STUDIES ON IMMUNITY TO *TRICHINELLA SPIRALIS* IN MICE.

A thesis submitted for the degree of Doctor of Philosophy from the Faculty of Medicine of London University.

by,
E.R. James, B.Sc., M.Sc.,
from the Department of Medical Helminthology,
London School of Hygiene and Tropical Medicine,
Keppel Street, (Gower Street),
London WC1E 7HT.

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To those mice, rats and rabbits

which science has consumed.

We need another and a wiser and perhaps a more mystical concept of animals. Remote from universal nature, and living by complicated artifice, man in civilisation surveys the creatures through the glass of his knowledge and sees thereby a feather magnified and the whole image in distortion. We patronise them for their incompleteness, for their tragic fate of having taken form so far below ourselves. And therein we err, and greatly err. For the animal shall not be measured by man. In a world older and far more complete than ours they move finished and complete, gifted with extensions of the senses we have lost or never attained, living by voices we shall never hear. They are not brethren; they are not underlings; they are other nations caught with ourselves in the net of life and time, fellow prisoners of the splendour and travail of the earth.

Henry Beston. The Outermost House.

ABSTRACT.

A 'dual antibody' basis for acquired immunity has been suggested for Trichinella spiralis infections. Adult T. spiralis recovered from mouse intestines were cultured for 24 hours in medium 199 with serum. Newborn larvae born during this time were injected intravenously into mice to produce infections of the parenteral stages. 'Anti-adult' immunity was produced by eliminating adult worms with methyridine before newborn larva production commenced. The immune state of the host was gauged from the numbers of muscle larvae encysting from a challenge complete infection or from the numbers and size of adult worms in the intestines. 'Anti-adult' immunity was 86-95% effective against a challenge complete infection but 0-16% effective against a parenteral challenge infection. Adult worms in immune mice were stunted and expelled earlier. 'Anti-parenteral phase' immunity was 74-100% effective against a parenteral challenge but 27-63% effective against a complete infection, and no obvious effects of immunity occured against adult worms in the intestines. Newborn larvae were shown to be antigenic by their capacity to react with antibody in in vitro culture and in the IFAT, however, attempts to immunise mice with antigen prepared from newborn larvae or by cambendazole abbreviated newborn larva infections were unsuccessful. Attempts to detect the occurence of stage specific cell mediated responses were inconclusive but IFAT and in vitro serum culture techniques indicated a stage specific difference between the three life cycle stages tested. Adult worm and newborn larva cuticular antigens appeared to cross-react and to be different from those of muscle larvae while newborn larva and muscle larva E and S antigens appeared to cross-react and to be different from those of adult worms. Mice challenged with newborn larvae injected 14 days after a normal infection contained more muscle larvae than the challenge and the infected/non-challenged controls added together. It is speculated that the intestinal and parenteral phases could interact to partially suppress the immune response developing in a normal infection.

Acknowledgements.

I would like to express my gratitude to Professor G.S. Nelson, in whose department this work was undertaken, for his understanding and encouragement. This thesis owes much to Dr. David Denham to whome I am especially grateful, who devoted much time, interest and advice, and who also largely facilitated my attendance at the Third International Conference on Trichinellosis.

Money is an essential ingredient for any research and to this end I am grateful to the Medical Research Council for their financial support throughout my study and also to the British Council and Dutch Government.

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Many people, whittingly or not, have contributed either technical assistance or necessary encouragement, or both, and here I would like to thank particularly the late Dr. Spooks Duckett and also Barry Andrews, Tony Daniels, Rob Holdstock, Brenda Moult, Dr. Rosy Murphy, Dr. Kingsley Opuni, Dr. Thivi Ponnudurai, Dr. Mary Smith and Dr. Chris Teesdale, and all those who won't be offended to be included as 'others'.

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Typing has proved to be no small problem; this task was initiated by Chris McCauley while the major part was coped with admirably by Renita Somers.

One's work has the habit of occasionally following one around and becoming a burden to others, for these times I would like to thank my parents and especially Rosey, for their love help and understanding.

CONTENTS.

Key to abbreviations used.

ALS	-	anti lymphocyte serum
ATS	-	anti thymocyte serum
B cells	-	'B' lymphocytes, similar to Bursa of Fabricius derived cells of birds.
BSA	-	bovine serum albumin
CFT	-	complement fixation test
CMI	-	cell mediated immunity
E and S	-	excretions and secretions
FAT	-	direct fluorescent antibody test
FITC	-	fluorescein isothiocyanate
GAR	-	goat anti rabbit
HA	-	haemagglutination test
ID	-	intradermal test
IFAT	-	indirect FAT
Ig	-	immunoglobulin (A,G,M,D,E)
IP	-	intraperitoneal
IV	-	intravenous
PBS	-	phosphate buffered saline
PCA	-	passive cutaneous anaphylaxis
pH	-	$-\log_{10} cH^{+}$
PI	-	post infection
PTFE	-	poly tetrafluoroethylene
RAD	-	unit of absorbed dose of ionising radiation
SPF	-	specific pathogen free
T cells	-	'T' lymphocytes, thymus derived
UV	-	ultraviolet

PART 1.

INTRODUCTION

FOREWORD

Trichinosis is of minor importance as a helminth disease of man, but it does provide a very convenient laboratory model to study various aspects of the immunology of helminth infections. Nematode infections usually comprise both a phase of larval migration and an adult propagative phase. The life cycle of Trichinella spiralis is roughly divisible into the intestinal and parenteral phases, each of which may play a different role in the immunology of a natural infection.

This thesis is presented as a study of some of the specific and the interacting immunological mechanisms associated with the phases of the life cycle.

The introductory chapters present reviews of the literature relating to the current understanding of the life cycle of the parasite (chapter 1) and of the immunology of the disease (chapter 2), while a short review of the more recent immunological advances in trichinosis introduces chapter 7.

The results reported in each experimental chapter in the results section are discussed individually and all findings are summarised at the end of that section.

An attempt has been made to present a fairly lucid 'materials and methods' section and thus some of the minor digressions, into the experimental reasons why certain techniques were employed, have been removed to the appendices in section 5.

The experimental work was designed with the following main objectives:

1. To confirm that protective immunity will develop to the intestinal phase without the presence of the parenteral phase.
2. To demonstrate that protective immunity develops to the parenteral phase in the absence of the intestinal phase.
3. To determine whether or not the immunity developed to each phase is stage specific.
4. To ascertain whether the migratory phase is antigenic/immunogenic.
5. To determine whether stage specific CMI and humoral responses can be detected during the course of a normal infection.
6. To determine whether there is a co-operative interaction between different life cycle stages in the host which may act to the benefit of the parasite.

The experimental evidence presented here indicates that objectives 1 - 4 have been completed. Objectives 5 and 6 have not been completely achieved, but a basis for considerable further research has been set.

CHAPTER 1 - LIFE CYCLE

Trichinella spiralis was first described in 1835 when James Paget, a medical student of St. Bartholomew's Hospital, observed the cysts in the muscles of an Italian who had died of pulmonary tuberculosis. The parasite was named Trichina spiralis in that year by Owen and later re-named Trichinella spiralis by Railliet in 1895.

The cycle of infection in most countries is restricted mainly to pigs and rats with sporadic infections of man, however, feral life cycles can exist as in Germany and Poland, where foxes, badgers, mink and other forest animals are implicated as reservoir hosts. Up to 26% of wolves have been found to be infected in Poland. The Arctic region has a dominantly feral cycle of transmission and arctic foxes, wolves, seals and walruses have all been found to harbour T. spiralis while the chief source of infection for man in this region is the polar bear. Since the first recorded occurence of human trichinosis in Africa in 1959 (Forrester, Nelson and Sander, 1961) the sylvatic reservoir hosts of the parasite have been found to include the bushpig, lion, hyena, jackal, serval and leopard, (Nelson, Guggisberg and Mukundi, 1963).

With the existence of these different and geographically divided zoonoses, it is not surprising that T. spiralis is considered to incorporate several strains. Britov and Boev (1972 and 1973) and Garkavi (1972) have suggested that the genus may comprise four sibling species -

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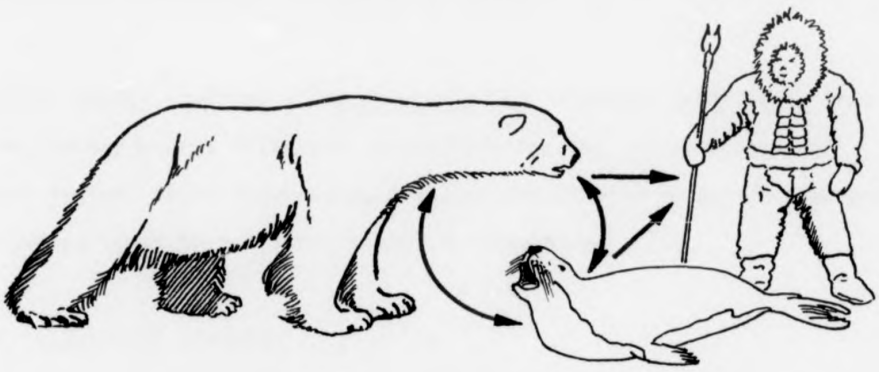
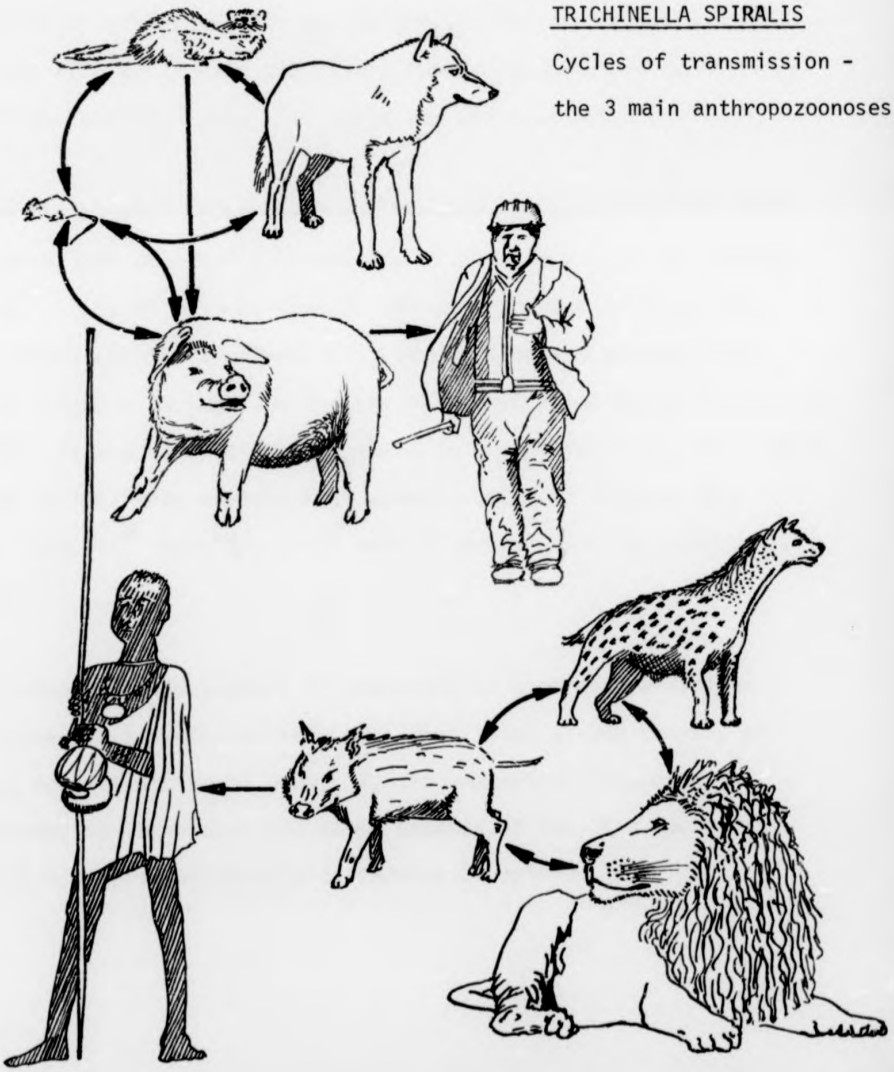




TRICHINELLA SPIRALIS

Cycles of transmission -

the 3 main anthropozoonoses

spiralis, nativa, nelsoni and pseudospiralis. Although Trichinella from one region may have a different infectivity for the sylvatic hosts of another region, it is interesting that the infectivity to man is apparently retained at least in the first three of these 'species'.

The Life Cycle of Trichinella spiralis

Because a large part of the experimental work which follows in later chapters is concerned with the interrelationships of the different stages of the life cycle with the host's immune response, it is necessary to describe the stages of the life cycle and their development.

From the studies of Leuckart and Virchow, it was discovered that encysted larvae are released by digestion of the capsule in the stomach of a new host. The worms then move to the small intestine where they mature and mate, and the female worms give birth to second generation larvae which migrate through the tissues to the striated muscles where they encyst. This is the bare outline of the life cycle, and since the studies of Leuckart in 1857 many workers have added details to improve this knowledge; however, there are still several areas where the evidence is conflicting.

The stages of development of nematodes in general are divided from each other by a series of ecdyses. The timing of the ecdyses of T. spiralis and the status of each stage in comparison with other parasitic nematodes is uncertain, and these aspects of the life cycle are still the objects of considerable difference of opinion.

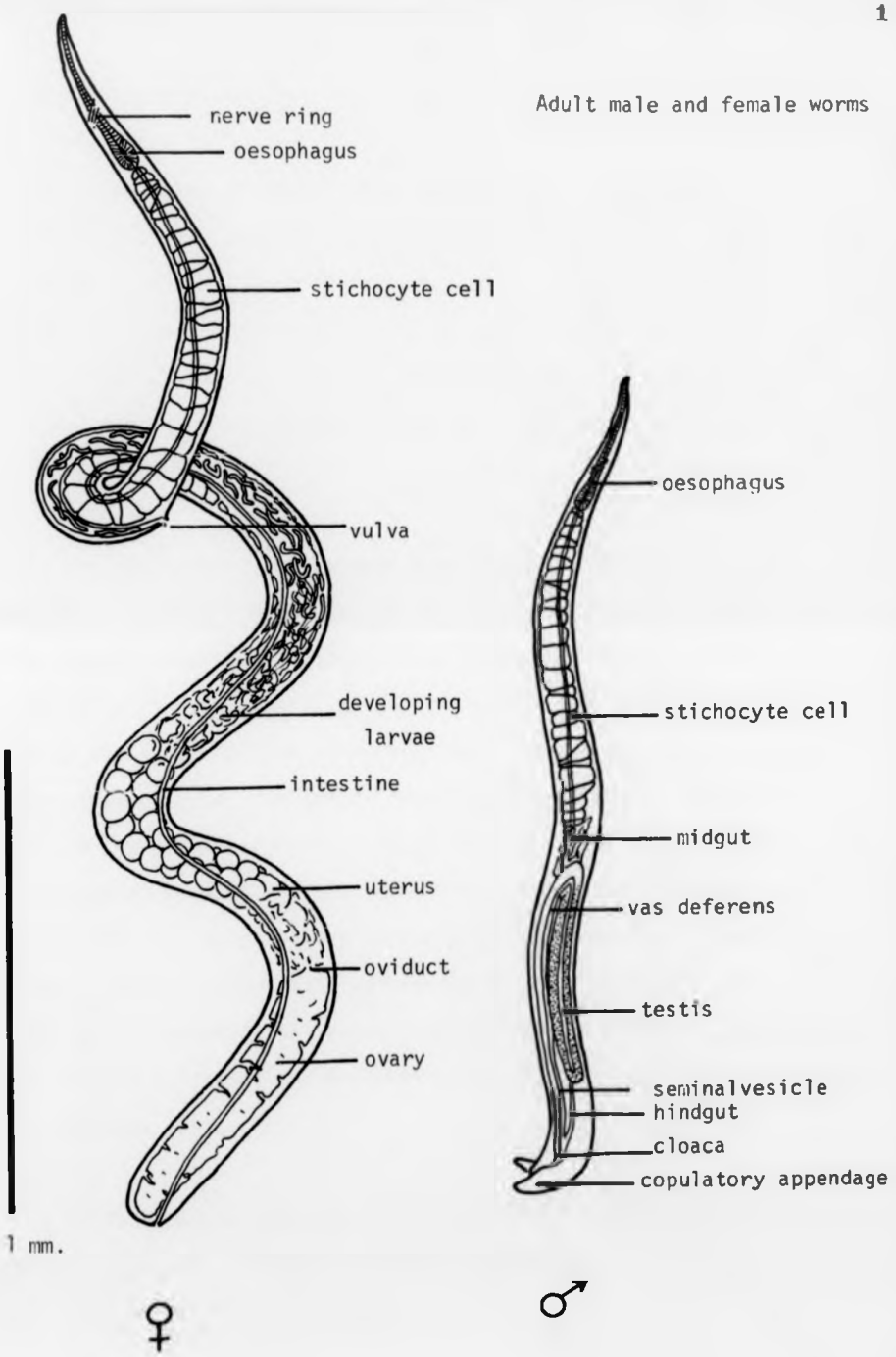
The Intestinal Phase:

The action of pepsin with hydrochloric acid in the stomach of the host digests the infected muscle and cyst wall, and the muscle larvae are liberated. These larvae resist peptic digestion and are infective if they have been encysted longer than 17 to 21 days (Nolf and Edney, 1935; Phillipson and Kershaw, 1960; Thomas, 1965; Ali Khan, 1966; Shanta and Meerovitch, 1967b).

Berntzen (1965) regarded the life cycle as typical for a nematode as he claimed there were three moults prior to, and one moult during the intestinal phase; but his evidence differs from that of Villella (1958), Ali Khan (1966), and Kozek (1970 and 1971) who found four intestinal moults. The moults (Villella, 1958) were observed to occur in females at approximately 6, 12, 18 and 24 hours and in males at 12, 18, 24 and 30 hours. Thus infective larvae which enter the intestine can be regarded as 1st stage larvae or 3rd stage larvae according to whether any ecdyses are considered to have occured in the pre-intestinal development. Gursch (1949) found that after one hour the excysted larvae moved from the stomach to the small intestine and that after two hours had penetrated the intestinal mucosa, and had reached the muscularis by three hours. By 20 hours most worms had moved back into the lumen and from there they re-penetrated the mucosa sometime around 48 hours and came to lie between, and towards the base of, the villi near the crypts of Leiberkuhn.

The ratio of female to male worms varies considerably both during the course of an infection and in the reports from different authors, but a generally accepted belief is that early in an infection

Adult male and female worms



the ratio is approximately 1 to 2 female worms to 1 male worm.

Insemination of female worms commences between 30 and 32 hours after infection and both insemination and ovulation appear to have occured by 37 hours (Wu and Kingscote, 1957). Insemination of female worms is believed to occur more than once (Kozek, 1972). During insemination, Gould, Gomberg, Villella and Hertz (1957) found that the posterior regions of the female and male worms were free of the mucosa and lying between the villi.

The adult worms continue to grow and the females, at least, reach their maximum size at around the 10th day, whereafter the effect of the immune response (Chandler, 1936a; Semrad and Coors, 1951; Shikobalova, 1953; Campbell, 1955; Kim, 1957; Denham, 1969), and possibly the effect of the production of larva (Kozek, 1972 - personal communication) cause a slight decrease in their size. The adult worms remain in the intestines for a length of time which depends on the interaction of several factors affecting the intestinal environment (see part 2), but particularly on the developing acquired immunity of the host. Thus in previously uninfected mice or rats the usual length of stay of the worms in the intestines is between 12 and 18 days (Gursch, 1949; Larsh, Gilchrist and Greenberg, 1952; Larsh, Race and Jeffries, 1956; Denham, 1968).

It is probable that the adult intestinal T. spiralis feed to a varying extent both on the host cells and on the contents of the intestinal lumen.

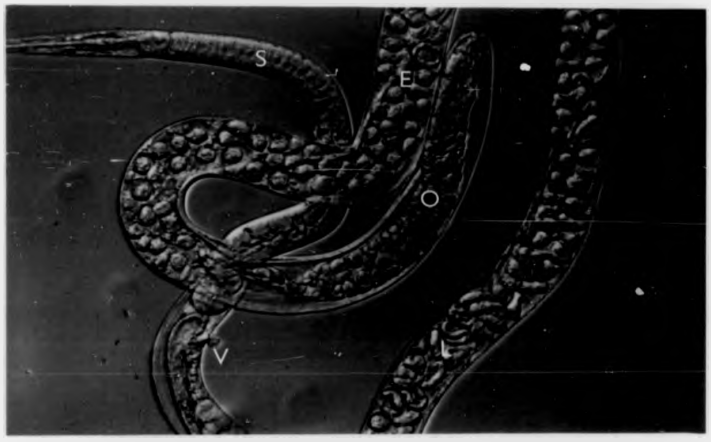


Plate 1. Adult female T. spiralis x150 showing ovary (o), developing embryoes (e), unborn larvae (l), vulva (v) and stichosome (s).

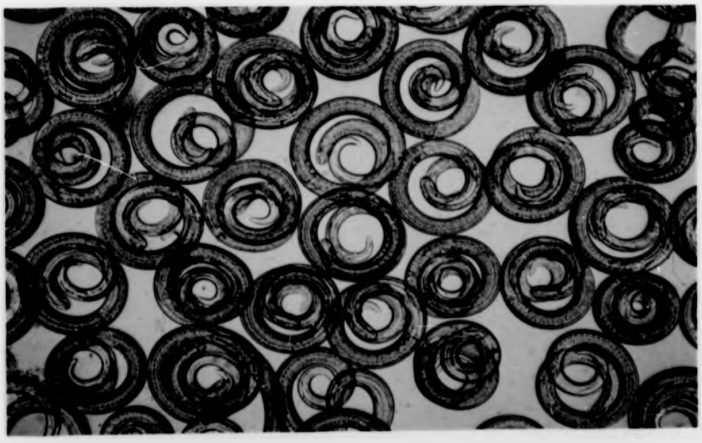
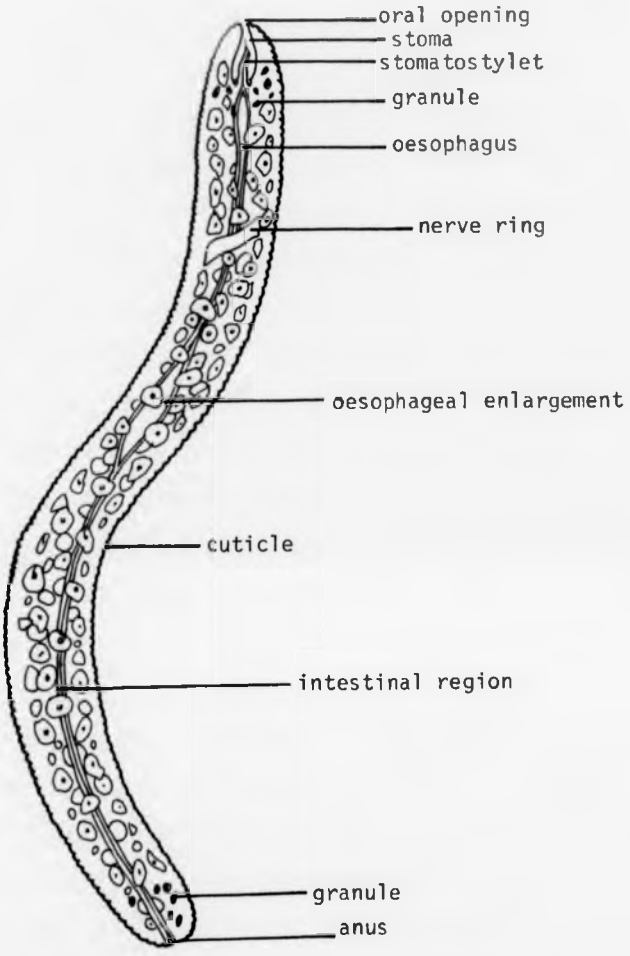


Plate 2. Muscle larvae excysted from artificial digestion x100.

The term 'pre-adult' has been applied to worms up to 48 hours after ingestion into a host by Denham (1965), Larsh (1970), and Harley and Gallicchio (1971a) while 'adult' has been used frequently to describe the mature intestinal worms, although Shanta and Meerovitch (1967a) have suggested the 'adults' could be sexually mature 3rd stage larvae which have evolved through neoteny. These terms, together with 'infective larvae' (excysted larvae at the time of ingestion), are embraced by the synonymous terms enteral phase and intestinal phase. The terms pre-adult, adult, and intestinal phase will be used throughout where these are appropriate.

Newborn Larvae

T. spiralis is ovoviviparous; the embryo develops inside its egg shell to become a juvenile or nymph and hatches from the egg in utero before being born. Berntzen (1965) maintains that the development of these juveniles includes one ecdysis within the egg shell. As the embryos pass anteriorly along the uterus they progressively develop and hatch, and the first 'newborn larva' emerges from the vulva sometime around the 5th day after infection. Again, the many factors which must affect the local environment in the intestine must produce variations in maturation times of the female worms for the time of the commencement of larviposition has been claimed to be 80 to 90 hours by Heller (1933), 96 hours by Gould et al. (1955), and sometime between the 5th and 6th days of infection by Britov (1962). The experiments of Denham and Martinez (1970) using methyridine to abbreviate the infection in mice and those of James (1971) suggest that at least in certain strains of mice the release of newborn larvae starts just before 120 hours post infection. The birth and migration of these larvae initiates the



Newborn larva (after Harley, 1970).

migratory stage of the parenteral phase of trichinosis.

The newborn larvae are liberated from the female worm at an average rate of one every half hour (cited by Gould, 1945), but this rate of production fluctuates during the course of an infection (see appendix 6). The number of larvae produced per female again depends on several variable conditions and consequently different authors have claimed figures ranging from 91 to 625 larvae per female worm by Thomas (1965), 200 to 400 larvae by McCoy (1932), 1300 to 1500 by Roth (1939) and up to 10,000 by Braun (1895, cited by Villella, 1970). Wolffhugel (1938) infected a rat with one female and one male worm and recovered 200 second generation larvae from its muscles, while Nolf (1937) recovered 1,112 larvae from a rat which had received one transplanted gravid female worm.

At birth the larvae measure 5 to 6 micrometers (μm) in width and are on average 95.06 μm in length (Harley and Gallicchio, 1971b). They are cylindrical, have blunt ends and an anterior indentation or stoma through which the buccal stylet or stomatostylet is actively protruded and retracted in live specimens (Harley, 1971c). Apart from the anterior region, Ali Khan (1966) considered newborn larvae to be similar to microfilariae and to be composed mainly of undifferentiated cells and lacking a fully developed alimentary canal. However, Wu (1955) found a muscular oesophagus was present, and Harley (1971c) found evidence in his studies for the presence of a complete alimentary canal.

During the course of their migration, some of the morphological

features develop further, particularly the oesophagus, and Harley and Gallicchio (1971a) found that the larvae progressively increased in length during their migration and were on average 106.60 µm long when they reached the skeletal muscles. This contrasts with the statements of Levin (1941), Gould (1945) and Phillipson and Kershaw (1960) who said that the larvae do not grow until they reach the muscles - although no length measurements were made by them.

Thus it would appear that the newborn larva is not a simple undifferentiated intermediate migratory stage, but rather that it is highly motile, equipped with a 'boring apparatus' and an alimentary canal; it can actively burrow through host tissues and it does grow and differentiate during this brief period.

Gravid adult female worms are considered by Harley and Gallicchio (1971b) to contact the capillary networks of the intestinal mucous coat, or those surrounding the central lacteals. They penetrate the villi, or less likely the crypts of Lieberkuhn, and there they deposit the newborn larvae. The larvae then make their way to the voluntary muscles by any one, or all, of three different routes.

Route 1: The newborn larvae penetrate the lymph capillaries and via the thoracic duct go to the jugular vein where they join the blood system and are disseminated to all parts of the body.

Route 2: They pass directly into the venous capillaries and are carried via the hepatic portal vein to the liver, thence to the heart and all parts of the body.

Route 3: This route involves continuous direct active migration on the part of the larvae which burrow through the muscularis mucosa and through the abdominal cavity to the striated muscles.

Most reports (Cerfontaine, 1895; Staubli, 1909; Chandler et al., 1941; Matoff, 1967; Harley and Gallicchio, 1971b) favour route 1 as the primary route of migration, and consider the others to be secondary routes. Berntzen (1965) and Shanta and Merrovitch (1967b) claimed that the majority of newborn larvae migrated by way of the connective tissues to the voluntary muscles, but as Harley and Gallicchio (1971b) point out....'the mere presence of migratory larvae in any given body site does not warrant the conclusion that this is their main pathway'.

During the early part of the intestinal infection, small numbers of newborn larvae were isolated from the body cavities (Matoff, 1940; Berntzen, 1965; Shanta and Meerovitch, 1967) and from the blood (Matoff, 1943; Gould et al., 1955; Phillipson and Kershaw, 1961) of host animals. Berezantsev (cited by Villella, 1970) found haemorrhages in the lungs, brain, muscles, heart and other organs of mice during the period of larval migration, while Kershaw (1954) has stated that central nervous system involvement is not uncommon in human patients. Berezantsev considered that the migrating larvae burrowed out of these tissues and returned to the venous portion, but Gould (1945) noted that the diameter of these larvae was less than that of a human erythrocyte, so presumably they should easily be able to pass through a capillary network.

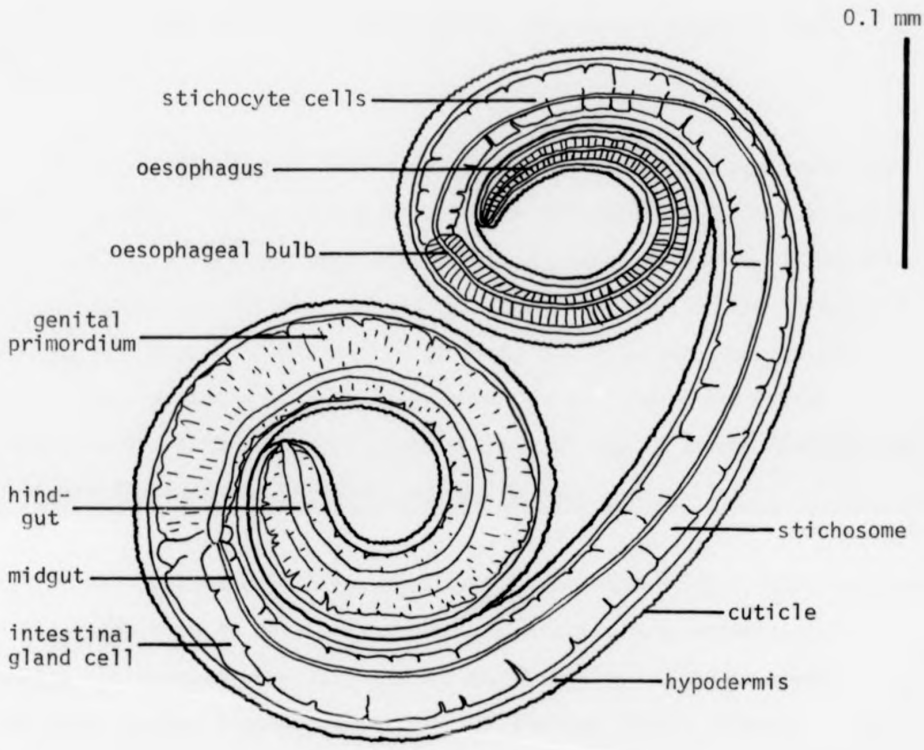
Harley and Gallicchio (1971b) performed a comprehensive study of the possible migration routes of the newborn larvae and concluded that route 1 via the thoracic duct to the blood accounted for 70% of the distributed larvae and that both the other routes together accounted for the remaining 30%. In their studies of the differences in infectivity between strains of T. spiralis, Nelson, Blackie and Mukundi (1966) found up to 237 migrating larvae/ml of blood from rats infected with 5000 Polish strain T. spiralis.

Dennis, Despommier and Davis (1970) showed that 70% of newborn larvae injected intraveinously (IV) into rats reached the muscles, while 9% of intraperitoneally injected larvae did so.

The terms newborn larvae and migratory larvae will be used throughout in descriptions of this phase of the life cycle.

Muscle Larvae: The reasons for the selection of striated muscles by the migratory larvae as a site for encystment is unknown but it has been suggested that some aspect of the metabolism of striated muscle may 'attract' the larvae (Vogel, cited by Villella, 1970). Ogielski (1949) considered that larvae show a prediliction for certain of the striated muscles because of the 'exceptional' density of the capillary networks of these muscles. Apart from lesions and haemorrhages and the occasional recovery of migratory larvae from other body tissues (Harley, 1971c) including cerebro-spinal fluid (Lintz, 1916), 'muscle' larvae have been recorded as developing normally exclusively in the striated muscle.

Migratory larvae have been reported from the muscles as early as day 5 post infection by Harley and Gallicchio (1971d) and by day 6 to 7 by Britov (1962). In theory the invasion of the muscles continues to occur while the female intestinal worms remain fecund. All striated muscles are involved, but among those generally agreed to be the most often parasitised are the



Mature female muscle larva 35 days PI recovered from digest

muscles of the neck, tongue, larynx, the extraoc ular muscles, the masseters, intercostals, diaphragm and deltoid muscle (Nelson and Mukundi, 1963, Gould, 1970a).

The migratory larvae leave the blood capillaries and penetrate the muscle sarcolemma. Their length on entering the muscle cell is approximately 107 µm (Harley, 1971d) and they grow slowly at first attaining 126 µm after 4 days in the muscle cell and 203 µm by 7 days. Growth is then more rapid until the 18th day and practically ceases by the 20th day after muscle invasion (approximately 25 days post infection (PI)) when the fully developed muscle larvae measure approximately 930 µm in length (Harley, 1971d) and between 35 and 40 µm in width (Gould, 1970a).

It was suggested by Ribas-Mujal and Rivera-Pomar (1968) that the host cell helps the worm to obtain nutrients and expel waste products. Purkerson and Despommier (1972) support this idea and their electron microscopic studies together with those of Tepemma (1973, personal communication) indicate a very close association between the parasite and the host cell cytoplasm. Soon after invasion the host cell myofilaments become disorientated and lost, the endoplasmic reticulum becomes much enlarged and the mitochondria become enlarged and more numerous. Ribosomes increase in number and are found free in the cytoplasm and on the endoplasmic reticulum, the nuclei also slightly increase in number and the 'T system' of transport tubules becomes distorted and expanded. Purkerson and Despommier (1972) found that the inner double unit cell membrane completely surrounded the worm and closely adhered to its cuticle. They found no evidence of active feeding of the larva or of any specialised area of the host cell in the oral region of the larva, and they speculated that the muscle larva absorbs nutrients through its cuticle. Thus the function of the parasitised muscle cell is transformed and Despommier (1972) believes that it

can be more correctly thought of as a 'nurse cell' ministering to the requirements of the developing muscle larva. This idea was insinuated also by the work of Stoner and Hankes (1955) and Hankes and Stoner (1958) who found an exchange of metabolites between larvae and host and by Zarzycki, Kozar and Czechowicz (1970) in their studies of the developing capsule and ^{131}I uptake.

With histochemical techniques Bruce (1970) detected rudiments of the capsule wall forming 8 days after penetration of the muscle cell by the larva. About 14 days (Tepemma, 1973, personal communication) after muscle cell penetration the sarcolemmal membranes separate and vacuoles and filaments thought to be 'collagen precursors' appear between them. This area continues to expand and collagen begins to surround the outside of the host cell in a connective tissue matrix. By day 32 after penetration the outer sarcolemmal membrane is lost or occluded and a well developed capsule exists round the parasitised cell. Despommier (1972) considered that the capsule acted as a barrier to 'wandering immunocytes', but Tepemma has observed small numbers of monocytes and macrophages penetrating the capsule wall and also present in the host cell although they appeared not to have attacked the larva which by then was fully developed. Ogielski (1949) noticed that after encystment the parasitised muscle cells were almost completely surrounded by networks of 1st, 2nd and 3rd order capillaries which had developed from pre-capillaries.

Encystment of the muscle larvae completes the life cycle, and in natural transmission cycles these larvae lie dormant to await ingestion by a new host.

CHAPTER 2.

Stage Specific Immunity

There have been a considerable number of publications concerned with the immunological response of the host to T. spiralis infections. Gould (1945 and 1970) has reviewed the broader aspects of the knowledge of T. spiralis, while the immunological and serological aspects have been reviewed by Kagan (1960), Larsh (1963, 1968 and 1970b), Kozar, Z (1969), Kozar, M (1973), Kagan and Norman (1970) and Duckett (1971).

The experimental work presented below is largely related to the immune response which develops to the different stages of the life cycle of T. spiralis. The intended purpose of this chapter is to review the literature which bears relevance to the existence of a stage specific immune response to T. spiralis infections.

The Dual Antibody Hypothesis.

The first evidence for the immunogenicity of T. spiralis was presented by Sulzer (1916), while Ducas (1921, cited by Chute, 1956) is credited with demonstrating that a primary infection could stimulate an immune reaction, and he considered that the immunity was primarily directed against the adult worms. That the immune reaction is directed against the establishment of new adults was the generally held belief (cited by Oliver Gonzalez, 1940), but Shtchoupakov (1935) had suggested that there were two defence mechanisms, one of the intestine and one of the musculature.

Oliver Gonzalez (1940) showed that serum from rabbits immunised

with homogenated larvae induced death in over 50% of larvae incubated in this serum, while there was little or no effect on adult worms. He considered that his results taken together with those of Heller (1933) and Taliaferro and Bloom (cited by Taliaferro, 1940) indicated that there was definitely an effect of immune serum, previously absorbed with homogenised r scle larvae (Oliver Gonzalez, 1941), an in vitro effect was produced around adult worms but not around muscle larvae cultured in this serum. Conversely after absorption on homogenised adult worms it would not form precipitates at the openings of adult worms but would do so for muscle larvae. To account for these observed effects Oliver Gonzalez (1941) proposed the theory of a 'dual antibody' response to T. spiralis infections.

Oliver Gonzalez considered that the life cycle stages involved in this response were the adult worms and the 'mature' muscle larvae. He considered that the 'immature' larvae (newborn migratory larvae and pre-encystment muscle larvae) resembled the adult worms antigenically and gradually acquired new antigenic characteristics as their source of food changed from the lamina propria of the intestine to the muscle cells. Most of the reactivity of the anti-adult antibody occurred from 25 to 35 days after infection, while the peak of anti-larval antibody activity occurred at approximately 50 days after infection and the level remained relatively high up to 120 days. The anti-muscle larva immune serum gave no precipitate with larvae up to 5 days old, but thereafter increased for 15 and 20 day old larvae, becoming very marked against 65 day old larvae. However, he found that 'hyperimmune' serum from rats infected 4 times did produce an in vitro effect on the 5 day old larvae. By passively transferring this hyperimmune serum from rabbits to rats, a degree of immunity to infection was conferred, and this passive immunity was still transferred when the serum had been absorbed with muscle larvae:

Therefore, passive transfer of protection was found to be independent of the anti-larval antibody.

Hendricks (1952) used T. spiralis larvae irradiated with 3,700R to infect mice, and he found that the resulting serum antibody titres to muscle larvae were lower than those for mice which had received normal non-irradiated infective larvae; the antibody titres to adult worms remained unchanged. If there is an independent antigenic stimulus from the muscle larvae then these results would indicate support for Oliver Gonzalez (1941) as a dose of 3,700 R sexually sterilises the female worms.

Support for the dual antibody hypothesis of acquired immunity was given by the work of Ivey (1956) and Oliver Gonzalez and Levine (1962). Roth (1943) considered that 'true acquired immunity' developed only as a result of the intestinal phase of infection, but he believed that the immunity could be enhanced by the migratory and muscle larvae phases.

Oliver Gonzalez and Levine (1962) obtained 2 precipitation bands in Ouchterlony tests against muscle larva antigen using immune rabbit serum and 3 bands for hyperimmune serum. With adult worm antigen 1 and 2 bands were produced respectively. There also appeared to be no lines of identity between the larval and adult precipitates to the hyperimmune serum. When the sera were absorbed with either adult or muscle larva antigens only the homologous antibodies were absorbed as the antisera still retained precipitating antibodies to the antigen which had not been used for absorption. Larval metabolic products used as antigen were found to give the same number of precipitin bands as macerated larval antigen and reactions of identity occurred between them both.

Taliaferro, Woolridge and Benditt (1949) conducted a study on how low protein diets affected development of immunity to trichinosis in rats and they found that anti-larva antibody titres were lowered when a reduced protein regime was started 7 days before infection, but the anti-adult titres were not. They ascribed the differential response to the fact that muscle larvae provide antigenic stimulation later in the infection. When the low protein diet commenced 33 days before infection both antibody titres were lowered.

Ross (1952) reported that during a primary infection the serum antibody titres against muscle larva antigen rose more rapidly than did the titres to adult antigen, and that they were consistently higher; she could find no advantage in using adult antigen for achieving early detection of T. spiralis. She does mention that it is difficult to prepare pure antigen from adult worms as the adults will contain young larvae and embryos in utero, although Oliver Gonzalez (1941) suggested that young larvae are not antigenic until born.

There have been several reports which have claimed to refute the theory of a dual antibody hypothesis. Chute (1956) could find no evidence to support the dual antibody hypothesis. He used injections of killed muscle larvae to induce an immune response which was effective against the adult worms and considered that the dual antibody theory of Oliver Gonzalez might represent a quantitative rather than a qualitative antigenic difference between the two stages. However, he did present evidence that excretions and secretions of the muscle larvae are immunogenic and also that an immune response will act directly on migrating larvae as well as on adult worms, for, when rabbit immune serum was given to rats during the migratory period the muscle larvae which resulted were stunted although not reduced in numbers.

In the sera from normally infected rabbits, Jackson (1959) could find no distinct anti-larval or anti-adult antibodies, and like Oliver Gonzalez (1940) he could find no precipitating complexes on newborn larvae. Some fluorescein labelled immune sera reacted with virtually all worm tissues in section, the major exception being the cuticle w hich Jackson held to be non-immunogenic. However, many reports now agree that the cuticle is highly immunogenic (see part 3).

Stages stimulating an immune response.

The intestinal phase - pre-adult and adult worms.

Several workers have stated that the pre-adult (Chipman, 1957; Kim, 1957; Larsh, Race and Goulson, 1959; Ewert and Olson, 1960) and adult worms (Ducas, 1921; Anderson and Leonard, 1940; Culbertson, 1942; Roth, 1943; Chipman, 1957; Kim, 1957) are the stages 'primarily responsible' for inducing an immune response in host animals, while other workers (Campbell, Hartman and Cuckler, 1963; Campbell, 1965; Denham, 1965 and 1966) have shown that the intestinal phase is at least capable of inducing a strong immune response.

The study of the role of the intestinal phase in stimulating immunity has been studied by several methods.

Single sex infections.

Roth (1943) separated the sexes of infective larvae and was able to induce a pronounced immune response in guinea pigs with only male worms which he claimed was true acquired immunity. Anderson and Leonard (1940) and Anderson (1941) also employed single sex infections; they allowed the worms to develop for 24 hours in a host by which time they would be more easily distinguishable into males and females and between 200 and 700 worms of one sex were transplanted by duodenal tube into a new host. The numbers of muscle larvae developing from a challenge infection

were significantly reduced compared with controls. Both male and female worms apparently stimulated the same degree of immunity.

Irradiation.

Irradiation can be used to sexually sterilise infective larvae so that no parenteral larval migration follows the intestinal infection, or, when the radiation dose is increased the worms can be prevented from developing past the pre-adult stage (Gould, Gomberg, Bethell, Villella and Hertz, 1955). Intestinal infections produced by giving irradiated infective larvae to animals have been shown to stimulate an immune response (Levin and Evans, 1942; Hendricks, 1950 and 1952; Hartung and Becht, 1960; Zaiman, Howard and Miller, 1961). Kim (1957) used various doses of X-irradiation to alter the growth and maturation of the intestinal worms and after 3 stimulating infections of these altered worms he measured the degree of immunity to a normal challenge infection by counting the numbers of adult worms present on the 7th day post challenge and measuring the length of the female worms. From the results he concluded that pre-adult and adult worms stimulated an immune response, while migratory larvae and muscle larvae did not appear to contribute to the total immunity.

Hendricks (1952) found the anti-T. spiralis antibody titre for mice which had received irradiated worms was lower than for mice which were given normal worms. Larsh, Race and Goulson (1959) irradiated infective larvae to prevent development past the pre-adult stage and found that 5 stimulating infections produced a level of immunity, measured by counting adult worms and muscle larvae, almost equivalent to that produced by non-irradiated larvae.

Denham (1966b) demonstrated that a strong immunity to reinfection in mice could be produced by both a single or double transplant of

irradiated adult worms. Their collection from the intestines of rats was preceded by administration of thiabendazole to eliminate the few underdeveloped pre-adults which remained as a result of the irradiation. As transplanted worms were shown to be sterile the immunity produced in the mice receiving transplanted worms was due entirely to adult worms.

Chemotherapy.

The use of anthelmintics to chemically abbreviate infections has proved to be probably the most convenient and precise method of controlling the duration of the intestinal phase of T. spiralis. The effectiveness of most of the anthelmintics against T. spiralis was the subject of a review by Zaiman (1970).

Campbell, Hartman and Cuckler (1963) induced immunity with an infection terminated with thiabendazole after 14 days, but although thiabendazole is active against the parenteral stage there would have been a considerable invasion by migratory larvae of the host muscle before that time. They were also able to stimulate an immune response by giving several repeated immunising infections terminated after 1 day. Campbell (1965) regulated the dose of thiabendazole to 0.1% of the feed of mice to induce sterility in the female worms. The immunity in treated mice caused a 69% reduction of adult worms and a 91% reduction of muscle larvae. A single large infection terminated after 20 hours reduced the number of adult worms from a challenge infection by 54% and muscle larvae by 53%, and when 6 abbreviated immunising infections were used the reduction in muscle larvae from the challenge infection reached 99%. Campbell (1965) thus suggests that not only are the pre-adults immunogenic but that if they are given in large enough numbers the maturation into adult worms does not add substantially to the induced immunity.

Methyridine was used by Denham (1966a) to terminate intestinal infections of T. spiralis after 7 hours, 48 hours and 72 hours. He found no immunity would develop after either a single or three immunising infections of 7 hours duration unless these infections were particularly heavy. A 7 hour infection is composed entirely of pre- adults(or phase I - Kim, 1957 and Larsh, 1963). He suggested that because the pre-adult stage is present for only a short time in the intestine it does not induce any immunity, but that this stage acts more to sensitise the host to antigens produced by later stages of the worm; however, he did suggest that the pre-adults produce small amounts of immunising antigens and that this stage can be immunogenic when present in the intestine in abnormally large numbers, or for a long time period as occurs when irradiated infective larvae are used to infect animals. A 48 hour infection (which includes the moults from 3rd stage pre-adult larvae to 5th stage adult worms - Berntzen, 1965) was not immunogenic, and he concluded that the chief stimulus for the immune response in the intestine comes from the adult worms.

The expulsion of adult worms from the intestines of hosts in primary and secondary infectionshas been claimed to be a phenomenon of immunity by McCoy (1931, 1932 and 1940), Culbertson (1942), Fischthal (1943), Larsh, Gilchrist and Greenberg (1952), Larsh and Race (1954), Larsh, Race and Jeffries (1956), Larsh, Race and Goulson (1959), Larsh, Goulson and Van Zandt (1962), and Larsh (1963 and 1967). The intestinal inflammation which precedes expulsion has been considered, particularly by Larsh and his co-workers, to be linked to it. Larsh (1963) suggested that vascular changes associated with inflammation could be caused by an antigen-antibody reaction and that this inflammation coupled with other factors such as decreased oxygen tension and increased carbon dioxide and organic acids (Larsh, 1963

and 1968) produce a reduction in the worm burden as a result of the unfavourable intestinal environment. Coker (1955) and Markell (1958) found that the longevity of adult worms was considerably extended in mice which had been treated with cortisone. However, cortisone is a general immunosuppressant drug and Campbell (1968) found that while mice treated with anti-inflammatory steroids did not lose their intestinal worms, mice which were treated with anti-inflammatory non-steroids did. He concluded that intestinal inflammation plays no part in the expulsion of the worms.

Larsh and Hendricks (1949) found most adult worms were located in the anterior half of the small intestine in old mice and Larsh, Gilchrist and Greenberg (1952) observed a posteriorly directed migration of adult worms down the small intestine before expulsion occured, and they considered that this migration was associated with 'self cure'. This term describes the expulsion of an intestinal worm burden which is of an immune nature (Denham, 1968). Podhajecky (1962) and Denham (1968) could find no evidence of adult worm migration while the migration observed by Duckett (1970) could be largely attributed to host strain, and by Campbell (1967) to many factors including type of feed, intestinal emptying time, intestinal flora and host stress.

Denham and Martinez (1970) used methyridine to chemically abbreviate the intestinal infection and allow only the migratory larvae born up to that time to develop into muscle larvae. By treating groups of mice in sequence with the drug they ascertained that larval production ceased to occur some time before the host expulsion of the adult worm population from the intestines. They also found that in previously infected mice the cessation of larval

production by female worms occurred much earlier than in previously uninfected mice and they suggested that T. spiralis adult worms are similar in this respect to most other intestinal nematodes.

Similar effects have been reported by several workers: Semrad and Coors (1951) and Rappaport and Wells (1951) noted that diminished fecundity and stunting of female worms occured in reinfections- these are two of the criteria commonly used to assess the effect of the immune response on the intestinal phase. Mougeot and Lancastre (1973) from a transplantation procedure similar to that used by Chandler (1936a) found that interruption of larval production can be prevented by transplanting the adult worms after 10 days from mice but not after 18 days. They found the deleterious effects of immunity on 18 day worms to be permanent.

The parenteral phase - muscle larvae.

T. spiralis differs from other nematode parasites whose life cycles have intestinal and parenteral stages in the same host, for the basic reason that the adult intestinal phase of T. spiralis precedes the parenteral larval phases while for other nematodes it is the migrating larval stages which come first, and these are usually limited to a few days in the time taken for development and migration. Although the migratory phase of T. spiralis is of relatively short duration the 'mature' muscle larvae remain encysted in the host's musculature for an exceedingly long time and represent more than just a transitory stage of the life cycle. Because of the length of time of encystment, this phase of the disease represents a host - parasite relationship which is independent of the intestinal phase (except that these larvae are products of the intestinal phase).

In stating that the dual antibody hypothesis may represent a quantitative rather than a qualitative immune phenomenon Chute (1956) suggested that the immunity to T. spiralis might act directly on the larvae during migration or development in the muscles as well as on the intestinal stages, and Mauss (1940) showed that infective larvae incubated in immune serum were subsequently of lower infectivity.

Denham (1969) treated mice with homologous anti-T. spiralis serum and could not find any adverse effect on muscle larvae resulting from a challenge infection. Campbell (1965) similarly found no evidence to suggest that immune mice possessed a protective mechanism directed specifically against the parenteral phase of a challenge infection.

Phillipson and Kershaw (1960) and others have pointed out that trichinosis is not usually diagnosed in human patients until the symptoms produced by muscle invasion have started, while it is recognised that the extreme immunopathology of the muscle can lead to death in very severe infections.

Apart from the evidence of Oliver Gonzalez (1940 and 1941), Oliver Gonzalez and Levine (1962), and of Hendricks (1952) who used irradiated intestinal infections to induce an immune response exhibiting higher antibody titres to adult worm antigen than to muscle larva antigen, there had been little to suggest that the muscle larvae contributed to the immune response and the main immunogenic phase of T. spiralis was generally considered to be the intestinal phase.

However, there is recent evidence to suggest that the muscle

larvae do contribute to the immune response. Despommier and Wostman (1968) sealed muscle larvae into the peritoneal cavities of mice. With a normal challenge infection the muscle larva burden was reduced by 73% compared with unoperated controls. This level of immune reduction closely mimics the level induced by a primary complete infection, so here an immunity comparable to that produced by a complete infection was produced solely by muscle larvae. When rats were immunised with only the parenteral phase (Despommier, 1971) by injecting newborn larvae IV the numbers of muscle larvae resulting from a challenge infection were reduced by 95%.

The parenteral phase - newborn larvae.

Larsh (1970a) stated that little was known of the immunity produced by phase III larvae (which includes newborn and muscle larvae) and he suggested that work on this phase was long overdue.

Studies on the immunogenicity of the newborn larvae have been delayed until recently by the inability to obtain them in large enough numbers. A method of substantially overcoming this problem has been devised by Dennis, Despommier and Davis (1970) (see chapter 3) involving the *in vitro* culture of adult worms.

By this method Despommier (1971) was able to produce sufficient newborn larvae, which after freeze-thaw killing, were inoculated into rats. An injection of 2000 killed larvae produced no effective immunity in rats as judged by the number of muscle larvae resulting from a normal challenge of 600 infective larvae, whereas 6000 viable newborn larvae allowed to develop to encysted muscle larvae produced a 95% reduction of muscle larvae due to a complete challenge infection.

Rats which had been immunised to muscle larvae, by implantation of diffusion chambers containing this stage, did not exhibit any reduction in the numbers of muscle larvae due to a challenge of 9000 newborn larvae given I.V. (Despommier, 1971). He concludes that no immunity is developed by, or to, the migratory larvae until they have penetrated the host muscle and he suggests that this can be accounted for by the lack of a stichosome in this stage of the worm - highly immunogenic granules have been isolated from the stichocytes of both adult worms and muscle larvae by Despommier and Muller (1969 and 1970).

Jackson (1959) in his fluorescent antibody studies could find no precipitates around newborn larvae cultured in vitro and neither could he (1960) for recently hatched N. muris which had been similarly cultured.

Functional antigens.

Virtually the whole of the body of nematodes has been reported to be antigenic to some extent, but as the contact of most tissues with the host is normally restricted when the parasites are alive, very few of these antigens are functional in stimulating an immune response.

The cuticle of the intact nematode functions antigenically and this tissue is employed as the antigen in most of the indirect fluorescent antibody techniques (see chapter 7).

Soulsby (1963) cites evidence that the ability of the cuticle of Ascaris and Nippostrongylus to fix antibody increases with age and larval development and he suggests that the antigenicity of the cuticle may increase with time for these parasites.

The antibody binding sites of T. spiralis muscle larvae with ferritin-conjugated hyperimmune sera were found by Despommier, Kajima and Wostman (1967) to be specifically the outer cuticle surface. Jackson (1959) however found that the cuticle did not react with fluorescein conjugated antisera, but he observed the formation of fluorescent precipitates which formed at the external orifices of worms incubated in immune serum and in the gland cell at the base of the stichosome. These observations were confirmed by Catty (1969).

Oliver Gonzalez (1940 and 1941) used the formation of precipitates around the oral, anal and vulval orifices of worms in vitro to demonstrate an immune reaction: The reacting antigens were excretions and secretions (E and S) of the worm. Campbell (1955) injected E and S antigens obtained by incubating encysted muscle larvae in vitro for 5 days; the immune response which was generated caused a more rapid loss of adult worms from the intestine and a stunting of the adult females, and a lower number of muscle larvae were subsequently recovered. Mills and Kent (1965) could distinguish 3 active fractions in E and S antigens by immunoelectrophoresis and Ouchterlony diffusion tests.

Oliver Gonzalez (1963) discussed the possibility that as larval and adult helminths usually occur in different habitats the adaptation of a worm from one environment to another may induce a change in the enzymes necessary to one stage to those of the other stage, and he considered that the antigenic differences observed between larvae and adults may be due to enzymatic alterations.

Several methods have been used to extract somatic antigens

from T. spiralis particularly from the muscle larve (see chapter 3) and these have been analysed by several workers. Olson, Richards and Ewert (1960, cited by Catty , 1969) found 9 agar precipitin bands forming between antiserum and sonicated infective larval material, and 4 bands which did not cross react with the somatic antigens were produced when live larvae were incubated in contact with agar so that the metabolites could diffuse directly into the agar. Campbell (1955) was able to induce immunity in mice by injecting the E and S of 'mature' muscle larvae, and Ewert and Olson (1961) confirmed this observation.

Tanner and Gregory (1961 and 1963a) using a technique of immunoelectrophoresis in agar were able to discern 11 antigenic fractions from muscle larva material and (1963b) identified 1 fraction as a major antigen with a mucoprotein structure. Catty (1969) found 16 separate antigenic fractions.

Purified isolates of muscle and cuticle of A. lumbricoides injected into mice (Oliver Gonzalez, 1960) were able individually to elicit an immune response, but little work has been performed with specific tissue extracts of T. spiralis. Despommier and Muller (1969 and 1970) extracted alpha and beta granules, distinguishable by size and density, from the stichocyte cells which occur in both muscle larvae and in adult worms, and purified the suspensions of these granules on an isopycnic sucrose gradient. When injected into mice the smaller beta granules proved highly immunogenic and reduced by 80% the number of muscle larvae developing from a challenge infection.

Despommier and co-workers are of the opinion that the stichocyte granules are the major functional antigens and that normal acquired

immunity to T. spiralis develops as a result of stimulation by these granules. The function of the stichosome is unknown but Zarzycki, Kozar and Czechowicz (1970) suggest that it may be an endocrine gland and they found that the largest uptake of ^{131}I occured in the stichocyte cells of encysted muscle larvae. Wu (1955) suggests that each stichocyte cell is a unicellular binucleate gland.

Despommier (1971) considers that the secreted functional antigens are localised in the $beta_1$ granules of the stichocyte cells and that only the stages of the parasite which contain these granules are immunogenic. The 'mature' muscle larvae and adult intestinal worms possess these granules (Richels, 1955; Wu, 1955; Despommier, 1971) while Despommier (1971) states that the 'immature' migratory larvae do not, and he considers that the absence of these granules from this stage would account for the lack of the immune response to migratory larvae observed in his studies.

The evidence discussed in this chapter and chapter 1 indicates that most work has been concentrated on the adult stage of T. spiralis. This would seem logical if T. spiralis is to be used solely as a research tool to help determine the immunological responses of a host to enteric nematode infections in general. If a parallel can be drawn between the intestinal phase of trichinosis and other intestinal nematode diseases then surely an equally justifiable parallel can be drawn between the parenteral stages of other helminth infections. But work on T. spiralis should not be directed only to this end for the parasite does cause a disease in its own right and as debilitation and mortality from T. spiralis infections are often a result of the invasion of the hosts' muscles, the work on the parenteral stages of this parasite has been, to date, disproportionately small.

As previously stated, the refined culture technique for harvesting large numbers of newborn larvae has made work with the parenteral stages, without the involvement of the adult worms, possible. It is the immunological studies with the parenteral stages and the interrelationships of all the life cycle stages in the immune response which form the majority of the work reported below.

PART 2

GENERAL MATERIALS AND METHODS.

CHAPTER 3.

1. Animals.

Trichinella spiralis : The strain of T. spiralis used in these experiments is the 'London strain' (Nelson and Mukundi, 1963). It was isolated from an infected cat from Penrith, Cumberland, in 1939 by Professor R. T. Leiper and has been maintained since then in the London School of Hygiene and Tropical Medicine by passage through laboratory rats, and since 1968, also through mice. It has also been used to infect domestic pigs (Nelson and Mukundi, 1963; Taylor, 1973 - personal communication).

Laboratory hosts : The mice used both for maintenance of the stock infection and for experimentation were females of the T.O. (Tizer Original, Tuck and Co.) strain purchased when 3½ to 4 weeks old. For stock maintenance or for the production of adult worms the mice were of various ages, but for the experiments 7 to 8 week old mice were used, except where otherwise stated. When cage space was available old male Wistar rats weighing approximately 200 grams were used for the production of adult worms.

New Zealand wnite rabbits were used to produce stage specific anti-T. spiralis serum.

2. Infection with excysted muscle larvae.

Digestion : Mice infected for stock usually harboured 100,000 or more muscle larvae each when digested. It was found that the infective larvae recovered from the digestion of only one mouse at least 5 weeks after infection were normally sufficient for all infections on any one day. The animal was killed by cervical fracture, skinned and eviscerated, and the feet, tail, muzzle and excess fatty tissue

were removed from the carcass. A household hand meat mincer (National 25, Spong) was then used to mince the mouse carcass which was passed through the mincer twice to achieve a uniform consistency (see plate 3).

In early experiments the mince was then blended with a small quantity of digestion fluid in an M.S.E. 'atomix' for approximately one minute, but with the use of a more concentrated digestive fluid this step was found to be of no advantage, and particularly when individual digestions of large numbers of mice were performed (no. 3 below) the additional time and potential additional error and loss of viability of the larvae made blending slightly disadvantageous.

The digestion mixture was prepared fresh for each digestion and consisted of:

Pepsin 1:2,500 BDH	15gm.
Hydrochloric acid (technical) 31% w/v BDH	15ml.
Warm tap water	1000ml.

The single minced carcass was added to 1 litre of this digestion fluid in a 1 litre square section specimen jar and the constituent parts of the hand mincer were washed in the fluid in this jar to utilise all pieces of infected meat and to clean the mincer at the same time. The contents of the jar were then dispersed uniformly by hand stirring with a glass rod and the jar was placed in a 37^{o}C water bath.

Initially, an electrically powered mechanical stirer was used to stir the mixture, but this was later superceeded by a pump (Hyflow model B, Medcalf Bros. Ltd.) which, by blowing air

through the mixture, agitated it continuously (see no. 3 below).

Sieving : After 5 to 6 hours the mixture was poured through a 50 mesh/inch Endecote sieve (pore size - 297 µm) in series with a 200 mesh/inch Endecote sieve (pore size - 73 µm). The technique of 'bumping' reported by Duckett (1971) was used on occasion to assist the passage of the fluid through the sieves. The muscle larvae were recovered from the lower 200 mesh sieve by washing them to the side of the sieve with a fine jet of tap water and then emptying them into a 50 ml. specimen jar.

The suspension of infective larvae recovered by the above digestion technique was free of other particulate material and it was not found necessary to wash them further before using them to infect mice.

Counting. : A quantity of normal saline was added to the suspension in the jar to make the volume up to between 40 to 50 ml. A teflon coated magnet was used to stir the suspension on a magnetic stirer (Voss). Campbell (1965) found the use of a magnetic stirrer essential to give reproducible counts of aliquots removed for counting. A MacMaster type (Hawksley) counting chamber with the ruled areas on the floor of the chamber was used. The total volume above the two ruled areas was 0.3 ml. (see diagram **6**).

The suspension of larvae was often too concentrated for easy counting and then 1:10 or 1:20 dilutions were prepared. 5 sequential counts of live larvae were made from aliquots in the counting chamber under a dissecting microscope and the mean was used to calculate the volume of the original or diluted suspension necessary for infection.

Larsh, McKenzie, Greenberg and Campbell (1953) added egg

embryo extract to their suspensions to enhance the infectivity of the larvae. This was found to be unnecessary and throughout these experiments the larvae were suspended only in normal saline.

Infection : Infections of mice were made orally with a blunted 1½ inch gauge 18 hypodermic needle attached to a fixed volume spring loaded syringe (Beckton-Dickinson pattern), and the volume of the suspension normally given was between 0.3 and 0.5 ml. Rats were also infected orally but with a 4 inch gauge 15 blunted needle using volumes of between 1 and 2 ml.

Because there was some variability in the worm counts made by this method it was decided to try and find a more precise infection technique.

Direct transplantation of counted female or male worms was used by Anderson and Leonard (1940), Anderson (1941), Roth (1943) and Denham (1966).

Exact numbers of mature 6 day old female worms were counted and administered by duodenal tube to mice. Muscle larva burdens of the mice following digestion had considerably greater variation than normally infected mice, this method was also extremely time consuming when used for large numbers of mice. Counting individually infective excysted larvae in drops of saline and administering per os was similarly found to be very time consuming.

Embedding the larvae in warm agar plated on to glass slides was found to be easier and the area of agar with the counted number of larvae could be cut and rolled up. The difficulty occured in administering the agar pellet to mice;

Plate 3. Apparatus used to mince mice.

Plate 4. Apparatus used to digest mice individually. 'Hyflow' pump (p), air lines (a), 1 litre jars containing digestion fluid (j), water bath (w).

Plate 3. Apparatus used to mince mice.

Plate 4. Apparatus used to digest mice individually. 'Hyflow' pump (p), air lines (a), 1 litre jars containing digestion fluid (j), water bath (w).

it was too large to be drawn into a blunted hypodermic needle and administered orally and it was necessary to feed the pellet, held by forceps, to the mice. They showed no predilection for eating agar and there was no certainty as to whether all of the pellet had been swallowed. Consequently the method of counting and administering infective larvae from a magnetically stirred suspension, as previously described, was retained as the quickest, simplest and possibly most accurate method.

3. Determination of the individual muscle larva burdens of mice.

The digestion of individual mice was the same as the method described above, except that by using 2 water baths, 12 individual digestions could be performed simultaneously and all could be agitated by separate airlines from the air pump (see plate 4).

The digests were sieved as described previously and the larvae were washed into 50 ml. specimen jars.

The contents of the jars could be counted immediately, or by keeping sealed at 4°C, at the earliest convenient opportunity. They were normally kept overnight and counted during the time that the next 12 mice were being digested. The first jar from each group was briefly studied under the microscope; if only a few larvae could be seen then a total count of the larvae was made, while if there were many larvae present then aliquots were taken and counted from a magnetically stirred suspension. Total count method : The contents of the jar were emptied into a petri dish which was ruled into squares of approximately 1 cm. X 1 cm. on the inside. All larvae were counted and a reasonable correlation was found to exist between this method and the aliquot

count method at the region of overlap of the two methods (see appendix 1). This method was used when less than 500 larvae were present in the total suspension.

Aliquot count method : Aliquots were withdrawn by a pasteur pipette from a position approximately halfway to the vortex centre and midway between the surface and the base of the stirred suspension (see diagram 5, and appendix 2). The first aliquot was run into the MacMaster chamber then withdrawn with the pasteur pipette and 'discarded' into a graduated cylinder.

The second aliquot, taken with the same pipette (see appendix 3) was run into the counting chamber and those larvae in the two ruled areas counted with a tally counter. 5 consecutive counts were made (see appendix 4). When all the counts had been made the remaining suspension of muscle larvae was added to the graduated cylinder to determine the total volume of the sample. From the mean of the aliquot counts and the total volume of the sample, the total number of muscle larvae was calculated for each digested mouse.

4. Determination of the number of adult worms in the intestines of mice.

The adult worm counts for individual mice were made as follows: The mouse was killed, the required portion of intestine immediately removed and slit along its length with a pair of blunt-nosed scissors, then by holding with a pair of rat-tooth forceps it was shaken in a jar of warm normal saline to remove the excess digesta; Denham (1968) estimated the worm loss by this procedure at less than 1%. The slit intestine was then incubated at 37^{o}C in approximately 50 ml. of normal saline for 1½ hours in a 50 ml. specimen jar. After this time the contents of the jar were

Diagram 5.

Diagram showing the position (A) from which aliquots were taken in order to determine the number of excysted muscle larvae in the suspension.
(See appendix 2).

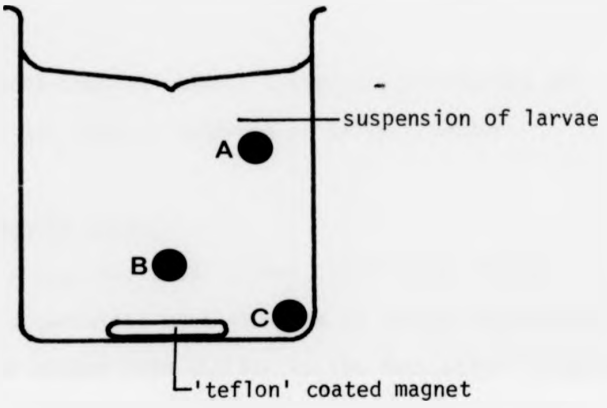


Diagram 6.

Diagram of the MacMaster counting chamber used for counting excysted muscle larvae - actual size. (Each ruled area is 10mm x 10mm and encloses a volume of 0.15 ml).

emptied through the 50 mesh and 200 mesh sieves held together and the intestine was washed in a fine jet of tap water and discarded. The jar was rinsed and the washings also passed through the sieves, then the adult worms held on the 200 mesh sieve were washed into the ruled petri dish.

The worms were counted under a dissecting microscope and the numbers of males and females recorded on tally counters.

5. Production of newborn larvae.

Adult recovery : In a previous study (James, 1971) (see figures 3.1 and 3.2) it was ascertained that the peak of larval production by adult female worms occurs from the 6th to 7th days after infection. This observation agrees with the findings of Denham and Martinez (1970), Despommier (1972 - personal communication) and Ruitenberg (1973 - personal communication).

Adult worms were obtained from the intestines of mice or rats as described above except that 15 to 25 mouse intestines depending on the number of mice killed were normally incubated together in 1 litre of normal saline.

After recovery the adult worms were added to a Baermann apparatus containing culture medium, and incubated for 1½ hours at 37°C. The worms migrated to the base of the Baermann apparatus and were run out into a conical centrifuge tube and allowed to settle. They were then cleaned by 3 washes lasting a total of 30 minutes in sterile culture medium with 1:20,000 merthiolate.

All further preparations for culturing the worms and the mixing of the culture medium were performed in sterile conditions.

Figure 3.1.

The rate of larval production by adult female T. spiralis during the course of an infection. (From James, 1971).

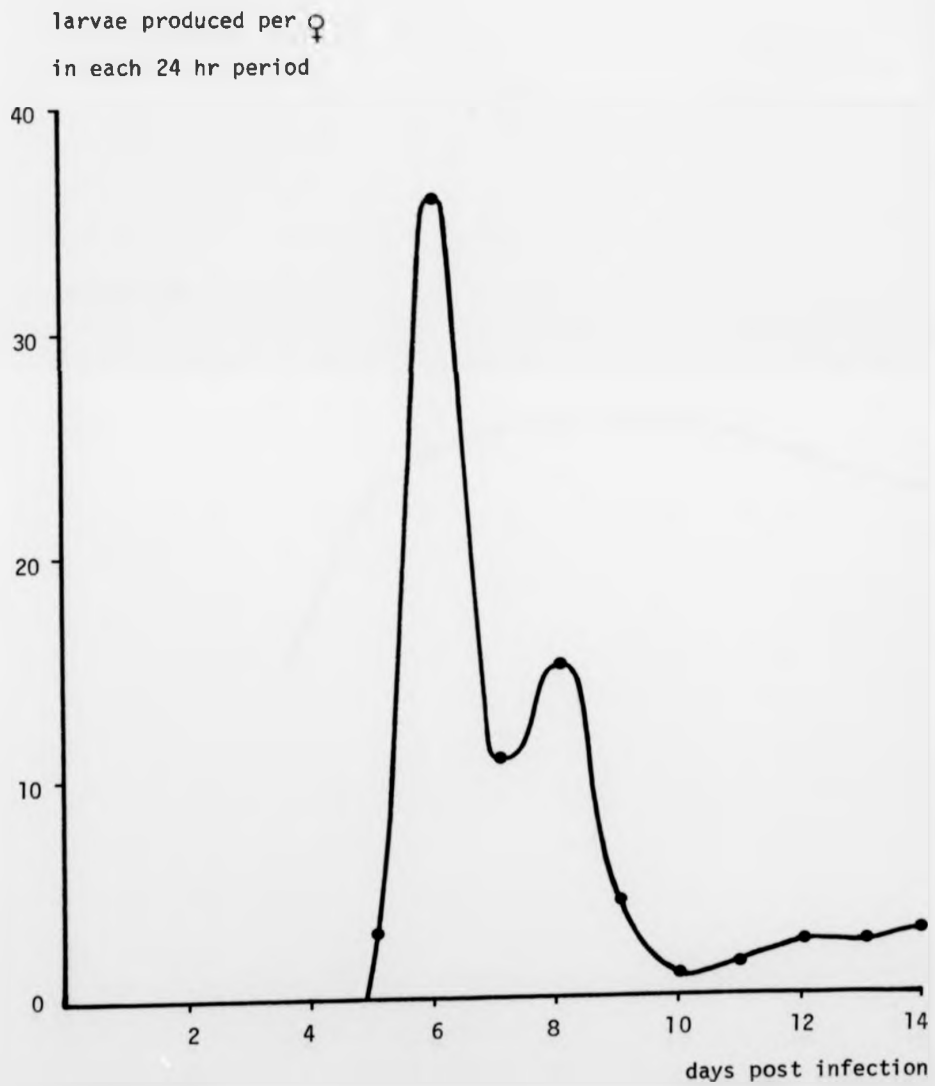
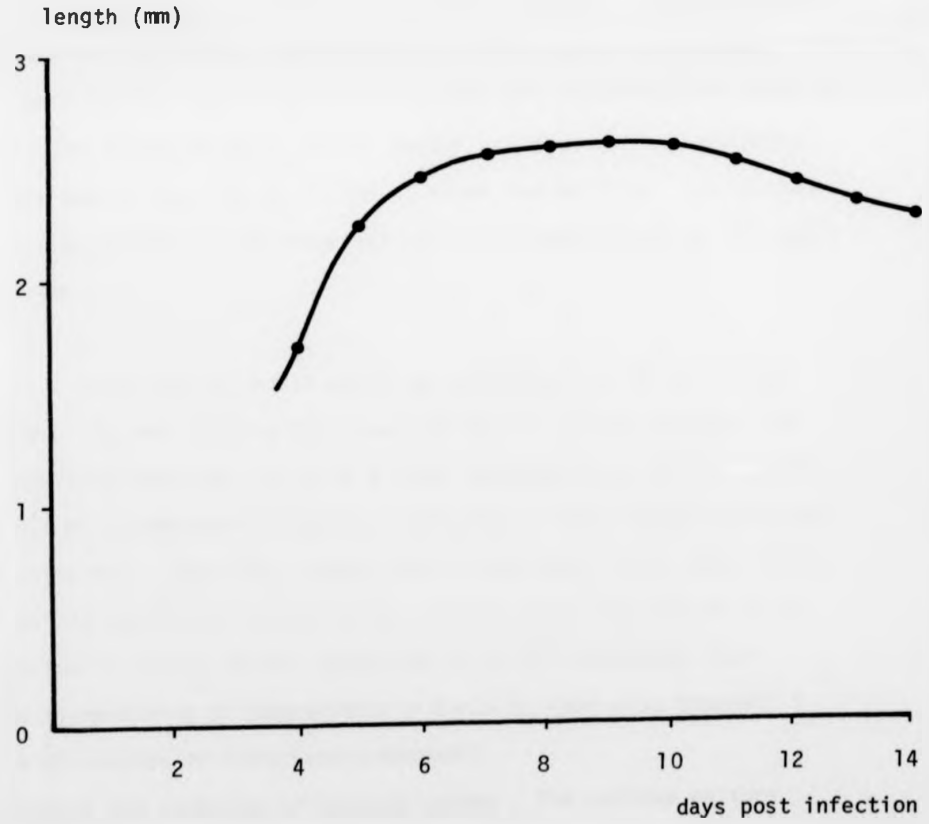


Figure 3.2.

The size of adult female T. spiralis obtained from mice during the course of an infection. (From James, 1971).



Culture of adult worms : The culture medium was prepared as follows:

Tissue culture medium 199 (10xconcentrated BDH)	100 ml
Sodium bicarbonate 4.4% w/v (59009 BDH)	20 ml
Sterile inactivated calf serum No.1 (Wellcome CS 08)	100 -200 ml
Sterile distilled water	780 ml

Initially approximately 10% serum in medium was used, but after discussing the culture technique with Ruitenberg and co-workers who used a medium containing 20% serum, it was decided to try a medium containing this quantity of serum. It was concluded that a serum content varying between 10 to 20% did not appear to alter the production of newborn larvae by the female worms in culture. 20 ml of culture medium was poured into each of two 20 ml screw top bottles. To the first was added 1000 mg of Penbritin (Ampicillin sodium, Beecham Research Labs.) and to the second was added 500,000 units of Mycostatin (Nystatin B.P., E. R. Squibb and Sons.) The remaining culture medium was stored in 100 ml screw top bottles. All culture media and antibiotic and chemical solutions were stored at $4^{o}C$ until required.

Culturing of adult worms was performed in 250 ml roller bottles. To each bottle was added 100 ml of culture medium, 1 ml of Penbritin solution (to give a final concentration of 0.5 mg/ml) and 0.5 ml of Mycostatin solution (to give a final concentration of 135 units/ml). Then the cleaned sterilised adult worms were added, the bottle was firmly sealed with a rubber bung, and placed on an electrically driven roller apparatus in a $37^{o}C$ incubator (see plate 5) revolving at approximately 8 r.p.h. (see also appendix 5 for a discussion of alternative methods).

Collection and counting of newborn larvae : The optimum culture period, judged from the number of active newborn larvae as a proportion of the total number recovered was found to be 24 hours.

Plate 5. Incubator containing roller apparatus and 3 culture vessels.

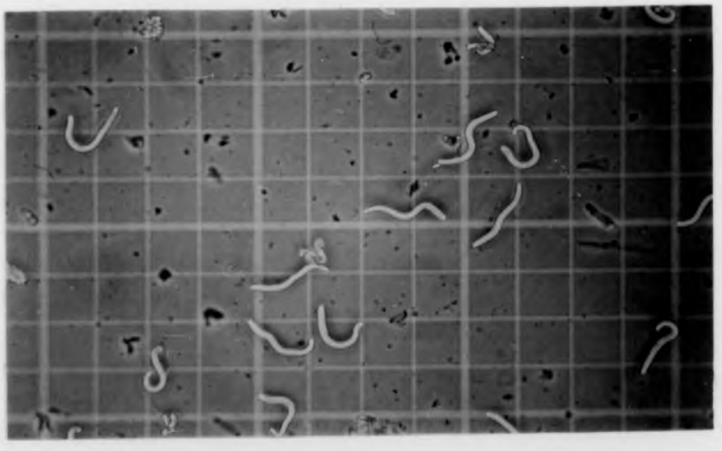


Plate 6. Newborn larvae on grid of 'bright-line haemocytometer'.

The contents of the roller bottles were passed through a 300 mesh/inch Endecote sieve (pore size - 54 μm) into a conical urine flask, while the adult worms were collected on the sieve. The culture medium containing the newborn larvae was then run into several 10 ml conical centrifuge tubes and the suspension was centrifuged for 5 minutes at 1000 r.p.m. The supernatant was discarded from each tube and the sedimented larvae were pooled. The larvae were washed twice in warm phosphate buffered saline (PBS) and resuspended in PBS.

The suspension was agitated with a pasteur pipette and samples withdrawn and run into a haemocytometer (Neubauer, improved 'bright line' 0.1 mm deep) (see plate 6). Larvae in each of the 9 large central fields (volume 0.004 ml) were counted for both chambers. This was repeated twice more and the mean of the 54 counts was the volume of the suspension to be injected into the mice.

Infection with newborn larvae : The suspension of newborn larvae was agitated as before with a pipette and the volume required was injected into the lateral tail veins of mice (see appendix 6) with a 1 ml. graduated disposable syringe fitted with a $\frac{3}{8}$ inch X 26 gauge needle.

Injection of the mice was facilitated by using a modified empty 78 gm stick deodorant container (Aramis Inc. Distr., or Brut, Faberge Inc. Distr.) (see plates 7 and 8). The volume of the interior of this container could be adjusted to accommodate mice or small rats of various sizes.

Infection of rabbits was accomplished by injecting the suspension into the marginal ear vein, these animals were docile

Plate 7. Chamber used for injecting mice.

Plate 8. Newborn larvae being injected into the lateral tail vein of a mouse.

and did not need to be restrained.

6. Preparation and estimation of antigens.

Antigen was prepared from muscle larvae, newborn larvae and adult worms. In each case the worms were cleaned by 3 washes in PBS and allowed to sediment or were centrifuged for 5 minutes at 1000 r.p.m. The worms were then resuspended in 1 to 2 ml of PBS and transferred by pipette to a Hughes type 'X Press' (AB Biox, Nacka Sweden, type X5) (see plate 9) which had been previously cooled to -20°C. The X press was placed in the deep freeze for 2 to 3 hours to ensure complete freezing of the worm material. After the material had been forced 4 times through the X press with the aid of a 'fly press', the press was dismantled, the antigen removed, and the X press returned to the -20°C freezer. The worm material was allowed to thaw and then re-pipetted into the X press, frozen and pressed again as before (see appendix 7).

After the second pressing the X press was dismantled and the worm material placed in a 20 ml screw top bottle. The material was thawed to 4°C and with continuous agitation was left for 24 hours for the soluble protein to extract (see plate 10). It was then centrifuged at 3000 r.p.m. for 10 minutes and the supernatant withdrawn and stored in 1 ml vials at -60°C until required.

The total protein content of the antigens was assayed using the method of Lowry, Rosenbrough, Farr and Randall (1951).

7. Collection of sera.

Mice were killed by ether fumes and exsanguinated by cardiac puncture. Rabbits were bled from the marginal ear vein.

Plate 9. Hughe's type 'X-Press'.

Plate 10. Continuous agitation apparatus used to extract soluble protein from antigen.

Figure 3.3.

The effect of ^{60}C radiation on the fecundity of adult female T. spiralis, calculated from the number of muscle larvae obtained by digestion divided by the original number of larvae used to infect the mice. (See appendix 8).

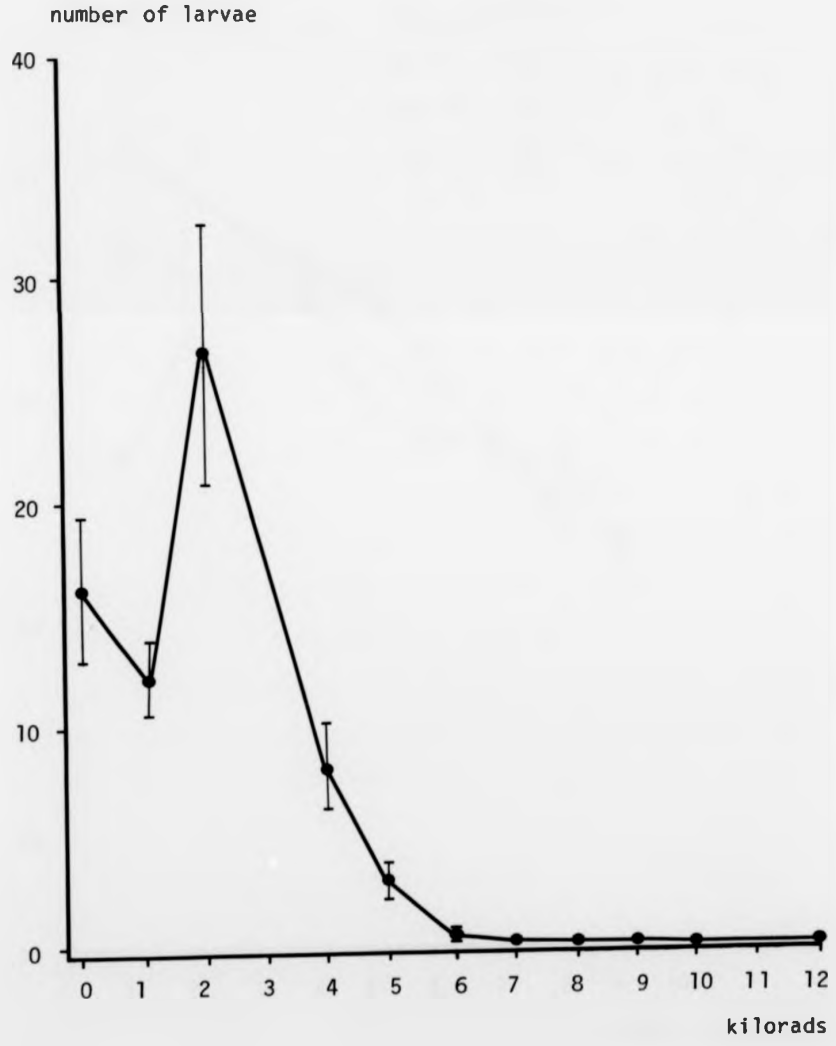
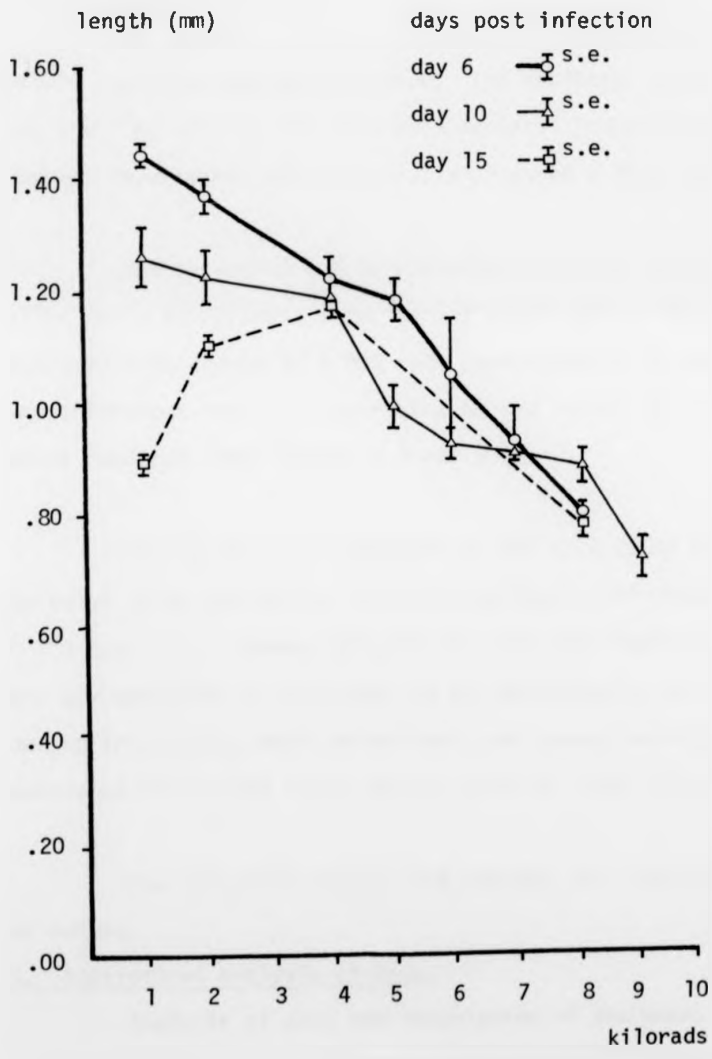


Figure 3.4.

The effect of ^{60}C radiation on the size of adult female *T. spiralis* obtained at 6, 10 and 15 days post infection. (See appendix 8).



Blood samples were allowed to clot at room temperature for 2 to 3 hours, and refrigerated at $4^{o}C$ to contract the clot. The samples were then centrifuged for 20 minutes at 3500 r.p.m., the serum was withdrawn and stored in 1 ml vials at $-60^{o}C$ until required.

8. Irradiation of worms.

In several experiments infective larvae were irradiated before injection per os into mice. The radiation source used was the ^{60}Co unit at the Middlesex Hospital Medical School, Physics Department, which initially produced a dose of 2500 rads/min.

The objective was to sexually sterilise the larvae but to allow their development into 'normal'adult worms. This was achieved with a dose of 8,500 rads (see appendix 8) and in this way infections could be restricted to the intestinal phase only where required (see figures 3.3 and 3.4).

The larvae were suspended in PBS in a 20 ml plastic conical bottomed screw top bottle (Sterilin products, universal container, P 128 type 'A'). Gamma radiation is hard and highly penetrative, and any variation in exposure due to sedimentation of the larvae during irradiation would be minimal, and anyway would have been accounted for in the final adopted dose of 8,500 rads.

The infective larvae were counted and injected per os as before.

9. Statistical analysis of data.

Analysis of data and calculation of the means and standard errors of samples was performed on an Olivetti Programma 101. Results are usually expressed as the mean and (standard error). The

significance of the results was assessed by the student 't' test from the geometric mean and variance derived from the $\log_{10}$ of the samples in each group. Results were considered to be significantly different when the probability (p) was less than 0.05.

PART 3

RESULTS.

Chapter 4.

Immunity to the intestinal phase of T. spiralis.

The experiments reported in this chapter attempted to restrict the 'immunising' infections of T. spiralis to the intestinal stages. This was accomplished with the anthelmintic methyridine which has an activity spectrum ideally suited to this purpose.

Methyridine has been shown to be virtually 100% effective in eliminating the adult phase of the infection (Schanzel and Hegerova, 1964; Denham, 1965; Campbell and Cuckler, 1968; Denham and Martinez, 1970), although it has a minimal effect on the newborn and developing muscle larvae. The studies of Gould et al. (1955), Denham (1965) and James (1971) indicate that the first larvae are born late on the 5th day after infection. Thus by removing the adult worms just prior to the release of the first newborn larvae, an immunity can be produced to the intestinal phase of T. spiralis.

Denham (1965) showed that single infections of 3 to 4 days duration did stimulate an immune response in mice. The purpose of the experiments in this chapter was to assess the specificity of immunity produced by intestinal worms, so usually 2 or 3 abbreviated intestinal infections were used for immunising the mice.

Experiment 4.1.

In this experiment mice immunised with abbreviated

intestinal infections were challenged with a normal complete infection. The infection and treatment schedule for this experiment was the same as for experiment 4.4.

15 mice were divided into 3 groups.

Group 1: these mice received 3 immunising doses of T. spiralis infective larvae per os - 480 (31), 480 (17) and 444 (26) larvae at intervals of 7 days. They were treated at approximately 110, 114 and 118 hours after each infection with methyridine injected sub-cutaneously 3 times at 500 mg/kg (a total treatment of 1500 mg/Kg for each infection). 2 additional mice were similarly infected and treated and killed the following day to determine if the treatment had been successful - no adult worms were found in their intestines.

Group 2: These mice were uninfected but received a series of injections of methyridine identical to group 1.

Group 3: These mice were uninfected and untreated.

7 days after the last immunising infection had been eliminated all groups of mice were challenged with 57 (2) infective larvae given per os. 30 days after the challenge infection the mice were killed and digested individually to recover the muscle larvae.

One mouse from group 1 died soon after the first injection of methyridine.

The results are presented in table 4.1.

In this first experiment an immunised unchallenged group of mice was not included. However, the two mice killed after treatment, and the results of experiment 4.4. suggest that

Table 4.1.

Immunity to the intestinal phase of a T. spiralis infection: The effect of this immunity on a challenge complete infection assessed by the number of larvae which have developed in the muscles of mice.

Numbers of larvae recovered from individual mice (experiment 4.1.).

Group 1 (immunised-challenged)	Group 2 (treated-challenged)	Group 3 (challenge controls)
707	3,746	6,327
780	6,920	7,967
1,863	11,117	8,092
2,038	13,419	10,116
	16,672	14,420
1,347 (351)	10,373 (2,295)	9,384 (1,395

immunisation - intestinal phase
challenge - complete infection

significance tests:

groups 1 & 2	$0.0025 \geq p \geq 0.0005$
groups 1 & 3	$0.0005 \geq p \geq 0.0$
groups 2 & 3	$0.4875 \geq p \geq 0.475$

the methyridine was effective in eliminating all the adult worms and that none of the muscle larvae present in the mice had resulted from the immunising infection. Group 2 is not statistically significantly different from group 3, but both groups 2 and 3 are significantly different from group 1.

The mean number of muscle larvae recovered from group 1 (1,347) is 14% of the mean for group 3 (9,384) indicating that immunisation with the intestinal phase has reduced a challenge complete infection by 86%. (A complete infection is one in which all normal stages of the life cycle are present and which is initiated by a *per os* injection of infective larvae).

Experiment 4.2.

This experiment was designed as a repeat of experiment 4.1 but included an immunised unchallenged group of mice, and the challenge dose of infective larvae this time was much higher.

The immunising infections were 300 (24), 302 (30) and 556 (33) infective larvae. The intestinal worms were removed as before by sub-cutaneous administration of methyridine at 110, 114 and 118 hours PI.

The challenge infection consisted of 299 (21) infective larvae given *per os* 7 days after the termination of the last immunising infection with methyridine. 26 mice were divided into 3 groups of 8, 10 and 8; one of the challenge control mice died before digestion. All mice were killed and individually digested 30 days after administering the challenge infection to determine their muscle larva burdens. The results are shown in table 4.2.

Table 4.2.

Immunity to the intestinal phase of a T. spiralis infection: The effect of this immunity on a challenge complete infection assessed by the number of larvae which have developed in the muscles of mice.

Numbers of larvae recovered from the muscles of individual mice (experiment 4.2.).

Group 1 (immunised-unchallenged)	Group 2 (immunised-challenged)	Group 3 (challenge controls)
0	117	34,472
0	144	40,506
0	149	40,552
0	324	44,007
1	737	56,356
2	1,281	61,707
3	1,848	73,459
13	2,789	
	5,139	
	10,948	
2.4 (1.6)	2,348 (1,079)	50,151 (5,310)

immunisation - intestinal phase
challenge - complete infection

significance tests:

groups 1 & 2	$0.0005 > p > 0.0$
groups 1 & 3	$0.0005 > p > 0.0$
groups 2 & 3	$0.0005 > p > 0.0$

Each of the groups shown in table 4.2 is highly statistically significantly different from the others.

The use in this experiment of a higher challenge infection has made the differences between group 2 (immunised-challenged)and group 3 (treated-challenge controls) even more apparent. The mean number of muscle larvae recovered from group 2 (2,348) represents less than 5% of the mean for group 3 (50,151) which indicates that the procedure of immunisation used here is 95% effective against a complete challenge infection when assessed by the number of muscle larvae which develop from this challenge infection.

Experiment 4.3.

This experiment was designed similarly to the two previous experiments (4.1 and 4.2) except that 2 immunising infections were given in this instance. Three groups of mice were used.

Group 1 : These mice received an immunising infection of 350 (45) infective larvae followed after 7 days by a second infection of 450 (33). Both infections were terminated by methyridine given at 100, 104 and 116 hours PI at doses of 500 mg/Kg (total 1,500 mg/Kg). This group remained unchallenged.

Group 2 : These mice received the same immunising infections and drug treatment as group 1, and 5 days after the last methyridine injection they received 145 (20) infective larvae per os as a challenge infection.

Group 3 : Mice in this group were not immunised and received the challenge infection only.

All mice were killed and digested individually after a further period of 28 days. The results are shown in table 4.3.

Table 4.3.

Immunity to the intestinal phase of a T. spiralis infection: The effect of this immunity on a challenge infection assessed by the number of larvae which have developed in the muscles of mice.

Numbers of muscle larvae recovered from individual mice (experiment 4.3.).

Group 1 (immunised-treated)	Group 2 (immunised-challenged)	Group 3 (challenge controls)
0	36	2,979
0	44	3,700
0	94	5,302
0	174	6,102
1	545	9,282
1	566	13,310
	3,681	18,445
0.3 (0.2)	734 (498)	8,446 (2,139)

All groups are statistically significantly different from each other.

immunisation - intestinal phase
challenge - complete infection

significance tests:

groups 1 & 2	$0.0005 > p > 0.0$
groups 1 & 3	$0.0005 > p > 0.0$
groups 2 & 3	$0.0005 > p > 0.0$

The results for all 3 groups are statistically significantly different from each other. This experiment has demonstrated that by previously immunising mice with only the intestinal phase of T. spiralis there has been a substantial reduction in the numbers of muscle larvae which encyst from a challenge infection as compared with previously unimmunised controls. The effective reduction in the challenge infection was 87%.

Experiment 4.4.

In this and the following 2 experiments the effectiveness of the methyridine abbreviated immunisation with the intestinal phase of T. spiralis was tested against a challenge infection of the parenteral stages. The immune state of the mice was assessed as before by the number of muscle larvae developing from the challenge infection.

This experiment was performed at the same time as experiment 4.1 and utilised the same immunising infections.

15 mice were divided into 3 groups.

Group 1 : These mice were infected 3 times at intervals of 7 days with 480 (31), 480 (17) and 444 (26) infective larvae. Each infection was eliminated by administering methyridine on the 5th day PI at 110, 114 and 118 hours as for experiment 4.1.

Group 2 : These mice received no immunising intestinal infections but were treated with methyridine as for group 1.

Group 3 : These mice received neither the immunising infections nor the methyridine treatment.

7 days after the last immunising infection had been

Table 4.4.

Immunity to the intestinal phase of a T. spiralis infection: The effect of this immunity on a challenge infection of the parenteral phase assessed by the number of larvae which have developed in the muscles of mice.

Numbers of larvae recovered from the muscles of individual mice (experiment 4.4.).

Group 1 (immunised-challenged)	Group 2 (treated-challenged)	Group 3 (challenge controls)
175	1,504	1,665
984	2,034	1,801
2,016	2,362	2,176
2,402		
2,412		
1,598 (441)	1,967 (250)	1,881 (152)

There is no statistically significant difference between the mean number of muscle larvae recovered from any of the 3 groups.

immunisation - intestinal phase
challenge - parenteral phase

significance tests:

groups 1 & 2	$0.25 > p > 0.20$
groups 1 & 3	$0.25 > p > 0.20$
groups 2 & 3	$0.45 > p > 0.40$

eliminated, all the mice in the 3 groups received a challenge infection of approximately 2,900 newborn larvae via the lateral tail vein. The mice were killed and individually digested 30 days after the injection of newborn larvae.

2 mice from both groups 2 and 3 died in the time between being challenged and digested.

The results are shown in table 4.4.

Although the differences between the groups are not significant, the mean of group 1 is 19% lower than that for group 2 and 16% lower than that for group 3. This reduction in group 1 is accounted for by 2 readings which are much lower, and clearly this group was not homogeneous in its response to a parenteral challenge. This experiment demonstrated that mice immunised to the intestinal phase are still largely susceptible to the parenteral phase.

Experiment 4.5.

This experiment was performed at the same time as experiments 4.3 and 4.7 and the immunisation schedule was identical.

Three groups of mice were used.

Group 1 : These mice were given immunising infections of 350 (45) and 450 (33) infective larvae and treated with methyridine at 100, 104 and 116 hours. This group received no challenge infection.

Table 4.5.

Immunity to the intestinal phase of a T. spiralis infection: The effect of this immunity on a challenge infection of the parenteral phase assessed by the number of larvae which have developed in the muscles of mice.

Numbers of larvae obtained from the muscles of individual mice (experiment 4.5.).

Group 1 (immunised-treated)	Group 2 (immunised-challenged)	Group 3 (challenge controls)
0	1,034	916
0	1,214	1,372
0	1,524	1,591
0	1,675	1,908
1	1,724	2,042
1		
0.3 (0.2)	1,434 (134)	1,566 (448)

immunisation - intestinal phase
challenge - parenteral phase

significance tests:

groups 1 & 2	$0.0005 > p > 0.0$
groups 1 & 3	$0.0005 > p > 0.0$
groups 2 & 3	$0.35 > p > 0.30$

Group 2 : Immunisation of this group was identical to group 1. 5 days after the last injection of methyridine a challenge infection of 3,200 newborn larvae was given by IV injection.
Group 3 : This group served as challenged controls and received only the injection of newborn larvae.

All the mice were killed and individually digested after a further period of 28 days, and the results are shown in table 4.5.

There is no statistically significant difference between the immunised - challenged and challenge control groups of mice in this experiment. The difference between these two groups is only 8% and therefore immunisation with the intestinal phase of T. spiralis has had an insignificant effect on a challenge infection of the parenteral phase.

Experiment 4.6.

The objective of this experiment was to determine if the numbers of muscle larvae from a challenge infection of the parenteral phase could be reduced by a previous immunisation with irradiated intestinal phase worms. Infective larvae were irradiated with 8,500 rads. of ^{60}Co radiation and were given in 3 separate infections per os at intervals of 7 days.

30 mice were used in 3 groups as follows:
Group 1 : These mice received 3 immunising infections of 284 (19), 286 (21) and 296 (20) irradiated infective larvae on days 0, 7 and 14 of the experiment. One mouse from this group died.

Table 4.6.

Immunity to the intestinal phase of a T. spiralis infection: The effect of an immune response stimulated by irradiated (sexually sterile) worms on a challenge infection of the parenteral phase assessed by the number of larvae which have developed in the muscles of mice.

Numbers of muscle larvae recovered from individual mice (experiment 4.7).

Group 1 (immunised-unchallenged)	Group 2 (immunised-challenged)	Group 3 (challenge controls)
0	2,007	1,875
0	2,069	2,068
0	2,281	2,401
0	2,339	2,454
2	2,388	2,486
2	2,423	2,496
4	2,464	2,500
	2,712	2,506
	2,878	2,510
	3,222	2,512
		2,745
		2,801
1.1 (0.6)	2,478 (117)	2,446 (73)

immunisation - irradiated intestinal phase
challenge - parenteral phase

significance tests:

groups 1 & 2	$0.0005 > p > 0.0$
groups 1 & 3	$0.0005 > p > 0.0$
groups 2 & 3	$0.45 > p > 0.40$

Group 2 : This group received the same immunising infections as group 1 and in addition on day 21 an IV injection of approximately 4,000 newborn larvae was given.

Group 3 : These mice received only the newborn larvae and served as challenge controls.

The results are presented in table 4.6.

There is no significant difference between the mean number of larvae recovered from groups 2 and 3, and hence, there appears to be no immunity conferred by the intestinal irradiated worms against a challenge infection of parenteral stages. This experiment also confirms that a dose of radiation of 8,500 rads. effectively sexually sterilises the intestinal worms.

Experiment 4.7.

This experiment attempted to determine what effect immunisation against the intestinal phase had on a subsequent population of intestinal worms.

2 groups of mice were used.

Group 1 : These mice received immunising infections of 411 (33), 464 (36) and 436 (27) infective larvae given at intervals of 7 days and were treated with 3x 250 mg/Kg of methyridine on the 5th day after each infection.

Group 2 : This group was unimmunised.

Both groups of mice received a challenge infection of 162 (11) infective larvae 14 days after the last methyridine treatment.

2 mice from each group were killed on the 6th, 8th, 10th,

13th and 15th days PI. Their intestines were removed and divided into large and small intestines and the adult worms extracted. Male and female worms recovered from both portions of the intestines were counted and the lengths of at least 5 females worms were measured using a calibrated eyepiece micrometer.

The results are presented in Figures 4.71 and 4.72.

Group 2 (unimmunised) shows a fairly normal expulsion of the adult worms from the intestine and a temporary rise in the percentage of the worms present in the large intestine. The length measurements of the female worms in this group also follow a fairly normal progression. Group 1 (previously immunised), however, shows no decrease in the number of adult worms present in the intestine up to the 13th day PI but then a sudden fall occurs. There is no elevation of the percentage of worms present in the large intestine and the length measurements for the female worms show an almost continuous decrease in size during the course of an infection from the 6th day onwards.

It could be assumed that while the adult worms of the challenge infection were present in the intestine there existed developing and encysting larvae which resulted from the 'immunising' infections in the muscles. Thus the expulsion of the adult worms which proceeded normally in the challenge controls (group 2), occured much later in the animals which had previously been 'immunised'. It is suggested that the parenteral infection may have induced a suppressive action on the immune response to the adult worms to produce these

Figure 4.71.

Immunity to the intestinal phase of a T. spiralis infection: The effect of this immunity on the intestinal phase of a challenge infection assessed from the mean number of worms obtained from the intestines of mice. (See appendix 9).

1 - group 1 (previously immunised)
2 - group 2 (unimmunised)

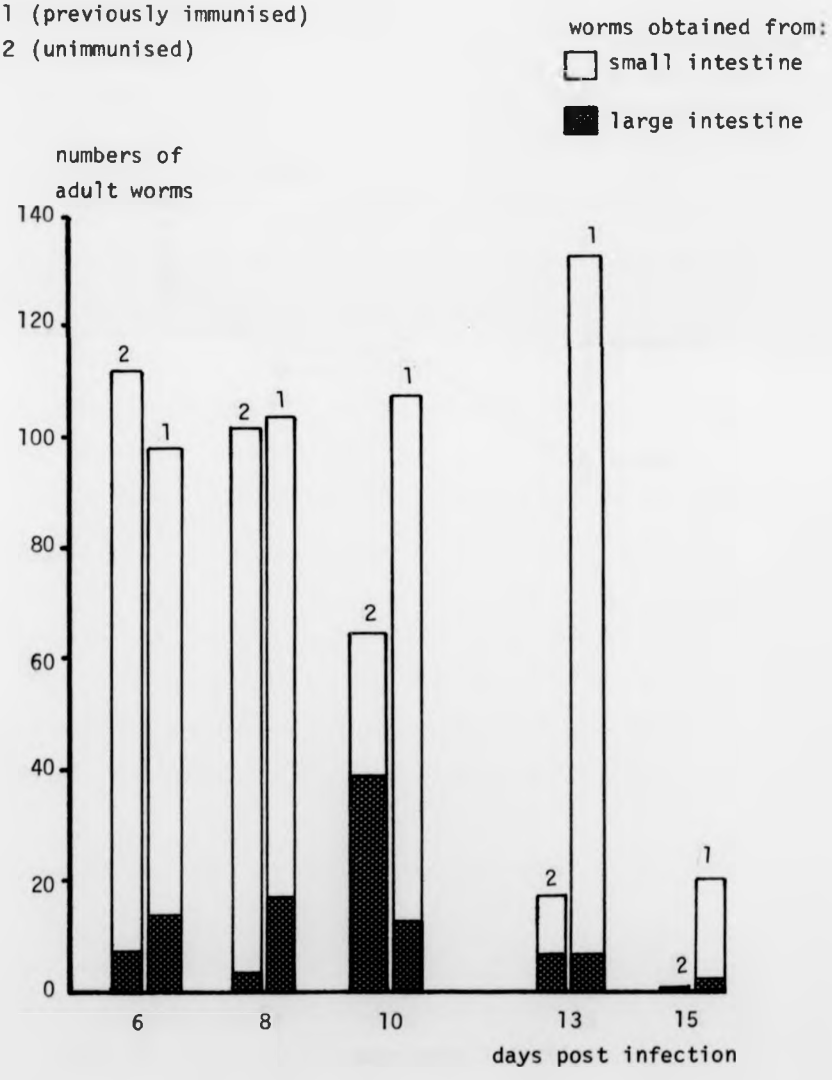
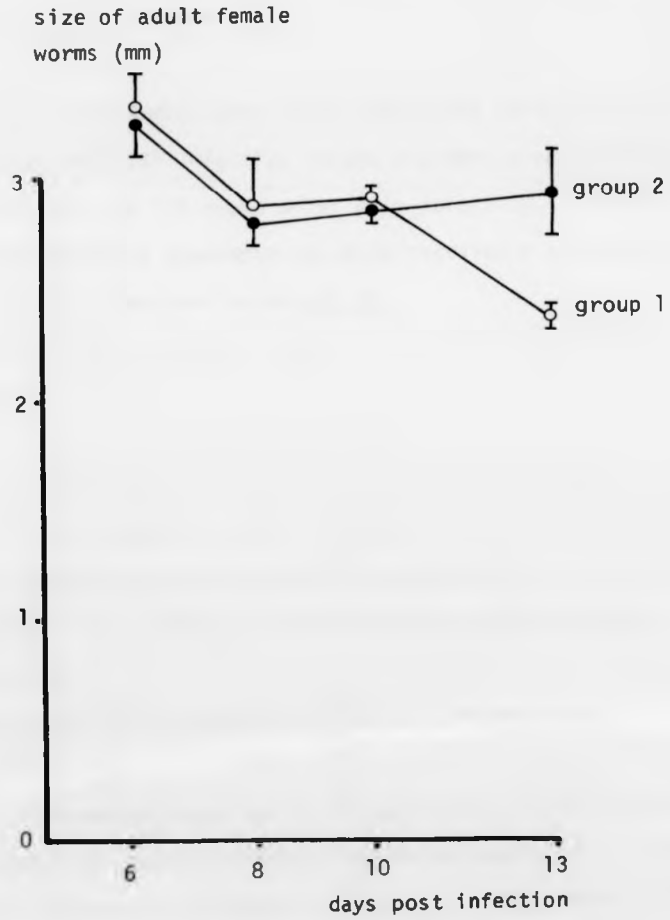


Figure 4.72.

Immunity to the intestinal phase of a T. spiralis infection: The effect of this immunity on the intestinal phase of a challenge infection assessed from the 'stunting' of the adult female worms.

1 - group 1 (previously immunised) ○ s.e.

2 - group 2 (unimmunised) ● s.e.



seemingly anomalous results. This will be discussed further after chapter 8.

Experiment 4.8.

This experiment was designed, similarly to the previous one, to test the effectiveness of the immune response to the intestinal phase against the adult worms of a challenge infection. 2 groups of mice were used.

Group 1 : These mice were given immunising infections of 350 (45) and 450 (53) infective larvae and were treated with methyridine at 100, 104 and 116 hours after each infection. 5 days after the last methyridine treatment the mice received a challenge infection of 245 (8) infective larvae per os.
Group 2 : These mice were not immunised and served as challenge controls.

Two mice were killed from each group on days 6, 10, 13 and 15 PI. The intestines were removed, the adult male and female worms were recovered from the large and small intestines and were counted. The lengths of the female worms, where found, were measured.
The results are presented in Figures 4.81 and 4.82.

An early expulsion of the adult worm population was observed for the previously unimmunised group 2 mice, nevertheless there had been a substantial reduction in the number of adult worms in the immunised group by the 6th day PI. Previously immunised mice harboured a mean of only 1 worm by the 10th day PI,

Figure 4.81.

Immunity to the intestinal phase of a T. spiralis infection: The effect of this immunity on the intestinal phase of a challenge infection assessed from the mean number of worms obtained from the intestines of mice. (See appendix 11).

1 - group 1 (previously immunised)
2 - group 2 (unimmunised)

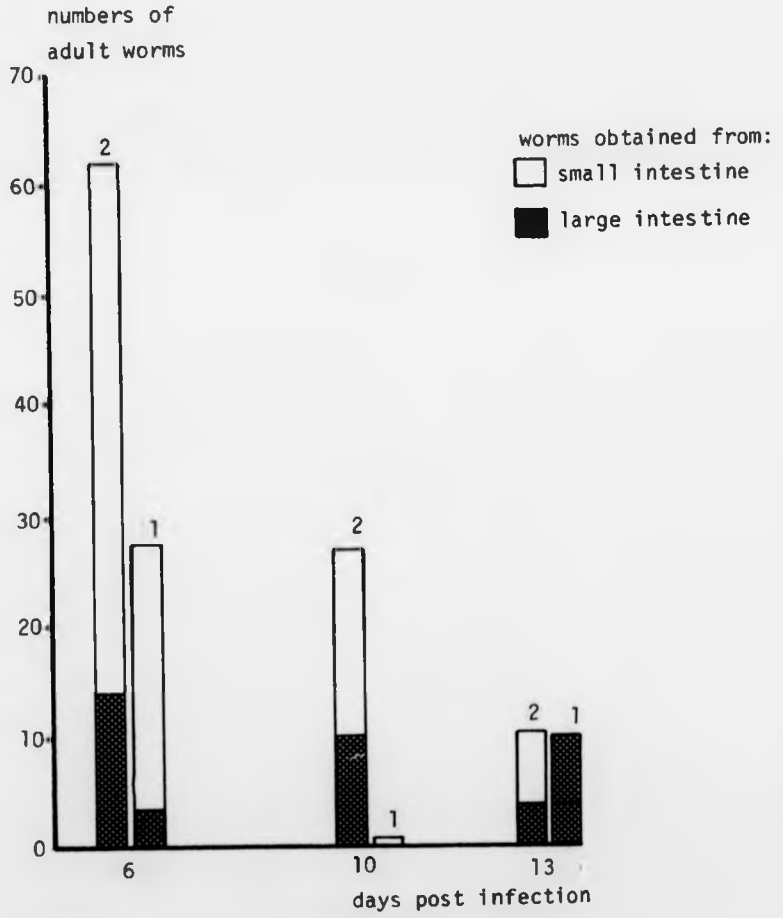
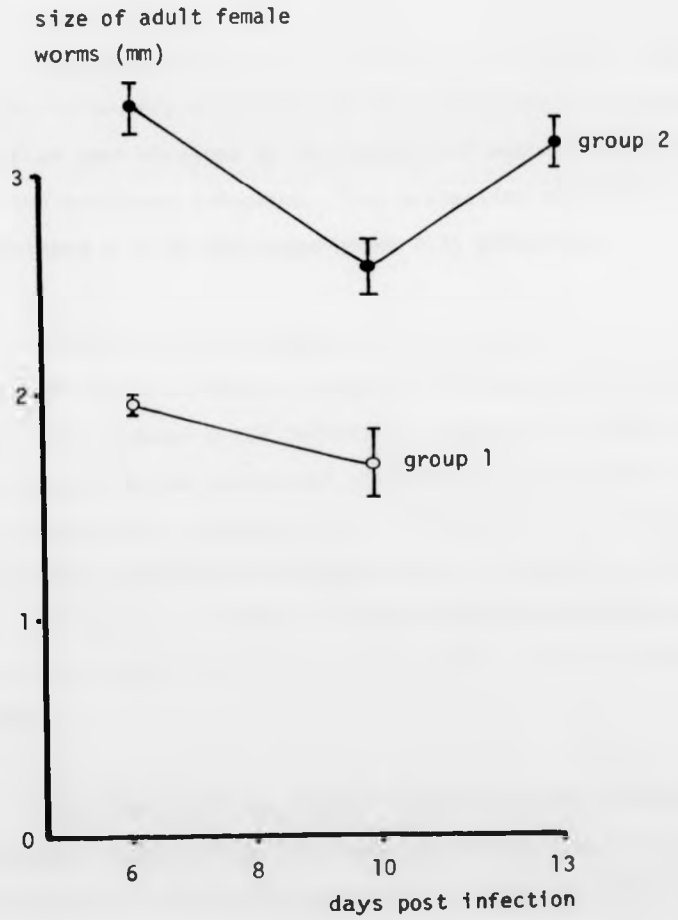


Figure 4.82.

Immunity to the intestinal phase of a T. spiralis infection: The effect of this immunity on the intestinal phase of a challenge infection assessed from the 'stunting' of the adult female worms. (See appendix 12).

1 - group 1 (previously immunised) ○ s.e.

2 - group 2 (unimmunised) ● s.e.



while the 11 worms from this group on the 13th day PI were all recovered from the large intestine of one mouse.

These results suggest that an earlier expulsion of adult worms from a challenge infection is effected in previously immunised mice.

Discussion

The immunisation of mice with only intestinal phase worms appears to be very effective against a complete challenge infection when assessed by the numbers of muscle larvae developing from the challenge infection. The protection afforded was 86% (experiment 4.1) to 95% (experiment 4.2) effective.

Conversely, this immunity gave no protection to a challenge infection of the parenteral stages: The methyridine abbreviated infections produced a 15% reduction in the muscle larva burden developing from the parenteral challenge in experiment 4.4 and an 8% reduction in experiment 4.5. Immunity stimulated by irradiated intestinal worms gave only a 2% reduction - experiment 4.6. Therefore, the action of immunity generated against the intestinal phase must be largely specific to the intestinal stages.

The effects of the immune response on adult worms in a challenge infection have been demonstrated by several workers (see chapter 2) and include an earlier expulsion of the adult intestinal population, stunting, and a lowered fecundity of the female worms.

Expulsion of the adult worms occurred earlier in immune mice than in unimmunised controls (experiment 4.8.). Although there may have been a complicating factor in the presence of muscle larvae in experiment 4.7, the stunting of the female worms in the immunised - challenged mice appeared to be more pronounced in this group than in controls irrespective of the resulting muscle larva burden being higher for this group.

The possibility that the worms produce continuous numbers of larvae which are subsequently destroyed en route to, or in, the muscles of an immune host is largely discounted (experiments 4.4 and 4.5), at least where this immunity has been stimulated by an intestinal infection, and Denham and Martinez (1970) found evidence which suggested that the adult worms ceased releasing larvae well before they were expelled from the intestine of previously immunised mice. The immunity to the intestinal worms thus does not appear to affect the migrating newborn larvae but must act on the adult worms' reproductive system directly or indirectly so that the newborn larval production is interfered with at its source.

Premature cessation of larval production by worms in immune hosts seems to play the major role in reducing the number of muscle larvae from a challenge complete infection, and it appears to be even more important than the phenomenon of expulsion. Experiment 4.8 has shown that although a few adult worms were present in the intestines up to the 13th day PI, experiment 4.3 has shown that these worms were practically sterile.

Immunity generated to the intestinal phase is specific to that phase and produces an earlier expulsion and stunting of the adult worms, but it operated primarily by reducing the fecundity of the female worms. Thus the 'desired' effect of limiting the invasion of the muscles of the host animal by parenteral larvae is accomplished (even without prior larval invasion) by prohibiting their production.

CHAPTER 5.

Immunity to the parenteral phase of T. spiralis.

The experiments in this chapter were designed to stimulate an immune response only to the parenteral phase by using the infection technique of Dennis et. al. (1970). The specificity of this immune response was tested against the intestinal and parenteral phases of infection.

Despommier (1971) considered that newborn larvae are not immunogenic as they do not possess stichocyte granules. These granules stimulate an immune response (Despommier and Muller, 1970 a and b) which can be detected by Ouchterlony diffusion studies and the immunity is highly effective in reducing the burden of muscle larvae in challenged mice.

Despommier and Wostman (1969) used muscle larvae sealed in diffusion chambers and implanted in mice to demonstrate that muscle larvae alone can stimulate an immune response. This immunity was effective in reducing the numbers of muscle larvae resulting from a challenge infection, and in addition they could detect some stunting of the adult worms and a very slight reduction in their numbers in the intestine. Thus with this method which precludes cell contact with the worms in the diffusion chambers there appeared to be some immune cross reactivity between the life cycle stages.

Experiment 5.1.

The purpose of this experiment was to immunise mice with the parenteral phase and then to challenge them with a normal complete infection and to assess the level of protection from the numbers of larvae encysting in the muscles.

Mice were immunised with 3 IV injections of newborn larvae at intervals of 7 days and challenged with a complete infection per os 14 days after the last immunising infection. It was intended to make the burden of muscle larvae from the challenge infection low and of a similar magnitude to that developing from the immunising infections; consequently an infection of 25 to 30 infective larvae was considered to be the correct challenge dose. 3 groups of mice were used as follows:

Group 1 : On days 0, 7 and 14 of the experiment these mice received IV injections of approximately 1,900, 2,100 and 2,400 newborn larvae. This group did not receive a challenge infection.
Group 2 : These mice, in addition to the immunising infections reported for group 1, were given 27 (2) infective larvae as a challenge infection per os on day 28 of the experiment.
Group 3 : These mice were the challenge controls and received only the challenge dose of infective larvae per os.

All the mice were killed after a further 30 days and digested individually to recover the muscle larvae: The results are shown in table 5.1.

Table 5.1.

Immunity to the parenteral phase of a T. spiralis infection: The effect of this immunity on a challenge complete infection assessed by the number of larvae which develop in the muscles of mice.

Numbers of muscle larvae recovered from individual mice (experiment 5.1.).

Group 1 (immunised-unchallenged)	Group 2 (immunised-challenged)	Group 3 (challenge controls)
3,675	2,870	0
3,920	4,102	209
4,469	4,144	383
6,002	4,687	549
	4,901	685
	5,195	846
	5,207	933
	5,331	1,160
	6,021	2,146
4,516 (1,044)	4,718 (305)	768 (211)

immunisation - parenteral phase
challenge - complete infection

significance tests:

groups 1 & 2	$0.35 \geq p \geq 0.30$
groups 1 & 3	$0.05 \geq p \geq 0.025$
groups 2 & 3	$0.025 \geq p \geq 0.0125$

There is no statistically significant difference between groups 1 and 2, but since the challenge infection produced so few larvae no useful conclusions could be drawn from this experiment save that either the number of infective larvae used in the challenge infection was far too small or that a more accurate method of infecting mice with known numbers of infective larvae needed to be developed (see chapter 3).

Experiment 5.2.

This experiment was designed as a repeat of the previous experiment but this time a larger challenge infection was used. 3 groups of mice were used as follows:

Group 1 : These mice received 3 immunising injections of newborn larvae, approximately 2,100, 1,600 and 1,900 larvae, on days 0, 7 and 14 of the experiment and were not challenged.
Group 2 : These mice were given immunising infections identical to group 1 and also a challenge infection of 316 (19) infective larvae *per os* on day 28 of the experiment.
Group 3 : This group served as the challenge control.

All the mice were killed and digested after a further 30 days: The results are presented in table 5.2.

This experiment shows the difficulties which were sometimes encountered in harvesting sufficient viable newborn larvae. The fault was considered to lie in the sieving and centrifugation of the larvae after culture and prior to injection into the mice, and an effort was made to remedy this in subsequent experiments. However, the immunising infections which produced this low number of muscle larvae

Table 5.2.

Immunity to the parenteral phase of a T. spiralis infection: The effect of this immunity on a challenge complete infection assessed by the number of larvae which develop in the muscles of mice.

Numbers of muscle larvae obtained from individual digestions (experiment 5.2.).

Group 1 (immunised-unchallenged)	Group 2 (immunised-challenged)	Group 3 (challenge controls)
325	21,226	10,144
463	23,733	31,328
473	35,640	44,720
508	45,387	66,800
546	52,787	69,894
561	58,880	74,752
621	67,787	99,877
657	89,010	102,114
713		107,570
847		
571 (41)	49,306 (8,089)	67,467 (11,198)

immunisation - parenteral phase
challenge - complete infection

significance tests:
groups 1 & 2 $0.0005 > p > 0.0$
groups 1 & 3 $0.0005 > p > 0.0$
groups 2 & 3 $0.25 > p > 0.20$

have reduced the burden of muscle larvae which resulted from the challenge infection by 27% in the immunised challenged group. These results are of minimal import since the mean numbers of larvae for groups 2 and 3 are not statistically significantly different, the p value being greater than 0.05.

Experiment 5.3.

This experiment was designed identically to experiments 5.1 and 5.2 and was performed in conjunction with experiment 5.7. A further attempt was made in this experiment to balance the level of the challenge infection with the levels of the immunising infections.

28 mice were divided into 3 groups and used as follows:
Group 1 : These mice received IV injections of approximately 2,000, 2,000 and 2,800 newborn larvae on days 0, 7 and 14 of the experiment respectively.
Group 2 : These mice were immunised as for group 1 and on day 28 of the experiment they received a per os infection of 101 (23) infective larvae.
Group 3 : This group served as the challenge only controls.

All mice were killed and digested as for the previous two experiments: The results are shown in table 5.3.

The results indicate that if the number of larvae due to the immunising infections (3,319) is subtracted from the mean number for the immunised challenged group (11,409), the remainder (8,090) must be due to the challenge infection. The

Table 5.3.

Immunity to the parenteral phase of a T. spiralis infection: The effect of this immunity on a challenge complete infection assessed by the number of larvae which develop in the muscles of mice.

Numbers of muscle larvae recovered from mice in experiment 5.3.

Group 1 (immunised-unchallenged)	Group 2 (immunised-challenged)	Group 3 (challenge controls)
1,186	4,125	7,020
1,792	5,238	9,245
2,378	6,507	10,076
3,136	7,471	18,236
3,219	8,818	19,808
4,479	9,325	19,887
5,023	14,483	24,366
5,340	14,867	25,475
	18,527	29,096
	24,724	50,065
3,319 (537)	11,409 (2,085)	21,327 (3,949)

immunisation - parenteral stages
challenge - complete infection

significance tests:
groups 1 & 2 $0.0005 > p > 0.0$
groups 1 & 3 $0.0005 > p > 0.0$
groups 2 & 3 $0.025 > p > 0.0125$

difference between the number of muscle larvae surviving in the immunised group (8,090) compared with the challenge controls (21,327) is statistically significantly different; the previous immunisation with the parenteral stages has reduced the number of muscle larvae developing from the challenge infection by 62%.

Experiment 5.4.

This experiment attempted to determine whether the immunity stimulated by a parenteral infection affected the intestinal adult worm population of a challenge infection. Mice were infected as for groups 2 and 3 of experiment 5.2 and 18 mice were used as follows:

Group 1 : 8 mice received injections of approximately 2,100, 1,600 and 1,900 newborn larvae on days 0, 7 and 14 of the experiment.

Group 2 : This group was unimmunised.

Both groups received a challenge dose of 316 (19) infective larvae per os on day 28 of the experiment and two mice from each group were killed on days 6, 8, 10, 13 and 15 post challenge. Adult worms were recovered from these mice and counted, and the lengths of the female worms were recorded: The results are presented in Figures 5.41 and 5.42.

The newborn larvae used were of low viability and largely as a consequence of this only 8 mice were immunised, consequently the results are of limited significance. The length measurements do not indicate sufficient evidence for stunting of the female worms. However, the numbers of worms recovered from the intestines of the mice in group 1

Figure 5.41.

Immunity to the parenteral phase of a T. spiralis infection: The effect of this immunity on the intestinal phase of a challenge infection assessed from the mean number of worms obtained from the intestines of mice. (See appendix 14).

1 - group 1 (previously immunised)
2 - group 2 (unimmunised)

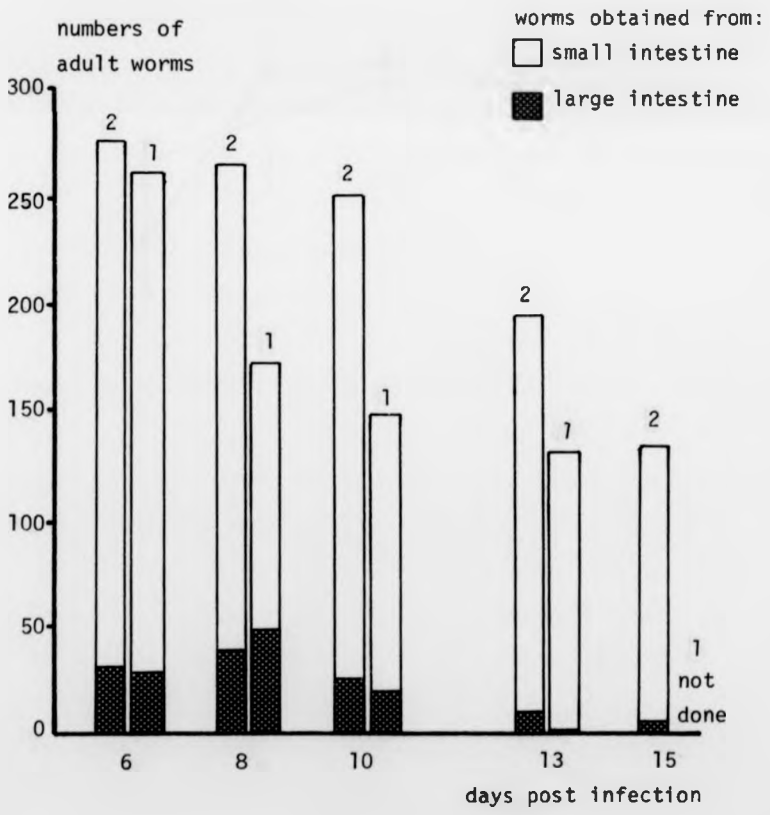
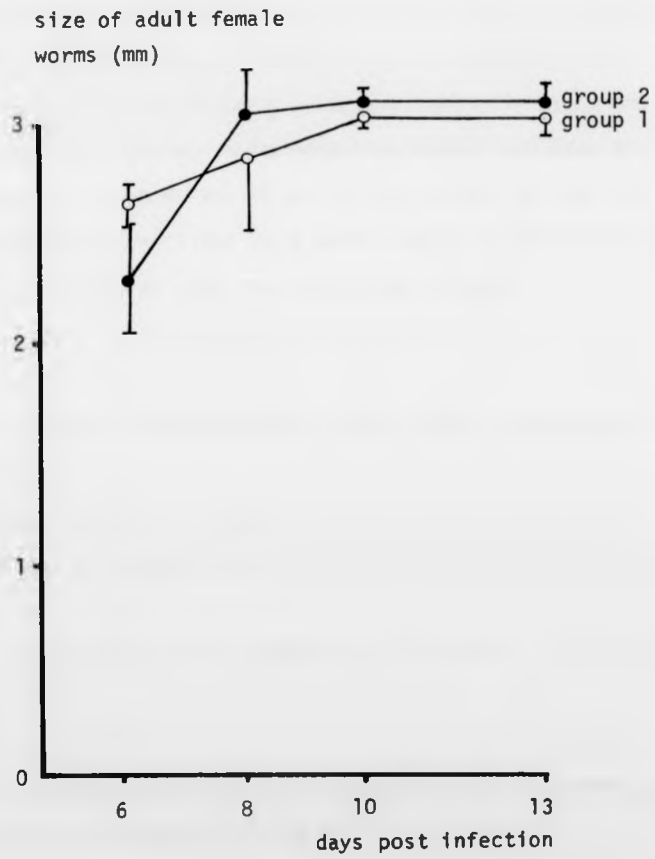


Figure 5.42.

Immunity to the parenteral phase of a T. spiralis infection: The effect of this immunity on the intestinal phase of a challenge infection assessed from the 'stunting' of the adult female worms. (See appendix 13).

1 - group 1 (previously immunised) ⊥ s.e.

2 - group 2 (unimmunised) ⊥ s.e.



(immunised challenged) do not appear to follow the usual population decline of a first infection which those from group 2 (challenge controls) do. But there is a very tentative indication that there could be a slightly earlier onset of adult worm expulsion in mice which have previously been immunised to the parenteral phase of the life cycle.

Experiment 5.5.

Experiment 5.3 had shown that it was possible to induce a 63% reduction in the burden of muscle larvae resulting from a challenge complete infection when mice had been previously immunised with the parenteral phase. This reduction could have been produced by the immunity acting on any or all of the stages of the life cycle. This experiment was the first in a series which attempted to immunise and then challenge with only the parenteral stages.

3 groups of mice were used as follows:

Group 1 : One dose of approximately 2,800 newborn larvae was injected IV into 5 mice.
Group 2 : These mice were immunised together with group 1, and 28 days later received a challenge injection of approximately 2,500 newborn larvae.
Group 3 : These mice were not immunised and served as the challenge controls.

All the mice were killed and digested after a further 24 days: The results are presented in table 5.5.

This was an early experiment designed to determine if a single immunising infection of the parenteral phase could induce immunity to

Table 5.5.

Immunity to the parenteral phase of a T. spiralis infection: The effect of this immunity on a challenge of the parenteral phase assessed by the number of larvae which develop in the muscles of mice.

Numbers of larvae recovered from the muscles of mice in experiment 5.5.

Group 1 (immunised-unchallenged)	Group 2 (immunised-challenged)	Group 3 (challenge controls)
1,621	1,322	290
1,637	1,330	302
1,709	1,371	318
1,753	1,408	329
2,629	1,506	330
	1,563	330
1,870 (191)	1,417 (40)	317 (7)

immunisation - parenteral phase
challenge - parenteral phase

significance tests:

groups 1 & 2	$0.01 > p > 0.005$
groups 1 & 3	$0.0005 > p > 0.0$
groups 2 & 3	$0.0005 > p > 0.0$

a challenge infection, and as such it failed. The results show that the challenge infection of newborn larvae was of low viability while a comparison of the mean numbers show that the immunised challenged group developed fewer larvae (1,417) than the group receiving only the immunising infection (1,870). A fault in the technique was considered to be most likely to account for this observation and it was largely as a result of this experiment that immunisation with the parenteral phase subsequently consisted of 2 or 3 injections of newborn larvae.

Experiment 5.6.

This experiment was designed as a repeat of experiment 5.5 but with 3 immunising injections of newborn larvae. Injections of approximately 2,000, 1,600 and 1,900 newborn larvae were given on days 0, 7 and 14 respectively to 2 groups of mice. One of these groups together with a group of uninfected control mice received a challenge injection of 2,200 newborn larvae on day 28 of the experiment. After a further 24 days the mice were killed and the muscle larvae extracted: The results are shown in table 5.6.

The possible reasons for the poor viability of the immunising infections has been mentioned previously. The difference in the number of muscle larvae recovered from immunised and immunised-challenged groups (425) means that when compared with the number recovered from the challenge controls (1,605) the previous immunising infections have caused a reduction of 74%, in the challenge infection of parenteral larvae.

The individual results from the immunised - challenged group suggest that the mice in this group have responded in two separate ways to the challenge infection - the first 3 or 4 mice have demonstrated

Table 5.6.

Immunity to the parenteral phase of a T. spiralis infection: The effect of this immunity on a challenge infection of the parenteral phase assessed by the number of larvae which develop in the muscles of mice.

Numbers of larvae recovered from digestion of the muscles of mice in experiment 5.6.

Group 1 (immunised-unchallenged)	Group 2 (immunised-challenged)	Group 3 (challenge controls)
325	320	1,330
463	324	1,393
473	523	1,469
508	907	1,621
546	1,019	2,212
561	1,484	
621	2,397	
657		
713		
847		
571 (41)	996 (282)	1,605 (159)

immunisation - parenteral phase
challenge - parenteral phase

significance tests:
groups 1 & 2 $0.15 > p > 0.10$
groups 1 & 3 $0.0005 > p > 0.0$
groups 2 & 3 $0.05 > p > 0.025$

an acquired immunity while the last 3 have not, and the muscle larva burdens for these are similar to those of the challenge control mice.

Experiment 5.7.

This experiment was designed as a repeat of the previous two experiments. 2 groups of mice were immunised with approximately 2,000, 2,000 and 2,800 newborn larvae on days 0, 7 and 14 respectively and one of these groups together with a previously uninfected group received a challenge of approximately 7,200 larvae on the 28th day. 56 days after the first immunising injection the mice were killed and the muscle larvae extracted: The results are shown in table 5.7.

The results indicate that, in this instance, the newborn larvae from the immunising infection were viable, and the immunity developed to this immunisation was 100% effective against a large challenge injection of newborn larvae - as there is no difference between the numbers of larvae obtained from the immunised only and the immunised - challenged groups.

Discussion

The results of these experiments have confirmed the evidence of Despommier and Wostman (1969) and Despommier (1971) that a parenteral infection which includes the muscle larva stage is immunogenic. This immunity has been shown to be 74% (experiment 5.6) to 100% (experiment 5.7) effective against a challenge infection of the same parenteral stage.

When the challenge consisted of a complete infection, which includes the intestinal phase, then the immunisation was 27% (experiment

Table 5.7.

Immunity to the parenteral phase of a T. spiralis infection: The effect of this immunity on a challenge infection of the parenteral phase assessed by the number of larvae which develop in the muscles of mice.

Numbers of muscle larvae recovered from the muscles of mice (experiment 5.7.).

Group 1 (immunised-unchallenged)	Group 2 (immunised-challenged)	Group 3 (challenge controls)
1,186	927	3,619
1,792	998	4,017
2,378	2,112	4,212
3,136	3,424	4,223
3,219	3,563	4,609
4,479	3,706	4,815
5,023	4,531	5,031
5,340	6,471	5,346
		6,249
		6,970
		7,557
3,319 (537)	3,217 (656)	5,150 (383)

immunisation - parenteral phase
challenge - parenteral phase

significance tests:

groups 1 & 2	$0.45 > p > 0.40$
groups 1 & 3	$0.0005 > p > 0.0025$
groups 2 & 3	$0.01 > p > 0.005$

5.2) to 63% (experiment 5.3) effective. The immunity may be acting exclusively on the parenteral phase - on the newborn and/or the muscle larvae - of this complete challenge infection. However, some indication that the adult intestinal worms may be adversely affected was shown by the marginally earlier expulsion time for the adult worm population of previously infected mice in experiment 5.4: By inducing an earlier adult worm expulsion this would cause fewer muscle larvae to develop from a complete challenge infection. Thus, the reduction in the muscle larva burden from a complete challenge infection could be by action on all the life cycle stages, but it is likely, considering the results of experiments 5.6 and 5.7, that immunisation with the parenteral phase is largely specific to this phase.

An alternative hypothesis, to account for the results of experiments 5.2 and 5.3 where mice immunised to the parenteral phase were challenged with a complete infection, could exist. The immunity to the parenteral phase might be highly effective in reducing the number of muscle larvae from a complete challenge infection but the presence of the adult worms in the intestines, during invasion by the muscle larvae, may act partially to suppress this immunity, and thus the protection is not as complete as when the challenge is solely parenteral.

The interrelationships of the life cycle stages with the possibility that one or more stages may actively suppress an immune response will be discussed in conjunction with chapter 8.

CHAPTER 6.

Studies on the antigenicity of newborn larvae.

The work reported in chapter 5 demonstrates that an immune response will develop to the parenteral phase of the life cycle. This parenteral phase incorporates both the migratory stage and muscle stage larvae. The muscle larvae have been shown to be capable of stimulating an immune response (Despommier and Wostman, 1969), but there have been no reports to suggest that the migratory larvae are able to.

This chapter reports various experiments to determine whether the newborn larvae are antigenic or immunogenic.

The immunogenicity of the newborn larvae of T. spiralis has been the subject of only one previous study. Despommier (1971) used 2,000 freeze-thaw killed newborn larvae to immunise rats and then challenged these rats and controls with 600 viable infective larvae given 30 days later. The numbers of muscle larvae recovered following digestion of the rats showed that no acquired immunity had been induced in the rats which had received the dead newborn larvae. However, the burden of muscle larvae resulting from a challenge infection in rats which had been immunised with 6000 viable newborn larvae was reduced by 95%. He suggested that the stimulation of acquired resistance occurred some time after the penetration of the host skeletal muscle and was not due to the migratory stage.

To support his assumption that the newborn larvae are not immunogenic Despommier (1971) cited the evidence of Despommier and

Muller (1969, 1970 a and b) that the $beta_1$ granules of the stichocyte cells contain strong functional antigens, and of Richels (1955), Wu (1955) and Despommier (unpublished observations) that these granules and cells are present in the adult worms and muscle larvae but not in the newborn larvae. He concluded that any stage of the life cycle which does not possess these granules is unable to induce immunity and thus escapes the effects of acquired immunity.

Experiment 6.1.
Immunisation with newborn larvae antigen.

Following the findings of the previous chapter and of Despommier (1971) it was considered that if dead newborn larvae were used as an antigenic stimulus, then the resulting immunity would be most likely to be directed against the parenteral phase.

An antigen was prepared (see chapter 3) from approximately 160,000 newborn larvae. 0.5 ml, amounting to approximately half of the antigen was added to 1.0 ml of Freunds compete adjuvant and homogenised using a glass syringe in a 25 ml bottle held in an ice cooled water bath. 7 mice were injected with 2x 0.1 ml of antigen with Freunds (total antigen for each mouse was derived from approximately 10,500 newborn larvae - total soluble protein content approximately 200 µg), into the thigh muscles of the hind legs.

14 days later the mice received a booster dose of 2x 0.1 ml of antigen (comprising 0.5 ml of antigen and 1 ml of normal saline) in both hind legs as before. After a further 8 days these mice with 6 unimmunised controls received an intrave nous injection of approximately 3000 newborn larvae.

28 days later the mice were killed and digested to recover the muscle larvae: The results are shown in table 6.1.

These results are statistically not significantly different. The immunisation has been shown to be ineffective in reducing the burden of muscle larvae resulting from a parenteral-only challenge. Thus dead newborn larvae (Despommier, 1971) and antigen prepared from newborn larvae do not appear to stimulate an immune response.

Experiment 6.2.
Immunisation with living newborn larvae.

Despommier (1971) found that exposure to living newborn larvae was necessary to induce an acquired immunity effective against a complete challenge infection. The objective of this experiment was to immunise mice with only living newborn larvae. This was attempted by injecting mice with newborn larvae and then treating with the drug cambendazole.

Cambendazole is active against both the intestinal and parenteral phases of T. spiralis (Duckett and Denham, 1970). They reported a partial activity of the drug against the migratory phase, and Duckett (1971) has shown that when given to mice at a dose of 50 mg/Kg/day for 7 days commencing on the 28th day PI the burden of muscle larvae was reduced by more than 90% compared with untreated controls and Duckett (1971) considered that the larvae were killed or damaged in the muscles by the drug and then actively removed by the host defence mechanism over a period of 14 days after treatment.

Because cambendazole has the ability to damage or destroy encysted muscle larvae it was thought that this drug would provide

Table 6.1.

Antigenicity of the newborn larvae of T. spiralis: The effect of immunisation with newborn larva antigen on a challenge infection of the parenteral phase as assessed by the number of larvae which develop in the muscles of mice.

Numbers of muscle larvae recovered from mice in experiment 6.1.

Group 1 (immunised - challenged)	Group 2 (challenge controls)
102	408
215	887
250	1,179
1,809	1,341
1,824	1,345
2,112	2,305
2,149	
1,209 (364)	1,244 (257)

immunisation - newborn larva antigen
challenge - parenteral phase

significance test: $0.25 > p > 0.20$

a useful means of limiting the immunising injection of parenteral larvae to the migratory stage. The LD_{50} of cambendazole in adult female mice is known to be approximately 1,500 mg/Kg (cited by Duckett and Denham, 1970).

From the evidence of Duckett and Denham (1970) that cambendazole is only partially active against the migratory larvae and from the growth measurements of muscle larvae of Harley (1971d) it was decided to commence drug treatment on the 9th day post injection.

At the same time it was decided to determine whether the presence of an intestinal infection (see chapter 8) in any way affected the course of the challenge infection, and for this, infective larvae were irradiated and administered to one group of immunised mice 6 days before the challenge infection of newborn larvae. The 6th day was chosen since the majority of newborn larvae are produced by the intestinal worms between the 6th and 7th days PI and this would correspond to the time of injection of newborn larvae.

4 groups of mice were used as follows:

Group 1 : These mice received approximately 4,200 newborn larvae by IV injection. A suspension of cambendazole in water was prepared and 0.1 ml of this suspension was given <u>per os</u> using a blunted 1½ inch gauge 18 hypodermic needle fitted to a 1 ml syringe on days 9, 10, 11 and 12 PI. (Total dose 800 mg/Kg for each mouse). These mice served as unchallenged controls to determine the effectiveness of the cambendazole treatment.

Group 2 : These mice were infected and treated as for group 1 and

also received a challenge infection of 8,000 newborn larvae 7 days after the last dost of cambendazole had been administered.
Group 3 : This group of mice received the same immunising infection, treatment and challenge as group 2 but in addition, they were given 560 (50) infective larvae which had been irradiated with 8,500 rads. 6 days prior to the newborn larval challenge.
Group 4 : These mice received only the challenge injection of newborn larvae and served as challenge controls.

30 days after this challenge the mice were digested to recover the muscle larvae: The results are shown in table 6.2.

The results show that the single cambendazole abbreviated immunising infection has been effective in reducing the numbers of muscle larvae developing from a parenteral challenge infection. Despite the dose of cambendazole used (4 doses of 200 mg/Kg), 34% of the injected larvae still survived and developed into muscle larvae.

This confirms the findings of Duckett and Denham (1970) that cambendazole is only partially effective against the migratory larvae.

To what extent the encysted muscle larvae developing from the incompletely 'abbreviated' parenteral infection affected the immune response to the challenge infection cannot be determined but assuming that the 1,682 larvae (3,121 less 1,439) in group 2 (immunised - challenged) were the result of the challenge infection then the immunity was shown to be 74% effective. This compares favourably with the results of chapter 5 as in this experiment only the one immunising infection of 4,200 larvae was used.

Unfortunately because the action of cambendazole does not

Table 6.2.

Antigenicity of the newborn larvae of T. spiralis: The effect of immunisation with a drug abbreviated infection on a challenge infection of the parenteral phase, assessed by the number of muscle larvae which develop in the muscles of mice.

Numbers of muscle larvae recovered from mice in experiment 6.2.

Group 1 (immunised-treated)	Group 2 (immunised-challenged)	Group 3 (as for group 2 with irrds.)	Group 4 (challenge controls)
14	935	2,586	5,330
104	2,002	2,962	5,645
695	2,090	4,006	5,995
814	2,301	5,301	6,206
1,371	2,364	5,933	6,762
1,708	3,711	6,333	6,899
2,181	4,114	7,091	6,977
2,243	7,452		7,182
2,275			
2,982			
1,439 (318)	3,121 (712)	4,887 (654)	6,375 (240)

The difference between all these results are statistically significant.

immunisation - newborn larvae
challenge - parenteral stages

significance tests:

groups 1 & 2	$0.05 > p > 0.025$
groups 1 & 3	$0.01 > p > 0.005$
groups 1 & 4	$0.0025 > p > 0.0005$
groups 2 & 3	$0.05 > p > 0.025$
groups 2 & 4	$0.0025 > p > 0.0005$
groups 3 & 4	$0.01 > p > 0.005$

totally remove the young 'immature' larvae reaching the muscles, the possibility of using this drug to abbreviate parenteral infections and allow immune stimulation by only the newborn migrating larvae and 'immature' muscle larvae does not appear to be possible; thus it is unlikely that the immunogenicity of the newborn larvae could be assessed by this type of infection.

The mice from group 3 harboured significantly more muscle larvae as a result of receiving irradiated infective larvae 6 days prior to challenge. It would therefore appear that the presence of an intestinal infection has in some way affected the immunity to the challenge infection of parenteral larvae. This possibility is dealt with more exhaustively in chapter 8.

Experiment 6.3.

This experiment was performed in conjunction with the previous experiment and the immunised groups of mice received the same infection and drug treatment as experiment 6.2. The challenge infection in this case however consisted of a complete infection of 49 (3) infective larvae given per os 7 days after the last cambendazole treatment.

The results following digestion of the mice are shown in table 6.3: The immunising parenteral infection is shown in this experiment to have been very effective in stimulating an immune response, the 2,189 (3,628 less 1,439) larvae from the immunised challenged (group 2) mice represents an 83% reduction compared with the challenge controls (12,973). But as stated for experiment 6.2 it is not known to what extent the newborn larvae are responsible for the demonstrated immune response.

Table 6.3.

Antigenicity of the newborn larvae of T. spiralis: The effect of immunisation with a drug abbreviated infection on a challenge complete infection, assessed by the number of muscle larvae which develop in the muscles of mice.

Numbers of muscle larvae obtained from mice after digestion (experiment 6.3.).

Group 1 (immunised-treated)	Group 2 (immunised-challenged)	Group 3 (challenge controls)
14	579	3,134
104	1,795	5,142
695	1,855	6,615
814	2,158	8,686
1,371	2,297	9,530
1,708	5,153	11,360
2,181	7,047	11,760
2,243	8,137	13,063
2,275		29,896
2,982		30,547
1,439 (318)	3,628 (983)	12,628 (3,033)

These results are all statistically significantly different from each other.

immunisation - newborn larvae
challenge - complete infection

significance tests:

groups 1 & 2	$0.05 > p > 0.025$
groups 1 & 3	$0.0005 > p > 0.0$
groups 2 & 3	$0.0025 > p > 0.0005$

Experiment 6.4.

The *in vitro* effect of immune sera on *T. spiralis*.

Oliver Gonzalez (1940) and Lukashenko (1960) have reported that serum from immunised animals has a very marked effect *in vitro* on *T. spiralis* adult worms and muscle larvae.

Oliver Gonzalez studied the action of immune and hyperimmune sera from rats, guinea pigs and rabbits in *in vitro* culture; he also reviewed some of the early work on this *in vitro* technique and the action of immune sera in other nematode infections. His results showed that sera from *T. spiralis* infected animals produced large masses of precipitates around the mouths of muscle larvae, these were associated with immobilisation, disintegration and death of many of the larvae. Oral, anal and vulval precipitates also formed around adult worms, and in all the cultures free precipitates could be found in the medium. The only difference between the reactions with immune (single infection) and hyperimmune (multiple infections) sera was that the precipitates occurred earlier in the latter, but in both cases the precipitation was well marked after 4 days incubation at $37^{o}C$. He found also that inactivated serum was as effective as unheated serum or serum with added complement.

Although immune sera reacted well against adult worms and muscle larvae, Oliver Gonzalez could find no reaction to the newborn larvae produced by female worms in the culture.

This experiment reports observations made on adult worms, newborn larvae and muscle larvae cultured in sera taken from rabbits immunised to different stages of the life cycle.

Sera F. This rabbit received a normal complete infection of approximately 10,000 infective larvae and a booster infection of 12,000 infective larvae 14 weeks later. Serum was collected at weekly intervals (see also chapter 7) and sera used here are F0 - taken immediately prior to the first infection, and F19, taken 5 weeks after the second immunising infection.

H. This rabbit received 3 injections of newborn larvae into the marginal ear vein. The infections consisted of approximately 12,000, 14,000 and 11,000 larvae given on weeks 0, 12 and 22. Serum HI was collected after a further 8 weeks. The larvae were allowed to 'mature' to muscle larvae so that this rabbit had effectively been immunised against the newborn and muscle larva stages of the life cycle.

R. An attempt was made to immunise this rabbit against the adult stage worms only. 10,000 excysted larvae were given per os and the rabbit was treated with methyridine at 110, 113 and 116 hours. A second infection of 12,500 larvae was given 7 days after the first and a third of 28,000 larvae after 14 days, both were similarly terminated by methyridine treatment. This infection schedule was originally adopted on another rabbit using doses of methyridine at 500 mg/Kg, but the treatment had proved fatal and so doses of 200 mg/Kg were used here. It is doubtful whether this treatment removed all the adult worms from the intestines so this rabbit was presumed to be immunised against the parenteral phase also.

Another rabbit was infected with newborn larvae with the intention of abbreviating the infection with cambendazole, and this

rabbit was to be used as a source of serum specific to the newborn larvae. Administration of cambendazole proved difficult, and there was no certainty that the rabbit had received the complete dose of the drug: It developed an unrelated acute 'middle ear' infection soon afterwards and the autopsy showed that the drug treatment had not been successful.

Incubation of worms: In an initial experiment 'macrophage cultivation' trays with 12 wells were used to contain the sera. This method required large quantities of serum while the results were difficult to observe under the microscope at high power owing to the thickness of the culture. Thus, glass microscope cavity slides were preferred. 2 drops of serum from a sterile pasteur pipette were put on to the slide after first ringing the well with vaseline. 1 drop of sterile medium 199 containing adult worms or infective larvae was added - this suspension also contained merthiolate, penbritin and mycostatin and was made up as follows: 199 with worms - 2 ml, merthiolate solution 1:20,000 - 2 ml, penbritin solution - 0.1 ml, mycostatin solution - 0.05 ml.

The cultures were observed at 15 minutes, 1½ hours, 6 hours, 24, 48 and 72 hours, and a scale of readings from + to 6+ was awarded as follows:

+ precipitate present as fine granules adhering to cuticle
2+ first appearance of small oral precipitate
3+ cuticular and oral precipitates well developed with possibly small anal precipitate, some free precipitate in culture
4+ large oral precipitates, increased cuticular precipitates, much free precipitate.

Plate 11. Precipitate around newborn larva cultured in immune serum 1+.

Plate 12. Precipitate around newborn larvae cultured in immune serum 4+, oral precipitate (o), cuticular precipitate (c), free precipitate in medium (f).

Plate 11. Precipitate around newborn larva cultured in immune serum 1+.

Plate 12. Precipitate around newborn larvae cultured in immune serum 4+, oral precipitate (o), cuticular precipitate (c), free precipitate in medium (f).

Plate 13. Muscle larvae cultured in immune serum, no precipitate.

Plate 14. Muscle larvae cultured in immune serum, precipitate 2+, oral precipitate (o), cuticular precipitate (c).

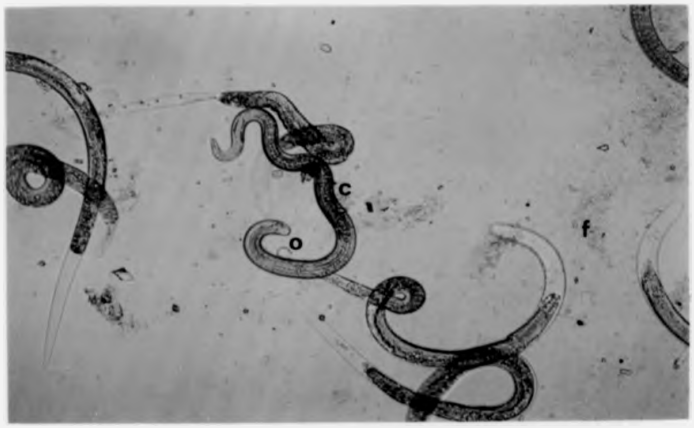


Plate 15. Muscle larvae cultured in immune serum, precipitate 3+ oral precipitate (o), cuticular precipitate (c), free precipitate (f).

Plate 16. Muscle larvae cultured in immune serum, precipitate 5+, oral precipitate (o), cuticular precipitate (c), free precipitate (f), cuticle sheath (s), disintigrating oesophagus (d).

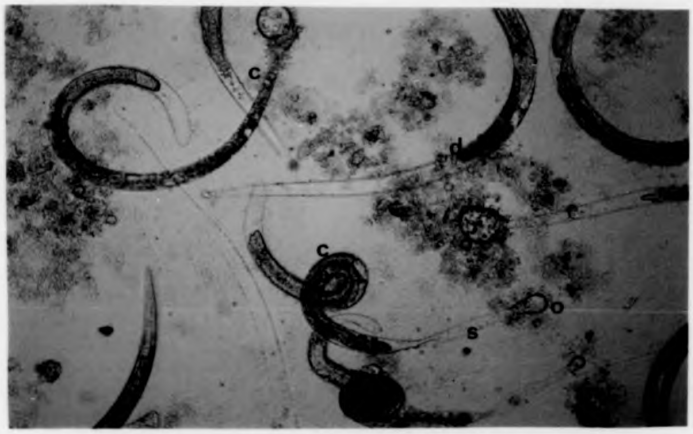


Plate 17. Muscle larvae cultured in immune serum, precipitate 6+, oral precipitate (o), cuticular precipitate (c), free precipitate (f), cuticle sheath (s), disintigrating oesophagus (d).

Plate 18. Adult worms cultured in immune serum, precipitate 4+, anal precipitate (a), cuticular precipitate (c).

5+ very large oral precipitate, large local masses of cuticular precipitate, much free precipitate in culture. For muscle larvae evidence for disintegration of the oesophageal region within the cuticle and/or moulted undiscarded sheath.

6+ presence of very large amount of all types of precipitates. Decreased motility of worms and disintegration of muscle larvae more advanced.

(see plates 11 to 18).

Not all the worms seen in each culture appeared as above, some were observed to lose their attached precipitates which had become too large and were dislodged as the worms moved, and thus some worms would appear without precipitates.

It was noticed that when the majority of worms were in the 6+ condition there were often 1 or 2 which did not have attached precipitates and which also appeared undamaged.

The results are presented in table 6.4.

The most interesting observation was that precipitates formed around newborn larvae as well as around the other 2 stages used. This observation had not previously been noted by Oliver Gonzalez (1940) and it strongly suggests that both the cuticle and excretions and secretions of newborn larvae are antigenic.

The precipitates appear to be of two basic types: The cuticular precipitate appeared first on adult worms and newborn larvae, but later became more noticeable on muscle larvae.

Table 6.4.

Antigenicity of the newborn larvae of T. spiralis: The effect of immune rabbit sera on adult worms, newborn larvae and muscle larvae in in vitro culture.

Record of precipitates forming on adult worms, newborn larvae and muscle larvae cultured in rabbit sera.

	adult worms				newborn larvae				muscle larvae			
time	F0	F19	HI	R3	F0	F19	HI	R3	F0	F19	HI	R3
15 mins.	-	-	-	-	-	-	-	-	-	-	-	-
1½ hrs.	-	+	-	+	-	+	-	+	-	2+	+	+
6 hrs.	-	2+	-	+	-	4+	-	+	-	4+	2+	4+
24 hrs.	-	5+	-	4+	-	6+	+	+	-	6+	4+	4+
48 hrs.	-	6+	-	6+	-	6+	+	+	-	6+	4+	4+
72 hrs.	-	6+	-	6+	-	6+	+	2+	-	6+	4+	4+

Key - see page

F0 serum from uninfected rabbit

F19 serum from rabbit given 2 complete infections

HI serum from rabbit infected 3 times with the parenteral phase.

R3 Serum from rabbit infected with adult worms.

Adult worms incubated in HI serum (immunised to parenteral stages) did not develop cuticular precipitates while newborn and muscle larvae did, this may indicate that the antigenicity of the cuticle of adult worms differs from that of the other stages or that it is masked in some way. Masking with host material in a similar way to that which occurs for Schistosoma mansoni is unlikely as the adult worms used in these cultures had been recovered after 6 days' growth from mice, and cuticular precipitates will form on adult worms in rabbit serum as was shown by F19 (twice infected).

The second type of precipitate, presumably generated by excretions and secretions, was typically the oral precipitate. This was totally absent from adult worms incubated in HI serum (immunised to parenteral stages) even after 4 days, while the oral precipitate on muscle larvae incubated in this serum was very pronounced. This suggests that the E and S of adult worms and muscle larvae may be different. Newborn larvae incubated in HI serum developed cuticular precipitates but no oral precipitate, and as oral precipitates were very evident on muscle larvae this indicates that the E and S of newborn larvae may also differ from those of muscle larvae. Unfortunately with the failure to immunise a rabbit against the newborn larval stage only, no comparison between the precipitates to E and S for adult worms and newborn larvae can be made.

The formation of oral precipitates on newborn larvae incubated in sera F19 (immunised to 2 complete infections) and R3 (immunised to adult worms) gives strong evidence for the assumption (chapter 1) that newborn larvae do feed and metabolise, and do secrete enzymes or excrete waste products, during their migration.

Experiment 6.5.

The indirect fluorescent antibody test (IFAT) for T. spiralis is a well established technique for determining the presence of antibodies in immune sera (see chapter 7), and it was used here to test the sera from experiment 6.4. This test is used extensively with muscle larvae , the binding sites for antibody occurring on the cuticle of the larvae; this experiment was designed to test the activity of sera against different stages of the life cycle in the IFAT.

The technique used by Jackson (1959) was a direct fluorescent antibody test. He incubated adult worms and muscle larvae in isocyanate labelled immune rabbit sera. He found that the internal organs, particularly the digestive tract and reproductive system, incorporated this labelled serum and so did some of the oral precipitates on the adult worms and muscle larvae. In no instance did he find the cuticles stained by fluorescence, nor also did he find fluorescence on newborn larvae. Oliver Gonzalez (1941) was of the opinion that the young larvae do not become antigenically distinct from adults until after they have been born.

In this experiment adult worms and muscle larvae were recovered from mice, washed 5 times in PBS and killed by suspending them for 10 minutes in 10% formol PBS. They were again washed 4 times before being used in the test. Newborn larvae were harvested from the culture of adult worms and similarly killed in formol PBS and washed before use.

Sections of adult worms were prepared by freezing approximately 10,000 adult worms in a small quantity of PBS on to a metal block.

Cryostat sections 7 μm thick were cut at -20°C and placed on coated multispot slides - 12 sections per slide (C. A. Hendley & Co., Essex). They were placed in a dessicator at 4°C for at least 24 hours and then fixed in acetone for 10 seconds immediately before use.

Whole worms were used in the IFAT tube technique as described in chapter 7. Sections of adult worms were treated as follows:

1. immune and control sera were put on to the sections in single drops and the slides were incubated for 30 minutes at 37°C.
2. slides were then washed in PBS 3 times, each wash lasting approximately 5 minutes.
3. discrete drops of fluorescein isothiocyanate conjugated (FITC) goat antirabbit (GAR) immunoglobulin (Nordic Pharmaceuticals) diluted 1:10 with a 0.25% evans blue solution were placed on the slides and these were incubated for 30 minutes at 37°C.
4. slides were washed 3 times in PBS and then immersed in acetone for 10 seconds to remove the excess evans blue stain.
5. the slides were washed 3 times more in PBS, PBS-buffered glycerine added with a coverslip and the sections studied for fluorescence under a UV microscope.

The results are shown in table 6.5: When sections of adult worms were used the most reactive portion was seen to be the cuticle/hypodermis. Some sections also contained fluorescing structures which could have been the cuticles of unborn larvae when the female worms were sectioned in the uterine region. Other sections showed a distribution of fine fluorescing particles in the general structure of the worms, and these sections conformed closely to the appearance of sections through the stichosome region viewed under normal transmitted light. It is interesting that the sections

Table 6.5.

Antigenicity of the newborn larvae of T. spiralis: The reactivity of whole newborn larvae used as antigen in the indirect fluorescent antibody test against immune rabbit sera compared with the other stages of the life cycle.

Titres of immune sera reacting to T. spiralis. Results are expressed as the reciprocal of the titre.

sera	adult worms		newborn larvae	muscle larvae
	whole	section		
F0	0	0	0	0
F13	16	512	32	1,024
F19	32	1,024	64	1,024
HI	0	32	32	128
R3	32	512	64	1,024

F0 serum from uninfected rabbit

F13 serum from rabbit given 1 complete infection

F19 serum from rabbit given 2 complete infections

H1 serum from rabbit infected 3 times with parenteral stages

R1 serum from rabbit infected with adult worm stages and other stages (see text)

of adult worms consistently gave higher titres than when the whole adult worms were used.

The reactivity of the cuticle of the newborn larvae to the IFAT test confirms the findings of the previous experiment that the cuticle of newborn larvae is antigenic.

Discussion

It has been shown that newborn larva antigen could not elicit a protective immune response (experiment 6.1) (and when this was tested by Ouchterlony diffusion in agar no precipitation lines formed to immune sera when the antigen was of a comparable protein concentration to muscle larva antigen which did react). However, both experiments 6.4 and 6.5 demonstrated that the cuticle of the newborn larva reacts with antisera, and experiment 6.4 has shown that the newborn larvae do excrete or secrete substances from the oral region and that these are antigenic.

Although there is strong evidence that newborn larvae (experiments 6.4 and 6.5) are antigenic, the immunogenicity of these larvae can only be presumed. The degree of exposure of the host to all antigens of newborn larvae in a normal infection must be very small, both because the larvae themselves are small and because their migration to the muscles is of short duration. The role of the newborn larvae in the development of the immune response to a T. spiralis infection is thus probably very small and is subsidiary to the roles of both the intestinal worms and muscle larvae.

CHAPTER 7.

Studies on stage specific immunological responses during an infection with T. spiralis.

Between the time of infection and the time of the encystment of the muscle larvae, mice have usually acquired an immunity which will prevent or diminish a subsequent challenge infection.

An inflammatory response begins in the intestine on about the 4th day post infection (PI) (Larsh and Race, 1954), becomes progressively more severe during the first week of infection, and then subsides by about the 10th day PI. Histological studies have revealed (Larsh and Race, 1954; Larsh, Goulson and Van Zandt, 1962; Race, Larsh, Martin and Pate, 1972) that during the early stages of intestinal inflammation there occurs a large infiltration of polymorphonuclear cells into the lamina propria which is superceeded as the inflammation becomes sub-acute by an infiltration consisting predominantly of lymphocytes but also of large mononuclear cells, plasma cells, eosinophils and neutrophils. There appears to be a rough correlation between the intestinal response to infection and the body weight changes of mice observed by Yarinsky (1962), and this is largely attributed to a decrease in food intake while inflammation occurs.

The work of Larsh, Goulson and Weatherly (1964a and b), Larsh, Race, Goulson and Weatherly (1966) and Larsh, Goulson, Weatherly and Chaffee (1969; 1970a and b) has been interpreted by them as supporting a cell mediated (CMI) basis for the immune reaction developing against an intestinal infection, and recent evidence with the administration of antithymocyte serum (ATS) to mice (Larsh, Weatherly, Goulson and Chaffee, 1972) lends some support

to their theory. The theory is that an antigen-antibody reaction acts as a trigger to the CMI response causing injury to cells and tissues of the small intestine resulting in the allergic inflammation. The changes produced by this inflammation - a decrease in oxygen tension and pH and increase in carbon dioxide tension - aid in the expulsion of the worms. Castro, Cotter, Ferguson and Gordon (1973) found that a decrease in pH adversely affected the worms, but that other changes did not; nevertheless they indicated support for this CMI basis of acquired immunity.

During this period of infection Ruitenberg (1972) and Ruitenberg and Duyzings (1972) from studies of the spleen and mesenteric and mandibular lymph nodes observed a steady increase in the number of immunoglobulin containing cells in SPF rats. They also detected an increase in the number of pyroninophylic blast cells in the paracortical (thymus derived) areas of the lymph nodes which increased in size and which thus indicated (from Oort and Turk, 1965) that a CMI response had been induced in addition to the humoral response.

Crandall and Crandall (1972) demonstrated a fall in serum immunoglobulin concentration soon after infection, followed by a rise towards the end of the first week PI, attributed mainly to the IgG_1 and IgM fractions although the responses of these immunoglobulins varied with the strain of mice used. They suggest that the early immunoglobulin fall may be due to a general hypoproteinemia related to inflammation and intestinal damage and the weight loss of infected hosts reported by Yarinsky (1962) and Larsh et al. (1962a) and that the subsequent rise may be due both to an increased synthesis stimulated by the loss of immunoglobulins and to the antigenic stimulation by the helminth antigens. IgG_1 is an anaphylactic agent

in mice (Nussenzweig, Merryman and Benacerraf, 1964) and the observed increase in this antibody paralleled the anaphylactic sensitivity to T. spiralis antigens reported by Briggs and De Giusti (1966). They also reported that from immunofluorescent studies early IgG_1 and IgG_2 reacted specifically to the contents of the stichocyte cells of the adult worms and suggest that these cells are the major source of antigens which induce this IgG response. The stichocyte cells have been confirmed as being immunogenic by Jackson (1959), Brzosko, Gancarz and Nowoslawski (1965), Catty (1969) and Despommier and Muller (1970a and b). The IgG_1 level in the intestinal contents was found by Crandall and Kozek (1972) to remain at a low level while perfused intestinal contents concentrated by radial immunodiffusion showed a level of IgA which increased during the course of infection. There was no correlation between serum and intestinal levels of IgA and they suggested that immunoglobulins of this class had been synthesised locally and secreted into the intestine. The significance of this finding was not known as the adult worms positioned within the villi should have been capable of contact by serum immunoglobulins (Crandall and Crandall, 1972). Fluorescent staining revealed that both IgA and IgM class antibodies developing early in the infection specifically reacted to the membranes of the worms.

Bazin, Andre and Heremans (1973) have suggested that antigens which are confined to the mucosa will tend to induce an 'intestinal-type' response based on IgA production and this may lead to a state where immunogens rendered tolerogenic by combination with excess local antibody produce a level of tolerance to the antigens, but what type of pathophysiological condition that may be produced was not specified by them.

Karmanska and Kozar (cited by Kozar, 1969) reported an experiment using direct immunoflorescence to detect immunoglobulin production. They found that the number of immunologically competent cells increased in the intestinal villi as early as 1 to 2 days PI and became most numerous between 3 and 10 days PI, but they did not define the nature of these cells. The cells were located in bands on the borders of the Peyer's patches proximal to the lumen. Injections of antigens into Peyer's patches are known to stimulate local antibody production (Pollard and Sharon, 1970) and while these lymphoid tissues do not appear to function as draining lymph nodes, the pre-adult and adult intestinal T. spiralis are found in close proximity to the Peyer's patches.

Mast cell sensitisation and degranulation is produced by certain antigen/antibody complexes for a T. spiralis infection as has been demonstrated by Briggs (1961) and this anaphylactic-type response can be induced by intrave nous (IV) injection of antigen as early as 5 to 7 days PI (Briggs and De Giusti, 1966). An expulsion of intestinal worms similar to that for T. spiralis and concurrent with a rise and fall in inflammation occurs for N. brasiliensis, and Miller and Jarrett (1971) have reported an increase in the mast cell population occuring at the time of worm expulsion. Karmanska, Kozar, Seniuta and Dlugiewica-Bulla (1972) found a clear correlation between mast cell stimulation and expulsion of adult T. spiralis. The strongest mast cell stimulation in mesenteric lymph nodes occurred at about 14 days PI. Karmanska et al. (1972) reported that antilymphocyte serum (ALS) induced an inhibition of mast cell mobilisation particularly in the small intestine and expulsion of worms occurred much later in ALS treated mice, as has been reported by Machinicka (1972).

There is much evidence to suggest that the developing immune response to intestinal T. spiralis may be predominantly humoral in nature but Soulsby (1970) has emphasised that further critical studies are needed on the CMI response to parasitic infections before the role of CMI can be evaluated.

The parenteral infection begins on the 5th day PI. The newborn larvae burrow through the intestinal mucosa and migrate largely via the lymphoid system and thoracic duct (Harley and Gallicchio, 1971b) and reach the striated muscles via the circulatory system. Some of the pathological changes to the muscle cell during and after penetration have been described in chapter 1.

The immunity which develops to the parenteral phase has not been monitored in nearly as much detail as has immunity to the intestinal phase, and until the technical problems of producing a parenterally stimulated immune response in the absence of the intestinal worms had been solved, it was not possible to ascertain the role that the parenteral phase played in the total immune response to a normal infection. That the parenteral phase was immunogenic and possessed some immunogens which were distinct from those of the intestinal phase had been suggested by Oliver Gonzalez (1940), but that the immunity which was stimulated solely by the parenteral phase was protective was not demonstrated until the studies of Despommier and Wostman (1969) and Despommier (1971) had showed that the burden of muscle larvae resulting from a challenge infection was reduced in hosts immunised to the parenteral stages.

From the time of invasion of the muscle fibres inflammatory and alterative changes are exhibited (Gould, 1970) in the muscle, with polymorphonuclear cells, lymphocytes and tissue histiocytes invading the interstitial connective tissues. Mast cells seldom occur. Eosinophils are observed to increase in number during this phase of the infection and the blood eosinophil level is elevated. The eosinophil response was generally believed to be caused by the parenteral phase but Lin and Olson (1972) recently showed that the intestinal phase was capable of inducing at least some increase in eosinophils.

There is some evidence that the development of eosinophilia in T. spiralis infections (Basten and Beeson, 1970; Basten, Boyer and Beeson, 1970) may depend on the presence of functioning lymphocytes - largely of T cells with possible B cell cooperation - but the precise interrelationships of these cells with eosinophils in infected animals remains to be elucidated.

By administering intraperitoneal (IP) injections of T. spiralis to mice Stankiewicz (1972) showed that of the adult worms, newborn larvae and muscle larvae, it was the muscle larvae which stimulated the most rapid and most extensive adherence of peritoneal exudate cells and that muscle larvae obtained by digestion from rats and injected into mice provoked a greater reaction than when they were injected into rats. He suggests that the muscle larvae may become adapted to the host in a manner which results in less interaction with cells. This view is consistent with the fact that the muscle larvae must remain within the host muscle for a considerable length of time to ensure continuation of the life cycle into a new host.

The larva within its cyst has been considered to be unassailable by antibodies or immunocytes, and it is interesting that in electronmicroscopic studies Tepemma (1973 - personal communication) has noticed small numbers of monocytes and macrophages within the capsule wall but which did not appear to have attacked the larva. However, it is the muscle larva antigen which is most reactive to hyperimmune serum (Oliver Gonzalez and De Sala, 1963) and it is this antigen which has been the subject of most immunological analysis (Tanner and Gregory, 1961 and 1963a and b; Catty, 1969).

Homocytotropic antibodies, identified with the immunoglobulin class IgE, appear during the 5th week PI (Mota, Sadun and Gore, 1968; Mota, Sadun, Bradshaw and Gore, 1969) and peak at 9 weeks PI. Mota et al., (1969) were able to distinguish by passive cutaneous anaphylaxis (PCA) tests two antibodies which had been separated on DEAE cellulose. The PCA reactions occurred 4 hours and 72 hours after injection of antigen and the serum concentration of these homocytotropic antibodies was capable of being elevated by 'dead' muscle larvae.

The IFAT is probably most useful for testing sera (see below) for the presence of antibody while for the detection of a delayed hypersensitivity response, Collins and Mackaness (1968) have reported that the 'footpad response' to small quantities of antigen is useful (see below).

Using antigens prepared from adult worms, newborn larvae and muscle larvae or whole worms of these stages, this chapter reports the experiments which attempted to determine the time of appearance of stage specific immune responses during the course of

a single infection with T. spiralis.

Experiment 7.1.

Cell mediated immunity - detection of a CMI response using the technique of granuloma formation in the lungs to T. spiralis antigens.

The principle behind this response is that the presence of antigen/antibody complexes can stimulate a CMI response (Uhr, Salvin and Pappenheimer, 1957). When whole viable eggs of S. mansoni are injected IV into infected or sensitised mice, a localized cellular infiltration and granuloma formation occurs around the eggs which have lodged in the microvasculature of the lungs. This has been termed the 'Von Lichtenberg method' by Warren and has been used to demonstrate the existence of an infection or a previous sensitisation to Schistosome eggs (Warren, Domingo and Cowan, 1967; Warren and Domingo, 1970) and even to demonstrate that a response can occur preferentially to certain fractions isolated from the eggs (Boros and Warren, 1970). Warren and his co-workers have demonstrated that the granuloma response around Schistosome eggs is a manifestation of CMI by their ability to induce the response in previously unsensitised mice by transfer of lymph node and spleen cells and to suppress the reaction in sensitised mice by neonatal thymectomy and the administration of ALS (Warren, 1969). However, Warren (1969) also found that mice infected with Friend virus leukemia, which depressed synthesis of circulating antibody, exhibited an enhanced granulomatous response.

Warren and Domingo (1970) have stated that 'humoral antibody mediated immediate hypersensitivity responses are relatively non

specific in comparison with cell mediated delayed hypersensitivity reactions': This experiment reports an attempt to adapt the lung granuloma response for use in a T. spiralis infection for the detection of stage specific immunity.

In a preliminary experiment the system was tested using mice sensitised or infected with S. mansoni and injected IV into the lateral tail vein with viable eggs which had been recovered by the technique of Smithers (1960). The mice from the infected and control groups were killed 8 days after egg injection, the lungs removed and fixed in 10% formol saline and sectioned and stained in haematoxylin and eosin. Granuloma responses were observed around viable ova in the lungs of previously infected mice but not in those of uninfected mice (see plate 19), and the results were similar to those reported individually by Von Lichtenberg and Warren.

Having confirmed that the technique would work for S. mansoni using eggs, it was then attempted with T. spiralis. Initially it was intended to determine whether T. spiralis muscle larva antigen, this being generally considered as the most reactive, coated on to bentonite particles of a size sufficient to be trapped in the lung capillaries, would induce a granuloma response. Then if this proved successful the test was to be repeated using antigens from adult worms and newborn larvae; and finally it was intended to determine the time of onset of the granuloma response to the different stages of the life cycle during the course of a single complete infection.

Following the technique of Warren and co-workers, bentonite particles were suspended in saline and passed through a 300 mesh

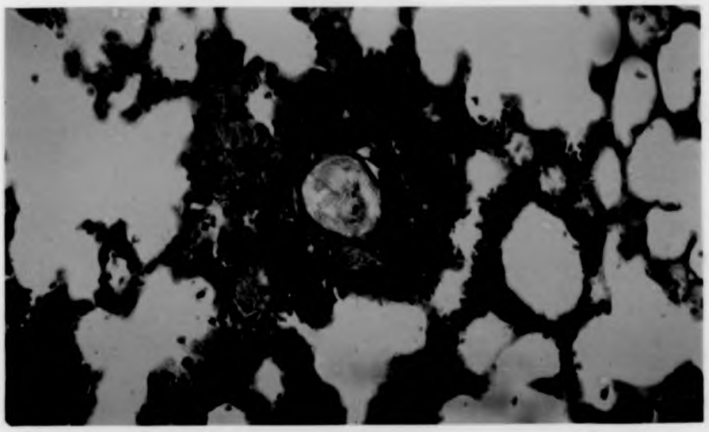


Plate 19. Granuloma response around a live Schistosoma mansoni egg in the lung of an infected mouse, x300.

Plate 20. Cellular adhesion on a bentonite particle coated with T. spiralis muscle larva antigen in the lung of a mouse on the 45th day of an infection, x400.

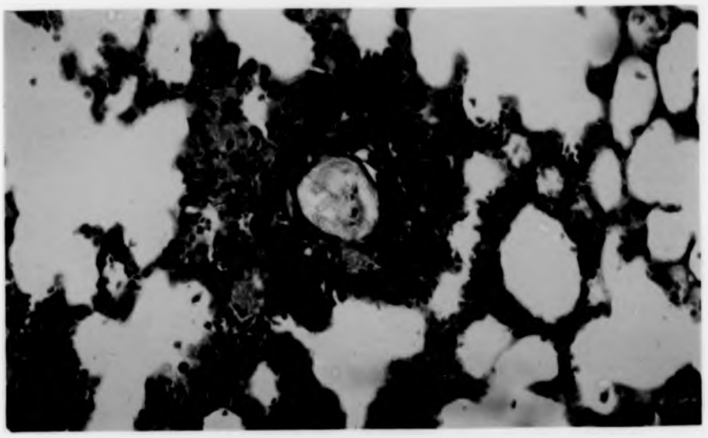


Plate 19. Granuloma response around a live Schistosoma mansoni egg in the lung of an infected mouse, x300.

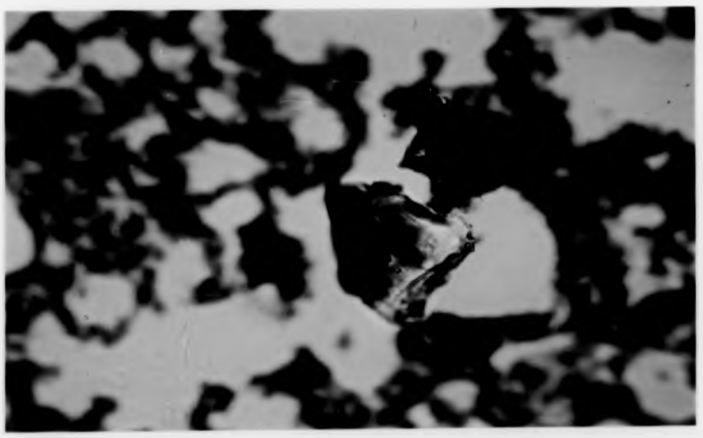


Plate 20. Cellular adhesion on a bentonite particle coated with T. spiralis muscle larva antigen in the lung of a mouse on the 45th day of an infection, x400.

per inch Endecote sieve. The particles were washed, sedimented and coated with antigen by incubation for 45 minutes at 37°C in muscle larva antigen, and washed 4 times in PBS to remove excess antigen. The particles were then injected IV into the lateral tail vein of mice on the 45th day PI when a cellular reaction in the muscles is normally well developed.

The first trial experiment failed as the bentonite particles proved to be too small to be trapped in the lungs. A repeat experiment using bentonite kindly supplied by Dr. K. Warren was successful at least in producing bentonite particles which could be observed in lung sections, but no granulomata were visible around them. It was interesting however that at x400 magnification flattened cells could be seen adhering to various faces of the trapped bentonite particles (see plate **20**).

The significance of this is not known - but when bentonite particles coated with muscle larva antigen were tested with the indirect FAT (see experiment 7.4) fluorescence was observed,which at low dilutions of immune rabbit serum,was greater than the autofluorescence induced by diffraction at the rough edges of the particles. Thus it was considered likely that the coating of antigen on to the bentonite was successful but that these particles were unable to induce a granuloma-type response as has been observed for S. mansoni.

The results of this experiment do not mean that a CMI response is absent from T. spiralis infections but that the applicability of the Von Lichtenberg technique for the determination of a delayed hypersensitivity response in T. spiralis infections was not successful.

Experiment 7.2.

Cell mediated immunity - an attempt to detect a stage specific CMI response by the footpad technique.

Bachman (1928) reported delayed-type skin reactivity in the intra dermal (ID) test for T. spiralis, and Kim (1966) found a typical delayed hypersensitivity reaction using muscle larva antigen which gave a peak response at 18 to 28 hours. Kim, Jamuar and Hamilton (1970) used an injection of 76 or 380 µg (protein content) muscle larva antigen to guinea pigs and found the skin test gave a delayed-type response 7 days later.

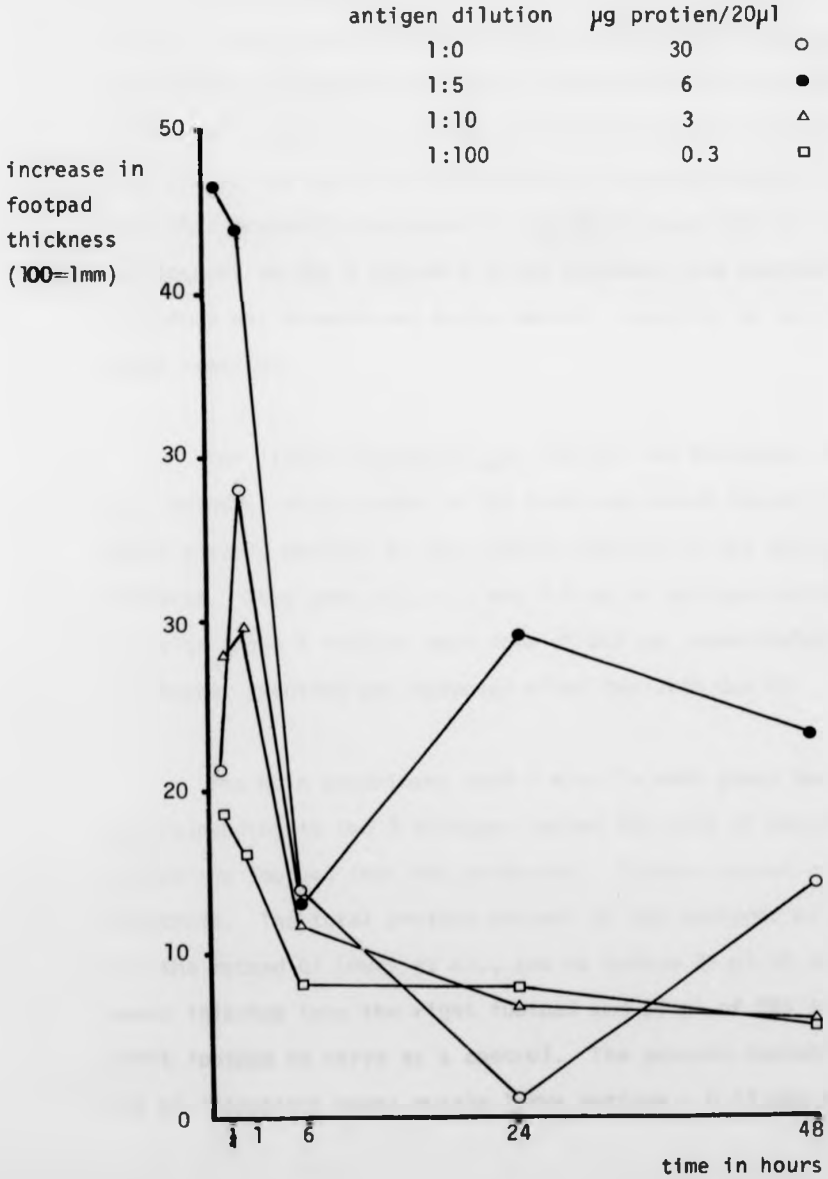
Collins and Mackaness (1968) used a footpad test (see below) to evaluate the role of delayed hypersensitivity and Arthus reactions to mice infected with Salmonella sp. They found clear evidence using that technique, and from previous studies, that the immunity to Salmonella was mediated by a predominantly cellular rather than humoral mechanism. This test was successfully applied by Opuni (1973) in the detection of a delayed-type response to the procercoides of Spirometra theileri. Collins and Mackaness (1968) stated that several facultative intracellular parasites have been noted to stimulate an immunity mediated by a cellular mechanism, and since the muscle larvae of T. spiralis can be regarded as intracellular parasites, and the studies of Larsh and co-workers and Kim (1966) have suggested a cell mediated response occurs to the adult worms and muscle larvae, it was decided to employ the technique of Collins and Machaness to study the appearance of cell mediated stage specific immune responses during a T. spiralis infection.

Materials and methods: Antigen prepared from muscle larvae was assessed for total protein content by the method of Lowry et al. (1951). Its reactivity was checked at different dilutions by the ID test for immediate sensitivity in a 6 week infected rabbit and compared to the result produced by the Trichinella Wellcotest (Wellcome reagents Ltd). Antigen dilutions of 1:10 to 1:100 produced an immediate response identical to the Wellcotest response. The Wellcotest sample contained 15 µg/ml total protein and this corresponded to the total protein content of the 1:100 antigen dilution.

Results: An initial experiment was performed using mice infected for 5 weeks with a complete infection to determine the optimal dilution of antigen for the delayed response in the footpad. 5 mice in 4 groups were given an injection of 20 µl of antigen into the right footpad with a 0.1 ml glass gyringe (710 Hamilton-Boneduz Schweiz) fitted with a 5/8 x 26 gauge needle; 20 µl of PBS was injected into the left footpad of each mouse to serve as a control. The thicknesses of the footpads were measured before injection and at 15 minutes, 6, 24 and 48 hours after injection using a micrometer (Mercer, St. Albans, England). The calculation of the size of the swelling due to the antigen was made as follows: The mean of the net thickness from the 5 mice (antigen less PBS control - i.e. right foot minus left foot) was calculated and the mean of the net measurements prior to injection subtracted from this, such that the mean pre-injection reading for each group was always zero. From this the optimum dilution of antigen which produced a marked footpad swelling at 24 hours was found to be 1:5. This corresponded to a total protein injection of 6 µg in 20 µl, Opuni (1973) used 6.2 µg (see appendix 15).

Figure 7.20.

Footpad response in infected mice to injections of T. spiralis muscle larva antigen of different protein concentrations; to determine the dilution of antigen which would elicit a 'delayed hypersensitivity' (CMI) response. (See appendix 15).



30 mice were infected with 212 (25) infective larvae *per os* and tested for the footpad response on days 6, 10, 14, 18, 22 and 26 PI using injections of 6 µg per 20 µl of antigen. 5 mice served as an uninfected control group.

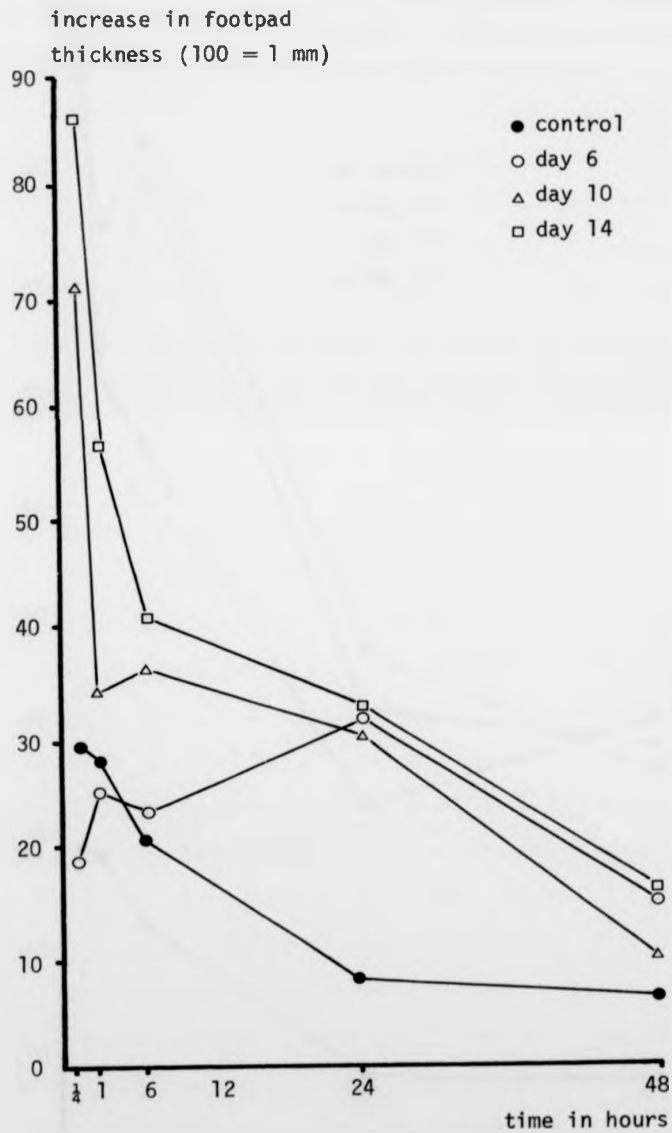
The results are shown in appendix 16 and figures 7.21 and 7.22: It can be seen that the only group of mice exhibiting a clear delayed reaction as tested by the footpad response are those tested on day 6 PI (the 24 hour response thus occurs on day 7 PI). At day 10 PI the immediate response became evident and this gradually increased to day 18 PI such that all results subsequent to day 6 showed a mixed response, and whatever CMI response was present was being masked - possibly by an Arthus-type reaction.

For living *Salmonella sp*. Collins and Mackaness found a CMI response which peaked at 24 hours and which became increasingly more clearly defined as the protein content of the antigen was reduced. They used 0.5, 1.0 and 2.5 µg of antigen and from their results chose a routine test dose of 0.5 µg, nevertheless an Arthus reaction was detected after the 11th day PI.

The main experiment used 4 mice in each group and 3 groups corresponding to the 3 antigens tested for each of the days for which the footpad test was conducted. 12 mice served as uninfected controls. The total protein content of the antigens was determined by the method of Lowry et al., and as before 20 µl of antigen were injected into the right footpad and 20 µl of PBS into the left footpad to serve as a control. The protein contents of the 20 µl injections were; muscle larva antigen - 0.53 µg; newborn

Figure 7.21.

Footpad response in infected mice to injection of 6μg of T. spiralis muscle larva antigen. The response on days 6,10 and 14 PI.



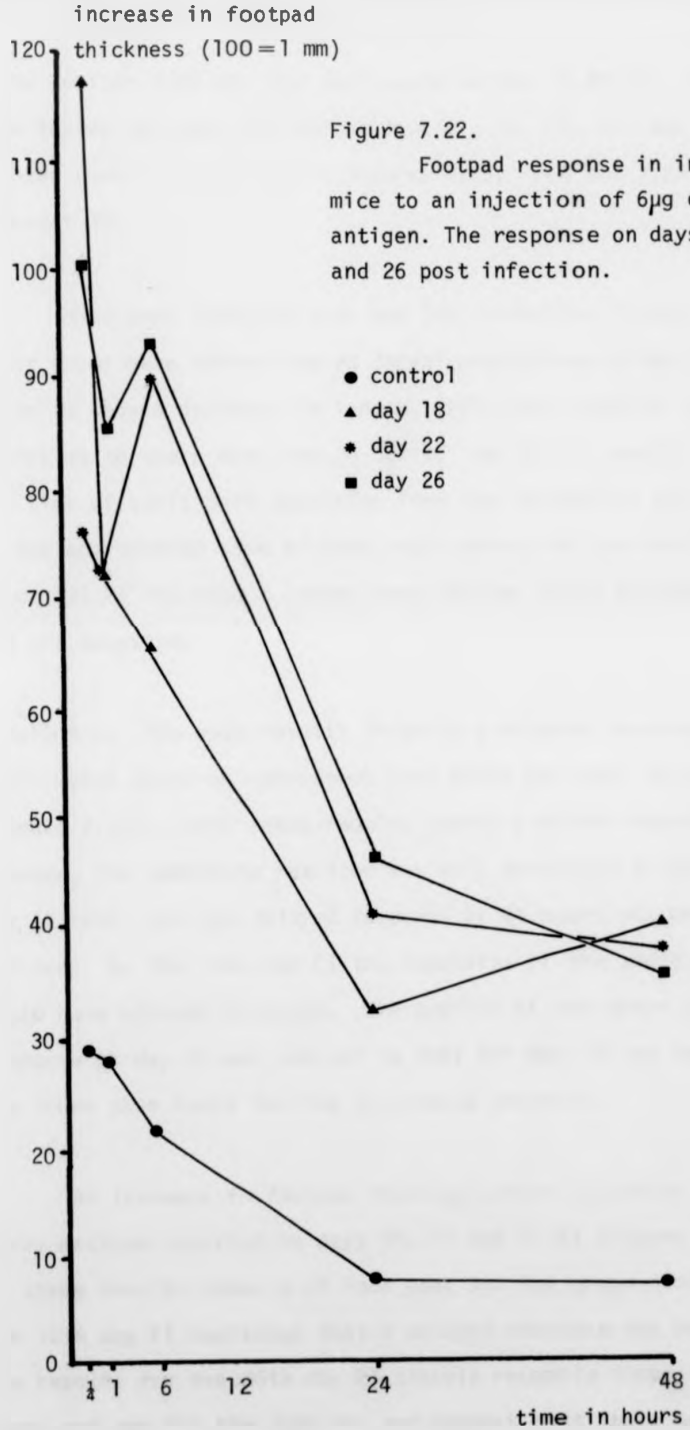


Figure 7.22.
Footpad response in infected mice to an injection of 6µg of T. spiralis antigen. The response on days 18, 22 and 26 post infection.

larva antigen 0.51 µg; and adult worm antigen 0.50 µg. The footpads were tested for each antigen on days 11, 15, 19, 25, and 32 PI, and the results are given in figures 7.23, 7.24 and 7.25 and in appendix 17.

Mice were injected with 464 (48) infective larvae. The adult worms have almost ceased larval production by day 11 PI and begin to show a decrease in length, while most newborn larvae are migrating to their host muscle cells. Day 15 PI usually marks the time of adult worm expulsion from the intestines and day 19 PI the approximate time of most rapid growth of the muscle larvae. By day 25 PI the muscle larvae have reached their maximum size and are encysted.

Conclusion: The only results in which a delayed response to the adult worms could be considered were those for days 15 and 19 PI (figure 7.23). Both these results showed a mixed response; However, the immediate reaction was well developed in the day 19 test, and the delayed response at 24 hours was more easily defined. By the 19th day PI the expulsion of the adult worms would have already occurred. The profile of the graph for the response on day 25 was similar to that for day 19 but the values are lower than those for the uninfected controls.

An increase in footpad thickness after injection of newborn larva antigen occurred on days 15, 19 and 35 PI (figure 7.24). None of these results shows a 24 hour peak but the graph profile for the 15th day PI indicates that a delayed response may be present. The results for the 35th day PI closely resemble those to muscle larva antigen for the 35th day and suggest that there may be a degree of antigenic cross reactivity.

Figure 7.23.

Footpad response in infected mice to an injection of 0.5µg of T. spiralis adult worm antigen.

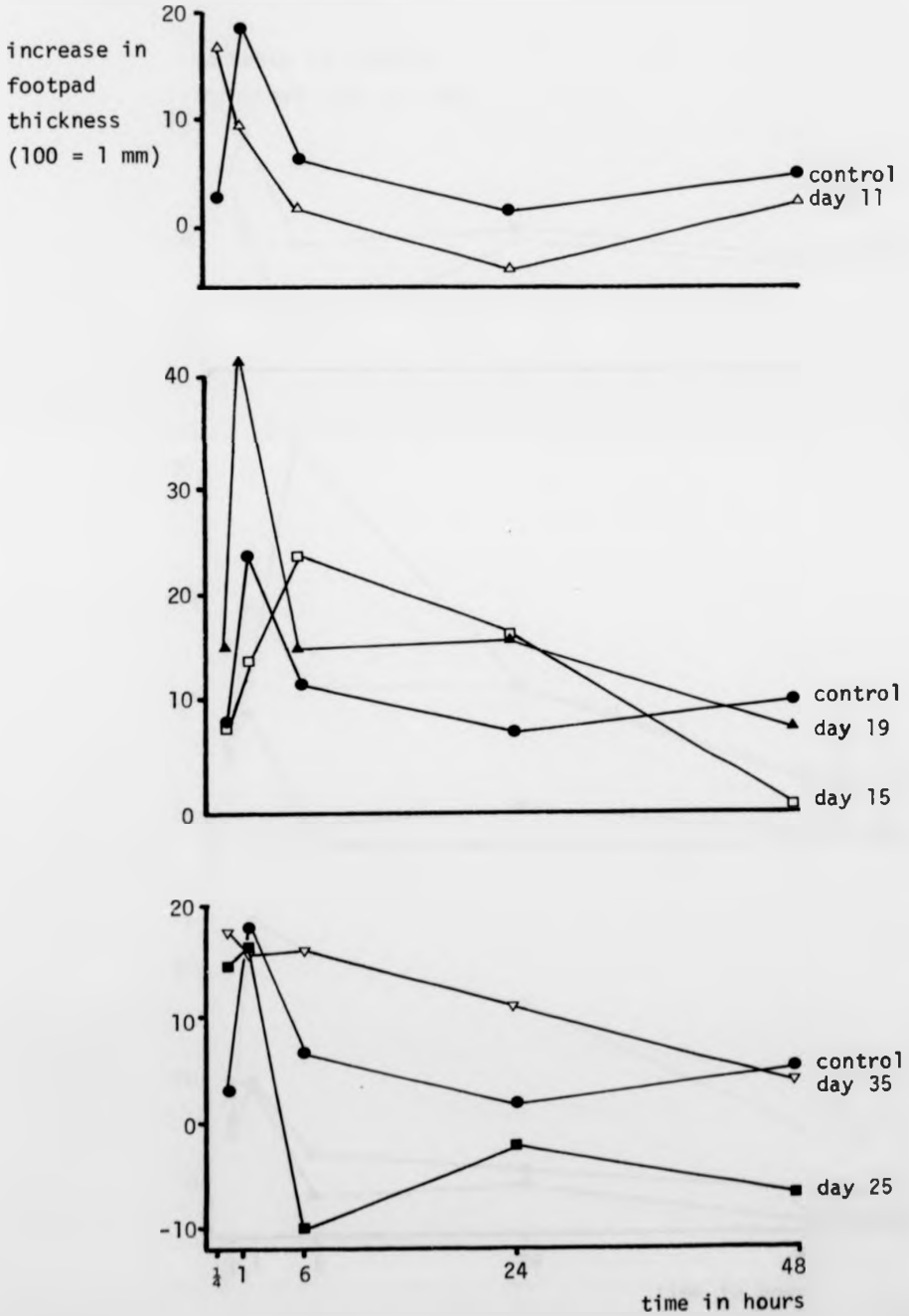
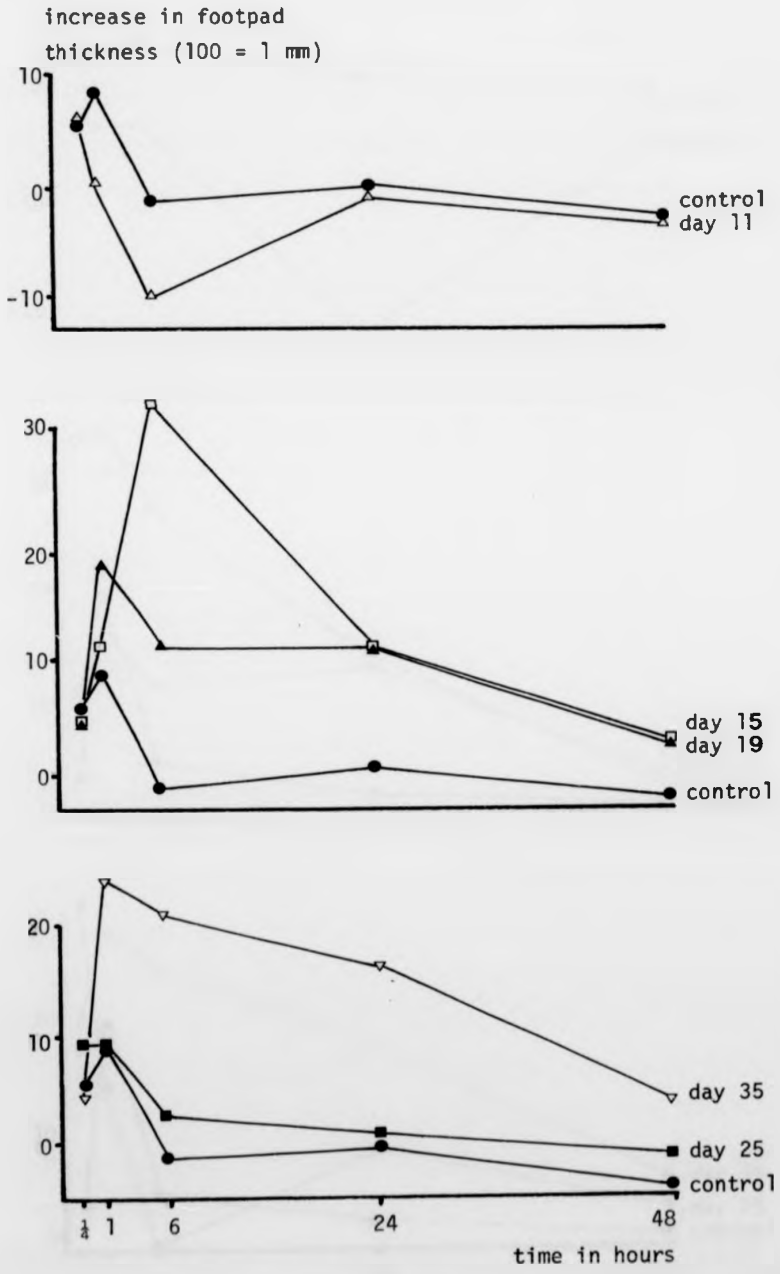


Figure 7.24.

Footpad response in infected mice to an injection of 0.5µg of T. spiralis newborn larva antigen.



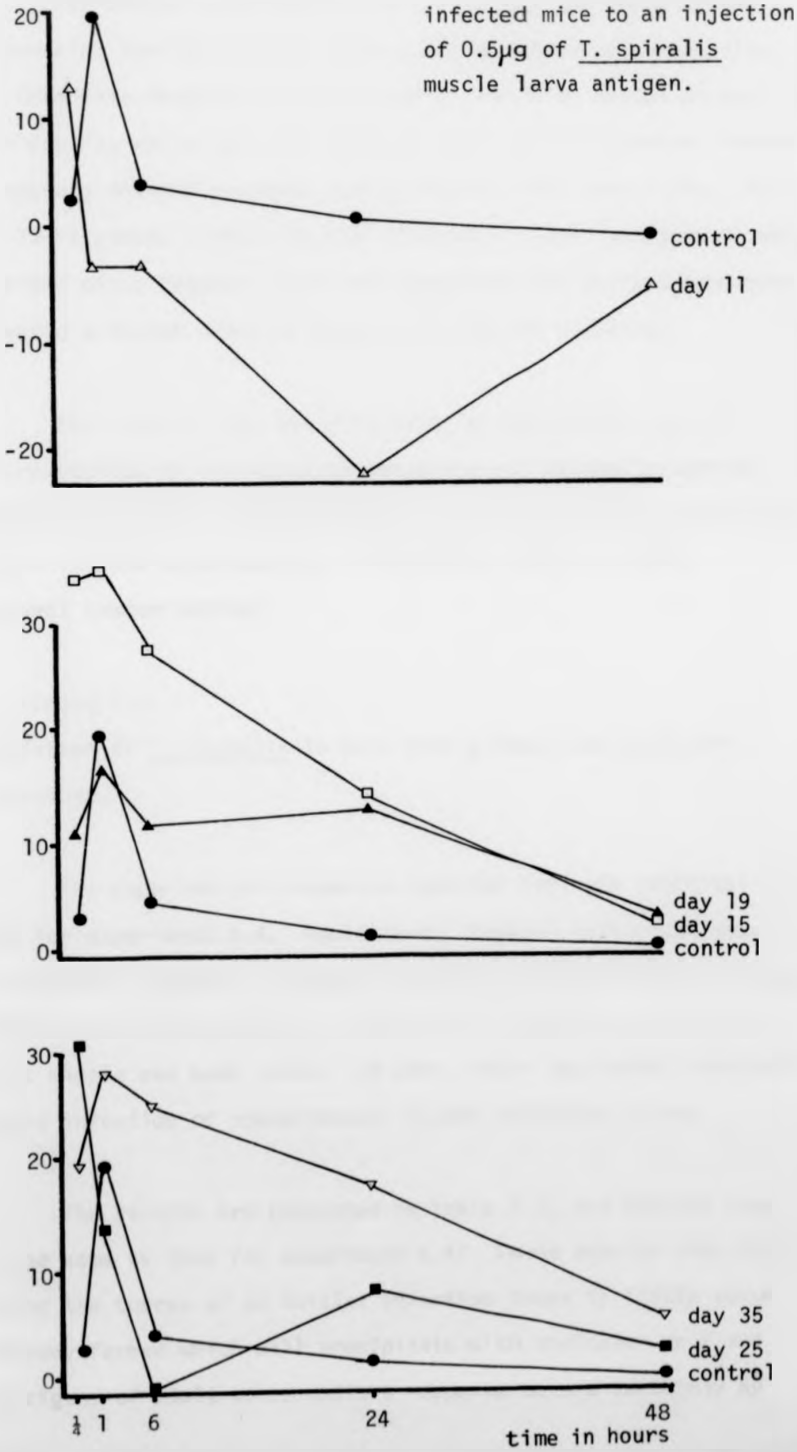


Figure 7.25. Footpad response in infected mice to an injection of 0.5μg of T. spiralis muscle larva antigen.

For muscle larva antigen (figure 7.25) the graph of the response on the 15th day PI shows a strong immediate reactivity. The immediate response was decreased in the mice tested on the 19th day PI, while both the 19th and 25th day PI responses showed a separate delayed response indicative of a CMI reactivity. The day 35 response, similar to that to newborn larva antigen, showed a strong mixed response with both immediate and delayed components elevated although separate peaks could not be discerned.

The results show the difficulty in obtaining a useful interpretation of the relative immediate and delayed responses on the strength of footpad testing alone, and any subsequent work should include supplementary histological studies (Kagan, personal communication).

Experiment 7.3.
Incubation of T. spiralis in sera from primary and secondary infections.

The experimental procedure reported here was identical to that for experiment 6.4. Adult worms, newborn larvae and muscle larvae were incubated in serum taken from a rabbit which had been infected with approximately 10,000 larvae immediately after the first sample had been taken. 14 weeks later the animal received a second infection of approximately 12,000 infective larvae.

The results are presented in table 7.3, and the key used is the same as that for experiment 6.4: These results show that during the course of an initial infection there is little serum antibody formed which will precipitate with cuticular or E and S antigens of adult worms while a response occurs certainly by

Table 7.3.

The stage specificity of the immune response: The production of precipitating antibodies to adult worms, newborn larvae and muscle larvae during the course of an infection.

Results of observed precipitate formation on worms incubated in serum from an infected rabbit. (Key - see experiment 6.4).

Adult worms

	serum (weeks after infection)															
time	0	1	2	3	4	6	7	10	13	15	16	17	18	19	20
15 m	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 hr	-	+	+	-	+	+	-	-	-	+	+	+	+	+	-
6 hs	-	-	-	-	-	-	-	-	-	2+	2+	+	+	2+	2+
24 hs	-	-	-	+	-	+	+	+	+	3+	3+	+	2+	5+	3+
48 hs	-	-	-	-	-	+	+	+	+	4+	5+	4+	5+	6+	3+

Newborn larvae

time															
15 m	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
1 hr	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-
6 hs	-	-	-	-	-	-	-	-	-	2+	2+	3+	2+	4+	+
24 hs	-	-	-	+	+	+	+	2+	3+	3+	4+	4+	3+	6+	4+
48 hs	-	-	+	+	+	+	+	2+	4+	4+	5+	4+	6+	6+	4+

Muscle larvae

time															
15 m	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 hr	-	-	-	-	+	+	2+	2+	2+	2+	2+	2+	2+	3+	+
6 hs	-	-	-	-	+	-	2+	2+	3+	4+	4+	4+	3+	4+	+
24 hs	-	-	-	-	+	4+	3+	3+	5+	4+	5+	5+	5+	6+	5+
48 hs	-	-	+	-	+	5+	4+	4+	5+	5+	5+	6+	6+	6+	5+

the 3rd week PI to newborn larval antigens and by the 4th week PI to muscle larval antigens. These results may indicate that a specific anti-newborn larva antibody acting from the 3rd week PI exists and that the heightened precipitating response to newborn larvae observed for the 10th and 13th weeks PI may be due to antibody produced to an antigen(s) common to both the newborn larva and muscle larva stages.

The results would appear to indicate that there is a distinct difference between the E and S antigens from adult worms and those from newborn larvae and muscle larvae, for whereas oral precipitates formed to muscle larvae in sera F6 to F13 and to newborn larvae from F10 to F13, none formed to adult worms in these sera. This suggests that the oral E and S, possibly functioning in a digestive capacity, are adapted for and specific to the life of the worms in the two distinct environments of the intestinal lumen and the muscle.

A secondary infection produces an immediate marked increase in anti-adult serum antibodies, the serum from the 15th week PI was in fact taken 6 days after the second infection. The reaction to newborn larvae and to muscle larvae increases from the 4th week after a second infection but not so markedly.

Thus distinct anti-adult and anti-muscle larva antibodies develop during the course of an infection and there is tentative evidence to suggest that an antibody is formed independently to newborn larvae; but there does appear to be significant antigenic cross reactivity between newborn larvae and muscle larvae.

The appearance of precipitating antibody to adult worms poses an interesting situation, and its absence (or at least its occurence

at a low titre) from the sera taken during the course of a first infection could be due to one or both of two reasons. First, there may be locally produced antibody secreted from the intestinal mucosa and acting on the worms; This possibility is suggested by Bazin et al. (1973), and in this case the antigen could induce an immunisation of the gut wall such that systemic penetration by immunogenic substances is prevented. IgA synthesis in the intestine was demonstrated for a T. spiralis infection by Crandall and Kozek (1972) and it was shown that this immunoglobulin has an affinity for the membranes of T. spiralis by Crandall and Crandall (1972). Thus the worms could presumably be damaged and their 'expulsion' from the intestine initiated or aided by local antibody production without the production of measurable amounts of serum antibody.

Secondly, the actual expulsion may, as Larsh and his co-workers have suggested, be cell mediated,following the formation of antigen-antibody complexes. But presumably as there is no precipitating reaction in sera from a first infection to the E and S of the adult worms (as distinct to the E and S of the muscle larvae) these antigens must all be complexed in the intestine with locally produced antibody or perhaps rendered tolerogenic, prior to systemic dissemination as Bazin et al. (1973) suggested.

Experiment 7.4.
Detection of stage specific antibodies with the indirect fluorescent antibody technique.

The IFAT is a double immunological reaction. Immune serum from an infected animal combines with the antigen(s) to form a complex and a fluorescein tagged anti-serum attaches to the immunoglobulin portion of this complex.

The immunoglobulin involved in the initial reaction is thought to be IgM (Kagan, Maddison and Norman, 1968) and the number of IgM sites which results from the initial reaction, and which will combine with the tagged antiserum, is very much greater than if a direct fluorescent technique had been used. Because the fluorescein tagged antiserum is available commercially and can be standardised easily the IFAT has become a very sensitive and reproducible test, and Scholtens, Kagan, Quist and Norman (1966) have reported that antibodies to T. spiralis can be detected with this technique as much as 2 weeks before they are demonstrable by haemagglutination (HA) or complement fixation (CFT) tests.

IFAT testing of sera of patients with suspected trichinosis has become a routine in Holland at least, and its reliability as a diagnostic tool has been evaluated by Ruitenberg, Kampelmacher and Berkvens (1966 and 1967), Ruitenberg, Kampelmacher, Berkvens and Duyzings (1970) and Ruitenberg, Duyzings and Kampelmacher (1972).

The source of antigen is usually the whole freshly excysted muscle larva (Sulzer, 1965; Brzosko and Gancarz, 1969) and immune serum reacts with the cuticle, which on addition of fluorescein isothiocyanate (FITC) labelled species specific immune serum, will fluoresce when exposed to UV illumination (see plates 21 and 22).

Materials and methods : Muscle larvae were recovered fresh before each test, as the reactivity of stored larvae deteriorates with time (Ruitenberg, 1973 - personal communication), and were washed 3 times in PBS. Ten minutes in 10% formol saline served to kill the larvae and they were washed 3 more times in PBS before use.

Insert FAT positive photograph here.

Plate 21. Excysted muscle larvae demonstrating a positive reaction in the IFAT. Seen under UV illumination, x100.

Plate 22. Excysted muscle larva demonstrating a negative reaction in the IFAT. Seen under UV illumination, x100.

Initially all tests were performed in small 5 ml round bottomed tubes but subsequently a far less tedious and time consuming modification was employed. This involved a further miniaturising of the technique which was performed in the round bottomed wells of haemagglutination trays.

2 drops (25 µl each) of serum to be tested were added to the first well and 1 drop of PBS into all other wells. Serial dilutions of sera were then carried out using microdiluters (Cooke Engineering Co.) as for the HA or CFT tests. The concentration of the suspension of muscle larvae was adjusted by addition of PBS so that each 25 µl drop contained about 20 - 25 larvae. 1 drop of the suspension was then added to each well, taking care not to fill more than 8 wells before reag itating the suspension of larvae and drawing a fresh sample into the dropper - this was to prevent an unequal distribution of the larvae among the wells produced if the larvae were allowed to settle in the dropper. The dilution of the serum in the first well was thus 1:2.

The tray was placed in an incubator at $37^{o}C$ for 30 minutes and then the serum was removed from the wells by 3 washes of PBS. Washing the larvae in wells was facilitated by using gravity fed drops of PBS from a 19 gauge needle connected to a PBS supply elevated above the work area. Removal of washings was accomplished with the aid of the Hyflow air pump which was also used to recover infective larvae in the digestion procedure (see chapter 3). In this case a tube was connected from the input of the pump to a medical flat bottle which served as a constant vacuum source. A second tube fitted with a 5/8 x 26 gauge needle served to remove the washings from the wells to the medical flat bottle and to regulate the speed of removal. It was found that larvae did not pass easily

into a 26 gauge needle especially when held to the side of the well, and the length of the needle prevented it from reaching the bottom of the well and sucking out the larvae, (see plate **23**). The larvae were thus left in approximately 10 µl of PBS after each washing of more than 100 µl.

A 1:10 dilution of species specific FITC antiserum (Nordic Pharmaceuticals) was prepared in PBS and 1 drop containing 25 µl added to each well. Thus the working dilution of FITC antiserum was 1:12. At least one positive control was incorporated in each test.

The trays were again incubated for 30 minutes at 37^{o}C, the FITC antiserum removed and the larvae washed 4 times in PBS before adding 1 drop of PBS buffered glycerol.

By this modified method the time taken for testing sera by the IFAT was considerably reduced, and it is estimated that between 5 to 10 times as many sera could be handled in any given time by this method compared to the trichinella tube test IFAT, without any loss of sensitivity. The use of haemagglutination trays also makes storing of the larval samples at -20^{o}C before observation very much easier. However, as there is as yet no UV microscope adapted to scan the wells of haemagglutination trays, the larvae must still be transferred to standard microscope slides and a coverslip applied in order to observe the results.

Adult worms were similarly tested but unfortunately newborn larvae are far too small to sediment as do muscle larvae and the tests for these had to be performed in tubes and the larvae centrifuged after each washing.

Plate 23. Modified IFAT apparatus showing $^{5}/_{8}$ x 26 g needle (n) being used to suck the washings from the haemagglutination tray containing the larvae into the medical flat bottle (b). Suction is supplied by the 'Hyflow' pump (p). The larvae were washed in PBS dispensed from the reservoir (r).

Table 7.4.

The stage specificity of the immune response: The reaction of immune sera to different stages of the life cycle of T. spiralis in the indirect fluorescent antibody test.

Serum antibody titres to specific stages assessed by IFAT, (expressed as the reciprocal of the dilution).

sera	whole muscle larvae	whole newborn larvae	whole adult worms	sections of adult worms
F0	0	0	0	0
F1	0	0	0	0
F2	8	0	0	8
F3	16	0	0	128
F4	32	nd	8	nd
F5	256	nd	8	nd
F6	512	2	8	nd
F8	1,024	16	16	128
F9	1,024	32	16	128
F10	1,024	nd	nd	512
F11	1,024	32	16	512
F13	1,024	32	16	512
F19	1,024	64	32	1,024
C -	0	0	0	0
C PBS	0	0	0	0
expt. 4.7. days PI.				
6	0	0	0	0
8	0	0	0	2
10	2	0	2	8
13	2	0	8	16
15	8	0	8	32

nd = not done

Results: Sera which had been taken from an infected rabbit at weekly intervals and the sera from mice in group 2 of experiment 4.7 which had received a single infection of 162 (11) larvae and which had been killed in pairs on days 6, 8, 10, 13 and 15 PI were used: The results are shown in table 7.4.

The antigenicity of the newborn larvae has been demonstrated here for the rabbit sera, but no positive results were obtained to newborn larvae with the sera from experiment 4.7 mice.

Because the titres of immune sera to the cuticles of muscle larvae are consistently higher than those to the cuticles of newborn larvae and adult worms, it is possible that there may be antigenic differences between the cuticle of muscle larvae and those of other stages. The location of these stages in the host differs, and thus the cuticle may be adapted to protect each stage in its environment.

The results using sections of adult worms indicate that the hypodermis and the other internal tissues are antigenic and react in the IFAT. The internal tissues of the adult worms may react with immune sera either because these have been exposed to the host's immune system or because the adult worm tissues cross react antigenically with those of muscle larvae.

Anti-adult worm antibodies could be detected (in section) in mouse serum from the 8th day PI, and by the 10th day had increased to a titre of 1:8. Anti-muscle larva antibodies were detected (to whole worm cuticle) between the 10th and 13th days PI and reached a titre of 1:8 by the 15th day PI. Because the muscle larvae would still be rather small on the 15th day PI and no reaction is produced

with newborn larva antigen (whole worm) by that time, there must be some cross reactivity between the adult worms and muscle larva stages.

The most consistently reactive antigens in this test were the cuticle of whole muscle larvae and sections of the adult worms. The cuticles of whole adult worms and newborn larvae were much less antigenic, and newborn larva cuticle gave a positive titre of 1:32 by the 9th week PI compared with 1:128 for adult worm sections and 1:1,024 for whole muscle larvae.

Studies are at present being undertaken with the cross over electrophoresis test, and electrophoresis/diffusion against specific anti-immunoglobulins, in an attempt to determine more precisely the nature of the serum antibodies produced during an infection, and their specificity.

Discussion

In this chapter evidence has been sought to support the theory that immunity develops independently to different life cycle stages during the course of an infection.

Most of the methods which could have been used for the detection of stage specific immune responses need large quantities of antigen, and it would have been necessary for newborn larvae production to be reserved almost completely for antigen preparation before an adequate amount of antigen could be obtained for most tests. Killed newborn larvae (Despommier, 1971) and soluble newborn larva antigen (experiment 6.1) are not immunogenic and a preliminary study with Ouchterlony diffusion was negative also. Thus it could be considered that newborn larvae produce and/or

contain only poor immunogens, or antigens which do not stimulate, or may even suppress, an immune response (see the next chapter).

Experiment 7.1 indicated that while the granuloma response in the lungs was useful to determine the occurrence of a CMI response to S. mansoni eggs, it was not applicable to infections of T. spiralis. Experiment 7.2, which employed the footpad response in mice as a possible indicator of a CMI reaction did prove to be of some use. However, although the existence of a CMI response was indicated, particularly for days 15, 19 and 25 PI for adult worm antigen, 11 and 15 days for newborn larva antigen and 15, 19 and 25 for muscle larva antigen, there was also a humoral response possibly occurring as an Arthus reaction.

An experiment was performed in conjunction with R.P. Holdstock using the technique of lymphocyte transformation to attempt to detect a differential response during the course of an infection to antigens of the three life cycle stages. The results were inconclusive. However, both the antibody producing population of B cells and the T cells which are concerned with CMI may be transformed while there may also be T and B cell cooperation in the immune response to an antigen, and thus lymphocyte transformation cannot be used to discriminate humoral and CMI responses completely.

The macrophage migration inhibition test has until now required a relatively large amount of antigen, but with the recent development of a micro-technique in agar this modified test may be useful in studying the development of CMI responses to antigens from different stages during an infection.

Tests using the immunoelectroosmophoresis (IEOP - or cross over electrophoresis) technique have so far not yielded any useful results, but as the quantity of antigen (0.5 to 3 µl) required for this test is relatively small it may yet prove to be useful in determining whether a stage specific antibody production occurs. The two techniques employed in this chapter to detect the presence of serum antibodies both used whole worm antigen (and worm sections), which had previously been shown to be reactive in chapter 6.

The IFAT in experiment 7.4 demonstrated that all stages of the life cycle were antigenic, and that the cuticle of the muscle larvae reacts more strongly (as in chapter 6) than do the cuticles of newborn larvae or adult worms. Sections of adult worms were shown to give higher positive titres than whole worms. The results of the IFAT with sera of mice from experiment 4.8 taken during the first 15 days of infection show that anti-adult worm antibody is able to be detected earlier than anti-muscle larva antibody.

Incubating worms in immune sera proved to be one of the most useful tests, and strong evidence for the existence of an immune response specific to adult worms and muscle larvae, and possibly also to newborn larvae, was produced.

The experiments reported in this chapter have largely been preliminary investigations, and in order to determine the relative roles of CMI and humoral responses to different life cycle stages during the course of an infection very much more detailed work is necessary.

CHAPTER 8

Studies on the interaction of newborn larvae with a complete infection.

This chapter attempted to investigate the apparent anomaly noted in experiment 4.7 and in the first of these experiments. The first experiment of this series was attempted to determine if an IV injection of newborn larvae given during the course of a normal infection would affect the subsequent numbers of muscle larvae which encysted. The possibility that an immunosuppressive interaction between stages of the life cycle may occur will be discussed later.

Experiment 8.1.

2 groups of mice each received 280 (14) infective larvae given per os. 14 days later one of these groups together with a previously uninfected group received an IV injection of approximately 4,000 newborn larvae, and after a further 28 days all the mice were digested to recover the muscle larvae: The results are presented in table 8.1.

From these results it can be seen that the number of muscle larvae recovered from the mice in group 1 (complete infection plus newborn larvae) is greater than both groups 2 and 3 added together. Although the result is not statistically significant it appeared to be rather unusual, and the experiments which follow were performed in an attempt to repeat and to clarify these results.

Experiment 8.2.

This experiment was aimed at repeating experiment 8.1, except

Table 8.1.

The immunological interaction between different stages of the life cycle of T. spiralis: The effect of injecting newborn larvae IV on the 14th day of a complete infection.

Numbers of muscle larvae obtained from mice in experiment 8.1.

Group 1 (complete with newborn larvae)	Group 2 (complete only)	Group 3 (newborn larvae only)
25,440	25,401	2,034
39,199	29,761	2,056
49,920	41,578	2,515
67,092	45,968	2,520
95,970	47,851	2,780
123,203	58,901	2,802
134,820	86,640	2,932
145,600	89,587	3,072
167,960	104,750	3,151
170,662	125,764	3,350
		3,466
98,338 (18,659)	65,618 (10,747)	2,789 (143)

significance tests:

groups 1 & 2	$0.10 > p > 0.05$
groups 1 & 3	$0.0005 > p > 0.0$
groups 2 & 3	$0.0005 > p > 0.0$
groups 1 & 2+3	$0.15 > p > 0.10$

that the newborn larvae were injected on the 30th day PI. 233 (11) infective larvae were given per os to initiate the complete infection and approximately 3,000 newborn larvae were injected IV as the challenge infection: The results are presented in table 8.2.

Whatever 'mechanism' was operating in experiment 8.1 when newborn larvae had been injected on the 14th day PI, it did not operate in this experiment when the larvae were given on the 30th day PI.

Experiment 8.3.

This experiment was designed similarly to the previous 2, but the newborn larva injection in this case was given on the 10th day PI. The initial infection consisted of 70 (4) infective larvae given per os and the challenge consisted of approximately 3,200 newborn larvae: The results are shown in table 8.3.

The results from this experiment indicate that the effect observed for experiment 8.1 has not been repeated here where the newborn larvae were injected on the 10th day PI.

In summarising these 3 experiments the 'suppressive' effect caused by injecting newborn larvae IV was not seen to occur during the early part of the intestinal infection (day 10 PI - experiment 8.3) or after the time of adult worm expulsion (day 30 PI - experiment 8.2). But this effect did occur when newborn larvae were injected at, or just before, the time of expulsion of the intestinal adult worms (day 14 PI - experiment 8.1).

Table 8.2.

The immunological interaction between different stages of the life cycle of T. spiralis: The effect of injecting newborn larvae IV on the 30th day of a complete infection.

Numbers of larvae obtained from the muscles of mice (experiment 8.2.).

Group 1 (complete with newborn larvae)	Group 2 (complete only)	Group 3 (newborn larvae only)
48,642	13,196	1,762
52,535	37,786	1,846
53,825	44,755	1,867
80,487	50,856	1,955
86,889	76,560	2,000
86,963	81,295	2,066
90,604	82,263	2,193
96,671	93,166	2,227
	114,308	2,238
	114,643	2,600
74,573 (6,908)	70,882 (10,580)	2,075 (78)

significance tests:

groups 1 & 2	$0.40 > p > 0.35$
groups 1 & 3	$0.0005 > p > 0.0$
groups 2 & 3	$0.0005 > p > 0.0$
groups 1 & 2+3	$0.45 > p > 0.40$

Table 8.3.

The immunological interaction between different stages of the life cycle of T. spiralis: The effect of injecting newborn larvae IV on the 10th day of a complete infection.

Numbers of larvae obtained from the muscles of mice in experiment 8.3.

Group 1 (complete with newborn larvae)	Group 2 (complete only)	Group 3 (newborn larvae only)
9,899	10,853	1,087
15,573	20,770	1,216
20,533	29,387	1,261
36,080	32,884	1,463
40,040	44,807	1,477
44,296	49,749	2,051
47,907	53,669	2,320
50,700	55,132	2,382
61,768	55,760	2,628
78,155	71,340	2,689
40,495 (6,683)	42,432 (5,858)	1,854 (197)

significance tests:

groups 1 & 2	$0.40 > p > 0.35$
groups 1 & 3	$0.0005 > p > 0.0$
groups 2 & 3	$0.0005 > p > 0.0$
groups 1 & 2+3	$0.35 > p > 0.30$

The following 2 experiments sought to repeat the findings of experiment 8.1.

Experiment 8.4.

Experiment 8.1 had used a heavy intestinal infection (280 (14) infective larvae) and as the number of muscle larvae resulting from this infection was large in comparison to the number developing from the newborn larval challenge, this experiment attempted to resolve this incompatibility and the initial infection thus consisted of 18 (1) infective larvae given per os. The challenge injection of approximately 6,300 newborn larvae was given on the 14th day PI. The results are presented in table 8.4.

The large standard errors for the results of groups 1 and 2 mean that the difference between groups 1 and 2+3 is not statistically significant; however, the same effect is indicated here as occurred in experiment 8.1, and the number of muscle larvae developing in mice where both infections were given (17,318) is larger than the addition of these individual infections (14,246).

Experiment 8.5.

In this experiment the initial infection comprised 24 (2) infective larvae given per os, then 14 days later approximately 2,000 newborn larvae were injected IV: The results are presented in table 8.5.

Unfortunately the newborn larvae used in this experiment were obviously of low infectivity, nevertheless the group of mice which received both the complete and challenge infections developed a larger number of muscle larvae (15,071) than did the other 2 groups added together (12,641).

Table 8.4.

The immunological interaction between different stages of the life cycle of T. spiralis: The effect of injecting newborn larvae IV on the 14th day of a complete infection.

Numbers of muscle larvae obtained from mice (experiment 8.4.).

Group 1 (complete with newborn larvae)	Group 2 (complete only)	Group 3 (newborn larvae only)
8,007	671	2,892
9,263	1,385	4,077
11,390	2,053	4,080
12,971	5,461	4,355
13,219	11,383	4,563
16,787	14,232	4,721
17,069	17,671	4,836
19,917	18,194	
20,655	19,197	
43,902		
17,318 (3,244)	10,028 (2,570)	4,218 (247)

significance tests:

groups 1 & 2	$0.025 > p > 0.0125$
groups 1 & 3	$0.0005 > p > 0.0$
groups 2 & 3	$0.25 > p > 0.20$
groups 1 & 2+3	$0.20 > p > 0.15$

Table 8.5.

The immunological interaction between different stages of the life cycle of T. spiralis: The effect of injecting newborn larvae IV on the 14th day of a complete infection.

Numbers of muscle larvae obtained from mice (experiment 8.5.).

Group 1 (complete with newborn larvae)	Group 2 (complete infection only)	Group 3 (newborn larvae only)
7,837	8,081	30
10,346	9,018	46
12,582	9,982	46
15,003	10,789	60
16,234	11,909	62
30,826	12,514	84
	13,814	84
	14,160	86
	16,442	104
	19,032	
15,071 (2,829)	12,574 (1,074)	67 (8)

significance tests:

groups 1 & 2	$0.25 > p > 0.20$
groups 1 & 3	$0.0005 > p > 0.0$
groups 2 & 3	$0.0005 > p > 0.0$
groups 1 & 2+3	$0.30 > p > 0.25$

The difference between these results is not statistically significant.

Discussion

The experiments reported in this chapter, although by no means conclusive, do indicate that by injecting newborn larvae on the 14th day of a complete infection, the resultant burden of muscle larvae is higher than would be expected by adding the results of the 2 separate infections together. These observed results could be accounted for by some mechanism of immune unresponsiveness or immunosuppression although the mode of action of this is undefined and is speculative.

The 14th day PI when the 'enhancing' effect to an infection by a parenteral injection of newborn larvae is maximal coincides with the approximate onset of 'expulsion' of the intestinal worm burden during self cure. Because the burden of muscle larvae due to an infection and challenge, as shown in these experiments, is much larger than could be produced by the parenterally injected newborn larvae, the demonstrated effect must be predominantly on the existing infection.

Immunological unresponsiveness broadly defined is the failure of an animal's immune system to respond to contact with antigen. One form in which unresponsiveness can be manifested is tolerance. It is known that adult mice given repeated injections of small quantities of bovine serum albumin (BSA) become tolerant and do not respond to a final large dose of BSA given in a highly antigenic form with Freunds adjuvant. This occurs as low zone or high zone tolerance according to the level of antigen predosage. Both T and B lymphocytes become tolerant to 'high zone' antigen stimulation while T cells alone do so at low doses (Weigle, cited Roitt, 1973). Immunosuppressive agents can aid the establishment of

tolerance in adult mice.

Soluble antigens are more tolerogenic than particulate or bound antigens and it has been suggested (Roitt, 1973) that this may be because they react directly with lymphocytes and are not first processed by macrophages. It has also been suggested by Bazin et al. (1973) that orally ingested tolerogenic antigens, or immunogens rendered tolerogenic by combination with excess local antibody (essentially based on IgA), may lead to permanent immunological hyporeactivity or tolerance.

But the results of chapters 4 and 5 suggest that immunity develops largely specifically to the two phases of the life cycle (intestinal and parenteral) and that these two immune responses may be independent. There must thus be another mechanism, possibly acting in addition to a mechanism of immune tolerance, which could account for the results observed in this chapter. Immunosuppression and antigen competition are two such possible mechanisms; the roles of both of these have been discussed by Faubert and Tanner (1971) in relation to inhibition of haemagglutinin production to sheep red blood cells (SRBC) in mice infected with T. spiralis. They do not say to what stage the infection had advanced before injections of SRBC were given, but presumably the T. spiralis infection was given 7 days before the SRBC injection and therefore 14 days before exsanguination. They could find no evidence of circulating antigens in infected animals although Bozicevitch and Detre (1940) claimed to have done so. Svet Moldavsky et al., (1969 and 1970) suggested that T. spiralis secretes an immunosuppressive factor and Faubert and Tanner (1971) consider that their findings might lend support to this suggestion.

If the results from this chapter are to be explained by the action of an immunosuppressive mechanism this mechanism must act within the host-

parasite system between different stages of the life cycle. Since the superimposition of a parenteral infection on an existing infection produces these results, it must be the injected newborn larvae which exert this immunosuppressive activity. The adult worms are thus induced to give birth to more newborn larvae prior to their imminent expulsion from the intestine. The effect of these parenteral injections on the adult worms in the intestinal environment has not been investigated here; however, since it has been suggested (Denham, 1968) that the first major effect of a developing immune response against adult T. spiralis is an interference with the fecundity of the female worms, an immunosuppressive 'agent' from the injected newborn larvae could be acting on this facet of the host immune response.

Although the intestinal and parenteral phases of T. spiralis are capable of independently inducing specific protective immunity (chapters 4 and 5) there could be a measure of antigenic competition (Adler, 1964) between these two phases. Whether immunosuppression and/or competition of antigens occurs, a speculative hypothesis can be constructed to account for the results in this chapter.

The action of the immune response of a host to a first infection acts initially to reduce the fecundity of the female worms, but since a large number of newborn larvae have been produced by that time the suppressive or competitive action between these 2 stages prevents the fecundity from being reduced completely to zero but allows a continued low level of production of newborn larvae until the immune response finally 'expels' the adult worms. With a sudden and massive artificial introduction of newborn larvae before the adult worm expulsion the immune balance between these two stages is disturbed in favour of the parasite with the result that more larvae are produced by the female worms.

If antigenic competition occurs between these two stages then, presumably, it may be mutual and not only will the migratory larvae be assisting the further production of newborn larvae, but the presence of the adult worms in the intestine will be assisting the parenteral migration and invasion of the muscles by the larvae. This hypothesis remains purely speculative; but the results of experiment 6.2, where in the immunised and challenged group of mice more muscle larvae developed when irradiated adult worms were present in the intestines, tend to add some weight to this possibility.

CHAPTER 9.

Summary and Conclusion.

The results from this work indicate that the immune response which develops against a T. spiralis infection is comprised of at least two stage specific components.

1. Confirmation that protective immunity will develop to the intestinal phase in isolation and that this immunity is largely stage specific was given in chapter 4. Treatment with methyridine on the 5th day of an infection removed the intestinal adult worms before the parenteral phase of infection had begun. The immunity which developed was shown to be effective against a challenge of the intestinal phase in experiment 4.8. The adult worms were stunted and expulsion from the intestines occurred earlier in immunised mice. The fecundity of the adult worms was adversely affected as was shown when the challenge consisted of a complete infection, the resultant muscle larva burdens were reduced by between 86% (experiment 4.1) and 95% (experiment 4.2). This 'anti - adult' immunity had very little effect on a challenge of the parent ral phase, and the muscle larva burden was only reduced by between 16% (experiment 4.4) and 0% (experiment 4.7).

2. The results from chapter 5 have demonstrated that it is possible to stimulate an immune response to the parenteral phase in the absence of the intestinal phase and that this immunity is largely stage specific. The immunisation of mice was performed by injecting newborn larvae intravenously. There was little evidence that this immunity produced an adverse effect against adult worms; expulsion of the intestinal worm population was more gradual but no stunting occured (experiment 5.4).

However, the immunisation was between 27% (experiment 5.2) and 63% (experiment 5.3) effective in reducing the number of muscle larvae encysting from a complete challenge infection, and it is suggested that this was probably due to the immune response acting on the parenteral phase alone. The resistance to a parenteral challenge in immunised mice was well marked and varied from 74% (experiment 5.6) to 100% (experiment 5.7).

3. From chapters 4 and 5 it has been demonstrated that the immune response to T. spiralis consists of 'anti-intestinal phase' and 'anti-parenteral phase' components which are stage specific in their action.

4. Chapter 6 attempted to demonstrate that migratory larvae are antigenic and/or immunogenic. They were shown to be antigenic by their ability to react with antibody in the in vitro culture technique (experiment 6.4) and in the indirect fluorescent antibody test (experiment 6.5). It was not possible to demonstrate the immunogenicity of this stage and the results obtained from cambendazole abbreviated infections of migratory larvae were inconclusive (experiments 6.2 and 6.3). This stage may yet be shown to be immunogenic using infections of newborn larvae which have received a dose of radiation to prevent their development into 'mature' muscle larvae.

5. Attempts to detect the occurrence of stage specific cell mediated immune responses by the 'Von Lichtenberg lung granuloma technique' (experiment 7.1) and by the 'footpad technique' (experiment 7.2) were inconclusive. Experiment 7.4 demonstrated that the cuticle of muscle larvae reacted more strongly in the indirect fluorescent antibody test with immune sera than did the cuticles of the other stages; sectioned adult worms reacted better in the IFAT than did

whole worms. Using sera from mice which had been given a normal complete infection, the adult worms were the first to demonstrate a response in the IFAT on the 8th day, followed by muscle larvae on the 10th day. Incubation of worms in rabbit immune sera showed that a response occured to all the life cycle stages but that it developed most strongly to muscle larvae and then to newborn larvae in a first infection. There was a sudden and almost immediate increase in the reactivity of adult worms in this test after a second infection. A difference was indicated between the antigenicity of the cuticles of adult worms and newborn larvae and those of muscle larvae. It is suggested that an antigenic difference may also exist between the excretions and secretions of adult worms and those of newborn larvae and muscle larvae. It is speculated that these antigenic differences may occur because the life cycle stages are normally located in different environments within the host and that their protective and nutritional requirements must consequently be different.

6. Intravenous injection of newborn larvae into mice on the 14th day of a complete infection produced larger numbers of muscle larvae than would have been expected from the addition of the primary and challenge infections (experiments 8.1, 8.4 and 8.5). In mice previously immunised to the parenteral phase, the presence of sexually sterile adult worms during a challenge with parenteral phase worms appeared to reduce the effectiveness of the immunisation (experiment 6.2). It is tentatively suggested that the parenteral phase may 'suppress' the response which normally limits the fecundity of the adult female worms; and conversely it is suggested that the adult intestinal worms may 'suppress' the immunity to the migratory larvae in a normal infection facilitating their passage to the muscles.

PART 4

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Appendix 1.

Comparison of the 'total count' method with the 'aliquot count' method for assessing the numbers of muscle larvae recovered from the digestion of individual mice.

Aliquot count

	1	2	3
larva	16	4	20
counts	16	10	20
from	10	19	22
0.3 ml	11	15	25
aliquots	12	15	13
	13.0 (1.3)	12.6 (2.6)	20.0 (2.0)
sample volume	10.0	15.0	19.5
total	433 (43)	630 (130)	1299 (130)

Comparative results from counting the samples completely -

384	532	1425

Appendix 2.

Comparison of different sampling points in the larval suspension. (See diagram 5).

Muscle larva counts in 0.3 ml of suspension.

position A	position B	position C
307	238	176
330	330	259
362	352	321
367	432	389
418	447	426
mean		
357 (19)	360 (38)	314 (45)

The mean of the samples from position A was closest to the mean for all the samples taken (344 (20)) and the variation in the readings from position A (s.e. (19)) was smaller than for the other sampling points. There is no statistically significant difference between any of these groups.

positions A & B	$0.4975 > p > 0.495$
positions A & C	$0.25 > p > 0.20$
positions B & C	$0.25 > p > 0.20$

Appendix 3.

Because of the necessity to count very large numbers of aliquots, an experiment was performed to determine whether the MacMaster counting chamber needed to be cleaned and dried after each aliquot count.

Counts were made in the order A1 to A5, B1 to B5, A6 to A10 and B6

to B10. Group A aliquots were taken as follows: The first aliquot was drawn into the pasteur pipette and run into the counting chamber; it was then withdrawn without being counted, and 'discarded' into a graduated cylinder and the pipette used to suck out all traces of liquid. The second aliquot was drawn into the pipette, run into the counting chamber and counted under a dissecting microscope; it was then removed as before and 'discarded'. This procedure was repeated for the subsequent counts. From the mean of the aliquot counts and the total volume of the sample, the total number of muscle larvae is calculated for each digested mouse.

Group B aliquots were taken as follows: A fresh pasteur pipette was used for each aliquot and after each had been counted the counting chamber was cleaned and dried by passing a wedge of filter paper through it.

Muscle larva counts in 0.3 ml aliquots.

	group A	group B
1	202	265
2	221	215
3	207	204
4	205	244
5	235	225
6	239	235
7	200	211
8	243	308
9	256	229
10	265	260
mean	227.3 (7.5)	239.6 (9.9)
sample volume	81.7	81.7
total	61,828 (2,043)	65,251 (2,696)

There is no statistical difference between these two groups ($0.20 > p > 0.15$) while the standard error for group A is slightly less than for group B.

The method employed for group A represents a substantial saving in time and as this was the method used for the aliquot counts in Experiment 4.1 it was decided to use this method for all future aliquot counting.

Appendix 4.

A further substantial saving of time would be effected if only 5 aliquots were counted for each sample instead of 10, so this experiment was performed to determine whether this would produce any loss of accuracy.

The counts were analysed statistically as follows: For each sample the mean of the total larval counts using the first 5 aliquots was compared with the mean of the total larval counts using the last 5 aliquots and both were compared with the cumulative mean for all 10 aliquots (see table A.4).

Table A4a

Muscle larva counts in 0.3 ml aliquots.

	1	2	3	4	5	6	7	8	9	10	mean (s.e.)	total vol.
1	14	12	23	16	19	19	27	15	20	35	20.0 (2.2)	30.5
2	18	20	21	17	24	21	20	17	18	23	19.9 (0.8)	31.0
3	20	22	21	22	13	19	21	27	23	16	20.4 (1.2)	37.0
4	28	31	27	21	30	14	34	16	14	25	24.0 (2.3)	31.5
5	31	31	35	41	39	25	29	41	46	37	35.5 (2.1)	23.5
6	22	26	19	14	25	24	17	18	16	24	20.5 (1.3)	41.0
7	34	33	27	36	30	30	42	33	39	28	33.2 (1.5)	26.5
8	34	27	32	27	25	21	23	30	29	27	27.5 (1.2)	33.5
9	23	22	22	27	30	30	27	38	27	24	27.0 (1.5)	35.0
10	32	34	36	27	24	36	32	37	32	24	31.4 (1.5)	32.0
11	37	32	37	34	26	22	29	25	21	34	29.7 (1.9)	35.0

Table A.4b

	total (s.e.)	means of counts 1-5	means of counts 6-10
1	2034 (224)	1708 (193)	2359 (336)
2	2056 (83)	2067 (124)	2046 (114)
3	2515 (148)	2417 (210)	2615 (234)
4	2520 (242)	2877 (178)	2163 (410)
5	2780 (164)	2773 (157)	2789 (298)
6	2802 (178)	2897 (301)	2706 (232)
7	2932 (133)	2827 (141)	3039 (239)
8	3072 (134)	3238 (190)	2903 (190)
9	3151 (175)	2893 (187)	3407 (280)
10	3350 (160)	3264 (235)	3435 (245)
11	3466 (222)	3873 (233)	3057 (280)
total mean	2789 (143)	2804 (178)	2774 (139)

significance tests:

counts 1-10 vs 1-5	$0.4875 > p > 0.475$
counts 1-10 vs 6-10	$0.4875 > p > 0.475$
counts 1-5 vs 6-10	$0.495 > p > 0.49$

Appendix 5.

Harvesting of newborn larvae.

The experiments reported here have usually only been able to use a barely adequate number of newborn larvae, and it is almost impossible to obtain sufficient newborn larvae to make much high protein content antigen. Consequently a different method of obtaining newborn larvae, which is much less extravagant of animals (mice or rats used to grow the adult worms for 6 days), and which is much less time consuming is highly desirable.

The continued presence of adult worms in the intestines of the host

animal is dependent on an equilibrium, which apart from a developing immune response, can be altered by such variables as the sex of the host, the age, genetic strain (Duckett, 1971) and the psychological stress of the host, diet and intestinal emptying time, total worm burden, the intestinal flora or the presence of an intercurrent infection.

Cortisone: There are several reports that cortisone (Coker, 1955, and 1956a and b; Markell and Lewis, 1957; Markell, 1958; Ogilvie, 1965; Wakelin, 1970), Imuran (Kozar 1969), cyclophosphamide (Sollod and Allen, 1971), splenectomy (Silakova and Pustovgar, 1971), and antilymphocyte serum (Dinetta, Katz and Campbell, 1972; Machinicka-Rugusta, 1971; Machinicka, 1972) either prevent or reduce the immune response to T.spiralis and other helminths. Cortisone treatment was selected as a possible aid to production.

A very heavy infection of adult worms - 1,500 to 3,000 in mice - causes acute diarrohea in these animals and their death 4 or 5 days after infection, but with regular injections of cortisone the mice develop no symptoms and do not die before the 6th day PI.

16 mice were each infected with 2,500 infective larvae and 8 of these were treated with 0.08 mg/day of cortisone (Betnesol, Glaxo Labs.) given in 0.2 ml intra muscular injections to the left and right hind legs alternately. By the 6th day only 2 of the untreated control mice were alive and these died early that day. There was one fatality among the treated mice and these were killed and their intestines removed (see part 2) to recover the adult worms. A total of 13,420 adult worms were recovered from these 7 mice (1,917 each). These worms were then cultured for 24 hours to harvest the newborn larvae, but few larvae were recovered and counting them was not considered expedient.

The use of cortisone would thus appear to allow larger numbers of

adult worms to be grown in individual mice, but the productivity of these worms is very low for the above level of infection. This is unlikely to be due to any toxic effects of cortisone on the worms. However, it could conceivably be a result of crowding of the adult worms in the intestine or of metabolic toxins, produced by the worms, exceeding a critical level and thus the worms might adversely affect each other in a crowded environment. Both of these factors could act in nature to partially self-limit an infection. It has been noticed that in a first infection the numbers of second generation larvae encysting in the muscles do not increase in proportion to the infecting dose as the dose increases.

An experiment was performed to determine the effect of cortisone on a lower level of infection. 18 mice were each given 500 infective larvae; 9 of these mice received injections of cortisone on days 0, 1, 4 and 5 PI while the remainder were untreated. On the 6th day PI each mouse was killed, the adult worms recovered from the intestines and counted. These were then pooled for each of the 2 groups, passed through a Baermann apparatus, cleaned, and cultured overnight to determine the rate of production of newborn larvae per female worm. The results were as follows:

	treated mice	untreated mice
infection dose	500	500
adult worms recovered	143	104
	234	156
	244	195
	325	195
	364	195
	494	309
	520	357
	578	403
	795	683
mean (s.e.)	377 (51)	289 (59)
% recovery	75%	58%
significance	$0.10 > p > 0.05$	
male : female	0.94 : 1	0.90 : 1
newborn larvae : ♀	13.8 : 1	4.2 : 1

The adult worm recovery is certainly improved by treating the mice with cortisone, but the level of newborn larva production per female for untreated mice may have been low as there was evidence of contamination of the culture of these worms.

It would be a disadvantage to have to treat a hundred or more mice every day (ideally) and the simple procedure of using more mice in the production of adult worms was adopted, so the use of cortisone was not continued.

When rats could be obtained these were used in conjunction with mice for the production of newborn larvae.

Culture techniques: If it were possible to culture infective excysted larvae through to healthy adult worms which produced newborn larvae then this would form the method of choice.

It is not yet possible to culture large enough portions of mammalian intestine in which to grow infective larvae to adult worms (De vogt van de Straaten, 1973 - personal communication) and the problems of tissue cultures alone are great in themselves. In vitro culture of infective larvae has been attempted by Levin (1940), Weller (1943), Kim (1961 and 1962), Berntzen (1962, 1965 and 1966), Tarakanov (1964 and 1965) and Meerovitch (1965a and b) and reviewed by Denham (1967) and Meerovitch (1970), but these have only been of limited success. The infective larvae have usually grown, moulted several times, often 5 times, and reached some level of sexual differentiation without becoming morphologically recognisable as adult worms or becoming sexually mature.

The only exception to this has been the claim in a short abstract by Berntzen (1962) to have produced sexually mature adult worms which copulated

and produced embryos and juveniles; but there has been no work published since to substantiate his claim.

Prinz (1973 - personal communication) has used an 'artificial rumen' with a continuous throughput of nutrients mimicing the in vivo situation in order to study rumen dwelling ciliates, and although this is a long way from an artificial intestine, this form of development could lead to successful in vitro cultivation of T. spiralis (and other intestinal helminths) from infective larvae to mature adult worms. It is realised that this would be a complete study in itself and here the use of in vitro cultivation is only a means to an end in the production of newborn larvae.

Appendix 6.

Comparison of the IP and IV routes of injection of newborn larvae.

Dennis et al. (1970) and Despommier (1971) favoured the IV route for newborn larvae and their decision was based on the results of a comparison of the numbers of muscle larvae recovered from IP and IV injected rats.

9 mice each received an injection of approximately 3,000 newborn larvae - 5 IV via the lateral vein and 4 IP. The mice were digested individually after 30 days and the number of muscle larvae resulting from this infection were assessed. One of the IP injected mice died before its time for digestion.

Numbers of muscle larvae recovered from mice:

IV group	IP group
1028	24
1050	94
1109	145
1441	
1634	
1253 (121)	88 (35)

The number of muscle larvae recovered from mice injected IP represented only 7% of the number given and although the significance is diminished by the small sample numbers, this experiment does confirm the indication that the IV route of injection is substantially superior.

Appendix 7.
Antigen preparation.

Silverman (1963) described 3 methods of preparing antigens from worm material; the Hughes press, tissue grinder and ultrasonic vibration. He considered that ultrasound produced the most complete, and the Hughes press the most incomplete, disintigration of worms of these 3 methods. The disadvantage of manual and mechanical tissue grinders was the amount of heat generated at the grinding surfaces and the variable efficiency of disintigration.

Ponnudurai (1971) favoured the modified Hughes press method for obtaining antigen and found that with the modified Hughes press - the 'X' press - the particle size of the crushed worm material obtained was smaller than when a tissue grinder or sonic disintigration method was used. From this, and considering the simplicity and ease of operation, it was decided to use the X press method.

An experiment was performed to ascertain if the total protein content of an antigen could be increased by a different routine of pressing.

Approximately 300,000 infective larvae were recovered by artificial peptic digestion, washed and sedimented 3 times in PBS, and transferred to the X press (see chapter 3, section 6) in 3 ml of PBS.

Normally the material would be left to extract for 24 hours at $4^{o}C$

but on this occasion approximately 0.5 ml of the thawed suspension only was withdrawn by pipette and allowed to extract (Ag 1); the remainder was put back into the refrigerated X press and left for 3 hours at -20°C to be re-pressed. Again 0.5 ml of the thawed worm material was removed from the X press (Ag 2) and the remainder refrozen in the X press. This was repeated 4 times until 5 samples of soluble antigen were obtained after extraction and centrifugation.

These 5 samples were assayed for total protein concentration by the Lowry method:

Antigen	No. of presses	Protein content
Ag 1	1	3,500 µg/ml
Ag 2	2	5,000
Ag 3	3	4,300
Ag 4	4	4,300
Ag 5	5	4,200

No explanation can be given for the drop in soluble protein content between Ag 2 and Ag 3, but as can be seen there was no improvement after the 3rd press (Ag 3 to Ag 5). There does appear to be a significant advantage in passing the worm material through the X press more than once, and on the strength of the above results it was decided to employ 2 pressings of the worm material (as described in chapter 3, section 6) before extracting the antigen.

Appendix 8.

Irradiation of infective larvae.

Tyzzer and Honiej (1916), Schwartz (1921), Semrad (1937), Evans, Levin and Sulkin (1941), Levin and Evans (1940 and 1942), Stowens (1942), Alicata and Burr (1949), Alicata (1951), and Gould, Gomberg, Villella and

Hertz (1957) have studied the effect of various types of radiation on the development of T. spiralis. Many other workers have used irradiation to alter the development of the parasite for the purposes of their experiments.

^{60}Cobalt was adopted as the source of choice as high energy gamma radiation is less susceptible to the various physical variables encountered in these studies, than is the photon emmission from an X ray or U.V. source (Evans, 1970).

The effect of radiation on infective larvae produces a sigmoidal curve for the percentage survival with time in the host intestine vs. radiation dose while the survival vs. dose curve for the gametes of T. spiralis is exponential (Evans, 1970, from Evans et al. 1941 and 1942).

The experiment reported here was performed to determine the dose of radiation from a ^{60}Co source required to sterilise the adult intestinal phase of T. spiralis while otherwise restricting the development of these worms as little as possible.

50 mice in 11 groups received infective larvae of T. spiralis which had been irradiated with different doses of ^{60}Co radiation from 0 to 20,000 rads. These mice were killed after 30 days and individually digested to determine the numbers of encysted muscle larvae. In addition 3 mice from each group were infected to be killed on days 6, 10 and 15 PI to determine the number of adult worms present, the length of the adult female worms and the presence or absence of intrauterine larvae. Length measurements shown are the mean of the measurements of at least 5 female worms, except where 5 could not be found at the higher dose levels.

Table A8.2 shows the results of the recovery of muscle larvae from individually digested mice.

Conclusion: Only a few second generation larvae resulted from an infection which had been treated with 8,000 or 9,000 rads. For both of these dose rates adults could be demonstrated in the intestines of mice 10 days after infection although these adult worms were reduced in size. Because the results of many of the experiments were to be assessed by the numbers of muscle larvae recovered by digestion, a dose rate which minimised the number of larvae born to the irradiated worms was chosen. This dose rate was 8,500 rads.

TableA8.1 : Recovery of adult worms from the intestines of mice.

Day 6 post infection.

radiation dose rads	infective larvae given	adults recovered	% recovery	length of females (mm)	intrauterine larvae observed
0	191(8.0)				
1000	166(8.5)	107	65	1.43(0.02)	+
2000	184(12)	136	74	1.36(0.03)	+
4000	188(18)	165	88	1.21(0.04)	+
5000	227(18)	159	70	1.17(0.04)	+
6000	217(21)	93	43	1.04(0.10)	+
7000	196(11)	70	36	0.92(0.06)	ova
8000	173(17)	150	87	0.79(0.01)	-
9000	187(14)	nd	nd	-	-
12000	242(16)	0	0	-	-
20000	183(14)	0	0	-	1

Day 10 post infection.

1000		70	42	1.25(0.05)	+
2000		176	96	1.21(0.05)	+
4000		138	73	1.13(0.08)	+
5000		120	53	0.98(0.04)	+
6000	as	156	72	0.91(0.02)	-
7000	above	78	40	0.90(0.02)	-
8000		61	35	0.87(0.03)	-
9000		7	4	-	-
12000		0	0	-	-
20000		0	0	-	-

Day 15 post infection.

1000		186	112	0.87(0.03)	+
2000		86	47	1.09(0.03)	+
4000		57	30	1.16(0.02)	+
5000		nd	nd	-	
6000	as	nd	nd	-	
7000	above	nd	nd	-	
8000		128	74	0.77(0.02)	-
9000		nd	nd		
12000		0	0	-	-
20000		0	0	-	-

TableA8.2.

Numbers of muscle larvae recovered from individually digested mice.

control (0 rads)	1000 rads	2000 rads
12,917	9,648	10,041
24,700	18,603	44,268
29,097	22,172	53,314
31,670	24,336	69,403
55,440	25,019	71,332
30,765 (6957)	19,956 (2809)	49,677 (11,117)

4000 rads	5000 rads	6000 rads
6,675	231	485
7,520	553	714
8,769	6,076	1,174
17,472	9,893	
18,690	10,313	
29,850		
14,824 (3,670)	5,414 (2,179)	791 (203)

7000 rads	8000 rads	9000 rads
3	0	
95	0	5 mice
153	0	total
165	0	3 larvae
	0	
	32	
	103	
104 (37)	27 (20)	0.6

No larvae were recovered from the 12,000 and 20,000 rad groups.

Appendix 9.

(see experiment 4.7)

Numbers of adult worms obtained from the intestines of mice from group 1. (immunised - challenged)

days post challenge	small intestine male	small intestine female	small intestine total	large intestine male	large intestine female	large intestine total	total
6	33.5	51.0	84.5	1.5	12.0	13.5	98.0
8	31.0	55.0	86.0	3.5	13.5	17.0	103.0
10	39.5	55.5	95.0	4.0	9.0	13.0	108.0
13	33.0	93.5	126.5	0.5	6.5	7.0	133.5
15	4.0	14.5	18.5	0.5	2.0	2.5	21.0

Numbers of adult worms obtained from the intestines of mice from group 2 (challenge controls).

days post challenge	small intestine male	small intestine female	small intestine total	large intestine male	large intestine female	large intestine total	total
6	48.0	56.5	105.0	1.0	6.0	7.0	112.0
8	30.0	67.5	97.5	0.5	2.5	3.0	100.5
10	8.5	17.0	25.5	18.5	20.5	39.0	64.5
13	9.5	1.0	10.5	4.5	2.5	7.0	17.5
15	0	0	0	1.0	0	1.0	1.0

Appendix 10.
(see experiment 4.7)

Lengths of female worms (mm) recovered from intestines of mice.

Group 1. (immunised - challenged)

6	8	10	13	15
4.19	3.87	3.30	2.61	2.97
3.89	3.56	3.30	2.58	2.89
3.86	3.53	3.23	2.44	2.70
3.57	3.47	3.22	2.40	2.65
3.29	2.81	3.14	2.40	2.44
3.11	2.36	2.91	2.39	2.43
2.94	2.32	2.79	2.32	2.40
2.91	2.30	2.58	2.31	2.37
2.75	2.24	2.56	2.30	2.22
2.71	2.17	2.41	2.04	
3.32 (0.17)	2.86 (0.21)	2.94 (0.10)	2.38 (0.05)	2.56 (0.08)

Group 2. (challenge controls)

6	8	10	13	15
3.84	3.27	3.22	3.42	
3.69	3.22	3.10	3.31	
3.64	3.02	3.03	3.04	
3.25	2.87	3.02	2.60	
3.24	2.82	2.95	2.31	
3.16	2.74	2.87		
3.12	2.70	2.79		
3.12	2.68	2.75		
2.64	2.46	2.72		
2.36	2.26	2.67		
3.21 (0.14)	2.80 (0.10)	2.91 (0.06)	2.94 (0.21)	n.d.

Appendix 11.
(see experiment 4.8.).

Numbers of adult worms recovered from the intestines of mice.

Group 1. (immunised - challenged)

days post challenge	small intestine male	small intestine female	small intestine total	large intestine male	large intestine female	large intestine total	total
6	1.5	22.0	23.5	0.5	3.0	3.5	27.0
10	1.0	0	1.0	0	0	0	1.0
13	0	0	0	7	4.0	11.0	11.0
15							

Group 2. (challenge controls)

days post challenge	small intestine male	small intestine female	small intestine total	large intestine male	large intestine female	large intestine total	total
6	21.0	27.0	48.0	3.0	11.0	14.0	62.0
10	5.0	12.0	17.0	3.5	6.5	10.0	27.0
13	2.5	5.0	7.5	1.5	2.5	4.0	11.5
15							

Appendix 12.
(see experiment 4.8.).

Lengths of female worms (mm) obtained from the intestines of mice.

Group 1. (immunised - challenged).

days post challenge

6	10	13	15
2.10	1.84		
2.06	1.52		
2.05			
2.00			
1.99			
1.97			
1.93			
1.90			
1.90			
1.68			
1.96 (0.04)	1.68 (0.16)		

Group 2. (challenge controls)

6	10	13	15
3.62	3.17	3.44	
3.61	3.00	3.41	
3.51	2.68	3.11	
3.49	2.60	2.96	
3.39	2.53	2.85	
3.31	2.50		
3.30	2.21		
3.26	2.03		
3.25			
2.32			
3.31 (0.12)	2.59 (0.13)	3.15 (0.11)	

Appendix 13.
(see experiment 5.4.).

Lengths of female worms (mm) recovered from the intestines of mice.

Group 1. (immunised - challenged)

days post challenge

6	8	10	13
2.83	3.86	3.42 3.03	3.35 2.95
2.53	3.48	3.29 3.01	3.34 2.94
2.52	2.51	3.22 2.90	3.26 2.93
2.50	2.25	3.07 2.81	3.16 2.80
2.29	2.23	3.04 2.75	2.96 2.66
2.53 (0.09)	2.87 (0.34)	3.05 (0.07)	3.04 (0.07)

Group 2. (challenge controls)

days post challenge

6	8	10	13	15
3.00	3.49	3.33	3.45	2.76
2.70	3.19	3.28	3.31	2.72
2.30	3.18	3.24	3.29	2.57
2.00	3.06	3.24	3.21	2.51
1.50	2.40	3.15	3.21	2.38
		3.11	3.20	2.31
		3.10	3.18	2.18
		3.04	3.00	2.17
		2.78	2.89	2.15
		2.78	2.50	2.08
2.30 (0.26)	3.06 (0.18)	3.12 (0.06)	3.12 (0.08)	2.38 (0.08)

Appendix 14.
(see experiment 5.4.).

Numbers of adult worms obtained from the intestines of mice.

Group 1. (immunised - challenged)

days post challenge	small intestine male	small intestine female	small intestine total	large intestine male	large intestine female	large intestine total	total
6	103.0	129.0	232.0	3.5	25.5	29.0	261
8	36.0	87.5	123.5	16.0	32.5	48.5	172
10	56.4	73.0	129.5	7.5	12.0	19.5	149
13	62.5	69.0	131.5	0	0.5	0.5	132
15							

Group 2. (challenge controls)

days post challenge	small intestine male	small intestine female	small intestine total	large intestine male	large intestine female	large intestine total	total
6	96.5	147.0	243.5	15.0	16.5	31.5	275.0
8	87.0	138.0	225.0	14.5	25.0	39.5	264.5
10	72.0	152.0	224.0	11.5	15.0	26.5	250.5
13	83.0	101.5	184.5	4.5	5.0	9.5	194.0
15	39.5	89.0	128.5	4.0	1.5	5.5	134.0

Appendix 15.
(see experiment 7.2.).

Mean net footpad measurements less mean net measurements pre-injection.

antigen concentration	0	15 mins.	1 hr.	6 hr.	24 hr.	48 hr.
1:0	0.000	0.116	0.280	0.040	-0.090	0.040
1:5	0.000	0.46	0.440	0.003	0.193	0.130
1:10	0.000	0.18	0.197	0.017	-0.033	-0.046
1:100	0.000	0.083	0.060	-0.021	10.023	10.047

Appendix 16.
(see experiment 7.2.).

Mean net footpad measurements less mean net measurements pre-injection.

Days PI	time				
uninfected controls	15 mins.	1 hr.	6 hr.	24 hr.	48 hr.
	0.29 (.09)	.28 (.03)	.21 (.04)	.08 (.03)	.07 (.01)
6	0.19 (.04)	.25 (.04)	.23 (.03)	.32 (.12)	.15 (.05)
10	0.71 (.07)	.34 (.06)	.36 (.05)	.30 (.01)	.10 (.02)
14	0.86 (.14)	.56 (.09)	.41 (.04)	.33 (.08)	.16 (.05)
18	1.17 (.10)	.72 (.08)	.66 (.11)	.32 (.11)	.40 (.09)
22	0.76 (.08)	.73 (.11)	.90 (.14)	.41 (.07)	.38 (.07)
26	1.00 (.05)	.85 (.22)	.93 (.16)	.46 (.11)	.36 (.08)

Appendix 17.
(see experiment 7.2.).

Mean net footpad measurements less mean net measurements pre-injection.

Adult worm antigen.

uninfected controls	time 15 mins.	1 hr.	6 hrs.	24 hrs.	48 hrs.
	.032 (.065)	.185 (.029)	.065 (.012)	.047 (.030)	.060 (.036)
day 11	.166 (.056)	.096 (.039)	.018 (.069)	-.032 (.033)	.030 (.013)
day 15	.073 (.095)	.138 (.067)	.235 (.024)	.158 (.024)	.004 (.009)
day 19	.144 (.037)	.412 (.035)	.149 (.044)	.157 (.032)	.072 (.024)
day 25	.140 (.042)	.158 (.025)	-.100 (.031)	-.022 (.031)	-.062 (.061)
day 35	.174 (.016)	.157 (.061)	.159 (.013)	.104 (.025)	.035 (.017)

Newborn larva antigen.

uninfected controls	time 15 mins.	1 hr.	6 hrs.	24 hrs.	48 hrs.
	.057 (.027)	.085 (.047)	-.013 (.009)	.000 (.011)	-.035 (.013)
day 11	.065 (.063)	.005 (.030)	-.100 (.047)	-.010 (.049)	-.040 (.103)
day 15	.040 (.236)	.155 (.093)	.330 (.056)	.111 (.000)	.017 (.030)
day 19	.042 (.041)	.185 (.066)	.112 (.035)	.110 (.043)	.047 (.014)
day 25	.092 (.055)	.090 (.024)	.027 (.024)	.012 (.012)	-.130 (.042)
day 35	.038 (.083)	.242 (.055)	.212 (.024)	.165 (.031)	.038 (.013)

Muscle larva antigen

uninfected controls	time 15 mins.	1 hr.	6 hrs.	24 hrs.	48 hrs.
	.012 (.010)	.195 (.017)	.042 (.012)	.008 (.025)	.000 (.019)
day 11	.127 (.071)	-.033 (.032)	-.033 (.039)	-.225 (.083)	-.048 (.072)
day 15	.335 (.034)	.343 (.021)	.275 (.022)	.141 (.031)	.023 (.023)
day 19	.105 (.042)	.165 (.040)	.115 (.017)	.120 (.035)	.030 (.021)
day 25	.308 (.054)	.140 (.066)	-.012 (.035)	.080 (.046)	.020 (.031)
day 35	.192 (.077)	.282 (.060)	.250 (.005)	.180 (.025)	.055 (.031)