intestinal mucosa. Thus LIU (1965) reported that by the second day of infection haemorrhages were apparent in the intestinal walls, by the third day the local mucosa was inflamed and contained numerous neutrophils. As the larvae developed they caused extensive necrosis of the adjacent tissues and damage to the capillaries. However, by days 6 to 7 when the worms began to emerge and return to the intestinal lumen, the leucocytic infiltration of the cysts in the submucosa was extensive. As the worms left their sites of development they left behind cuticle from the L3 to L4 moult and excretory products which attracted leucocyte and macrophage infiltration into these sites. It is therefore interesting that irradiated larvae, very few of which complete development, caused a significantly lower neutrophilia and monocytosis than normal larvae (Fig. 4). Presumably a proportion of irradiated larvae remains at the late 3rd stage of development and hence the host is not subjected to the damage caused by the considerably larger 4th stages.

There can be little doubt that the pronounced leucocytosis in the mice infected with *N. dubius* represents the mobilization of these cells and their migration to the intestinal wall. As in the other species which have been studied, the haematological picture of infected mice returned to near normal levels during the third and fourth weeks after infection with the obvious difference that in the case of *Nematospiroides dubius*, unlike in *T. spiralis*, *Nippostrongylus brasiliensis* and *S. ratti* the adult parasites survived for a considerable length of time. Therefore, the leucocytosis which was observed early in the infection did not constitute an effective host protective response. Adult worms helped maintain a leucocytosis, especially the neutrophilia since the removal of adult parasites or infection by irradiated larvae resulted in a faster return to normal levels than in infected animals which had retained the full worm burden (Fig. 4).

In contrast to the primary infection, in immune mice the larvae are inhibited and eventually destroyed in the granulomata which form around their sites of development (JONES & RUBIN, 1974). Although neutrophils, lymphocytes and macrophages are present in the lesions in immune mice, it is the eosinophils which are particularly common, and which undergo a dramatic rise in the peripheral circulation (HURLEY & VADAS, 1983). Fig. 5 shows that eosinophilia does also occur during a primary infection. However, no worms are lost during the first month of a primary infection so if eosinophils are the main cell type involved in the killing of trapped larvae in immune mice (PROWSE *et al.*, 1978; HURLEY & VADAS, 1983), they appear to be ineffective in this role during the primary infection. A possible explanation of this phenomenon may be that eosinophil mediated killing in mice is lgG_1 dependent (PRITCHARD *et al.*, 1983; RAMALHO-PINTO *et al.*, 1979). WILLIAMS & BEHNKE (1983) demonstrated that host protective antibody does not appear during a primary infection, and that a period of two to three weeks is required before these antibodies are generated in multiply infected mice. PRITCHARD *et al.*, (1983) also established that host protective antibody was lgG_1 and they suggested that lgG_1 dependent eosinophil mediated killing constitutes the main form of antiparasite immunity in immune animals. In a primary infection this mechanism does not operate because the worms escape to the gut lumen before the granulomata are completed and because of the absence of host protective antibody in these animals. However, even within such granulomata in immune mice, the larvae are not easily disposed of by the host. BEHNKE & PARISH (1979) demonstrated that the larvae may become arrested and can be revived to continue their development when

immune mice are treated with cortisone two to three weeks after a challenge infection. This suggests that the larvae may also have a mechanism of resistance to the granulomatous response developing around them, and that during a primary infection this mechanism coupled with the absence of host protective antibody, allows all the worms to complete development and to escape from the host reaction that might otherwise trap the parasites in the intestinal walls.

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