THE IMMUNE RESPONSE OF THE MOUSE TO *DIPLOSTOMUM PHOXINI* AND CERTAIN CESTODES IN THE INTESTINAL LUMEN

THESIS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY

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In thanks to my parents.

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Summary

This thesis was concerned with the study of gut immunity to an intestinal trematode. The parasite used was the strigeid *Diplostomum phoxini*. Adult *D. phoxini* normally parasitise the intestine of fish eating birds, but will readily establish and reach sexual maturity in laboratory infected mice. After reaching sexual maturity in about 4 days, the worms are lost shortly afterwards, and there is strong evidence that this loss is immunologically mediated by the host. The aims of this thesis were to establish the kinetics of the infection in mice and to investigate aspects of the immune response of the host to the parasite.

The first chapter was concerned with the kinetics of establishment and rejection of both a primary and secondary infection of *D. phoxini*. The kinetics of heavy (200 metacercariae) and light (20 metacercariae) infections were compared in an attempt to economise on the number of parasites required, especially with regards to immunization schedules. Results showed that in NIH mice, at least 80% of the administered worms would establish in the intestine. This worm population remained stable until rejection, the onset of which was related to the level of infection, i.e. heavy worm burdens were expelled earlier than light infections. The most important experiment with regards to immunity was the demonstration that a secondary infection was expelled earlier than a primary infection, showing that loss was probably immunologically mediated and that 'memory cells' were established as a result of a primary infection in a mouse.

The remaining chapters were concerned with aspects of the response of the host to the infection. Chapter 2 dealt with the lymphocyte response and it was shown that high rates of cell division occurred in the mesenteric lymph node during infection. The blast cells were probably responsible for the successful transfer of adoptive immunity because cells recovered from donors after blast activity had declined eg. day 12 immune mesenteric lymph node cells, could not transfer immunity. Interestingly, enriched B cell populations were also able to transfer a significant degree of protection, but they were still contaminated with T cells.

The third chapter was concerned with the antibody response. The successful transfer of passive protection was achieved with secondary immune serum, but not with primary immune serum, although the former was not as efficient as the immune mesenteric lymph node cells in accelerating rejection. Circulating antibody titre was measured by an enzyme-linked immunosorbent assay and increased titers were recorded during infection with higher titers during a secondary infection. An indirect fluorescent antibody test using living <u>D. phoxini</u> as target antigen subsequently showed that most of this antibody was IgG with some IgM.

The presence of antibody on the tegument of the worms was investigated by direct fluorescence. This was carried out as it is reasonable to assume that if antibody has any direct effect on the worm, then this is likely to be directed against the tegument. The presence of IgA, IgG and IgM was recorded and the evidence suggested that at least the IgG and

IgM was specific anti worm antibody.

The final chapter was concerned with the non-specific responses of the mouse. The intra epithelial globule leukocyte, lamina proporia mast cell and goblet cell response to infection was investigated. Both the intra epithelial globule leukocytes and lamina proporia mast cells increased in numbers in response to infection, with the greatest response observed in the intra epithelial globule leukocyte population. No increase in goblet cell numbers was observed. The transfer of immune mesenteric lymph node cells in the presence of D. phoxini also accelerated the increase in the intra epithelial globule leukocyte and lamina proporia mast cell populations which suggests that these cells may be under lymphocyte control.

Finally the results of the experiments on D. phoxini were discussed in relation to the more well known, but still far from well understood, nematode and cestode models.

Abbreviations

Ab	Antibody
C	Complement
^{o}C	degree centigrade
CI	Cytotoxic index
CMLNC	Control mesenteric lymph node cells
c.p.m.	counts per minute
CS	Control serum
ELISA	Enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
g	gravity
GāChIg/FITC	Goat anti-chicken immunoglobulin/FITC
GALT	Gut associated lymphoid tissue
$G\bar{a}MC_3/FITC$	Goat anti-mouse C_3/FITC
GāMIg/FITC	Goat anti-mouse immunoglobulin/FITC
GāMIgA/FITC	Goat anti-mouse immunoglobulin A/FITC
GC	Goblet cell
GPC	Guinea pig complement
HBSS	Modified Hanks balanced salt solution
I	Iodoacetamide
IEGL	Intra epithelial globule leukocyte
IFAT	Indirect fluorescent antibody test
Ig(s)	Immunoglobulin(s)
IMLN	Immune mesenteric lymph node cells
i.p.	intraperitoneally
IS	Immune serum
ISC	Immune spleen cells
ITDL	Immune thoracic duct lymphocytes
J	Joining chain

LI	Labelling index
LPMC	Lamina propria mast cell
m	metacercariae
MC	Mast cell
MLN(C)	Mesenteric lymph node (cell)
M199	Medium 199
NaF	Sodium fluoride
NaN_3	Sodium azide
PBS	Phosphate buffered saline
PEC	Peritoneal exudate cell
p.i.	post infection
RabāMIgG/FITC	Rabbit anti-mouse immunoglobulin G/FITC
SC	secretory component
ShāMIgM/FITC	Sheep anti-mouse immunoglobulin M/FITC
sIg^-	surface immunoglobulin negative i.e. T cell
sIg^+	surface immunoglobulin positive i.e. B cell
v.c.u.	villous crypt unit
v/v	volume to volume ratio
w/v	weight to volume ratio
W.L.E.P.	The Wellcome Laboratories for Experimental Parasitology
<	less than
>	greater than
$\bar{x} \pm$ s.d.	mean $\pm$ standard deviation
2-4 DNP	2,4-dinitrophenol
1^o	Primary
2^o	Secondary
3^o	Tertiary
$[^{125}I]$-Udr	5-$[^{125}I]$-iodo-2'-deoxyuridine
% Δ	% depression

GENERAL INTRODUCTION

The work involved in this thesis was undertaken to study the intestinal immune responses of mice to an intestinal lumen dwelling trematode.

Very little work has been done on intestinal lumen dwelling trematodes. This is because in general, intestinal trematodes, although common parasites of man and other mammals as well as other vertebrates, are of much less importance than blood and liver trematodes.

The blood trematodes, schistosomes for instance, have been recorded as infecting 125 million people in 1972 (Wright, 1972), and between 200 and 300 million people at present (Butterworth, address R.S.T.M. Scot. 1983). Almost certainly this represents a real increase in incidence associated with the spread of irrigation and the failure of snail control and chemotherapy to contain the disease. Intensive investigations into snail control and the discovery of more effective and better tolerated schistosomacides continue, and these are still the only effective form of control, but the possibility still exists that an anti-schistosome vaccine could be produced. Prospects for vaccines against helminths tend to 'ebb and flow' but the development of monoclonal antibody techniques permitting the identification of specific antigens has caused the vaccine tide to flow in recent years.

Whether the study of the immune response against an intestinal trematode will be of relevance to schistosome immunity is just not known, on balance probably not, as the effector cells of the host's attack are almost certainly

different. This is possibly reflected in the fact that the tegument of an intestinal fluke is the normal trilaminate membrane, not the heptalaminate of the blood fluke (McLaren, 1980). Nevertheless, study of an intestinal fluke/mammal model is of great importance, not only as a biological phenomenon but (i) it should increase our general understanding of the way the intestine limits worm infections and (ii) the results would be of specific interest to medical and veterinary staff investigating intestinal trematode infections like Fasciolopsis buski, Heterophyes heterophyes, Metagonimus yokogawai and various echinostomes which occur in both man and his domestic animals. There is very little information on immunity to these parasites (Muller, 1975) although Christensen et al (1981) have done some work on resistance to Echinostoma revolutum in Schistosoma spp. infected mice. These authors showed that schistosomes induced complete cross resistance to E. revolutum.

The majority of work on gut immunity to parasites has been done on nematodes such as Nippostrongylus brasiliensis, Trichinella spiralis, Trichuris muris, Nematospiroides dubius and others. Also much work has been done on the response of the gut to infection with cestodes such as Hymenolepis diminuta.

There are several reasons for using these parasites in gut immunology studies. The parasites can be easily maintained in the laboratory, and are therefore convenient to use. They also provide excellent models for the study of diseases of humans and domestic animals such as trichinosis and trichuriasis under controlled laboratory conditions. Similarily they

provide good models for the study of gut responses to infection. Also, there is a large volume of literature on these parasites to provide excellent background information.

This thesis attempts to investigate the expression of immunity to an intestinal dwelling trematode. The work attempts to investigate how the mammalian gut brings about expulsion of the trematode and compares the mechanism of expulsion with known nematode and cestode models.

The parasite used in the present study is the strigeid Diplostomum phoxini. Adult D. phoxini occur in the intestine of fish eating birds. The life cycle of D. phoxini was demonstrated by Arvy and Buttner (1954) and by Rees (1955; 1957). It is a complex life cycle involving two intermediate hosts. The first intermediate host in France is the snail Lymnea auricularia (L) (Arvy and Buttner, 1954) but in Wales it is Lymnea peregra var. ovata (Rees, 1957). In Glasgow, Berrie (1960a) found Lymnea peregra to be the intermediate host. The second intermediate host in Britain is the minnow Phoxinus phoxinus. The metacercariae are located in the brain.

Adult D. phoxini have been recovered from experimentally infected ducks (Rees, 1955; Berrie, 1960a). Berrie (1960a) showed that the worms grew to a greater size in gulls than in other hosts, but of significance to the present study is that he managed to recover fully mature adults producing eggs and mature sperm from mice.

Extensive histochemical, light and electron microscopic

investigations have also been carried out on the nature of the host-parasite interface, especially the role of the pseudosuckers and holdfast organ (Lee, 1962; Erasmus, 1969, 1970b and 1970d).

Both Lee's and Erasmus' work suggests that the host-parasite interface between D. phoxini and the intestine wall is very close. Erasmus (1969) states "that there seems to be little doubt that attachment to the host tissue is muscular and that close host-parasite contact occurs in the region of the lappet". He also recognised the existence of three host-parasite interfaces, (a) the general tegument, (b) that covering the adhesive organ and (c) the lappets which are paired glandomuscular complexes.

Ohman (1965) working with Diplostomum spathaceum in gulls recorded some lysis of host epithelium where the lappets were attached and in the vicinity of the ventral sucker and adhesive organ. Probably similar damage occurs to the mouse intestine where D. phoxini is attached.

Erasmus (1970b) demonstrated the presence of spines on the sides of the adhesive organ in D. phoxini although the surface of the organ is devoid of spines. These spines could produce considerable erosion of host tissue.

Various enzymes have been demonstrated in the adhesive organ, forebody gland cells and lappet cells in both D. phoxini and D. spathaceum by Lee (1962) and Ohman (1965) respectively. They demonstrated the presence of esterases,

acid phosphatase and alkaline phosphatase but neither worker could demonstrate the presence of proteolytic enzymes. Ohman (1965) also demonstrated the secretion to the exterior of acid phosphatase and esterase in D. spathaceum. The function of these enzymes in the parasites are unknown, as is their effect on the host gut. However as the secretion of these proteins has been demonstrated, it is possible that they could be absorbed by the host, providing a source of parasite antigen.

Host epithelial tissue could be disrupted by both enzyme and mechanical means. This damage could result in another pathway for antigen uptake by the host as well as by the normal antigen sampling routes of the gut, the Peyers patches and the membranous epithelial cells (M-cells) covering the Peyers patches (Walker, 1981). and possibly across or between normal columnar epithelium.

Together with the light and electron microscopy and histochemical studies, further work has been done on the development of D. phoxini both in vivo, in the domestic duck Anas boschas domestica, and in vitro using different culture media (Bell and Hopkins, 1956; Bell and Smyth, 1958), and attempts to replace the yolk constituent with synthetic media composed of serum and amino acids (Wyllie, Williams and Hopkins, 1960). The most satisfactory medium found so far for the in vitro development of D. phoxini is a egg yolk/ albumen/yeast extract which provides a medium of a consistency suitable for ready ingestion by the worm (Kannangara and Smyth, 1974). Metacercariae of D. phoxini can also be stored in vitro in NCTC 135 at $4^{o}C$ for at least 2 months without loss of viability (Kannangara and Smyth, 1974).

These combined studies provide a useful basic framework of knowledge about the parasite.

In the following work, the kinetics of the D. phoxini - mouse model were first investigated to confirm the results of Mawdsley (1983). It is important to establish the kinetics of both primary (1^{o}) and secondary (2^{o}) infections to allow further work on characterising the immune response.

After establishing the kinetics of the host-parasite relationship, the response of lymphocytes, especially the immune mesenteric lymph node cells (IMLNC), using cell transfer, cell fractionation and cell division assay techniques, during infection was investigated. The response of antibody, both in serum and on the tegument of the worm was studied to determine if antibody was involved in the expulsion mechanism of the parasite.

Finally an investigation of histological changes in the small intestine during infection was undertaken with emphasis on the response of goblet cells (GC) and mucosal mast cells (MMC) to the presence of the worm.

The possible role, if any, and interaction of these effector mechanisms in the expulsion of D. phoxini is discussed. The results are also discussed and compared in relation to the better known, but still far from well understood enteral responses to nematodes and cestodes.

GENERAL MATERIALS AND METHODS

1) Animals

Mice

The majority of experiments were carried out on inbred NIH mice. These were bred in the Wellcome Laboratories and maintained under conventional animal house conditions.

The C_3H and C57 mice used in some experiments were bred and maintained as above.

In one experiment NIH mice were obtained from Hacking and Churchill Ltd.

Fish

Minnows, Phoxinus phoxinus were obtained from Mugdock and Craigmaddie reservoirs near Glasgow. The fish were maintained in a large covered aquarium outside. This was provided with copper free running water. Throughout the Winter the minnows were fed once a week with dried Daphnia.

Parasite

Obtaining metacercariae and infecting mice

The metacercariae were obtained from the brains of minnows. The brain was removed and placed in modified Hanks balanced salt solution (HBSS) at room temperature. The

metacercariae were then dissected out, and the required number for infecting one mouse were counted into a watchglass.

Before infecting, mice were starved overnight on wire grid floor cages, but were provided with water.

The metacercariae were administered by stomach tube. This apparatus was composed of a 1.0 ml plastic syringe with a 25 G 5/8" needle connected to an appropriate polythene tubing (Portex Ltd). The tubing was at least 60 cm long, allowing sufficient length to prevent the metacercariae from entering the syringe.

The parasites were administered in a volume of 0.2 ml of HBSS while the mouse was under light ether anaesthesia.

Recovery of adult worms

Mice were killed by an overdose of ether vapour. Starting at the pyloric end, the small intestine was removed and placed on a 30 cm plastic ruler. This prevented shrinkage by contraction. The anterior 20 cm of the small intestine was cut into four 5 cm sections. Each section was placed in a 5 cm glass petri dish containing HBSS at 37°C. Each section was opened longituidinally and incubated at 37°C for 1 hour.

After incubation the majority of worms could be collected from the bottom of the petri dish. The gut was

however shaken vigorously to free any worms remaining on the gut.

In early experiments mucosal scrapings were carried out after incubation. Usually no worms were recovered suggesting that incubation was successful in recovering the worms. Gut scrapings were therefore stopped.

2) Anthelmintic

Mice were routinely treated with piperazine citrate (Citrazine, J. M. Loveridge Ltd) at 3 g/l in their drinking water for two weeks and then given normal drinking water for a week before being used in experiments. This was to remove any nematodes which may be present.

3) Preparation of cell suspensions

The appropriate organ (mesenteric lymph node, thymus or spleen) was removed from the mouse, cleaned of fat and placed in plastic test tubes containing cold ($4^{o}C$) HBSS and kept on ice. The organs were placed in nylon sieves, diced with scissors and squeezed through the fine mesh nylon sieve with a 5 ml syringe stopper. The cells were squeezed into a 10 cm plastic petri dish containing medium 199 (M199) (Gibco, Europe) with L-glutamine and Hepes buffer (25 mM), supplemented with 5% v/v heat inactivated foetal calf serum (Gibco, Europe) and 10 i.u./ml of heparin (B.D.H. Chemicals Ltd). The debris was allowed to settle for 5 minutes, the supernatant was then removed, and centrifuged at 200 g for

5 minutes. The cells were resuspended in a known volume of fresh medium. Cell viability was tested using the Trypan blue dye exclusion test. One drop of the cell suspension was mixed (1 in 20) with a 2% w/v solution of Trypan blue in HBSS, and the number of live cells counted in a haemocytometer. The concentration of cells was then adjusted to give the required number in a volume not greater than 0.5 ml. The cells were injected intra-peritoneally (i.p.).

4) Protein Assay

Protein content of crude antigen was measured by the method of Schacterle and Pollack (1973) which is a modified method of Lowry et al (1951).

A series of dilutions of the test antigen were prepared. One ml of the alkaline copper reagent (see solutions) was mixed with 1.0 ml of the antigen sample and the test tube was allowed to stand undisturbed for 10 minutes at room temperature. Four ml of the phenol working solution (see solutions) was then added forcibly and rapidly and the test tubes incubated in a water bath at 55°C for 5 minutes. They were then cooled rapidly in cold tap water for 1 minute. The results were read in a spectrophotometer at 650 n.m.

The standard used for calibration was Bovine serum albumin, Fraction V (Sigma Chemical Co).

5) Statistics

The students' 't' test was used to measure the statistical significance of differences between mean values in results. P values$<$ 0.05 were considered significant.

6) Solutions

Modified Hanks' Balanced Salt Solution (HBSS) (Hopkins and Stallard, 1974)

The Hanks' solution was modified by excluding glucose and $NaHCO_3$ and increasing the remaining salts to an osmotic pressure of 300 m-osmole.

Solution 1

NaCl	168 g
KCL	8 g
KH_2PO_4	2 g
Na_2HPO_4	4 g
0.02% Phenol red	200 ml

Made up to 2.0 l with de-ionized water.

Solution 2

$CaCl_2.2H_2O$	3.92 g
$MgCl_2.6H_2O$	2.0 g

Made up to 2.0 l with de-ionized water.

105 ml of solutions 1 and 2 were mixed and made up to 1.0 l with de-ionized water (final pH 7.2).

Phosphate buffered saline (PBS, pH 7.2)

Na_2HPO_4	2.84 g
$NaH_2PO_4.2H_2O$	2.76 g
NaCl	9.0 g

Made up to 1.0 l with distilled water.

Trypan blue

The stock solution, 2.0 g trypan blue (B.D.H. Chemicals Ltd) was made up to 100 ml in distilled water and diluted 1:20 in HBSS for use in the dye exclusion test for cell viability.

Alkaline copper reagent

A solution of sodium hydroxide (0.5 N) containing 10% sodium carbonate, 0.1% potassium hydrogen tartrate and 0.05% copper sulphate. This reagent has been found to be stable for at least 30 days at approximately 24°C.

Phenol reagent

The Folin-Ciocaltea phenol reagent was obtained as a 2 N solution (B.D.H. Chemicals Ltd), and diluted to 1 N with distilled water. A working solution was prepared by

making a 1:9 dilution (distilled water) of the 1 N solution.

The following reagents were used in the ELISA.

Wash buffer (PBS, pH 7.2)

To 1.0 l of distilled water the following were added:

Na_2HPO_4 (anhyd)	1.07 g
$NaH_2PO_4.2H_2O$	0.39 g
NaCl	8.5 g
Tween 20	0.05% v/v

Coating buffer (Carbonate - Bicarbonate, pH 9.6)

The following were made up to 1.0 l with distilled water.

Na_2CO_3	1.5 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g

The coating buffer should be kept at 4°C and made fresh every 2 weeks.

Diluting buffer

To the wash buffer add 0.25% w/v Bovine serum albumin and 0.01% w/v NaN_3.

CHAPTER 1

THE <u>DIPLOSTOMUM PHOXINI</u> - MOUSE MODEL SYSTEM

Introduction

It is of fundamental importance when investigating any laboratory host - parasite relation to work out the kinetics of infection. Once this is achieved one is able to investigate and characterise the immune response further by experimental manipulation.

The kinetics of the D. phoxini - NIH mouse system have been investigated by Mawdsley (1983) who observed that expulsion of a 200 metacercariae (m) primary (1^{o}) infection began on day 6 post infection (p.i.) and was complete by day 11 p.i. A 20 m 1^{o} infection however was expelled later, rejection beginning after day 8 p.i. and being complete by day 12 p.i. In both cases, the mice were found to be fully immunized against a challenge infection of 200 m. Mawdsley further demonstrated, that such a challenge infection began to be expelled after day 2 p.i. and was complete by day 5 p.i.

The following experiments were therefore designed to (i) enable the author to gain experience in handling the system, (ii) to determine whether the earlier work was reproducable and (iii) to extend the scope of the earlier experiments.

It is of importance to know the exact duration of a 1^{o} infection so that one can measure the effect of different treatments on expulsion eg. the adoptive transfer of

lymphocytes from previously infected mice. If expulsion of a 1^{o} infection could begin earlier than day 6, (as some preliminary experiments had suggested) it would make the time difference between a 1^{o} and a secondary (2^{o}) response very narrow. This would make it difficult to determine the extent to which experimental treatments were having on immunizing effects. It might be advantageous therefore to develop a regime which would prolong the duration of a 1^{o} infection. This would allow greater time for experimental manipulation during the infection and might make identification of partially effective components of the immune response easier.

In the D. phoxini - mouse model there are several possible ways of prolonging the duration of a 1^{o} infection. The two methods investigated were (i) by reducing the worm burden and (ii) by using a different strain of mouse.

Several workers have shown that the rate of rejection is related to the worm burden eg. Haley and Parker (1961) using Nippostrongylus brasiliensis in rats (Quoted from Ogilvie and Jones, 1971), Harris and Turton (1973) using Hymenolepis diminuta in rats, Hopkins and Stallard (1974) using Hymenolepis citelli in mice and Befus (1975) using H. diminuta in mice. A threshold level may exist in these models below which worm rejection does not occur as shown by Wakelin (1973) in the Trichuris muris - mouse system.

Previous work carried out in this laboratory has

shown that different strains of mice react differently to the same parasite. Wakelin (1975b) showed that inbred NIH showed less variation in worm burden and greater uniformity in worm expulsion than random bred CFLP mice when infected with T. muris.

Wakelin also compared establishment and expulsion in other inbred strains. He found that the ability to expel T. muris was common to all strains investigated except strain DBA_2, but the time at which expulsion took place was a strain characteristic. Expulsion of T. muris from C_3H and C57 mice commenced later than in NIH mice.

Two experiments were designed to compare the response of NIH and C_3H and NIH and C57 mice.

As the response to infection can vary between inbred strains it was important to know whether the response could vary within the strain, especially as the NIH were bred at the laboratory (W.L.E.P.). This is important as it is known that sublines of a strain gradually diverge due to environmental factors or genetic segregation (Festing, 1979). Green (1953) showed that sublines of C_3H even differed in their number of lumbar vertebrae, either having 5 or 6, and a few appeared asymmetrical.

A comparison of the primary response of W.L.E.P. NIH and NIH from a commercial supplier was therefore done.

The loss of the 1^o infection does not necessarily imply

that it is immunologically mediated. Hopkins (1980) states "The single most important experiment which indicates that rejection is immunologically mediated is the demonstration of memory". Immunological memory can be expressed in many ways and has been demonstrated in most host parasite systems.

The most striking effect of immunological memory is the faster rejection of an established (2^{o}) infection of the homologous parasite as occurs with Trichostrongylus colubriformis in guinea pigs (Gordon et al, 1960; Herlich, 1963), Trichuris muris in mice (Wakelin, 1967), Trichinella spiralis in mice (Wakelin and Lloyd, 1976; Alizadeh and Wakelin, 1982), both Nippostrongylus brasiliensis (Jarret et al, 1968) and Strongyloides ratti in rats (Moqbel and Denham, 1977) and Apatemon gracilis minor in ducks (Blake, 1973). Behnke and Wakelin (1977) showed that challenge infections of Nematospiroides dubius, a trichostrongyle nematode which causes chronic infections in mice would be expelled if the 1^{o} immunizing infection was terminated with anthelmintic.

In the H. diminuta - mouse system the most striking effect of memory is the stunting of worm growth which occurs (Hopkins et al, 1972; Befus, 1975). Stunting of H. diminuta 2^{o} infections also occurs in rats (Andreassen and Hopkins, 1980). Roepstorff and Andreassen (1982) demonstrated the faster rejection of a 2^{o} infection of H. diminuta in C57 mice but they used heavy worm burdens, i.e. 100 cysticercoids.

Stunting has also been observed in T. spiralis (Rapport

and Wells, 1951), T. muris (Wakelin, 1967), N. brasiliensis (Ogilvie and Hockley, 1968) and S. ratti (Moqbel and Denham, 1977).

In other host parasite models the 2^{o} infection worms fail to establish resulting in lower burdens as with Fasciola hepatica in the rat (Hayes et al, 1972, 1973; Rajasekariah and Howell, 1977), Strongyloides ratti (Moqbel and Denham, 1977) and both Echinostoma revolutum (Sirag et al, 1980) and N. dubius (Behnke and Wakelin, 1977) in mice.

Perez et al (1974) and Sher et al (1974 a) showed that fewer schistosomula of Schistosoma mansoni were recovered from the lungs of immune rats and mice respectively than from non-immunized controls. They concluded that 'a significant amount of the schistosomula were eliminated in the lungs or at an earlier point in the pathway of migration'.

Immunological memory can also effect the parasite in other ways. Loss of fecundity has been demonstrated in 2^{o} infection of T. spiralis (Denham and Martinez, 1970; Kennedy, 1980) and in N. brasiliensis (Ogilvie and Hockley, 1968). Gray (1973) showed that 2^{o} infections of the poultry cestode Railletina cesticillus did not reach patency and worms lost their strobilae by day 14 p.i.

Other effects of memory include earlier morphological damage as observed in N. brasiliensis (Ogilvie and Hockley, 1968) and also altered distribution of the parasite as seen

with S. ratti (Moqbel and Denham, 1977), and with 50 cysticercoid infections of H. diminuta in the rat (Andreassen and Hopkins, 1980).

The object of the present work was to determine the longevity of D. phoxini in a 2^{o} infection, and the extent to which varying the number of worms in the 1^{o} immunizing, infection affected this.

Materials and Methods

Both infection and worm recovery are described in the general material and methods.

Results

Survival of D. phoxini in heavy (200 metacercariae) and light (20 metacercariae) primary infections.

Twenty, 8 - weeks - old, female, N_{IH} mice, caged four per box were infected with 200 m (Group A) and 25 (caged 5 per box) with 20 m (Group B). One box of each group was killed on the days shown (Fig 1.1) between days 2 and 12 p.i.

To economise on mice, no group B mice were killed on day 4 p.i. because Mawdsley's results had shown that loss of worms did not begin until day 8 p.i. The kill on day 2 p.i. was a check on establishment. Similarily no group A mice were killed on day 10 p.i. because Mawdsley had shown that expulsion was virtually complete by then. A group was killed on day 12 to try and ascertain whether expulsion was total.

Establishment of worms, as measured by recovery on day 2 p.i., was over 80% in both groups (Table 1.1, Fig 1.1). The worm population in group A remained stable until day 4 and this was followed by a decline. The exact day that the decline began was not known but there was no significant difference in the rate of worm loss in the two groups up to day 6. Expulsion of group B worms proceeded at a slightly slower rate after day 6. The % loss of worms was significantly greater ($P < 0.02$) in group A mice on day 8 p.i. and all worms had been expelled by day 12 p.i., whereas 10% still remained in the group B mice, i.e. the mice which had

received the light infection (Table 1.1, Fig 1.1). The rate of rejection of the 20 worm infections was apparently much quicker than in Mawdsley's mice (1983). It was decided, therefore, to repeat the 20 m infections, to determine whether this difference was due to an experimental error (due to lack of experience) or whether the mice were now rejecting more acutely to light infections. In order to gain new information at the same time, it was decided to investigate the effect of age of the mice on their ability to reject light infections.

The effect of age of mouse on the course of a primary infection.

Thirty female, NIH, 14 - weeks - old, and thirty female, NIH between 5 - 6 weeks of age, caged 5 per box were infected with 20 m. One box of mice of each cage was killed 2, 4, 6, 8, 10 and 12 days p.i.

Rejection of D. phoxini by the young and old mice used in this experiment was almost identical (Fig 1.2). There was no statistically significant difference between any two cages killed on the same day (Table 1.2).

Of interest however is that more flukes were recovered on day 4 than on day 2. This was probably because the flukes were larger and easier to find, but it could also be that more flukes were actually present. Recovery on day 4 p.i. is therefore a better measure of establishment in light (20 m) infections.

Higher worm burdens were obtained on days 6 and 8 p.i. than in the previous experiment (Table 1.1), i.e. rejection was slower; the time course agreeing closely with Mawdsley's results (1983).

Comparison of the duration of a primary infection in (i) NIH and C_3H mice and (ii) NIH and C57 mice.

The design of both these experiments was similar; one is described.

Thirty, male NIH and 28 male, C_3H (females were used in (ii)) were caged in groups of 6, each cage containing 3 NIH and 3 C_3H mice, except groups killed on day 4 p.i. which contained 3 NIH and 2 C_3H mice. Half the groups were infected with 20 m on day 0 and the remainder on day 1. The first kill was made 4 days p.i. and subsequently every two days until the end of the experiment. The reason for dividing the experiment was to allow enough time to recover the worms because of the light infections used. The results were pooled.

The results show that both C_3H and C57 were less suitable hosts for D. phoxini compared with NIH. There was a wide variation in establishment, as measured on day 4 p.i. and also rejection was faster in C_3H and C57 than in NIH, (Figs 1.3 and 1.4; Tables 1.3 and 1.4).

Of importance is the fact that 80% of the worms were recovered from the NIH on day 8 p.i. in both experiments,

These two experiments showed different responses between strains, the next experiment was to investigate if there were different responses within the NIH strain.

Comparison of a primary infection of 200 metacercariae in W.L.E.P. NIH and commercially bred NIH mice.

Four star category, female, NIH were purchased from Hacking and Churchill Ltd. Mice were caged in groups of 6, each cage containing 3 W.L.E.P. and 3 commercially bred mice. The infection and recovery schedule were the same as used in the light infections, except that the mice were infected with 200 m. Because rejection was expected to be underway by day 6, a kill on day 5 was included to determine whether rejection started in either or both sub-lines between day 4 and 5, or day 5 and 6. To economise, a day 10 kill was excluded as it was doubtful whether it would reveal significant information.

Expulsion of parasites in both groups was virtually identical (Fig 1.5, Table 1.5); there was no statistically significant difference between the groups.

It had been noted in previous experiments that a posterior shift in position of the worms was associated with expulsion, so position of the worms was also measured in the experiment. The data shown in Fig 1.6 was obtained from the groups of 3 W.L.E.P. NIH infected on day 0 of the experiment with 200 m.

Up to day 4 p.i. virtually all the worms recovered were located in the anterior 10 cm of the gut, i.e. the duodenum and first few cm of jejunum. By day 5 p.i. a slight posterior shift had begun and by day 6 p.i. less than 50% of the worms recovered were found in the anterior 10 cm. From day 8 onwards, too few worms were recovered to draw conclusions about their distribution.

Effect of a heavy (200 metacercariae) and light (20 metacercariae) immunizing infections on the duration of a challenge infection of 200 metacercariae.

Twelve, 8 - weeks - old, female NIH were infected with 200 m (Group A) and caged in groups of 4. Fifteen age matched female mice were infected with 20 m (Group B) and caged in groups of 5. All mice were challenged on day 27 p.i. with 200 m and a cage of each group was killed 2, 4 and 6 days after challenge.

The results show that establishment of the worms was not affected by immunization (Fig 1.7). 80% of the worms were recovered on day 2 p.i. from both groups. However, very few worms were recovered from both groups on day 4 p.i. and expulsion was complete by day 6 p.i. Rejection was identical in both groups.

A comparison of the longevity of a 200 m 2^{o} infection (0 Fig 1.7) with the longevity of a 200 m 1^{o} (Fig 1.1, Fig 1.5) shows that expulsion is very much faster (Table 1.6), being almost complete by day 4 p.i. in the challenge worms.

Table 1.1

The % survival of flukes in a primary infection in NIH mice initially infected with 200 (Group A) or 20 (Group B) metacercariae

Day p.i.	Group A % $\bar{x} \pm$ s.d.	Group B % $\bar{x} \pm$ s.d.
2	85 ± 7	90 ± 3
4	90 ± 5	n.d.
6	55 ± 10	61 ± 9
8	11 ± 4[a]	24 ± 7[a]
10	n.d.	16 ± 7
12	0	11 + 8

n.d. = not done

a = means significantly different,

$P < 0.02$

Group A n = 4

Group B n = 5

Table 1.2

The effect of host age on the % survival of a primary infection of 20 metacercariae in NIH mice

Day p.i.	Group A 14 week mice % $\bar{x} \pm$ s.d.	Group B 5-6 week mice % $\bar{x} \pm$ s.d.
	(n)	(n)
2	(5) 81 ± 2	(5) 78 ± 3
4	(5) 84 ± 7	(5) 84 ± 7
6	(5) 82 ± 3	(4) 73 ± 12
8	(4) 59 ± 8	(5) 46 ± 7
10	(5) 26 ± 7	(4) 16 ± 3
12	(5) 0	(5) 10 ± 6

(n) number of mice per group used for calculating $\bar{x} \pm$ s.d.

There was no significant difference between groups A and B on any day.

Table 1.3

Comparison of % survival of a 20 metacercariae primary infection in NIH and C_3H male mice

Day p.i.	NIH % $\bar{x} \pm$ s.d.	C_3H % $\bar{x} \pm$ s.d.
	(n)	(n)
4	(6) 83 ± 3[a]	(4) 48 ± 27[a]
6	(5) 81 ± 2	(6) 64 ± 18
8	(5) 81 ± 2[b]	(6) 33 ± 27[b]
10	(6) 42 ± 13[c]	(6) 17 ± 19[c]
12	(6) 8 ± 10	(6) 3 ± 4

The following means are significantly different

a $P < 0.02$

b $P < 0.01$

c $P < 0.05$

(n) number of mice/group used to calculate $\bar{x} \pm$ s.d.

Table 1.4

Comparison of % survival of a 20 metacercariae primary infection in NIH and C57 female mice

Day p.i.	NIH % $\bar{x} \pm$ s.d.	C57 % $\bar{x} \pm$ s.d.
	(n)	(n)
4	(6) 83 ± 3	(4) 54 ± 33
6	(6) 81 ± 2[a]	(6) 38 ± 21[a]
8	(6) 81 ± 2[b]	(6) 33 ± 20[b]
10	(6) 29 ± 16[c]	(6) 3 ± 4[c]
12	(6) 9 ± 7[d]	(6) 1 ± 2[d]

The following means are significantly different

a $P < 0.001$

b $P < 0.001$

c $P < 0.01$

d $P < 0.05$

(n) number of mice/group used to calculate $\bar{x} \pm$ s.d.

Table 1.5

Comparison of % survival of a 200 metacercariae primary infection in W.L.E.P. NIH and commercially bred NIH female mice

Day p.i.	Group A W.L.E.P. NIH		Group B Commercial NIH	
	(n)	% $\bar{x} \pm$ s.d.	(n)	% $\bar{x} \pm$ s.d.
2	(6)	82 ± 2	(6)	82 ± 2
4	(6)	82 ± 3	(6)	84 ± 3
5	(6)	67 ± 17	(6)	67 ± 14
6	(6)	50 ± 9	(5)	50 ± 15
8	(6)	4 ± 4	(6)	7 ± 5
12	(6)	0	(6)	0

(n) number of mice/group mean based on.

There was no significant difference between groups A and B on any day.

Table 1.6

Comparison of the % survival of a primary infection (Group A, Table 1.1) of 200 metacercariae with a secondary infection of 200 metacercariae (Group A, Fig 1.7)

Day p.i.	1° infection $\bar{x} \pm$ s.d.	2° infection $\bar{x} \pm$ s.d.
2	85 ± 7	80 ± 0
4	90 ± 5[a]	6 ± 4[a]
6	55 ± 10	0

a Mean significantly different between groups.

$P < 0.001$

Figure 1.1

Comparison of a 200 and 20 metacercariae primary infections in NIH mice

Key:

○ 200 metacercariae

● 20 metacercariae

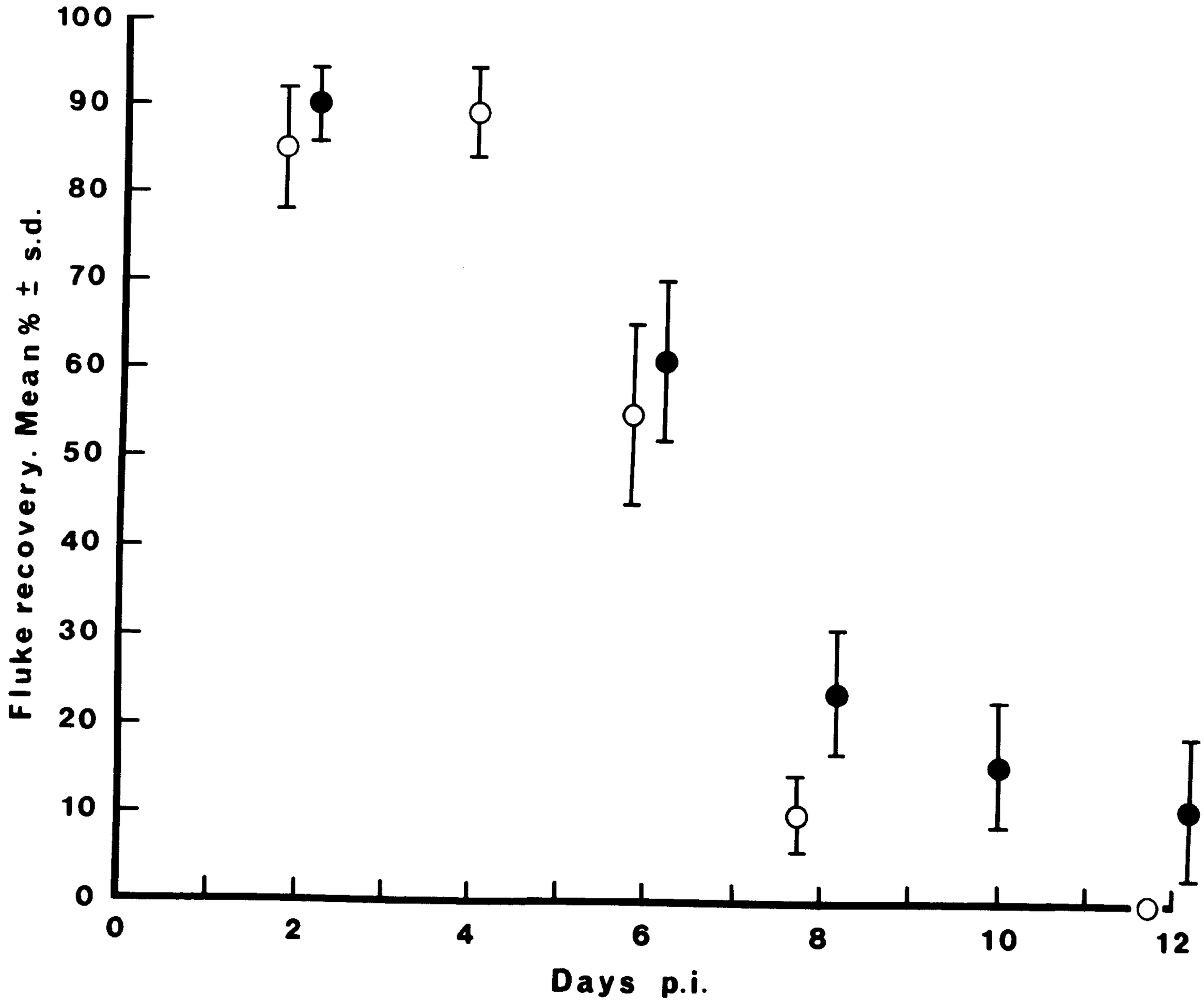

Fluke recovery. Mean % ± s.d.
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Days p.i.

Figure 1.2

Duration of a 20 metacercariae primary infection in old ● (14w) and young ○ (5 -6w) NIH mice. (▲△) not included in $\bar{x} \pm$ s.d.

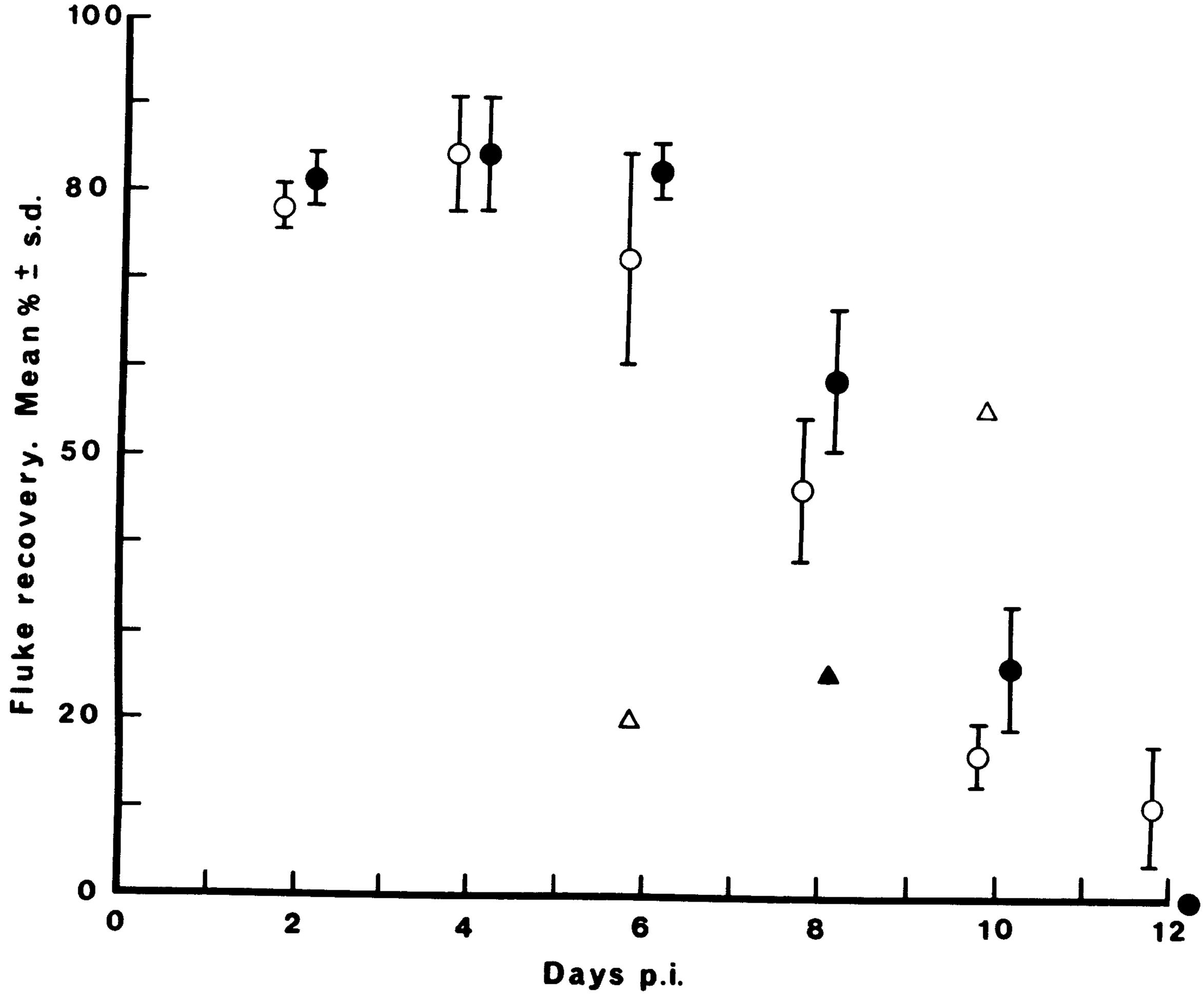

100
80
50
20
0
Fluke recovery. Mean % ± s.d.
0
2
4
6
8
10
12
Days p.i.

Figure 1.3

Comparison of a 20 metacercariae primary infection in NIH and C_3H mice

Key:

○ NIH Means of 6 mice except day 8 p.i.

● C_3H n = 6 except day 4 p.i. when n = 4

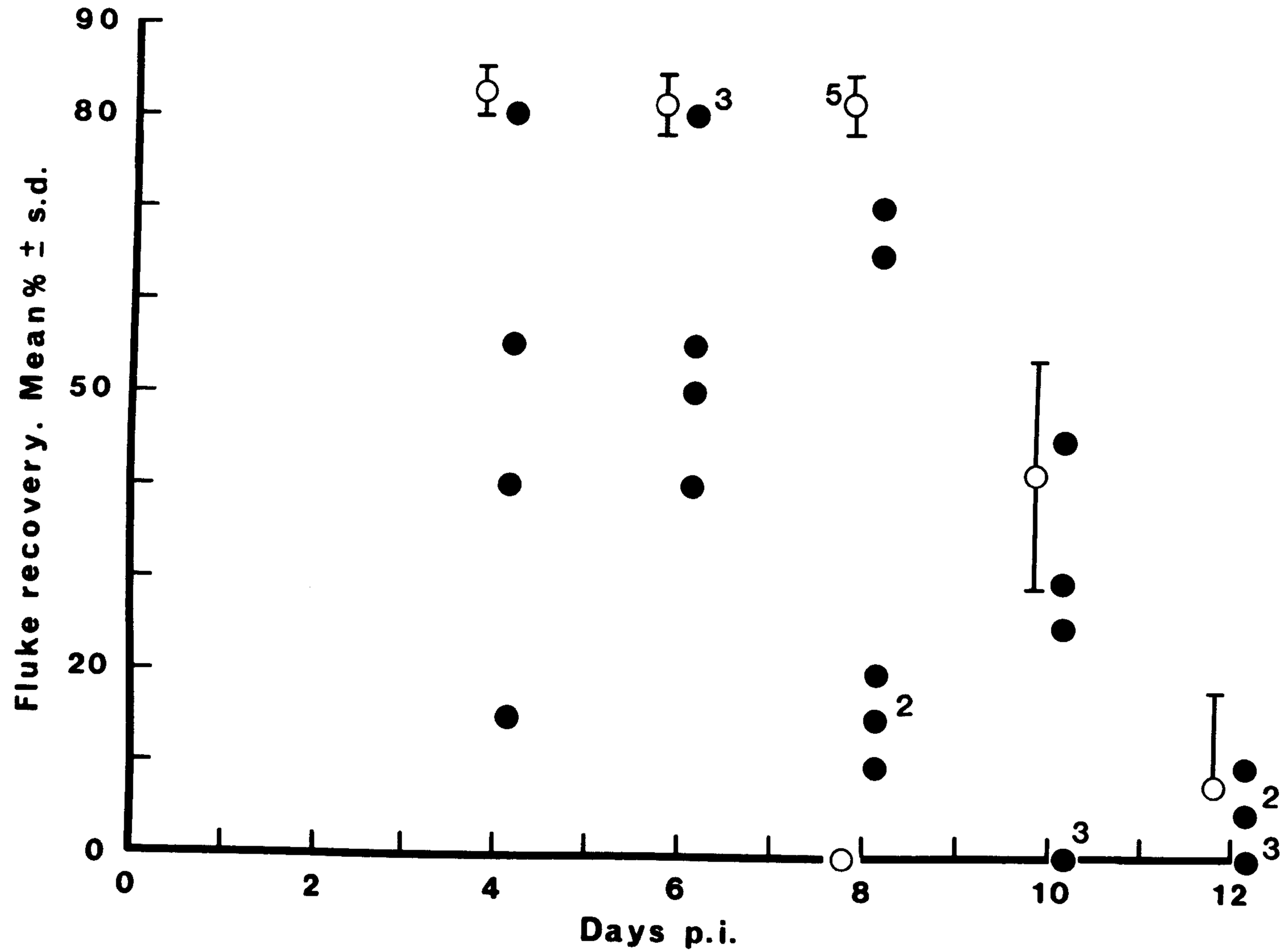

90
80
50
20
0
Fluke recovery. Mean% ± s.d.
0
2
4
6
8
10
12
Days p.i.
3
5
2
3
2
3

Figure 1.4

Comparison of a 20 metacercariae primary infection in NIH and C57 mice

Key:

○	NIH	Means of 6 mice
●	C57	n = 6 except day 4 p.i. when n = 4

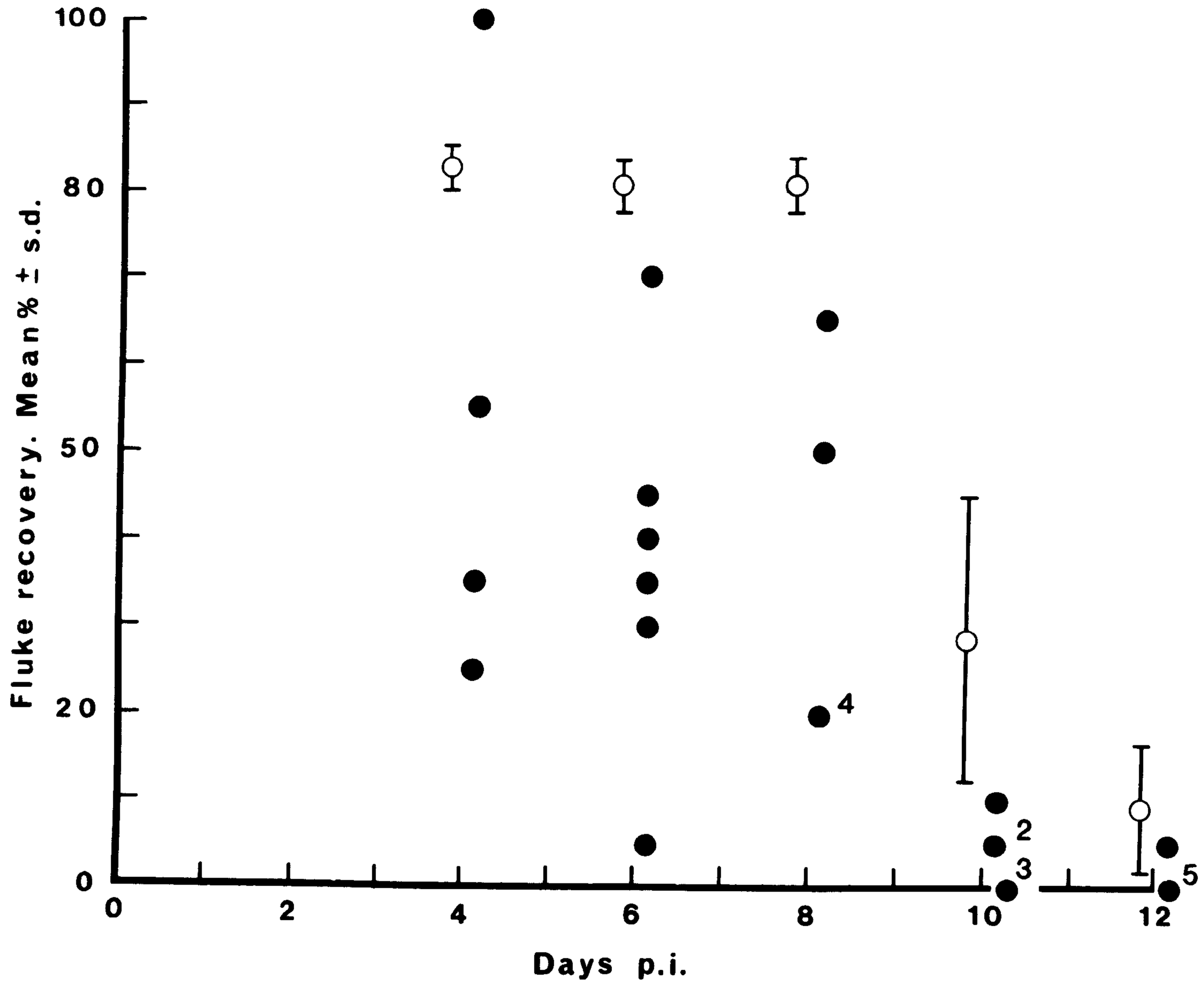

100
80
50
20
0
Fluke recovery. Mean % ± s.d.
0
2
4
6
8
10
12
Days p.i.
2
3
4
5

Figure 1.5

Comparison of a heavy (200 metacercariae) primary infections in W.L.E.P. NIH and H&C NIH

Key:

○ W.L.E.P. NIH

● H&C NIH

▲ Not included in $\bar{x} \pm$ s.d.

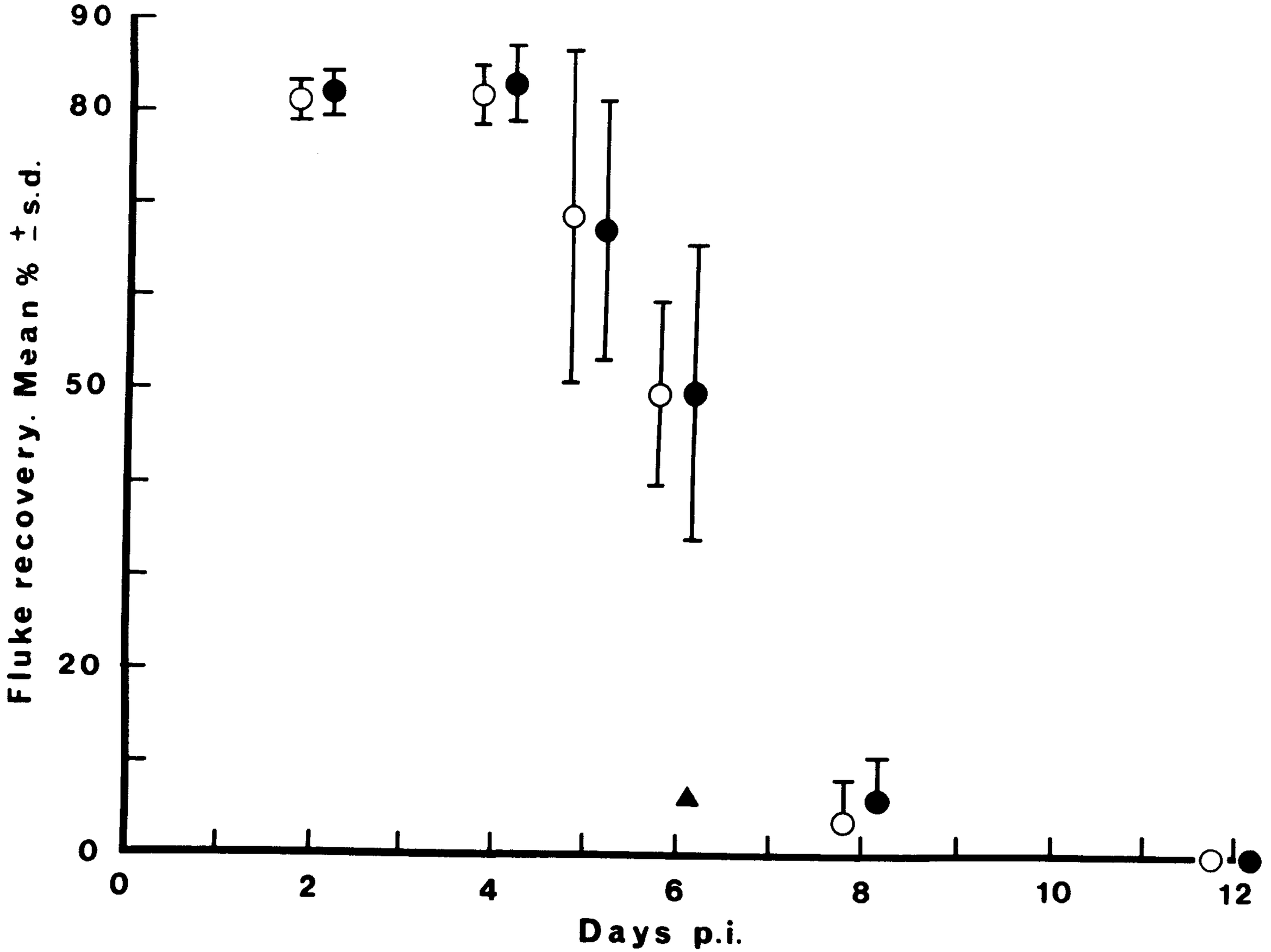

90
80
50
20
0
Fluke recovery. Mean % ± s.d.
0
2
4
6
8
10
12
Days p.i.

Figure 1.6

Posterior shift of *D. phoxini* in the intestine during the course of an infection

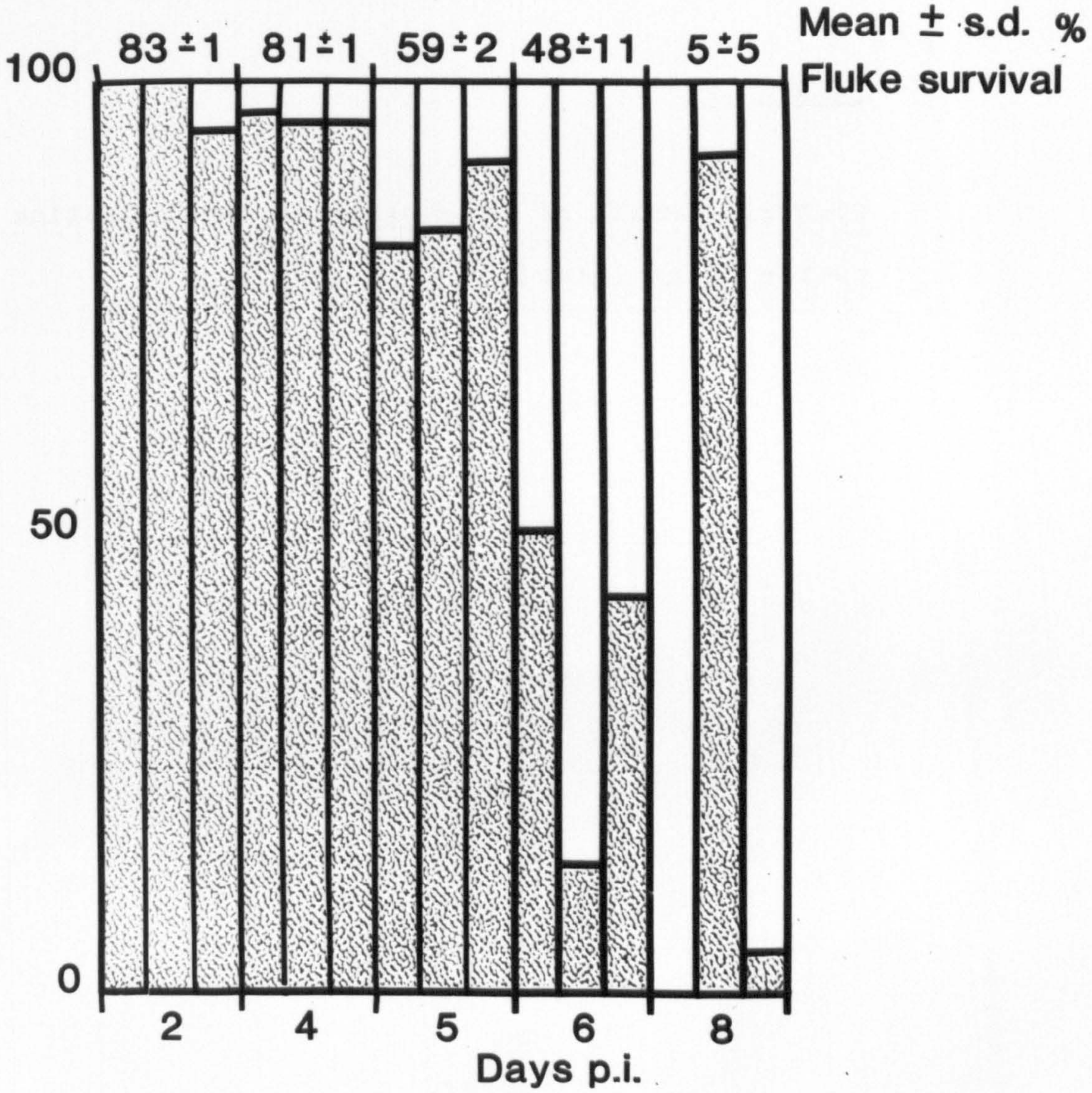
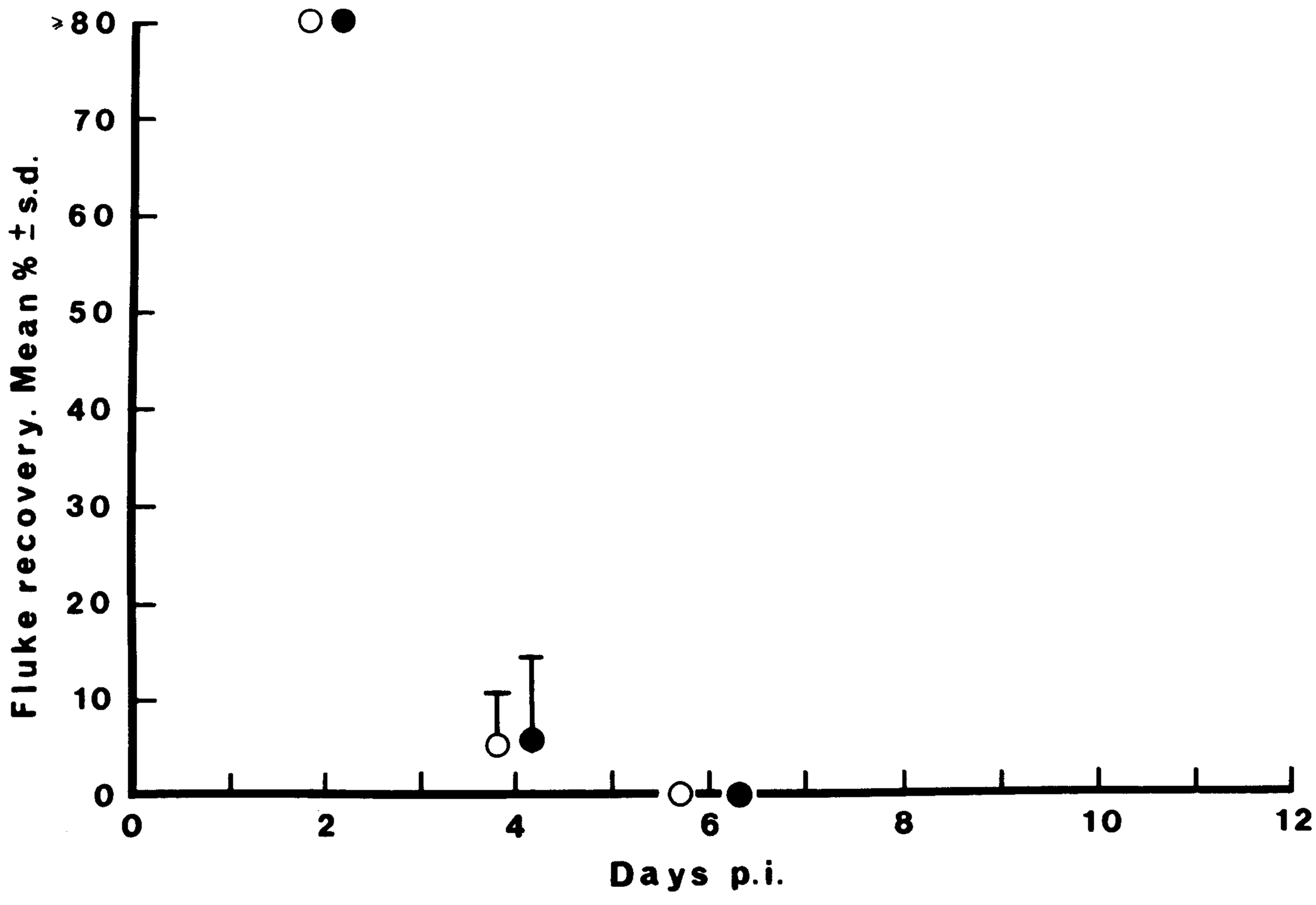

Mean ± s.d. %
Fluke survival
100
83 ± 1
81 ± 1
59 ± 2
48 ± 11
5 ± 5
50
0
% of the recovered flukes in the anterior 10 cm of the intestine.
2
4
5
6
8
Days p.i.

Figure 1.7

Course of a secondary infection of 200 metacercariae after immunization with 200 metacercariae (○) or 20 metacercariae (●)


Fluke recovery. Mean % ± s.d.
≥80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Days p.i.

Discussion

Expulsion of *D. phoxini* from the mouse is of the 'spontaneous cure' type. This is in common with many parasite models studied eg. *T. colubriformis* (Gordon *et al*, 1960), *N. brasiliensis* (Jarrett *et al*, 1968), *T. muris* (Wakelin, 1967), *R. cesticillus* (Gray, 1972), *H. diminuta* (Hopkins *et al*, 1972), *Apatemon gracilis minor* (Blake, 1973) and T. *spiralis* (Wakelin and Lloyd, 1976; Love *et al*, 1976).

The pattern of the 1^{o} infection is similar in all the named models. Establishment in the intestine is followed by a period of stability when worm numbers remain virtually the same. This is then followed by loss of worms. The time scale and other factors differ between models though.

In the *D. phoxini* model, over 80% of the infection establishes in the duodenum, as measured by recovery on days 2 or 4 p.i. depending whether 200 or 20 m were used. In nematode systems and some cestode systems the percentage of the administered worms establishing is much less, around 50% or even less.

Jarrett *et al* (1968) working on the kinetics of *N. brasiliensis* infections in rats found that of 3000 larvae given by subcutaneous inoculation, only 40% reached the intestine. The majority of the remaining 60% were lost within a day or so after infection. Although no evidence for the cause of this loss was presented, it was assumed to occur at the site of injection or during the course of

migration to the lungs. The percentage of worms lost in this way was a relatively fixed proportion of the infective dose ranging between 40% and 60%, and Jarrett et al (1968) suggested that the differences within this range were probably due to minor variables in handling technique which affected larval infectivity.

Similarily Moqbel and Denham (1977) attributed loss of S. ratti larvae to being killed in the lungs, although this is based on 2 dead larvae recovered from the lungs of 5 challenged rats. This suggests that the larvae may be killed elsewhere in the migration route. There is no real evidence of where and how this loss occurs during 1^{o} infections. Approximately 50% of the inoculum of a 1^{o} infection of S. ratti arrive in the rat intestine when administered subcutaneously.

A similar low establishment of worms occurs with T. muris (Wakelin, 1967), T. spiralis (Wakelin and Lloyd, 1976; Alizadeh and Wakelin, 1982), with the cestode R. cesticillus (Gray, 1972) and with A. gracilis minor (Blake, 1973). All of these latter parasites were administered directly into the gut, as is D. phoxini, thus avoiding a parenteral phase. The lower establishment is probably due to several reasons although the exact cause remains unknown (Alizadeh, 1981). Possible reasons may be poor selection of infective larvae especially during the counting of nematode larvae, failure of cysts to hatch or a failure of newly hatched parasites to maintain an adequate adhesion to the gut wall before being

swept out of the gut by peristalsis (Blake, 1973) and failure of cysticercoids to be activated in R. cesticillus infections.

Establishment in the intestine is followed by a plateau phase as described by Jarret et al (1968), when the worm burden remains almost constant. In D. phoxini infections, this phase lasts for at least 4 days in 200 m infections (Figs 1.1 and 1.5) and can last up to 8 days in 20 m infections (Figs 1.3 and 1.4). The plateau phase is followed by worm loss which is complete by day 12 following a heavy infection (200 m) and almost complete following a light infection (20 m).

That this loss is immunologically mediated is suggested by the enhanced rate of rejection of 2^{o} infections (Fig 1.7). The results are similar to those recorded by Mawdsley (1983), who also showed that treatment of mice with the immunosuppressive drug cortisone, greatly prolonged worm survival. It is reasonable, therefore to assume that the loss of D. phoxini is immunologically mediated.

Comparing the time course of the rejection of D. phoxini by mice with that of many other species of intestinal worms that evoke rejection by the host, reveals that it is rather quicker than in most other species studied, eg. N. brasiliensis is rejected by rats after 10 days (Jarret et al, 1968), T. muris by mice in 16 days (Wakelin, 1967), H. diminuta in mice in 9 - 12 days (Hopkins et al, 1972), S. ratti in rats between 14 and 18 days (Moqbel and Denham, 1977) and T. spiralis in mice between days 8 and 11 (Lash, 1963; Denham, 1968; Wakelin and

Lloyd, 1976), depending on mouse strain (Wakelin and Lloyd,1976).

The differences in rejection time is quantitative rather than qualitative. Heavy infections of H. diminuta (100 cysticercoids) are expelled after 4 days in mice (Roepstorff and Andreassen, 1982). Why D. phoxini is more rapidly expelled than most helminths studied is probably due to a combination of factors. The evidence that 200 m infections are more rapidly expelled than 20 m infections (Figs 1.1, 1.3 and 1.4) fits with the observation that 100 H. diminuta infection was expelled more rapidly than 1 - 6 worm infections. This is presumably because of increased antigenic stimulation shortening the response time.

There are other factors however, for instance, the very close apposition of parasite and host tissue, and the disruption of the hosts mucosa by the lytic action of the adhesive gland secretions as suggested by Erasmus (1970b) and demonstrated in the Diplostomum spathaceum - gull model by Ohman (1965), and the abrasive action of the heavily spined lateral surface of the named organ (Erasmus, 1970b) almost certainly enhance antigen entry.

It may be concluded therefore that there is no reason to suppose that we are dealing with a fundamentally different regime against D. phoxini to that observed in many other worm / host systems, merely one towards the acute end of a spectrum due probably to strong antigen stimulation and a very effective attack mechanism.

The effectiveness of the attack mechanism may partially

be due to the position of the worm in the small intestine. D. phoxini appears to be concentrated into the anterior 10 cm of the small intestine (Fig 1.6) and once displaced does not survive long. The posterior shift of the worms from the site of preference is probably a result of the hosts immune response, the worms either crawling away or being carried down by peristalsis. It is well established that blood flow to the duodenal region is appreciably greater than to the ileal region and hence blood born cells of the efferent arm of the immune response are more likely to arrive in this region.

Almost complete expulsion occurs by day 8 p.i. and rejection is complete by day 12 p.i. in heavy (200 m) infections (Figs 1.1 and 1.5). No residual threshold level occurs as with T. muris (Wakelin, 1967), N. brasiliensis Jarrett et al, 1968) and A. gracilis minor (Blake, 1973), all the worms are expelled even in the low (20 m) infections (Mawdsley, 1983). This suggests that D. phoxini is very antigenic in the mouse.

The D. phoxini - mouse model can therefore be proposed as a model of an early immune response against an intestinal parasite.

Figure 1.2 shows that both young (5 - 6 weeks) and old (14 weeks) mice had identical expulsion patterns. There is no significant difference between groups. This is in contrast to Wakelin and Lloyd (1976) who showed that 15

weeks old NIH mice expelled T. spiralis 1 or 2 days earlier than 6 - 8 weeks old NIH. The results show that the young NIH used in the present experiments were therefore immunologically mature. These were bred under conventional conditions at the laboratory, being exposed to a rich antigenic environment. It is possible that the mice used by Wakelin and Lloyd (1976) which were bred by a commercial supplier were not as immunologically mature as the mice bred in the laboratory, possibly due to the former being raised in more sterile conditions, but this is unlikely. However, many workers have found that neonates are unable to reject worms efficiently because of their immature immune system (Ogilvie and Jones, 1967; Jarret et al, 1968), so this may be a reason if the 6 - 8 week old mice were raised under sterile conditions.

Attempts to prolong the duration of a 1^{o} infection by using different mouse strains failed (Figs 1.3 and 1.4; Tables 1.3 and 1.4). Establishment of the worms in both C_3H and C57 strains was highly variable when compared with NIH controls and rejection was also faster, especially in C57 (Fig 1.4 and Table 1.4). It is not possible to say whether poor establishment of the worms in C_3H and C57 is immunologically based or because of more adverse environmental conditions in the intestine as compared with NIH. However Tables 1.3 and 1.4 show that there is a significant difference between means of C_3H and NIH, and C57 and NIH during the course of rejection. The difference between the latter group is statistically highly significant (Table 1.4).

The expulsion of D. phoxini by the C_3H is interesting because some seem to reject at the same time as NIH and some before. However this reflects the poor establishment in C_3H. As rejection of a 20 m infection would not be expected to have begun until after day 6, we can see that by combining the days 4 and 6 recoveries (Fig 1.3) that 40% of the C_3H gave good recoveries, i.e. 80% worm recoveries, and 60% gave poor worm recoveries. On day 8 p.i. 2 mice gave good recoveries and 4 gave poor recoveries, i.e. a ratio of 33% to 67% which is not significantly different from the mice on days 4 and 6. However the worm recoveries are slightly lower in both the 2 good mice and 4 bad mice on day 8 p.i. suggesting that rejection had begun.

Similarily in combining the days 8 and 10 results, we see that 5 of the 12 mice (i.e. 42%) have similar worm recoveries to the NIH and the remaining 7(i.e. 58%) correspond to the poorly infected C_3H, the worm recoveries being lower though than on days 4 and 6. The ratios are remarkably close.

The problem with this hypothesis is it assumes that the mice which have rejected earliest are the ones which initially had lower worm burdens due to poor establishment.

This does not correspond with the earlier argument of greater worm burdens being expelled quicker than low worm burdens due to greater antigenic stimulation. However with regard to total worm numbers, the effect of the different

quantity of antigen produced by 16 worms, i.e. 80% of a 20 m infection, compared with eg. 8 worms, i.e. 40% of a 20 m infection or even fewer worms is probably negligible as far as stimulating an immune response is concerned, as compared to the different quantity of antigen produced by 160 worms, i.e. 80% of a 200 m infection and 16 worms. Therefore the argument may not be valid when using such low numbers of worms.

The fact that in some of the C_3H the worms failed to establish properly whereas in the NIH establishment was above 80% in this experiment, suggests that the metacercariae used were viable and infective and that the poor establishment in some C_3H was due to some physiological reason.

A similar phenomenon has been demonstrated by Wakelin (1975b)who showed that some DBA_2 mice were capable of expelling T. muris while others were not. However due to the low number of mice used it is advisable not to extrapolate too much from data based on a single experiment.

In conclusion, neither the C_3H nor C57 mice appeared to offer any advantage over NIH for experimental work with D. phoxini.

Reducing the infecting dose from 200 to 20 metacercariae had, initially, little effect on longevity of the worms (Fig 1.1), but repeat experiments showed that, in fact, longevity was extended considerably (Figs 1.3 and 1.4). There can be

little doubt that the earlier result was wrong due to lack of expertise in recovering flukes in small infections. During recovery, a small number of flukes are obviously missed by remaining on the intestine. This number does not vary a great deal between light and heavy infections, but missing eg. 10 in a 200 m infection makes little difference to the % recovery, but in a 20 m infection it is disasterous. There are other difficulties arising from using 20 m infections, such as taking longer to recover the worms. Because of these difficulties, if metacercariae were abundant, it seemed best to continue to use heavy infections unless the experimental design demanded a more prolonged 1^{o} infection.

Figure 1.5 shows that there was no difference in rejection between the mice bred at the W.L.E.P. and commercially bred NIH mice.

A point of importance in this experiment is that expulsion has begun on day 5 p.i. and this is reflected in the large standard deviation obtained showing that the worms were undergoing some sort of crisis.

The pattern of rejection and time course is similar to the result shown in Figure 1.1 and therefore serves to confirm the duration of a 200 m 1^{o} infection. It is possible that the mice in Figure 1.1 (0) had also begun to reject on day 5 p.i. because the rejection pattern is so similar to the mice in Figure 1.5.

The fact that rejection can begin on day 5 underlines the little difference between rejection of a 200 m 1^{o} infection in which rejection begins between days 4 and 5 (Fig 1.5) and rejection of a 200 m 2^{o} infection where rejection begins between days 2 and 3 p.i. (Fig 1.7).

Memory in this model is expressed by the faster rejection of the challenge infection (Fig 1.7). However establishment of the parasite is not affected as shown by recovery on day 2 p.i. which is 80%. The faster rejection of the challenge infection is in common with the various host parasite models named in the introduction, although there is little conclusive evidence on whether establishment is affected in some of the named parasites.

A similar response occurs with T. colubriformis in guinea pigs. Herlich (1963) showed that T. colubriformis did establish equally well in control and immunized guinea pigs, there being little difference between the groups on day 2 p.i., but by day 5, rejection of the 2^{o} infection was underway.

Similarily the establishment of a 7 day old H. diminuta transferred into immunized mice is unaffected, growth being normal over the first 48 hours, but between 48 and 120 hours, most of the worms destrobilate or are lost (Hopkins, 1982). This is similar to the expulsion of a 2^{o} infection of D. phoxini. It therefore appears that it takes 48 hours for the host to mobilise its defense mechanisms.

The other important conclusion drawn from this experiment is that 20 m are as successful as 200 m in immunizing mice against a challenge infection. The rejection of the challenge infection in both groups is identical (Fig 1.7). This is in agreement with Wakelin (1973) who showed that 10 T. muris eggs would immunize a mouse against a 1000 egg challenge infection just as efficiently as a 100 or 1000 egg 1^{o} infection. Also Wakelin & Lloyd (1976) showed that a 1^{o} infection of 45 T. spiralis would fully immunize mice against a challenge infection of 500 larvae.

Therefore on the basis of this result it can be concluded that memory is induced by a 1^{o} infection of D. phoxini and is expressed by a faster rejection of a 2^{o} infection and finally 20 m are as good as 200 m as an immunizing dose.

The work in this chapter has confirmed Mawdsley's results, that D. phoxini can establish in the intestine of NIH mice and stimulate an immune response resulting in its expulsion. Therefore the D. phoxini - mouse model provides an adequate system for the further study of gut immune responses to an intestinal trematode and the results obtained so far do not appear to differ significantly from the more intensly studied but still far from well understood, nematode and cestode models.

Summary

1) The course of a 1^{o} infection has been established; rejection of a 200 m infection starts within 6 days and is complete by 12, whereas rejection of a 20 m does not start until 8 days, being later than a 200 m infection.

2) Young (5 - 6 weeks) NIH mice rejected D. phoxini as efficiently as 14 weeks old mice.

3) A challenge infection given on day 29 p.i. is expelled faster than a 1^{o} infection which is good evidence for the presence of immunological memory.

4) 20 m are as efficient as 200 m in immunizing mice.

CHAPTER 2

THE ROLE OF LYMPHOCYTES IN THE PROTECTION OF MICE AGAINST D. PHOXINI

Introduction

With the establishment of the model system it was possible to proceed with experimental work to investigate aspects of the expulsion mechanism.

This chapter is concerned with experiments to transfer immunity adoptively using immune lymphocytes and to characterise the cells responsible.

The role of lymphocytes in parasite expulsion was suspected in the sixty's and seventy's when experiments using cortisone derivatives (Ogilvie, 1965), thymectomy (Ogilvie and Jones, 1967), anti lymphocyte serum (Kassai et al, 1968), irradiation (Jones and Ogilvie, 1971; Keller and Keist, 1972) and neonatal thymectomy and anti lymphocyte treatment (Keller and Keist, 1972) showed that egg production and duration of a 1^{o} infection of N. brasiliensis could sometimes be prolonged in rats and in mice (Kassai et al, 1968).

Attempts to transfer immunity with lymphocytes during this period were not very successful. Hunter and Leigh (1961) failed to transfer immunity to Nippostrongylus muris, i.e. N. brasiliensis using immune mesenteric lymph node cells (IMLNC) and immune spleen cells (ISC) simultaneously, and Ogilvie and Jones (1968) had variable results in their attempts to transfer immunity adoptively to N. brasiliensis in rats.

Successful transfer of protection was achieved by Dineen and Wagland (1966) using IMLNC in their T. colubriformis - hamster model. Larsh et al (1964 b) also demonstrated the earlier rejection of T. spiralis from mice by giving peritoneal exudate cells (PEC) to recipients, but failed with various lymph node cells. Lang et al (1967) also achieved a significant reduction of F. hepatica in groups of mice given PEC from isologous donors as compared with groups not given cells during infection with two metacercariae.

The successful transfer of immunity with IMLNC in the N. brasiliensis - rat system was shown by Kelly and Dineen (1972). Using highly inbred Lewis rats they managed to lower egg production, reduce the number of eggs per uterus in gravid females and cause the earlier expulsion of worms in recipients.

Keller and Keist (1972) showed that syngeneic transfer of mesenteric lymph node cells (MLNC) from uninfected adult donors would restore the expulsion system in irradiated adult and young (40 day) rats which had 'immunologically' damaged N. brasiliensis transplanted into the intestine. Irradiated rats are deficient in functional lymphocytes and young rats probably lack a fully mature immune system and the transfer of MLNC restored the deficiency. The experiments showed that the lymphocyte was an essential agent in the expulsion of adult worms.

Since this period, other workers have demonstrated the ability to confer protection to a homologous parasite

challenge by transferring lymphocytes from an immune host to a syngeneic recipient in several models eg. N. brasiliensis in rats (Dineen et al, 1973; Ogilvie et al, 1977), T. muris (Selby and Wakelin, 1973), T. spiralis (Wakelin and Lloyd, 1976) and N. dubius (Cypess, 1970; Behnke and Parish, 1981) in mice and S. ratti (Moqbel and Wakelin, 1981) and Angiostrongylus cantonensis (Young and Dobson, 1982) in rats. Miller (1967) transferred protection to Ancylostoma caninum allogeneically in dogs using IMLNC.

Transfer of adoptive immunity in cestode models has proved less successful. Bland (1976) failed to transfer any protection to H. diminuta using IMLNC and ISC in mice. Christie (1979) only managed to transfer protection with large numbers of cells eg. 1.7×10^8 IMLNC (cf. 4×10^7 IMLNC used by Wakelin and Lloyd (1976) to accelerate the expulsion of T. spiralis from mice) and even then his results were inconsistent.

The most striking effect on nematode infections of IMLNC transfer is their earlier rejection. However expulsion is dependant on the number of cells transferred. Wakelin and Wilson (1977) showed that by transferring low numbers of cells eg. 4×10^6 IMLNC, fecundity of T. spiralis was depressed but earlier expulsion did not occur. Moqbel and Wakelin (1981) showed that by transferring small numbers of cells expulsion of S. ratti did not occur, but the worms were shorter, less fecund and had moved posteriorly when compared with controls.

In the N. brasiliensis - rat system antibody damaged

worms were expelled by IMLNC much quicker than 'normal' worms transplanted into the gut (Dineen et al, 1973). However Ogilvie et al (1977) showed that immune thoracic duct lymphocytes (ITDL) were more efficient than IMLNC in expelling damaged N. brasiliensis. They also showed that removal of surface immunoglobulin bearing cells (sIg^+) i.e. B cells, did not cause a significant reduction in the ability of the remaining cells to cause expulsion.

Nawa and Miller (1978) also found that ITDL conferred greater protection than IMLNC against N. brasiliensis and also succeeded in accelerating the expulsion of 'normal' transplanted worms with ITDL.

In most of the systems studied, parasite expulsion is known to be thymus dependant.

Although the importance of the thymus to parasite expulsion had been recognised for several years, the development of athymic 'nude' mice aided the investigation of the role of the thymus. Ruitenberg and Steernberg (1974) showed that T. spiralis remained for longer in 'nude' (nu/nu) mice than in thymic (nu/+) littermates and Jacobson and Reid (1976) similarily showed that nu/nu mice could not expel 'damaged' N. brasiliensis.

The expulsion of H. diminuta from athymic mice has been shown to be dependant on worm numbers. Andreassen et al (1978) observed that athymic mice could expel five - cysticercoid - infections, although slower than nu/+ mice, but Bland (1976)

observed that single cysticercoid infections remained at least until day 33 p.i. in athymic mice.

T cells, i.e. sIg^- cells are usually more efficient than B cells in transferring protection in most models eg. N. brasiliensis in rat (Ogilvie et al, 1977; Nawa et al, 1978) and T. spiralis in mice (Wakelin and Wilson, 1979). However Despommier et al (1977) described the faster rejection of T. spiralis from irradiated rats given B cells rather than T cells.

To achieve successful transfer of immunity, it has been recognised for some time that cells have to be taken from donors within a defined period. This period differs between experimental models, and is probably dependant on several factors. These include the time taken for the cells to become sensitized to the antigenic stimulus, and the time which the sensitized cells are present in sufficient quantity in the organs used for transfer.

Wakelin and Wilson (1977) therefore could only transfer immunity to T. spiralis with IMLNC taken between days 4 and 8 p.i. and similar results have been observed in the N. brasiliensis - rat system (Ogilvie et al, 1977; Nawa and Miller, 1978), S. ratti - rat system (Moqbel and Wakelin, 1981) and T. colubriformis - hamster model (Adams and Rothwell, 1977).

In the present system Mawdsley (1983) showed that cells capable of transferring immunity to D. phoxini appeared in the mesenteric lymph node (MLN) as early as day 2 p.i., but

she could not transfer immunity with cells taken from the MLN between days 8 and 12 p.i. Of interest however is that she managed to transfer immunity with day 21 IMLNC. The ability of day 21 IMLNC to transfer immunity is investigated further in the present work.

An important variable in the D. phoxini - mouse model is the time the transferred cells require in the recipient before causing a response.

Wakelin and Wilson (1977) showed that IMLNC required at least 6 days in the recipient before causing accelerated expulsion of T. spiralis. In the S. ratti model the cells required 10 days (Moqbel and Wakelin, 1981).

Mawdsley (1983) failed to transfer immunity with IMLNC when transferred on the day of challenge or after challenge. She was successful however in causing the accelerated expulsion of D. phoxini when the cells were given 9 days before challenge. This probably reflect the time taken for enough of the cells to establish in the intestine or in the circulation.

Attempts have been made to correlate the ability of cells to transfer immunity with changes in the organ of cell origin. Grencis and Wakelin (1982) showed that cells capable of transferring protection were present in the MLN at the time of peak cell numbers and blast activity during T. spiralis infection in mice. In contrast, in the S. ratti system the cells capable of transferring protection are present after

the cells have reached their peak numbers and the population is in decline (Moqbel and Wakelin, 1981). Mawdsley (1983) also showed high rates of cell division in the MLN during D. phoxini infection but found no correlation with the ability of the cells to transfer immunity.

The present work was designed to verify some of Mawdsley's preliminary results on the ability of IMLNC to transfer immunity, especially day 21 p.i. cells. It was also hoped to clarify the relationship between the ability of cells to transfer immunity, blast activity and total cell numbers in the MLN. Further work on fractionation of cell populations was carried out to clarify the role of B cells in the transfer of immunity.

Experiments to see whether 'light' infections could be substituted for 'heavy' infections eg. in immunizing regimes were further investigated. This work became particularly important as the metacercarial infections in minnows was lighter than in previous years. (This may have resulted from a successful on-going project to scare gulls from the reservoirs from which the fish came, this in turn may have had an adverse effect on the life cycle of D. phoxini by reducing the number of definitive hosts).

Materials and Methods

Animals

All mice used in these experiments were male NIH. Maintenance and infection of mice, and recovery of parasites was as described in the general materials and methods.

Preparation of cell suspensions and cell transfer

Cell suspensions were prepared and transferred into recipients as stated in the general materials and methods.

Assay for Blast cell activity *in vitro*

Cell suspensions were prepared as normal but suspended in the culture medium, RPMI 1640 (without L - glutamine and $NaHCO_3$:Gibco Europe). To 100 ml of the RPMI 1640, the following were added, 0.8 ml of sodium bicarbonate (7.5% solution), 1.0 ml of L - glutamine (200 mM), 1.0 ml of Penicillin/streptomycin (10,000 units/ml) and 0.5 ml of heat inactivated new born calf serum, all of which were obtained from Gibco Europe. Sterile technique was observed during preparation.

Four ml of the culture medium was placed in sterile plastic tissue culture grade tubes (Sterilin) and 1.0 ml of medium containing 2×10^7 lymphocytes was added. To each tube, 2 μCi of 5-[^{125}I]-iodo-2'-deoxyuridine, ([^{125}I]-UdR), specific activity 5 Ci/mg (Amersham International), a thymidine analogue, made up in sterile PBS in a volume of 0.2 ml was added. One tube without cells was prepared similarily as an isotope

control. All tubes were incubated for 2 hours at 37°C, after which they were washed 3 times in M199 (4.0 ml).

The activity in each tube was counted for 1 minute in a Packard Tricarb liquid scintillation spectrometer.

Background readings were also taken at the same time using three clean unused vials. The mean of the background count was subtracted from the experimental and control group counts before further analyses.

The results were expressed as a labelling index:

$$LI = \frac{\bar{x} \text{ c.p.m. experimental group}}{\bar{x} \text{ c.p.m. control group}} \times 100$$

Enrichment of T and B cell populations

Cell suspensions prepared as normal were separated into non adherent T cell enriched (surface immunoglobulin - (sIg^-)) and adherent B cell enriched (surface immunoglobulin + (sIg^+)) cells by a nylon wool separation technique adopted from Julius et al (1973), Trizio and Cudkowicz (1974) and Handwerger and Schwartz (1974).

Ten ml plastic disposable syringes were loosely packed with glass wool, which had been repeatedly washed in HBSS to reduce toxicity, to the 5 - 6 ml mark. 30.0 ml plastic disposable syringes were packed with 2.5 g of nylon wool (Fenwel Laboratories) to the 25 ml mark. Both were sterilized

by autoclaving.

The columns were set up on stands with needles (18 G 1½") attached. A suitable length (eg. 14 cm) of appropriate polythene tubing (Portex Ltd) was attached to the needle and a smaller length (2.5 cm) of a wider diameter tubing was added to the free end, to allow efficient clamping of the apparatus. The nylon wool columns were washed through with prepared medium (medium 199, containing L - glutamine and Hepes buffer (25 mM) supplemented with 5% v/v heat inactivated newborn calf serum and 10 i.u./ml of heparin, B.D.H. Chemicals Ltd) taking care to ensure that no air bubbles were left, sealed with Nescofilm and pre-incubated at 37°C for 1.5 hours. 200 ml of the prepared medium was also incubated at 37°C.

The cell suspension was prepared as normal and passed down the glass wool column to remove debris, cell clumps and macrophages. The cells were caught in beakers and counted.

A maximum of 5×10^8 cells in a volume of 10 - 15 ml was added drop-wise to the nylon wool column, the column was resealed and incubated for 45 minutes at 37°C.

After incubating, the non adherent T cells were first recovered by washing through the syringe with fresh medium (37°C), allowing the medium to escape at a drop-wise rate and adding the fresh medium at the same rate. The adherent fraction was recovered by placing the nylon wool in a 100 ml crystallizing dish, covering with cold (4°C) medium and

teasing apart with clean forceps for 10 minutes. The nylon wool was then replaced in the syringe and the remaining medium was squeezed out by compression, into the dish.

Both suspensions were washed 3 times, counted, and the ratio of B to T cells in each fraction determined by direct fluorescence.

Direct fluorescence of cells

Eight drops of M199, 1 drop of 0.1% w/v NaN_3 and 1 drop of 1:10 v/v fluoroscein conjugated goat anti mouse immunoglobulin (GāMIg/FITC Nordic Laboratories) reconstituted in HBSS were added together in a centrifuge tube. Two drops of cell suspension were added and the tubes incubated at $4^{o}C$ for 30 minutes in the dark. After incubation, the cells were washed twice in cold ($4^{o}C$) HBSS and finally resuspended in 0.25 ml of 50% v/v glycerol in HBSS. The cells were examined under water immersion with a Leitz Ortholux 1 microscope fitted with a Ploem Incident light fluorescent system.

Complement mediated cytotoxicity

This technique was used to eliminate contaminating T cells to enrich B cell populations. A Thy 1.2 monoclonal antibody (Clone F7D5, IgM) which is cytotoxic to cells bearing this surface antigen was obtained from Olac 1976 Ltd. A preliminary experiment with unadsorbed guinea pig complement (C) (Fresh guinea pig serum) (Wellcome reagents) was first carried out to assess its cytotoxic titre.

Cytotoxicity assay

A thymocyte suspension was prepared from the thymi of NIH mice in the same manner as the preparation of IMLNC suspensions. The suspension was adjusted to 2×10^7 cells/ml and 0.5 ml was added to 0.5 ml of anti Thy 1.2 antibody at various concentrations in M199. The suspension was incubated at room temperature (~ 21°C) for 30 minutes and centrifuged at 200 g for 5 minutes. The suspension was resuspended in a 1:10 dilution of C and incubated at 37°C for 40 minutes. After washing twice in M199 cell viability was assessed by the dye exclusion test (see p 12). Appropriate controls (see Table 2.1) were prepared and treated accordingly.

The cytotoxic titre of the antibody was expressed as a cytotoxic index:

$$CI = \frac{\% \text{ dead (test)} - \% \text{ dead (control)}}{100 - \% \text{ dead (control)}} \times 100$$

The assay was repeated and both results are shown in Table 2.1. An antibody concentration of 1:500 with a C concentration of 1:10 killed over 90% of thymocytes, therefore these concentrations were used in further experiments.

% Depression

In cell transfer experiments the effectiveness of the cells to protect was expressed as % depression (%Δ) which was calculated from the following equation:

$$\% \Delta = \frac{\% \text{ worms in controls} - \% \text{ worms in experimental mice}}{\% \text{ worms in controls}} \times 100$$

Table 2.1

Complement mediated cytotoxic assay of anti Thy 1.2 antibody against thymocytes of NIH mice

Antibody concentration	Cytotoxic Index Sample 1	Sample 2
No treatment	-	-
Ab 1:100 only	2.4	-
C 1:10 only	6.7	.5
Ab 1:100 + C	72.3	89.0
Ab 1.250 + C	58.2	91.2
Ab 1:500 + C	90.2	92.5
Ab 1:1,000 + C	31.6	89.7
Ab 1:10,000 + C	81.6	87.8

Results

The effect of adoptively transferred IMLNC on the course of a primary *D. phoxini* infection

Twenty seven, 10 - week - old, male NIH were divided into two groups of donors. Group X (12 mice) were infected with 200 m and caged in groups of 4 and group Y, (15 mice) were not infected and caged in groups of 5.

Four days p.i., the donors were killed and their MLNC transferred into the recipients. Five, male 8 - week - old, NIH (Group A) were given 4×10^7 IMLNC i.p., 3 similar mice (Group B) were given 4×10^7 control MLNC (CMLNC) i.p. and (Group C) 4 mice were given 0.5 ml of the incubating medium, i.e. M199.

The cell transfer was given on day -9 and on day 0 the recipients were infected with 200 m. All mice were killed on day 5 p.i. This was the time scale used by Mawdsley (1983).

The results (Fig 2.1, Table 2.2) show that day 4 IMLNC were clearly capable of transferring protection in this experiment. Both control groups, i.e. groups B and C had over 60% of the flukes present but the group given IMLNC had only 22% of their worms present (Table 2.2). The difference between groups A and C, and A and B are highly significant ($p<0.001$).

Because CMLNC did not affect worm survival, this

control group was excluded from other experiments (partly because collecting enough cells from uninfected mice was very time consuming).

With the ability of day 4 IMLNC to transfer immunity confirmed, the next experiment was designed to establish whether MLNC from mice immunized with 20 m were capable of transferring protection and when was the optimum time to take them.

Ability of day 4 and day 6 IMLNC, recovered from mice infected with either 200 m or 20 m, to adoptively transfer protection

Twenty four, 10 - week - old, male, NIH were infected with 200 m and caged in groups of 6, (Group X), and 24 similar mice were infected with 20 m and caged in groups of 6, (Group Y). Twelve mice from each group were killed 4 days p.i. and the remainder 6 days p.i. The recipients, 4, 8 - week - old, male NIH per group were either given 4×10^7 day 4 or day 6 IMLNC from donors immunized with 200 m or 20 m. Two control groups, 4 mice per group, were given 0.5 ml incubating medium.

Infecting of recipients and recovery of worms occurred on the same time scale as in the previous experiment.

The results show that both day 4 and day 6 IMLNC, from mice immunized with 200 m or 20 m were capable of transferring a significant degree of protection (Table 2.3).

The most potent cells on day 4 were those from mice

immunized with 200 m, reducing the worm burden by 65% compared with the control group, whereas the cells from mice immunized with 20 m reduced the worm burden by only 38%. Cells taken from immunized mice on day 6 p.i., however had the opposite effect, those from the 20 m infected mice being more potent (Table 2.3), reducing the worm burden by 68% compared with the controls. The cells from donors infected with 200 m reduced the worm burden by 38% as compared with the controls.

To complement this experiment, a further experiment was designed to investigate the effect of immunizing donors with 50 m, and transferring cells on days 4 and 6 p.i. This experiment may seem unnecessary due to the significant amount of protection transferred with cells taken from donors immunized with 20 m, but in theory, 50 m should provide greater antigenic stimulation than 20 m, therefore ensuring more successful transfer of immunity.

<u>The efficiency of day 4 and day 6 IMLNC to transfer immunity from mice immunized with 50 m</u>

Ten, 10 - week - old, male, NIH mice were infected with 50 m and caged in groups of 5. A further 10 mice were similarily infected two days later. Four days after this infection all the mice were killed and their cells transferred into recipient mice. The recipients, 12, 8 - week - old, male, NIH were divided into 3 groups of 4. Group A were given 0.5 ml of incubating medium, group B were given 4×10^7 day 4 IMLNC and group C, 4×10^7 day 6 IMLNC.

Infecting of recipients and worm recovery was as in previous experiments.

Both day 4 and day 6 IMLNC transferred a significant degree of protection in this experiment (Table 2.4). However the large deviations obtained in both the recipient groups makes further interpretation difficult. It is probable that the '0' obtained in the recipients of day 4 IMLNC (Fig 2.2) is a rogue result and could arguably be ignored. If the 2 low results from day 6 are equally ignored, because they appear to be rogue results also when taken in context with the whole experiment, this would leave day 4 with n = 3 and day 6 with n = 2. By combining these results, i.e. day 4 + 6 = n = 5, with a $\bar{x} \pm$ s.d. of 47 ± 10, which is statistically significantly lower ($P<0.01$) than the controls. From the results it is possible that the day 6 (n = 2) IMLNC were slightly less effective than the day 4 (n = 3) IMLNC, the former inducing 28% protection compared with 45% protection induced by day 4 cells as compared to the controls.

As the previous experiments had shown that days 4 and 6 IMLNC were capable of transferring protection, the work was extended to see if cells taken later from a 1^{o} infection could also transfer protection.

<u>The ability of day 4, day 12 and day 21 IMLNC to transfer protection</u>

An interesting result observed by Mawdsley (1983) was that IMLNC taken 21 days after a 1^{o} immunizing infection

could transfer immunity although day 12 IMLNC failed to confer protection, time, however prevented her from repeating the experiment.

It was decided, therefore to re-investigate this rather surprising result. Although the experiments had the same aims, their design differ slightly.

Thirty nine, 10-12 - week - old, male, NIH were divided into one group (X) of 15, and two groups (Y & Z) of 12 donors. All donors were infected with 200 m, group X on day -21, group Y, day -13 and group Z on day -6 resulting in a staggard design. Cell transfers were carried out on day 0 (Group X), day -1 (Group Y) and day -2 (Group Z).

The recipient mice, 8 - week - old, NIH, males were divided into three groups of five mice and there were the same number of control mice in each group. Each group of recipients were given 4×10^7 IMLNC/mouse i.p. of either day 21, day 12 or day 4 cells. The controls were given 0.5 ml of incubating medium at the same time. All mice were challenged with 200 m 9 days after the cell transfer and the worms were recovered 5 days after challenge as in previous experiments.

The results show that day 21 IMLNC were the most efficient in transferring protection, inducing 54% protection as compared with controls (Table 2.5a). Days 12 and 4 IMLNC induced 20% protection as compared with their controls, the

amount of protection being statistically significant ($P<0.05$ and $P<0.01$ respectively).

Due to the poor expulsion caused by day 4 IMLNC, the experiment was repeated using a slightly different design.

Thirty six, 10-12 - week - old, male, NIH were caged four per box to serve as donors. All mice were infected with 200 m, 12 on days -21, -12 and -4. Cell transfer was carried out as normal on day 0. The recipients were composed of three groups of five, 8 - week - old NIH, males and each group was given either 4×10^7 day -21 day -12 or day -4 IMLNC i.p. A control group of four mice were given 0.5 ml of incubating medium per mouse i.p. All mice were infected with 200 m and worm recovery was as described in previous experiments.

The day 4 IMLNC caused the accelerated expulsion of D. phoxini, the result being highly significant ($P<0.001$) (Table 2.5b and Fig 2.3). Mice which had received day 21 or day 12 IMLNC yielded fewer worms than the controls but the reduction was not statistically significant.

Because of the contradictory results between this experiment and the previous experiment it was again repeated, with the addition of groups of mice to which immune spleen cells (ISC) were transferred instead of IMLNC. This was to determine how localized the response was.

The experimental design for transferring IMLNC was identical to the above experiment. The ISC were transferred

on the same days as the IMLNC, but the recipients were composed of four, 8 - week - old mice rather than five mice. Recipients of ISC and their controls were challenged with 200 m, 10 days after cell transfer. Worms were recovered 5 days p.i.

Day 4 IMLNC successfully transferred immunity ($P<0.02$) (Table 2.5c), but both day 21 and day 12 IMLNC did not confer any significant protection. Of the five mice in groups given day 21 and day 12 IMLNC, two had no worms in the former group and one had no worms in the latter. Another mouse in this group had one worm. These mice were excluded from the means of the groups.

Immune spleen cells did not affect worm numbers at any time (Table 2.5c). In the group given day 21 ISC, two mice again had no worms and this group was excluded from any statistical test.

<u>The effect of transferring immunity with enriched B or T cell fractions</u>

With the ability of day 4 IMLNC to transfer adoptive immunity demonstrated in the previous experiments, the following were designed to provide evidence of the nature of the cells responsible.

Thirty six, 12-14 - week - old, male NIH were infected with 50 m on day -13 and caged in groups of 6. Twelve 8 - week - old male NIH were caged in groups of four to serve as

recipients.

All donor mice were killed 4 days p.i., their MLN removed and pooled and a cell suspension prepared. The B to T cell ratio of the cell population was measured by a direct fluorescent antibody test and then four of the recipient mice were injected i.p. with 4×10^7 IMLNC.

The remaining cells were passed down nylon wool columns to enrich the B and T cell fractions. After separation the B to T cell ratio in each sample was measured as above, and then five mice of the recipients were given 4×10^7 enriched T cells and three given 4×10^7 enriched B cells i.p. (only three due to the lack of B cells). The remaining four mice served as controls and were given 0.5 ml of incubating medium i.p.

Nine days after the cell transfer, all the recipients were infected with 200 m and killed 5 days p.i.

On day 5 p.i., >70% of the flukes remained in the controls, but recipients given IMLNC (T - B cell ratio 52% - 48%) and enriched T cells (T - B cell ratio 55% - 45%) had significantly lower worm populations, ($P<0.001$). The group given enriched B cells (T - B cell ratio 30% - 70%) had slightly lowered their worm burden, but because of the small group (n = 3) and large deviation no conclusion could be drawn (Table 2.6a and Fig 2.4).

The experiment was repeated to confirm the results and

to remove the worry one always has when mice have no worms. (Was it due to strong protection or faulty infection?).

Again mice given IMLNC (T - B cell ratio 54% - 46%) had significantly lower numbers of flukes than controls ($P<0.01$) as did those given enriched T cells (T - B cell ratio 58% - 42%) ($p<0.01$) (Table 2.6b) although the protection was only 42% ($\bar{x}$) compared with 91% ($\bar{x}$) in the first experiment.

Few cells were recovered from the enriched B cell population (T - B cell ratio 28% - 72%) permitting only two mice to be given 4×10^7 enriched B cells. This was a pity as the B cell enriched fraction, albeit based on only two mice, were the most successful at expulsion of worms (Table 2.6b).

Because of the problems with the nylon wool separation, such as poor enrichment of T cells, poor recovery of enriched B cells and other criticisms of the technique eg. Corrigan et al (1979) showed that separation of cells by the nylon wool technique caused stripping of certain molecules from the cell membrane, it was decided to use another and hopefully better method for obtaining T and B cell fractions. The method used was the in vitro antibody mediated complement dependant lysis of selected cells which thereby enriches the remaining cell populations. This is the negative selection method.

The ability of IMLNC treated with Thy 1.2 cytotoxic monoclonal antibody and C, to transfer immunity

Because of the possible ability of enriched fractions of B cells (sIg^+) to transfer immunity (Table 2.6b), the following experiment was designed to try and determine whether it really was B cells that were important or whether this fraction still contained the important T sub-sets.

The experimental design was identical to the previous experiment but the procedure on the day of cell transfer differed. There were also only three recipient groups composed of five mice each. One group was given 0.5 ml of incubating medium and another was given 4×10^7 IMLNC as normal. The remaining cells were treated with the anti Thy 1.2 monoclonal antibody and C to remove the Thy 1.2 antigen bearing cells, i.e. T cells. After treatment the remaining mice were given 4×10^7 Thy 1.2 & C treated cells i.p. which were composed of 85% B cells.

The results were inconclusive because six mice, three in the control and three in the IMLNC recipient mice were uninfected. However four of the five mice receiving B cells contained only 25% ($\bar{x}$) of their worms, a figure similar to the mice receiving IMLNC (30% ($\bar{x}$)) which was well below the two controls (70%) ($\bar{x}$), a normal recovery (Table 2.7a).

The experiment was repeated, using the same design.

Due to the lack of enriched B cells (T - B cell ratio 14% - 86%) only three mice were given 4×10^7 enriched B cells.

The results (Table 2.7b) show that mean recovery for

the control group was 75% (n = 5) and there was a significant reduction in both groups given immune lymphocytes or enriched B cells ($P<0.001$).

Because the number of mice receiving T cell depleted, i.e. B cell enriched IMLNC was so small, and the implication of the results, that T cells were not essential for adoptive protection, being so important, it was decided that the experiment must be repeated.

The design was similar to the above experiment but larger recipient groups were used. They were composed of groups given:

a	Incubating medium	n = 6
b	4×10^7 IMLNC	n = 4
c	4×10^7 enriched B cells	n = 8
d	2×10^7 enriched B cells	n = 4

Challenge of recipients and recovery of worms was described in previous experiments.

The results again proved disappointing with several zero recoveries (Table 2.7c). Because of these zero recoveries, the two lowest numbers in groups a, c and d were excluded from further calculations. The lowest figure only was excluded from group b, leaving n = 3, to allow further statistical analysis. Recipients of IMLNC (T - B cell ratio 46% - 54%) and 4×10^7 enriched B cells (T - B cell ratio 20% - 80%) had statistically significantly lower numbers of worms ($P<0.02$ and $P<0.01$ respectively) than the controls. Recipients

of 2 x 10^7 enriched B cells showed a large variation in their recoveries. Although most of the worm numbers were below those of the control mice (Table 2.7c), statistical analysis was not possible.

However because of the wide deviation seen in the recoveries it is suggested the results ought to be treated with caution.

<u>The rate of cell division in the MLN of mice during the course of a 1^o *D. phoxini* infection of 200 m</u>

In association with the cell transfer experiments, further work was done to characterise the cells responsible for transferring immunity. Experiments were therefore designed to measure the rate of cell division in the MLN by the incorporation of [^{125}I]-UdR. Initial experiments were first carried out to familiarise oneself with the technique and solve difficulties, before attempting the experiments described.

Forty eight, 8-10 - week - old, male, NIH were caged in groups of six. Three mice in each cage were infected with 200 m, the other three served as non-infected controls. Two cages of mice were killed on days 2, 4, 8 and 12 p.i. for assay. The cells recovered from the infected mice in each cage were pooled, as were cells from the controls. Two replicate samples were incubated from infected cells, except one group on day 4 p.i. which had one sample, and only one sample was incubated from the control cells.

The results expressed as a L.I. (Table 2.8, Fig 2.5) show a rapid increase in blast activity peaking on day 4 p.i. By day 8 p.i. the rate of cell division had decreased, so that by day 12 p.i., cell division had almost returned to control levels.

The c.p.m. measured in the infected group also follows this pattern with peak activity on day 4 p.i. However c.p.m. values in the control groups show a wide variation and tend to increase with time. On both days 4 and 8 p.i. there is one high and one low value in the controls, in retrospect it would have been better to have three controls to distinguish the odd result.

<u>The rate of cell division in the MLN of mice during the course of a 2^{o} infection of 200 m</u>

Forty eight, male, NIH aged between 8 and 10 weeks were divided into four groups of 12 mice, and caged in boxes of six. A cage of mice in each group were infected with 200 m on day -27 and challenged with 200 m on day 0. Three of the remaining non infected mice in each group were also infected on day 0 with 200 m to provide the 1^{o} infection control. The remaining mice served as non infected controls. A group of mice were killed on days 2, 4, 6 and 10 p.i. for assay of blast activity. Four pools of cells were obtained for each day, i.e. control, 1^{o} infection and two pools of 2^{o} infection. Two samples of cells from each pool were assayed if there were sufficient cells.

The results confirm that peak blast activity during a 1^{o} infection occurs on day 4 p.i. (Table 2.9, Fig 2.6) and this is followed by a decline in the rate of cell division. In this experiment blast activity on day 2 p.i. (1^{o} infection) is lower than the control value. This is certainly due to the high c.p.m. value measured for the control group on day 2 (Table 2.9) which is almost double the value observed for the controls on the other days. This has obviously affected the L.I. for both 1^{o} and 2^{o} infection on day 2 p.i. If the L.I. for this day is ignored, the c.p.m. results show that blast activity during the 1^{o} infection is similar to the other control values, but during the 2^{o} infection, blast activity is much higher than in the 1^{o}. Blast activity during the 2^{o} infection continues to increase up to day 6 p.i. and then declines by day 10 p.i. reaching similar values to the 1^{o} (Table 2.9, Fig 2.6).

The effect of varying the immunizing dose on blast activity in the MLN as measured by incorporation *in vitro* of ^{125}I -UdR on days 4, 6 and 8 p.i.

Previous work had shown that 20 m were as efficient as 200 m in immunizing mice and that IMLNC transferred from mice immunized with 20 m or 50 m could transfer immunity. This experiment was therefore done to compare the blast activity in mice infected with 20, 50 or 200 m.

Forty five, male, NIH aged between 8 and 10 weeks were caged in groups of five. All mice were infected on day 0, 15 with 200 m, 15 with 50 m and the remainder with 20 m.

Twelve age matched male NIH caged in groups of four were also set up, as non infected controls.

Nineteen mice, five infected with 200 m, five with 50 m, five with 20 m and four non infected controls were killed on days 4, 6 and 8 p.i. Cell suspensions were prepared as in previous experiments, and cells within groups were pooled. Incubation of cells with isotope and counting were as described in previous experiments.

The L.I. shows that peak blast activity in all groups occurred on day 4 p.i., with the highest level of blast activity occurring in the mice infected with 200 m. However the c.p.m. values show a wide variation in the controls between days 4, 6 and 8 p.i. and this affects the L.I. The results of c.p.m. values in the experimental groups suggest there is very little difference between values on day 4 and day 8 p.i., but on day 6 p.i., values were lower. It is interesting that mice infected with 200 m had higher c.p.m. values than those infected with 50 m which were greater than those infected with 20 m (Table 2.10).

Table 2.2

The effect of adoptively transferred day 4 immune mesenteric lymph node cells on a primary *D. phoxini* infection

Group	% worm survival on day 5 $\bar{x} \pm$ s.d.	% depression	(n)
A 4×10^7 IMLNC	22 ± 4	69	(5)
B 4×10^7 CMLNC	62 ± 3	11	(3)
C Incubating medium	70 ± 11		(4)

(n) = number of mice/group

Means significantly different between:

A v C = $P < 0.001$

A v B = $P < 0.001$

Table 2.3

The effect of immunizing mice with 200 or 20 metacercariae on the ability of day 4 and day 6 immune mesenteric lymph node cells to transfer immunity

Day 4 donors	% survival on day 5 $\bar{x} \pm$ s.d.	% depression	(n)
No cells (control)	74 ± 5[a]		(4)
Donors			
IMLNC (Inf 200 m)	26 ± 3[b]	65	(4)
Donors			
IMLNC (Inf 20 m)	46 ± 3[c]	38	(4)

Day 6 donors	% survival on day 5 $\bar{x} \pm$ s.d.	% depression	(n)
No cells (control)	69 ± 2[d]		(4)
Donors			
IMLNC (Inf 200 m)	43 ± 10[e]	38	(4)
Donors			
IMLNC (Inf 20 m)	22 ± 19[f]	68	(4)

(n) = number of mice/group

Means significantly different between:

a v b $P < 0.001$; a v c $P < 0.001$

d v e $P < 0.05$; d v f $P < 0.01$

Table 2.4

Comparison of day 4 and day 6 immune mesenteric lymph node cells from mice immunized with 50 metacercariae to transfer immunity

Group	Treatment	% survival on day 5 $\bar{x} \pm$ s.d.	% depression	(n)
A	Incubating medium	75 ± 5		(4)
B	4×10^7 day 4 IMLNC	31 ± 22	59	(4)
C	4×10^7 day 6 IMLNC	27 ± 30	64	(4)

(n) = number of mice/group

Means significantly different between group:

A v B = $P < 0.02$

A v C = $P < 0.05$

Table 2.5a

The effect of transferring day 21, day 12 and day 4 immune mesenteric lymph node cells on a primary infection of 200 metacercariae of D. phoxini in NIH mice

Treatment	% worm survival on day 5 $\bar{x} \pm$ s.d.	% depression	(n)
Control	74 ± 4		(4)
		54	
4×10^7 day 21 IMLNC	34 ± 9		(4)
Control	78 ± 8		(4)
		20	
4×10^7 day 12 IMLNC	62 ± 9		(5)
Control	83 ± 1		(5)
		20	
4×10^7 day 4 IMLNC	66 ± 7		(4)

(n) = number of mice/group

Donors received 200 metacercariae.

Significance of difference on:

day 21 $P < 0.001$

day 12 $P < 0.05$

day 4 $P < 0.01$

Table 2.5b

Comparison of the effect of transferring day 21, day 12 and day 4 immune mesenteric lymph node cells on a primary infection of 200 metacercariae of D. phoxini in NIH mice

Treatment	% worm survival on day 5 $\bar{x} \pm$ s.d.	% depression	(n)
Control : 0.5 ml Incubating medium	72 ± 2[a]		(4)
4×10^7 day 21 IMLNC	60 ± 11[b]	17	(5)
4×10^7 day 12 IMLNC	66 ± 12[c*]	8	(3)
4×10^7 day 4 IMLNC	27 ± 11[d]	63	(5)

(n) = number of mice/group

Donors received 200 metacercariae.

* One mouse died during experiment and one gave a zero recovery and was excluded from $\bar{x} \pm$ s.d.

Significance of difference between means:

a v b = not significant

a v c = not significant

a v d = $P < 0.001$

Table 2.5c

Comparison of the effect of transferring day 21, day 12 or day 4 immune mesenteric lymph node cells or immune spleen cells on a primary infection of 200 metacercariae of D. phoxini

Treatment	% worm survival on day 5 $\bar{x} \pm$ s.d.			
	No cells	Day 21	Day 12	Day 4
4×10^7 IMLNC	73 ± 9^a (4)	61 ± 3^b (3)	67 ± 1^c (3)	44 ± 18^d (5)
% depression		16	8	40
4×10^7 ISC	74 ± 3^e (4)	61, 0, 0, 49.5 $\bar{x}$ not calculated	76 ± 4^f (4)	72 ± 8^g (4)
% depression				3

(n) = number of mice/group

Donors received 200 metacercariae.

Significance of difference between means:

a v b = not significant

a v c = not significant

a v d = $P < 0.02$

e v f = not significant

e v g = not significant

The large deviation in (d) was due to the following recoveries:

55%, 79%, 63.5%, 34% and 47%.

Table 2.6a

The effect of transferring immune mesenteric lymph node cells and enriched B and T cell fractions on a primary infection of D. phoxini (200 metacercariae) in NIH mice on day 5 p.i.

Treatment	% worm survival $\bar{x} \pm$ s.d.	% depression	(n)
Control : 0.5 ml Incubating medium	76 ± 2[a]		(4)
4×10^7 IMLNC	8 ± 16[b]	89	(4)
4×10^7 enriched T cells	5 ± 7[c]	93	(5)
4×10^7 enriched B cells	46 ± 31[d]	39	(3)

(n) = number of mice/group

Donors received 50 metacercariae.

Significance of difference between means:

a v b = $P < 0.001$

a v c = $P < 0.001$

Table 2.6b

The effect of transferring immune mesenteric lymph node cells and enriched B and T cell fractions on a primary infection of *D. phoxini* (200 metacercariae) in NIH mice on day 5 p.i.

Treatment	% worm survival $\bar{x} \pm$ s.d.	% depression	(n)
Control : 0.5 ml Incubating medium	71 ± 10[a]		(3)
4×10^7 IMLNC	37 ± 8[b]	47	(4)
4×10^7 enriched T cells	45 ± 8[c]	37	(6)
4×10^7 enriched B cells	22[d]	69	(2)

(n) = number of mice/group

Donors received 50 metacercariae.

Significance of difference between means:

a v b = $P < 0.01$

a v c = $P < 0.01$

Table 2.7a

The effect of transferring immune mesenteric lymph node cells treated with Thy 1.2 monoclonal antibody and complement on a primary infection of 200 metacercariae of D. phoxini

Treatment	% worm survival per mouse on day 5	% depression
Control : 0.5 ml Incubating medium	0, 0, 81, 60, 0.	
4×10^7 IMLNC	0, 0, 38, 21.5, 0.	58
4×10^7 enriched B cells	79.5, 30.5, 29.5, 22.5, 80.	49

Donors received 50 metacercariae.

Table 2.7b

The effect of transferring immune mesenteric lymph node cells treated with Thy 1.2 monoclonal antibody and complement on a primary infection of 200 metacercariae of D. phoxini

Treatment	% worm survival on day 5 $\bar{x} \pm$ s.d.	% depression	(n)
Control : 0.5 ml Incubating medium	75 ± 5^a		(5)
4×10^7 IMLNC	7 ± 7^b	91	(5)
4×10^7 enriched B cells	21 ± 6^c	72	(3)

(n) = number of mice/group

Donors received 50 metacercariae.

Significance of difference between means:

a v b = $P < 0.001$

a v c = $P < 0.001$

Table 2.7c

The effect of transferring immune mesenteric lymph node cells treated with Thy 1.2 monoclonal antibody and complement on a primary infection of 200 metacercariae of D. phoxini

Treatment	% worm survival on day 5 $\bar{x} \pm$ s.d.	% depression	(n)
Control : 0.5 ml Incubating medium	0*, 0*, 67, 60, 36.5, 52.5[a]		
	54 ± 13		(4)
4×10^7 IMLNC	0*, 27.5, 29, 12[b]		
	23 ± 9	57	(3)
4×10^7 enriched B cells	0*, 8.5, 3, 39.5, 35.5, 11, 1.5* 21[c]		
	20 ± 15	63	(6)
2×10^7 enriched B cells	18, 59.5, 0*, 0*, .5[d]		
	26 ± 30	52	(3)

* not included in $\bar{x} \pm$ s.d.

(n) = number of mice used to calculate $\bar{x} \pm$ s.d. Two lowest numbers in groups a, c and d were excluded and lowest number from group b only, leaving n = 3 for statistical analysis.

Significance of difference between means:

a v b = $P < 0.02$

a v c = $P < 0.01$

Table 2.8

The rate of cell division in the mesenteric lymph node of mice as measured by the incorporation of 5- [^{125}I] -iodo-2'-deoxyduridine during the course of a primary infection of 200 metacercariae

(n) = number of pools of cells, and 1 vial in background.

Labelling Index:

$$L.I. = \frac{\text{c.p.m. experimental group}}{\text{c.p.m. control group}} \times 100$$

Mean of background values on each day was subtracted from the original c.p.m. values of both control and experimental groups.

Days p.i.	Control (n) c.p.m.	Infected (n) $\bar{x}$ c.p.m.	Labelling Index	(n) Background	Isotope Control Count
2	(1) 17,306 (1) 19,061	(2) 33,698 (2) 25,155	195 132	(1) 299 (1) 290	900,000 in 40 sec
4	(1) 15,807 (1) 28,672	(2) 109,644 (1) 152,044	694 530	(1) 290 (1) 321	900,000 in 36 sec
8	(1) 29,805 (1) 19,502	(2) 67,915 (2) 63,801	228 327	(1) 311 (1) 318	900,000 in 34 sec
12	(1) 30,108 (1) 27,206	(2) 35,318 (2) 35,725	117 131	(1) 290 (1) 301	900,000 in 36 sec

Table 2.9

The rate of cell division in the mesenteric lymph node of mice during a primary or secondary infection of D. phoxini as measured by incorporation of 5-[^{125}I]-iodo-2'-deoxyuridine

(n) = number of pools of cells otherwise n = 1. When n = 2 the result is the mean.

Both primary and secondary infections were of 200 metacercariae.

Labelling Index:

$$L.I. = \frac{\text{c.p.m. experimental group}}{\text{c.p.m. control group}} \times 100$$

Mean of background values on each day was subtracted from the original c.p.m. values in each group.

Days p.i.	Control c.p.m.	Primary Infection c.p.m.	Labelling Index	Secondary Infection c.p.m.	Labelling Index	Background	Isotope Control Count
2	20,792	11,981	56	63,525 53,823	305 258	202 187	900,000 in 32 sec
4	11,611	(2) 100,771	867	(2) 58,359 (2) 62,858	503 541	188 200	900,000 in 33 sec
6	14,203	(2) 72,996	513	(2) 136,136 (2) 104,096	958 732	196 202	900,000 in 34 sec
10	13,630	(2) 52,937	388	(2) 44,203 (2) 66,804	324 490	178 217	900,000 in 33 sec

Table 2.10

The effect of varying the size of the primary infection on blast activity in the mesenteric lymph node of NIH mice

(n) = 3 alliquots of cells in controls except on day 8 p.i. when n = 2.

4 alliquots in all other groups.

Labelling Index:

$$L.I. = \frac{\text{c.p.m. experimental group}}{\text{c.p.m. control group}} \times 100$$

Mean of background values on each day was subtracted from the original c.p.m. values.

Level of Infection	Days p.i.					
	4		6		8	
	$\bar{x} \pm$ s.d. c.p.m.	L.I.	$\bar{x} \pm$ s.d. c.p.m.	L.I.	$\bar{x} \pm$ s.d. c.p.m.	L.I.
Control (No Infection)	35,145 ± 1,620	100	61,526 ± 1,575	100	41,105	100
20	67,009 ± 1,902	191	57,625 ± 4,813	94	63,454 ± 2,228	154
50	82,898 ± 3,618	236	65,098 ± 6,496	106	82,246 ± 4,761	200
200	95,101 ± 7,267	271	74,945 ± 6,145	121	96,303 ± 6,890	234
Background	215 197		As for day 4		194 189 196	
Isotope Control Count	900,000 in 38 sec		900,000 in 38 sec		900,000 in 36 sec	

Table 2.11 a and b

Number of cells recovered from the mesenteric lymph node of (a) mice infected with a primary infection of 200 metacercariae and (b) a secondary infection of 200 metacercariae following a primary infection of 200 metacercariae

Key:

C_1 & C_2 = Two groups of control mice

1^o_1 & 1^o_2 = Two groups of mice with primary infections

2^o_1 & 2^o_2 = Two groups of mice with secondary infections

(a)

Group	Days p.i.			
	2	4	8	12
C_1	3.5	3.2	3.2	2.5
C_2	3.0	4.0	3.3	2.6
1^o_1	6.1	6.9	10.4	7.7
1^o_2	6.1	10.0	12.3	10.1

(n) = 3 mice/group

No. of cells x 10^{-7}

(b)

Group	Days p.i.				
	1	2	4	6	10
C_1	8.5	5.2	14	6.2	4.8
$1^o{}_1$	7.9	11.2	20.4	18.9	13.6
$2^o{}_1$	7.3	8.2	24.4	24.0	9.2
$2^o{}_2$	7.0	9.1	16.8	22.8	-

(n) = 4 mice/group

No. of cells x 10^{-7}

Figure 2.1

The effect of adoptively transferring 4×10^7 immune mesenteric lymph node cells (IMLNC), 4×10^7 control mesenteric lymph node cells (CMLNC) or 0.5 ml of incubating medium on worm recoveries on day 5 of a primary infection of *D. phoxini* (200 metacercariae) in NIH mice

Key:

▦ = $\bar{x} \pm$ s.d.

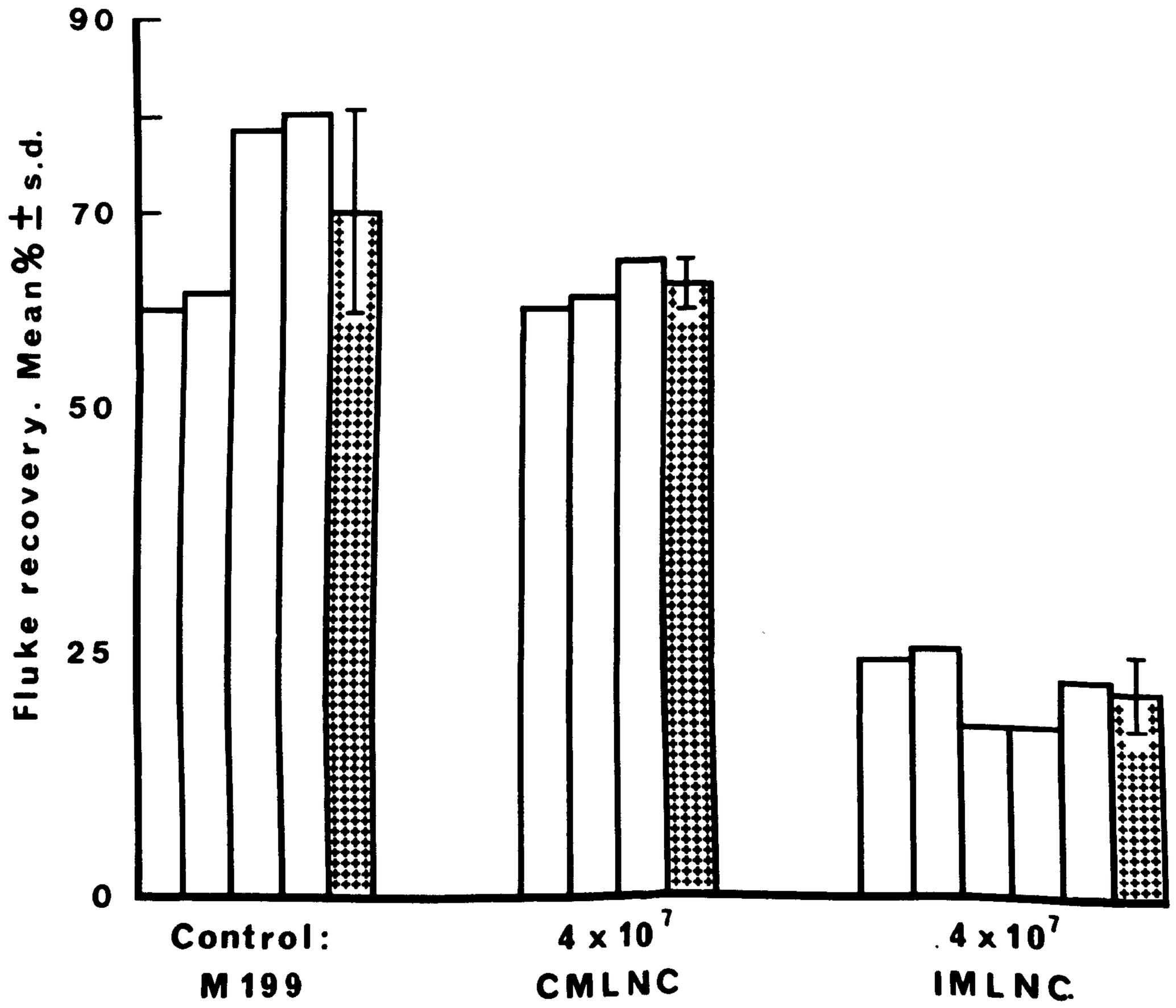

Fluke recovery. Mean% ± s.d.
90
70
50
25
0
Control:
M199
4 x 10 7
CMLNC
4 x 10 7
IMLNC

Figure 2.2

The effect of adoptively transferring 4×10^7 immune mesenteric lymph node cells, from mice immunized with 50 metacercariae for 4 or 6 days, on the worm burden on day 5 of mice infected with 200 metacercariae of D. phoxini

Key:

▦ = $\bar{x} \pm$ s.d.

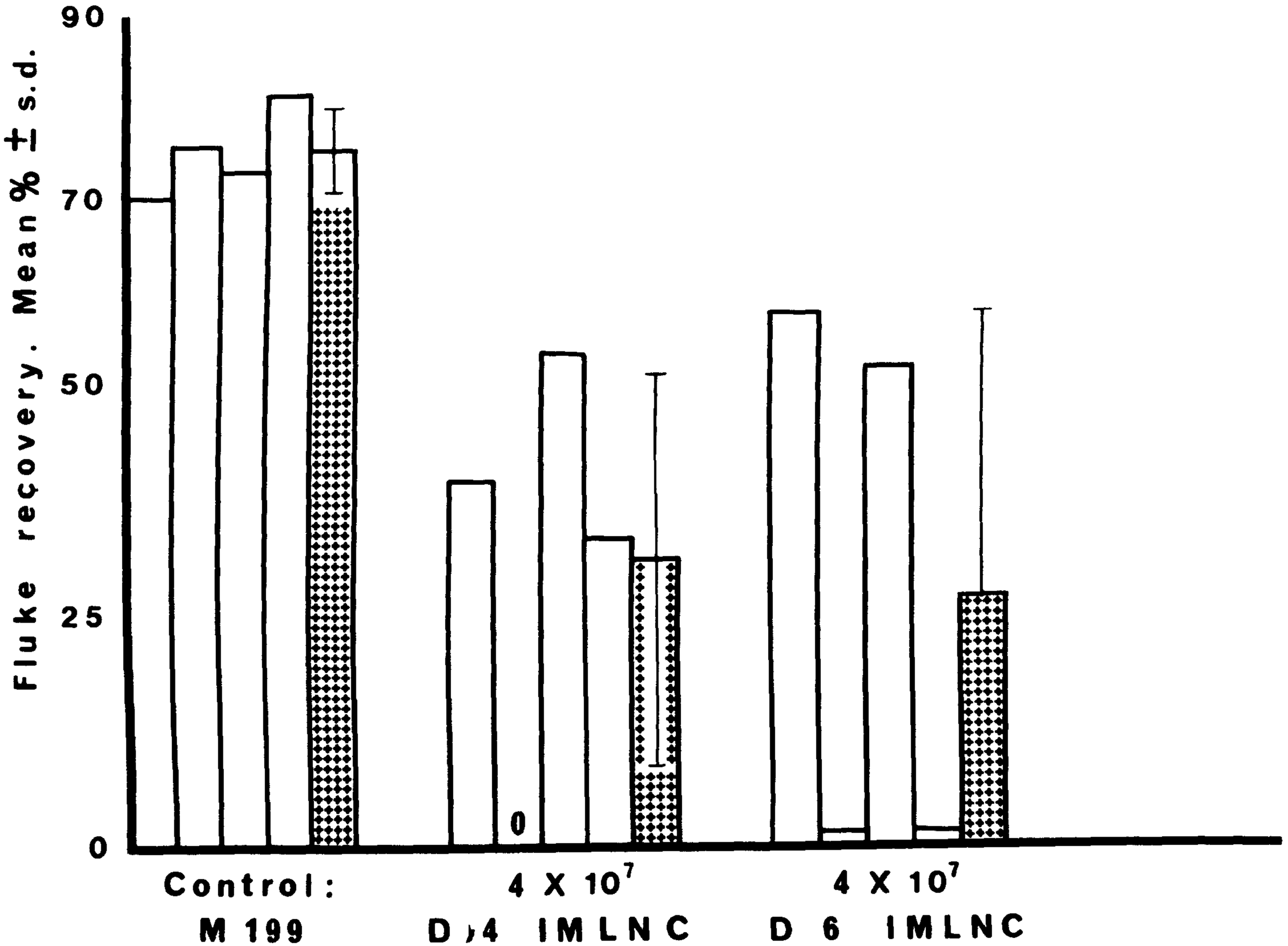

Fluke recovery. Mean % ± s.d.
90
70
50
25
0
0
Control :
M 199
4 X 10^7
D 4 IMLNC
4 X 10^7
D 6 IMLNC

Figure 2.3

The effect of adoptively transferring 4 x 10^7 immune mesenteric lymph node cells from donors immunized with 200 metacercariae for 4, 12 or 21 days, on the worm burden of recipient mice infected with 200 metacercariae of D. phoxini.

Key:

▦ = $\bar{x} \pm$ s.d.

0 = no worms recovered (excluded from mean)

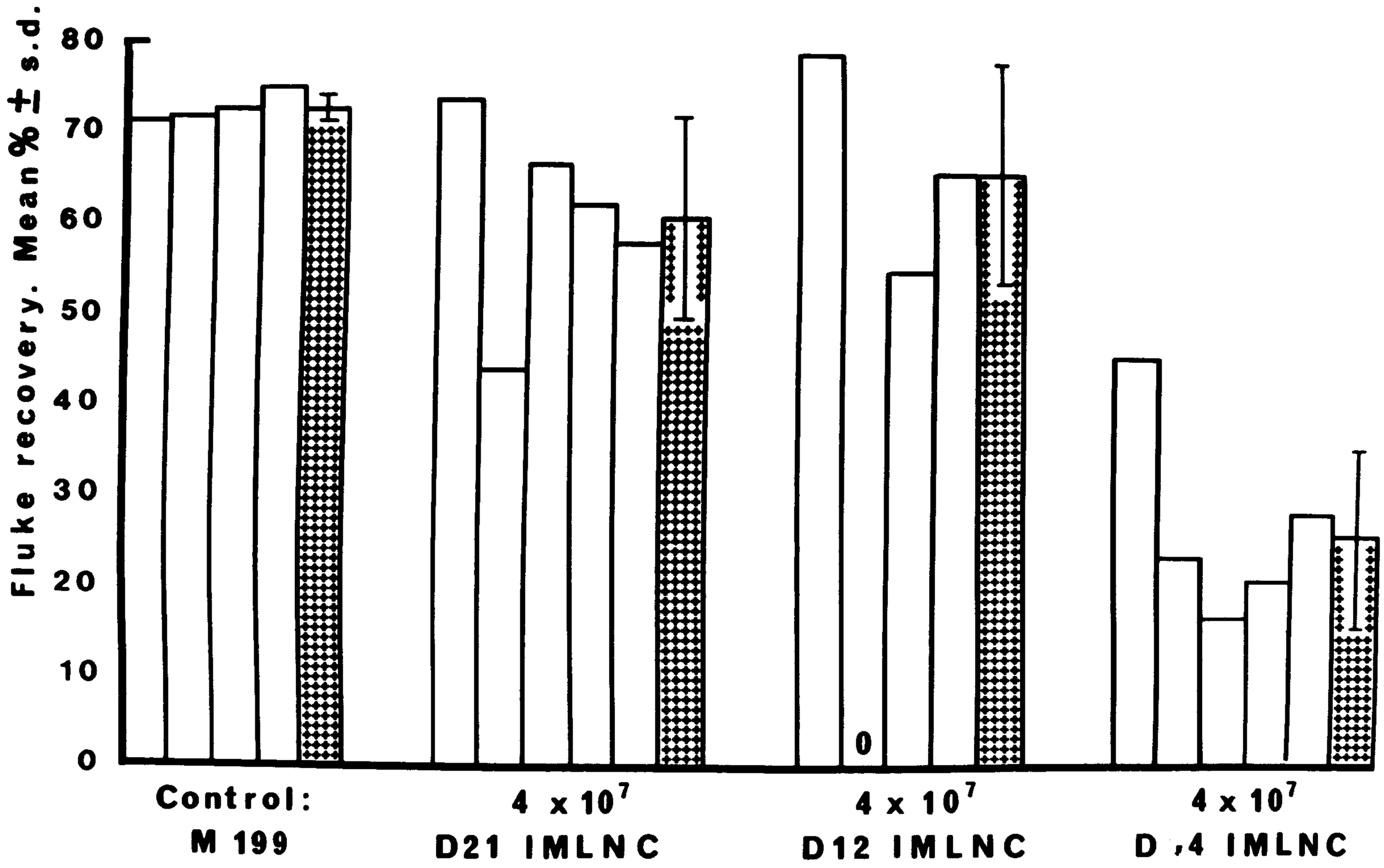

Fluke recovery. Mean % ± s.d.
80
70
60
50
40
30
20
10
0
0
Control:
M 199
4 x 10^7
D21 IMLNC
4 x 10^7
D12 IMLNC
4 x 10^7
D ,4 IMLNC

Figure 2.4

The effect of transferring 4×10^7 immune mesenteric lymph node cells, 4×10^7 enriched sIg^- (T) cells or 4×10^7 enriched sIg^- (B) cells from donors infected with 50 metacercariae for 4 days, on the primary infection of 200 metacercariae in recipient NIH on day 5 post infection

Key:

++++ = $\bar{x} \pm$ s.d.

0 = no worms recovered

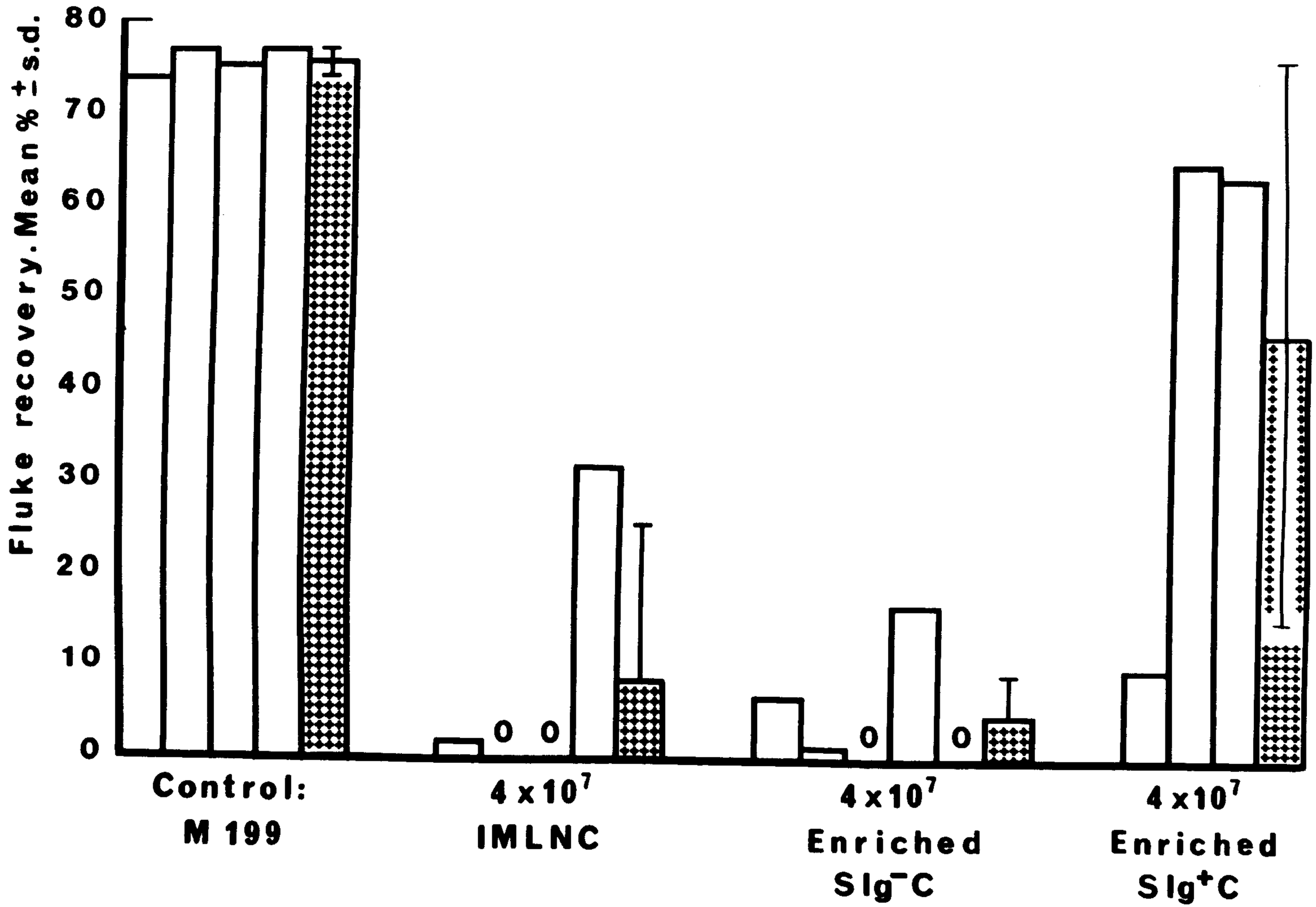

Fluke recovery. Mean % ± s.d.
80
70
60
50
40
30
20
10
0
0 0
0
0
Control:
M 199
4 x 10^7
IMLNC
4 x 10^7
Enriched
Sig⁻C
4 x 10^7
Enriched
Sig⁺C

Figure 2.5

Blast-cell activity in the mesenteric lymph node of NIH mice during a primary infection of 200 metacercariae as measured by incorporation of 5-[^{125}I]-iodo-2'-deoxyuridine

Key:

Each bar (except controls (C)), shows a result based on two pools of cells from 3 mice

Control (C) = 100%

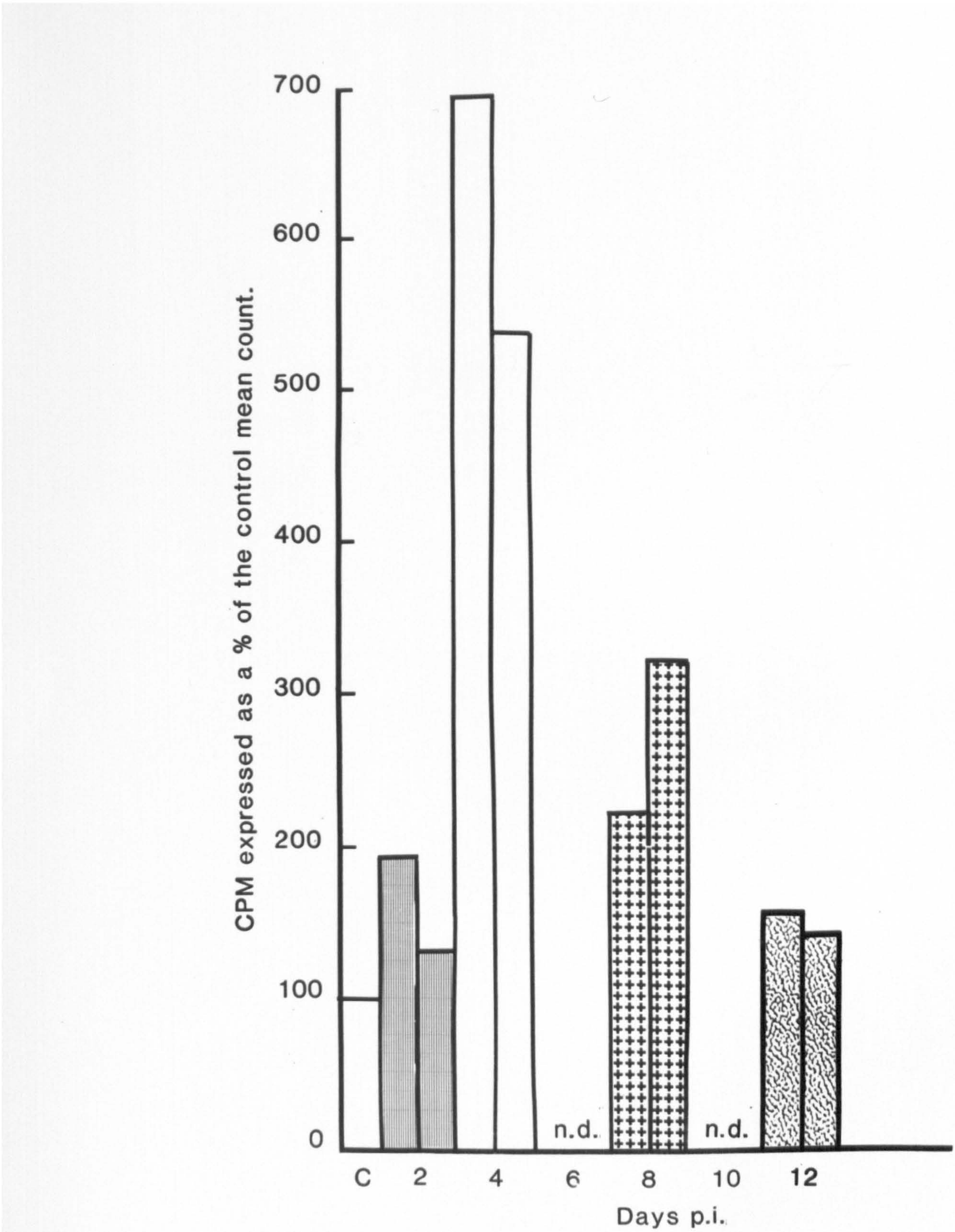

700
600
500
400
300
200
100
0
CPM expressed as a % of the control mean count.
n.d.
n.d.
C
2
4
6
8
10
12
Days p.i.

Figure 2.6

Blast activity in the mesenteric lymph node of NIH mice during a primary or secondary infection of 200 metacercariae as measured by incorporation of 5-[^{125}I]-iodo-2' -deoxyuridine

Key:

(C) = Control

++++ Primary infection

□ Secondary infection

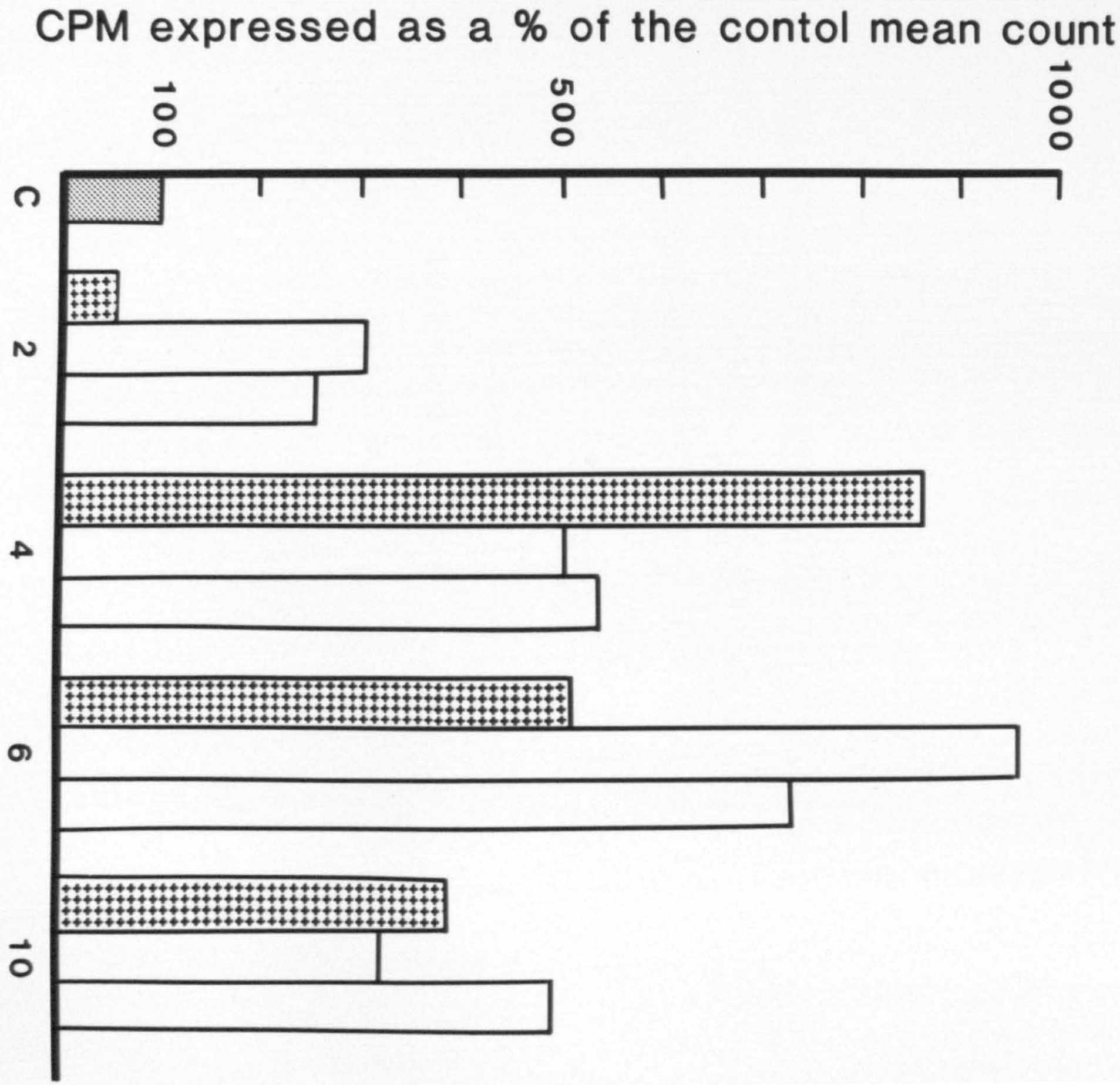

CPM expressed as a % of the contol mean count
100
500
1000
C
2
4
6
10
Days p.i.

Figure 2.7

Graph demonstrating the hypothesis of the effectiveness of immune mesenteric lymph node cells to transfer adoptive immunity after immunization with either 20 or 200 metacercariae of D. phoxini

Key:

——— 200 metacercariae

– – – 20 metacercariae

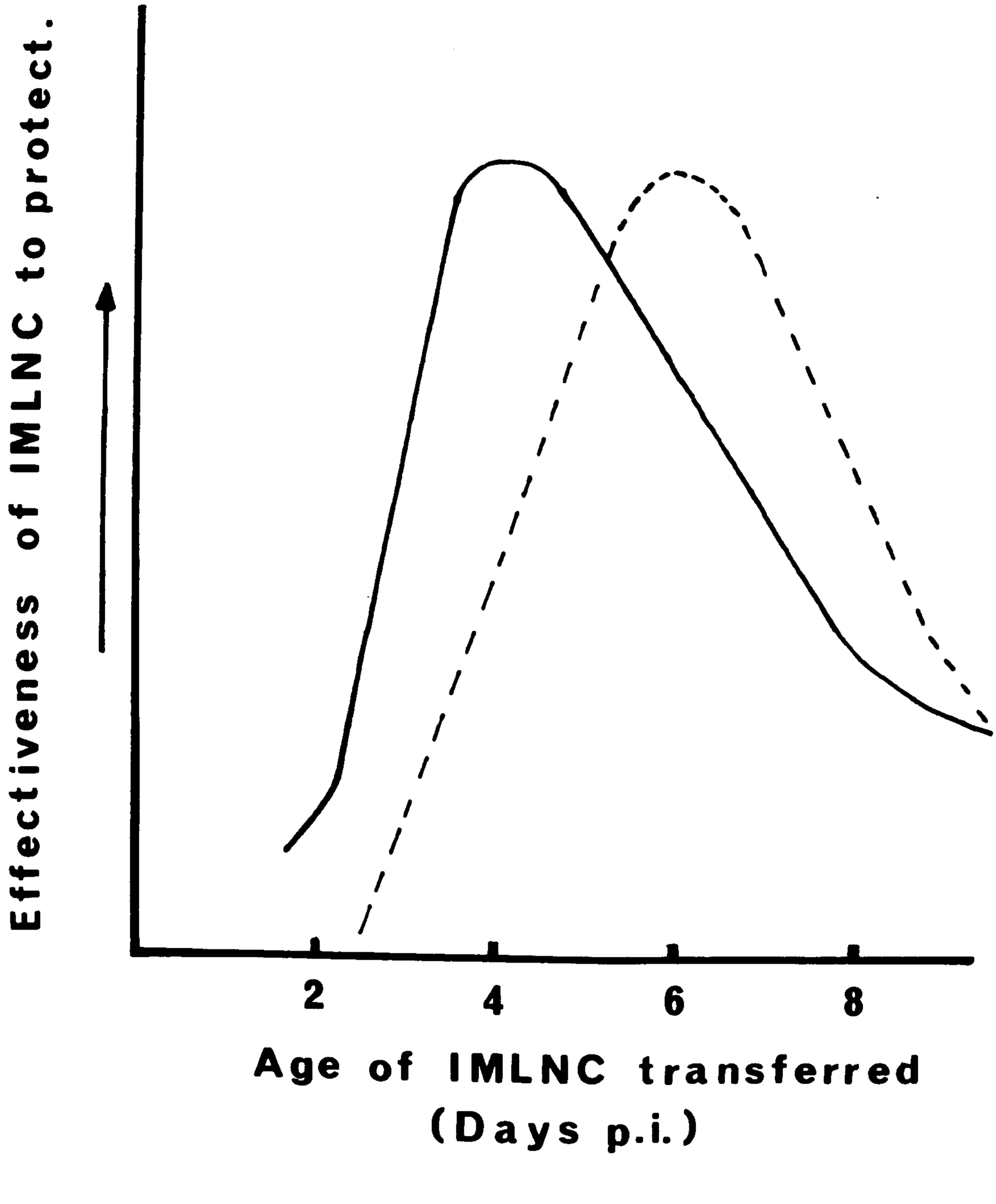

Effectiveness of IMLNC to protect.
2
4
6
8
Age of IMLNC transferred
(Days p.i.)

Figure 2.8

A) Graph comparing total cell numbers expressed as a % of the control, recovered from NIH mice during a primary infection of 200 metacercariae of D. phoxini

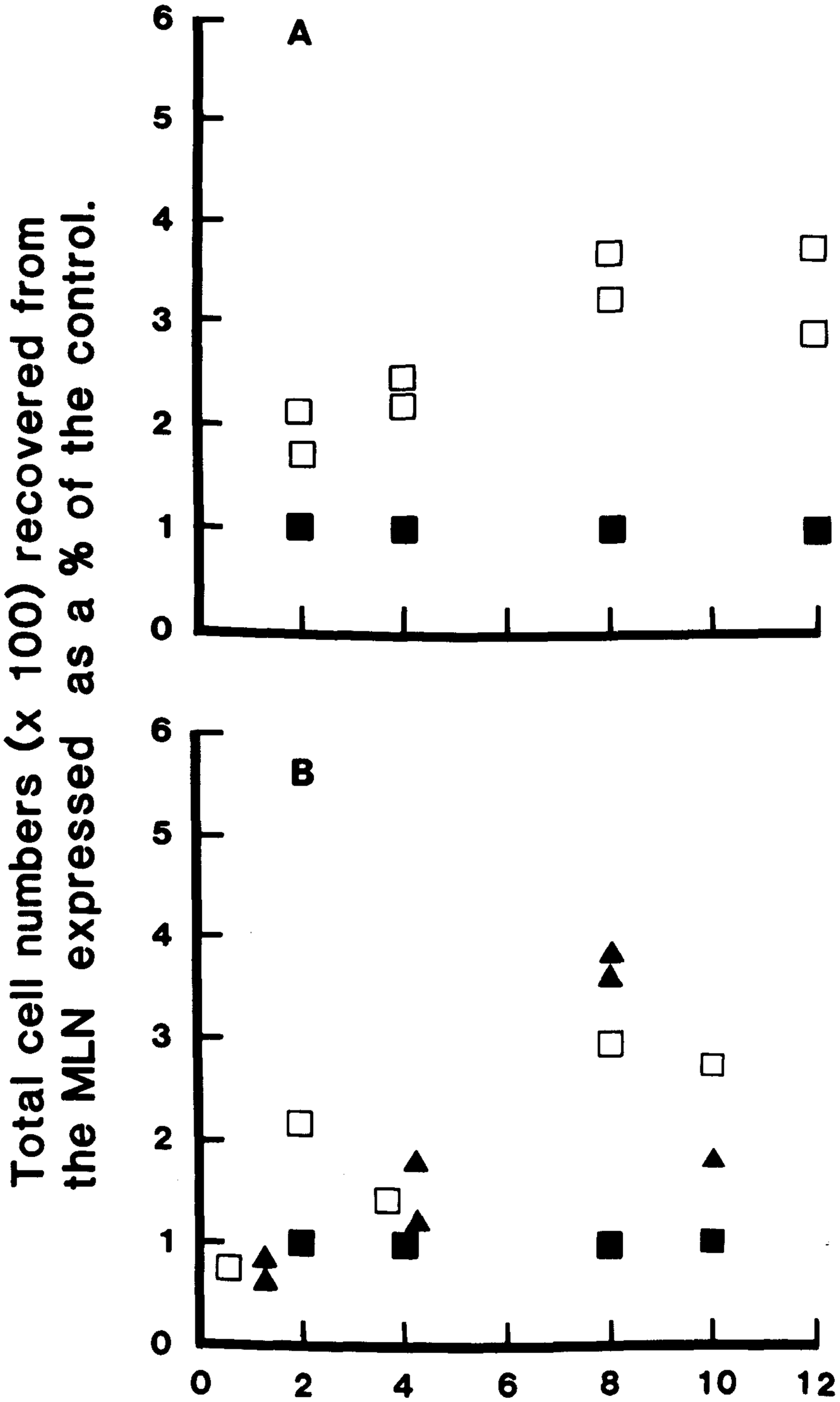
B) Graph comparing total cell numbers expressed as a % of the control recovered from NIH during a primary or secondary infection of 200 metacercariae of D. phoxini

Key:

□ Primary infection

■ Control

▲ Secondary infection


A
B
Total cell numbers (x 100) recovered from the MLN expressed as a % of the control.
0
1
2
3
4
5
6
0
2
4
6
8
10
12
Days p.i.

Discussion

The ability to accelerate the rejection of an established *D. phoxini* infection by adoptive transfer of immunity with IMLNC has been confirmed. Day 4 IMLNC transferred into naive recipients caused almost a 50% reduction in worm burdens by day 5 p.i. as compared with controls given CMLNC from uninfected mice or incubating medium only (Tables 2.2, 2.3 and 2.4).

Although expulsion was not as rapid as expulsion of a 2^{o} infection, it was significantly accelerated. This is in common with many nematode models studied such as *N. brasiliensis* in rats (Kelly and Dineen, 1972), *T. muris* (Selby and Wakelin, 1973), *T. spiralis* (Wakelin and Lloyd, 1976) and *N. dubius* (Cypess, 1970 and Behnke and Parish, 1981) in mice, and *S. ratti* in rats (Moqbel and Wakelin, 1981).

In the current work the effectiveness of the cells was measured as % worm recovery on day 5 p.i. and also as % depression (%Δ). The number of worms recovered on day 5 was used because rejection of a 1^{o} infection would not be expected to be underway on this day (see chapter 1), therefore the controls would be expected to have high levels of worms present. The present results (Tables 2.2, 2.3, 2.4, 2.5 and 2.6) certainly suggest this with normally 70% of the infected dose (200 m) present in control mice on day 5 p.i.

In all 11 experiments (Tables 2.2 - 2.7c), using various levels of m to immunize donors, IMLNC recovered on day 4 p.i. were found to significantly depress worm survival, with the %Δ being usually above 38% and normally much higher. On one occasion the %Δ was quite low, %Δ= 20% (Table 2.5a) and in this experiment an exceptional high result was obtained using day 21 IMLNC (%Δ = 54%). In the other two experiments (Tables 2.5b and c) a value of 20% was observed by transferring day 21 IMLNC and this suggests that a mistake was made during harvesting of cells or labelling of cages resulting in mixed groups. Under the circumstances it seems best to ignore this result (Table 2.5a).

Although the effectiveness of day 4 IMLNC to adoptively protect is beyond doubt, the value of cells taken later in infection has been less well established. Day 6 IMLNC were also effective in reducing the worm burden (Table 2.3 and 2.4), but excluding the one odd result already mentioned, IMLNC taken on days 12 or 21 (Tables 2.5a, b and c) were not very efficient in reducing the worm burden with %Δ=20% as measured by worm recovery on day 5 p.i. Although a slight reduction in worm burdens did occur, % worm recoveries were similar to recipients given CMLNC (Table 2.2).

The effect of varying the number of worms in the infection of donor mice is possibly of more importance than was realised at the time. Day 4 IMLNC from 20 m donors (Table 2.3) gave poor protection (38%) compared with day 6 (68%). At the time it was thought this might have been a

chance result, but it could result from the peak in effective cells in the MLN occurring later (Fig 2.7).

By day 6 p.i., IMLNC from 200 m donors were already declining in effectiveness to protect (Table 2.3), whereas 20 m are more protective then. The 50 m infections tend to confirm this interpretation. In Table 2.4, there was little difference between days 4 and 6 IMLNC in their ability to protect, both being good, but neither very good, suggesting cells may have been recovered from each side of the peak of effectiveness. This might also explain why in later experiments (Tables 2.6a - 2.7c), day 4 IMLNC from 50 m infected donors varied considerably in their ability to depress worm survival (% Δ in recipients varied between 47% and 91%). Very slight differences in the speed of the response, time of recovery of cells etc. could therefore make a big difference in effectiveness of the cells if they were recovered at a time of rapid proliferation or sensitization (Fig 2.7). The differences observed in the effectiveness of cells taken from donors with differing immunizing doses may reflect different antigenic levels of stimuli with 200 m providing a greater stimulus than 20 m, resulting in earlier and possibly greater sensitization of the cells.

The presence of similar peaks of effectiveness of cells to transfer adoptive immunity may occur in other models. Certainly, cells have to be recovered from donors within defined periods to be able to accelerate rejection in recipients. This period varies between models, eg. Wakelin and Wilson (1977)

observed that IMLNC taken 2 days p.i. from mice infected with T. spiralis did not accelerate rejection whereas days 4 and 8 IMLNC did. Ogilvie et al (1977) showed that cells capable of accelerating the rejection of N. brasiliensis from rats were present in the TDL and MLN by days 7 - 8 p.i., but that cells taken later eg. days 10 - 11 were more efficient and cells taken on days 13 - 14 were still better. This may correspond with the increase to the peak, as hypothesized for D. phoxini. Similar observations were recorded by Moqbel and Wakelin (1981) in their S. ratti - rat model, IMLNC taken before day 16 did not accelerate rejection but cells taken after day 20 did, with day 32 p.i. IMLNC being the most efficient. None of these authors investigated the efficiency of cells taken later in infection.

Grencis and Wakelin (1982) did show a similar pattern of IMLNC effectiveness in their T. spiralis - NIH model. The most efficient cells at causing the accelerated rejection of the worms were days 4 and 8 IMLNC whereas those taken on days 12 and 22 p.i. failed to accelerate rejection, which is similar to the D. phoxini model.

The ability of cells taken at different times p.i. to transfer protection reflects the cell composition of the population on that day. The inability of days 12 and 21 IMLNC to accelerated rejection is evidence that the population differs from that on day 4 p.i.

On day 4 p.i. the population is composed of rapidly

dividing cells (Figs 2.5, and 2.6; Tables 2.8 and 2.9) and peak blast activity occurs on this day. Also the population is increasing in numbers (Fig 2.8A and B). The results in Table 2.11a and b differ with regards to the 1^{o} infection in that in (b) a large number of cells were recovered on day 4 (group 1^{o}_{1}) but also a large number of control cells were recovered as compared to the other days. In ignoring this group the pattern of cell numbers is similar to Table 2.11(a) throughout the 1^{o} infection. It is this population of day 4 IMLNC which transfer the highest degree of protection.

By day 12 p.i. the cell population is in decline and blast activity is only slightly elevated above control values (Table 2.8 and Fig 2.5). This population of cells may lower the worm burden but it is not statistically significant.

Blast activity on day 21 p.i. was not measured and there are no cell counts, but from trends of available data it would appear that blast activity would be at control levels and the numbers of cells in the MLN would also be near control levels or in decline.

The evidence from the day 4 and day 12 transfers implies that it is the blast cells which are responsible for transferring protection. On day 6 p.i. of a 200 m 1^{o}, blast activity is in decline (Table 2.9, Fig 2.6) as is their ability to transfer protection (Table 2.3), and this also suggests that blast cells are important in transferring immunity. The cells present in the MLN on day 6 p.i. are probably the immediate progency of the blast cells together

with blast cells and it is probably these populations which transfer immunity.

Evidence for lymphoblasts being responsible for transferring immunity comes from Grencis and Wakelin (1982) and Wakelin _et al_ (1982) who showed that cells capable of transferring protection to _T. spiralis_ in mice had the characteristics of dividing lymphocytes. They were large cells which were readily labelled with [^{125}I]-UdR and whose capacity to function in adoptive transfer was suppressed after exposure to the mitotic inhibitor vinblastine.

The ability of day 12 IMLNC to induce a small but not significant amount of protection may also be due to the blast cells which are present on day 12, but form a smaller percentage of the total cell population than on day 4. The inability of day 12 IMLNC to induce significant protection in recipients could therefore be a quantitative phenomenon and it would be interesting to isolate the blast cells from day 12 IMLNC to see if they are capable of transferring protection.

The ability of day 21 IMLNC to transfer protection may also be quantitative phenomenon but it is possible that a different cell type may be involved than on days 4 and 12 p.i., possibly a memory cell because blast activity would be expected to be low.

Blast activity was also measured during a 2^{o} infection and a more rapid response to infection was observed. The

highest levels of activity were observed on day 6 (Fig 2.5, Table 2.9), later than in the 1^{o}. No attempt was made to transfer immunity with these cells as Mawdsley (1983) had shown that cells taken 12 hours after giving a 2^{o} infection could transfer immunity.

The inability to ISC to transfer protection (Table 2.5c) was not surprising, and this suggests the response is localized to the MLN. Wakelin and Wilson (1977) similarily failed to transfer immunity to T. spiralis in mice with ISC showing the response was restricted to the gut associated lymphoid tissue. However Larsh et al (1969) did manage to protect mice against T. spiralis with ISC but they were derived from donors immunized with a homogenate of T. spiralis larvae and spleen involvement would therefore be expected.

The measurement of blast activity in the MLN of mice with varying 1^{o} infections (Table 2.10) appears to reflect the amount of antigenic stimulation. The larger the infection the larger the activity was in the node, and this is probably due to the greater antigenic stimulation presented by the larger infections. The course of blast activity was similar in all the groups.

Because of the results of this experiment and the results of the cell transfer experiments after varying the immunizing dose of the donors, it was decided that 20 m could be safely used to immunize donors in further experiments, especially if a 2^{o} infection was given, but 50 m would probably be better due to the greater amount of antigenic stimulation.

These experiments were important as it appeared that fish stocks were not as heavily infected as in the first year. This reduction in parasite numbers could have been due to a concurrent project in the Department which involved reducing the roosting gull population on the reservoirs, which would have a severe effect on the parasite population.

The remaining experiments involving the nylon wool separation of cells into enriched B and T cell populations showed that the T cells were as efficient as the IMLNC in transferring protection, but some immunity could also be transferred with the enriched B cells (Table 2.6a and b, Fig 2.4).

The importance of T cells in transferring protection is well established, although their exact role is still unknown. Wakelin and Wilson (1979) showed that both T and B cell enriched populations could transfer immunity to T. spiralis in mice, but T cells were more consistent in their ability to confer protection. Also the ability of B cells to transfer immunity was severely hampered if the contaminating T cells were reduced.

Ogilvie et al (1977) showed that removal of cells bearing surface immunoglobulin, i.e. B cells did not cause a significant reduction in the ability of the remaining TDL to cause expulsion of N. brasiliensis from rats. This was confirmed by Nawa et al (1978) who did however show that a small but significant effect was transferred with B cells separated 1 and 5 weeks after a 3^{o} infection of N. brasiliensis

in rats. T cells were the more efficient though. The present work is open to criticism. Enrichment of T cells was not very successful, the ratio of T to B cells being only slightly greater than in unseparated cells. Although the B cells were quite enriched, recovery was poor, so large groups of mice could not be used. Also separation of cells by this method has been shown to strip certain molecules from the cell surface (Corrigan et al, 1979).

In further experiments the B cells were enriched by lysing contaminating T cells. The results showed that these enriched B cell populations did significantly reduce the worm burden (Tables 2.7a, b and c).

The ability of B cells to transfer protection is highly disputed. Some protection can usually be transferred but T cells are usually more efficient. However, Crum et al (1977) showed that B and T cells had an equal protective capacity against T. spiralis in the rat and in the accompanying paper, Despommier et al (1977) showed that enriched B cells isolated from TDL were more efficient than enriched T cells in accelerating T. spiralis expulsion.

Both these authors used four drug abbreviated infections to immunize their donors and Nawa (1978) showed that multiple immunization of donors may give rise to B cell populations which are qualitatively different from those derived from 1^{o} infections.

In the present work, although the enriched B cells were

capable of transferring immunity, the results usually have a large deviation and should be treated with caution. This large deviation is due to several zero recoveries, even in control groups. It is not known whether the flukes failed to establish or whether they were expelled quicker, but it is certain that an unrecognised variable was affecting the results.

Because of the large variation in recoveries, especially in control mice, this work was terminated. It had been hoped to analyse the cells responsible for transferring protection further by using a fluorescent activated cell sorter (FACS), but it would have been difficult to obtain enough enriched cells for transfer by this technique because of the large number of cells required and the slow rate at which cells can be sorted.

Summary

1) The ability to transfer immunity resulting in the accelerated expulsion of D. phoxini has been confirmed. Cells taken on day 4 p.i. from donors were the most efficient and day 12 cells did not transfer protection.

2) Low worm burdens, i.e. 20 m or 50 m can be used successfully to immunize mice although the response is not as great after immunization with 200 m and also the response may not be as rapid. This may be related to the antigen exposure.

3) High blast activity occurs in the MLN during a 1^{o} infection with peak activity on day 4. The response is accelerated as a result of a 2^{o} infection but peak values are reached later. It may be assumed that these cells are the important cells in adoptive protection.

4) Whether T or B cells transfer immunity is still ambiguous although enriched B cell populations did lower the worm burden significantly.

CHAPTER 3

THE HUMORAL RESPONSE OF MICE TO INFECTION WITH *D. PHOXINI*

Introduction

The transfer of passive protection using serum from immunized hosts has usually proved less successful and less consistent than cell transfers.

Wagland and Dineen (1965) failed to confer protection to *T. colubriformis* in guinea pigs and Larsh *et al* (1964a) failed to transfer protection to *T. spiralis* in mice although circulating antibody titres increased as a result of infection in both models.

Ogilvie and Jones (1968) did manage to transfer some protection to *N. brasiliensis* in rats with serum from hyperimmunized donors. However only 12 out of 40 pools of serum successfully conferred protection as measured by early worm expulsion. Similar inconsistent results were obtained by Wakelin and Lloyd (1976) in their *T. spiralis* - mouse model. They also suggested that host strain may be important in transferring protection.

In contrast to the above work, Miller (1980) was consistently successful in transferring high degrees of protection using hyperimmune serum in rats infected with *N. brasiliensis*. He demonstrated the importance of immunizing schedules and protocols. Miller showed that hyperimmune serum from rats given one or two challenge infections was more effective than immune serum (IS) i.e. from rats given one immunizing infection, and the former could be as effective in causing the early expulsion of worms as immune thoracic

duct lymphocytes (ITDL) (Nawa and Miller, 1978). The effect of serum was found to be dose dependant, i.e. the more he gave the greater the number of worms expelled, and also serum given on day 0 of an infection was more effective than serum given on day 4 p.i., or, the same amount divided into four doses given between days 4 and 7 p.i.

Other models where the transfer of passive protection with immune serum has met with some success include Ancylostoma caninum in dogs (Miller, 1967), Obeliscoides cuniculi in rabbits (Sollod and Allen, 1971), T. muris in mice (Selby and Wakelin, 1973) and N. dubius in mice (Behnke and Parish, 1979).

Even with the successful transfer of protection with IS, the role of antibody in the expulsion of intestinal parasites remains controversial.

In the rat - N. brasiliensis system it was originally thought that worm expulsion occurred because of a local anaphylactic reaction damaging the intestinal mucosa and thus inducing a 'leak lesion' in the intestine, allowing anti-worm antibody to pass into the lumen and attack the worms resulting in their expulsion (Mulligan et al,1965; Urquhart et al, 1965; Barth et al, 1966).

Jones and Ogilvie (1971) disagreed with this, suggesting that antibody first damaged the worms, making them susceptible to expulsion (Ogilvie and Hockley, 1968; Ogilvie and Love, 1974). Expulsion was then dependent on lymphocytes (Keller

and Keist, 1972) and also inflammatory processes could be involved (Jones and Ogilvie, 1971).

A similar diphasic model for parasite expulsion has been proposed for T. muris in mice (Wakelin, 1975; Wakelin and Selby, 1976) and T. spiralis in rats (Love et al, 1976).

Further evidence for increased permeability of the intestine during N. brasiliensis infections in the rat came from Nawa (1979). Although he failed to relate increased leakage of Evans blue into the gut with the rapid phase of worm expulsion or rise in mast cell numbers, he suggested that the increased leakage due to increased permeability of the small intestine was a function of the mechanical or toxic damage to the epithelial cells and/or the vascular bed caused by the parasites themselves and/or their metabolites.

It has been assumed that antibody activity is the direct cause of the cytopathalogical damage seen in N. brasiliensis (Ogilvie and Hockley, 1968; Lee, 1969) and T. spiralis (Love et al, 1976) before spontaneous cure, but there is no evidence that this is so (Wakelin, 1978).

Evidence against the importance of antibody damage comes from Nawa and Miller (1978) who showed that thoracic duct lymphocytes from immunized donors were capable of expelling 'normal' (i.e. worms not damaged by antibody) N. brasiliensis from rats. Jacobson and Reed (1976) also showed that athymic 'nude' mice could not damage worms and concluded that worm damage was also thymus dependant and Jacobson et al (1977)

found that mice lacking antibody production potential could still expel their worms. Finally the damage thought to be caused by antibody can also be induced by maintaining N. brasiliensis in vitro (Love et al, 1975).

In the present work the efficiency of 1^{o} immune serum (1^{o} IS) and 2^{o} immune serum (2^{o} IS) in transferring passive protection was examined. The protective effect of transferring cells and serum was investigated to determine whether together they gave a greater degree of protection than separately. Circulating antibody was measured using the urease conjugate enzyme-linked immunosorbent assay (ELISA), and serum IgG and IgM levels to exposed surface antigens of D. phoxini were measured during the course of both 1^{o} and 2^{o} infections by an indirect fluorescent antibody test (IFAT).

Materials and Methods

Animals

All mice used in these experiments were NIH strain. Maintenance and infection of mice, and recovery of parasites was as described in the general materials and methods.

Preparation of cell suspensions and cell transfer

Cell suspension and transfer were carried out as stated in the general materials and methods.

Preparation of serum

Blood was recovered from mice by cardiac puncture while under ether anaesthesia. Usually over 1.0 ml of blood could be recovered with a 1.0 ml plastic syringe and a 25 G 5/8" needle.

The blood was placed in conical centrifuge tubes and allowed to clot at room temperature for 2 hours. The clot was then separated from the side of the tube with a 21 G 1½" needle and left in a refrigerator overnight to allow contraction.

The following morning the tubes were spun at 80 g for 10 minutes in a Chillspin centrifuge. The serum was decanted into a clean tube and spun for 0.5 hours at 750 g to remove any remaining cells.

After centrifugation the serum was aliquoted and stored at -40°C.

Preparation of Antigen

A crude antigen preparation was prepared from 5 day old *D. phoxini*. Adults were recovered from mice and washed five times in PBS. The worms were homogenized in a glass tissue homogenizer in PBS and the resulting homogenate was allowed to extract overnight at 4°C.

The homogenate was then spun at 80 g for 5 minutes to remove coarse particulate matter and then spun for a further 30 minutes at 750 g to remove finer particulate matter in an MSE Chillspin centrifuge.

The protein content of the supernatant was determined and adjusted with PBS, usually to 1.0 mg/ml, before aliquoting and storing at -40°C.

Urease-antibody conjugate enzyme-linked immunosorbent assay (ELISA)

The urease conjugate ELISA technique was developed for the rapid screening of specific antibody in the culture supernatant used for growing hybridoma cell lines.

The assay works on the principal of a pH shift in the substrate solution giving rise to a highly visible colour change from yellow through grey-blue to purple in the presence of a positive result.

The urease substrate solution consists of urea in a solution of the pH indicator bromocresol purple. In the presence of the enzyme urease the urea is hydrolysed to liberate ammonia which causes a rise in pH, causing the colour change.

20 µl of blood was collected from the tail of each mouse using a non heparinised capillary tube. The tubes were sealed at one end by heating and the blood was allowed to clot for 4 hours at room temperature. After clotting, the capillary tubes were spun in a microhaematocrit centrifuge for 5 minutes to separate the serum. The serum was recovered and stored at -40°C until required.

Flat bottomed microelisa plates (Dynatech) were used in the assay. The wells were coated with 50 µl of D. phoxini antigen at an appropriate protein concentration, diluted in coating buffer (see General Materials and Methods). The plates were sealed with clear adhesive tape and incubated at 37°C for 2 hours.

After incubation the solution was aspirated from the solid phase and the plates were washed three times with wash buffer (see General Materials and Methods). 50 µl of diluting buffer (see General Materials and Methods) were added to all wells except the first vertical row. 50 µl of serum at a predetermined dilution were added to the first two vertical rows of wells and a doubling dilution prepared along the plates. The plates were re-sealed and incubated at 37°C for 30 minutes.

Post incubation, the solutions were aspirated and the plates washed three times as above. 50 µl of a 1 : 100 CSL urease conjugated rabbit anti-mouse IgG immunoglobulin (Sera-lab) in diluting buffer was added to all wells, the plates re-sealed and incubated at 37°C for 30 minutes.

After incubation, the washing procedure was repeated with a further three washes in distilled water. 50 µl of CSL urease substrate solution was added to each well, and the plates were re-sealed and incubated at 37°C. After 1 hour the plates were read visually and the titration end point was taken as the last well to turn purple.

To determine the concentration of antigen and serum to use in the experiments to eliminate non specific reactions, two concentrations of antigen and two dilutions of seara were first tested using the above technique. Antigen was tested at concentrations of 5 µg/ml and 4 µg/ml, and the serum doubling dilutions began at 1 : 10 or 1 : 100. Day 8 1° IS, day 4 2° IS and control serum (CS) were used from earlier experiments.

All wells in the 1 : 10 sample gave positive reactions indicating high non specific activity, and serum used at this concentration was useless. A good titration effect was observed with the 1 : 100 sample with end points observed in the 2° IS at both antigen concentrations and in the 1° IS at an antigen concentration of 5 µg/ml. No positive results were observed in the controls (Table 3.1). On the basis of these results it was decided to use an antigen concentration

of 4 µg/ml and a serum dilution commencing at 1 : 50.

Indirect Fluorescent Antibody Test (IFAT)

To determine the class of the antibody response directed against exposed surface antigens of living D. phoxini an IFAT was used.

Five - day - old D. phoxini were recovered from mice as normally and washed five times in HBSS. Five worms were placed in LP3 tubes (Plastic disposable tubes measuring 40 mm x 10 mm diameter), containing 0.5 ml of either 1^{o} IS or 2^{o} IS in HBSS in a range of doubling dilutions from 1 : 50 to 1 : 51,200 or CS at 1 : 50.

The worms were incubated for 1 hour on ice and then washed five times in HBSS at $4^{o}C$. They were then incubated for a further 30 minutes in 0.5 ml of either rabbit anti-mouse-immunoglobulin G, (RabāMIgG) conjugated to fluorescein isothiocyanate (FITC) (Sigma Chemical Company) or sheep anti-mouse-immunoglobulin M/FITC (ShāMIgM/FITC) (Miles) diluted 1 : 50 in HBSS. They were kept on ice throughout.

After incubation the worms were washed seven times in cold HBSS and then viewed under a Leitz Ortholux II microscope using a x25 water immersion lens with eyepieces of magnification x10 giving a total magnification of x250.

The titration end point was taken as the last tube where all worms had no detectable surface fluorescence.

Titration of conjugate

The conjugate dilution used was determined in a preliminary experiment where worms were incubated directly in conjugate for 30 minutes as described above. This was to determine the end point of non specific uptake of the conjugate. The conjugates were goat anti-mouse IgA/FITC (GāMIgA/FITC) (Cappell Laboratories), RabāMIgG (Sigma Chemical Company) and ShāMIgM (Miles), both conjugated to FITC. The conjugate dilutions were 1 : 10, 1 : 20, 1 : 40, 1 : 50 and 1 : 100 in HBSS.

Because of the results obtained (Table 3.2) it was decided to use the conjugates at a dilution of 1 : 50. No non specific surface fluorescence occurred at this dilution.

Table 3.1

Titration end points of antigen and serum assay

Antigen concentration	Serum doubling dilutions beginning at:				
	1 : 10		1 : 100		
	CS	1° IS 2° IS	CS	1° IS	2° IS
5 µg/ml	All wells gave a		0	1 : 400	1 : 3,200
4 µg/ml	positive reaction		0	0	1 : 200

Table 3.2

Titration end points of non-specific uptake of fluorescein isothiocyanate conjugate by D. phoxini

Conjugate	End point
GāMIgA/FITC	1 : 50
RabāMIgG/FITC	1 : 50
ShāMIgM/FITC	1 : 40

Key:

GāMIgA/FITC : Goat anti-mouse-immunoglobulin A conjugate.

RabāMIgG/FITC : Rabbit anti-mouse-immunoglobulin G conjugate.

ShaMIgM/FITC : Sheep anti-mouse-immunoglobulin M conjugate.

Results

A preliminary experiment to transfer passive immunity

Thirty six, 10-12 - week - old, male, NIH were infected with 200 m and caged in groups of six. Eighteen mice were bled 8 days p.i. and their serum pooled to form the day 8 1^{o} IS pool. The remaining mice were challenged with 200 m 27 days p.i. and killed 4 days later. The serum was pooled forming the day 4 2^{o} IS pool.

Twenty one, 8 - week - old, male, NIH were caged in three groups of four (1^{o} IS recipients) and three groups of three (2^{o} IS recipients). The mice were given either 1^{o} IS, 2^{o} IS or HBSS i.p. at a dose of 0.5 ml on days -1, 0, 1, 2, 3 and 4 p.i. (Group A) or 1.0 ml on either day 2 (Group B) or day 4 p.i. (Group C).

All the recipients were infected with 200 m on day 0 and killed on day 5 p.i. for worm recoveries.

The results showed that day 8 1^{o} IS given between days -1 and 4 did reduce the worm burden slightly below the control levels although no test of significance could be done because of the low numbers of mice. Both groups B and C had virtually no difference between recipients of 1^{o} IS or HBSS (Table 3.3).

The recipients of 2^{o} IS had much lower worm burdens than the recipients of 1^{o} IS. However one of the two control mice in each group also had a low worm burden, further complicating the result (Table 3.3).

The evidence suggests that 2^{o} IS may be able to confer protection and is worthy of further investigation.

<u>The ability of day 4 2^{o} IS to transfer passive immunity</u>

Forty five, female, 10-12 - week - old, NIH were infected with 20 m and caged in groups of five. Twenty seven days p.i. they were all challenged with 200 m and bled for serum 4 days later. Serum recovered from groups of three mice was pooled, giving 15 pools.

Fourteen, 8 - week - old, female, NIH were caged in two groups of four (HBSS), and two groups of three (2^{o} IS) and were given serum or HBSS i.p. at a dose of 0.5 ml on days -1, 0, 1, 2, 3 and 4 p.i. (Group A) or 1.0 ml on day 4 p.i. (Group B).

All the recipients were infected with 200 m on day 0 and killed 5 days later.

The results (Table 3.4) show a 20% difference between means of groups given 2^{o} IS and their controls.

<u>An experiment to investigate the combined effect of transferring IMLNC and 2^{o} IS</u>

Day 4 2^{o} IS was prepared as in the previous experiment except that the mice were challenged with 50 m.

The serum was stored at $-40^{o}C$ until required.

Eighteen, female, 10 - week - old NIH were infected with 50 m and caged in groups of six. They were killed 4 days p.i., their MLN removed and cell suspensions prepared as normally.

Sixteen, 8 - week - old, female, NIH caged in groups of four served as recipients. Group A were given 0.5 ml of HBSS on day -9 and 1.0 ml of HBSS on days 2 and 4 p.i. They were the controls. Group B were given 4×10^7 IMLNC i.p. on day -9, group C were given 1.0 ml of 2^o IS i.p. on days 2 and 4 p.i. and finally group D were given 4×10^7 IMLNC i.p. on day -9 and 1.0 ml of 2^o IS on days 2 and 4 p.i.

All recipients were challenged with 200 m on day 0 and killed on day 5 p.i.

The results (Table 3.5; Fig 3.1) show that IMLNC were far more efficient than 2^o IS in expelling the worms, although the latter did significantly reduce the worm burden. As the IMLNC alone were so effective, reducing the worm burden by over 60%, no combined effect could be measured by giving both cells and serum.

With the ability of 2^o IS to transfer significant degrees of protection shown, the next experiment was designed to determine when the antibodies, which it is assumed were the important protective component, appeared in the serum and to quantify them.

The measurement of circulating antibody in response to a 1^{o} or 2^{o} infection of D. phoxini by ELISA

Serum antibody titres were measured using an urease conjugate ELISA.

Twenty eight, 8 - week - old, male, NIH were caged in four groups of five (experimental) and two groups of four mice (control). Each mouse was individually numbered by ear piercing.

Ten experimental mice were infected with 20 m on day -27 and challenged with 50 m on day 0. The remaining 10 experimental mice were also infected with 50 m on day 0. The controls were left uninfected.

All the mice were bled from the tip of the tail on day 2 p.i. and every two days until day 14 p.i. and finally on day 24 p.i. The ELISA was carried out as stated in the materials and methods with an antigen concentration of 4 µg/ml and a serum doubling dilution range of 1 : 50 to 1 : 102,400.

The response of each individual mouse was followed throughout the experiment and the results are expressed as a mean $\log_2$ titre of the experimental and control groups.

In response to the 1^{o} infection an antibody titre was first detected on day 4 p.i. ($\log_2$ 3.0) and this increased rapidly until after day 6 when antibody levels reached a

plateau. Levels continued to increase slightly until day 12 p.i. (log_2 9.3) but no distinct peak was detected (Table 3.6; Fig 3.2). In response to the 2^o infection titres were observed much more quickly, being present on day 2 (log_2 6.7). The titres increased rapidly, reaching higher values than during the 1^o infection. The highest titres were observed on day 8 (log_2 15.2) but the titres remained fairly stable at least until day 14. The titre then declined to day 24 (log_2 8.9) at the termination of the experiment (Table 3.6; Fig 3.2).

Afer quantifying the circulating antibody response a further experiment was done to attempt to identify the antibody class involved in the response.

<u>The measurement of circulating antibody titres by IFAT and investigation of the class involved during a 1^o and 2^o infection of *D. phoxini*</u>

In this experiment circulating IgG and IgM titres were measured by IFAT. These are the predominant circulating antibodies involved in passive protection and therefore IgA was not looked for.

Serum left over from the previous experiment was used and pooled within the groups. The response was measured on days 2, 6, 8, 12, 14 and 24 p.i. corresponding with the days of highest and lowest titres in the previous experiment.

Ten, female, 8 - week - old, NIH were infected with

100 m each and caged in groups of five on day 0. A further 10 mice were infected on day 1. All the mice were killed 5 days after they were infected and the assay carried out.

No specific IgM could be demonstrated in 1^{o} IS until day 12 p.i. at a titre of 1 : 200. This was maintained until day 14 p.i. but had disappeared by day 24 p.i. In response to a 2^{o} infection, IgM titres appeared earlier, by day 6 (titre 1 : 800) and remained at similar levels until day 14, after which the titre declined.

Specific IgG appeared earlier than IgM after a 1^{o} infection with a titre of 1 : 200 on day 6 p.i. This increased until day 14 (titre 1 : 6,400) and then declined. IgG was recorded earlier in response to the 2^{o} infection, a titre of 1 : 400 was observed on day 2 and this increased to a plateau by day 6 p.i. (titre 1 : 25,600) although a titre of 1 : 51,200 was observed on day 12 p.i. (Table 3.7). The same data in $\log_2$ scale is shown in Fig 3.3.

Worms incubated in CS did not show fluorescence throughout the experiment.

Table 3.3

The effect of transferring primary or secondary immune serum on the course of a primary infection of 200 metacercariae of D. phoxini

Group	% worm recovery on day 5 p.i.			
	1° IS	HBSS	2° IS	HBSS
A				
(0.5 ml : Days	72	82.5	48	80.5
-1, 0, 1, 2, 3 and 4)	66.5	80		55
B				
(1.0 ml : Day 2)	61.5	76.5	54.5	80
	67	70.5		56
C				
(1.0 ml : Day 4)	80	80	41.5	39
	77	80		70

(n) = 1

Key:

1° IS = Primary immune serum (day 8).

2° IS = Secondary immune serum (day 4).

HBSS = Modified Hanks balanced salt solution.

Donors were immunized by either a primary infection of 200 metacercariae or both a primary and secondary infection of 200 metacercariae.

Table 3.4

The effect of transferring day 4 secondary immune serum on the course of a primary infection of 200 metacercariae of D. phoxini

Group	Control : HBSS		2° IS	
	% worm recovery	$\bar{x} \pm$ s.d.	% worm recovery	$\bar{x} \pm$ s.d.
A (0.5 ml : Days -1, 0, 1, 2, 3 and 4)	84 80.5 75 80.5	80 ± 4[a]	72 42.5 61.5	59 ± 15[b]
B (1.0 ml : Day 4)	75.5 90 85 80	83 ± 6[c]	74.5 50.5 63	63 ± 12[d]

Key:

2° IS = Secondary immune serum.

HBSS = Modified Hanks balanced salt solution.

Donors were immunized with a primary infection of 20 metacercariae followed by a challenge of 200 metacercariae.

Means significantly different between groups:

a v b $P < 0.05$

c v d $P < 0.05$

Table 3.5

The combined effect of transferring 4×10^7 immune mesenteric lymph node cells and secondary immune serum on the course of a primary infection of 200 metacercariae of D. phoxini

Key:

2^{o} IS = Secondary immune serum.

IMLNC = Immune mesenteric lymph node cells.

HBSS = Modified Hanks balanced salt solution.

Serum donors were immunized with a primary infection of 20 metacercariae followed by a secondary infection of 50 metacercariae.

Cell donors were immunized with a single infection of 50 metacercariae.

Means significantly different between groups:

A	v B	$P < 0.001$	A	v D	$P < 0.001$
A	v C	$P < 0.05$	B	v C	$P < 0.001$
			B	v D	not significant

Group	% worm recovery on day 5	$\bar{x} \pm$ s.d.
A (0.5 ml HBSS : Day -9 1.0 ml HBSS : Days 2 and 4 p.i.)	70 70 70 72	71 ± 1
B (4 x 10^7 IMLNC : Day -9)	0 11.5 8 0	5 ± 6
C (1.0 ml 2^o IS : Days 2 and 4 p.i.)	39 55.5 62.5 0*	52 ± 12 (n) = 3 * not included
D (4 x 10^7 IMLNC : Day -9 1.0 ml 2^o IS : Days 2 and 4 p.i.)	0 14 10.5 24.5	12 ± 10

Table 3.6

Mean ± s.d. (log_2) of circulating antibody titre of mice infected with a primary or secondary infection of 50 metacercariae of D. phoxini compared with non-infected controls as measured by an enzyme-linked immunosorbent assay

(n) = 8 in control serum group.

(n) = 10 in both primary and secondary immune serum groups.

Significance of difference between means of groups:

a v b = P < 0.001

c v d = P < 0.02 c v e = P < 0.001 d v e = P < 0.001

f v g = P < 0.001 f v h = P < 0.001 g v h = P < 0.001

i v j = P < 0.001 i v k = P < 0.001 j v k = P < 0.001

l v m = P < 0.001 l v n = P < 0.001 m v n = P < 0.001

o v p = P < 0.001 o v q = P < 0.001 p v q = P < 0.001

r v s = P < 0.001 r v t = P < 0.001 s v t = P < 0.001

u v v = P < 0.001 u v w = P < 0.001 v v w = P < 0.001

Day p.i.	Control Serum	Primary Immune Serum	Secondary Immune Serum
2	0^{a}	0	6.74 ± 0.56^{b}
4	0^{c}	3.02 ± 3.2^{d}	11.54 ± 0.87^{e}
6	0^{f}	7.5 ± 1.1^{g}	14.04 ± 1.1^{h}
8	0^{i}	8.24 ± 0.8^{j}	15.24 ± 0.8^{k}
10	0^{l}	8.94 ± 1.4^{m}	13.44 ± 0.4^{n}
12	0^{o}	9.34 ± 0.9^{p}	14.24 ± 0.6^{q}
14	0^{r}	8.84 ± 1.4^{s}	13.24 ± 0.5^{t}
24	0^{u}	4.6 ± 2.4^{v}	8.94 ± 0.9^{w}

Table 3.7

Titration end point of circulating IgG and IgM during both primary and secondary infections of 50 metacercariae of D. phoxini as measured by an indirect fluorescent antibody test

Key:

CS = Control serum.

1° IS = Primary immune serum.

2° IS = Secondary immune serum.

	IgG			IgM		
Day p.i.	CS	1° IS	2° IS	CS	1° IS	2° IS
2	0	0	1 : 400	0	0	0
6	0	1 : 200	1 : 25,600	0	0	1 : 800
8	0	1 : 200	1 : 25,600	0	0	1 : 400
12	0	1 : 3,200	1 : 51,200	0	1 : 200	1 : 800
14	0	1 : 6,400	1 : 25,600	0	1 : 200	1 : 1,600
24	0	1 : 1,600	1 : 12,800	0	0	1 : 50

Figure 3.1

The effect of transferring 4×10^7 immune mesenteric lymph node cells, secondary immune serum or combined cell and serum on a primary infection of 200 metacercariae in NIH mice

Key:

++++ ++++ ++++ $\bar{x} \pm$ s.d.

0 no worms recovered

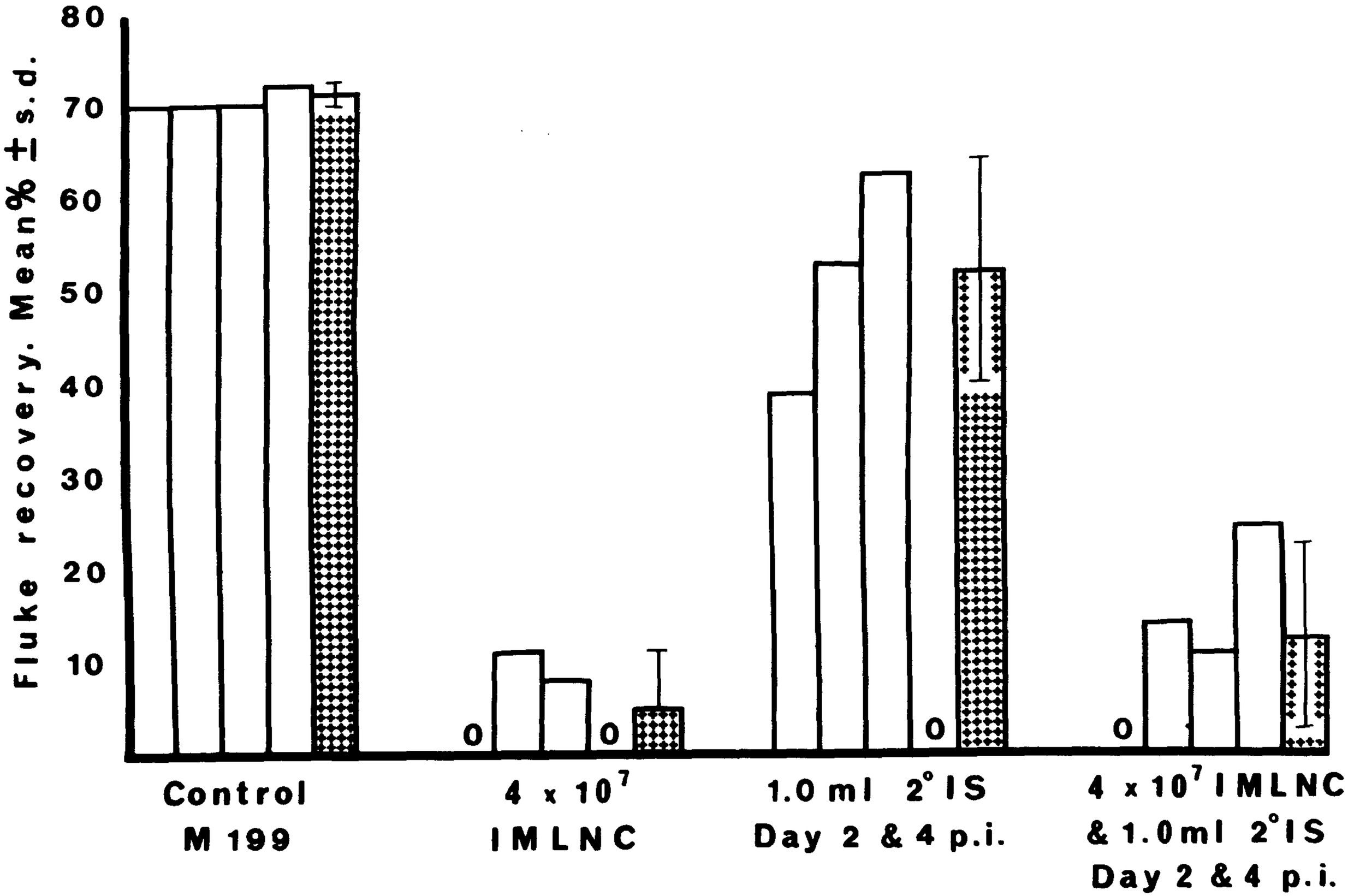

Fluke recovery. Mean% ± s.d.
80
70
60
50
40
30
20
10
0
0
0
0
Control
M 199
4 x 10^7
IMLNC
1.0 ml 2° IS
Day 2 & 4 p.i.
4 x 10^7 IMLNC
& 1.0 ml 2° IS
Day 2 & 4 p.i.

Figure 3.2

The circulating antibody response in NIH mice during a primary and secondary infection of 50 metacercariae of *D. phoxini* as measured by an enzyme-linked immunosorbent assay

Key:

◆ Secondary infection

◇ Primary infection

Control group not shown on graph = 0

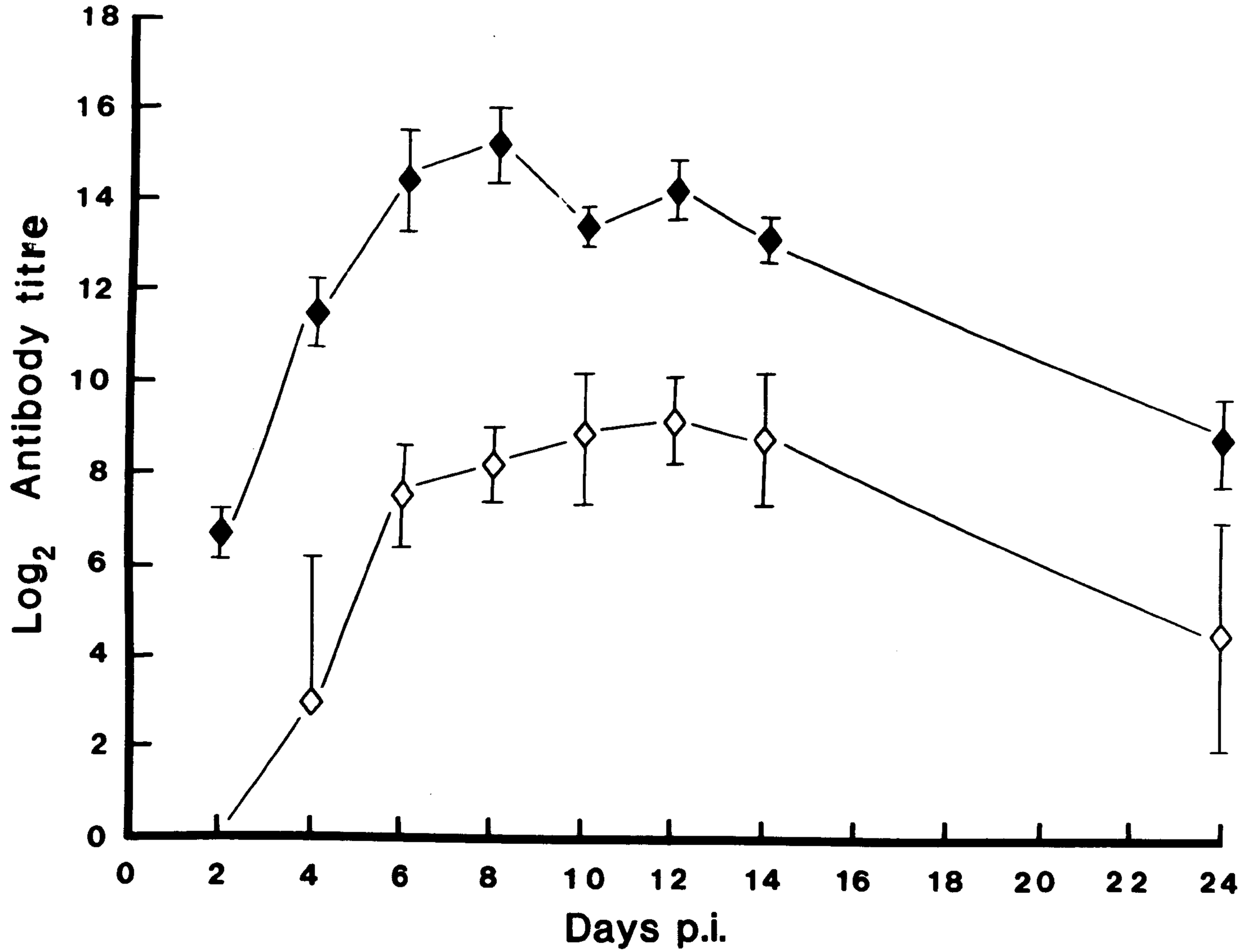

18
16
14
12
10
8
6
4
2
0
Log2 Antibody titre
0
2
4
6
8
10
12
14
16
18
20
22
24
Days p.i.

Figure 3.3

Circulating IgG and IgM levels during a primary and secondary infection of 50 metacercariae of D. phoxini as measured by an indirect fluorescent antibody test

Key:

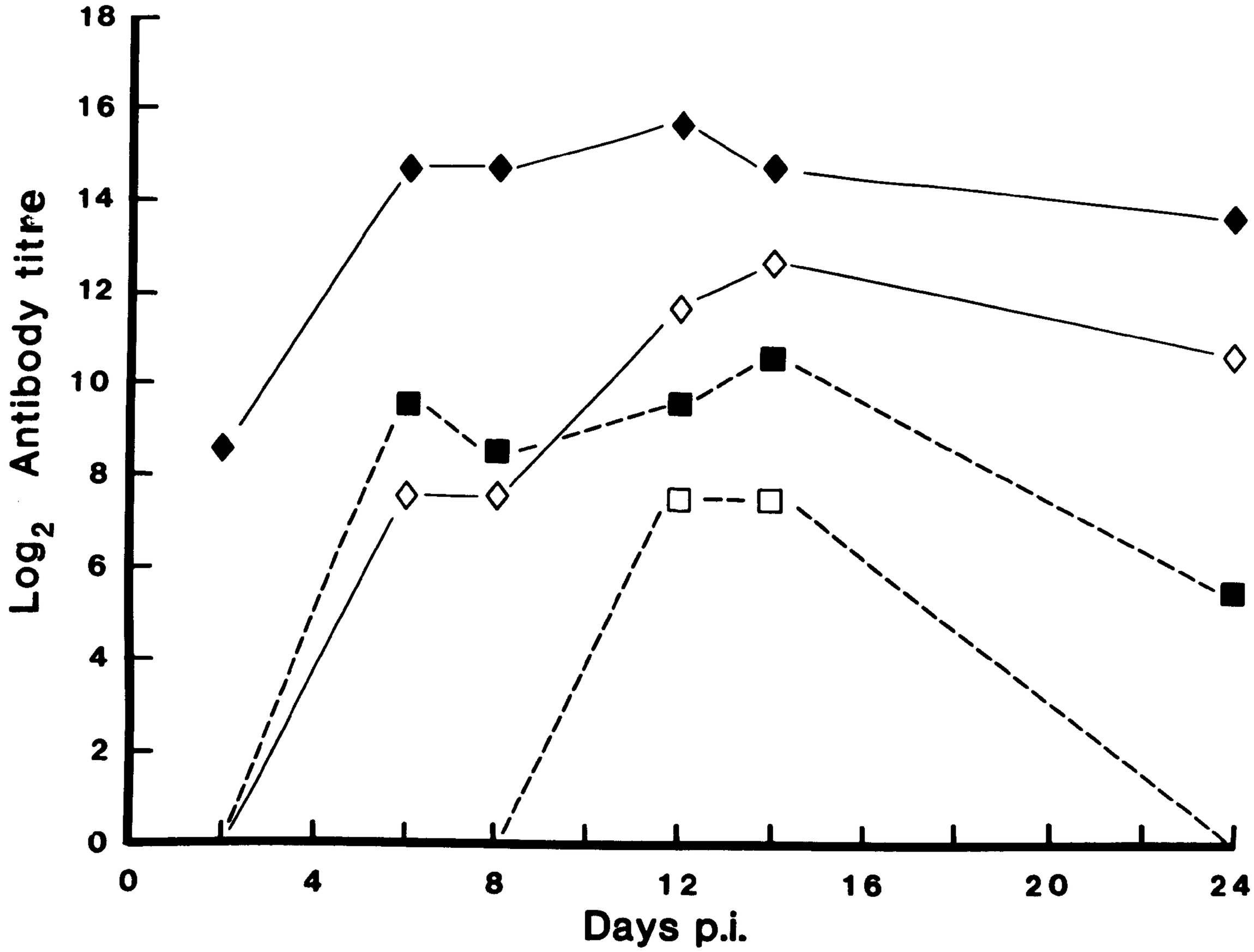
◆ IgG Secondary infection

◇ IgG Primary infection

■ IgM Secondary infection

□ IgM Primary infection

Controls not shown on graph = 0


18
16
14
12
10
8
6
4
2
0
Log2 Antibody titre
0
4
8
12
16
20
24
Days p.i.

Discussion

Day 4 p.i. 2^{o} IS was found to passively transfer a significant degree of protection, resulting in lower worm burdens in recipient mice on day 5 p.i. (Tables 3.4 and 3.5). The efficiency of the serum was not affected by either the volume administered or time of administration, (Tables 3.3 and 3.4). This is in contrast to the work of Miller (1980) who did show that in the N. brasiliensis - rat system, the effect of serum transfer was dose dependant, i.e. the greater the volume he gave, the greater the number of worms expelled. Also Miller showed that the time he gave the serum was important. Serum given on day 0 of an infection was more effective than serum given on day 4 p.i. or the same volume divided into four doses given between days 4 and 7 p.i. This probably reflects that the transferred serum may have been more potent against the developing larvae than against the adults which establish in the intestine by day 4 p.i. Also 'normal worms' transferred into passively protected rats were expelled faster than in controls, which is evidence that the serum was effective against lumen stages of the parasite.

The inability of day 8 1^{o} IS to significantly accelerate expulsion in the D. phoxini model confirms Mawdsley's observations (1983), that day 8 1^{o} IS is ineffective.

In models where 1^{o} IS has successfully transferred immunity, as measured by accelerated expulsion, the serum has usually been recovered late in the infection as in T. muris in mice (Wakelin, 1975) who used day 21 serum, or even after

the parasite has been expelled as demonstrated by Wakelin and Lloyd (1976), who showed that day 21 1^{o} IS would significantly reduce T. spiralis infections in mice.

Ogilvie and Jones (1968) and Miller (1980) successfully accelerated expulsion of N. brasiliensis from rats with 1^{o} IS. Miller (1980) observed that the efficiency of the 1^{o} IS increased with time p.i., the most efficient sera being taken between days 10 and 12 p.i. This probably reflects the time taken to produce enough specific antibody.

The fact that day 8 1^{o} IS failed to accelerate rejection in the D. phoxini model does not necessarily mean that 1^{o} IS cannot transfer immunity, the result may simply be due to a quantitative reason, that not enough antibody was present on day 8 and if serum would have been taken later in the infection, when antibody levels were higher (Table 3.6; Fig 3.2), then 1^{o} IS may have had some success in accelerating expulsion.

That serum antibody levels were low on day 8 of a 1^{o} infection is bourne out in (Table 3.6; Fig 3.2). By day 10 p.i. the mean titre had almost doubled over the day 8 level and levels continued to increase until day 12 (Table 3.6; Fig 3.2). In retrospect, it might have been better to have taken the serum on either of these days, after worm expulsion had commenced and was almost complete, to see if 1^{o} IS was capable of accelerating expulsion.

Comparison of the 1^{o} IS circulating antibody titre with 2^{o} IS levels (Table 3.6; Fig 3.2) show that the latter are at

a far higher level with differences being statistically highly significant. Again in retrospect it would have been interesting to see if serum taken between days 8 and 12 p.i. after a 2^{o} infection would have stimulated greater protection than the day 4 2^{o} IS. Days 10 and 12 p.i. of the 2^{o} infection were the days that the highest antibody titres were recorded, being much higher than on day 4 (Table 3.6; Fig 3.2). Again peak levels occur after worm expulsion.

The pattern of the circulating antibody response to D. phoxini reflects a classical response with 2^{o} IS showing higher titres than the 1^{o} IS and remaining at higher levels for longer. Antibody levels also remain high, after the parasite has been expelled. This is a feature of other systems such as N. brasiliensis in rats (Sinski and Holmes, 1977) and Hyostrongylus rubidus in piglets (Smith and Herbert, 1976).

As measured by IFAT, the IgG response was much greater than the IgM response. Measurable IgG appeared earlier and reached higher titres, and remained higher for longer than IgM (Table 3.7; Fig 3.3).

Protective immunity has been associated with IgG in several models eg. N. dubius in mice (Williams and Behnke, 1983) who found protective antibodies in mouse serum only after multiple immunization and not after a 1^{o} infection. They associated this protection with the increase in IgG antibody. Di Conza (1969) observed that the protective capacity of whole serum of mice infected with Hymenolepis nana

was associated with the IgG fraction. Jones et al (1970) also suggested that passive protective immunity in the N. brasiliensis - rat model was associated mainly with IgG, but also showed that IgM and IgA contained some protective antibodies but these contributed little to protection when compared with the IgG fraction. In view of the large increase in IgG in the D. phoxini model, it is possible that passive protection is associated with the IgG class. Although there is no evidence of this, it does merit further investigation.

Passive protection with 1^{o} IS has also been demonstrated in the Taenia taeniaformis - mouse system and again protection was associated with IgG. No protection was associated with serum IgA or IgM (Musoke and Williams, 1975a). Lloyd and Soulsby (1978) confirmed that 1^{o} IS IgG could protect mice from challenge with T. taeniaformis, but also observed that colostrum IgA administered intraduodenally would protect the mice. IgG isolated from intestinal secretions or tissues did not transfer any protection. The role of intestinal immunoglobulins is the subject of the next chapter.

Although 2^{o} IS did reduce the worm burden in the D. phoxini system, it was not as efficient as IMLNC (Table 3.5; Fig 3.1). Behnke and Parish (1981) had similar results with N. dubius in mice. Both IS and IMLNC significantly reduced the worm burden in recipients, but IMLNC caused a slightly greater expulsion of worms, although the difference in worm burden between these groups was not statistically significant.

In other systems a comparison of the efficiency of IS and immune cells is not as clear. Miller (1980) managed to confer the same degree of resistance against 1000 13 N. brasiliensis in rats with hyperimmune serum as Nawa and Miller (1978) did with 1 x 10^8 day 10 ITDL. Both Love et al (1976) and Wakelin and Lloyd (1976) working with T. spiralis in rats and mice respectively found that the efficiency of IS or immune cells varied between experiments. This variability probably reflects the importance of the immunization protocol used as shown by Wakelin (1975). Selby and Wakelin (1973) observed that cells transferred immunity most consistently but the greatest degree of protection was achieved with serum in the T. muris - mouse model.

Most workers however do agree that there is a synergistic effect when IMLNC and IS are transferred together (Wakelin and Lloyd, 1976; Love et al, 1976; Behnke and Parish, 1981; Williams and Behnke, 1983). Love et al (1976) suggested an additive rather than a synergistic effect for the expulsion of T. spiralis by rats whereby the function of the cells was simply to produce antibody. However they did consider it likely that cells did contribute separately to the expulsion process.

In the current work no synergistic effect was recorded because the IMLNC were very efficient in reducing the worm burden (Table 3.5; Fig 3.1). In retrospect it might have been better to reduce the number of cells transferred.

In conclusion, although the transfer of some protection with day 4 2^o IS has been demonstrated, and a circulating

antibody response shown, the role of antibody in the expulsion of *D. phoxini* remains to be elucidated. If antibody has a direct effect against the worm, it is reasonable to assume that it would act against the tegument and therefore should be located there. The next chapter is concerned with investigating whether antibody does appear on the tegument of *D. phoxini*.

Summary

1) Day 4 2^{o} IS was found to be capable of significantly reducing the D. phoxini worm burden, but was not as efficient as IMLNC.

2) A circulating antibody response occurred in response to the infection. Antibody titres were first recorded on day 4 p.i. and these continued to increase reaching a plateau around day 10. After day 12 the titre began to decrease. In response to a 2^{o} infection, titres appeared sooner and reached higher levels than during the 1^{o} infection. This is a classical anamnestic response.

3) Most of this circulating antibody was composed of IgG with a small amount of IgM. IgA was not measured.

CHAPTER 4

AN INVESTIGATION OF THE PRESENCE OF ANTIBODY ON THE TEGUMENT OF D. PHOXINI

Introduction

In the previous chapter a large increase in circulating antibody was recorded during D. phoxini infection. Most of this antibody was of the IgG class.

There is much evidence that serum antibody levels do not correlate well with parasite expulsion. Serum antibody levels rise after expulsion has begun in N. brasiliensis in rats (Poulain et al, 1976; Sinski and Holmes, 1977; Jarrett and Bazin, 1977). In the first two of these papers, the authors showed that intestinal haemagglutinins (thought to be IgA) and local anti-worm IgA respectively showed better correlation with parasite expulsion than serum antibody.

As local antibody therefore appears to correlate better with expulsion, the work in this chapter was designed to look for immunoglobulin (Ig) and specific antibody on the tegument of D. phoxini.

If antibody is directly involved in the response against D. phoxini, then it is reasonable to assume that the antibody would attack the tegument of the worm and should be detectable there.

The most common Ig normally found in the gut lumen is IgA. Parrott (1976) states that 90% of plasma cells in the lamina propria of rats are actively producing IgA. There is ample evidence that IgA is actively secreted via epithelial cells (Brandtzaeg, 1973) into the intestinal lumen and also via hepatocytes into the bile (Hall and Andrew, 1980) and

reaching the intestine in the bile. This IgA is in the form of a 11S dimer conjugated to J (joining) chain which allows specific combination with secretory component (SC). It is this IgA_2JSC that is the major form of IgA in mucosal secretions (Befus and Podesta, 1976). IgM is similarily thought to be selectively secreted by the same system into the lumen, but in smaller quantities (Brandtzaeg, 1973; Allen et al, 1976).

IgG has been shown to pass into the lumen by intercellular passive leakage, as has IgA (Brandtzaeg and Baklien, 1972), but the former is present in less quantity than IgA. This is because less IgG is produced in the intestinal wall and IgG is highly susceptible to proteolysis in the lumen (Befus and Podesta, 1976).

In the presence of inflammation however large amounts of IgG can pass into the gut and this is derived from local increases in IgG producing cells (Befus and Podesta, 1976), and increased leakage from the serum due to enhanced vascular permeability.

As host immunoglobulins (Igs) have been demonstrated on the surface of Schistosoma mansoni (Kemp et al, 1978), and on the tegument of Hymenolepis diminuta and H. microstoma (Befus, 1977), experiments were done to look for the presence of Igs, and determine whether they were specific antibodies, on the tegument of D. phoxini.

This was done by incubating the worms in some general

antimetabolites and/or at various temperatures to reduce or prevent surface turnover. The tegument of D. phoxini has been shown to be cytoplasmic in nature and contains a variety of secretory bodies (Erasmus, 1969). It broadly resembles the tegument of Fasciola hepatica as described by Threadgold (1984), and this has been shown to be metabolically active and to turnover (Hanna, 1980a, 1980c). If Ig is present on the tegument in vivo, it is important to prevent surface turnover when the worm is recovered to retain the Ig on the tegument, at least long enough for it to be detected in vitro. Detection was done by direct fluorescence using FITC conjugated anti mouse Ig.

Due to problems with obtaining enough D. phoxini, the detection of tegumental Igs by direct fluorescence was applied to cestodes which were maintained in the laboratory. The cestodes used were H. diminuta and H. microstoma.

An investigation of the presence of Ig on the tegument of these worms has been published by Befus (1977). He demonstrated the presence of IgA, IgG_1, IgG_2 and IgM on the tegument of H. diminuta grown in mice. The Igs appeared sooner on worms recovered from mice given six cysticercoids, being present on day 9 p.i. as compared with day 12 p.i. on worms from two cysticercoids infection, although IgA was present by day 10 p.i. This reflects the earlier expulsion of the larger infections.

IgA was the most abundant Ig found on H. microstoma, being present on day 8 p.i. on the section of the worm in the

bile duct, but not on the section in the intestine. By day 12 p.i. IgA occurred on all worm sections. The other Igs were not detected until day 20 p.i.

Materials and Methods

Animals

All mice used in conjunction with D. phoxini were male NIH. Maintenance and infection of mice, and recovery of worms was as described in the general materials and methods.

H. microstoma were obtained from male C_3H mice which had long standing infections and were maintained as above.

Cysticercoids of H. diminuta were obtained from Tribolium confusum, by dissecting the beetle in HBSS. Female C_3H were used in these experiments and were infected by stomach intubation as described for D. phoxini.

Recovery of H. diminuta was by flushing the intestine with the antimetabolite solution or HBSS as described by Hopkins and Barr (1982). H. microstoma was recovered by placing the intestine and bile duct in a wax bottom dish, covering with antimetabolite solution or HBSS and then cutting longituidinally along the gut and bile duct. The scolex was tugged from the tissue with forceps.

Antimetabolites

Four general antimetabolites were used at the following molarities (Smith et al, 1981).

2,4-dinitrophenol	10 mM (2-4 DNP)
Sodium fluoride	5 mM (NaF)

Iodoacetamide	0.25 mM (I)
Sodium azide	8 mM (NaN_3)

They were made up in either HBSS or M199.

Direct fluorescence

D. phoxini were recovered in the normal manner except that the HBSS was replaced by M199 or antimetabolite solution. The worms were washed six times in the relevant medium at the relevant temperature, 2°C or 37°C.

After washing, five worms were placed in plastic disposable LP3 tubes containing the FITC conjugate. The following conjugates were used, goat anti-mouse-Ig/FITC (GāMIg/FITC, Nordic Immunological Laboratories), rabbit anti-mouse-IgG (whole molecule)/FITC (RabāMIgG/FITC, Sigma Chemical Company), goat anti-mouse-IgA (α chain specific)/FITC (GāMIgA/FITC, Dynatech), sheep anti-mouse-IgM (μ)/FITC (ShāMIgM/FITC, Miles), goat anti-mouse-C_3/FITC (IgG fraction) ($GāMC_3$/FITC, Cappel Laboratories) and goat anti-chicken-Ig/FITC (GāChIg/FITC, Nordic Immunological Laboratories).

The worms were incubated in conjugate for 30 minutes and then washed six times either in M199 or antimetabolite solution before being viewed as shown by the protocol in Table 4.1.

The cestodes were treated similarily, except that usually two worms were placed in each tube.

Viewing was as described in chapter 3. Photographs were taken on Kodak Professional Ektachrome, 200 ASA.

Scoring of fluorescence ranged from (-) which was no detectable fluorescence and excluding autofluorescence, through (+) to (++) and finally (+++) being the brightest. The scores should only be compared within an experiment and not between experiments, as brightness of fluorescence was compared between worms within an experiment and not to a particular standard.

Preparation of antiserum

Ten, male, 8 - week - old C_3H were infected with five cysticercoids of H. diminuta on day 0. They were then challenged on day 12 p.i. with a further five cysticercoids and their blood recovered 8 days later. The serum was prepared as described in chapter 3.

Chicken anti-Railletina cesticillus serum was prepared by infecting 5 days old chicks with 50 cysticercoids of R. cesticillus. The cysticercoids were administered in gelatine capsules. The blood was recovered 15 days p.i., by exposing the heart and severing the vessels leading to and from it. Serum was prepared as described in chapter 3.

Mouse anti-D. phoxini serum was raised as described in chapter 3.

Results

A preliminary experiment to determine the survival of flukes in antimetabolites and to determine whether there was any sign of Ig on the tegument

Five, male, 10 - week - old, NIH were infected with 50 m and killed 5 days p.i. for worm recovery. Treatment of the worms was as shown (Table 4.1). After recovery, the worms were only incubated on melting ice and not at 37°C. The conjugate used was GāMIg/FITC at a dilution of 1 : 50.

The results showed that some fluorescence occurred on the tegument of *D. phoxini* when incubated in NaF, I or 2-4 DNP but no fluorescence occurred on the flukes incubated in NaN_3 or M199 (Table 4.2).

The fluorescence occurred as a faint line surrounding the worms denoting that fluorescence occurs over the surface (Plate 4.1). This suggests that Ig was present on the tegument.

The flukes were not killed by the antimetabolites at the molarities used, although activity was reduced.

The experiment was repeated and the effect of temperature on conjugate binding was investigated.

The effect of antimetabolites and temperature on binding of conjugate to the tegument of *D. phoxini*

Six, male, 10 - week - old mice were infected with

50 m. The worms were recovered on day 5 p.i. and the treatment of the worms followed the protocol described in Table 4.1.

A further control group was added, composed of worms maintained at 37°C or 2°C in M199 throughout the period of the experiment to serve as autofluorescent controls. The conjugate was the same as in the previous experiment.

The experiment was repeated twice. In the final experiment, a further control was added where worms were incubated in day 4 2° IS for 1 hour after the first wash, they were then re-washed before being incubated in conjugate as shown in Table 4.1.

The results (Table 4.3) showed a variation in levels of fluorescence in worms incubated in antimetabolites. Fluorescence ranged from (-) to (++) but usually only a faint line of fluorescence surrounding the periphery of the worm could be seen. No one antimetabolite seemed to be better and no consistent temperature response was seen, although NaN_3 and NaF incubated flukes gave different responses in E2 and E1 respectively, with fluorescence being present at 37°C but absent at 2°C. In contrast, flukes incubated in 2-4 DNP in E2 showed brighter fluorescence when incubated at 2°C than at 37°C.

However in the control group (C2) incubated with conjugate only, a temperature response was observed. No surface fluorescence occurred when the flukes were incubated at 2°C, but fluoresence did occur at 37°C. Worms incubated in 2° IS before incubation in conjugate gave the brightest fluorescence (Plate 4.2).

Some autofluorescence was seen in the group (C1) kept in M199 throughout the experiment. This was restricted to the gut caeca and eggs (Plate 4.3).

The effect of I in E2 was not investigated because no worms were recovered.

As fluorescence did not occur in group C2 at 2°C, but did occur in most of the groups incubated in antimetabolites at the same temperature, it suggests that Ig was present on the worm surface.

The next experiment was designed to investigate the class of Ig found on the tegument using class specific conjugates.

An experiment to determine the presence of IgG, IgA and IgM on the tegument of D. phoxini

In this experiment flukes were incubated in either RabāMIgG, GāMIgA or ShāMIgM FITC conjugates. As the presence of Ig on the tegument was shown in the previous experiment, this experiment was also designed to provide evidence as to whether the Ig was specific anti-worm antibody.

This was done by incubating both day 1 and day 5 flukes in conjugate to see if the fluorescence was identical in both age groups.

Twelve, male, 8 - week - old, NIH were caged in two boxes of six mice. One cage of mice was infected with 100 m on day 0 and the other with 100 m on day 4. All mice were killed on day 5 p.i.

Worms were incubated at 2°C throughout the experiment, i.e. after the initial recovery at 37°C. No control group incubated in 2° IS was included, otherwise the experimental protocol was as shown in Table 4.1.

The results showed no fluorescence in the control groups incubated in conjugate, showing that no Ig was present on the surface and no adsorption of conjugate had occurred (Table 4.4) in either day 1 or day 5 worms.

Of the day 1 worms incubated in antimetabolites, all showed faint surface fluorescence when incubated in GāMIgA/ FITC, but no fluorescence occurred with the other two conjugates.

The day 5 flukes incubated in NaN_3 showed no fluorescence at all, but those incubated in the remaining antimetabolites, showed faint fluorescence with all conjugates except worms incubated in NaF with ShāMIgM/FITC which showed no fluorescence.

Fluorescence when present, did not cover the entire worm, and was very faint. It had a patchy distribtuion, being present in discrete clumps on the surface.

A further investigation of the effect of temperature on the binding of conjugate to the tegument of D. phoxini

This experiment was designed to investigate further the very interesting and unexpected observation that conjugate in the absence of antimetabolites, bound to the tegument of flukes incubated at 37°C but not at 2°C.

Five, male, 8 - week - old, NIH were infected with 100 m and killed 5 days p.i. The worms from two mice were recovered in 2-4 DNP in M199 and the worms in the remaining mice were recovered in M199 as normal.

The experimental protocol after recovery is shown in Table 4.5. Incubation was carried out at both 2°C and 37°C.

The rationale behind each group was that fluorescence on G1 worms could only be derived from autofluorescence and therefore serve as a control.

Fluorescence on G2 worms was expected to be similar to

the pattern observed by workers using different models, i.e. specific fluorescence occurring at low temperature but not at high temperature due to membrane turnover (Vetter and Klaver - Wesseling, 1978; Smith et al, 1981). However results of earlier experiments had indicated the reverse in the D. phoxini model with fluorescence occurring at 37°C but not at 2°C. One is therefore not certain what to expect.

Group 3 worms, maintained in antimetabolites throughout the experiment should show specific fluorescence at both temperatures because membrane turnover at 37°C would not be expected to occur and therefore any Ig on the tegument would be expected to remain.

Worms in G4 incubated at 2°C should show specific fluorescence because turnover would not be expected. A similar situation to the worms in G3 kept at 2°C. It is not certain what the result of worms in G4 incubated at 37°C should be. In accordance with the work of other people, fluorescence should not occur due to turnover during the 1° incubation (Table 4.5). However fluorescence could occur due to the same reason for the fluorescence observed in worms incubated in M199 at 37°C in previous experiments. The significance of recovering G4 worms in 2-4 DNP is that surface turnover during worm recovery should not occur, therefore any Ig on the surface should remain there, at least until the worms are incubated in M199 and conjugate when turnover would be expected to resume. In comparison it is probable that G2 worms, recovered from mice in M199, lose a significant quantity of their surface Ig during recovery, due to turnover, so that

none or an indetectable quantity remains when the worms are incubated in conjugate.

If fluorescence is observed on G4 worms incubated at 37°C it is possible that it could be specific or non specific. There is no way of differentiating.

Worms in G5, incubated in GāChIg/FITC should show no surface fluorescence at either temperature. The group serves as a control for non specific uptake of the conjugate.

Group 6 worms are the positive controls and should show the greatest amount of fluorescence at either temperature as observed in previous experiments when worms have been incubated in MāD.p. 2° IS.

Flukes in G7 should show no fluorescence. If flukes do fluoresce then this will be due to non specific uptake of the conjugate and possibly the chicken Ig.

The results are shown in Table 4.6. Some autofluorescence was observed in flukes in G1 but no surface fluorescence. Worms in G2 responded as in previous experiments showing surface fluorescence when incubated at 37°C but not at 2°C. Flukes in G3 also responded as predicted, showing surface fluorescence at both 37°C and 2°C when maintained in 2-4 DNP throughout the experiment.

The worms in G4 which were maintained at 2°C did show surface fluorescence as predicted but interestingly the worms

maintained at 37°C showed brighter fluorescence. The worms in G5 and G6 showed the pattern of fluorescence predicted, with worms incubated in MāD.p. 2° IS showing the brightest fluorescence. Again, worms kept at 37°C showed brighter fluorescence than those kept at 2°C in G6. No surface fluorescence was observed after incubating the worms in GaChIg/FITC (G5), but interestingly some fluorescence was observed in worms incubated in chicken serum, and then GaChIg/FITC at 37°C but not at 2°C (G7) (Table 4.6).

Interestingly, in four groups, G2, G4, G6, and G7, the flukes incubated at 37°C showed greater fluorescence than those incubated at 2°C which suggests that uptake of the conjugate, or in G6 and G7, Ig and conjugate, could also occur by a temperature dependant method resulting in the greater fluorescence observed.

An investigation of the rate of surface membrane turnover in D. phoxini

Evidence from the previous experiment has suggested that the membrane of D. phoxini may be active at 37°C, but not at 2°C when incubated in M199. This experiment was designed to provide data on the rate of membrane turnover.

Three, 8 - week - old, male, NIH were infected with 50 m and the worms recovered on day 5 p.i. The worms were recovered in M199, I or 2-4 DNP. The incubation protocol followed that of G6 (Table 4.5) for the worms incubated in M199.

The remaining flukes were maintained on antimetabolites throughout the experiment. The day 4 2^{o} IS was diluted 1 : 50 in antimetabolite solution or M199 depending on the group and the protocol follows Table 4.1 after incubation in 2^{o} IS for 1 hour. GāMIg/FITC at a dilution of 1 : 50 was the conjugate used. The flukes were maintained in the dark throughout the experiment.

Complete membrane turnover only occurred with the worms kept in M199 at $37^{o}C$. This took 5 hours, for all surface fluorescence to disappear (Table 4.7). Worms kept at $2^{o}C$ in M199 retained their fluorescence.

Iodoacetamide prevented loss of fluorescence at $37^{o}C$, but 2-4 DNP only appeared to slow down turnover, as some loss of fluorescence was observed at $37^{o}C$ (Table 4.7).

An investigation of the presence of C_3 on the tegument of D. phoxini

Six, male, 8 - week - old, NIH were infected with 50 m. The mice were killed on day 5 p.i. and the worms from two mice each were recovered in M199, NaN_3 or 2-4 DNP.

The protocol followed that in Table 4.1., but incubation was only carried out at $2^{o}C$. Five worms were incubated in LP3 tubes containing $GāMC_3$/FITC conjugate diluted in M199 or antimetabolites in the following range of dilutions: 1 : 10, 1 : 20, 1 : 40, 1 : 80, 1 : 100, 1 : 200 and 1 : 400.

Flukes incubated in conjugate and M199 or NaN_3 showed faint fluorescence up to a dilution of 1 : 40, but were negative after (Table 4.8). This was taken as the titration end point for non specific adsorption of the conjugate. Flukes incubated in 2-4 DNP showed fluorescence up to 1 : 100 and this is interpreted as indicating specific fluorescence for C_3.

An investigation of the presence of mouse Igs on the tegument of H. microstoma and H. diminuta

Eight, 8 - week - old, female C_3H were infected with six cysticercoids of H. diminuta. Eight days after infection the mice were killed for worm recovery. Forty days old H. microstoma were recovered at the same time.

Worms from four infected mice were recovered in HBSS and the worms from the other four were recovered in an antimetabolite solution prepared in HBSS as shown in Table 4.10.

This was repeated with the H. microstoma infected mice until enough worms were present for the experiment.

The worms were incubated in GaMIg/FITC following the protocol shown in Table 4.1., except M199 was substituted by HBSS. Two groups of H. diminuta were first incubated in 2° IS at 2°C or 37°C before being incubated in conjugate. The H. microstoma however were only incubated at 2°C.

The results are shown in Table 4.9 (E1) for H. microstoma and Table 4.10 (E1) for H. diminuta. No fluorescence occurred on any worms incubated in HBSS. The H. microstoma showed similar levels of fluorescence between all groups. Incubation in antimetabolites made no difference when compared with the group incubated in HBSS and conjugate.

In the H. diminuta groups, worms incubated in NaN_3 and NaF and conjugate did show more fluorescence than worms incubated in HBSS and conjugate at 2°C and at 37°C (Table 4.10 (E1)). No temperature effect was observed in this experiment as compared with D. phoxini.

Both experiments were repeated. H. microstoma were also incubated at 37°C in the repeat. The results (E2) (Tables 4.9 and 4.10) were similar to those seen in (E1). Some H. microstoma showed brighter fluorescence when incubated at 37°C than at 2°C when incubated in NaF or I, but no such increase was observed in the worms incubated in HBSS and conjugate at 37°C. This may represent a temperature response but should therefore have occurred in the worms incubated in HBSS and conjugate.

The H. diminuta showed little variation between groups in the level of fluorescence (Table 4.10 (E2)). As in E1, the worms incubated in 2° IS showed the brightest fluorescence. Again there was no difference in fluorescence between worms incubated in HBSS and conjugate at the two temperatures. Incubation in antimetabolites at both temperatures did not affect the fluorescence, nor did it increase fluorescence above

the worms incubated in HBSS and conjugate.

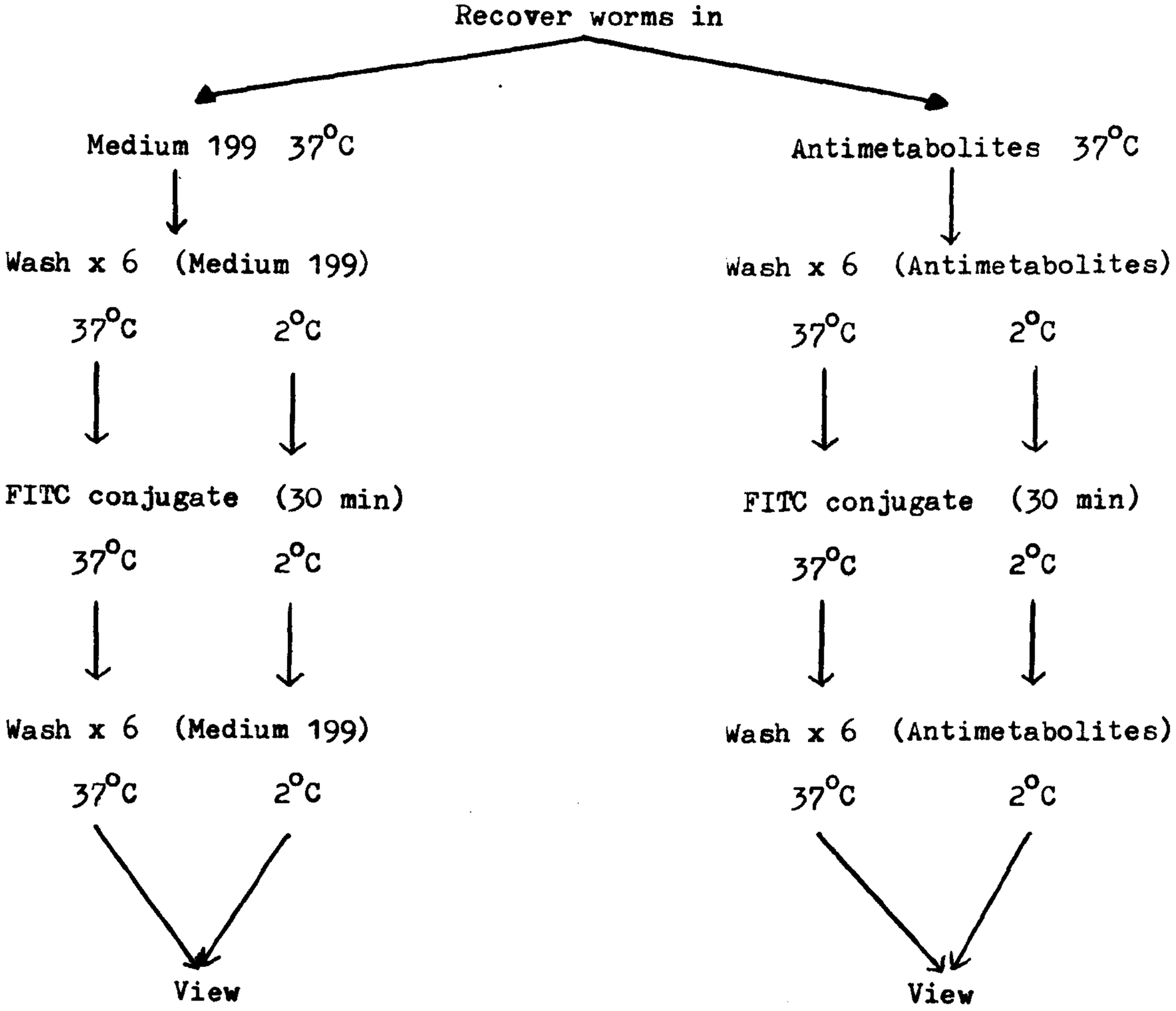
The H. diminuta section of the experiment was again repeated with the additional control for non specific uptake of conjugate, using worms incubated in GāChIg/FITC at 1 : 50 dilution v/v in HBSS.

The results were similar to the previous two experiments (Table 4.10 (E3)). No one antimetabolite seemed to be better than any other. Of importance is that worms incubated in the GāChIg/FITC did not show any fluorescence at either 2°C or 37°C.

This is interpreted as no non specific uptake of conjugate occurring, which suggests that the GāMIg/FITC binding is specific, due to the presence of surface Ig.

Table 4.1

Experimental Protocol



Worms kept at 2° were held over melting ice throughout incubation.

Table 4.2

Preliminary result of incubating worms in GāMIg/FITC at 2°C to determine the presence of immunoglobulin on the tegument of D. phoxini

Incubating Medium	Fluorescence
Medium 199 & conjugate	-
NaN_3 & conjugate	-
NaF & conjugate	+
Iodoacetamide & conjugate	+
2,4-dinitrophenol & conjugate	+

Table 4.3

The effect of temperature and antimetabolites on the presence of immunoglobulins on the tegument of D. phoxini

Key:

C	=	Control.
E_1, E_2, E_3	=	Repeated Experiments.
Conjugate	=	Goat anti-mouse-Ig/FITC.
n.d.	=	Not done.

Incubating medium		Fluorescence		
		E_1	E_2	E_3
C_1 Medium 199	2°C	-	-	-
	37°C	-	-	-
C_2 Medium 199 & conjugate	2°C	-	-	-
	37°C	+	+	+
C_3 Medium 199, 2° IS & conjugate	2°C	n.d.	n.d.	+++
	37°C	n.d.	n.d.	+++
NaN_3 & conjugate	2°C	++	-	+
	37°C	++	+	+
NaF & conjugate	2°C	-	+	+
	37°C	+	+	+
Iodoacetamide & conjugate	2°C	-	n.d.	+
	37°C	-	n.d.	+
2,4-dinitrophenol & conjugate	2°C	-	++	+
	37°C	-	+	+

Table 4.4

An investigation of the presence of IgG, IgA and IgM on the tegument of one day old and five days old D. phoxini

Key:

RabāMIgG/FITC = Rabbit anti-mouse-IgG.

ShāMIgM/FITC = Sheep anti-mouse-IgM.

GāMIgA/FITC = Goat anti-mouse-IgA.

Day 1 = One day old worms.

Day 5 = Five days old worms.

Incubating medium			Fluorescence Day 1	Fluorescence Day 5
Medium 199			-	-
Medium 199	&	RabāMIgG/FITC	-	-
		ShāMIgM/FITC	-	-
		GāMIgA/FITC	-	-
NaN_3	&	RabāMIgG/FITC	-	-
		ShāMIgM/FITC	-	-
		GāMIgA/FITC	+	-
NaF	&	RabāMIgG/FITC	-	+
		ShāMIgM/FITC	-	-
		GāMIgG/FITC	+	+
Iodoacetamide	&	RabāMIgG/FITC	-	+
		ShāMIgM/FITC	-	+
		GāMIgG/FITC	+	+
2,4-dinitrophenol	&	RabāMIgG/FITC	-	+
		ShāMIgM/FITC	-	+
		GāMIgA/FITC	+	+

Table 4.5

Experimental protocol for investigating the effect of temperature on the binding of conjugate to the tegument of *D. phoxini*

Key:

G1 - G7	=	Experimental groups.
M199	=	Medium 199.
2-4 DNP	=	2,4-dinitrophenol.
GāMIg/FITC	=	Goat anti-mouse-Ig/FITC.
GāChIg/FITC	=	Goat anti-chicken-Ig/FITC.
MāD.p. 2^{o} IS	=	Day 4 mouse anti-D. phoxini secondary immune serum.
ChāR.c. 1^{o} IS	=	Day 15 chicken anti-Railletina cesticillus primary immune serum.
n.d.	=	Not done.

Protocol	G1	G2	G3	G4	G5	G6	G7
Recovery Medium	M199	M199	2-4 DNP	2-4 DNP	M199	M199	M199
Wash	x 6 M199	x 6 M199	x 6 2-4 DNP	x 3 in 2-4 DNP x 3 in M 199	M199	M199	M199
Primary Incubation	M199	GāMIg/ FITC	GāMIg/ FITC	GāMIg/ FITC	GāChIg/ FITC	MāD.p. 2° IS	ChāR.c. 1° IS
Wash	x 6 M199	x 6 M199	x 6 2-4 DNP	x 6 M199	x 6 M199	x 6 M199	x 6 M199
Secondary Incubation	n.d.	n.d.	n.d.	n.d.	n.d.	GāMIg/ FITC	GāChIg/ FITC
Wash	n.d.	n.d.	n.d.	n.d.	n.d.	x 6 M199	x 6 M199

Table 4.6

The effect of temperature on binding of conjugate to the tegument of five days old D. phoxini

Group	Fluorescence	
	2°C	37°C
1	-	-
2	-	+
3	+	+
4	+	++
5	-	-
6	++	+++
7	-	+

The results are based on five D. phoxini in each group.

For key to groups, refer to Table 4.5.

Table 4.7

The effect of antimetabolites and temperature on the rate of surface membrane turnover in D. phoxini

Time (hours)	M199 (Control)		Iodoacetamide		2,4-dinitrophenol	
	2°C	37°C	2°C	37°C	2°C	37°C
0	++	++	++	++	++	++
0.5	++	+	++	++	++	+
1	++	+	++	++	++	+
2	++	+	++	++	++	+
5	++	-	++	++	++	+

Key:

M199 = Medium 199

Results are based on five worms in each group.

Table 4.8

An investigation of the presence of the C_3 component of complement on the tegument of D. phoxini

Conjugate dilution	Medium 199 & Conjugate	NaN_3 & Conjugate	2,4-dinitrophenol & Conjugate
1 : 10	+	+	+
1 : 20	+	+	+
1 : 40	+	+	+
1 : 80	-	-	+
1 : 100	-	-	+
1 : 200	-	-	-
1 : 400	-	-	-

Key:

Conjugate = Goat anti-mouse-C_3/FITC.

The results are based on five worms in each group.

Table 4.9

The effect of antimetabolites and temperature on the presence of immunoglobulins on the tegument of H. microstoma

Incubating medium	Fluorescence		
	E_1	E_2	
	2°C	2°C	37°C
HBSS	-	-	-
HBSS & conjugate	++	++	++
NaN_3 & conjugate	+++	++	++
NaF & conjugate	++	++	+++
Iodoacetamide & conjugate	++	+	++
2,4-dinitrophenol & conjugate	++	++	++

Key:

E_1 & E_2 = Experiment numbers.

HBSS = Modified Hanks balanced salt solution.

Conjugate = Goat anti-mouse-IgG/FITC.

Table 4.10

The effect of antimetabolites and temperature on the presence of immunoglobulin on the tegument of H. diminuta

Key:

$E_1 - E_3$ = Experiment numbers.

HBSS = Modified Hanks balanced salt solution.

2^o IS = Day 8 mouse anti-*H. diminuta* secondary immune serum.

GāChIg/FITC = Goat anti-chicken-Ig/FITC.

Incubating medium	Fluorescence					
	E_1		E_2		E_3	
	2°C	37°C	2°C	37°C	2°C	37°C
HBSS	-	-	-	-	-	-
HBSS & conjugate	+	+	+	+	+	+
HBSS, 2^o IS & conjugate	+++	+++	+++	+++	n.d.	n.d.
NaN_3 & conjugate	++	++	+	+	++	+
NaF & conjugate	++	++	++	+	+	+
Iodoacetamide & conjugate	+	+	+	+	+	+
2,4-dinitrophenol & conjugate	+	++	+	+	++	++
HBSS & GāChIg/FITC	n.d.	n.d.	n.d.	n.d.	-	-

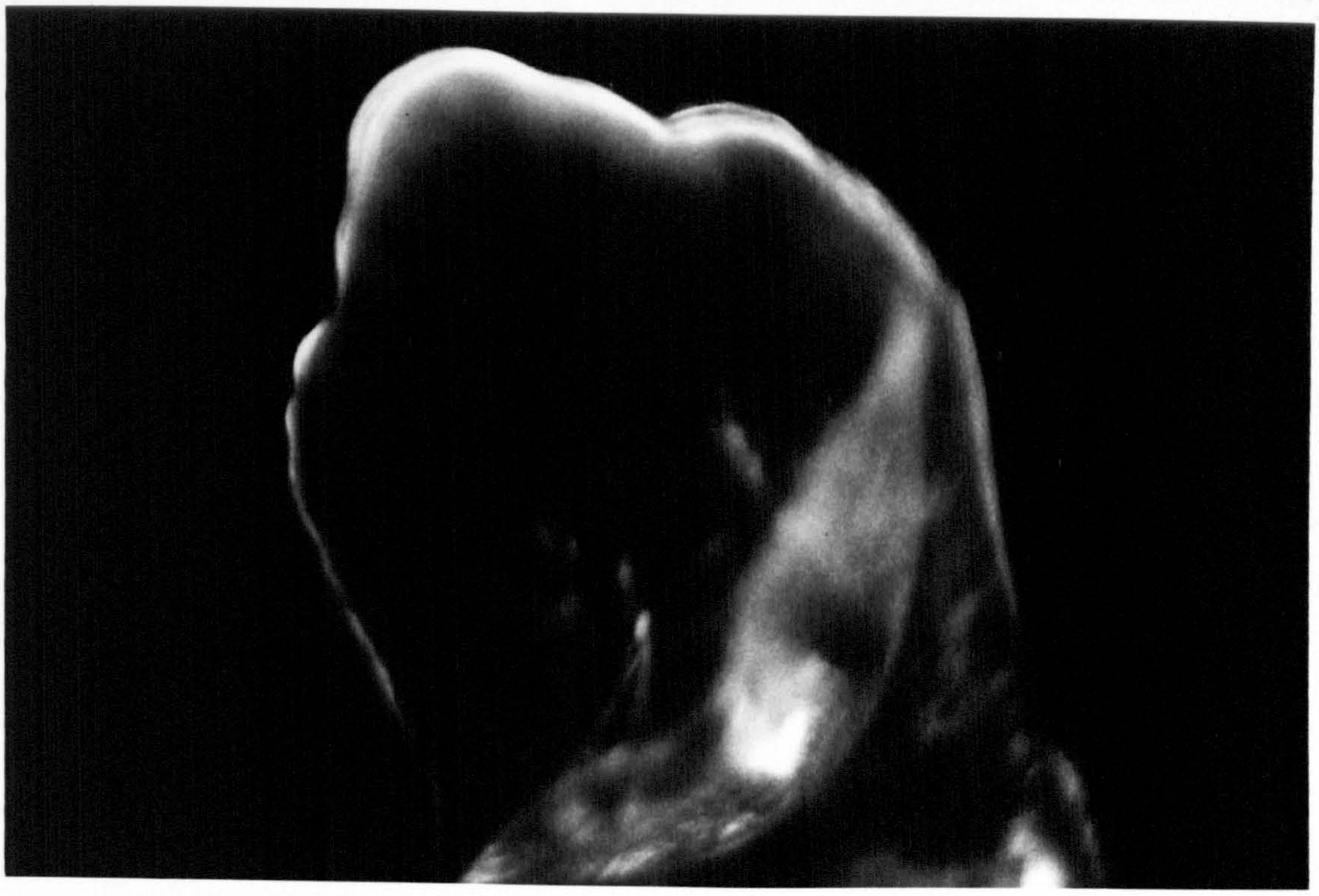
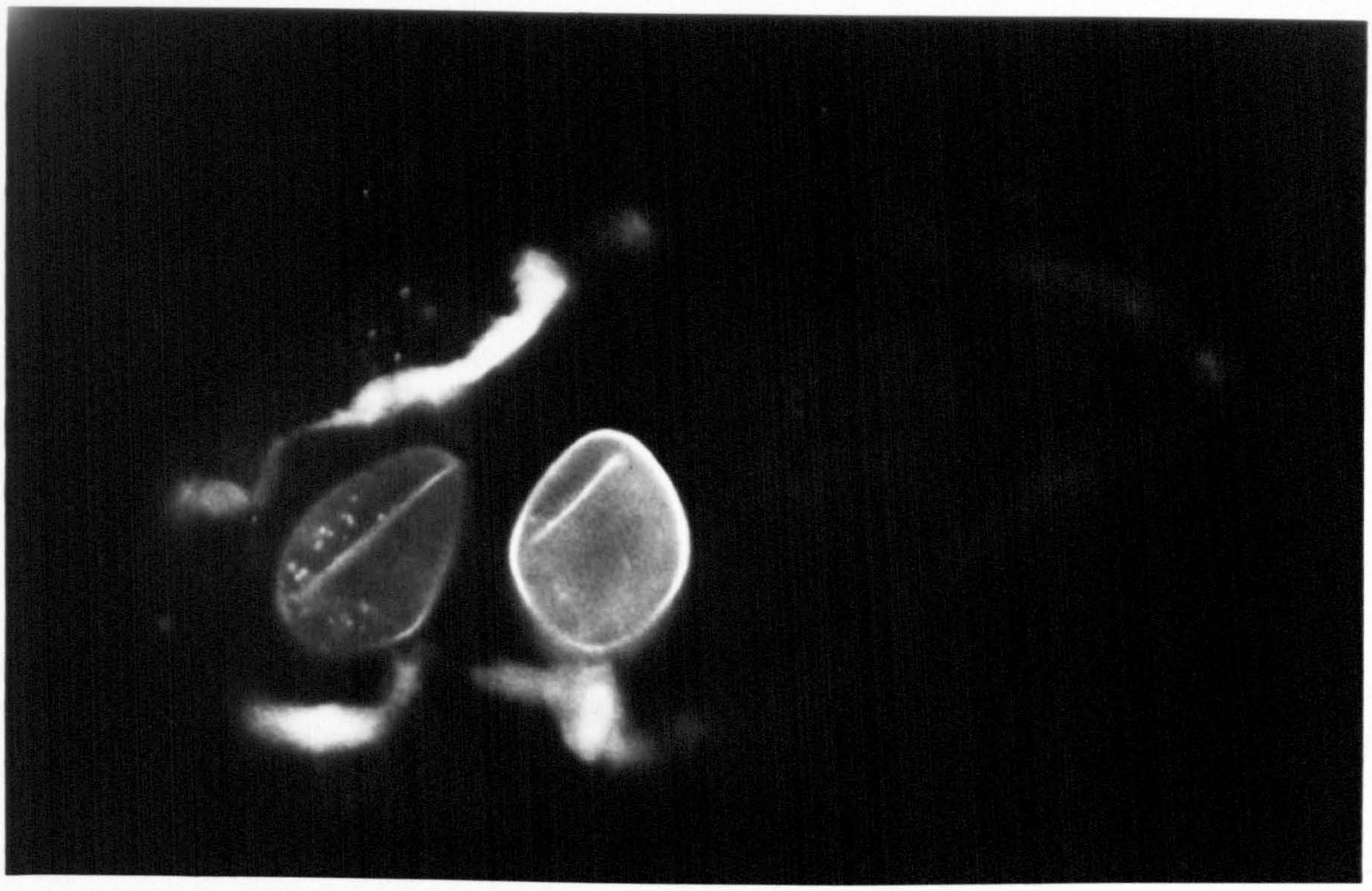


Plate 4.3

A living D. phoxini showing autofluorescence of eggs and gut caeca after incubation in medium 199 only at $2^{o}C$ (x 250)



Discussion

The evidence presented here suggests that specific anti-worm antibody is present on the tegument of D. phoxini, at least on day 5 p.i.

By incubating the worms at 2°C in antimetabolites, a faint line of fluorescence was consistently seen around the worm, denoting surface fluorescence. No fluorescence was visible on worms incubated in M199 and conjugate or in NaN_3 and conjugate (Table 4.2). In further experiments similar results were observed (Table 4.3). Flukes incubated in conjugate in the presence of antimetabolites usually showed surface fluorescence, although it was faint. It was not as bright as the positive controls, where flukes were incubated in day 4 2° IS, before being incubated in conjugate. These flukes showed bright overall fluorescence.

Temperature did not usually affect binding of the conjugate if the worms were incubated in antimetabolites. However on two occasions, flukes did show surface fluorescence at 37°C but not at 2°C in the presence of antimetabolites, suggesting that some membrane activity was occurring at 37°C. On one occasion flukes incubated at 2°C showed greater fluorescence than at 37°C. The results (Table 4.3) however do suggest that the antimetabolites inhibited surface turnover.

A temperature response was consistently seen with flukes incubated in M199 and conjugate (Tables 4.3 and 4.6). At 2°C the flukes showed no fluorescence, showing that no

conjugate was binding to the tegument, either specifically or non specifically. At 37°C, surface fluorescence did occur and this was consistently seen.

This is interpreted as non specific uptake of conjugate by the fluke at 37°C. The interpretation relies on the probability that any Ig on the tegument of the worm whilst in the gut, would have been removed by membrane turnover during the recovery incubation which was carried out in M199 at 37°C for 30 minutes. There is no real evidence of this, but measurements of the rate of membrane turnover in D. phoxini show that more fluorescence was lost in the first 30 minutes of incubation than in the next 90 minutes (Table 4.7). It is possible that during the recovery in M199, most or all of the surface Ig could be lost, so that wnen these worms were incubated in conjugate, no specific binding could occur, but at 37°C, membrane activity was maintained resulting in temperature dependant non specific uptake of the conjugate. This would also explain why some flukes incubated in antimetabolites and conjugate at 37°C occasionally had higher levels of fluorescence than those incubated at 2°C.

Further evidence that surface Ig may be lost during recovery of the worms in M199 comes from result of the worms in G4 (Table 4.6). These worms were recovered in 2-4 DNP, therefore Ig should remain on the tegument. On transferring the worms to M199 for incubation with conjugate, the worms incubated at 2°C did show fluorescence, showing the presence of Ig on the tegument. The worms incubated at 37°C showed greater fluorescence, possibly due to a combination of specific

binding and non specific uptake of conjugate, although loss of surface Ig and conjugate would also be expected to proceed.

According to this theory, the worms incubated in GāChIg/FITC should also have shown surface fluorescence at 37°C but they did not (Table 4.6 (G5)). This is an important control which suggests that the fluorescence observed in other groups could be specific. However, worms incubated in chicken serum before being incubated in GāChIg/FITC at 37°C did show fluorescence, but not when incubated at 2°C. The reason for this is possibly that chicken Ig was adsorbed on to the tegument allowing enough conjugate to bind, giving the positive result.

The exact nature of this non specific uptake of Ig is unknown. It does appear to be temperature dependant, and could therefore be absorbed on to the tegument, however there appears to be little evidence of macromolecular uptake by the trematode tegument in the literature although an absorptive function has been implicated for the strigeoid adhesive organ (Threadgold, 1984). To determine whether adsorption of the Fc portion of the conjugate molecule occurred, the use of $F(ab')_2$ conjugates, which lack the Fc portion may have provided the answer. They should certainly be used in any further investigation.

The non specific adsorption of heterologous molecules from the incubating medium has been shown in other trematode models, particularly with schistosomes. Clegg et al (1971) and Dean et al (1974) showed that schistosomula of S. mansoni

incubated *in vitro* would adsorb host blood group antigens on to their tegument. Kemp *et al* (1977) showed that adult *S. mansoni* possessed host Ig on their tegument, which were lost when the worms were incubated in Eagle's minimal essential medium, but if the worms were incubated in normal or immune mouse serum the worms would regain their surface Ig.

It is probable that adsorption of Ig occurs with *D. phoxini* in the absence of antimetabolites and at 37°C.

The results of better fluorescence at 37°C than at 2°C is at variance with similar work done on other parasites, such as the nematodes *Ancylostoma caninum* (Vetter and Klaver - Wesseling, 1978), and *Toxocara canis* (Smith *et al*, 1981). In these two models incubation of the parasite in IS and then conjugate, resulted in surface fluorescence at 0°C or 2°C but not at 20°C or 37°C.

Both workers concluded that the metabolically active state of the cuticle of the nematodes prevented the binding of antibody. This was due to the turnover of the molecules to which the antibodies bind (Smith *et al*, 1981).

The presence of non specific Ig on the tegument of *D. phoxini* is suggested by the appearance of IgA on the tegument of day 1 worms (Table 4.4) maintained in antimetabolites. It is highly improbable that specific IgA antibody would have been produced by day 1 of the infection. Whether the IgA found on the tegument of day 5 worms is specific IgA or partly composed of specific IgA cannot be determined. However it is

possible that specific IgA could have been produced by then. Andrew and Hall (1982) demonstrated the presence of specific agglutinins which were mainly IgA in the bile of rats 5 days after immunizing with killed Brucella abortus, so specific IgA can certainly be produced by day 5.

By day 5 p.i. both IgG and IgM were detected on the tegument of most of the D. phoxini when incubated in antimetabolites. As these Igs were not detectable on day 1, it suggests that these may be specific antibodies which are certainly present in the serum by day 5 p.i. (Fig 3.2). The antibodies could have gained access into the lumen by the increased permeability of the intestinal epithelium caused by the mechanical and chemical disruption of these cells caused by the worm as envisaged by Erasmus (1970b).

There is no direct evidence that these Igs are specific antibodies but the evidence tends to support this idea.

Specific C_3 fluorescence was also detected on the tegument of flukes when incubated in 2-4 DNP but not in NaN_3 (Table 4.8). The reason for this is unclear. Complement has also been demonstrated on the tegument of H. diminuta (Befus, 1977) and Tarleton and Kemp (1981) provided evidence for the presence of a C_3 receptor, but not of its origin, on the tegument of S. mansoni. They demonstrated this by showing that Ag-F(ab')$_2$-C complexes would bind to surface receptors whereas Ag-F(ab')$_2$ complexes could not. They suggested that the receptor could act by binding C_3b and inactivating it, preventing further amplification of the C system.

Even though the presence of C and/or C receptors has been demonstrated on the surface of several worms, there is no evidence to date, that C_3 bound to the tegument is relevant to host resistance (Befus and Bienenstock, 1982).

Investigation of the rate of turnover showed that flukes incubated in 2^{o} IS and conjugate shed their surface fluorescence into the incubating medium at $37^{o}C$. Incubation in antimetabolites or at $2^{o}C$ reduced or prevented the loss of fluorescence.

The time of complete turnover compares well with other systems. Kemp et al (1977) observed that complete loss of surface Ig from S. mansoni occurred in 2.5 hours after incubation in medium at $37^{o}C$. Smith et al (1981) showed that T. canis larvae would shed surface fluorescence completely after 3 hours incubation and Vetter and Klaver - Wesseling (1978) observed that A. caninum larvae would shed surface fluorescence completely after 3 hours incubation at $20^{o}C$ in PBS. The authors of both papers also showed that surface fluorescence would remain on the larvae if they were kept in antimetabolites at $37^{o}C$ and $20^{o}C$ respectively.

The importance of membrane turnover to the parasite would be to remove any antibody on the surface which would prevent antibody dependant cell adhesion reactions (Smith et al, 1981) and also antibody dependant C mediated reactions. Befus and Threadgold (1975) demonstrated the presence of dark areas on the tegument of H. diminuta in rats and mice and suggested they were in response to the developing immunity of

the host. The dark areas completely disappeared when the worms were incubated for 4 hours at 37°C, but not in worms maintained at 4°C, suggesting that a metabolic response was involved in the repair. Again, the removal of these dark areas correlates well with the rate of membrane turnover in D. phoxini.

Membrane turnover in D. phoxini can therefore be envisaged as a survival aid, however as with H. diminuta in mice, survival would depend on equilibrium between the immune response and turnover and it is possible that with D. phoxini as in H. diminuta in mice the balance of equilibrium changes in favour of the host resulting in expulsion of the worms. This statement suggests that antibody is involved in the expulsion of D. phoxini. There is no evidence of this, although antibody is produced in response to infection.

An investigation of the presence of Ig on the tegument of H. diminuta and H. microstoma using the same technique as used for D. phoxini was not as successful.

The results can be interpreted as showing that Ig is present on all the worms, because all worms incubated in GāMIg/FITC show surface fluorescence (Tables 4.9 and 4.10). Evidence for this is that the worms incubated in GaChIG/FITC did not fluoresce showing that no non specific adsorption of conjugate was occurring (Table 4.10 (E3)).

As stated in the introduction, Befus (1977) demonstrated the presence of Ig on the tegument of formalin fixed 9 days

old H. diminuta recovered from mice infected with six cysticercoids and on the tegument of day 40 p.i. H. microstoma. The present work on living worms confirms the presence of Ig on the tegument of both H. diminuta and H. microstoma but as Befus (1977) states 'the results does not prove that they are specific antibodies'.

In conclusion the results do suggest that Ig is present on the tegument of both D. phoxini and the cestodes. It is probable that some of this Ig is specific antibody in the case of D. phoxini. However the work does not prove this and does not provide any answers to the possible role, if any, of antibody in the expulsion of D. phoxini.

Summary

1) The presence of IgG, IgM and IgA was demonstrated on the tegument of 5 days old *D. phoxini*.

2) On day 1 *D. phoxini*, IgA only could be demonstrated, and this suggests that at least some of the Ig on day 5 worms was specific antibody.

3) The surface tegument of *D. phoxini* is metabolically active and is continually turning over and being released into the incubating medium.

4) The rate of turnover is at a similar rate to surface turnover in other parasites including nematodes.

5) The presence of antimetabolites or incubation at $2^{o}C$ reduced or prevented turnover.

6) The presence of Ig on the tegument of *H. diminuta* and *H. microstoma* was observed.

CHAPTER 5

AN INVESTIGATION OF THE MUCOSAL MAST CELL AND GOBLET CELL RESPONSE IN THE MOUSE DURING AN INFECTION OF *D. PHOXINI*

Introduction

Previous chapters have been concerned with the role of specific immune responses against *D. phoxini*. Although roles for lymphocytes and antibody have been hypothesized in expulsion of intestinal helminths, and evidence of these roles presented, there is also strong evidence that actual expulsion can be due to non specific effector mechanisms represented by intestinal inflammatory changes. Inflammation is often but not always associated with intestinal helminth infections. This is reflected both in cellular changes in the mucosa itself and in altered physiochemical conditions in the intestinal lumen (Wakelin, 1978).

Inflammatory changes in the intestine can be caused by interaction between immunologically specific components eg. antibody and lymphocytes, and non immunological non specific components eg. complement, myeloid cells and biologically active factors (Wakelin, 1978), and also by physical and chemical damage brought about by the parasite itself. This type of damage could be particularly important with *D. phoxini* where host tissue damage is thought to be caused by a combination of mechanical erosion by the heavily spined lateral surface of the adhesive organ and by secretion of histolytic substances (Erasmus, 1970b).

Changes recorded in the intestine of rats during infection with *N. brasiliensis* involve a mixed inflammatory response of plasma cells (discussed in chapter 4), lymphocytes, mononuclear cells, macrophages and eosinophils, and also

occurrences of large numbers of connective tissue basophils (Taliafero and Searles, 1939), mast cells and globular leukocytes. Structural changes in the same model involve an increase in epithelial turnover rate with a corresponding change in the nature of the epithelial cells and a reduction in the size of the villi, which become shorter and blunter whilst the crypts enlarge (Ogilvie and Rose, 1977). Similar structural changes have also been recorded in rats infected with T. spiralis. Recently, attention has also focused on the protective capacity of mucus in the intestine and also of the goblet cells (GC) that produce it.

Although many cells are involved during inflammatory changes, one of the most important and most studied is the mast cell (MC).

Mast cells are characterised by possessing metachromatic granules which contain a matrix of sulphated glycosaminoglycan and protein to which low molecular weight substances such as histamine are bound (Wingren and Enerback, 1983). Three cell lines share these fundamental characteristics, these being connective tissue mast cells (CTMC), mucosal mast cells (MMC) and blood basophils. Basophils have other characteristics in common with MC, such as cell surface receptors for the Fc portion of IgE but there are also important differences such as their distribution, basophils which are polymorphonuclear leukocytes are commonly found in progressive stages in bone marrow, are present in blood and are not normally found in the extravascular tissue (although they may be recruited into tissues in response to

occurrences of large numbers of connective tissue basophils (Taliafero and Searles, 1939), mast cells and globular leukocytes. Structural changes in the same model involve an increase in epithelial turnover rate with a corresponding change in the nature of the epithelial cells and a reduction in the size of the villi, which become shorter and blunter whilst the crypts enlarge (Ogilvie and Rose, 1977). Similar structural changes have also been recorded in rats infected with T. spiralis. Recently, attention has also focused on the protective capacity of mucus in the intestine and also of the goblet cells (GC) that produce it.

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parasitic infection) whereas true MC (which are monocytes) are not found in progressive stages in bone marrow (although precursor stages for some MC may originally arise from cells in bone marrow), or in blood, but are normally found in extravascular tissue (Askenase, 1980).

It is well established that CTMC and MMC differ morphologically and histochemically from each other (Miller, 1981) forming two distinct populations of MC, at least in rats and mice (Askenase, 1980). It is the MMC which are most involved with responses against intestinal helminths and therefore CTMC and basophils will not be discussed further.

Controversy also exists as to whether the MMC population is composed of two separate cell populations, one residing in the lamina propria, referred to as lamina propria mast cells (LPMC), and the other found in the epithelium of the villi called intra epithelial globule leukocytes (IEGL).

Intra epithelial globule leukocytes differ from LPMC in the rat in that they contain less glycosaminoglycan and monoamine, but Miller (1981) believes they are the same cell, and that the IEGL are simply LPMC discharging their granule contents.

Ruitenberg et al (1979) provided evidence suggesting that IEGL proliferation was thymus and antigen dependant but their origin may be thymic independant, and that LPMC proliferation was antigen independant but their origin was

thymic dependant. This suggested that they were two distinct populations in the rat.

The work of Ruitenberg et al (1979) can be criticised however, as pointed out by Askenase (1980) who cautioned, that the precursor of the IEGL could be thymus dependant but not stainable and therefore 'invisible' under the staining technique used by Ruitenberg et al (1979).

There is other strong evidence that the IEGL and LPMC are the same in the rat, in that they have similar morphological and staining properties. In the rat therefore the evidence strongly suggests that both cell types are related. In the mouse, the relationship of the IEGL and LPMC is much less well defined (Miller, 1981).

The role of MMC in the expulsion of intestinal parasites is not clearly understood though thoroughly reviewed by Askenase (1980). In brief, three hypotheses were proposed.

a) Basophils recruited to inflamed areas would release mediators eg. histamine and seretonin, which increase vascular permeability and together with possibly parasite induced epithelial damage would allow antibody and effector cells eg. eosinophils, neutrophils and macrophages to reach the worms and damage them. At a later stage of expulsion T cell dependant local proliferation of MMC would then occur, releasing mediators eg. histamine and prostoglandins which

could regulate effector leukocytes and stimulate peristalsis.

b) Mucosal mast cells may be predominantly involved in hyperimmune rejection and not primary rejection. This is based on the observation that large numbers of these cells are present after expulsion of the 1^{o} infection as in N. brasiliensis in Lewis rats (Kelly and Ogilvie, 1972; Befus et al, 1979), and T. spiralis in Wistar rats and NIH mice (Alizadeh and Wakelin, 1982). Rapid expulsion of T. spiralis in rats and mice (Alizadeh and Wakelin, 1982), and N. brasiliensis in rats (Miller et al, 1981) can occur within a few hours of parasite challenge. This immediate worm expulsion may be more dependant upon release of anaphylactic mediators from local MMC or basophils (Askenase, 1980).

c) Mucosal mast cells could play a role in expulsion of nematode parasites through an influence on intestinal epithelial goblet cells, leading to enhanced mucus secretion. It is possible that T cells could induce differentiation of GC directly or via mediators secreted by MMC, under possible regulation by T cells.

These hypotheses were proposed on the basis that they drew together current knowledge from several systems, however each is open to criticism (Askenase, 1980).

The importance of mucus in protecting the host against challenge infections was demonstrated by Lee and Ogilvie (1980) using T. spiralis in rats, challenge infections of which are rapidly expelled within a few hours of infection (Alizadeh

and Wakelin, 1982).

Lee and Ogilvie (1980) observed that far more challenge larvae were trapped within the mucus layer and more characteristically within mucus globules than in control rats. Also a smaller number of challenge larvae reached the deep mucus and epithelial layers in the immune rats as compared with controls.

Further evidence for the involvement of GC and mucus in parasite expulsion was provided by Miller et al (1980). They showed a significant increase in incorporation of D-(1-^{14}C) glucosamine into mucin over the period of a N. brasiliensis 1° infection in rats, with peak incorporation corresponding with the day of expulsion and the period of largest GC numbers in the intestine as shown by Miller and Nawa (1979).

Strong evidence exists that T cells are involved in some way in the generation of both MMC and GC. The transfer of either IMLNC (Befus and Bienenstock, 1979) or ITDL (Nawa and Miller, 1979) caused an accelerated mucosal mastocytosis in rats infected with N. brasiliensis and transfer of ITDL caused an accelerated increase of GC in the same model (Miller and Nawa, 1979). Miller et al (1979) demonstrated that the ability of transferred ITDL to increase MMC and GC production resided in the sIg^- cell population, i.e. T. cells.

However Miller and Nawa (1979) and Befus and Bienenstock (1979) also demonstrated that transfer of 2° or

teritary (3^{o}) IS or 1^{o} IS respectively would also accelerate GC and MMC productions in the N. brasiliensis - rat model. The effect of serum is not known but Miller and Nawa (1979) suggested that it acts indirectly because its effect was abrogated by corticosteroids or reserpine which inhibits GC differentiation and depletes MC amines.

In the present work the course of IEGL, LPMC and GC hyperplasia during a 1^{o} and 2^{o} infection of D. phoxini was investigated. The IEGL and LPMC were counted as two separate cell populations solely on the difference of their distribution. Also, the effect of adoptive transfer of IMLNC on the response was investigated.

Materials and Methods

Animals

All the mice used in these experiments were male and female NIH. Maintenance and infection of mice, and recovery of worms was as described in the general materials and methods.

Preparation of cell suspensions and cell transfer

Cell suspensions were prepared and transferred into recipients as stated in the general materials and methods.

Histology

Mice were killed by ether anaesthesia followed by cervical dislocation. The small intestine was removed and placed on a plastic ruler to prevent shrinkage. Starting with the pyloric end, the intestinal segments between 5 and 10 cm and 10 and 15 cm were removed and immediately placed in Carnoy's fixative. The segments were opened longitudinally and then shaken gently to remove gut contents. Segments were then rolled on to a piece of plastic 1.0 ml syringe plunger with the mucosal surface outermost (the Swiss role technique of Reilly and Kirsner, 1965). The tissue was fixed for 6 hours at room temperature.

After fixing, the plunger was removed, the tissues were dehydrated and embedded in polywax (Difco Laboratories).

The specimens were sectioned at 5 um forming a ribbon which was placed on slides.

The intestinal segment removed from between 5 and 10 cm was stained for IEGL and LPMC and the other segment for GC.

Staining of IEGL and LPMC was by the Astra Blue/ Safranin O technique using the following schedule:

1)	Rehydrate sections through xylene and graded ethanol to water	
2)	1% w/v Astra Blue (Gurr) in 0.7 MHCL	30-45 minutes
3)	0.7 MHCL	10 minutes
4)	0.5% w/v Safranin O (Searle) in 0.125 MHCL	5 minutes
5)	Distilled water	Rinse
6)	70% ethanol	1 minute
7)	90% ethanol	1 minute
8)	Absolute ethanol i	1 minute
9)	Absolute ethanol ii	1 minute
10)	Xylene i	5 minutes
11)	Xylene ii	5 minutes
12)	Mount in DPX	

Goblet cells were stained by the periodic acid - Schiff method, using the following schedule:

1) Rehydrate sections through xylene and graded ethanol

2) 1% w/v Alcian Blue 8GX (Gurr) in 3% Glacial acetic acid and 97% distilled water solution	30 minutes
3) Wash in water	5 minutes
4) Oxidise in 1% w/v Periodic acid in distilled water	5 minutes
5) Wash in water	5 minutes
6) Schiff reagent (B.D.H. Chemicals)	10-20 minutes
7) Three changes of 0.5% w/v Sodium metabisulphate in distilled water	Rinse
8) Wash in water	10 minutes
9) Mayer's haemalum	2 minutes
10) Dehydrate in graded ethanol 30% - 90%	3 minutes in each
11) Absolute ethanol i	5 minutes
12) Absolute ethanol ii	5 minutes
13) Xylene i	5 minutes
14) Xylene ii	5 minutes
15) Mount in DPX	

For quantitative purposes, all Astra Blue positive cells in the epithelium of the gut were recorded as IEGL all Astra Blue stained cells in the lamina propria were counted as LPMC. Goblet cells were counted as the mucus producing cells present in the epithelium of the crypts and villi.

In each intestinal segment, 20 villous crypt units (v.c.u.) were counted at random. A v.c.u. represents the

portion of intestinal mucosa lying between two gland crypts and the lamina propria of the villous above (Miller and Jarrett, 1971).

Solutions

1) Carnoy's fixative

Absolute ethanol	60 ml
Chloroform	30 ml
Glacial acetic acid	10 ml

2) Mayer's haemalum

1 g of haematoxylin was added to 1 l of distilled water, together with 50 g of potassium alum ($KAL(S_{04})^2.12H_2$) and 0.2 g of sodium iodate. The solution was heated and brought to boil. It was then allowed to cool overnight and on the following day 1 g of citric acid and 50 g of chloral hydrate were added. The stain was then ready for use.

Results

An experiment to investigate the course of the intra epithelial globule leukocyte, lamina propria mast cell and goblet cell hyperplasia during a primary or secondary infection of 20 metacercariae

Ninety six male, NIH aged between 8 - and - 10 weeks were caged in groups of 4. The cages were divided into 6 groups composed of A) 8 cages, (1^{o} infection); B) 5 cages, (2^{o} infection); C) 3 cages, (1^{o} control); D) 3 cages, (2^{o} control); E) 3 cages, (1^{o} infection check) and F) 2 cages, (2^{o} infection check).

All mice in groups B and F were infected with 20 m on day -27, the immunizing dose. On day 0, all mice in groups A and E were given a 1^{o} infection of 20 m and mice in groups B and F were challenged with 20 m. Groups C and D served as non infected controls.

A cage from each group was killed on the days shown below:

A: Days 2,4,6,8,10,12,16 and 20 p.i.

B: Days 2,4,6,8 and 12 p.i.

C: Days 2,10 and 20 p.i.

D: Days 2,6 and 12 p.i.

E: Days 4,8 and 12 p.i.

F: Days 2 and 6 p.i.

The guts from mice in groups A, B, C and D were prepared for histological examination and worms were recovered from the

mice in groups E and F to serve as a check on establishment.

Worm recoveries (Table 5.1) show that establishment and course of infection was as expected for a 20 m 1^{o} infection and a challenge infection as described in chapter 1. This is good evidence that infection was similar in the other groups.

There was no significant difference in GC numbers between infected and control mice throughout the duration of the experiment, (Fig 5.1, Table 5.2 and Plate 5.1 and 5.2). Goblet cell numbers remained fairly constant throughout the experiment.

The greatest rise in numbers was observed in the IEGL. Until day 4 p.i. numbers remained near control values and there was no statistically significant difference between groups. By day 6 p.i. a sharp increase had begun, the difference between controls being statistically highly significant ($P<0.001$).

The IEGL numbers continued to increase reaching a peak on day 12 p.i., with cell numbers being almost 30 times greater than on day 2 p.i. There then followed a decline until day 20 p.i., the last day of the experiment, but with the increase in cell numbers, above controls, still being statistically highly significant, $P<0.001$, (Fig 5.2, Table 5.2 and Plate 5.3 and 5.4).

During the course of the 2^{o} infection there was a

statistically highly significant difference ($P<0.001$) between means of control and infected mice throughout the experiment. Peak values were reached on day 8 p.i., earlier than in the 1^{o} infection, when cell numbers were similar to those found at the peak of a 1^{o} infection on day 12 (Fig 5.2).

Lamina proporia mast cell numbers also increased during the infection but did not reach the high numbers of the IEGL (note different scale on 'y' axis, Fig 5.2 and 5.3). The course of the increase of the LPMC was similar to that of the IEGL, with values remaining at control levels up until day 4 p.i. in a 1^{o} infection and then increasing.

After day 6 p.i. the difference between infected and control mice remained statistically highly significant ($P<0.001$ except day 10 when $P<0.01$). Highest cell numbers were seen on day 12 p.i. with the cells remaining stable until day 16 p.i. followed by a decline to day 20 p.i. (Fig 5.3 and Table 5.2).

During the 2^{o} infection the difference between means of control and infected mice was statistically highly significant ($P<0.001$) throughout the experiment. Peak values were reached on day 8 p.i. being earlier and also higher than the highest values in the 1^{o} infection.

Because of the increases observed in the IEGL and LPMC populations during infection it was decided to investigate whether the response could be transferred using IMLNC.

Plate 4.1

A living D. phoxini incubated in goat anti-mouse Ig/FITC conjugate in the presence of iodoacetamide at 2°C, showing a characteristic pattern of fluorescence around the periphery of the worm usually observed after incubation in antimetabolites (x 250)

Plate 4.2

A living D. phoxini incubated in day 4 secondary immune serum before being incubated in goat anti-mouse Ig/FITC at 2°C showing characteristic overall bright fluorescence (x 250)

As it is known that day 4 IMLNC can significantly accelerate expulsion of D. phoxini, the following experiment was designed to investigate whether the effect of the IMLNC on the worms was caused via the IEGL and LPMC populations.

The effect of immune mesenteric lymph node cell transfer on the lamina propria mast cell, intra epithelial globule leukocyte and goblet cell hyperplasia

Thirty two, 14 weeks old female NIH were infected with 50 m each on day -13 and caged in groups of 4. A further 24 age and sex matched mice were caged in groups of 6, to serve as donors of CMLNC.

Four days after infecting, on day -9, a mesenteric lymph node cell population was prepared from each of the donor groups, and 4×10^7 cells were transferred into each of the mice as shown below.

The recipients, 40 female NIH aged between 8 - and - 10 weeks, and caged in groups of 4 were given the following treatments:

Group	Cells transferred	Challenged
A & G	-	-
B & H	IMLNC	-
C & I	CMLNC	+
D & J	-	+
E & K	IMLNC	+

On day 0, the recipients were challenged with 200 m of D. phoxini as shown above. All mice in groups A - E were killed on day 6 p.i. and the remainder on day 10 p.i. Intestine segments were prepared for histological examination as in the previous experiment.

Goblet cell numbers remained fairly constant between all groups on both days. There was no significant difference between means of control and experimental mice (Fig 5.4 and Table 5.3).

The IEGL had increased above control numbers (A), on day 6, in all infected groups (C, D and E) with the difference in each case being statistically highly significant ($P<0.001$), but group E which had received 4×10^7 IMLNC gave far and away the highest response (Fig 5.5). The difference between group E and the other infected groups being statistically highly significant ($P<0.001$). It is also interesting that mice given CMLNC before challenge had a statistically significant ($P<0.01$) higher mean number of IEGL than those only given D. phoxini (DvC).

On day 10, both groups G and H, the controls, had similar numbers of IEGL as the corresponding groups on day 6. As on day 6 groups I, J and K had means significantly higher ($P<0.001$) than group G. However IEGL numbers had increased in groups I and J, (corresponding to groups C and D on day 6) and decreased in group K, as compared with the corresponding groups on day 6. The means of groups I and J were significantly higher than the mean of group K, ($P<0.01$

and P<0.02 respectively) (Table 5.3).

An almost similar result was observed with the LPMC (Fig 5.6). Control levels, groups A, B, G and H remained similar on both days. Levels were highest in group E (4×10^7 IMLNC & 200 m <u>D. phoxini</u>) but the means of groups C and D were also statistically, significantly higher (P<0.01) than the mean of group A. Also group C mice had a higher mean of LPMC numbers than group D mice but this was not significant (Table 5.3).

By day 10 p.i. group K had similar numbers of LPMC to the corresponding group E on day 6. However, groups I and J, LPMC levels had increased above those of day 6 reaching similar high levels to group K. The differences betweem the means of groups I, J and K were not significant.

Table 5.1

Table showing % worm recoveries of a 20 metacercariae primary and secondary infection in NIH mice

Group/Day p.i.	% worm recovery $\bar{x} \pm$ s.d.
E 1° infection 20 m 4	80 ± 0
E 1° infection 20 m 8	80 ± 0
E 1° infection 20 m 12	7.5 ± 2.8
F 2° infection 20 m 2	80 ± 0
F 2° infection 20 m 6	0

(n) = 4

Table 5.2

Table showing $\bar{x} \pm$ s.d. of lamina proporia mast cell (LPMC), intra epithelial globule leukocyte (IEGL) and goblet cell (GC) numbers from 20 villous crypt units (v.c.u.) per mouse during a primary or secondary infection of 20 metacercariae in NIH mice compared with uninfected control mice

Days p.i.		1° Infection (Group A) $\bar{x} \pm$ s.d.			1° Control (Group C) $\bar{x} \pm$ s.d.		
		GC	IEGL	LPMC	GC	IEGL	LPMC
2	$\bar{x}$	389	28.7	5.7	460	32.7	9
	s.d.	125.5	7.0	2.5	52.7	3.3	4
4	$\bar{x}$	380.5	28.0	5.5			
	s.d.	36	10.1	0.5			
6	$\bar{x}$	428.7	113.7	24			
	s.d.	44.6	35.3	4.9			
8	$\bar{x}$	411.5	327	33.2			
	s.d.	47.1	48.1	8.3			
10	$\bar{x}$	422	582	33	418.2	40	7.2
	s.d.	109.8	26.9	26.3	84.8	6.4	2.2
12	$\bar{x}$	416.5	800.5	68			
	s.d.	29.9	109.9	11.9			
16	$\bar{x}$	374.7	501.5	69.7			
	s.d.	60.9	206.4	19.1			
20	$\bar{x}$	382.7	318.2	39.5	392	38.7	11.5
	s.d.	8.7	50.5	13.6	51.2	10.4	5.5

(n) = 4 in Group A and Group B

In significance tests the control $\bar{x} \pm$ s.d. was calculated by combining all 12 control mice, i.e. the 4 killed on days 2, 10 and 20 (Group C) and days 4, 8 and 12 (Group D), hence n = 12.

Group C : $\bar{x} \pm$ s.d.

GC = 423.4 ± 65.5

IEGL = 37.1 ± 7.4

LPMC = 9.25 ± 4.1

Group D :

GC = 377.1 ± 55.4

IEGL = 32.5 ± 8.05

LPMC = 10.5 ± 6.3

Means significantly different :

Group A v Group C

Day p.i.	GC	IEGL	LPMC
2	n.s.	n.s.	n.s.
4	n.s.	n.s.	n.s.
6	n.s.	P < 0.001	P< 0.001
8	n.s.	P < 0.001	P < 0.001
10	n.s.	P < 0.001	P < 0.01
12	n.s.	P < 0.001	P < 0.001
16	n.s.	P < 0.001	P < 0.001
20	n.s.	P < 0.001	P < 0.001

Days p.i.		2° Infection (Group B) $\bar{x} \pm$ s.d.			2° Control (Group D) $\bar{x} \pm$ s.d.		
		GC	IEGL	LPMC	GC	IEGL	LPMC
2	$\bar{x}$	386.2	168	40	405	28.5	17.5
	s.d.	94.4	33.2	8.9	41.9	10.3	6
4	$\bar{x}$	393.7	398.7	58.5			
	s.d.	37.8	124.8	37.1			
6	$\bar{x}$	417.2	449	82	384	37.7	7.7
	s.d.	80.0	181.3	41.8	62.9	8.0	0.9
8	$\bar{x}$	336.7	756	107.5			
	s.d.	82.5	141.4	25.7			
12	$\bar{x}$	377.2	297.2	50	342.5	31.2	6.2
	s.d.	84.4	108.2	28.5	53.2	2.2	3.2

Means significantly different :

Group B v Group D

Day p.i.	GC	IEGL	LPMC
2	n.s.	$P < 0.001$	$P < 0.001$
4	n.s.	$P < 0.001$	$P < 0.001$
6	n.s.	$P < 0.001$	$P < 0.001$
8	n.s.	$P < 0.001$	$P < 0.001$
12	n.s.	$P < 0.001$	$P < 0.001$

n.s. not significant

Table 5.3

Table showing the effect of transferring 4 x 10^7 immune mesenteric lymph node cells (IMLNC) on the number of lamina proporia mast cells (LPMC), intra epithelial globule leukocytes (IEGL) and goblet cells (GC) from 20 villous crypt units (v.c.u.) per mouse, compared with immunized NIH and control NIH mice on days 6 and 10 post infection

Group/Treatment		Day 6 p.i. $\bar{x} \pm$ s.d.			Day 10 p.i. $\bar{x} \pm$ s.d.		
		GC	IEGL	LPMC	GC	IEGL	LPMC
A & C							
Uninfected	$\bar{x}$	383.5	41	8.25	366	33.2	10
Control	s.d.	38.4	5.2	1.25	35.9	5.2	5.9
B & H							
4×10^7 IMLNC	$\bar{x}$	350.7	36.5	10	325	36.7	7.5
Non infected	s.d.	46.9	8.2	4.6	35.9	5.2	3.6
C & I							
4×10^7 CMLNC &	$\bar{x}$	374	337.2	34.7	338.5	1200.5	95
200 m <u>D. phoxini</u>	s.d.	22	70	11.7	47.5	106.4	3.8
D & J							
200 m <u>D. phoxini</u>	$\bar{x}$	391.2	196.5	22	341.2	1133.7	76
	s.d.	32	15	6.3	41	90.6	24.7
E & K							
4×10^7 IMLNC &	$\bar{x}$	396.7	1249.5	97	344.2	932	95.5
200 m <u>D. phoxini</u>	s.d.	18.5	144.2	14.4	32.7	72.9	26.5

(n) = 4 in all groups

Means significantly different

Day 6 p.i.				
		GC	IEGL	LPMC
A	E	n.s.	P < 0.001	P < 0.001
A	D	n.s.	P < 0.001	P < 0.01
A	B	n.s.	n.s.	n.s.
A	C	n.s.	P < 0.001	P < 0.01
D	E	n.s.	P < 0.001	P < 0.001
D	C	n.s.	P < 0.01	n.s.
C	E	n.s.	P < 0.001	P < 0.001

Day 10 p.i.				
		GC	IEGL	LPMC
G	K	n.s.	P < 0.001	P < 0.001
G	J	n.s.	P < 0.001	P < 0.01
G	H	n.s.	n.s.	n.s.
G	I	n.s.	P < 0.001	P < 0.001
J	K	n.s.	P < 0.02	n.s.
J	I	n.s.	n.s.	n.s.
I	K	n.s.	P < 0.01	n.s.

n.s. not significant

Figure 5.1

Graph showing $\bar{x} \pm$ s.d. (n = 4) from 20 villous crypt units per mouse, of goblet cell numbers during a primary and secondary infection of 20 metacercariae in NIH mice

Key:

○ Infected

● Non infected

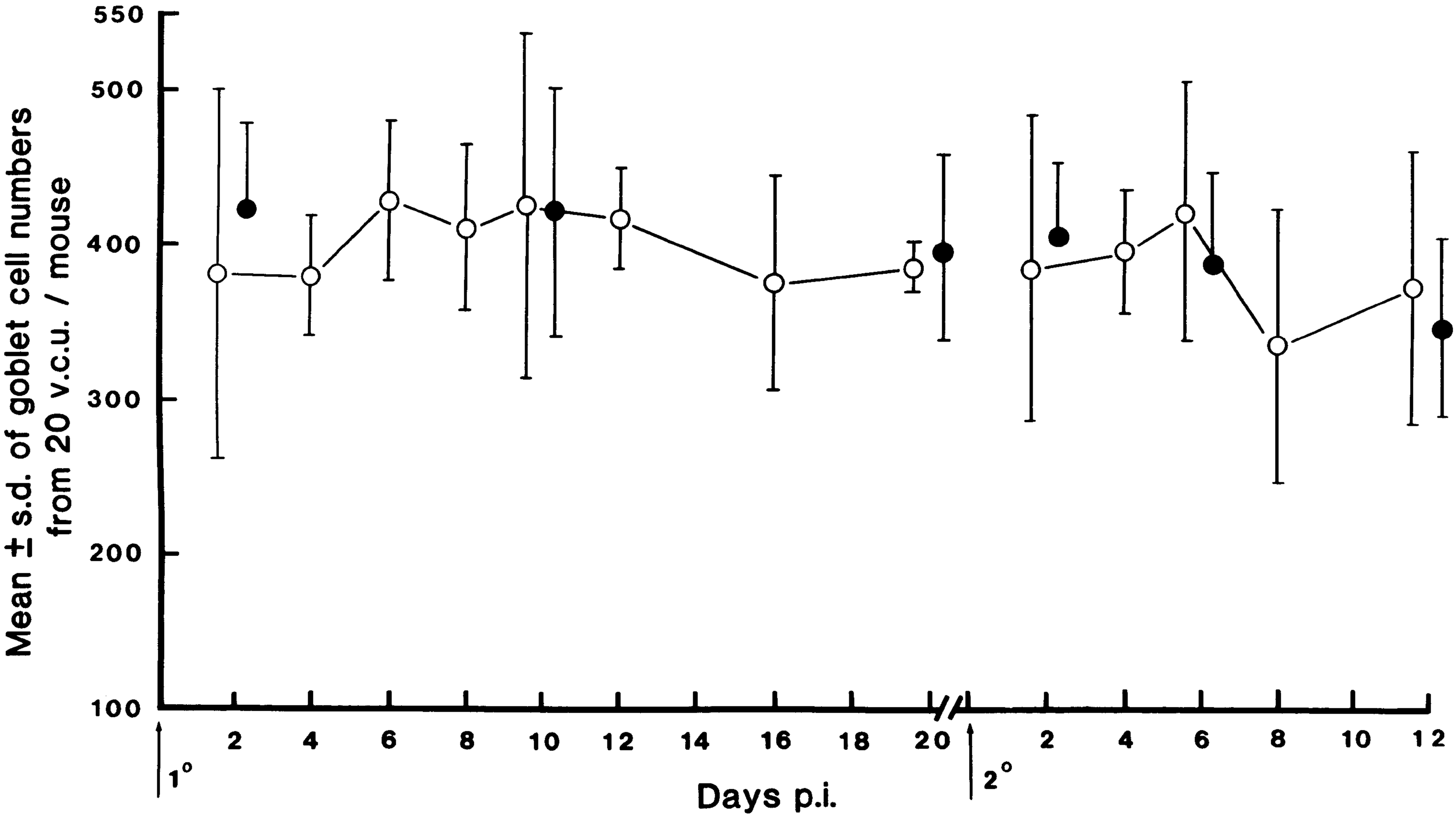

Mean ± s.d. of goblet cell numbers
from 20 v.c.u. / mouse
550
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
2
4
6
8
10
12
1°
2°
Days p.i.

Figure 5.2

Graph showing $\bar{x} \pm$ s.d. (n = 4) from 20 villous crypt units per mouse, of intra epithelial globule leukocyte numbers during a primary and secondary infection of 20 metacercariae in NIH mice

Key:

○ Infected

● Non infected

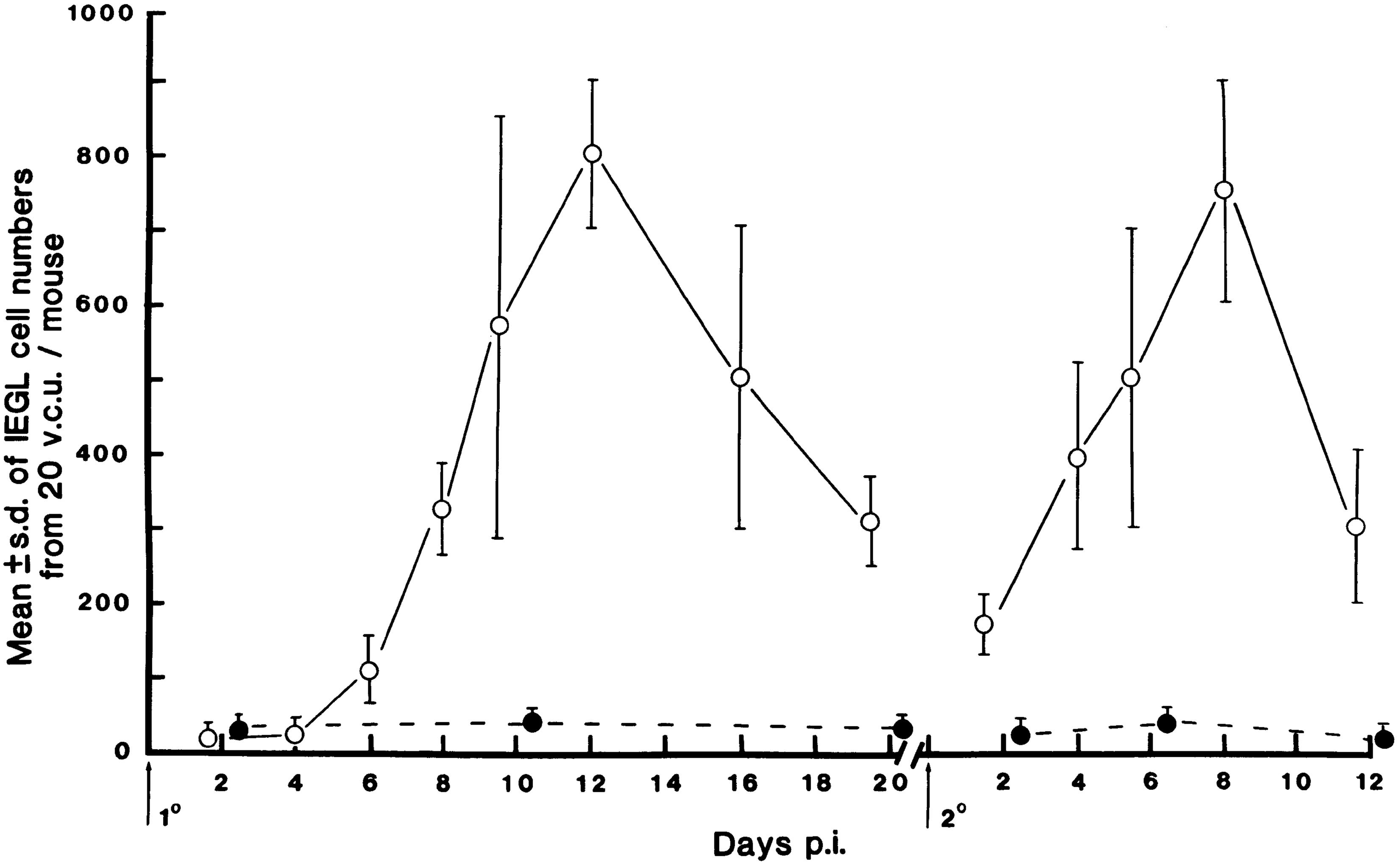

Mean ± s.d. of IEGL cell numbers from 20 v.c.u. / mouse
1000
800
600
400
200
0
2
4
6
8
10
12
14
16
18
20
1°
2°
Days p.i.

Figure 5.3

Graph showing $\bar{x} \pm$ s.d. (n = 4) from 20 villous crypt units per mouse, of lamina propria mast cell numbers during a primary and secondary infection of 20 metacercariae in NIH mice

Key:

○ Infected

● Non infected

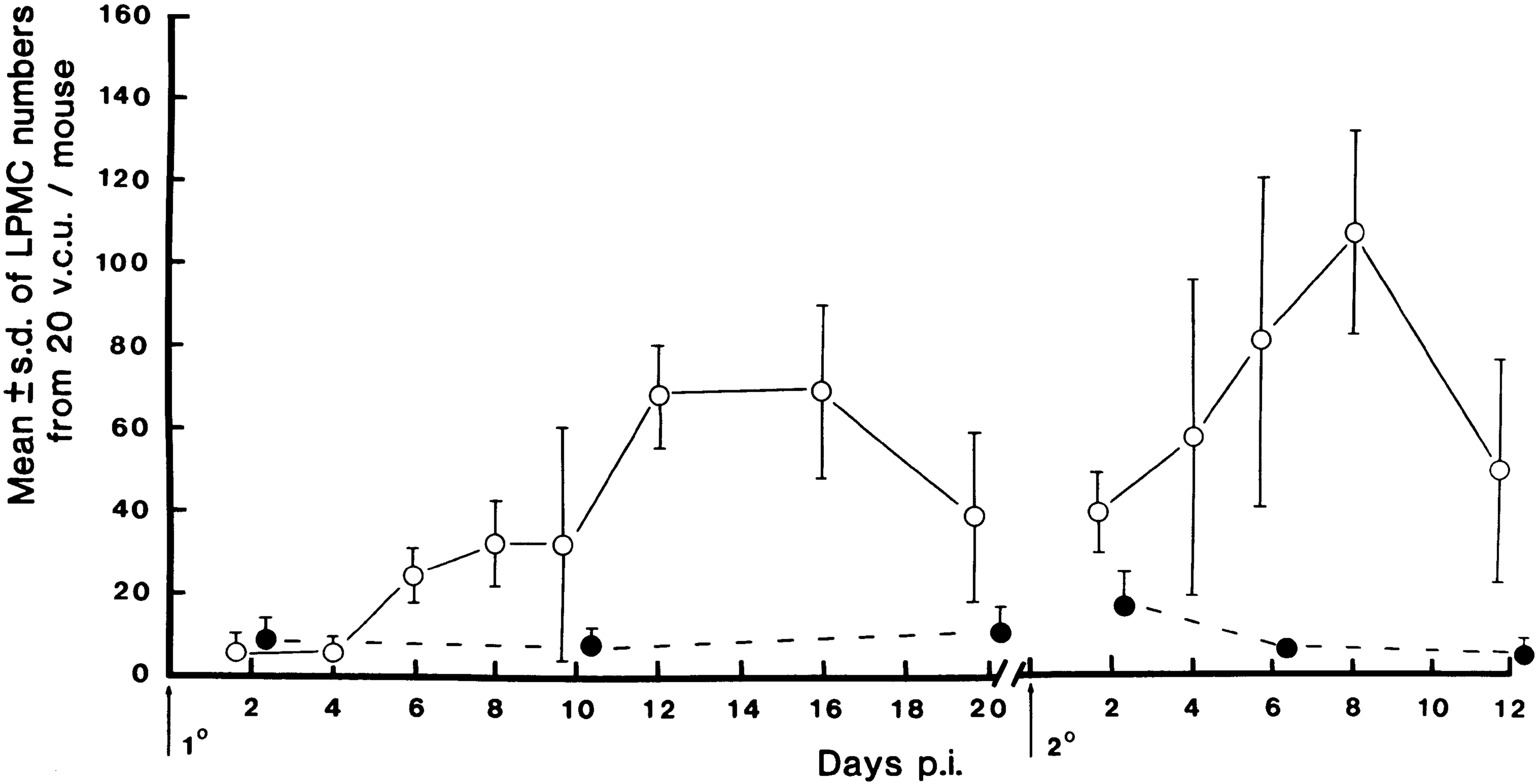

Mean ± s.d. of LPMC numbers
from 20 v.c.u. / mouse
160
140
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
2
4
6
8
10
12
1°
2°
Days p.i.

Figure 5.4

Histogram showing the effect of transferring 4×10^7 immune mesenteric lymph node cells or control mesenteric lymph node cells on goblet cell numbers as compared with control infected and uninfected mice. Results are expressed as $\bar{x} \pm$ s.d. of 20 villous crypt units from each of 4 mice

Key:

A & G	Uninfected control mice
B & H	Uninfected mice which had received 4×10^7 IMLNC
C & I	Infected mice which had received 4×10^7 CMLNC
D & J	Infected mice, no cells transferred
E & K	Infected mice, which had received IMLNC

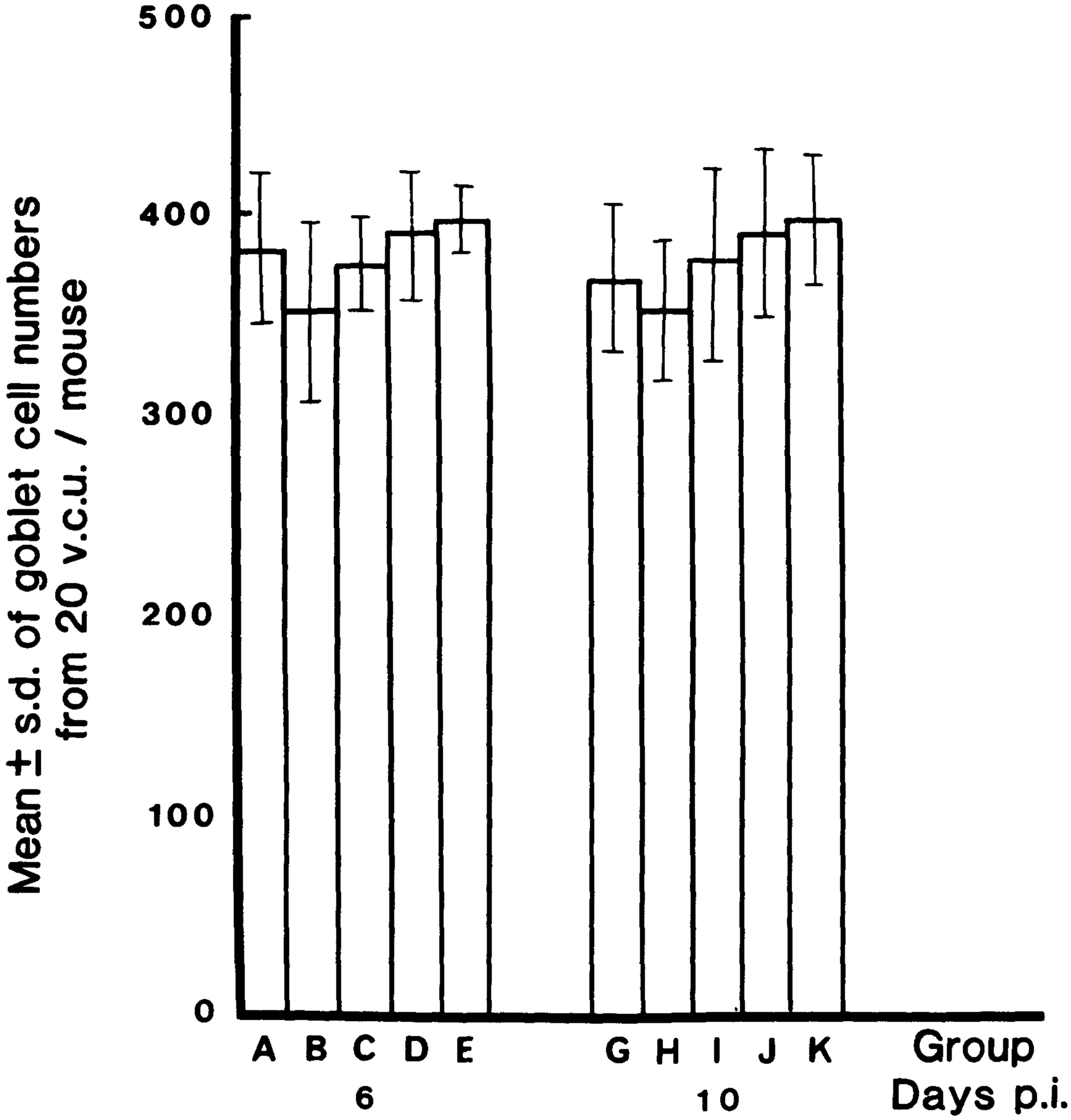

Mean ± s.d. of goblet cell numbers from 20 v.c.u. / mouse
500
400
300
200
100
0
A B C D E G H I J K
Group
6
10
Days p.i.

Figure 5.5

The effect of transferring 4×10^7 immune mesenteric lymph node cells or control mesenteric lymph node cells on intra epithelial globule leukocyte numbers as compared with control infected and uninfected mice. Results are expressed as $\bar{x} \pm$ s.d. of 20 villous crypt units from each of 4 mice

Key:

A & G	Uninfected control mice
B & H	Uninfected mice which had received 4×10^7 IMLNC
C & I	Infected mice which had received 4×10^7 CMLNC
D & J	Infected mice, no cells transferred
E & K	Infected mice, which had received IMLNC

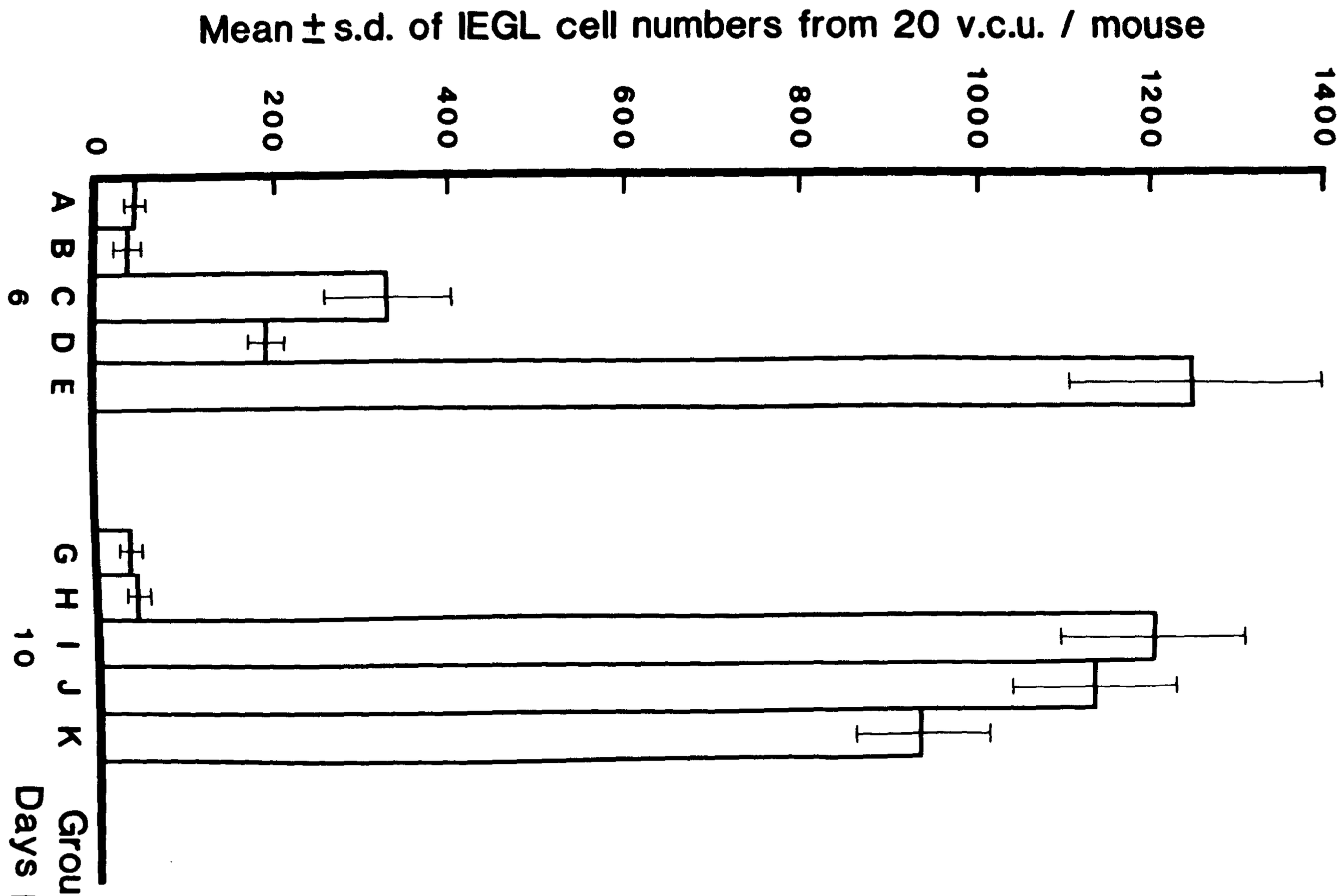

Mean ± s.d. of IEGL cell numbers from 20 v.c.u. / mouse
0
200
400
600
800
1000
1200
1400
A
B
C
D
E
G
H
I
J
K
Group
6
10
Days p.i.

Figure 5.6

Histogram showing the effect of transferring 4×10^7 immune mesenteric lymph node cells or control mesenteric lymph node cells on lamina propria mast cell numbers as compared with control infected and uninfected mice. Results are expressed as $\bar{x} \pm$ s.d. of 20 villous crypt units from each of 4 mice

Key:

A & G	Uninfected control mice
B & H	Uninfected mice which had received 4×10^7 IMLNC
C & I	Infected mice which had received 4×10^7 CMLNC
D & J	Infected mice, no cells transferred
E & K	Infected mice, which had received IMLNC

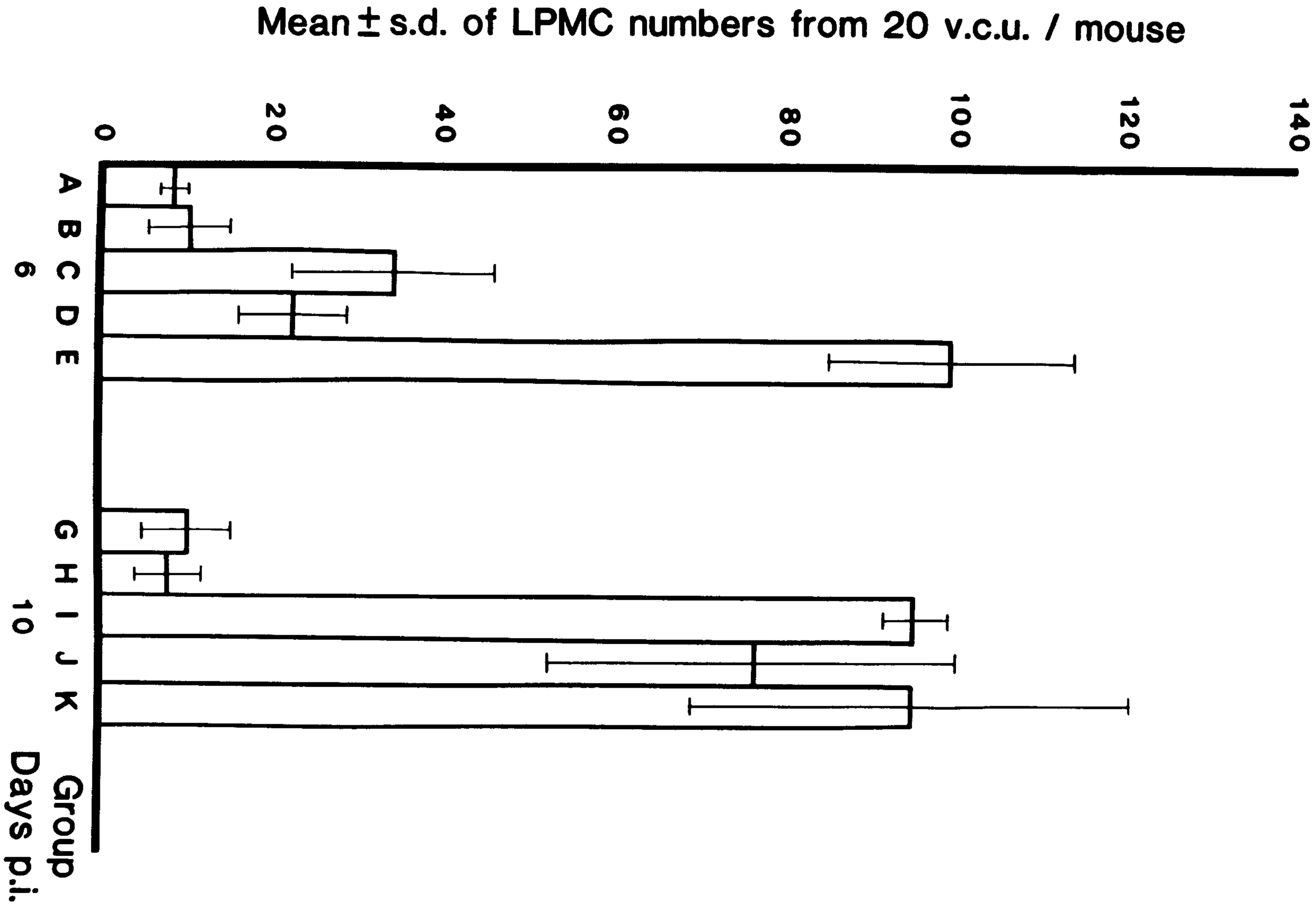

Mean ± s.d. of LPMC numbers from 20 v.c.u. / mouse
0
20
40
60
80
100
120
140
A
B
C
D
E
G
H
I
J
K
Group
6
10
Days p.i.

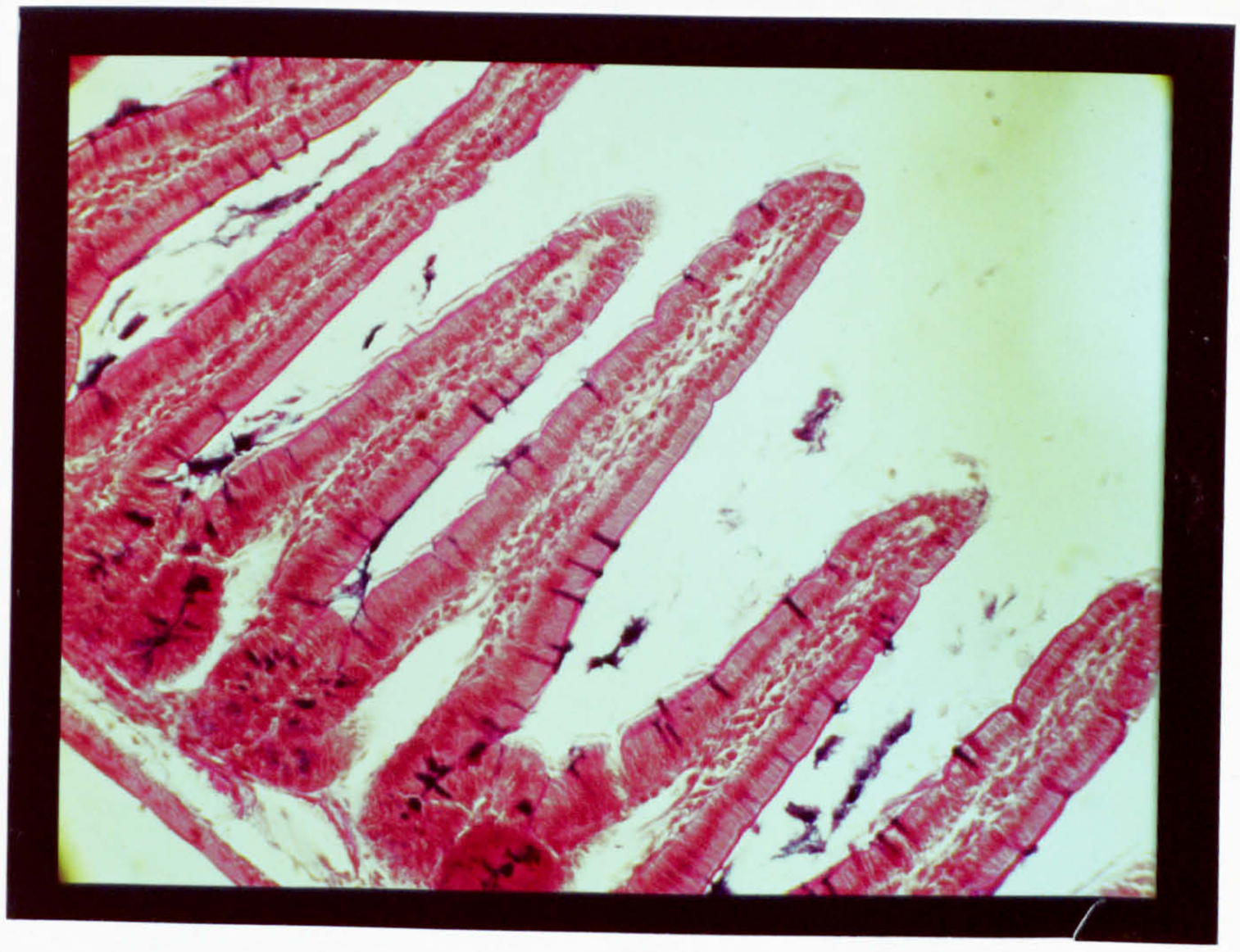
Plate 5.1

Section of uninfected mouse intestine stained for goblet cells (x 200)

Plate 5.2

Section of infected mouse intestine, stained for goblet cells and also showing section of D. phoxini (x 250)

(⟶ shows one goblet cell)



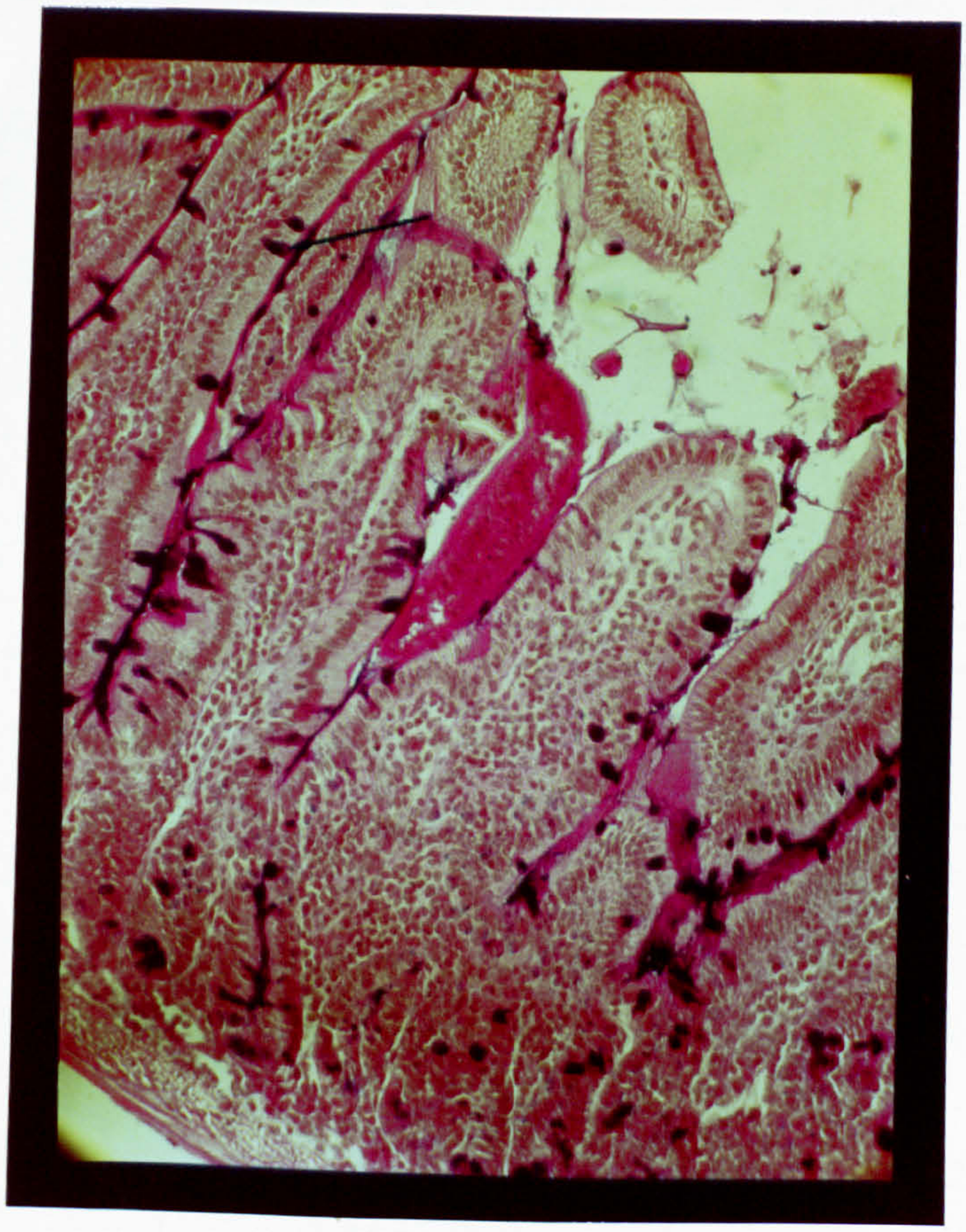


Plate 5.3

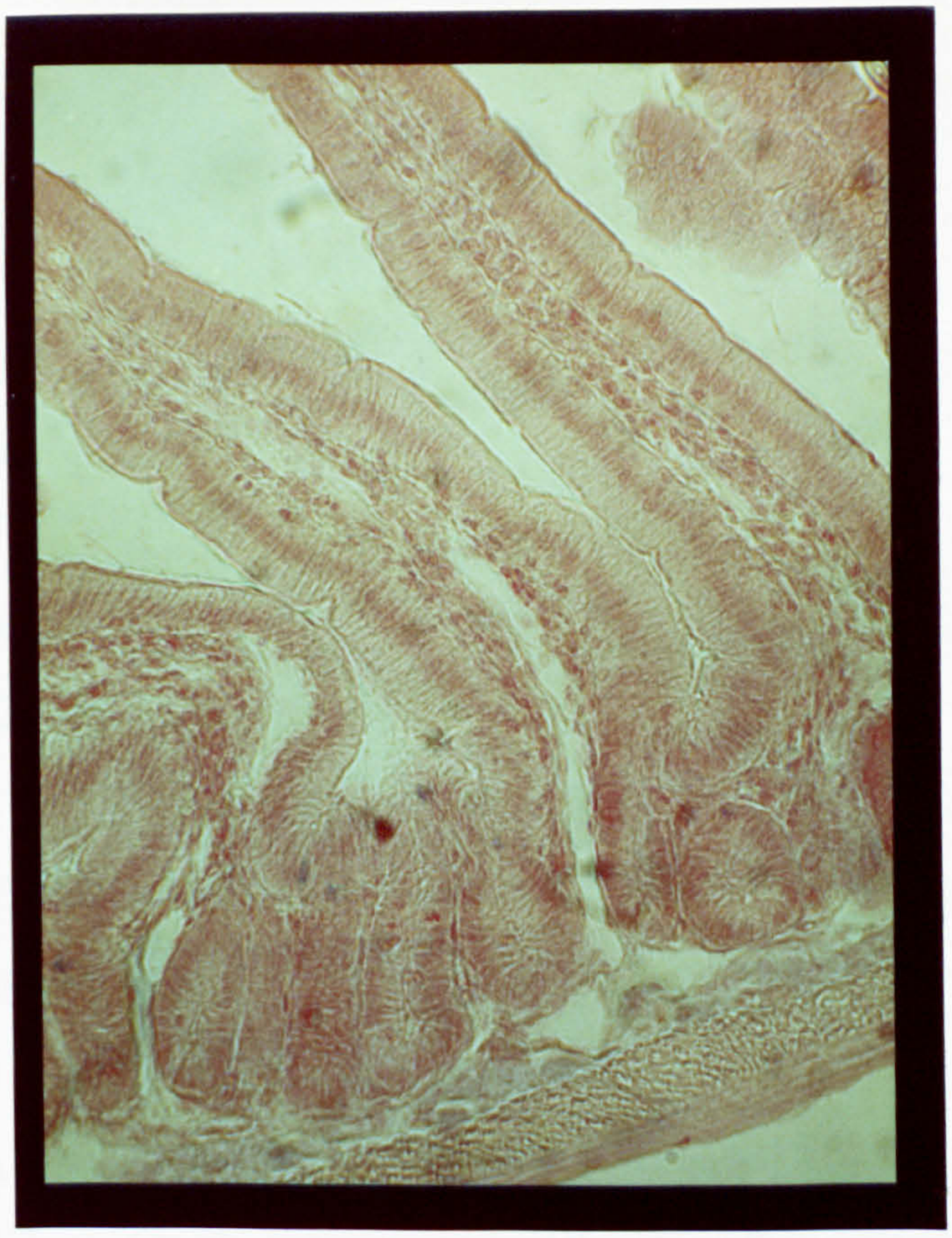
Section of uninfected mouse intestine stained for lamina proporia mast cells and intra epithelial globule leukocytes (x 250)

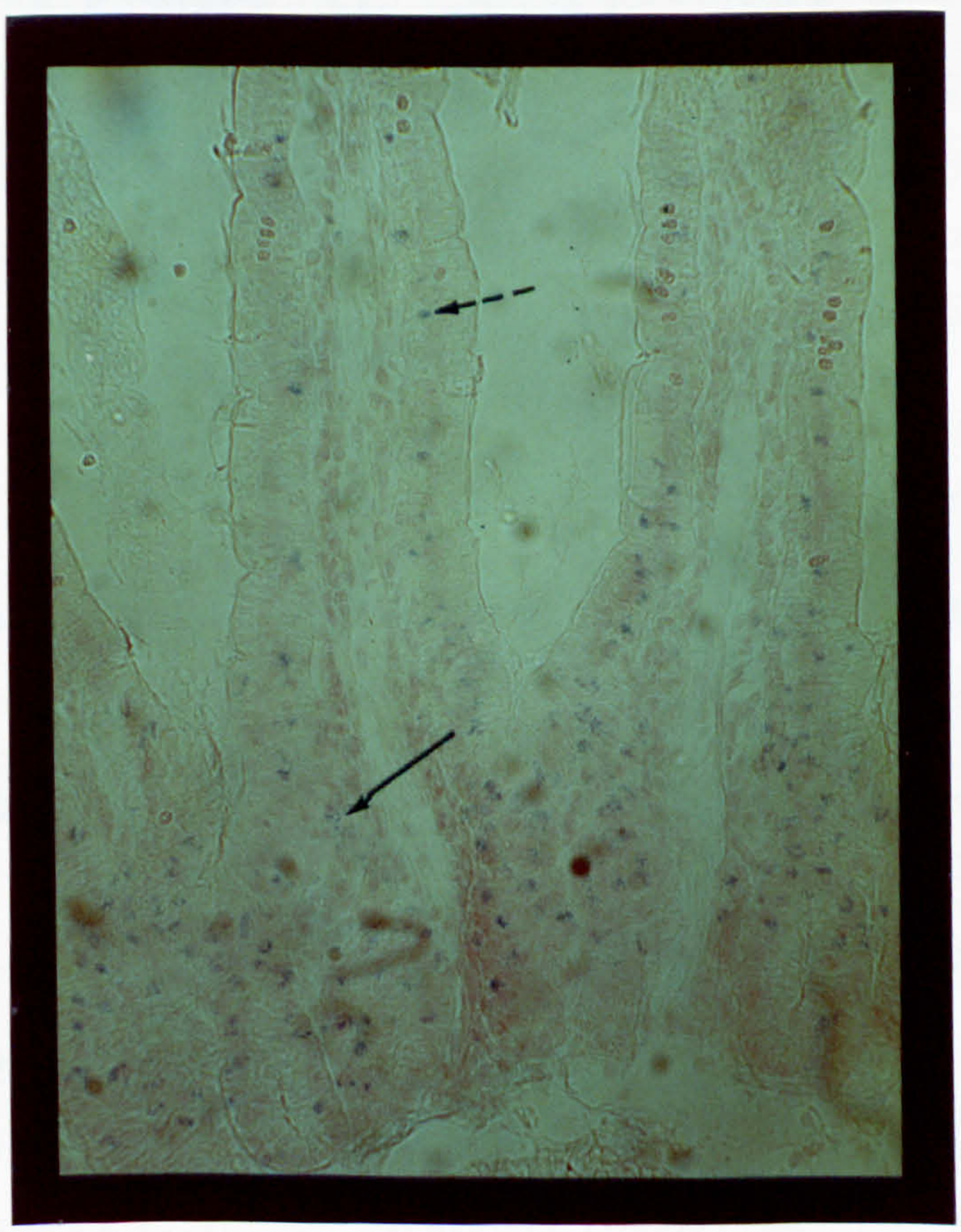
Plate 5.4

Section of infected mouse intestine (day 8 p.i.; 20 metacercariae) stained for lamina proporia mast cells and intra epithelial globule leukocytes (x 250)

(⟶ shows one lamina proporia mast cell and

⇢ shows one intra epithelial globule leukocyte)





Discussion

Both a LPMC and an IEGL hyperplasia was observed to occur during a 20 worm *D. phoxini* infection, but no GC response was detected.

The number of GC remained quite constant in infected mice, at a level similar to that in uninfected control mice (Table 5.2, Fig 5.1, Table 5.3 and Fig 5.4 groups A, D, G and J). This confirms preliminary observations made by Mawdsley (1983) who also could detect no statistically significant increase in GC numbers during a 1^{o} infection of 20 m, 200 m or in a 2^{o} infection of 200 m of *D. phoxini*, although there was a slight (but not significant) increase in GC numbers between days 4 and 12 of a 1^{o} infection of 200 m. As no such increase was observed in the current work, it would appear that this could reflect the different quantity of antigen present during infection with 200 m as compared with 20 m.

The whole question of GC response to helminth infections remains confused. Nawa and Miller (1979) presented very convincing evidence of hyperplasia during the rejection of *N. brasiliensis* by rats, and Alizadeh and Wakelin (1982) observed a similar hyperplasia in the acute phase of *T. spiralis* rejection by mice and rats. On the other hand Mimori *et al* (1982) did not detect an increase in their *S. ratti* - rat model. In view of the results of Mawdsley (1983) and the current results, a GC hyperplasia is not always associated with parasite expulsion. It should be stressed

that the absence of hyperplasia does not mean that there is not an increase in GC activity. A measure of mucus production such as that calculated from the rate of D-(1-^{14}C) glucosamine incorporation into an acid-precipitable glycoprotein fraction in the intestine (Miller et al, 1981) might be a better measure of GC activity.

The transfer of IMLNC prior to infecting with D. phoxini also had no effect on GC numbers. The transfer of immune lymphocytes in other models has caused an accelerated increase in GC numbers, as observed by Miller and Nawa (1979) using ITDL in their N. brasiliensis - rat model, and Miller et al (1979) has shown that the response was (sIg$^-$) i.e. T cell dependant.

The most striking response to the D. phoxini infection was seen in the MMC population, particularly in the IEGL population. Although LPMC numbers increased significantly above control values, the IEGL population increased to much higher numbers. This is in common with other parasite - mouse models. Ruitenberg and Elgersma (1976; 1979) showed a greater increase in IEGL numbers than in LPMC numbers in nu/+ Balb/c mice infected with T. spiralis. The IEGL numbers were also greater than LPMC numbers in the uninfected control mice and this is similar to the NIH controls in the present work. Andreassen et al (1978) also observed a greater IEGL response in nu/+ mice than LPMC response during infection with H. diminuta, and Alizadeh and Wakelin (1982) observed a large increase in the MMC population in NIH mice infected with T. spiralis. They did not differentiate between LPMC and IEGL

because of the controversy surrounding the identity of these cells (Askenase, 1980).

NIH mice infected with D. phoxini therefore show a characteristic response to an intestinal helminth infection, in that the major increase in the MMC population is due to the IEGL and not the LPMC. The situation is different in rats infected with nematodes, where sub epithelial mast cells are the more frequent responders (Askenase, 1980).

After infection with D. phoxini, both IEGL and LPMC remained stable until day 4 p.i. and then began to increase in number. There was no initial decline in the MMC population as has been observed in rats infected with N. brasiliensis (Keller, 1971; Befus et al, 1979) and with mice infected with T. spiralis (Ruitenberg and Elgersma, 1976). Both Keller (1971) and Ruitenberg and Elgersma (1976) suggested the decline was caused by a MC degranulating factor released by the worm as observed with Ascaris suis (Uvnas and Wold, 1967). No such decline has been observed in rats infected with S. ratti (Mimori et al, 1982) and the authors concluded that such a degranulating factor may not exist in S. ratti. This is possibly true for D. phoxini.

After day 4 p.i., both the IEGL and LPMC numbers continue to increase and both reach peak numbers at the same time, although there is no distinct peak in the LPMC population during the 1° infection. In contrast, Ruitenberg

and Elgersma (1979) observed that IEGL reached peak numbers on day 7, earlier than the LPMC which reached their peak values on day 13. The fact that in the D. phoxini model, both cell populations peak at the same time suggests that the cells are not two different populations, but that the IEGL are simply degranulating LPMC as suggested by Miller (1981), and both cell types are responding to the same stimulus. Ruitenberg and Elgersma (1979) concluded that both cell types were different.

Maximum values during the 1^{o} and 2^{o} infections were attained after the worms had been expelled. This situation has been observed in several other models, as in NIH mice infected with T. spiralis (Alizadeh and Wakelin, 1982), mice infected with H. diminuta (Andreassen et al, 1978), S. ratti in rats (Mimori et al, 1982) and with N. brasiliensis in rats (Keller, 1971; Kelly and Ogilvie, 1972; Befus et al, 1979). This may suggest that these cells are not of importance in the expulsion mechanism. This argument has continued since the findings of Keller (1971) who failed to relate intestinal histamine and MC levels with expulsion of N. brasiliensis in rats. However it is possible that the MMC important to parasite expulsion may arise and degranulate immediately, and may not be recognisable by the staining technique used, especially in the early stages of expulsion, or that the MMC may be involved in the later stages of expulsion and basophils may be involved in the earlier stages (Askenase, 1980). Basophil infiltration which precedes mucosal mastocytosis in N. brasiliensis infected rat intestine is only detectable in plastic embedded tissues (Miller, 1982).

The MMC response is now known to be host strain dependant and Miller and Jarrett (1971) did show that the MMC increase paralleled expulsion of N. brasiliensis in Hooded Lister rats. Keller (1971) used the Osborne-Mendel strain and Kelly and Ogilvie (1972) and Befus et al (1979) used Lewis rats, which show a different MMC response to the Hooded Lister strain.

A similar strain difference has been observed in mice infected with T. spiralis (Alizadeh and Wakelin, 1982), who observed that BlOG mice had a lower MMC response when compared with NIH or DBA_1, and also the increase in MMC numbers in the BlOG occurred later than in the other two strains. BlOG are known to be 'slow responders', expelling T. spiralis later than NIH (Wakelin and Donachie, 1981).

It may therefore be possible to obtain a mouse strain where a temporal relationship between D. phoxini expulsion and MMC production occurs. The fact remains that in NIH, the MMC continue to increase after worm expulsion, and although they respond to the infection no evidence is given as to their role in the expulsion mechanism.

The IEGL and LPMC response to a challenge infection of D. phoxini are characteristic of a 2^o response with cells reaching peak numbers earlier than in the 1^o. However the IEGL did not reach higher numbers in the 2^o infection. Again, as in the 1^o infection peak numbers were reached after the worms were expelled.

Both IEGL and LPMC increased in numbers due to transferring IMLNC. The effect of the cell transfer was to accelerate the increase in the IEGL and LPMC response by about 3 - 4 days, but this response was dependant on the presence of D. phoxini. This has also been observed in rats given ITDL. Unless they were challenged with N. brasiliensis, no MMC response occurred (Nawa and Miller, 1979) and similar results were recorded by Befus and Bienenstock (1979) in the same model but transferring IMLNC, and with T. spiralis in NIH mice given IMLNC (Alizadeh, 1981).

The role of the transferred IMLNC is speculative, but there is definitely a factor present in the cell population capable of stimulating MMC production in the presence of the homologous worms. Befus and Bienenstock (1979) demonstrated that worm antigen did not account for the mastoppietic activity of IMLNC on the basis that antigen administered with concurrent infections of N. brasiliensis did not influence mastocytosis and also antigen could not be detected in the cell suspension. Their evidence suggests that the IMLNC population is a rich source of MMC precursors and MMC have been cultured from IMLNC more readily than from CMLNC (Denburg et al, 1980). However it is also possible that 'helper cells' may be present in the IMLN after infection (Befus and Bienenstock, 1979) which are involved in MC differentiation and the results of Nawa and Miller (1979) support this hypothesis. They showed that the ability of day 10 ITDL to transfer the MMC response to recipient rats resided in the sIg^- i.e. T cell population after fractionation, and is compatible with the theory that MC are derived from T

cells (Burnet, 1977), but because the recipient animals were not irradiated, it is possible that the transferred sIg^- cells acted as 'helper cells' for the in situ differentiation of MC from host stem cells (Nawa and Miller, 1979).

The transfer of CMLNC also seemed to stimulate a slight IEGL and LPMC response in the presence of D. phoxini. This has also been observed by Befus and Bienenstock (1979) who showed that rats which had received CMLNC had significantly higher MMC levels than those given medium alone when challenged with N. brasiliensis. This suggests that the mastopoietic factor is present in the CMLNC but not in as great a quantity and/or as active as in IMLNC.

As IMLNC transferred into recipients which were not challenged with D. phoxini had no effect on the IEGL or LPMC numbers in the recipients, it shows the importance of the presence of the worm to the response, and that the effect of the mastopoietic factor in the IMLNC population cannot proceed without further antigenic stimulation from the worm. This is evidence that the response of the recipient is also important, and that the transferred cells interact with the recipient cells to accelerate MMC productions.

In conclusion no evidence is given as to the role of the IEGL and LPMC in the expulsion of D. phoxini. The cells however do respond to the infection by increasing their number, and the pattern of this response is similar to other

nematode models, i.e. the cells reach their peak after worm expulsion has occurred. Also, the response can be accelerated by the transfer of IMLNC, but only in the presence of the worms. This is again similar to the situation observed in the more common nematode models.

Summary

1) No measurable increase in GC numbers occurred during infection with *D. phoxini*, even after the transfer of IMLNC.

2) A large increase in IEGL numbers in response to infection was observed, and this response reached its peak after worm expulsion.

3) A slight but significant increase was observed in the LPMC population during infection, again the highest number of cells were present after expulsion.

4) Both these responses occurred earlier in response to a 2^{o} infection but the response was not necessarily greater.

5) The transfer of IMLNC accelerated the IEGL and LPMC response but only in recipients challenged with *D. phoxini*.

GENERAL DISCUSSION

The work presented in this thesis has demonstrated that *D. phoxini* in the mouse can be used successfully as a model for the study of intestinal immunity to adult trematodes.

The work in chapter 1 demonstrated that *D. phoxini* can establish successfully in the mouse intestine, grow and reach sexual maturity, which is good evidence that conditions in the mouse gut are quite suitable for the parasite. The parasite is then lost, usually between days 5 and 6 p.i. in heavy (200 m) infections but later, after day 8 p.i. in light (20 m) infections. The most important observation in this chapter with regards to the presence of an immune response by the host was that a 2^{o} infection of *D. phoxini* was expelled faster than a 1^{o} infection, although establishment of the parasite was not affected. This was good evidence that loss was immunologically mediated and that 'memory cells' exist in the system.

The remaining chapters were therefore designed to investigate the various components of the response of the host to the infection. Aspects of the lymphocyte, antibody and myeloid cell responses were each investigated in turn, to determine the response of each component to the infection.

Chapter 2 was concerned with the role of lymphocytes, particularly those of the MLN in rejection. Lymphocytes which were either present in the MLN at the time of infection or which had arrived as blast cells from the intestine as a response to the worms, divided and increased their numbers. At least some of these cells were capable of accelerating

rejection when transferred into syngeneic recipients. However, the ability of the MLN cell population to transfer immunity declined with time, and cells taken on day 12 p.i., a time when cell division was low, were not able to accelerate rejection in recipients, suggesting that the blast cells were important in transferring adoptive immunity.

In most host-parasite systems studied, it is normally T cells that are important in the responses especially as far as transferring immunity adoptively is concerned. However cell fractionation studies suggested that enriched B cell populations could transfer a significant degree of protection. These populations still contained large numbers of T cells which were also probably involved in the response. Although the IMLNC were capable of transferring immunity, no evidence of how they were involved in the immune response was provided in this chapter.

The levels of antibody in the serum during infection was also investigated and this showed the pattern of a classical immune response. During the 1^{o} infection, an increased titre was observed reaching peak values after expulsion had occurred. The antibody titre then declined. In response to a 2^{o} infection the antibody titre increased to a higher level than during a 1^{o} infection and reached its peak earlier. The IFAT showed that this antibody response was composed mainly of IgG although some IgM was involved. As in the previous chapter no evidence for the role of this antibody was provided.

The presence of antibody on the tegument of <u>D. phoxini</u>

was demonstrated in chapter 4. This was probably specific antibody as neither IgG nor IgM could be demonstrated on the tegument of 1 day old worms but was observed on the tegument by day 5. IgA was demonstrated on the tegument of both 1 day and 5 day old worms and presumably therefore some of this IgA was non specific and probably played little part in the expulsion mechanism.

The antibody on the tegument of day 5 worms could arrive in the intestine from the serum, presumably by some sort of pathatopic potentiation or/and by damage to the gut epithelium caused by the worms themselves, by both physical and chemical means. It is also probable that local increases in IgG and IgM production by plasma cells was responsible for some of the IgG and IgM observed on the tegument of day 5 worms. No evidence was obtained of any role for this antibody and indeed antibody has not been shown to directly cause the expulsion of parasites, but may perhaps by metabolic interference render worms susceptible to subsequent components of the protection response (Wakelin, 1978).

The role of non specific effector mechanisms was investigated in the final chapter. Hyperplasia of MMC was observed in response to both a 1^{o} and 2^{o} infection and the greatest response was observed in the IEGL populations. Both IEGL and LPMC reached their peak numbers after expulsion of the parasites had occurred, although numbers were increasing during the infection. The transfer of IMLNC accelerated the IEGL and LPMC response, resulting in larger cell numbers than in the controls which had been infected but had not received

IMLNC.

There is therefore, in response to infection a lymphocyte, antibody and myeloid cell response by the host, responses which are common to several other host-parasite models. The response is therefore a complicated one and it is probable that other factors not investigated in the present work such as eosinophils, neutrophils, basophils, oedema and other inflammatory changes of the intestine and biologically active factors eg. lymphokines and prostoglandins may be involved in the response.

There is probably a great deal of interaction between each of these systems to bring about expulsion, as shown by the ability of transferred IMLNC to accelerate production and increase the number of IEGL and LPMC. The transfer of IS has been shown to accelerate production of MMC in N. brasiliensis infected rats (Befus and Bienenstock, 1979), and it is possible that antibody produced in response to D. phoxini may act indirectly on the worms possibly via the MMC or other myeloid cells.

Both the lymphocytes and antibody could also act in other ways. By producing lymphokines, T cells are able to attract other effector cells to the areas of inflammation eg. neutrophils, macrophages, basophils and eosinophils and the arrival of these cells would provide many of the elements known to damage the worms in vitro (Askenase, 1980). However there is little evidence of their action in vivo. T cells can also control antibody production via plasma cells.

The presence of antibody on the tegument could provide a means of attack for antibody dependant killing mechanisms via C or cells eg. eosinophils (Butterworth et al, 1975) or neutrophils (Dean et al, 1974). Although such mechanisms may operate in vitro, even against intestinal nematodes such as N. brasiliensis and T. spiralis (McLaren et al, 1977) there is no evidence that the same mechanisms operate in vivo.

The problems which need to be overcome to elucidate the rejection mechanism of D. phoxini appear to be common to nematode and cestode models eg. the role of antibody, the post rejection increase of the IEGL and LPMC populations and their role. As these problems are similar, it is probable that the answers to such problems will also be found to be common to each model. There are however aspects of the response of the mouse to D. phoxini which do merit further investigation, in particular the role of B cells in adoptive immunity. Also the role of GC should be further investigated especially to see if mucus production is increased, particularly as some flukes were observed entrapped in balls of mucus during recovery as observed during N. brasiliensis infection in rats (Miller et al, 1981) and T. spiralis infection in rats (Lee and Ogilvie, 1980). Also oedema of the intestine was observed in many but not all mice during rejection, particularly on days 5 and 6 p.i. during heavy (200 m) infections. During oedema the intestine appeared turgid and the lumen was full of fluid and appeared quite clean, i.e. it was devoid of food particles. This could prove to be a fruitful area for further investigation.

Before proceeding with further research, there is an obvious need to re-establish the model, as in later work, problems arose with parasite establishment which did not improve with time. This suggests that the cause was not seasonal as first thought, and could therefore be due to some genetic mutation in the NIH mice bred at the laboratory. A further investigation of the kinetics of D. phoxini in different strains of mice and particularly in other sub strains of NIH may prove fruitful. Equally the problem could be with the parasite, and related to the decline in the metacercarial infection of the minnows which was observed over the 3 years. Possibly a change in fishing location may be the answer, otherwise there is very little one can do to solve the problem.

In conclusion, if D. phoxini can be successfully re-established in mice, it can provide a good model for the further study of host immune responses to an intestinal parasite and in particular expand the very limited knowledge of gut immunity to intestinal trematodes. Some of the host responses observed in the current work are common to other well known systems, but some are not and all merit further investigation to help unravel the extremely complicated response of the host to a parasite infection.

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