HOST-PARASITE INTERACTIONS OF LARVAL CESTODE INFECTIONS

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The work described in this thesis is original and has not been submitted in any form to any other University.

It was carried out by the author in the Centre for Tropical Veterinary Medicine, University of Edinburgh, under the supervision of Dr. M.M.H. Sewell.

Claire P. Nichol

ABSTRACT

This work is a study of the ability of three metacestode species: *Taenia crassiceps*, *Taenia taeniaeformis* and secondary infections of *Echinococcus granulosus* to interfere with the host's immune response.

Both mice and gerbils infected with secondary hydatidosis were found to have a low antibody response to a somatic preparation of *E. granulosus* as detected by ELISA throughout infection despite, in some cases, the presence of a large cyst burden. The possibility that this was the result of suppression of the host's immune response was investigated by studying the response of mice infected with secondary hydatidosis and also the murine models of metacestode disease *T. crassiceps* and *T. taeniaeformis*, to a subsequent infection of the haemoprotozoan, *Babesia microti*. The host susceptibility to the secondary infection was assessed by the percentage number of red blood cells that were infected and the serological response to *B. microti*, as detected by IFAT, throughout the infection. All three metacestode species were found to have enhanced parasitaemias and consistently lower antibody titres to *B. microti* than the *Babesia* only infected controls. The rate of decline in the parasitaemia from peak was markedly slower in the concurrently infected mice indicating that the suppression not only affected the development of the infection but also the speed of the host's ability to resolve it.

Metacestode extracts prepared from the surface of the parasites have been shown to cause a degree of cytotoxicity when added to a lymphosarcoma cell line culture. These same extracts and excretory/

secretory products of the metacestodes also depressed the normal Con A blastic response of MLNC from both naive and infected donor mice, to a significant extent. When living hydatid cysts are placed in culture with MLNC the normal Con A blastic response is again depressed. The MLNC from infected donors showed a greater depression of the Con A response than the cells from naive donors. The longer the period of culture of the MLNC with the hydatid cyst, the greater the depression of the Con A response. The reverse situation was found when _T. crassiceps_ metacestodes were cultured with MLNC, as a greater depression of the Con A blastic response was found when the cells were exposed to the metacestodes for a shorter period of culture. This result was difficult to account for and required repetition.

Various mechanisms were proposed for the generalised suppression induced by metacestode disease. These include antigenic competition, direct cytotoxic effects of parasite-derived factors and interference by parasite secreted substances with lymphocyte function. It is likely that several mechanisms account for the observed immunosuppressive effects of metacestode infections on the host but without further investigation of the nature of the suppressive factors, and the target cells they act on, no defined interaction between host and parasite can be postulated.

LIST OF ABBREVIATIONS AND SYMBOLS

Con A	Concanavalin A
CO_2	carbon dioxide
°C	degree Celsius (temperature)
cm	centimetre
CnBr	cyanogen bromide
DEAE	Diethylaminoethyl
ELISA	Enzyme-linked immunosorbent assay
ES	excretory-secretory or metabolic antigens
FCS	foetal calf serum
g	(acceleration due to) gravity
HEPES	4-2(2-hydroxyethyl)-1-piperazine ethane
^{3}H-Tdr	Tritiated methyl-thymidine
HRPO	horseradish peroxidase
i.e.	that is
IFAT	indirect fluorescent antibody test
IgG	immunoglobulin class G
IgG_1	immunoglobulin G subclass one
IgG_2	immunoglobulin G subclass two
IgM	immunoglobulin class M
IU	international units
l	litre
log	logarithm to the base 10
µg	microgram
µl	microlitre
µm	micrometre
mg	milligram

ml	millilitre
MLNC	mesenteric lymph node cells
mm	millimetre
mmol	millimole
nm	nanometre
NMS	normal mouse serum
N-O-G	N-octyl glucoside
PBS	phosphate buffered saline pH 7.3 (Dulbecco "A", Oxoid)
PHA 1	Phytohaemagglutin
PST	parasitaemia
RPMI 1640	Rosewell Park Memorial Institute cell culture medium 1640 (Gibco)
SA	somatic antigen
SI	Stimulation index
SRBC	sheep red blood cells
TRIS	Tris-(hydroxymethyl)-aminomethane
Ul^{-1}	International units per litre
v/v	volume per volume
w/v	weight per volume
$\bar{x}$	mean
%	percentage

SECTION I

CHAPTER ONE

INTRODUCTION

Cestodes of medical and veterinary importance have indirect life-cycles, generally involving a predator-prey relationship between the definitive and intermediate host. While hygienic, cultural and economic factors play a role in influencing the incidence and prevalence of tapeworm infections in animal and human populations, the longevity of the larval form in the intermediate host is a major prerequisite for successful transmission of these parasites. The dependence of the tissue phase of the cestode life-cycle upon evasion of immunity is particularly striking when the effective acquired resistance to challenge infection is considered. The characteristics of the immune response and its significance in concomitant immunity has been an area of extensive research but this study was devised to investigate the possibility that metacestodes are capable of interacting with the host by modulation of the immune response.

The main cestode infection in this study is *Echinococcus granulosus* causing hydatid disease but in addition to this species, two other metacestode infections were studied; *Taenia taeniaeformis* and *T. crassiceps*.

1.1 Hydatid Disease

Hydatid disease caused by the intermediate stage of the cestode *Echinococcus granulosus* is recognised as being one of the world's major zoonoses. Its distribution is normally highest in underdeveloped countries or those with a rural population where the necessary public health measures are difficult to apply or enforce (e.g. Africa, Australia).

The general pattern of the life-cycle of E. granulosus is well established; the eggs of the adult worm in the dog develop into hydatid cysts when ingested by man or a wide range of ungulates including sheep, cattle, pigs, goats, horses, camels and buffaloes.

The life-cycle is completed by dogs eating carcases infected with hydatid cysts. Man is considered a dead-end host with the possible exception of the Turkana tribe in northern Kenya, where traditionally exposed human cadavers are scavenged by dogs, jackals and hyaenas. On average, ten operations for surgical cyst removal on Turkana tribesmen are performed every month in the endemic area (MacPherson, 1983, personal communication). The situation in Europe is by no means as severe with the exception of Sardinia and Greece.

Human hydatidosis is relatively uncommon in Britain, the Public Health statistics giving an annual overall average of 160 cases per year. In Britain cases occur sporadically and mainly in two areas, the central counties of Wales (Walters, 1984) and the islands off the north and west of Scotland (Chisholm, MacVicar and Williams, 1983). In some sheep-raising countries much higher prevalence data have been reported (e.g. New Zealand) where 43.5 cases per million inhabitants were reported in man ten years after World War II (Smyth, 1976). In parts of Kenya, where man-dog contact is exceptionally close or where other epidemiological conditions are especially favourable, there is an even higher prevalence.

Hydatidosis has never been considered a major health hazard in Britain, and it is not at present notifiable, although this may change with the present attempts to eradicate the parasite in Mid-Wales and the increasing concern in the Western Isles about the higher incidence

of human cases being reported with the possibility of its spread to the mainland from Skye.

There are two variants of hydatid disease in Britain, often now regarded as being caused by two distinct strains of *E. granulosus*, which can be separated on morphological, biological and physiological grounds (Smyth, 1977; McManus and MacPherson, 1984).

The sheep strain of *E. granulosus* is relatively common in Mid Wales and the Western Isles and is mainly transmitted through a sheep to farm dog cycle, with man as a zoonotic host.

The other strain that occurs in the U.K. is the equine strain which is transmitted largely by a horse/hunting dog cycle. This strain is not known to cause human disease. *In vitro* studies have shown that the strobilar stage of the horse and sheep strains have different nutritional and/or physiological requirements. If these differences are reflected in the cystic stage this may account for the differing infectivity to man (Smyth, 1977). A difference can also be shown biochemically in that the isoenzyme patterns of the two strains are distinct and can be used for identification (McManus and Smyth, 1979). Little is known concerning the strain characteristics of hydatids from other intermediate hosts outside the U.K. such as the camel and goat. Work on camel-derived material by Dailey and Sweatman (1965) showed that *E. granulosus* of both the camel/dog and cattle/dog origins were infective for sheep, camels and the long-tailed macaque (*Macaca iris*) but not for donkeys. They concluded that the cattle and camel forms were identical and resembled the ovine strain. However, subsequent strain analysis by electrophoresis has indicated that there are clear differences between the

camel-derived and cattle-derived material (McManus, 1984). Areas where camels are regarded as important intermediate hosts such as Iran, Syria and Egypt are also areas where human hydatidosis is a problem, thus identification of the particular strain of E. granulosus found in the camel is of relevance to control.

1.1.1 Secondary hydatidosis - life-cycle and biology

Research on hydatid infection in the definitive host carries an inherent risk of accidental infection for laboratory personnel. The interval between infection and clinical disease in man is long, which may tend to lead to carelessness in handling infective Echinococcus material. One of the reasons for the need for extreme care is that, theoretically, one egg may result in infection (WHO, 1981).

The dog is the essential laboratory host for rearing the strobilate stage of Echinococcus spp. The time required to produce infective eggs is about six to nine weeks. However, the faeces of such dogs will contain large numbers of eggs which are infective for man, necessitating very high grade handling facilities and safety procedures. In order to circumvent the necessity for handling these eggs, infections can be initiated by intraperitoneal inoculation of protoscolices derived from sheep or horse hydatid cysts into laboratory mice or gerbils. The resultant cysts can then be maintained by serial passage of protoscolices from fertile cysts or small intact sterile cysts by surgical transplantation. Both strains can be maintained in inbred Balb/c mice (Pauluzzi, 1969; Connor, 1980) and the Mongolian gerbil, Meriones ungulatus (Schwabe, Kilejian and Lainas, 1970; Thompson, 1976). When mice were examined at various

intervals following infection by the intraperitoneal route with 2,000 protoscolices, heavy burdens were found in the liver and spleen at 12 weeks (Connor, 1980). By the seventh month, the abdomens were greatly distended. Age resistance to secondary hydatidosis has been reported in mice, which necessitates the use of much higher infecting doses (up to 10,000 PS) in mature animals than in those less than 35 days old which received 1,000 PS (Schwabe, Schinazi and Kilejian, 1959). The use of immature mice, with doses of 1,000 PS, resulted in consistent infections (Schwabe *et al.*, 1970). These authors discussed the limited use that had been made of this model in hydatid research because the secondary cysts which developed from injected protoscolices were invariably sterile and thus presented a problem in continuing subpassage. This serious limitation of *E. granulosus* infections in rodents has been obviated in several ways. Firstly, by infecting gerbils rather than mice, since the former hosts can support the infection for the longer period necessary for at least some cysts to reach a sufficient size for protoscolex development. The peritoneal cavity of the gerbil is considerably larger than that of the mouse, permitting greater cystic development before interference with the normal functions of the host becomes too adverse. Sweatman and Williams (1963) suggested that protoscolex production in primary hydatid cysts seemed to be related more to cyst size than to duration of infection and this has also been found to hold true for secondary cysts (Schwabe *et al.*, 1970).

Secondary cysts produced intraperitoneally in mice and gerbils from both equine strain and ovine strain protoscolices have displayed highly variable rates of growth (De Rycke and Pennoit-de Cooman, 1978; Connor, 1980). With an inoculum of 1,000 to 3,000 protoscolices, where one to ten protoscolices develop into cysts, these would typically be relatively large and fertile (1 to 5.5 cm diameter) in mice. However, if 10 to 200 protoscolices develop, they would produce smaller sterile cysts of varying sizes, while most of the protoscolices in the inoculum would apparently be overcome by the immune response of the host (Schwabe et al., 1970).

The variable rate of development posed a problem to workers on secondary hydatidosis as it was difficult to judge when any one infected rodent contained individual cysts of sufficient size to be fertile, so that much potentially valuable material was wasted. In addition, many of the animals containing large numbers of sterile cysts reached such a girth that necropsy was necessary. Therefore, a second mode of passage was developed. This involved the establishment of secondary hydatidosis in "breeder" rodents with subsequent surgical transplantation of small individual cysts, usually at that time sterile, into a second series of rodents (Pennoit-de Cooman and De Rycke, 1978). These were found to have an increased rate of development in the second host and developed into larger cysts showing fertility and daughter cyst formation at autopsy 9-14 months after transplantation. Thus, a ready supply of hydatid-bearing rodents and/or protoscolices can be maintained in the laboratory. Schwabe et al. (1970) believed that such a system could produce large numbers of gerbils each containing one large fertile

cyst (3 to 5 cm in diameter) two years after the initiation of the "breeder" group. One of the major advantages of this technique is that it makes it possible to isolate genetically identical parasite strains, which would be invaluable for sound comparative studies such as drug trials and immunological mechanisms in the host (De Rycke and Pennoit-de Cooman, 1978).

The present study was initially designed to utilise this relatively safe procedure in studies on the immune response to secondary echinococcosis. The other taeniid parasites used in the study were introduced in order to allow the necessary techniques to be developed during the inevitably prolonged period in which the culture of secondary hydatidosis was being established.

1.2 Taenia crassiceps

1.2.1 Life-cycle and biology

Taenia crassiceps has been found to be easily maintained as a laboratory model for metacestode infections (Chernin, 1977) as well as of use as a source of antigen for serodiagnosis of cysticercosis (WHO, 1981).

Life-cycle: *T. crassiceps* (Zeder, 1800; Rudolphi, 1810) is a common cestode of the red fox (*Vulpes vulpes*) in Europe and U.S.A. The metacestode stage, *Cysticercus longicollis* (Rudolphi, 1810) has been reported in a variety of species of small rodents including the mole. The adult worm occurs in the middle third of the small intestine of the definitive host, and the eggs are detectable in the faeces as early as 32 days post infection. Metacestode burdens develop in the intermediate hosts following ingestion of eggs (Freeman, 1962).

Laboratory infection of albino mice with eggs by the oral route resulted in cystercerci in the subcutaneous tissues as well as in the body cavities (Albert, Schueler, Panuska and Ingling, 1972).

In contrast to most other taeniid parasites which form cysticerci, reproduction of the metacestode occurs by exogenous budding and more rarely by endogenous budding from the abscolex pole.

1.2.2 Establishment and maintenance of laboratory cultures of *Taenia crassiceps*

T. crassiceps metacestodes can easily be maintained by direct passage from one intermediate host to another by subcutaneous, intrapleural or intraperitoneal injection. The course of development and asexual reproduction is similar to that following infection by egg ingestion (Freeman, 1962). A series of trials on the three different routes of inoculation produced the conclusion that the intraperitoneal route favoured a higher rate of reproduction. Metacestode passages were maintained by serial intraperitoneal sub-inoculation at 50 day intervals through 23 generations without any significant change in the rate of exogenous budding (Freeman, 1962).

The Toi strain used in the present study was originally isolated from a wild rodent in Canada in 1962 and the metacestodes transferred to a laboratory mouse. Reino Freeman's pet dog Toi (a Chow) caught and ate the infected mouse. The eggs voided from the adult worms were collected and used to orally infect a large group of laboratory mice. The metacestodes from these infections were harvested and passaged intraperitoneally. This strain was brought to England in 1962 by Dr. A. Taylor and was later used in a series of studies by Chernin (1975) who found that mice act as a convenient

laboratory host for this strain of T. crassiceps metacestodes, whereas rats are suitable hosts for the ORF strain and that both strains can be serially passaged by intraperitoneal inoculation. The sex of the murine host may affect the growth and development of the metacestodes as Freeman (1962) showed that male mice were more refractory to oral infection than female mice, although both sexes appeared equally susceptible to intraperitoneal passage. In contrast, Chernin (1975a) found that with three different mouse strains, CRS, LACA and CFLP, intraperitoneally passaged larvae grow and multiply more rapidly in females than in males although both sexes are equally susceptible to the initial infection. He postulated that female hosts either provide a metabolically more favourable environment for the growth of the metacestodes or that their resistance is less than that of the males. He further theorised that a growth stimulant, possibly of hormonal origin, was present only in the female host or inhibitive factors were present in the male only. It is known that groups of female mice housed closely together induce an hormonal state of pseudopregnancy (Nicholls, 1984). The hosts chosen for the present study were female CF1 mice for routine maintenance and female inbred Balb/c mice for the experimental studies; in order to minimise variation in host response.

1.3 Taenia taeniaeformis

1.3.1 Life-cycle and biology

Taenia taeniaeformis (Batsch, 1786) is a common cestode of the felidae with a rodent as the intermediate host. This parasite provides an easily maintained laboratory model for the two parasites

of major zoonotic importance in taeniasis cysticercosis, *Taenia saginata* and *Taenia solium*.

1.3.2 Establishment and maintenance of the laboratory cultures of Taenia taeniaeformis

Ingested *T. taeniaeformis* eggs, derived from tapeworms in a cat, the definitive host, penetrate the intestinal wall of rats or mice, as oncospheres, and develop into cysts containing strobilocerci in the liver.

The source of these parasites is of great practical importance since Heath and Elsdon-Dew (1972) and Ambu and Kura (1980) found that their respective "strains" of *T. taeniaeformis*, which had been originally isolated from rats, were infective for rats but not mice, while Williams and Oriol (1976) were unable to infect a variety of laboratory animal species, including rats and mice with *T. taeniaeformis* obtained from cats in their locality. Clearly, experiments concerning resistance to *T. taeniaeformis* require care in standardization of both host and parasite. In these studies the Belgian strain has been chosen as this can be inexpensively maintained in mice and easily infects inbred Balb/c mice used in the experimental section. This *Taenia taeniaeformis*-mouse system, being the natural host-parasite relationship, unmodified by extensive laboratory passage, makes it an ideal model (Mitchell, 1982).

1.4 Aims

The objectives of this study were to investigate the possibility that metacestode infections, and in particular hydatid disease, are capable of modulating the immune response of the host as a means

of evasion. The response and ensuing parasitaemia of mice infected with either of the three metacestode species described to a superimposed infection of *Babesia microti* was evaluated as an indication of the level of immune responsiveness during larval cestode disease. In addition to the *in vivo* analysis of the host-metacestode interaction, *in vitro* studies were carried out on the immune responsiveness of lymphocytes derived from both naive and infected mice to metacestode extracts, live metacestodes and/or the known plant mitogen Concanavalin A.

The results are discussed in the context of an analysis of the regulatory mechanisms of the host in the presence of the parasite, with the future possibility that therapeutic alteration may allow a shift of the regulatory balance in favour of the host.

CHAPTER TWO

LITERATURE REVIEW

MODULATION AND EVASION OF THE IMMUNE RESPONSE DURING LARVAL CESTODE INFECTIONS

2.1 Introduction

Hydatidosis, cysticercosis and coenuriasis are terms for the larval cestode infections by those parasites belonging to the family Taeniidae which have as their metacestodes the asexually multiplying hydatid cyst and the single non-multiplying cysticercus respectively.

The recognition that immunity in the intermediate host plays a central role in determining the natural patterns of transmission of cestodes (Lawson and Gemell, 1983) has stimulated interest in defining the innate and acquired mechanisms of resistance and of the parasite's ability to evade this host response.

Characterization of innate resistance would greatly facilitate the assessment of research on acquired immunity and will hopefully lead to the ability to select resistant strains of hosts. Detailed analysis of the immune response of the host could potentially lead to improved vaccination strategies; in particular, studies on the established metacestode which is capable of surviving for long periods of time in the host. Information on the complex host-parasite interactions in such chronic infections may lead to new strategies in prophylaxis to thwart evasion of the immune response.

The potential now exists to dissect out the mechanisms underlying evasion (Mitchell, 1982; Dixon *et al*., 1982; Maleckar and Kierszenbaum, 1983) and thereby identify those immunogenic components which are either involved in defence or interfere directly or indirectly with the immune response. Such analysis carries with it the possibility of shifting the regulatory balance in favour of the host (Cox, 1984) so resulting in the destruction of the parasite. This could be of benefit in both disease control and in the alleviation or prevention

of clinical signs of infection (Rickard and Williams, 1982).

This review will discuss the state of knowledge concerning innate and acquired resistance to larval cestode infections, including serological detection with particular emphasis on parasite modulation and evasion of the host response.

2.2 Immunodiagnosis

Many grazing herbivores are exposed to infection with several larval cestode species including *E. granulosus*, *Taenia hydatigena*, *Taenia multiceps* and *Taenia ovis*; therefore in order to assess the incidence of a particular species and design the necessary control programme a species specific test is necessary (Yong and Heath, 1984).

Hydatid disease is widespread and is now being reported in areas previously thought to be free of infection (Matossian, Rickard and Smyth, 1977). Matossian *et al.* (1977) suggested that this had resulted from the importation of live, infected sheep into previously hydatid-free areas.

One of the major reasons for the present unreliability of serodiagnosis of hydatid infection in herbivores is the high degree of cross-reactions (false positives) that occur not only with other metacestode infections, but with closely related helminth infections including *Fasciola hepatica* (Yong and Heath, 1984; Hilwig and Cramer, 1983).

The immunodiagnosis of hydatid disease in man is relatively effective, probably largely due to the fact that larval cestode infections are relatively uncommon in man so cross-reactions are rare. The literature on the various serological tests and their benefits

has been reviewed by Kagan (1974) and Rickard (1979).

The most significant problems preventing the development of reliable diagnostic tests to detect livestock or man harbouring larval metacestodes such as the cysticerci of Taenia solium or T. saginata and hydatid disease have not only been the lack of specificity but also the lack of sensitivity of many of the tests (Harrison, 1977). This problem has been particularly noted in man where a significant percentage of non-reactors (false negatives) later found to have hydatid disease is cause for concern. Thus, T. solium cysticercosis did not stimulate a strong serological response in man, rendering such infections difficult to detect (Flisser et al., 1980). This problem of weak reactions in serological assays could be caused either by poor antigen preparations or weak and non-specific antibody production by the host. The fact that, even when using known sensitive tests such as ELISA, the response is only weak does tend to suggest that the problem may lie in low antibody production by the host.

This phenomenon has prompted the pursuit of the alternative diagnostic procedure designed to detect the presence of parasite products in the circulation.

The use of recently developed techniques in immunochemistry such as SDS-PAGE gels have increased the knowledge of the complex of antigens in metacestode extracts (Bowtell et al., 1983; Harrison and Parkhouse, 1984), and which subsets cross-react with other related parasites. This approach must continue if specific antigens are to be identified and purified for routine serodiagnostic use.

2.3 Modulation and Evasion of the Host Response by Intrinsic Factors

2.3.1 Breed and strain of the host

The genetically determined ability of the host's immune system to recognise antigens and mount an immune response must determine the outcome of many host-parasite interactions (Ogilvie and Wilson, 1976). One means by which larval cestodes are able to persist is by passively benefiting from genetic variation of the population.

The presence of low responders within any given host population will enhance the parasite's chances of survival. Inbred strains of mice have been used as models for this genetic variation. A relatively simple mechanism has been found to underlie the genetically mediated difference in resistance to primary infection with *T. taeniaeformis* between C57BL/6 and C3H/He mice (Mitchell, 1982). The oncosphere and early larvae are only susceptible to complement fixing-antibody mediated attack for a few days after infection prior to the development of anti-complementary protective mechanisms by the parasite. Several days are also required for the production of complement-fixing antibodies. The C57BL/6 mouse is able to produce these antibodies over a shorter period of time than the C3H/He mouse thus rendering them more resistant to infection. Thus, the genetically determined rate of antibody production is crucial in the race between host resistance to the parasite and parasite resistance to the host's response.

Good *et al*. (1980) implanted three strains of mice, Balb/c, BDF1 and CH3 with primary and challenge infections of *T. crassiceps* metacestodes. The CH3 mouse strain mounted a stronger antibody

response to secondary infection with implanted metacestodes than did Balb/c or BDF1 mice but their ability to encapsulate the cysts, mediated by cellular components was slower.

However, the combined effects of host resistance to secondary infection were similar in all three strains indicating that the genetically determined differences had altered the balance between the various effector mechanisms without substantially altering the overall host resistance.

Very little is known about host strain susceptibility to secondary echinococcosis in mice. Several research groups have successfully established hydatid cysts by inoculating protoscolices from either equine or ovine strain infections in a variety of mouse strains. However, it is very difficult to deduce if any of these murine strains are genetically high or low responders because of the large amount of intra-strain variability as regards the proportion of the cysts in the inoculum that become established and the rate of development (De Rycke and Pennoit-de Cooman, 1978; Connor, 1980).

Some efforts have recently been directed towards the selection of livestock animals highly resistant to other helminths (Windon et al., 1980) but little progress has been reported with respect to cestode infections.

2.4 Modulation and Evasion by Cysticerci

The intermediate hosts of all larval cestodes manifest an impressive level of acquired immunity to reinfection which appears to depend on circulating antibody. Evidence for this is extensively reviewed by Rickard and Williams (1982) and discussed in a recent

study of E. multilocularis by Deuben and Tanner (1983). This level of resistance is not found in primary infections and the long term survival of metacestodes is thought to be facilitated by a combination of several immune evasion mechanisms (Rickard and Williams, 1982).

2.4.1 Antigenic shifts

Post-oncospheral stages soon outgrow their susceptibility to antibody (Heath, 1973; Musoke and Williams, 1975; Mitchell et al., 1977) and in primary infections this ability has to be achieved rapidly before the host response identifies and destroys them. The differing patterns of serological response to antigenic preparations of oncospheres and metacestodes of T. ovis and T. hydatigena (Craig and Rickard, 1981) suggest that there are unique oncosphere determinants. Morphological evidence of changes in the limiting membrane of developing metacestodes from the extensive cytoplasmic folds of the oncosphere (Niedland, 1968) to the microthrix layer (Engelkirk and Williams, 1983) in the metacestode T. taeniaeformis along with the identification of stage specific host-protective antigen in oncospheral membranes (Rajesekariah et al., 1982) support Craig and Rickard's findings.

2.4.2 Sequestration

After the brief period of pre-establishment migration taeniid larvae become sessile. The firm, fibrous capsule which surrounds an established T. taeniaeformis and E. granulosus metacestode in their natural intermediate hosts has been cited as a barrier to the influx of host cells, though antibody is not denied access (Varela-Diaz

and Coltorti, 1973; Kwa and Liew, 1978). Nevertheless, this fibrous tissue barrier may contribute to evasion of the host response by physically separating the antigenic mass of the metacestode from the circulating immune cells (Rickard and Williams, 1982). In favour of this view is evidence that metacestodes of E. granulosus, E. multilocularis and T. taeniaeformis are susceptible to complement-mediated lysis in vitro when exposed to normal sera (Herd, 1976; Kassis and Tanner, 1977). This appears to be directly contrary to the survival and enhanced growth rate of secondary hydatid cysts when transplanted into the peritoneal cavity of naive mice. It may be some form of closely adherent capsule to the cyst that is retained round the cyst during transplantation. Indeed, histological studies by Richards, Armé and Bridges (1983) would tend to suggest just such a situation. T. taeniaeformis strobilocerci removed from their capsules and exposed in the peritoneal cavity of the same host do not survive (Rickard and Williams, 1982). Such evidence suggests that sequestration may have an important role along with other evasive mechanisms.

2.4.3 Immunosuppression

Immunosuppression may be regarded as impairment in the production or action of suppressor T cells or of the soluble factors involved in the regulation of immune responses and this might be expected to enhance the survival of a variety of parasites. Some of the best documented cases of immunosuppression caused by parasitic diseases occur in the clinically important protozoa; trypanosomiasis, malaria and Leishmania. African trypanosomiasis is accompanied by

a generalised immunosuppression that affects both B and T cell responses to many unrelated antigens (Mansfield, 1981) and is thought to be as a result of non-specific proliferation of lymphoid populations leading to spontaneous Ig secretion and the progressive depletion of antigen-reactive B cells as these change into secretor cells, finally resulting in unresponsiveness. This process is known as clonal exhaustion (Clayton et al., 1979). However, the effects of protozoan and metazoan parasites on the immune response of the host differ in the extent that the various immune cell populations are involved and that a consideration of such studies of the mechanisms of immunosuppression in protozoal disease may not provide much clarification of the situation with respect to helminth diseases.

Nevertheless, non-specific immunosuppression of the host is a well established phenomenon in parasite immunology for helminth as well as protozoal infections (Ogilvie and Wilson, 1977; Mitchell, 1982) but its precise survival value for the parasites involved is still uncertain in most cases.

However, studies on some helminth infections have recently proved instructive concerning the mechanisms of immunosuppression and how they effect immune evasion. Thus, the prolonged survival of adult Nematospiroides dubius is effected by depression of homologous immunity at the intestinal level in the mouse (Behnke et al., 1983). Primary infections of N. dubius led to depression of the immune response to concurrently administered unrelated antigens (Ali and Behnke, 1984) and a delay in the expulsion of a concurrent infection of Trichinella spiralis (Behnke, Wakelin and Wilson, 1978).

Spleen lymphocytes of jirds infected with the filarial worm, *Brugia pahangi* have a reduced responsiveness to parasite antigens when cultured *in vitro* (Lammie and Katz, 1984). This was thought to be the result of an increase in the involvement of antigen-specific suppressor T cells in the immune response. This cell population appears to be distinct from the non-specific suppressor cells which were shown to have diminished responsiveness to plant mitogen in a lymphocyte transformation assay. Until recently there was very little evidence that either specific or non-specific immunosuppression resulted from metacestode infection.

However, intraperitoneal infection of mice with larvae of *T. crassiceps* has been found to result in depression of both primary and secondary antibody responses to sheep erythrocytes *in vivo* (Good and Miller, 1976). In both cases IgM and IgG responses were depressed, but IgG was depressed more than IgM in the secondary response. *In vitro* the secondary response to sheep erythrocytes were consistently depressed in both spleen and mesenteric lymph node cell preparations from infected mice whereas the primary *in vitro* response to sheep erythrocytes was consistently depressed in mesenteric lymph node cell preparations but not always in spleen cell preparations. The authors felt that these effects were consistent with antigenic competition playing a major role in the course of the infection.

The relationship between immunosuppression and parasite survival has been further investigated with the *T. crassiceps* mouse model (Good and Siebert, 1982). When excretory/secretory products collected from *T. crassiceps* larvae maintained in culture were

injected, they were found to elicit resistance to reinfection, a non-specific immunodepression and eosinophilia to a degree comparable to that produced by the living larvae *in vivo*. Depression of the host's antibody response to sheep erythrocytes occurs in established intraperitoneal infections with *T. crassiceps* larvae but not in subcutaneous infections or in individual mice which have overcome their intraperitoneal infections. These results suggest that the degree of immunosuppression is related to the high parasite burden.

In experimental *E. multilocularis* infections in mice, Ali-Khan (1978) found that a specific cellular unresponsiveness to *Echinococcus* antigen developed while delayed hypersensitivity reactions to unrelated determinants were at first reduced but eventually became normal. He attributed this phenomenon to a massive release of *E. multilocularis* antigen due to the high parasite burden found in the later stages of the disease. Interestingly, at certain stages of the infection the anti-sheep erythrocyte response of infected mice was significantly greater than the controls (Ali-Khan, 1979), perhaps due to enhancement of macrophage activity and antigen-processing capability.

Rats infected with *T. taeniaeformis* have been found to have depressed mast cell-mediated, hapten-specific reactions to heterologous antigens (Leid, 1977) as a result of parasite modulation of mast cell functions.

In addition to the possibility that the wholesale release of metacestode antigens may contribute to lymphocyte unresponsiveness there is some evidence that other parasite derived factors may

result in non-specific lymphocyte exhaustion similar to that demonstrated in trypanosomiasis (Clayton *et al.*, 1979). Thus, extracts of *T. solium* cause polyclonal B cell activation (Sullivan-Lopez *et al.*, 1980) and normal mouse lymphocytes undergo transformation in the presence of protoscolices of *E. granulosus* (Dixon *et al.*, 1982). This stimulation apparently occurs on contact between the lymphocytes and the protoscolices; there is no host-derived component nor any factor secreted by the protoscolices; the phenomenon is not diminished by culture of the parasite *in vitro*; is fully elicited only by the living organism and appears to be T cell dependent. It was thought by Dixon that these blastic responses may be the *in vitro* manifestation of an immunosuppression mechanism resulting in the elimination of reactive clones due to persistent stimulation. The physiochemical nature of the stimulator has yet to be determined.

Mesenteric lymph node cells from *E. granulosus* infected mice are highly suppressive of the normal host response to sheep erythrocytes when these cells are transferred into syngenic recipient normal-responder mice (Allan *et al.*, 1981).

There is also some evidence of a depression in lymphocyte reactivity mediated by metacestode antigens and factors circulating in the sera of hosts bearing larval cestode burdens. Annen *et al.* (1980) detected a low molecular weight substance in the cyst fluid of *E. granulosus* that suppresses the lectin responsiveness of lymphocytes. Similarly the addition of hydatid patients' sera to lymphocyte cultures resulted in significant depression of the normal lymphocyte responses to Con A and PHA (Chemtai, 1980).

2.4.4 Immunosuppression by tumour cells

Tumours, whether they are spontaneous or induced by carcinogens, are the progeny of transformed cells that are able to propagate and survive with their host. They are able to propagate thus, because transformed cells are no longer subject to normal cell regulatory mechanisms. There is evidence that cancer cells are able to subvert the immune system of the host and thus escape host rejection in much the same way as parasites appear to. (Plescia, 1980).

Inbred strains of mice, bearing tumours were tested at intervals during the course of tumour proliferation for immunological competence, in terms of their ability to develop cellular and humoral immunity to unrelated antigens. The level of immunocompetence of the tumour-bearing host declined with progressive tumour growth (Plescia, 1980). In vitro analysis of the immunosuppressive activity of tumours has shown that as few as one tumour cell added to 1,000 spleen cells suppressed the antibody response of the cells to SRBC and their mitogenic response to a T cell mitogen.

Much work has been carried out to determine which types of immunocytes are the targets of subversive tumour cells and their mechanism of subversion (Evans, 1972; Burk et al., 1975). There is evidence implicating T cells with a major role in tumour immunosuppression but in addition to this Fauve et al. (1974) found a tumour-derived factor that was capable of repulsing macrophages.

The evidence and lines of investigation used in the analysis of tumour-induced immunosuppression may be of benefit in unravelling the complex games of subversion and evasion that parasites play in

the host. Workers in the field of cancer biology have listed two main goals in order to prevent this escape which may be applicable to parasitology as well. These are (1) to block further subversion of the immune system and (2) to restore the depressed immunological system to full competence with non-specific immunostimulating drugs (Plescia, 1980).

The achievement of these goals necessitates in depth analysis of the immunoregulatory mechanisms involved in tumour-host interactions and parasite-host interactions, both of which appear to have a great deal of common ground.

2.5 Conclusion

The complexity of the mechanisms postulated to be involved in immunosuppression (Mitchell, 1982) and the apparent contradictory evidence as to whether stimulatory or inhibitory factors, or an interplay of both, are released during such infections, necessitates further investigations. In particular, it is of little value to simply demonstrate immunosuppression without evaluating its contribution to immune evasion. Such an evaluation will not only seek to define the suppressed components of the immune response that could be restored and might then result in the parasite's death but will also raise the possibility of directly interfering with the expression of the suppressive agents. The recently developed techniques for antigen isolation and purification will clearly aid in the analysis of the immune responses induced or suppressed by larval cestode infections.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental Animals

All mice and rabbits used were specific pathogen-free (SPF) animals obtained from the Centre for Laboratory Animals, University of Edinburgh. Two mouse strains were used: random bred CF1 for maintaining *Taenia crassiceps* and *Taenia taeniaeformis* and inbred Balb/c for secondary hydatidosis studies, immunosuppression experiments and lymphocyte transformation assays. New Zealand white rabbits were used to raise hyperimmune antisera. Suffolk cross lambs were also used to raise antisera to ovine strain hydatid antigens.

Cats were used to maintain the life-cycle of *Taenia taeniaeformis* and were obtained from the breeding colony at the above Centre. They were kept at the Wellcome Animal Research Unit, University of Edinburgh.

Outbred gerbils, *Meriones ungulatus*, were obtained from a breeding colony at Raigmore Hospital, Inverness. These animals were also used as secondary hosts for hydatid cysts.

3.2 Establishment of the Laboratory Culture of Secondary Hydatidosis

Hydatid cysts in sheep livers or lungs were obtained from Gorgie Abattoir, Edinburgh and Stornoway Abattoir, Isle of Lewis. Equine strain hydatid cysts were obtained from an abattoir near Midsummer Norton, Somerset.

Both strains of hydatid material were subjected to the same procedures prior to infection of rodents or *in vitro* culture.

The cyst surface was washed with 70% alcohol under a laminar flow cabinet (Microflow) and some hydatid fluid was withdrawn with

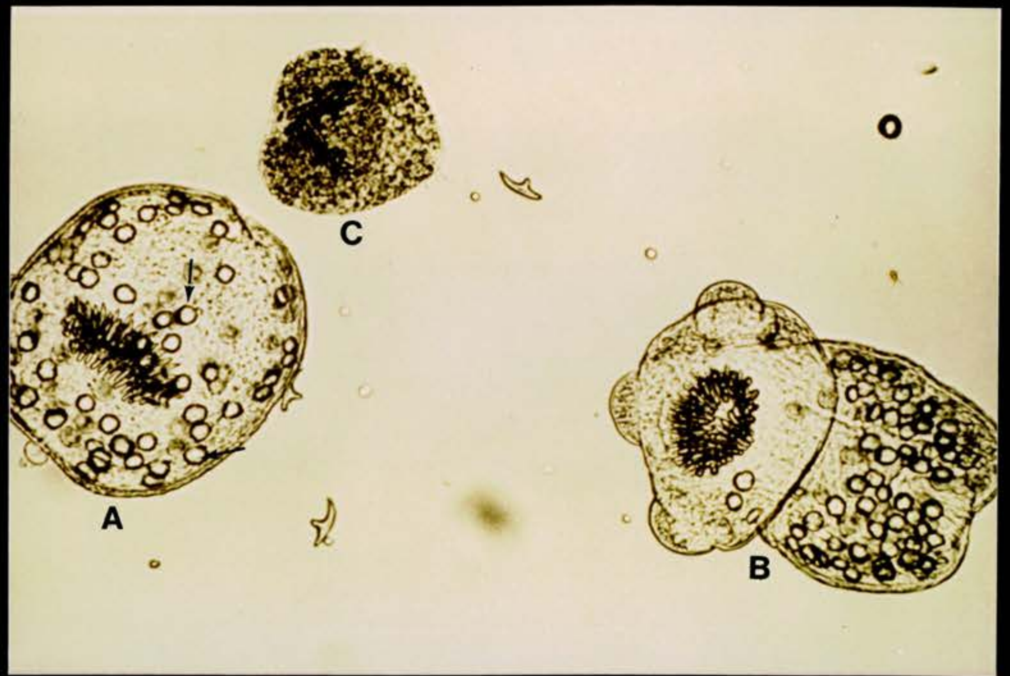
Plates 1 and 2 Protoscolices from equine hydatid cysts

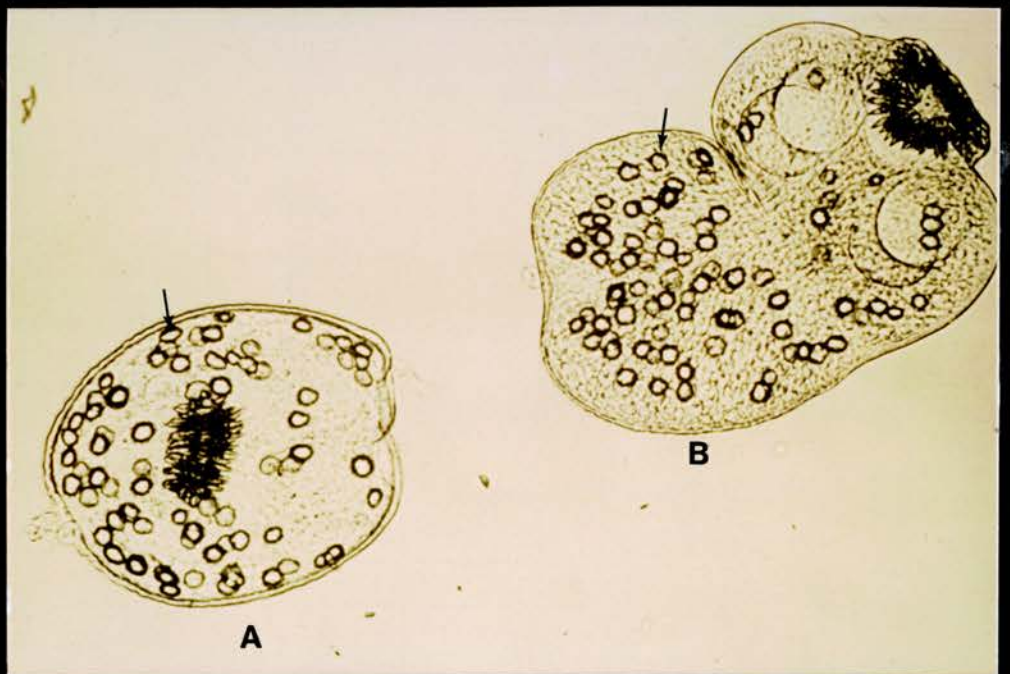
A = invaginated

B = Evaginated

C = Dead protoscolices which have taken up the dye of a 0.2% tryphan blue in PBS solution.

Arrows indicate the calcareous corpuscles.


C
A
B


B
A

a 50 ml disposable syringe (B-D Plastipak) through a 19G2 needle attached (Vale Microlance) to reduce the pressure in the cyst. The fluid was transferred to a sterile universal bottle. Brood capsules and protoscolices rapidly sediment if the cyst is fertile. The cyst was then opened using sterile instruments and the remaining cyst fluid and protoscolices were sucked up with a sterile pasteur pipette. This has the result of loosening brood capsules and protoscolices that are attached to the germinal epithelium. These bottles of cyst fluid and "sand" were centrifuged at 100 g for ten minutes, and the supernatant cyst fluid removed into sterile universals for antigen preparation (see Section 3.3). The remaining material was gently agitated by pipetting up and down with a sterile pasteur pipette in RPMI 1640 and antibiotics (for media constituents see Section 3.11) at 37°C to release any protoscolices still within their brood capsules. The protoscolices were centrifugally washed three times (100 g) in RPMI 1640 and antibiotics and then stored in universal bottles at 4°C for up to two days. The optimal concentration in this stock suspension is about one ml of settled protoscolices in 20 ml of medium (Smyth and Davies, 1974).

All batches of protoscolices used had a mean viability of at least 70%, as determined by the eosin dye exclusion test (Clegg and Smithers, 1972). This test is based on the fact that eosin is not absorbed by viable tissue, as intact cell membranes will exclude the dye. Non-viable tissues will absorb the dye and so turn pink indicating membrane damage and altered permeability. A few drops of 0.2% eosin in phosphate buffered saline (PBS, Oxoid Ltd.) were added to a 0.1 ml sample of the evenly suspended stock of protoscolices

on a slide and the proportion of protoscolices that had taken up the stain counted. The volume of the evenly suspended stock needed to give the required dose level could then be calculated.

Recipient mice and gerbils were inoculated intraperitoneally with the chosen dose level - 1,000, 2,000, 4,000 or 10,000 PS depending on experimental design - in 0.2 ml of media. Prior to inoculation the filled syringes plus needles were left to stand, point downwards, for ten minutes in order to allow the protoscolices to sediment and thus to be present in the first portion of the inoculum.

3.3 Preparation of Hydatid Cyst Fluid Antigen

Cyst fluid aspirated from lung or liver hydatid cysts was transferred to sterile plastic universal bottles and centrifuged at 2,500 g for 30 minutes at 4°C. The supernatant was removed and dialysed in Visking tubing (Medicell International Ltd.) against PBS for 48 hours with several changes of PBS. The cyst fluid was concentrated approximately five times by placing the Visking tubing in a plastic sandwich box and scattering polyethylene glycol ("Carbowax 6000", BDH) over the entire length of tubing. The Visking tubing cellulose membrane (Scientific Supplies) retains molecules in excess of about 20,000 molecular weight. The concentrated cyst fluid was assessed by the Warburg and Christian method of protein estimation (Section 3.12) and sterilised by passage through 22 μm pore size Millipore filtration apparatus.

3.4 Treatment of Protoscolices Prior to Culture for Excretory/ Secretory Antigen Production

The protoscolices were washed a further three times in RPMI 1640 medium and placed in sterile universal bottles at 4°C.

3.5 Establishment of Taenia taeniaeformis

Cats infected with a Belgian (mouse-adapted) strain of T. taeniaeformis (Brandt and Sewell, 1981) were already available, being held at the Wellcome Animal Research Unit, University of Edinburgh.

The proglottides expelled by the cats were collected daily by staff of the Wellcome Unit, delivered to the laboratory in normal saline plus antibiotics and fungizone (saline/antibiotic solution contained 100 IU penicillin, 100 μg streptomycin and 2 μg fungizone in one litre normal saline). On receipt, these proglottides were washed in fresh saline/antibiotic solution and held at 4°C in plastic storage pots. When required the proglottides were placed in a glass petri-dish and cut longitudinally with a scalpel so that the eggs were released into the dish. The entire contents of the petri-dish were washed into the top of a two-tier sieve system. This consisted of two 100 nm diameter test sieves (Endecotts) the upper sieve having a pore diameter of 53 μm and the lower sieve being a microplate of 20 μm pore size. The cut proglottides were pressed with a spatula and a spray of tap water was used to wash through the sieves followed by several washes with the saline/antibiotic solution. The lower sieve retained the eggs and its contents were poured and rinsed into a glass beaker. The suspension of eggs in 10 ml of saline was gently

centrifuged in 15 ml conical glass tubes at 250 g for ten minutes at room temperature. The supernatant was sucked off to leave 2 ml which was poured into a 10 ml glass beaker. The total number of viable eggs was then estimated by counting the number in several 0.1 ml aliquots. These aliquots were taken with a 1.0 ml syringe while the beaker was on a magnetic stirrer, placed on a slide and examined under 100 x magnification. The viability of the eggs was assessed from their morphology, those with thick brown shells having aligned hooks being considered viable. The eggs were then stored in saline/antibiotic solution at 4°C.

Batches of three-week-old CF1 mice were infected with 200 viable eggs in 0.1 ml of normal saline. The age of the mice was chosen on the basis of studies by Dow and Jarrett (1960) in which mice of this age were most susceptible to infection.

Only eggs that had been stored for less than two weeks were used. Eggs were administered using cannulae made by removing the bevel from a 23G one inch needle ("Vale microlance", Becton-Dickinson). The tip was smoothed and covered with a 30 mm length of silicone rubber tubing, half the length of tubing extending beyond the tip of the cannula.

Metacestodes from mice with three to six-month-old infections were used to infect the cats. The intermediate hosts were killed by cervical dislocation, their livers removed aseptically and the cysts dissected out. A few cysts from each liver were incised and the larvae removed to check for viability by their motility in warm saline (37°C). Each cat was starved for 24 hours and then given 15-20 intact cysts which were placed at the back of the tongue to encourage swallowing.

Proglottides were expelled by the cats one to two months after infection and collected daily. The cats were replaced with six-month-old females annually or when the level of egg production fell off, whichever occurred first.

3.6 Treatment of Metacestodes Prior to Culture

T. taeniaeformis metacestodes were obtained from CF1 mice infected for 12 weeks and killed by cervical dislocation. The livers of the mice were aseptically removed under a laminar flow cabinet (Microflow) while wearing sterile surgical gloves. Each cyst capsule was cut open with sterile scissors in a sterile plastic petri-dish and the larvae extruded by gentle pressure on the capsule. The larvae were washed until host material was no longer visible, with copious amounts of physiological saline in the sterile petri-dishes by refilling and decanting the fluid, while retaining the larvae. The larvae were then transferred through several disposable petri-dishes containing culture medium. A series of four to six further washes was carried out by transferring the larvae with sterile blunt forceps from one petri-dish to the next before placing them in a Nunc flask (Gibco). Care was exercised to avoid damage to the larvae during this procedure. All materials used in this procedure were sterile and solutions warmed to 37°C.

3.7 Routine Passage of Taenia crassiceps

T. crassiceps metacestodes (Toi strain) were maintained in female CF1 mice by intraperitoneal passage. Twenty metacestodes of less than 2 mm diameter in 0.2 ml of sterile PBS were injected into

each mouse through a 16G needle attached to a 2 ml syringe. Thereafter, the mice were weighed at weekly intervals and killed by cervical dislocation when they weighed 45-55 grams - i.e. had approximately doubled in weight. Under a laminar flow cabinet (Microflow) the hair from the ventral region was soaked in 70% alcohol prior to incision along the _linea alba_. The metacestodes were washed out of the peritoneal cavity with saline/antibiotics from a 20 ml syringe into a sterile glass beaker and then were washed at least three times with sterile PBS pH 7.6. Any opaque, white (dead) metacestodes were removed with sterile forceps. All the larvae used for either _in vivo_ or _in vitro_ experiments were taken from donor mice with infections of at least three months duration. Such infections permit sufficient numbers of larvae of uniform size and appearance to be selected from a single donor mouse for use in each experiment or passage group.

The viability of a sample of the recovered larvae was assessed by eosin dye uptake (Clegg and Smithers, 1972). Metacestodes that turned pink after incubation for 30 minutes at room temperature in 0.2% eosin and PBS were considered non-viable and the percentage viability calculated. The viability was rarely less than 98%.

3.8 Preparation of Taenia crassiceps Metacestode Somatic Antigen (defatted)

Metacestodes of _Taenia crassiceps_ recovered from mice which had been infected for three months were washed in PBS and drained. The cysts were then homogenised and the resultant suspension lyophilised. The dried powder is extracted with ethanol at 1g per cent w/v,

by stirring for two hours on a methanol-dry ice bath at 70°C and the supernatant removed by centrifugation at -20°C. The extraction is repeated twice more with diethyl ether. The defatted material is air-dried and stored dry.

Reconstitution is effected by the addition of PBS at 1:20 w/v. The antigen preparation was mixed for two hours at 37°C in an end-over-end mixer and then stirred overnight at 4°C. The resulting antigen was centrifuged at 15,000 g for 30 minutes and the supernatant removed for protein estimation (Section 3.13) and stored in aliquots of 0.2 ml in microcapped centrifuge tubes (Hughes and Hughes) at -20°C.

3.9 Preparation of Metacestode Somatic Antigen for ELISA

Metacestodes of *T. taeniaeformis* or secondary hydatidosis were obtained from infected Balb/c mice, washed in six changes of cold PBS and chopped finely with a pair of scissors. PBS + protease inhibitors + 1% NOG (9 volumes to 1 volume of parasite material) was added and the mixture was subjected to 4 x 3 minute treatments with a stomacher 80 Lab Blender (Seward Laboratories) and then homogenised for 4 x 1 minute periods with an MSE homogeniser. Throughout these procedures the parasite material was chilled in an ice-bath. The parasite extracts were then left to extract on ice for 30 minutes at 4°C. The supernatant was collected and dialysed in Visking tubing (Medicell International Ltd.) against PBS to give a final concentration of 0.03% NOG. The pellet was resuspended in a small volume of PBS and retained. The dialysed supernatant was centrifuged at 2,500 g for 30 minutes and 4°C and the resulting supernatant collected,

its protein content estimated from the optical density (Section 3.13) and stored in aliquots at -20°C.

3.10 Preparation of Metacestode Somatic Extracts for use in the Lymphocyte Transformation Assay

Metacestode extracts of *Taenia crassiceps*, *T. taeniaeformis* and secondary hydatidosis were prepared in the same way as in Section 3.9 except that protease inhibitors were not added to PBS as these would adversely affect the survival of the lymphocytes in culture.

3.11 In vitro Maintenance Procedure

The defined medium devised by Brandt and Sewell (1980) to support survival of metacestodes was used for maintaining the metacestodes. The following are the components of the medium:-

RPMI 1640 with 25 mmol l^{-1} HEPES buffer and L-glutamine (Gibco)
5 mmol l^{-1} sodium pyruvate (Gibco)
100 IU ml^{-1} sodium benzyl penicillin (Gibco)
100 μg ml^{-1} streptomycin sulphate (Glaxo)

As the activity of L-glutamine diminishes fairly rapidly with storage, even at 4°C, this was routinely added just before use at 300 mg l^{-1}.

T. crassiceps metacestodes (2-4 mm in size) for *in vitro* culture were washed a further six times in medium at 37°C. Groups of 15 metacestodes were then placed in universal bottles containing 15 ml of the culture medium at 37°C.

Metacestodes of T. taeniaeformis or E. granulosus were kept in disposable Nunc 25 cm^2 tissue culture flasks.

The T. taeniaeformis cultures were kept stationary in the dark at 37°C in an ungassed incubator. The E. granulosus cultures were maintained in a humidified 6.3% CO_2 incubator at 37°C. The medium of all cultures was changed every 24 hours for a period of seven days and the used medium was labelled and stored at -20°C in sterile universal bottles.

Occasional samples of used medium were taken before storage for bacterial and fungal examination. Smears of the debris after centrifuging samples of medium were Gram-stained and both aerobic and anaerobic blood agar cultures and aerobic nutrient broth cultures of medium and debris were set up. These cultures were kept for 2-4 days and examined for bacterial growth daily. Samples of used medium were also maintained at 37°C in 6.3% CO_2 in a gassed incubator for four days and regularly checked for fungal growth by viewing through an inverted microscope.

3.12 Excretory/Secretory Antigen Preparation

Pooled, used medium obtained daily from seven day cultures of live metacestodes was extensively dialysed at 4°C against phosphate buffered saline (Gibco) for 48 hours and concentrated against a solution of polyethylene glycol ("Carbowax 6000", BDH) in PBS. The Visking tubing cellulose membrane (Scientific Supplies) retains molecules in excess of about 20,000 molecular weight. The concentrated medium was finally sterilised by passage through 22 μm pore size Millipore filtration apparatus.

3.13 Protein Estimation

The method used was that developed by Warburg and Christian in 1941 (Dawson, Elliott, Elliott and Jones, 1967). This method measures the phenylalanine, tryptophane and tyrosine content of the sample. The technique is not particularly sensitive, but it is rapid, simple to perform and does not entail the destruction of precious antigen samples.

3.14 Affinity Chromatography Procedures

3.14.1 Principle

In affinity chromatography, components of samples are isolated and separated on the basis of their biological specificity as in antigen-antibody or enzyme-inhibitor systems. A ligand with specific affinity for the sample components of interest is covalently coupled to a solid matrix. When the sample is applied, components with specific affinity for the ligand are adsorbed to the gel matrix, the non-specific components being eluted. Disassociation of the bound proteins is later achieved by altering the pH and/or salt concentration of the column buffer so allowing the desired components to be recovered.

3.14.2 Preparation of the immunoadsorbent

The mouse-derived components in the E/S antigen preparations were removed by immunoadsorption. The adsorbent was prepared by coupling goat anti-mouse IgG to a Cn-Br activated Sepharose 4B (Pharmacia) as recommended by the manufacturer (Pharmacia, 1979).

The antibody chosen for the column should have an affinity for the Ig that will both permit stable binding of mouse IgG at the neutral pH of the column buffer and allow subsequent disassociation of the complex with dilute acid so that the absorbent can be reused. High affinity polyclonal antibodies obtained after hyperimmunisation regimens are not suitable as these bind antigen so firmly that the complexes cannot be disassociated. Accordingly, the goat anti-mouse IgG preparation used was obtained from an early stage in the immunisation regimen.

One ml of a 15 mg ml^{-1} solution of goat anti-mouse IgG prepared by precipitation with half saturated ammonium sulphate, was made up to 5 ml with a coupling buffer at pH 8.3 comprising 0.1M $NaHCO_3$ in 0.5M NaCl. This globulin was coupled to Ig of the activated Sepharose gel which had previously been washed and allowed to swell for 15 minutes in 200 ml of 10^{-3}M HCl solution. The ligand-gel mixture was decanted into a plastic universal bottle and mixed on a blood-cell suspension mixer (Eschmann) for two hours at room temperature.

The later stages in the process were performed with the ligand-gel mixture retained on a hardened filter paper (No. 54, Whatman) on the acrylic plate of a Hartley type 3-piece funnel of 50 mm internal diameter (Whatman).

Most of the unbound material was washed away in the coupling buffer. Any remaining active groups on the gel were blocked by exposure to 1M ethanolamine in coupling buffer, pH 8.0, for two hours. Three washing cycles were used to wash the gel and remove any remaining non-covalently bound protein, each cycle consisting of 15 ml volumes of 0.1M acetate buffer, in 0.5M NaCl pH 4.0 followed by a

15 ml wash at pH 8.0 with 0.1M borate buffer also in 0.5M NaCl. The prepared gel was stored in PBS plus sodium azide (0.02% w/v) ready for use.

3.14.3 Procedure

The prepared adsorbent was packed into a plastic column (Whatman) giving a 3.5 ml gel volume and allowed to warm to room temperature. After packing, the column was connected up to the pump, monitor and fraction collector. Samples and buffers were pumped through the column with a downward flow using Varioperspex peristaltic pump 12000 (LKB). The effluent from the column flowed through a Uvicord S optical unit 2138 (LKB) to measure the percentage transmission at 280 nm which was recorded on a continuous chart recorder unit 6520 (LKB) at a chart speed of 60 mm/hour^{-1}. The effluent was finally collected in 5 ml aliquots in 10 ml glass tubes on an Ultrorac fraction collector 7000 (LKB).

The newly packed column was washed with distilled water to remove the PBS/Azide, followed by 1-2 column volumes of 0.5M acetic acid rapidly and then by a wash with the chosen column buffer, PBS + 0.06% N-octyl-glucoside (w/v) (NOG, Sigma Chemical Company) pH 7.6. A sample of E/S antigen containing 5 mg of protein in 2 ml was centrifuged at 1000 rpm for 10 minutes and the supernatant applied to the column. The flow rate was 2-3 ml cm^{-2}/hour^{-1}. As the non-adherent fraction was required this was collected as the initial peak came off the column. The column was then washed until the optical density of the effluent returned to the baseline with PBS pH 7.6. The column was subsequently stripped of bound mouse immunoglobulin by passing

it through a one column volume of 0.5M acetic acid. The acid was immediately washed out with the PBS pH 7.6 and the pH of the effluent was monitored frequently with indicator paper (Whatman) until it rose to pH 7.6. The column was then ready for use again. This procedure was repeated three times with the same sample in order to remove all host immunoglobulins from the antigen preparations.

The final pooled non-adherent fraction was tested for any remaining traces of mouse IgG by ELISA. The pooled fractions were used to coat a microtitre plate at a protein concentration of 10 μg ml^{-1}. Rabbit anti-mouse IgG horseradish peroxidase conjugate (Nordic) was used in the second step, followed by the O-P-D substrate (see Section 3.20). Negative control wells were coated with ovine serum, albumin and positive control wells were coated with mouse IgG. Other control wells were also coated with samples of the antigen preparations prior to purification with the immunoadsorbent.

3.15 Lymphocyte Transformation Assay

3.15.1 Medium for lymphocyte cultures

The medium tested for this system was RPMI 1640 buffered with HEPES (N-2-hydroxyethyl piperazine-N-2: ethane-sulphonic acid) (25 mM), supplemented with 5% v/v foetal calf serum (FCS) and 2 mM L-glutamine all supplied by Gibco; 100 IU ml^{-1} benzyl penicillin sodium B.P. and 100 μg ml^{-1} streptomycin sulphate B.P. (Glaxo Laboratories), 5 x 10^{-5} M beta-mercaptoethanol (Sigma). The latter was added due to the constant improvement of cell viability which it seems to provide (Kristensen, Kristensen and Lazary, 1982). The pH of the medium was about 7.2 at 37°C and 6.3% CO_2.

3.15.2 Lymphocyte culture preparation

Infected or naive Balb/c mice were killed by cervical dislocation and their ventral surface soaked in 70% alcohol under a laminar sterile airflow cabinet (Microflow). Their spleens or mesenteric lymph nodes were excised aseptically and placed in culture medium in a petri-dish. Using sterile instruments, the tissue was chopped to release the cells and the material strained through a sterile plastic sieve of 100 μm pore size. The resulting cell suspension was washed three times centrifugally at 100 g for 10 minutes in fresh medium. Cells were kept at 4°C throughout the period of culture preparation.

Viability was assessed by adding a few drops of aqueous 0.2% eosin in PBS to a sample of the cell suspension and examining the cells in an improved Neubauer haemocytometer chamber by bright field microscopy at x 250 magnification. Only cell suspensions with a viability of at least 98% were used in this assay. The lymphocytes were finally suspended in medium at a final concentration of 2×10^6 ml^{-1}.

3.15.3 Mitogens

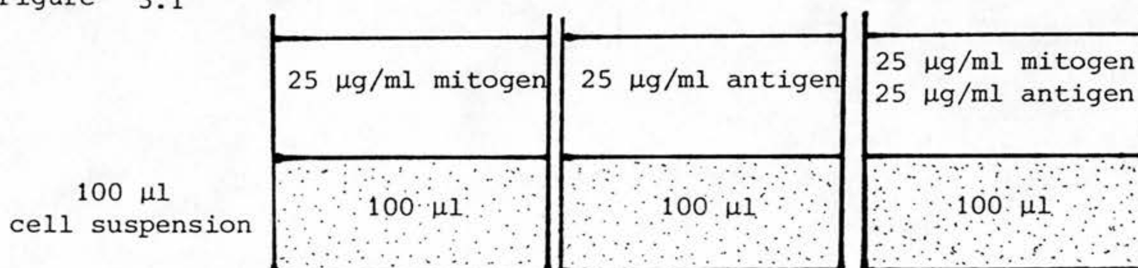
One type of mitogen was tested in the lymphocyte transformation assay. This was Concanavalin A (Con A) (Sigma) extracted from the jack bean, *Canavalia ensiformis*. The Con A crystals were dissolved in sterile PBS to make a stock solution at 1000 μg/ml. The solution was then dispensed in 1 ml aliquots and stored at -20°C until used.

3.15.4 Procedure

The procedure was based on that reported by Dixon, Jenkins and Allan (1982) and by Maleckar and Kierszenbaum (1983). The tests were done in 96-well flat bottomed microtitre plates (Nunc) with the exception of the experiments where the lymphocytes were cultured with live metacestodes, for the latter 24 well multi-dish plates (Nunc) were used. For each test an aliquot of a stock solution of the mitogen Concanavalin A (1000 μg ml^{-1} in medium stored at -20°C) was diluted to a concentration selected on the basis of initial titrations. The range of dilutions used were from 25 μg ml^{-1} to 400 μg ml^{-1}.

An aliquot of 100 μl of the cell suspension in tissue culture medium was added to each microtitre well followed by a 20 μl aliquot of mitogen solution. Test wells received 20 μl of parasite antigen with or without mitogen.

Figure 3.1



All wells were made up to 200 μl with media. Plates were incubated at 37°C for four days in 6.3% CO_2 in a humidified incubator. At 18 hours before harvesting each well received 20 μl of medium containing 18.5 k Bq of tritiated methyl-thymidine (^{3}H-TdR) (specific activity 185 M Bq $mmol^{-1}$; Amersham International). Cultures were

harvested with a semi-automatic Skatron Titertek multiple cell harvester (Flow Laboratories) onto glass fibre pads (Flow Laboratories). The filter pads were dried and transferred to 5 ml screw-capped glass vials. Each vial then received 5 ml of the toluene-based scintillating fluid made up of the following reagents:- 0.05g anhydrous (1,4-bis-2-(5-phenyloxazolyl)-benzene; phenyl-oxazolyl phenyl-oxazolyl-phenyl) (P.O.P.O.P.) (Sigma); 6.0g anhydrous, 2,5-diphenyloxazole (P.P.O.) (Sigma) in one litre of toluene.

Vials were capped and subjected to scintillation counting for two minutes on a liquid scintillation counter (Nuclear Enterprises). The degree of 3H-TdR incorporation into the DNA of the lymphocytes was expressed as both the arithmetic mean of counts per minute (cpm) of the replicate wells and as a stimulation index (SI). The stimulation index represents the ratio between the mean cpm incorporated by the cells in the test wells and that incorporated by the cells in the antigen and mitogen-free control cultures.

The lymphocyte transformation assays were assessed in these two ways because Schultz (1981), using bovine data, demonstrated that if the background counts differ greatly as between control and test animals, the data may be significant if presented as a stimulation index but not significant if presented as mean cpm. Most workers agree that data should be presented in both ways (Stobo, 1980; Schultz, 1981).

3.15.5 Lymphocyte transformation assays incorporating live metacestodes

Lymphocyte suspensions from spleen or lymph nodes were made up as described previously at a concentration of 2.0×10^6 cells ml^{-1}. This suspension was dispensed in 1 ml aliquots into each well of a

24 well multi-dish plate (Nunc). At least three replicates were set up for each test and control condition. A single live metacestode of Echinococcus or T. taeniaeformis or four metacestodes of T. crassiceps were added to each test well with or without the addition of 100 µl of mitogen. Again, the cultures were maintained for four days. Prior to pulse labelling, the metacestodes were removed from the wells and the cells evenly suspended using gentle agitation with a sterile pasteur pipette. Four 200 µl aliquots from each well were transferred into wells in a labelled 96-well tissue culture plate. To each well was added 20 µl of 3H-TdR (18.5 k Bq) and the plates were incubated for 18 hours before being harvested as described above. In addition to this, 50-100 µl aliquots of thoroughly mixed culture material were obtained from the large wells at the same time and used to prepare cytocentrifuge smears.

3.15.6 Assessment of cultures

Cultures were routinely examined on day 2 and day 4 of the assay for transformation. This was achieved both by examination of cytocentrifuge smears for the presence of blast cells and by the detection of an increased metabolic rate as indicated by the yellow colour of the medium.

3.15.7 Preparation of cytocentrifuge smears

To prepare cytocentrifuge smears, 20 µl aliquots of well mixed cell suspensions were taken from one well of each series of test replicates and controls and dispensed into the funnel attachment (Shandon) of each slide. These were spun at 1,000 rpm for five minutes in a cytospin (Cytospin 2; Shandon). The smears were then air-

dried, fixed in methanol for three minutes and stained with Giemsa as described for blood smears. They were then examined under bright field microscopy at x 1,000 magnification and the presence of blast and non-blast cells recorded.

3.16 Giemsa-staining of Blood Smears

Air-dried blood smears from the mice infected with *Babesia microti* were fixed in methanol for three minutes. This was followed by staining for 40 minutes with 5% Merck's Giemsa stain with added Azur II buffered at pH 7.2. The method for the preparation of the Giemsa stain was similar to that described by Shute (1966). The stain was diluted to a 5% solution with Giemsa buffer just before it was required and was used for only one set of slides. Stained smears were differentiated individually in Giemsa buffer pH 7.2 and blotted dry before being examined by light microscopy at x 1,000 magnification.

3.16.1 Evaluation

The blood smears of mouse red cells infected with *Babesia microti* were examined for the presence of piroplasms and the results expressed as percentage parasitaemia. At least 200 cells were examined per slide.

3.17 Preparation of Babesia microti Stabilates

Stabilates were made from the original stabilates obtained from the Institute for Research on Animal Diseases, Compton. Two mice with parasitaemias of 30-35% infected red blood cells were

exsanguinated into heparin. The mice were placed in a jar containing anaesthetic ether (May and Baker Ltd.) until adequately anaesthetized. They were then removed from the jar and pinned out on a cork board ventral side uppermost. A small pad of cotton wool soaked in ether was placed over the head to ensure continuing anaesthesia. The rib cage was reflected anteriorally, exposing the heart, and blood withdrawn from the right ventricle of the heart through a 25G $^{3}/8$" needle into a 1.0 ml sterile plastic disposable syringe containing 0.1 ml of heparin (10 international units) in PBS pH 7.2. The pooled blood was divided into 0.5 ml aliquots and to each of these was added 88 µl of a 50% solution of dimethyl sulphoxide (DMSO) in PBS pH 8.0 to give a final DMSO concentration of 7.5%. The mixture was then allowed to flow into sterile 2½" Wilkins capillary tubes until they were two-thirds full, the tube ends heat sealed and the capillary tubes placed in a cooling jacket and cooled at one degree per minute to -79°C in solid carbon dioxide as described in Lumsden _et al_. (1973). The capillary tubes were then placed in a liquid nitrogen vat and given a unique identifying number (TREU 1764).

3.18 Indirect Fluorescent Antibody Test

This test was used to assess the humoral response to _B. microti_. Heparinised red blood cells from the same pool used to initiate the infections under serological analysis, were centrifugally washed three times with phosphate buffered saline (Oxoid). The washed red blood cells were smeared across 15 well multi-spot slides

(Flow Laboratories) and air-dried. The smears were fixed in acetone for 15 minutes and, when dry, wrapped in foil in a moisture-free container to be stored at -20°C until required.

Test sera, positive and negative control sera were prepared as doubling dilutions in a microtitre plate (Nunc). Each animal's sera was applied to a single multi-spot slide as shown below.

O 1:40	O 1:80	O 1:160	O 1:320	O 1:640	Animal
O 1:1280	O 1:2560	O 1:5120	O 1:10240	O 1:20480	No. Bleed date
Θ	Θ	Θ	Θ	Θ	

Θ = negative

Twenty μl aliquots of sequential doubling dilutions from 1:40 to 1:20,480 in PBS pH 7.3 were applied onto the slide for 20 minutes at room temperature. The slides were then washed in three changes of PBS and excess fluid shaken off them. Twenty μl of 1:80 dilution goat anti-mouse IgG-FITC conjugate (Nordic Ltd.) prepared in PBS pH 7.3 was dropped gently onto each spot and left for a further 20 minutes. The slides were again washed in three changes of PBS, and excess fluid shaken off prior to counterstaining with a 1:10 dilution of Eriochrome black. The slides were allowed to stain for five minutes and then washed in a final three changes of PBS. The slides were mounted in glycerol and covered with a coverslip. They were then examined using an Orthoplan microscope (Leitz, Wetzlar, Germany) equipped for selective incident excitation of FITC at x 675 magnification. The observed fluorescence was visually assessed by determining the end-point titration.

3.19 Blood Sampling

The samples were taken by means of heparinized capillary tubes No. 442 (Sarstedt). The mice were held without the use of an anaesthetic, the tail was swabbed with 70% alcohol and a small snip removed from the tip of the tail with fine scissors. Gentle pressure was applied from the base of the tail to tip to stimulate flow and the blood was taken up into the capillary tubes. The tubes were centrifuged at 1500 g for 30 minutes at 4°C and the resulting plasma was removed with a Gilson pipette. The plasma was diluted 1:2 with PBS (pH 7.3) prior to storage at -20°C in labelled 0.5 ml microcapped centrifuge tubes (Hughes and Hughes). This was necessary as the volume of undiluted plasma was so low that it would suffer severe separation on freezing.

3.20 Enzyme-linked Immunosorbent Assay (ELISA)

MicroELISA was carried out according to the procedure and principles described by Harrison (1977) as shown in Table 3.1. The assays were performed in polystyrene plates (Mikrotiterplatte 96K F-form aus Immulon; Greiner labor technik).

The optimal dilutions for the antigen and antiserum were obtained by a series of chequer-board titrations which are described as preliminary experiments in each relevant chapter.

3.20.1 Solutions used in ELISA

Coating buffer - 0.1M Sodium carbonate buffer pH 9.6 with 0.02% w/v sodium azide (BDH).

PBS-Tween - PBS pH 7.3 (Dulbecco A) with 0.05% w/v Tween 20 (Polyoxyethylene sorbitan monolaurate; Sigma).

Conjugates - Commercial horseradish peroxidase conjugated to IgG against heterologous species IgG; namely goat anti-mouse IgG (Nordic) and rabbit anti-mouse IgG (Nordic).

Substrate - Ortho-phenylene diamine (OPD) (Sigma) 25 mg OPD in 100 ml 0.1M citric acid/phosphate buffer, pH 6.0, containing 0.05% H_2O_2.

Sulphuric acid solution - one part of 1M sulphuric acid in 11 parts of distilled water.

Washing solution - 0.9% w/v sodium chloride solution to which was added 0.05% w/v Tween 20.

Table 3.1 The stages in the ELISA for detecting antibody

Stage 1	Antigen diluted in coating buffer to the optimum concentration and adsorbed to the plate.
Stage 2	Wash
Stage 3	Serum or plasma diluted in PBS-Tween to the optimum dilution and added to plate.
Stage 4	Wash
Stage 5	Conjugate dilution in PBS-Tween to 1:1000 and added to plate.
Stage 6	Wash
Stage 7	Substrate added to plate.
Stage 8	The degree of colour produced by the substrate-enzyme reaction is measured on a spectrophotometer.

3.20.2 General procedure

At every stage, 150 µl aliquots of the relevant solution were added to the wells using an adjustable pipette ("Pipetman P" Gilson). The plates were covered with a plastic lid during incubation. The initial sensitization of the well took place over three hours at 37°C followed by storage at 4°C for periods not exceeding 48 hours. The duration of incubation for each subsequent step was one hour at a temperature of 37°C. In between each stage, the plates were washed at room temperature by emptying the wells, then refilling them all with washing solution. The solution was left in the wells for five minutes and the washing procedure repeated twice more. After the final wash, the plates were shaken dry and any remaining liquid was removed by tapping the inverted plates on tissues. The next stage was begun at once.

The substrate was prepared just prior to use. The plates were incubated in the dark at 37°C for the substrate reaction to develop. After 30-60 minutes, during which time the plates were checked regularly for colour change, the enzyme reaction was stopped by adding an equal volume of sulphuric acid solution. The reaction was stopped when the colour changes of the strongest reactions were strong but not unreadable. At the same time, an equivalent amount of the acid solution was added to the remaining substrate, which had also been left in the dark. The relative absorbance of the reaction product was read in a filterphotometer ("Vitatron", Fisons MSE) provided with a 450 nm filter. Zero absorbance was set against the substrate-acid mixture (blank). This procedure of "blanking" was repeated regularly throughout the reading of the plates.

3.20.3 Controls and interpretation

Every sample tested in the ELISA assay was carried out with at least four replicates and the average result calculated. A control for false positives was included which involved adding a saline buffer to four wells instead of serum. These wells were distributed randomly across the plate. The plan for the distribution of test samples, positive, negative and saline controls was determined using a table produced utilising random numbers. This plan was used in the ELISA assays carried out.

To take account of the fact that experiments involving a large number of sera samples had, of necessity, to be assayed on different days, a formula was designed to minimise day-to-day variation. This formula allowed the values obtained from different days to be standardized and had the added advantage of allowing comparisons to be made between results from one experiment to another. Samples consisting of sera from infected and normal mice and gerbils with high and low ELISA values respectively were separately pooled. Aliquots from these pools were preserved at -20°C and used in the subsequent assays. Dilutions of these reference sera were made at the same level as for the test sera and included in each group of tests. The ELISA values for these positive and negative sera assayed on a certain day (day 0) were taken as the constant or reference values and used in the following formula to standardize ELISA values from day to day.

$$\text{Corrected absorbance} = [Un - Nn] \times \frac{Po - No}{Pn - Nn}$$

where Un = test serum assayed on day n

Nn = negative serum assayed on day n

Pn = positive serum assayed on day n

No = negative serum assayed on day o

Po = positive serum assayed on day o

3.21 Statistical Analysis of Parasitaemia Data

The percentage parasitaemias were calculated for each mouse for each day by counting the number of both infected and uninfected red blood cells in three fields. This data was analysed by means of a two-way ANOVA with unequal but proportional subclass numbers (Sokal and Rohlf, 1969), the percentage parasitaemias of mice concurrently infected with a metacestode infection and B. microti being compared with those of mice infected with B. microti alone. The purpose of this analysis was to determine whether the measurement technique was accurate compared to normal variance and whether the differences demonstrated can be assigned to the different treatments. This analysis is therefore capable of indicating whether the only significant effect is due to difference in treatment.

In order to check that the parasitaemia data is normally distributed a Rankits test for normality using small samples (Sokal and Rohlf, 1969) was performed on the data obtained from a few randomly selected days observation in each experiment. This test was considered essential as small sample numbers and unequal groups made the performance of F and t tests more sensitive to the

assumptions that the populations from which samples are drawn are normally distributed and have equal variances.

3.22 Pattern of Decline of Average Parasitaemias in each Treatment Group

The day of the peak parasitaemia was ascertained and the differences in the rate of decline from this peak between the various treatments of an experiment were determined. Average readings of the three fields were used for each mouse as previous analysis by an F-test showed that the differences between the readings were not significant.

Regression analysis was used to study the decline of the parasitaemias observed in each experiment. Two forms of equation were used initially:

1. Log transformations adding 0.5 to all data to remove zeros e.g. Log $(P_F + 0.5) = \alpha + \beta \log (T + 0.5)$ which is derived from $P_T = 2T^B$ where PST = Parasitaemia on day T and T is days from peak parasitaemia.

2. Polynominal equations of the form $y = a + bx + cx^2 + dx^3 + ex^4$ with successive terms being added until the coefficients were not significantly different from zero.

Regressions were carried out to determine the functional form providing the best fit for treated mice and control mice separately. The best equations were used to determine the pattern of parasitaemia decline so that the differences between groups could be highlighted. Separate regressions on the data for each group provide the best

predictors of this pattern. It was essential to test whether the differences observed between treatments when the regressions were estimated separately, were in fact statistically significant. This was done by pooling the data and incorporating dummy variables to distinguish the separate treatment groups giving regressions of the following form:

$$\text{Parasitaemia} = C + \gamma D + \beta_1 T + \beta_{11} (T*D) + \beta_2 (T)^2 + \beta_{21}[(T)^2*D] + \beta_3 (T)^3 + \beta_{31}[(T)^3*D] \text{ etc.}$$

D is the dummy variable valued as follows:

D = O for the control mice and D = 1 for the concurrently infected mice

T = Days after peak parasitaemia

*The estimate of γ is a direct estimate of the differences between the peak parasitaemias of the different treatment groups within an experiment, while β_{11}, β_{21}, β_{31} directly estimate the differences in the profile of the decline and if the parameters are significant then the rates of decline are also significantly different. These can be interpreted by a mathematical analysis of the regression equations determined from the data for each experiment.

CHAPTER FOUR

SECONDARY HYDATIDOSIS IN MICE AND GERBILS

4.1 Aims

In order to monitor the ability of secondary hydatid infections to interact with and modulate the host response, experimental infections must be set up using protoscolices collected from naturally infected hosts. A major constraint on this study of hydatidosis is the danger of human infection, in particular when working with adult parasites and, to a lesser extent, protoscolices of the ovine strain. These dangers may be minimised by studying the larval form in mice and gerbils. However, to establish such secondary hydatidosis in rodents requires the inoculation of large numbers of protoscolices and even then only gives a low rate of cystic development (Williams and Oriol, 1976). In the present study, various dose levels were used to infect gerbils or mice and the resulting cyst burdens determined. In addition, the serological response was monitored using the ELISA.

Small non-fertile cysts obtained from mice infected originally with protoscolices from either the equine or the ovine strain have been shown to have an accelerated rate of development and to attain fertility when transplanted into young naive hosts (De Rycke and Pennoit-De Cooman, 1978; Connor, 1980).

4.2 Experimental Design

4.2.1 Gerbil infections (equine strain of hydatid)

Ten six-week-old female gerbils were infected with 4000 protoscolices (PS) and 10 more were infected at the lower dose level of 2000 PS. There were also five uninfected controls.

4.2.2 Murine infections

Both the equine and ovine strains of hydatid were used in this experiment to infect six-week-old Balb/c female mice. Each mouse received either 1000 or 500 PS intraperitoneally.

Serum collection from both gerbils and mice were taken before infection (week 0) and then every 2-3 weeks throughout the course of the experiment. At necropsy, the animals were killed by cervical dislocation. Blood was collected by cardiac puncture and the number of cysts within the peritoneal cavity was recorded. The diameter of each cyst was measured and whether or not protoscolices were present was also noted.

At the end of the experiment, all the serum samples were assayed for antibody levels by ELISA.

Chequer-board titrations of various protein concentrations of somatic and E/S antigens against pooled positive (infected) and pooled negative (normal) sera were carried out. As no difference had been found when antigens derived from ovine or equine strains of hydatid were used to detect secondary hydatids of equine origin and, initially, supplies of ovine strain material were more plentiful than equine strain, ovine strain hydatid material was used throughout the rest of this series of experiment as the source of the antigens. For the purpose of the serological analysis described in this chapter, only the somatic preparation was used as at the time there were only sufficient quantities of this antigen to carry out the complete series of ELISAs. However, the chequer-board titrations of the excretory-secretory preparation are included here as this antigen was used to analyse the humoral response in Balb/c mice harbouring transplanted cysts, detailed in Section II, Chapter Four.

Soluble antigen concentrations of 5, 10, 15, 20 and 25 μg/ml were titrated against 1:400, 1:800, 1:1000 and 1:1500 dilutions of serum. This series of titrations was carried out for both gerbil and mouse serum. The results were calculated as the difference between positive and negative sera ELISA values at each combination of ELISA values of antigen concentration and serum dilution and then used as the basis for the standard reference result corrected for day to day variation in the subsequent assays.

4.3 Results

Figures 4.1 and 4.2 show the ELISA values of the chequer-board titrations for sera from mice and gerbils respectively.

From this data the optimum antigen concentration and serum dilution were selected for use in all subsequent ELISAs. These were concentrations that would allow the maximum economy of the material in use consistent with good discrimination between positive and negative sera, minimal results with negative sera and a satisfactory ability to quantify the activity of the sera. For the murine sera an antigen concentration of 20 μg/ml for both preparations and a serum dilution of 1:1000 were chosen. For the gerbil sera, all assays were carried out using 1:400 serum as a larger volume of sera was obtainable at each bleeding as compared with the mice and an antigen concentration of 10 μg/ml was considered optimal for both preparations. Five replicates were included for each serum sample assayed.

Figures 4.1 and 4.2

The absorbance values obtained by ELISA from the chequer-board titrations of various concentrations of somatic and excretory/secretory hydatid antigens against four dilutions of both mice and gerbil sera. The results are expressed as the differences between positive and negative sera results for each dilution

X̄ CORRECTED ABSORBANCE VALUES

0·3
0·25
0·2
0·15
0·1
0·05
0

20
15 E/S
10
5 ug/ml

15
20 SOMATIC
10
5 ug/ml

1:400 1:800 1:1000 1:1500

Gerbil serum dilution

X̄ CORRECTED ABSORBANCE VALUES

0·2
0·15
0·1
0·05
0

15
20 E/S
10
5 ug/ml

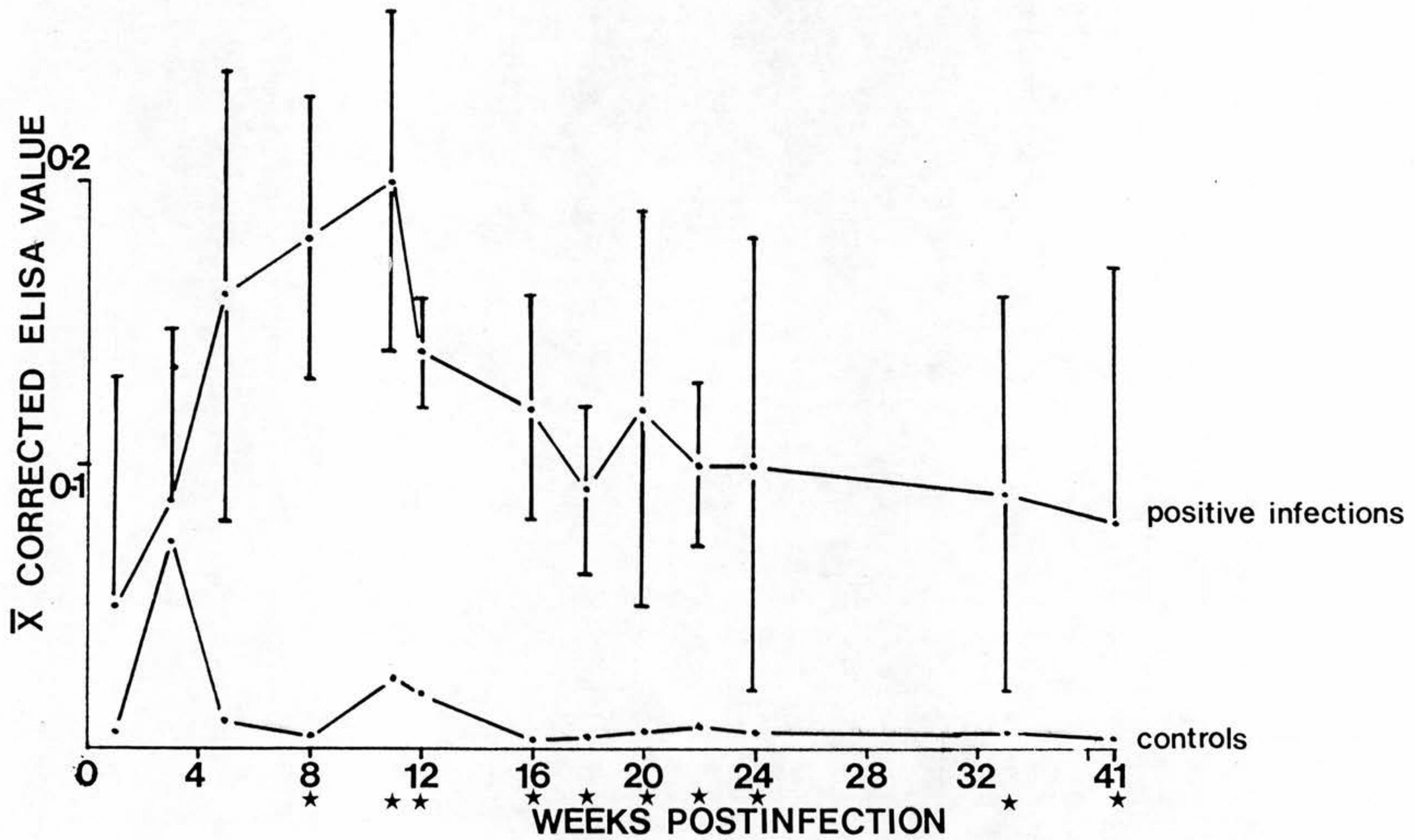
20
15 SOMATIC
5
10 ug/ml

1:400 1:800 1:1000 1:1500

mice serum dilution

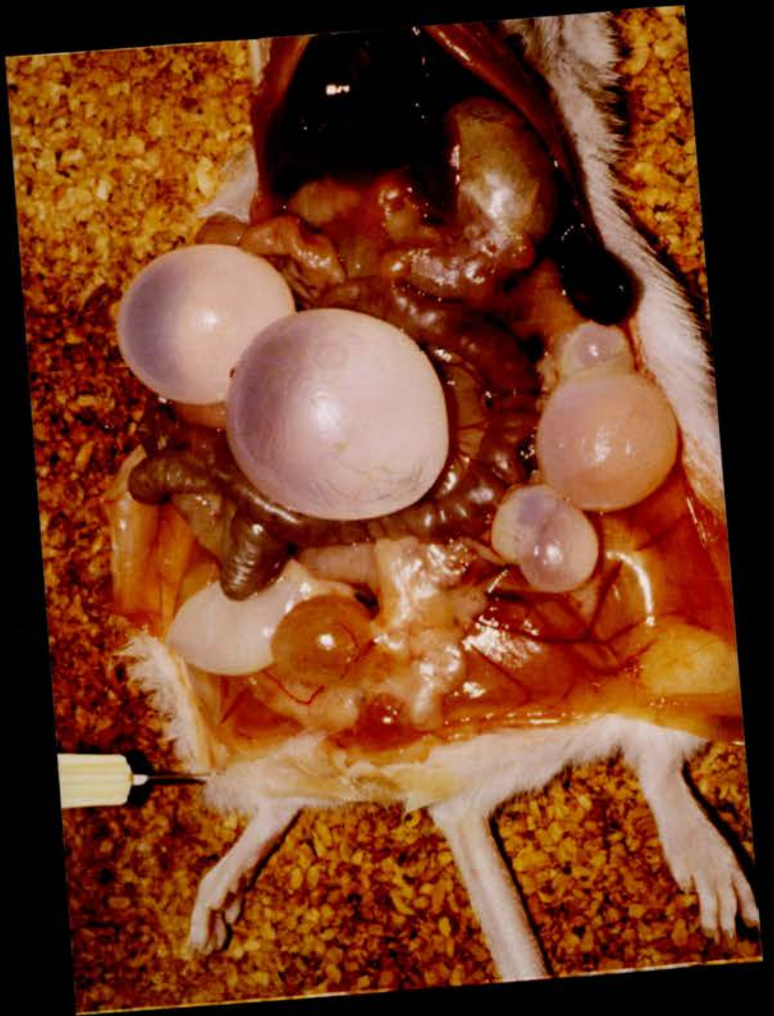
Figure 4.3

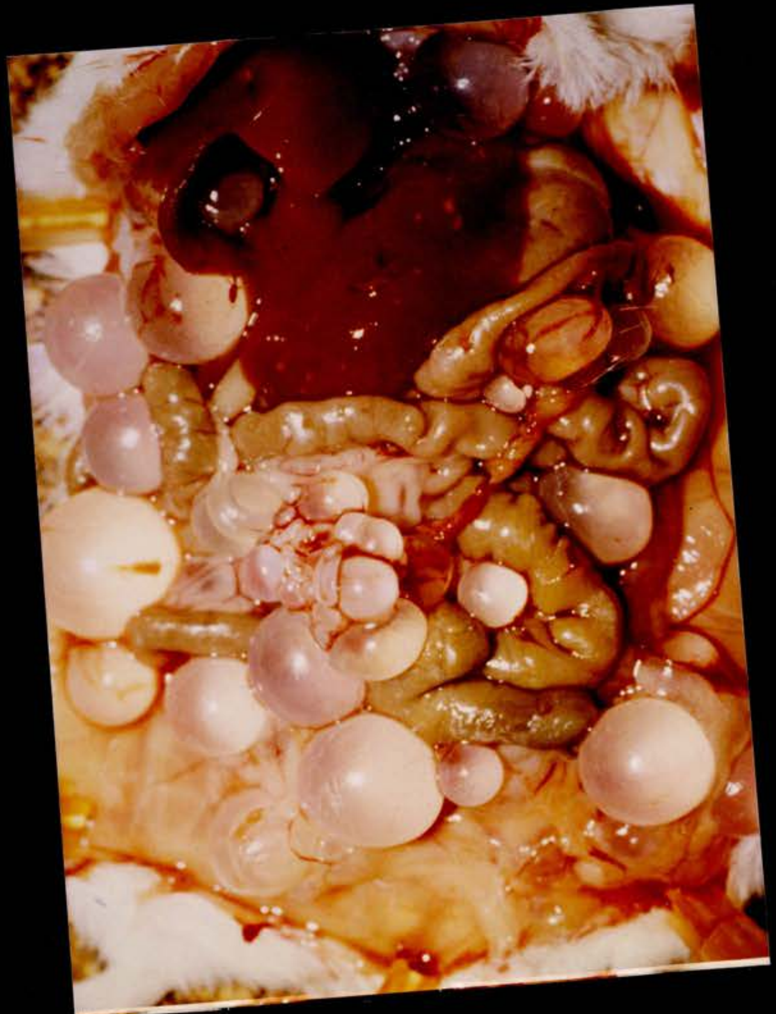
The mean antibody response of gerbils infected with 2000 PS (equine strain) detected by ELISA.



X̄ CORRECTED ELISA VALUE
0·2
0·1
0
4
8
12
16
20
24
28
32
41
WEEKS POSTINFECTION
positive infections
controls

Plates 3 and 4 14 month-old infections of secondary hydatidosis in Balb/c mice.





4.4 Secondary Hydatidosis (equine strain) in Gerbils

The number of cysts in the animals are shown in Table 4.1.

Table 4.1 Cyst burdens from gerbils infected with equine strain protoscolices

Gerbil No.	2000 PS		4000 PS	
	Total No. of cysts	No. of cysts over 10 mm diameter	Total No. of cysts	No. of cysts over 10 mm diameter
1	2	-	1	-
2	10	2	-	-
3	2	-	16	3
4	6	1	-	-
5	13	3	7	-
6	11	3	-	-
7	3	-	-	-
8	6	4	11	2*fertile
9	2	-	4	1
10	1	-	7	3
Total	56	13	46	9

The gerbils found to contain viable, hydatid cysts, which included all the animals in the 2000 PS group are referred to as positive infections and those in which no cysts were found being failed infections.

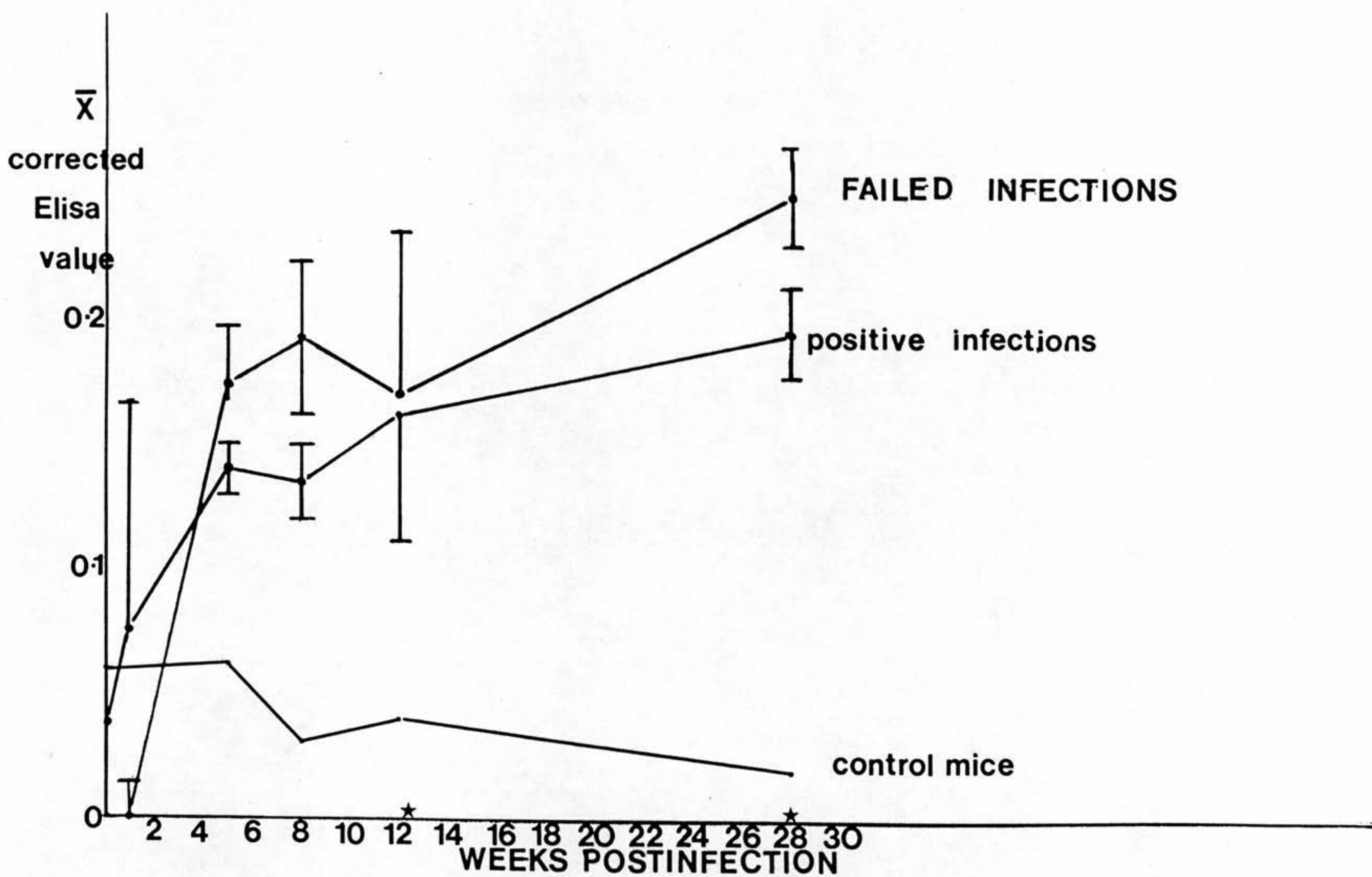
4.4.1 The humoral response

The mean ELISA values using somatic antigen obtained with sera from the gerbils which received 2000 PS are shown in Figure 4.3. The infected gerbils had a significantly greater antibody response than the controls from week 5 onwards. The serological response of the gerbils that had received 4000 PS is shown in Figure 4.4 and showed an initial rapid rise in the antibody levels of the positive infections over a period of five weeks with a plateau after the 12th week.

The sera from the failed infections showed a humoral response that was as strong and as persistent as those with the positive infections. Indeed, the mean values for each bleeding were not

Figure 4.4

The mean antibody response of gerbils infected with 4000 PS detected by ELISA.


X̄
corrected
Elisa
value
0·2
0·1
0
FAILED INFECTIONS
positive infections
control mice
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
WEEKS POSTINFECTION

significantly different by a Student's t-test. Both groups, however, differed significantly from the control from week 8 onwards. The overall take was better in the 2000 PS group but the mean percentages of the initial inoculum developing in each of the gerbils which were 0.31 ± 0.23% for the 2000 PS group and 0.26 ± 0.2% for the 4000 PS group were not significantly different. If the failed infections were included in the mean percentage of initial inoculum developing in the 4000 PS group the result was 0.12 ± 0.14% which was still not significantly different from the 2000 PS group. The only difference between the two dose levels was that at the lower dosage more animals became infected but in all the animals that parasites established themselves a similar burden was found.

Only two of the cysts examined at necropsy contained viable protoscolices and these were both from one animal in the 4000 PS group which contained 11 cysts in all. All cysts were immediately surgically transplanted into the peritoneal cavity of naive six-week-old gerbils.

4.5 Secondary Hydatidosis (equine strain) in Balb/c Mice

The number of cysts developing in each mouse in the two treatment groups were similar but when expressed as a proportion of the initial inoculum developing, the mean percentages were 1.5 ± 1.25% for the 500 PS group and 0.76 ± 0.47% for 1000 PS group, indicating that a lower but not significant proportion of the initial dose developed in the 1000 PS group. If the failed infections were included in these calculations the difference was more obvious and with the mean percentage developing in the 500 PS group being 1.08 ± 1.2%

and 0.36 ± 0.49% in the 1000 PS group. Again, it seemed that the only difference between the two dose levels was that a higher proportion of the animals inoculated had positive infections in the lower dose scheme but the mean proportions of the initial inoculum into developing cysts were not significantly different. The cysts were, in general, smaller than those removed from the gerbils and no fertile cysts were found. These cysts were also used for transplantation, in this case into six-week-old Balb/c mice.

Table 4.2 Cystic burdens from Balb/c mice infected with equine strain protoscolices

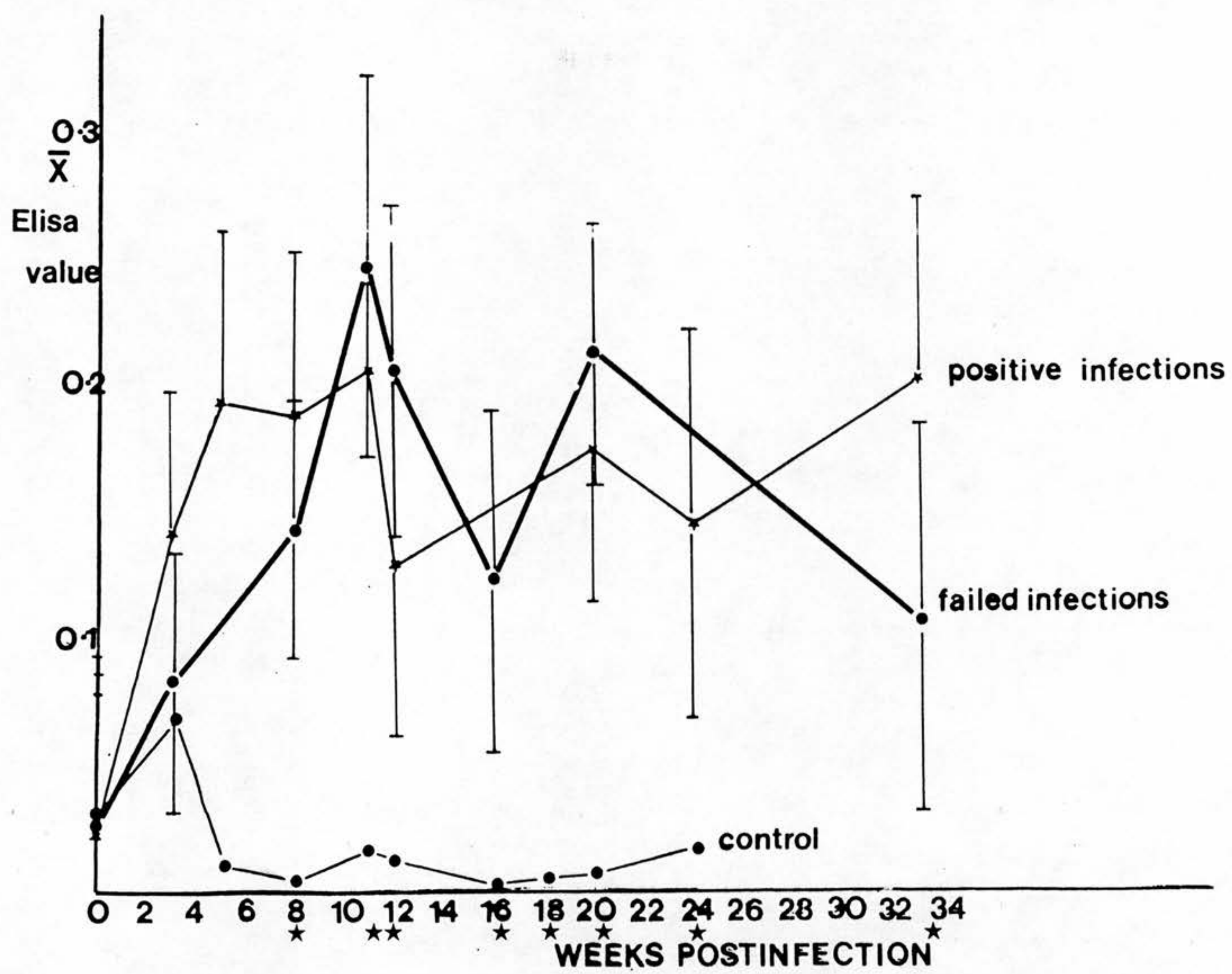
Mouse No.	500 PS		1000 PS	
	Total No. of cysts	No. of cysts over 10 mm diameter	Total No. of cysts	No. of cysts over 10 mm diameter
1	3	-	13	1
2	-	-	8	-
3	2	-	-	-
4	10	-	3	-
5	9	-	11	-
6	1	-	-	-
7	15	-	10	-
8	14	1	-	-
9	-	-	1	-
10	-	-	-	-
Total	54	1	46	1

4.5.1 The humoral response

Figure 4.5 shows the mean ELISA values using the somatic antigen of sera from mice inoculated intraperitoneally with 1000 PS and 500 PS. All the experimental groups - the positive 500 PS and

Figure 4.5

The mean antibody response of Balb/c mice infected with equine strain hydatid detected by ELISA.



0·3
$\bar{X}$
Elisa
value
0·2
0·1
positive infections
failed infections
control
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
WEEKS POSTINFECTION

1000 PS which have been grouped together in the graph, and the pooled failed infections showed a rapid rise in antibody level over the first four weeks of infection. As with the gerbil infections there was no significant difference between the antibody levels in positive infections and those in the animals which contained no cysts on necropsy. All infected mice gave significantly greater ELISA results than those from the control group from week 8 onwards.

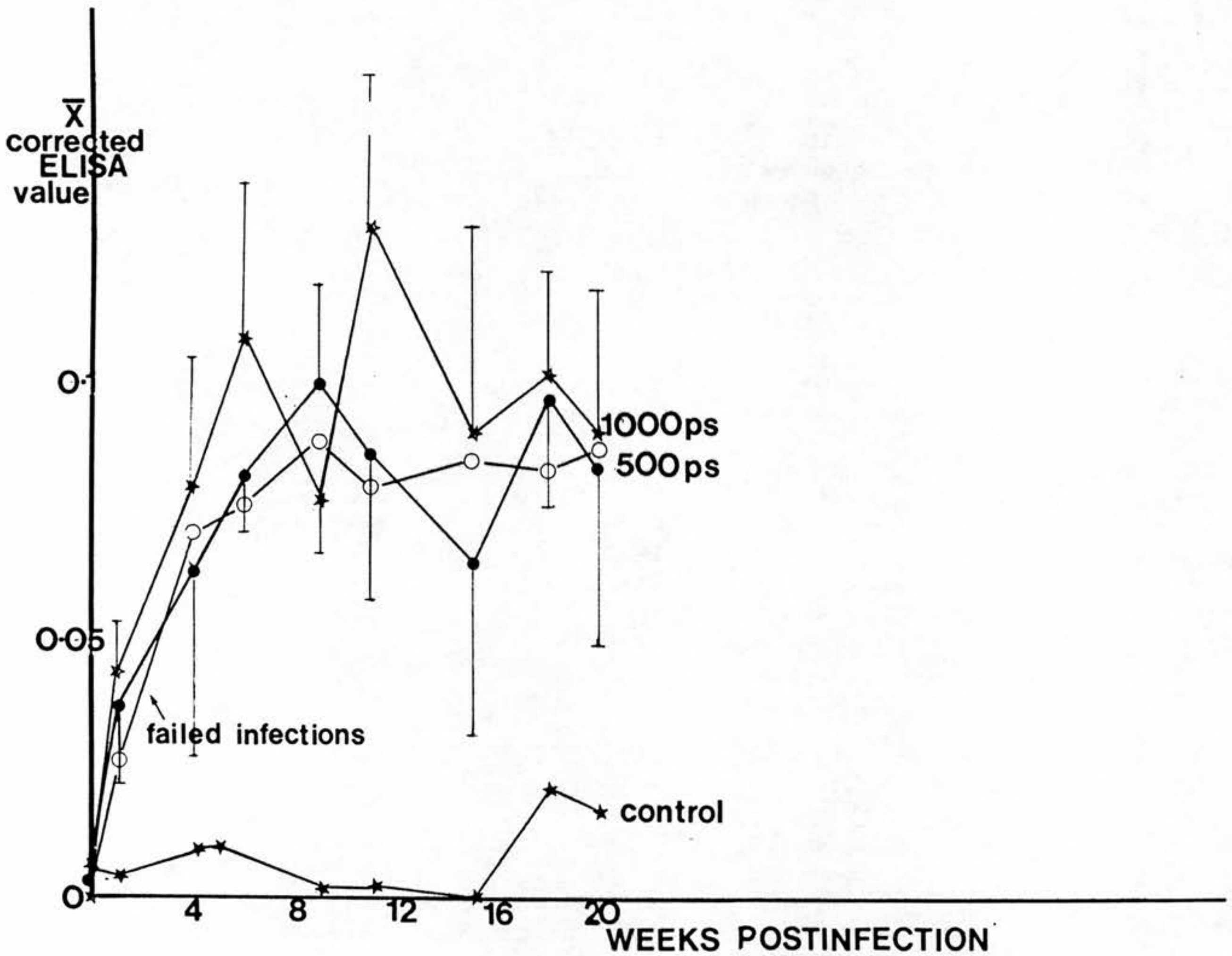
4.6 Secondary Hydatidosis (ovine strain) in Balb/c Mice

Table 4.3 shows that the lower dose level gave higher burdens in those mice in which cysts were able to develop but this was only in half of the mice originally inoculated. The number of mice developing infections in both groups was similar as was the proportion of initial inoculum developing in mice that did not reject the infection. The mean percentage in the 500 PS group was 1.56 ± 1.07% and for the 1000 PS group it was 0.47 ± 0.36%. If the failed infections were included the mean percentage of the 500 PS group was 0.78 ± 1.09% and 0.1 ± 0.16% for the 1000 PS group indicating no significant difference between the two dosages.

Only one of the cysts removed at necropsy and used for transplantation was found to be fertile.

Figure 4.6

The mean antibody response of Balb/c mice infected with ovine strain hydatid detected by ELISA.



X̄ corrected ELISA value
0·1
0·05
0
4
8
12
16
20
WEEKS POSTINFECTION
1000ps
500ps
failed infections
control

Table 4.3 Cyst burdens from Balb/c mice infected with ovine strain protoscolices

Mouse No.	500 PS		1000 PS	
	Total No. of cysts	No. of cysts over 10 mm diameter	Total No. of cysts	No. of cysts over 10 mm diameter
1	13	2	2	-
2	-	-	4	-
3	-	-	-	-
4	4	1*fertile	3	-
5	-	-	-	-
6	-	-	-	-
7	13	1	-	-
8	-	-	-	-
9	8	-	10	-
10	1	-	-	-
Total	39	4	19	0

4.6.1 The humoral response

Figure 4.6 shows the mean ELISA values of mice sera after successful intraperitoneal inoculation with 500 or 1000 PS and for the failed infections group. There was again an initial rise in antibody levels, over a period of six weeks followed by considerable fluctuation throughout the remaining course of the infections. The mice in which hydatids failed to develop had mean ELISA values indistinguishable from the successful infections. The two dose levels and the failed infections had mean ELISA values that were not significantly different from each other and were only significantly different from the control group on weeks 6, 11, 18 and 20. This appeared to be due to the high level of variation within the treatment groups.

4.7 Discussion

The results of the necropsies indicated that a larger proportion of the gerbils were susceptible to infection and allowed cysts to develop as compared to the proportion of mice. Williams and Oriol (1976) found the Mongolian gerbil to be the most susceptible host when laboratory infections were initiated in mice, hamsters and gerbils. They also found that the number of cysts developing in each gerbil was highly variable and comparable results were observed in this study. This uncontrollable variation represents a serious limitation to the use of secondary hydatidosis in further studies except by the use of surgical transplantation which enables infections with known numbers of cysts to be established (Varela-Diaz *et al.*, 1973).

The mice infected with either strain of hydatid showed lower overall susceptibility, but the mean proportion of protoscolices developing into cysts in the animals that did become infected was not significantly different from that for the gerbils. It seems that the mean number of cysts recovered in the positive infections was similar in mice and gerbils, regardless of infection level over the range of 500 to 4000 protoscolices per animal. This suggests that in animals that are receptive to the hydatid infection only a certain proportion of the initial dose will develop into cysts. Thus, an element of control appears to be involved in limiting parasite development. The nature of this limitation, whether it be host mediated or parasite mediated, to possibly avoid the competition for resources during overcrowding is unclear. Kassis and Tanner (1976) have proposed that although most protoscolices with hooks

(both invaginated and evaginated) would be lysed on exposure to complement in the hosts extracellular fluid of the initial inoculum developing, those larvae that have vesiculated and shed their hooks will survive. A few larvae would also be protected due to the presence of anticomplementary calcareous corpuscles released from the adjacent lysed protoscolices. Thus, it can be envisaged that this evasive mechanism will ensure that a small number of an inoculum will survive this initial stage. These same authors concluded that complement was required for the control of secondary hydatidosis infections (Kassis and Tanner, 1977) as significantly enhanced infections were obtained after treatment with cobra venom factor. Therefore, this apparent regulation of the number of protoscolices developing could be due to the combination of host interaction via complement and the release of anticomplementary substances by the parasite. This process may well occur in the failed infections but that parasite death, due to other immune mechanisms, was effected at a later stage.

Using this information, it was decided, in this study, to continue the investigation of secondary hydatidosis in Balb/c mice only as no obvious advantages were afforded by using the gerbils and indeed there were several disadvantages. Gerbils proved more difficult to handle for regular bleeding, they lacked the genetic advantages of an inbred mouse strain and were more expensive.

The serological response in both the mice and gerbils contained common features. All infections were characterised by an initial rapid rise in antibody levels over a period of 4-6 weeks after infection followed by a plateau which was maintained for several

months. The overall antibody response was low as the ELISA O.D. values rarely rose above 0.2. This, together with the similarity of the response to failed infections with positive infections and the fact that in some of the experiments rather high values were obtained with the negative control sera made it difficult to detect developing cysts in the host.

The high background values for the negative sera might have been reduced by purification of the antigen by passage through an immunoadsorbent column carrying goat anti-mouse IgG. This would remove traces of host immunoglobulins remaining in the antigen and cross-reacting with the goat anti-mouse IgG - HRPO conjugate in the ELISA. Reduced background levels might also be obtained by the addition of a blocking step of foetal calf serum or bovine serum albumin before the application of conjugate.

In later ELISAs of the mice infected with transplanted cysts described in Section II, Chapter Four, the antigen was purified by passage through an immunoaffinity column and this resulted in reduced background values. In addition to this, the antigen used in these subsequent ELISAs was an excretory-secretory preparation because it was felt that this may prove more successful in detecting developing cysts. It is possible that the failed infections gave as strong an antibody response to the somatic antigen as those with positive infections as both would contain a large amount of dead parasite material prior to elimination.

The low level of the humoral response to what, in some of the animals, amounts to a large biomass of foreign parasites tends to suggest that immunosuppression may play a role in hydatid disease

as suggested by Chemtai (1980) and Reuben and Tanner (1983). This possibility will be investigated in the following sections.

4.8 Observations on the Transplantation of Secondary Hydatid Cysts in Balb/c Mice

The transplantation of developing sterile hydatid cysts from "breeder" mice initially infected by intraperitoneal inoculation of protoscolices was carried out routinely to provide "stock" infected mice for subsequent experiments. When these animals were necropsied at the conclusion of the experiments described in Section II, Chapter Four, 12 weeks post-transplantation, all the cysts had doubled their size but few were fertile. In small secondary hydatid cysts, the cyst wall was sufficiently transparent to observe protoscolices and brood capsules in the intact cyst by the use of a lower power binocular microscope. Cysts that were not fertile and were still under 20 mm in diameter were used to infect additional six-week-old Balb/c mice.

In addition, five mice, each infected with a single cyst under 10 mm diameter, were necropsied 17 months post-transplantation. Each animal had a visibly distended abdomen and was found to contain a large fertile cyst between 50-55 mm in diameter. Examination of the aspirated cyst fluid under a dissecting microscope revealed many brood capsules and active protoscolices.

Thus, the procedure of transplantation in these studies confirms the findings of Schwabe _et al_. (1970) that the rate of development and attainment of fertility could be enhanced in the second and subsequent hosts.

The serological response of such infections is reported as part of this study in Section II, Chapter Four.

SECTION II

CONCURRENT INFECTIONS OF TAENIID METACESTODES WITH *BABESIA MICROTI*

CHAPTER ONE

INTRODUCTION

1.1 Aims

The aim of the work described in this section was to investigate the extent to which a metacestode burden is capable of modulating the normal host response to a superimposed infection of *B. microti*. The use of a concurrent infection to assess the degree of immunosuppression by a particular parasite has been used in several systems (Phillips and Wakelin, 1976; Livin, Last, Targett and Doenhoff, 1982) but there are no investigations of the effect of metacestodes on intercurrent infections to date.

B. microti has the advantage that it is easy to monitor the extent of the parasitaemia by simple analysis of blood smears, and that the antibody response can be quantified by IFAT. The piroplasm parasitaemia and the antibody response to it of the concurrently infected mice (TB group) will be compared to that of the *Babesia*-only (B group) infected controls.

1.2 Literature Review - Immunity to Murine Babesiosis

1.2.1 Introduction to the parasite and its life cycle

Babesia microti belongs to the family Babesiidae, order Piroplasmida, subclass Piroplasmia and class Sporozoea.

Babesia is a genus of parasitic protozoa which invade and multiply in the erythrocytes of various domestic and wild animals. *Babesia microti* is a species capable of infecting various wild and laboratory rodents and as such provides a useful model for the economically important *Babesia* species known to infect livestock. Individual strains of this piroplasm have been documented but they show no morphological or infection pattern differences (Shortt and Blackie, 1965).

The life cycle of a Babesia sp. has been particularly well documented for the economically important species B. bigemina but the same pattern can be found in all Babesia species. The vertebrate host, that is rodents in the case of B. microti, becomes infected from a feeding tick. The infective stages are released in tick saliva, and as far as is known, immediately enter the circulating red blood cells where they multiply eventually destroying the red cell. Within the red cell stage there are asexual multiplication forms and also, it is thought, gametocytes that are infective to the tick vector (Rudzinska, Spielman, Rick, Lewengrub and Piesman, 1979).

The natural vector of B. microti in Britain is believed to be Ixodes trianguliceps, whilst in North America it is Ixodes dammini, a tick commonly found on deer. The need for an arthropod host in laboratory infections of B. microti can be avoided by inoculation of parasitized erythrocytes using intravenous or intraperitoneal routes.

B. microti provided a useful model for babesiosis in the laboratory as the infection developed predictably and was rarely fatal (Cox and Young, 1969), but unlike the other rodent piroplasm, Babesia rodhaini which resulted in the death of the host within four days post infection.

The most susceptible rodent host for B. microti is the golden Syrian hamster, Mesocricetus auratus (Gleason, Healy, Western, Benson and Schultz, 1970) but other small laboratory rodents have also been used with success including outbred mice, jirds and rats (Ruebush, Cassaday, Marsh, Lisikir, Voorhees, Mahoney and Healy, 1977). Since many immunological studies require the use of inbred mice, investigations were carried out to compare the susceptibility of

various mouse strains to B. microti, but the adaptation of this parasite to inbred mice was prolonged and difficult (Ruebush and Hanson, 1980). However, intact C3H, B10A and Balb/c mice developed moderately high parasitaemias (Allison, 1979) and the mice recovered from the infection.

The course of B. microti infection depended largely on the size of the infective dose and the route of inoculation (Gray and Phillips, 1983). The intravenous inoculation of 10^6 parasitized red blood cells resulted in the appearance of piroplasms in the bloodstream after 3-6 days. The percentage parasitaemia increased rapidly to a maximum of 50-80% between days 11 and 17, thereafter declining to become undetectable 35-45 days after infection. Higher doses resulted in shorter prepatent periods and increased the height of the maximum percentage parasitaemias but the overall duration of the patent B. microti parasitaemia was not affected. It was known that after this acute phase there followed a long-lasting chronic and subpatent infection. This could be demonstrated for at least 100 days post infection because the subinoculation of tissues from animals with such chronic infections into uninfected recipients resulted in successful infections (Hussein, 1977). The onset of the effective immunity to challenge doses of B. microti in this state of premunition is being extensively studied to shed some light on the mechanisms of control and elimination of babesias and, equally relevant, the mechanisms whereby the parasite survives in the semi-immune host.

1.2.2 Immunity in the murine host

The immune system of the host is mobilised to generate cells which can control the parasitaemia by destroying parasites and/or parasitized red cells and possibly by interrupting the growth and multiplication of the parasite. The possible roles of the immuno-competent cells responsible for the production of antibody and lymphokines and the participation of phagocytic cells of the reticulo-endothelial system have been described in reviews of the acquired immune response to *Babesia* spp. (Zwart and Brocklesby, 1974; Aragon, 1976) and more recently by Phillips (1981). It is thought that circulating antibody is responsible for preventing penetration of extracellular babesia forms, and cytophilic antibodies or opsonins enhance phagocytosis of parasites or parasitized erythrocytes.

The humoral response in Swiss TO mice as determined using an indirect fluorescent antibody technique (Voller and O'Neill, 1971) showed a rapid increase in titre from day 14 after infection. IgM fluorescent antibody titres reached a peak on day 22 and IgG on day 36 (Purvis, 1977). Both immunoglobulin subclasses remained elevated long after patent infections were eliminated. Mice which have recovered from *B. microti* infections are resistant to challenge for the remainder of their lifespan. Despite this, many workers have tried and failed to transfer protection with serum (Mitchell *et al.*, 1978; Allison, 1979). This suggests that antibody plays, at most, a minor role in protective immunity to *Babesia*. The situation may be somewhat similar to that of rodent plasmodia in which high levels of protective activity in the serum have been found to be very short-lasting (Phillips *et al.*, 1972; McLean and Phillips, 1979).

Evidence of a role for antibodies in immunity to B. microti has come from Jayawardena and Kemp (1979). Using CBA/N mice, known to be defective in that they have a reduced number of splenic B-cells, they were able to show that B. microti infection resulted in a greatly extended parasitaemia. This infection had numerous relapses. In parallel with the reduced ability of the CBA/N mice to develop an effective immune response there was a reduced IgM and a delayed IgG anti-B. microti antibody response. These workers also suggested that another mechanism controlling the intraerythrocytic infection may be an auto-immune response directed at the altered determinants on the erythrocyte membrane.

By analogy with other intracellular parasites, it would be expected that cell-mediated immune mechanisms play a part in resistance to babesias (Gardener et al., 1974; Carson et al., 1977); but the evidence is by no means clear and this remains a contentious area. Nude (athymic) mice have exacerbated B. microti infections as do intact hamsters when treated with anti-thymocyte or anti-lymphocyte serum (Wolf, 1974; Clark and Allison, 1974). These observations suggested that acquired immunity to Babesia was thymus-dependent and involved T cells. Using thymectomized and lethally irradiated Balb/c mice, Ruebush and Hanson (1980) were able to show that T cells were essential for resistance and recovery from a primary infection. Experiments involving the reconstitution of these mice with syngeneic bone marrow cells, thymocytes, spleen cells depleted of B-cells or combinations of these cells revealed that the B-lymphocyte was less protective than T-cells when either

was given alone; but severely T-cell depleted mice still survived, which may indicate some element of T-independent resistance. The nature of this T-independent element was unclear but natural killer cells have been suggested (Eugui and Allison, 1980). Lately though, this theory has been shelved in favour of a more prominent role for the macrophage and its production of soluble factors mediating intraerythrocytic parasite death (Carson and Phillips, 1981). It is known that treatments, such as the inoculation of BCG, which enhance macrophage activity also lead to increased resistance to B. microti infection (Clark et al., 1977). The routes of administration of BCG which are known to cause the greatest production of soluble mediators also conferred the greatest protection against B. microti (Ruebush and Hanson, 1980). As these authors found that B- and T-cell depleted mice can be assumed to have equivalent amounts of macrophage activity and yet only B-cell depleted mice were able to resist high parasitaemias, it seems that if the macrophage is the immuno-effector cell, then it requires the contribution of T-cells for effective parasite destruction.

Future investigations will need to explore how these non-specific effector mechanisms interact with the specific acquired mechanisms, and such work will undoubtedly make use of the in vitro culture systems currently being technically refined to dissect out the nature of the immune response.

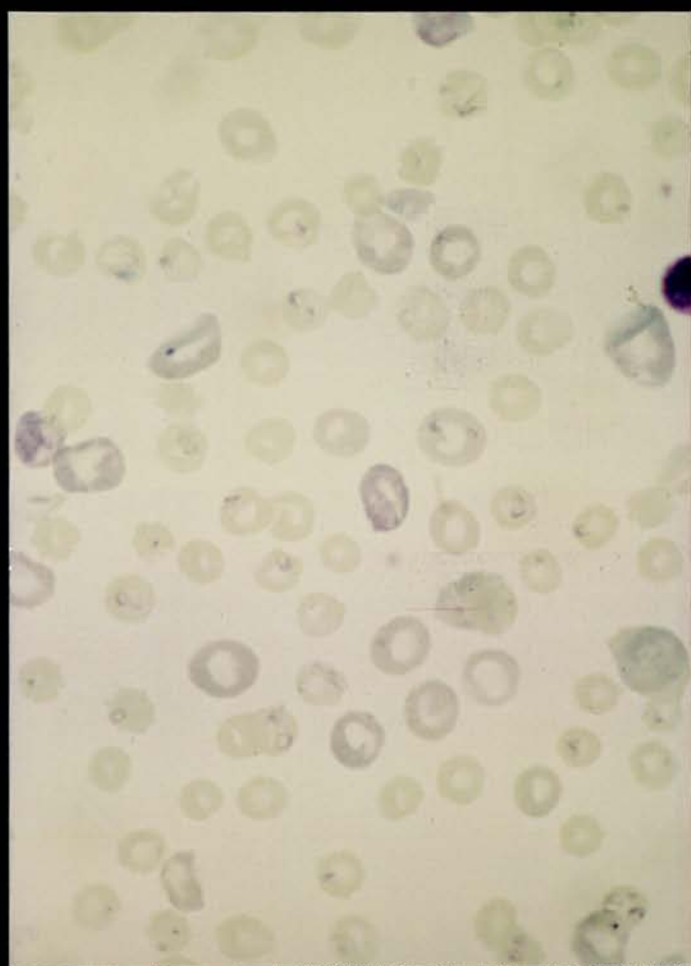
1.2.3 Immunosuppression during B. microti infections

A period of immunodepression to superimposed antigens is characteristic of a number of protozoal infections, including malaria (Greenwood, Playfair and Torrigiani, 1971) and trypanosomiasis (Murray, Jennings, Murray and Urquhart, 1974) and has now been demonstrated for *B. microti* (Purvis, 1977; Gray and Phillips, 1983). The maximum suppression of the antibody response to the heterologous antigen, sheep red blood cells,was associated with peak parasitaemia and the infection caused a small reduction in the expression of the secondary response (Gray and Phillips, 1983). This immunosuppression has been shown to alter the normal course of concurrent parasite infections (Phillips and Wakelin, 1976) so that if the acute phase of *B. microti* parasitaemia occurred immediately before the normal time for immune expulsion of the nematode, *Trichuris muris*, this expulsion was delayed.

The mechanisms underlying such immunosuppression are unclear but it was known to be a transient phenomenon (Gray and Phillips, 1983) associated only with the acute phase of the infection. The antibody response to heterologous antigens recovered fully after this period.

Babesia microti was chosen as a useful secondary infection with which to study the immunomodulatory effects of the metacestodes *Taenia crassiceps*, *T. taeniaeformis* and secondary echinococcosis, by monitoring their ability to depress the immune response to intercurrent *Babesia* infections. The extent to which a secondary infection was stimulated, particularly if this infection itself was capable of depressing the immune response at a specific phase, would indicate

Plate 5 Giemsa-stained blood smear taken from a Balb/c mouse infected with *Babesia microti*.



the degree of disruption caused by the metacestodes.

The antibody response to these three metacestodes can also be monitored to ascertain whether the presence of Babesia was influencing the normal host response to the metacestode burden.

1.3 Experimental Design

This study involved inoculating mice that had been previously infected with either T. crassiceps, T. taeniaeformis or secondary hydatidosis with red blood cells infected with B. microti and challenging them with the same piroplasm stabilate four weeks later. This routine infection schedule is shown in Table 1.1.

Table 1.1 Infection schedule for six-week-old CF1 mice

Week	Metacestode infected mice	Controls
0	Metacestode infection	-
5	10^6 B. microti infected red blood cells	
9	10^6 B. microti-infected red blood cells	

The mice were inoculated with the secondary infection at five weeks, as by this stage all three species of metacestodes had become established as a chronic burden. It is this model of chronic larval cestode infections and their means of evading the host response that this work aims to investigate. It should be possible to determine whether such hosts are immunosuppressed by monitoring both their host susceptibility and humoral response to the secondary infection. The host susceptibility was assessed by monitoring the percentage parasitaemia from Giemsa-stained blood smears. The humoral response to

B. microti was measured by an indirect fluorescent antibody test (IFAT) (see Methods). The antibody response to the larval cestode infections was measured using an ELISA test. Good and Miller (1976) found that when mice were infected with T. crassiceps larvae the secondary antibody response to SRBC was depressed to a greater extent than the primary response. Accordingly, a challenge dose of B. microti was administered in order to monitor the secondary response. The interval between the first inoculation of B. microti and the challenge inoculation was four weeks. This was determined from the time interval taken for the first Babesia infection to return to subpatent level and was also the time when a high titre of anti-Babesia antibody was found in the normal course of the murine disease (Carson and Phillips, 1981).

The optimum dose of B. microti was determined by inoculating groups of five control mice with 10^5, 10^6 and 10^7 infected red blood cells. The dose level of 10^6 B. microti infected red blood cells was chosen as in these mice piroplasms had appeared in the blood of all recipients by day 2 after infection, and the peak parasitaemias in these normal mice did not cause death. It was deemed essential for adequate serological analysis of the concurrently infected mice that the piroplasm infection did not kill the mice, thus allowing sufficient serum samples to be obtained throughout the primary and challenge phases of infection.

This routine experimental design was followed for all the experiments in Chapters Two, Three and Four. Any modifications to the design are detailed in the relevant chapters along with the rationale for such.

CHAPTER TWO

CONCURRENT INFECTIONS OF
TAENIA CRASSICEPS AND *BABESIA MICROTI*

ADMINISTERED INTRAPERITONEALLY

2.1 Aims

This series of experiments set out to determine whether mice infected with T. crassiceps metacestodes were capable of modulating the host response to a superimposed infection of B. microti. Good and Miller (1976) had previously shown that mice infected with this parasite had a depressed humoral response to an heterologous antigen - sheep red blood cells. This study was devised to see if this immunosuppression was capable of altering the normal course of B. microti infections in mice.

2.2 Experimental Design

Ten six-week-old Balb/c mice were inoculated intraperitoneally with 20 T. crassiceps metacestodes and five weeks later five of these mice (the TB group) and five uninfected Balb/c mice (B group) of the same age were inoculated intraperitoneally with 10^6 Babesia-infected red blood cells. All the mice were rechallenged on week 9 with 10^6 Babesia-infected red blood cells.

2.3 Results

The development of the parasitaemias in both treatment groups is shown in Figure 2.1. The data for each day were analysed separately to determine whether the mean parasitaemias for the two groups differed significantly. The ANOVA table comparing the peak results from both groups on the days of their respective peaks is shown in Table 2.1 and the remaining ANOVA tables are contained in Appendix 3.

Figure 2.1

The mean percentage parasitaemias of Balb/c mice infected with *Taenia crassiceps* and *Babesia microti* by the intraperitoneal route.

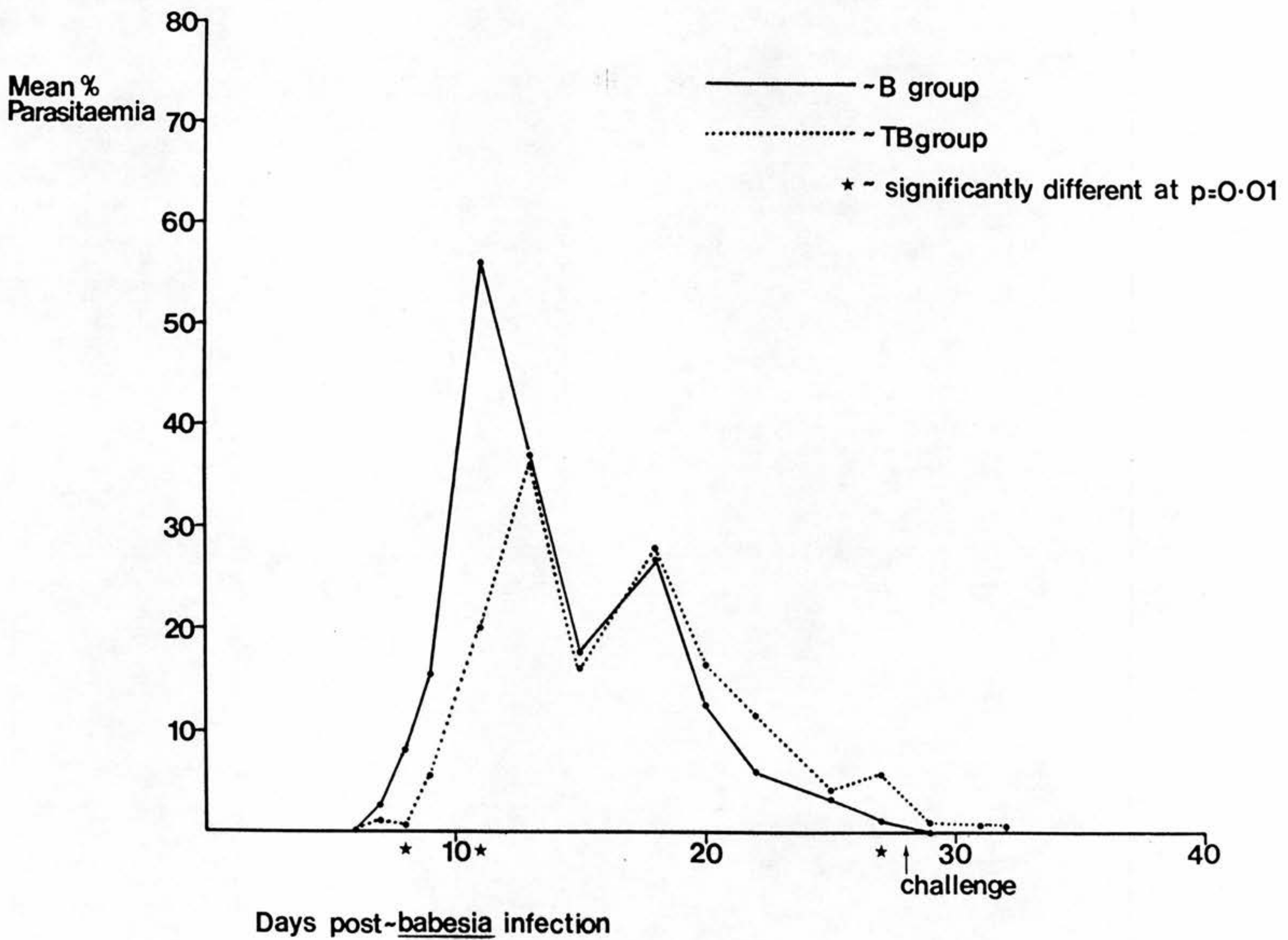

Mean %
Parasitaemia
80
70
60
50
40
30
20
10
~B group
~TBgroup
* ~ significantly different at p=0·01
* 10*
20
* 30
challenge
40
Days post~babesia infection

Table 2.1 ANOVA table comparing the peak results of TB and B groups

Source of variation	SS	df	MS	F
(1) Treatment	3,882	1	3882	28
(2) Field	196	2	98	0.7
(3) Interaction	232	2	116	0.8
ERROR	5,446	39	139.6	

This table shows that the parasitaemias of the two treatment groups are highly significant and that the effect of reading different fields and the interaction of the reading method with treatment is not. The results show that the peak parasitaemia was reached on day 11 for the B group and on day 13 for the TB group. The B group peak level was 56% and that for the TB group peak level was 37%; this difference was highly significant. The levels declined thereafter with a secondary peak on day 18. However, the two groups only had significantly different parasitaemias on days 8, 11 and 27.

Challenge on day 28 did not bring about any recrudescence of the parasitaemia.

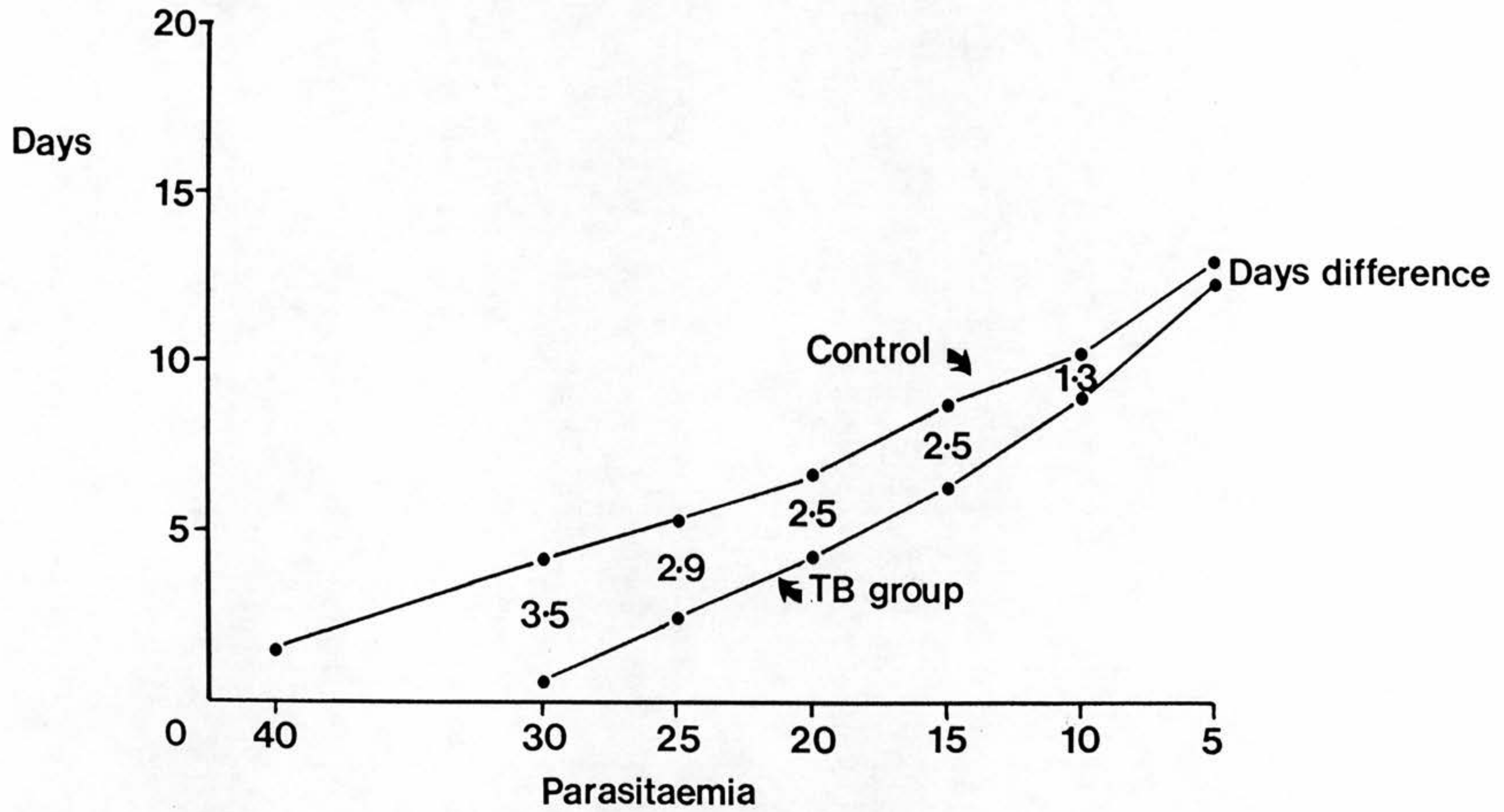
2.4 The Rate of Decline of the Parasitaemia from the First Peak

Regression analysis in the polynominal form was carried out on the data from both groups separately first, in order to obtain values for the parameters giving the closest fit to the individual data.

Terms with significant parameters were found up to the second power for both groups. The equation for the TB group took the form:

Figure 2.2

The rate of decline in parasitaemia expressed as the number of days above a given percentage parasitaemia each of the three groups remain.


Days
20
15
10
5
0
40
30
25
20
15
10
5
Parasitaemia
Days difference
Control
TB group
1·3
2·5
2·5
2·9
3·5

$$\text{PST} = 32.0 - 3.1T + 0.08T^2 \qquad R^2 = 0.74$$
$$\text{(t-value)} \quad (13.87) \quad (6.78) \quad (3.91)$$

and the equation for the B group was:

$$\text{PST} = 49.4 - 5.32T + 0.15T^2 \qquad R^2 = 0.84$$
$$\text{(t-value)} \quad (9.72) \quad (4.98) \quad (2.96)$$

One of the crucial assumptions for these regression models is the independence of the stochastic error. This is tested for by the use of Durbin-Watson statistics. The greater the deviation of the Durbin-Watson statistic from its expected value of two where there is no correlation, the higher the probability of serial correlation in the disturbances. The Durbin-Watson statistic for the TB group equation of 1.01 suggests serial correlation but that for the control group of 1.66 does not. Thus, the equations were chosen with this in mind and the TB equation has been corrected for serial correlation.

The estimated peak parasitaemia is 32% for the TB group and 49.4% for the B group, a difference of 17.5%. Thus, there was a significant difference between the peaks and in addition a significant difference in the rate of decline of the parasitaemia of these two groups of mice.

The equations of best fit were used to graphically describe the rate of decline as shown in Figure 2.2, which indicates the number of days above that each group of mice remains at given levels of parasitaemia. The rate of decline in the TB group was more rapid than in the B group until the lower levels of parasitaemia of 5-10%, when parasitaemia in the B group declined more rapidly than in the TB group.

Regression analysis on the combined data from the two groups showed that there was a significant difference in the rate of decline of the two groups. This regression is:

$$\begin{array}{lll} PST & = 49.6 & - 17.7T \\ (\text{t-value}) & (18.9) & (4.04) \end{array}$$

and has an R^2 value of 0.82. The values for peak parasitaemias are 49.62% for the B group and 32.3% for the TB group, a difference of 17.36%. These values were significant. When the slope differences were examined, significant values were obtained indicating that there are significant differences in the rate of decline of the two groups.

Once the average level of parasitaemia drops below 3%, the probability that some of the fields will include no parasitized cells increases rapidly from 0.09 at 3% to 0.2 at 2% and 0.45 at 1%. The chances of three separate fields having no parasitized cells increases correspondingly. The decline in parasitaemia at such low levels may therefore be measured by classifying the mice according to the number of positive fields they give on each day.

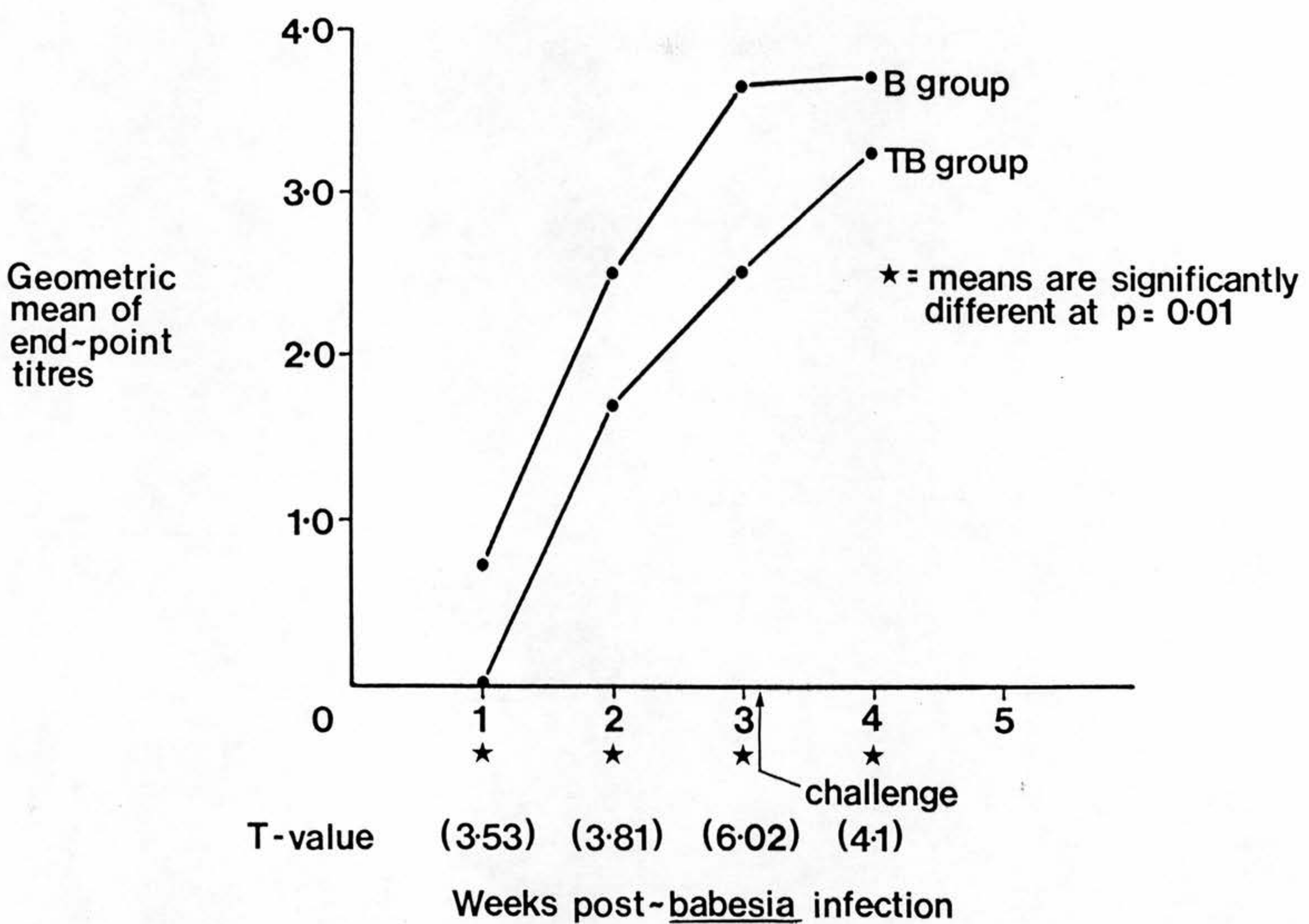
Table 2.2 The pattern of parasitaemia at low levels

Day	TB Group		B Group	
	Median number of positive fields	Mean parasitaemia	Median number of positive fields	Mean parasitaemia
27	3	5.4	1	1.4
29	2	0.6	1	1.0
31	2	1.1	2	0.77
32	2	0.7	0	0.12
34	2	0.8	0	0

On day 27 all three fields contained parasitized cells in only 2/5 of the B group mice whereas in the TB group, all three fields were positive

Figure 2.3

The mean antibody response to *B. microti* by mice infected with *Taenia crassiceps* and *Babesia* (TB) and the *Babesia* only controls (B) detected by IFAT.


4·0
3·0
2·0
1·0
0
Geometric
mean of
end-point
titres
B group
TB group
★ = means are significantly
different at p = 0·01
1
2
3
4
5
★
★
★
★
challenge
T-value
(3·53)
(3·81)
(6·02)
(4·1)
Weeks post-babesia infection

in all the mice. The mice in the TB group continued to give more infected fields except on day 31 until on day 34 the B group mice gave no positive fields, while 4/5 of those in the TB group still did so. This confirms the pattern of decline shown by the regression analysis that at low levels of parasitaemia the rate of recovery of the TB group was slower than that of the B group.

2.5 The Humoral Response to B. microti

This was assessed by the indirect fluorescent antibody test (IFAT). The end-point titres were expressed as log values. A one-tailed t-test was carried out as the hypothesis put forward was that the end-point titres of the treatment group would only be expected to be less than or equal to those of the mice infected with *Babesia* only, previous work by Good and Miller (1976) having indicated that metacestode burdens result in immunosuppression in the host. The results are shown in Figure 2.3.

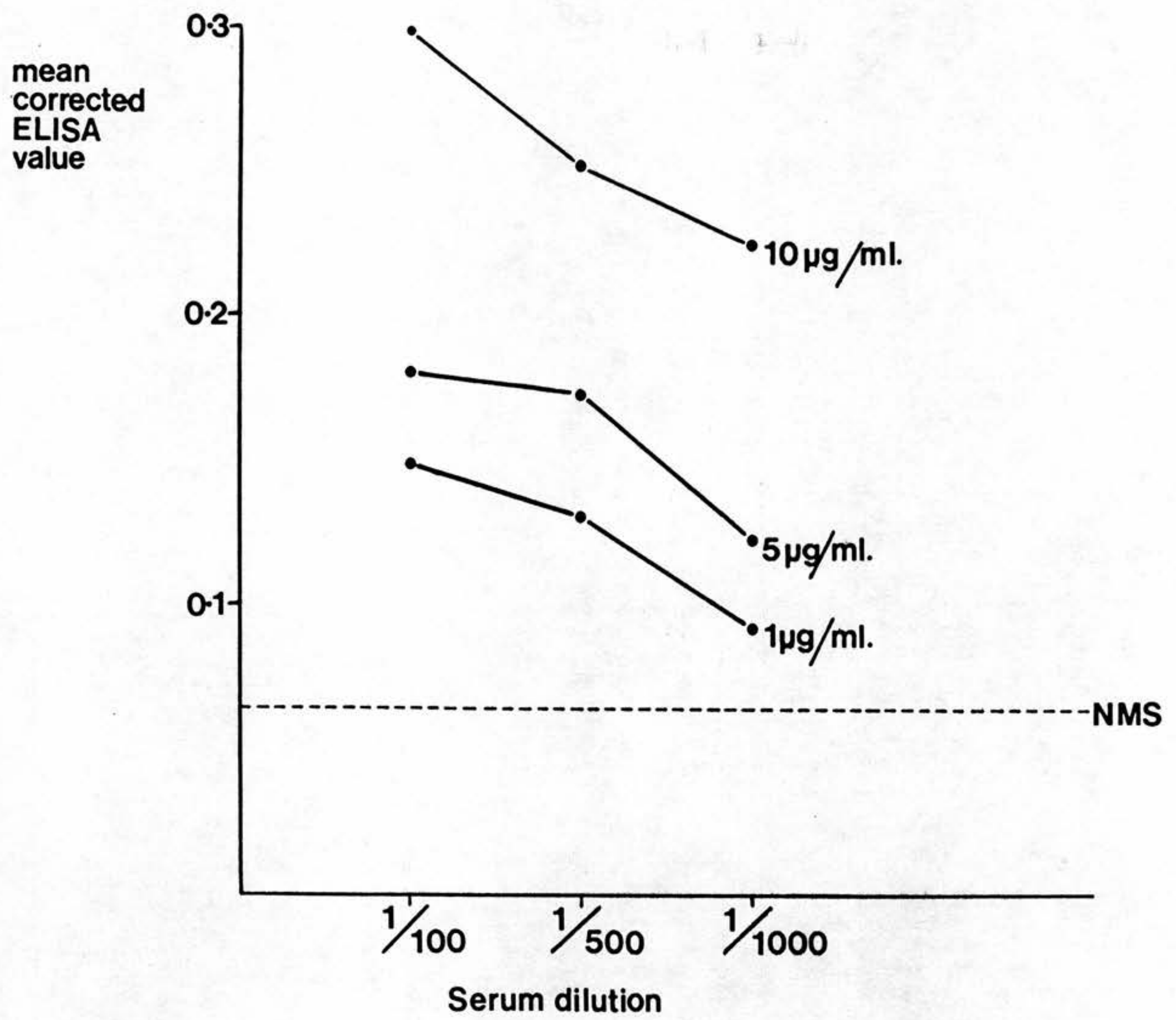
Following the inoculation of *B. microti*, the antibody levels in both groups rose rapidly. However, the TB group had a significantly lower antibody response to *B. microti* than the B group from week 1 to week 4. The inoculation of the challenge dose did not bring about any significant rise in the existing antibody levels to *B. microti*.

2.6 The Antibody Response to T. crassiceps

This was measured using the enzyme-linked immunosorbent assay (ELISA).

Figure 2.4

Absorbance values obtained by ELISA from the chequer-board titrations of various concentrations of the *Taenia crassiceps* delipidised antigen against three dilutions of sera. The results are expressed as the differences between the positive and negative sera results for each dilution.


mean
corrected
ELISA
value
0·3
0·2
0·1
10 µg/ml.
5 µg/ml.
1µg/ml.
NMS
1/100
1/500
1/1000
Serum dilution

2.7 Preliminary Chequer-board Titrations

Chequer-board titrations of various concentrations of the *Taenia crassiceps* delipidised antigen against positive (12 week old infections) and negative (normal) sera were carried out. Three antigen concentrations were used (1, 10 and 20 μg protein/ml) against three dilutions of sera (1/100, 1/500 and 1/1000). These high dilutions of sera were used in view of the small quantities of sera obtained from each mouse. Each serum sample had to provide for both IFAT and ELISA assays, with a small quantity left over in case any repetitions were necessary.

2.7.1 Results

Figure 2.4 shows the ELISA values from the chequer-board titrations and from this the optimal antigen concentration was selected. The results are expressed as the differences between the positive and negative sera results for each dilution. At a serum dilution of 1/500 and antigen concentration of 10 μg/ml, the difference between the mean ELISA values given by the positive and negative sera was 0.25. This was considered a usable difference for subsequent assays whilst allowing maximal economy of the available sera.

2.8 The Humoral Response to T. crassiceps in the Concurrent Infections

2.8.1 Results

Figure 2.5 shows the mean corrected ELISA values for the concurrently infected group (TB) and the *T. crassiceps* only control group.

Figure 2.5

The mean antibody response to T. crassiceps by mice infected with Taenia crassiceps and Babesia (TB) and the T.crassiceps only controls detected by ELISA.

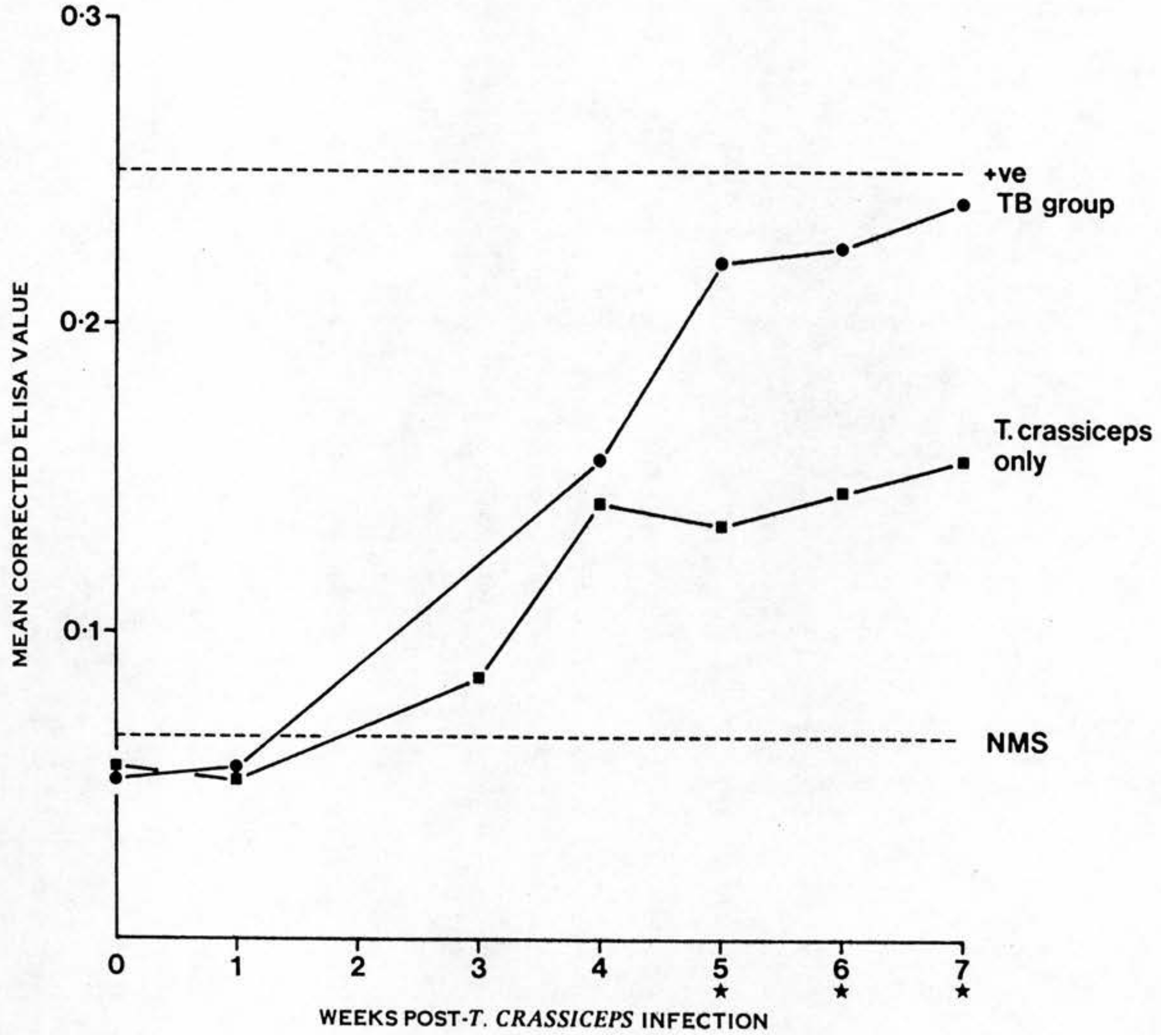

THE ANTIBODY RESPONSE TO T. CRASSICEPS
MEAN CORRECTED ELISA VALUE
0·3
0·2
0·1
+ve
TB group
T. crassiceps only
NMS
0
1
2
3
4
5
6
7
WEEKS POST-T. CRASSICEPS INFECTION

Table 2.3 shows the t-values obtained when the mean ELISA values of these two groups were compared by the Student's t-test.

Table 2.3

Weeks post *T. crassiceps* infections	t-value	Level of significance
0	0.22	Not significant
1	0.34	Not significant
4	1.29	Not significant
5	8.85	$P > 0.001$
6	7.93	$P > 0.001$
7	7.83	$P > 0.001$

There was a gradual rise in antibody response to *T. crassiceps* in both groups for four weeks after infection and during this time the mean ELISA values for the two groups were not significantly different from each other. Following the inoculation of *B. microti* the antibody levels in the TB group rose rapidly during week 4. The mean titre for this group then remained fairly constant and was consistently and significantly above the mean for the T group. This may be the effect of non-specific stimulation of the clones already responding to *T. crassiceps* by the unrelated *Babesia* antigen (Terry *et al.*, 1980)

2.9 Spleen/Body Weight Ratio

There was no significant difference in the spleen/body weight ratios between the two treatment groups, the mean ratio being 0.14 for both groups.

2.10 Infection of Nude (athymic) Mice with Babesia microti

2.10.1 Nude mice

The nude mouse arises from a mutation which has resulted in dysgenesis of the thymus and thus, provides a natural model of thymic malfunction (Pantelaine, 1968).

In general, nude mice:-

(a) are unresponsive to in vitro stimulation of spleen cells by the T cell dependent mitogens PHA and Con A;

(b) lack T helper cell functions in response to a specific antigen;

(c) are incapable of rejecting skin allografts and xenografts;

(d) apparently lack T suppressor cell function; and

(e) do not muster IgE or IgG responses thus precluding immediate hypersensitivity response.

On the other hand, nude mice have a B cell diversity indistinguishable from that of conventional mice (Cancro and Klinman, 1980), have increased numbers of natural killer cells (NK), exhibit enhanced macrophage activity and show increased circulating antinuclear antibodies compared with normal littermates. The nude mouse has served as a model for determining the T cell dependence of susceptibility and resistance to parasitic infections.

2.10.2 Aims

The purpose of this experiment was to verify that B. microti infections, using the stabilate TREU 1764, are enhanced in an immunodeficient host. This was felt to be desirable following the results in the previous experiment, in which intraperitoneal inoculation of

B. microti into mice containing T. crassiceps metacestodes resulted in a depressed parasitaemia as compared with mice without the taeniid infection. This is contrary to the observation by Good and Miller (1976) who claimed that mice containing T. crassiceps metacestodes exhibited a generalised immunosuppression. Previous work has shown that B. microti infections in nude mice are prolonged and ultimately prove fatal (Allison, 1979) but it was necessary to verify this with the particular stabilate of B. microti used in these experiments. It was felt to be most likely that this increased resistance to B. microti in mice harbouring T. crassiceps metacestodes was due to either a physical interaction between the two parasites or possibly to the action of the numerous macrophages located around the metacestode larvae in the peritoneal cavity. However, in order to rule out the possibility that the course of B. microti infections were unaffected in mice suffering from such immunodeficiency, infections were initiated in nude mice and compared with similar infections in their normal thymus-bearing littermates.

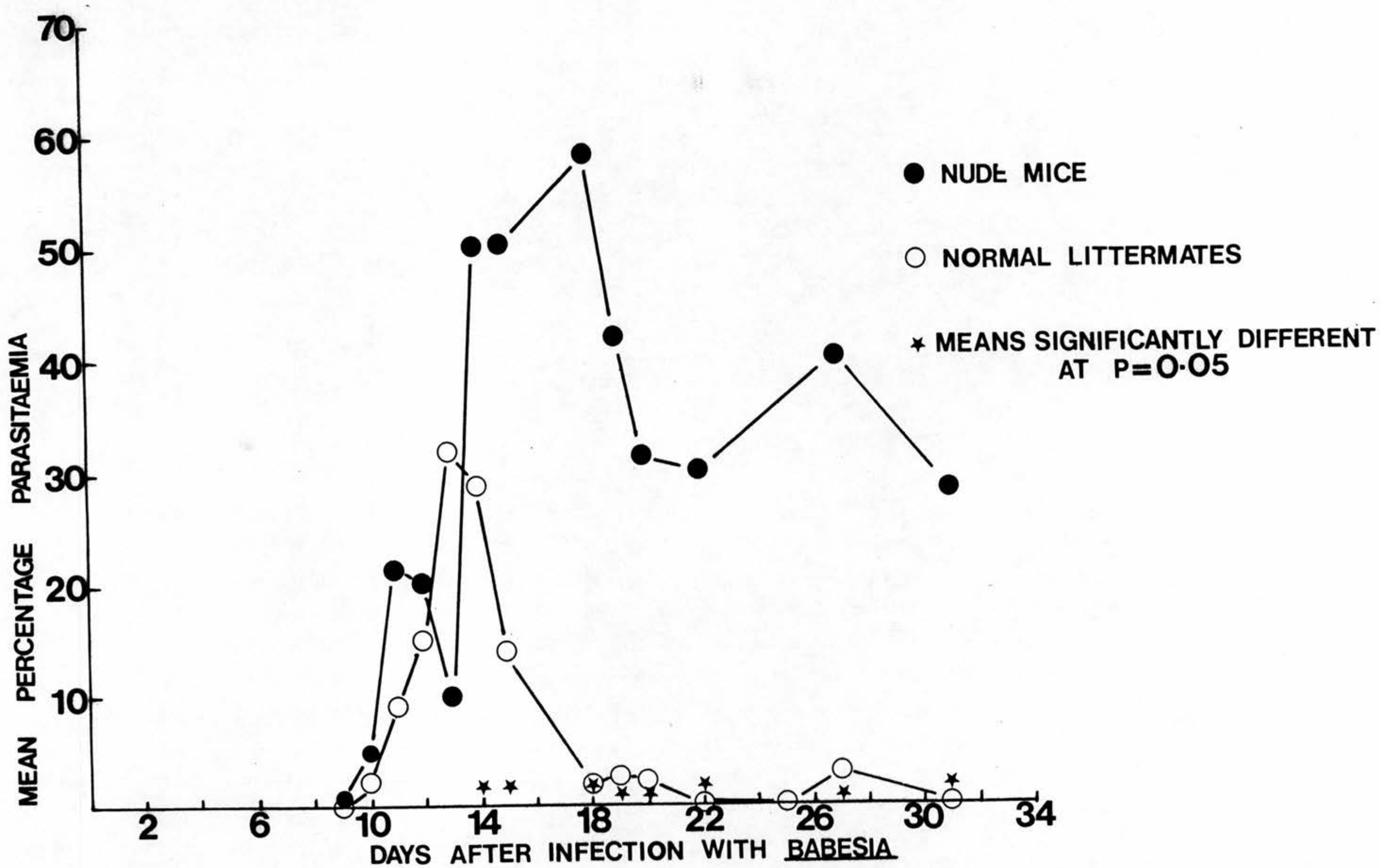
2.10.3 Experimental design

Eight nude (athymic) mice and eight normal littermates were inoculated intraperitoneally with 10^6 B. microti infected red blood cells. The animals were grouped together so that each cage of four mice contained two nude mice and two furred littermates. The purpose of this was to aid the maintenance of the body heat of the nude mice.

The mice were housed in an animal isolation chamber (Vickers Medical) at a temperature of 28°C in large animal constant temperature housing. The isolation chamber was sterilised prior to use by the

Figure 2.6

The mean percentage parasitaemias of nude (athymic) mice and their normal littermates when inoculated intraperitoneally with *B. microti*.


MEAN PERCENTAGE PARASITAEMIA
10
20
30
40
50
60
70
2
6
10
14
18
22
26
30
34
DAYS AFTER INFECTION WITH BABESIA
NUDE MICE
NORMAL LITTERMATES
★ MEANS SIGNIFICANTLY DIFFERENT AT P=0·05

application of 5% peracetic acid in a fine aerosol spray (Asda). This procedure was carried out 24 hours before the animals were put in the chamber, to allow toxic fumes to disperse.

Sterile bedding, feed and water were used throughout the experiment and the animals were handled for inoculation and collection of blood for smears by means of the gloves attached to the isolator.

The smears were made within the chamber and the accumulated slides were only removed from the chamber and stained with Giemsa at the end of the experiment. The experiment was brought to a close 28 days post infection. This period was determined by the practical considerations of sharing the use of the isolation chamber with other workers. Ideally, this time should have been extended.

2.10.4 Results

The mean percentage parasitaemias of nude and normal mice after infection with *B. microti* are shown in Figure 2.6. The data for each day after the appearance of piroplasms in the blood were analysed separately to determine whether the mean parasitaemias for the two groups differed significantly. The full ANOVA tables are in Appendix 3. A similar comparison for the two peaks is given in Table 2.4.

Table 2.4 ANOVA table comparing the *B. microti* parasitaemia peaks in nude mice and their normal littermates.

Source of variation	SS	df	MS	F
(1) Treatment	1,903.1	1	1,903.1	6.65*
(2) Field	321.3	2	160.35	1.1
(3) Interaction	377.33	2	188.35	1.3
Error	3,431.5	12	285.9	

It is clear that although the peak parasitaemia in the normal mice was earlier than that in the nude mice, a smaller proportion of the red cells were infected in the former. Following the peak, the level of parasitaemia dropped rapidly in the normal mice whereas it remained elevated in the nude group.

2.10.5 Spleen weights

At necropsy, the total body weight and spleen weight of each mouse was recorded and the spleen/body weight ratio was calculated.

Table 2.5 Spleen/body weight ratios

Treatment	Mean spleen weight (g) ± SD	Mean body weight (g) ± SD
Normal littermates	0.35 ± 0.16	28.0 ± 1.7
Nude (athymic)	0.48 ± 0.041	17.0 ± 1.2

2.10.6 Discussion

This experiment showed that a *B. microti* infection in athymic mice gave a greater parasitaemia and was not resolved as rapidly as in their normal littermates. Due to the relatively short duration of this experiment it is not clear whether the infection would be resolved at all or whether the hosts would have succumbed as reported by Allison (1979).

This suggests that the regulation of *B. microti* infection is probably thymus-dependent and involves T cells, so agreeing with the conclusions of Ruebush and Hanson (1980). These authors showed

that B lymphocytes conferred less protection than T cells when either was given alone to reconstituted thymectomized mice. On the other hand Mitchell (1979) found that athymic mice were more resistant to *B. rodhaini* than their normal littermates, a situation which is contradictory to the majority of studies on the role of T cells in acquired immunity to *B. microti*.

ADMINISTERED INTRAVENOUSLY

2.11 Aims

This experiment was designed to see if mice infected with T. crassiceps were capable of suppressing the immune response to B. microti when the piroplasm was administered by the intravenous route. In the previous experiment it had been shown that when both parasites were inoculated into the peritoneal cavity the metacestodes did not appear to induce immunosuppression similar to that observed by Good and Miller (1976). It was felt that this may be due to a physical interaction between these two parasites, since the presence of the large bulk of metacestodes in the peritoneal cavity might be slowing the rate at which the B. microti pass into the circulation or alternatively intraerythrocytic killing by the large numbers of macrophages associated with the metacestode infection. B. microti intracellular death is known to involve macrophages.

Accordingly, for this further experiment, the piroplasms were inoculated intravenously, which was the more usual route in experimental infections (Phillips, 1981) and also avoided this possible physical interaction.

2.12 Experimental Design

This study comprised four groups of six-week-old Balb/c mice.

Group TTC and B: Eight mice inoculated intraperitoneally with 20 intact metacestodes of less than 2 mm diameter.

Group BTC and B: Eight mice inoculated with 20 metacestodes that had been burst by forcing them through an 18G needle (Vale "Microlance"). The cyst fluid was washed away so that only tissues were

present in the inoculum. These burst metacestodes would mainly consist of the somatic antigen components of intact metacestodes and would lack or be defunct in any components which occur solely or mainly in the cyst fluid.

Group B: Twelve uninfected mice. Five weeks later eight of these mice were inoculated intravenously with 10^6 B. microti infected red cells. The subsequent parasitaemia was monitored by examining Giemsa-stained blood smears. Any mice which failed to show a parasitaemia by two days after inoculation were excluded as were mice that had not received the full dose of Babesia due to the needle becoming dislodged from the vein.

The group sizes following inoculation of B. microti were:

TTC and B = 5; BTC and B = 5; B = 6

2.13 Results

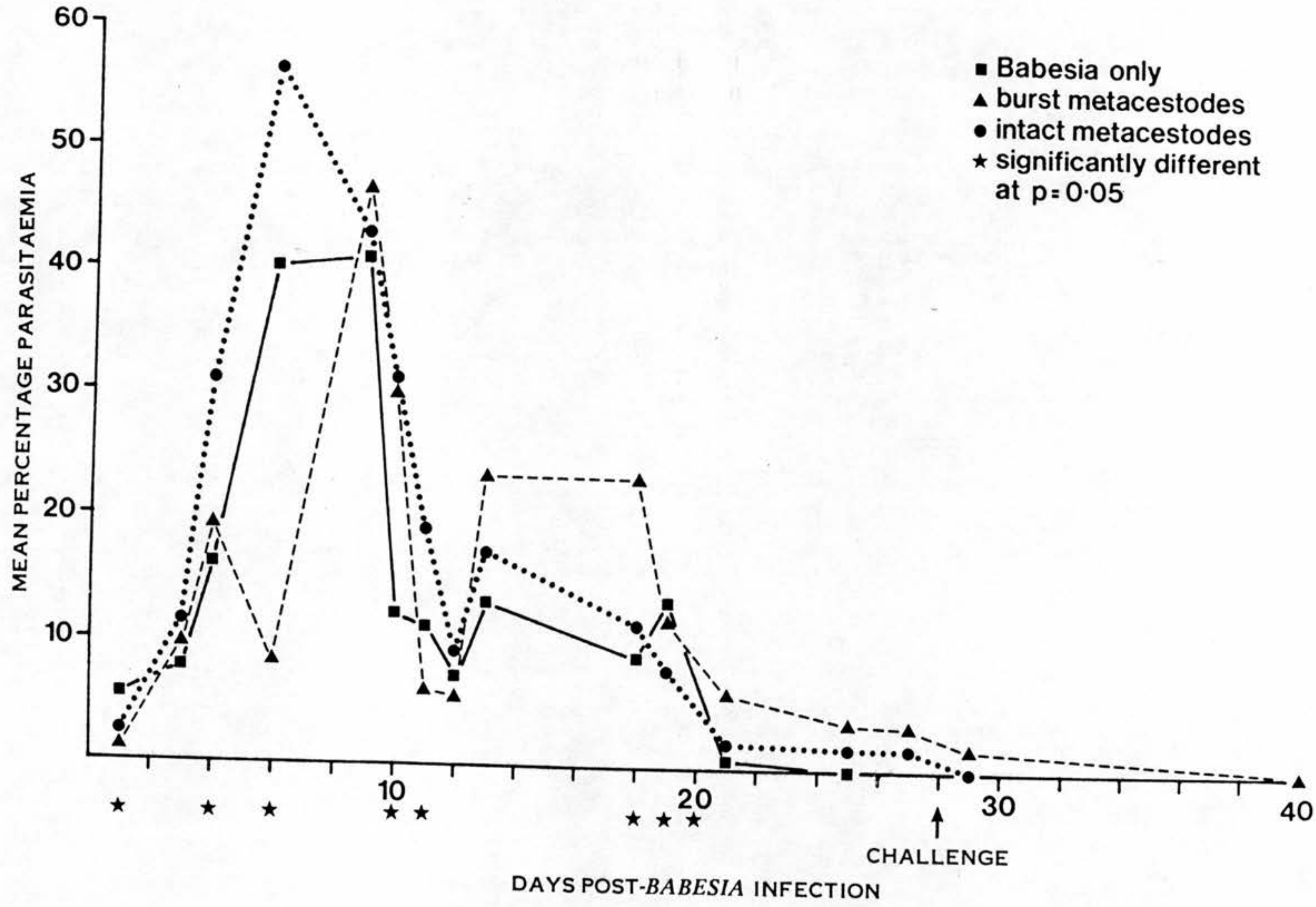
The development of the parasitaemias in each group after infection is shown in Figure 2.7. The ANOVA tables comparing the peaks for each group are shown in Table 2.6. The remaining daily ANOVA tables are contained in Appendix 3.

Table 2.6 ANOVA table for the peak results of the three treatment groups

Source of variation	SS	df	M	F
(1) Treatment	3255.4	2	1627.7	43.0*
(2) Field	50.14	2	25.0	0.66
(3) Interaction	745.0	4	186.2	5.0
Error	682.7	18	37.9	

Figure 2.7

The mean percentage parasitaemias of Balb/c mice infected with intact metacestodes of *Taenia crassiceps* and *Babesia* (TTC) burst metacestodes and *Babesia* (BTC) and *Babesia* only controls (B).


MEAN PERCENTAGE PARASITAEMIA
60
50
40
30
20
10
10
20
30
40
CHALLENGE
DAYS POST-BABESIA INFECTION
■ Babesia only
▲ burst metacestodes
● intact metacestodes
★ significantly different
at p=0·05

The TTC and B group had a peak mean parasitaemia on day 5 of 56% which is significantly greater than the peak in the other two groups. The remaining two groups reached their mean peak parasitaemias on day 9, which for the BTC and B group was 48% and for the B group was 43%. The parasitaemias in the three were not significantly different on this day. There was a secondary peak on day 12 in all the three groups, and the parasitaemia remained elevated until day 15 in the BTC and B group. Thereafter, the parasitaemias in all groups declined.

The differences between the treatment groups were not marked but the parasitaemia did appear to rise rapidly and to a higher peak in the TTC and B group.

No recrudescence of the *Babesia* infection was observed in any of the mice after challenge on day 28.

2.14 The Rate of Decline of the Parasitaemia from the First Peak

Regression analysis in the polynominal form was carried out on the data from each group separately. Terms with significant parameters were found up to the second power for the TTC and B group and up to the fourth power for the BTC and B group and the B group. These equations were:

$$PST = 56.1 - 6.6T + 0.2T^3 \quad \text{for the TTC and B group}$$
$$\text{(t-value)} \quad (14.93) \; (7.92) \; (5.21)$$

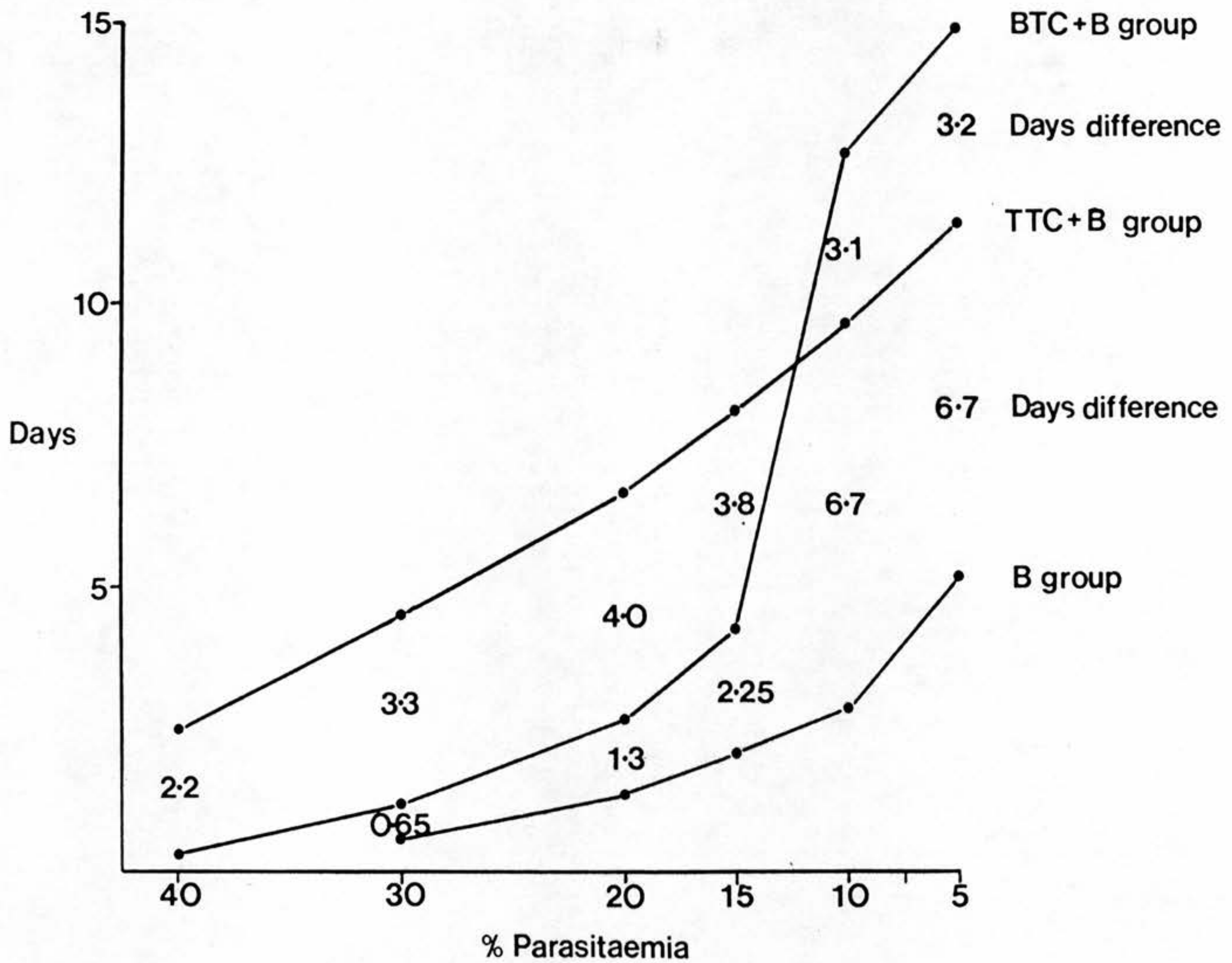
$$PST = 37.4 - 15.4T + 2.5T^2 - 0.2T^3 + 0.003T^4 \quad \text{for the BTC and B group}$$
$$\text{(t-value)} \quad (6.77) \; (3.73) \; (2.96) \; (2.6) \; (2.45)$$

$$PST = 43.5 - 13.6T + 2.2T^2 - 0.1T^3 + 0.003T^4 \quad \text{for the B group}$$
$$\text{(t-value)} \quad (12.4) \; (4.95) \; (3.8) \; (3.44) \; (3.21)$$

Thus, estimated peak parasitaemias for the TTC and B group was 56.1,

Figure 2.8

The rate of decline in parasitaemia expressed as the number of days above a given percentage parasitaemia each of the three groups remain.



15
10
5
Days
40
30
20
15
10
5
% Parasitaemia
BTC+B group
3·2 Days difference
TTC+B group
6·7 Days difference
B group
3·1
3·8
6·7
4·0
3·3
2·25
1·3
2·2
0·65

that for the BTC and B group was 37.4 and for the B group it was 43.5.

This analysis also showed a significant difference between the peak parasitaemias in the TTC and B group and the B group as well as a significant difference in the rate of decline in these two groups of mice. No significant difference was found between the peak levels of parasitaemia in the BTC and B group and in the B group or between the peak parasitaemias in the TTC and B group and the BTC and B group.

The equations of best fit obtained in this analysis have been used to graphically describe the rate of decline in Figure 2.8. Over the range of 40-15% of infected red blood cells, the parasitaemia in the mice in the TTC and B group remained elevated for longer than in either of the two other treatment groups; but over the range 15-5% the parasitaemia was more prolonged in the BTC and B group. This shows in Figure 2.7 as an extended secondary peak. The B group had a more rapid rate of decline than the other two groups, with the difference increasing as the parasitaemia level declined.

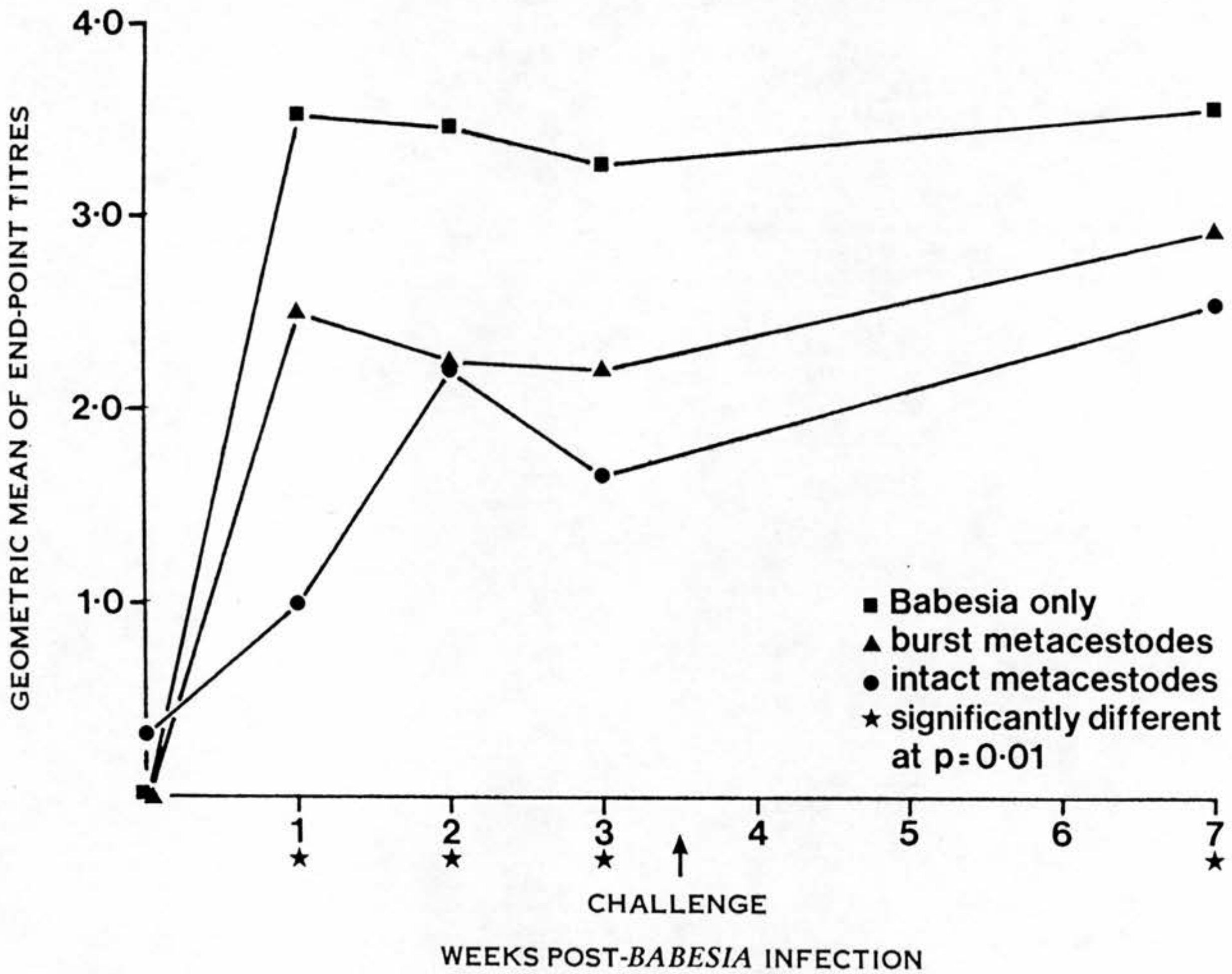
Regression analyses were also carried out on the combined data of the TTC and B group with the BTC and B group; the TTC and B group with the B group and of the BTC and B group with the B group. These analyses showed that there was no significant difference in the rate of decline of the two concurrently infected mice groups even though the separate regression analyses of this data indicated that the pattern of decline did differ significantly. It is probable that this was due to chance. There was however a significant difference

Table 2.7 Pattern of parasitaemia at low levels

Day	TTC and B Group		BTC and B Group		B Group	
	Median No. of positive fields	Parasitaemia	Median No. of positive fields	Parasitaemia	Median No. of positive fields	Parasitaemia
18	2	1.6	3	6.1	0.5	1
22	2	1.07	2	2.6	0	0
24	2	1.3	1	2.6	0	0
26	0	0	3	1.5	0	0
32	0	0	0	0	0	0

Figure 2.9

The mean antibody response to *B. microti* detected by IFAT.


THE ANTIBODY RESPONSE TO B. MICROTI
GEOMETRIC MEAN OF END-POINT TITRES
4·0
3·0
2·0
1·0
Babesia only
burst metacestodes
intact metacestodes
significantly different at p=0·01
1
2
3
4
5
6
7
CHALLENGE
WEEKS POST-BABESIA INFECTION

between the peak values which were 37.6% for the BTC and B group and 58.0% for the TTC and B group as calculated from the regression analysis.

When the data from the TTC and B group was analysed with that from the B group it was found that the difference between calculated peaks was 24% and this was significant. The rate of decline with these two groups also differed significantly.

When the BTC and B group and the B group were analysed by this same analysis there was no significant difference either in the peaks or rates of decline (Table 2.7). These results confirm the conclusion from the regression analyses that the rate of recovery by the BTC and B group mice was slower than the other concurrently infected group of mice.

2.15 The Humoral Response to B. microti

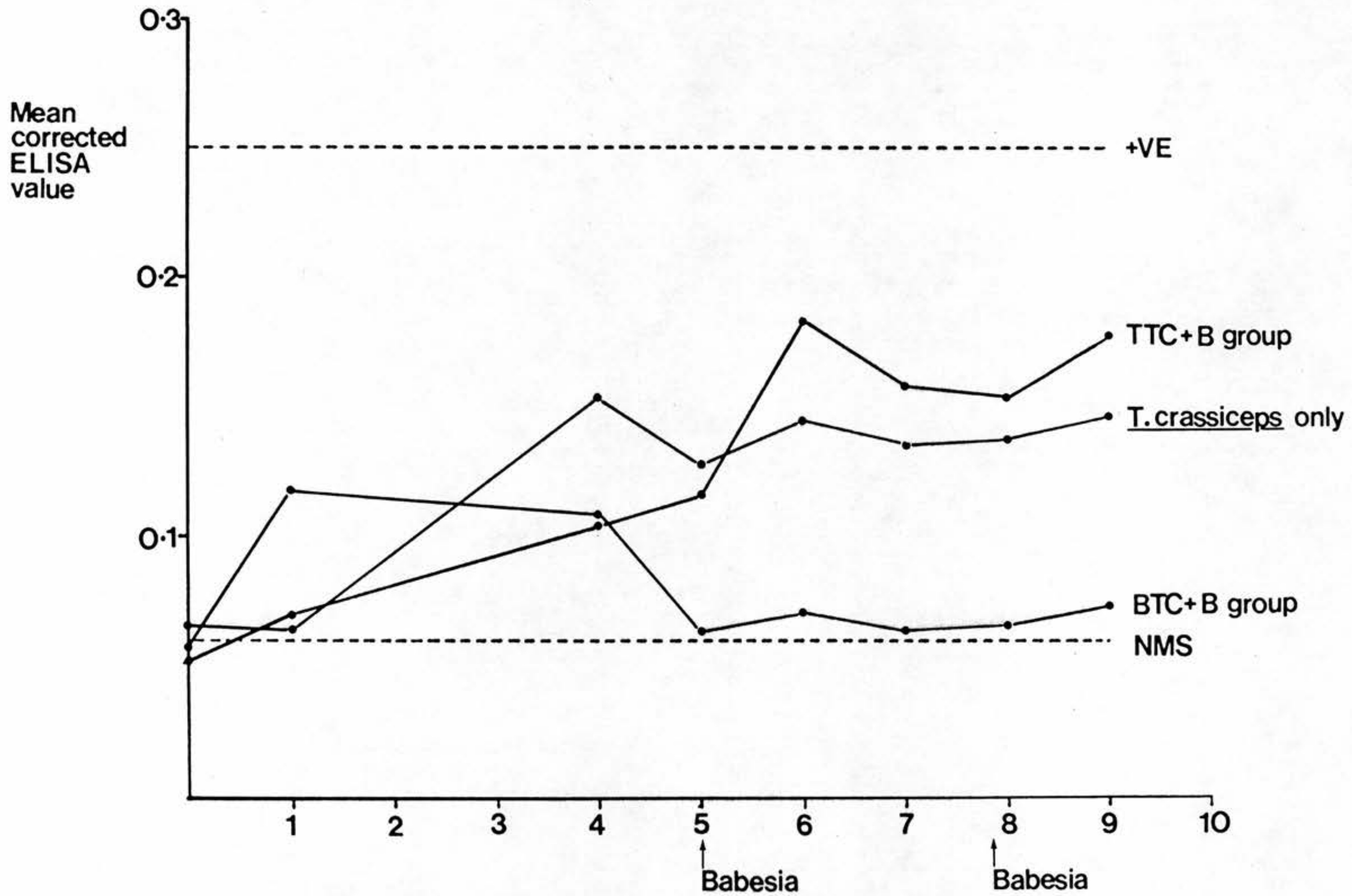
This was again assessed by the indirect fluorescent antibody technique. The end-point titres were expressed as mean log values and are shown in Figure 2.9. After week 1, the mice in the TTC and B group and the BTC and B group both had significantly lower antibody responses to *B. microti* than those of the control group.

Table 2.8 The t-values indicating the level of significance when the TTC and B group was compared with the B group

Week post *Babesia* infection	t-value	Level of significance
0	0.9	Not significant
1	4.53	$P < 0.01$
2	5.07	$P < 0.001$
3	2.33	$P < 0.05$
7	4.9	$P < 0.001$

Figure 2.10

The mean antibody response to *T. crassiceps* by mice infected with intact metacestodes and *Babesia* (TTC), burst metacestodes and *Babesia* (BTC) and *Babesia* only controls (B).



Mean corrected ELISA value
O·3
O·2
O·1
+VE
TTC+B group
T. crassiceps only
BTC+B group
NMS
1
2
3
4
5
6
7
8
9
10
Babesia
Babesia
Weeks post-infection with T. crassiceps

Table 2.9 The t-values indicating the level of significance when the BCT and B group were compared with the B group

Week post Babesia infection	t-value	Level of significance
0	0	Not significant
1	8.04	$P < 0.001$
2	3.85	$P < 0.001$
3	3.49	$P < 0.01$
7	2.08	$P < 0.05$

2.16 Spleen/Body Weight Ratios

Table 2.10

Treatment	Mean spleen/body weight ratio ± SD	Level of significance
TTC and B	0.0098 ± 0.0006	
BTC and B	0.0094 ± 0.0005	Not significant
B	0.0105 ± 0.0009	

2.17 The Humoral Response to T. crassiceps

The mean corrected ELISA results with *T. crassiceps* delipidised antigen for both concurrently infected groups and the *T. crassiceps* only control group are shown in Figure 2.10.

Table 2.11 shows the t-values obtained when the mean ELISA values of the TTC and B group and the BTC and B group were each compared separately with the B group.

<u>Table 2.11</u> TTC and B v T. crassiceps only

Weeks post *T. crassiceps* infection	t-value	Level of significance
0	0.39	Not significant
1	0.53	Not significant
4	0.21	Not significant
5	1.59	Not significant
6	2.51	$P < 0.05$
7	1.33	Not significant
8	0.64	Not significant
9	2.63	$P < 0.05$

There was a gradual rise in antibody titre over a period of 4-5 weeks to a plateau of about 0.15 absorbance units in both groups, except for weeks 6 and 9 when the TTC and B group had a slightly greater antibody level than the controls ($t = 2.51$ and 2.63 respectively, $P < 0.05$) or significant at $P < 0.05$ in both cases. This coincided with the inoculation of *B. microti* so may be due to short term non-specific stimulation of the clones already responding to *T. crassiceps* by the unrelated antigen.

<u>Table 2.12</u> BTC and B v *T. crassiceps* only controls

Weeks post *T. crassiceps* infection	t-value	Level of significance
0	0.23	Not significant
1	3.58	$P < 0.01$
4	0.26	Not significant
5	4.35	$P < 0.01$
6	6.31	$P < 0.001$
7	5.48	$P < 0.001$
8	6.5	$P < 0.001$
9	4.98	$P < 0.001$

In the BTC and B group there was an initial rapid rise in antibody titre against the T. crassiceps antigen following the inoculation of the burst metacestode preparation; but this then declined over a period of four weeks to reach a plateau level comparable with that of the negative control - normal mouse serum. From week 5 onwards the BTC and B group had significantly lower antibody levels than the T. crassiceps only controls.

CHAPTER THREE

CONCURRENT INFECTIONS OF

TAENIA TAENIAEFORMIS AND *BABESIA MICROTI*

3.1 Aims

This series of experiments set out to determine whether mice infected with T. taeniaeformis strobilocerci had a depressed immune response to Babesia microti as has been shown for T. crassiceps. The Babesia-infected red blood cells were inoculated by the intravenous route in all the following experiments due to the fact that it had already been shown with T. crassiceps that piroplasms appear in the bloodstream earlier when this route is used rather than the intraperitoneal route.

3.2 Experimental Design

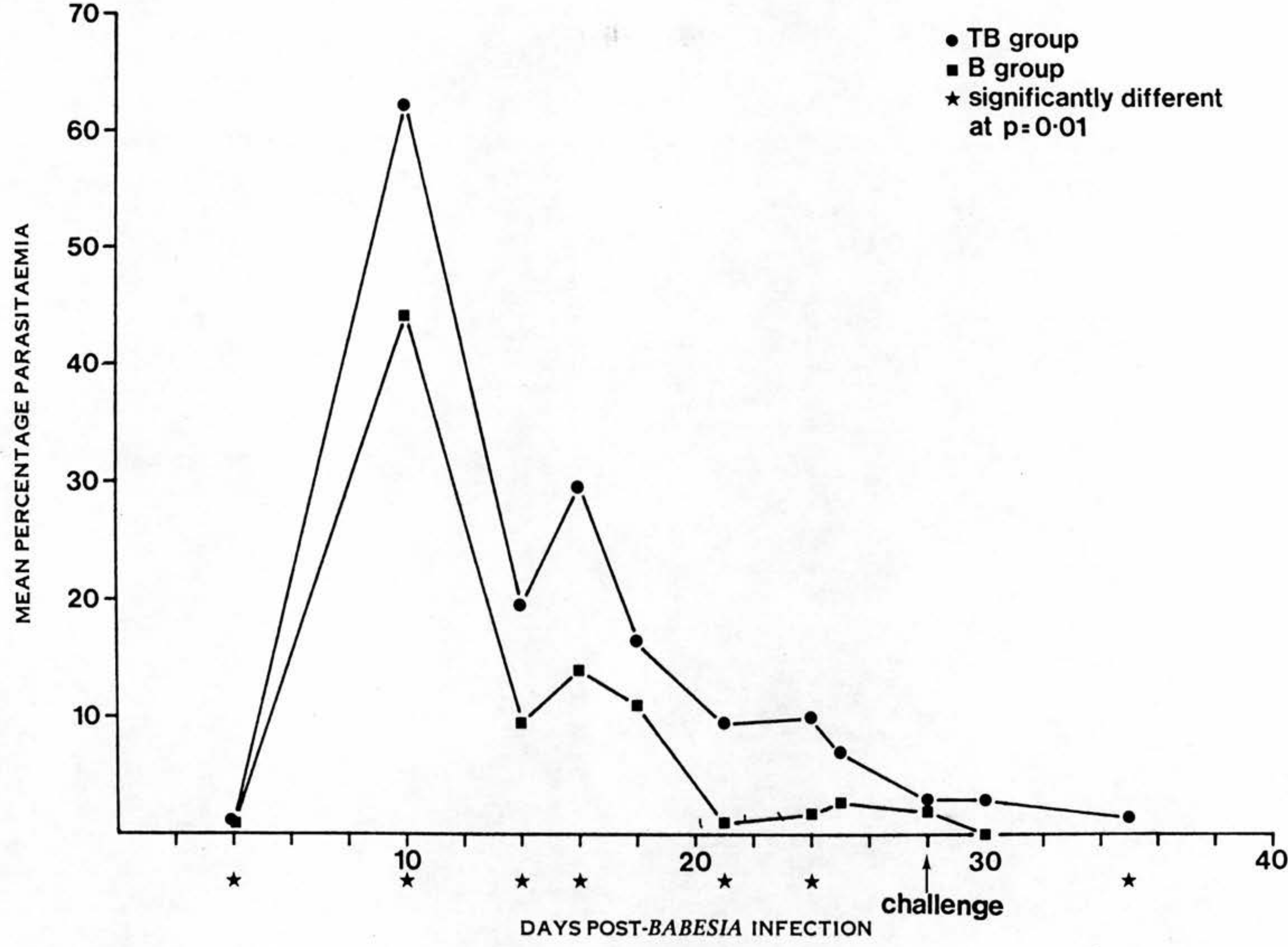
Following the same infection schedule, 15 six-week-old Balb/c mice were infected with 200 eggs of T. taeniaeformis (TB group) by mouth and five weeks later five of these mice and five uninfected Balb/c mice (B group) of the same age were inoculated intravenously with 10^6 Babesia-infected red blood cells. All the mice were re-challenged intravenously on week 9 with 10^6 Babesia-infected red blood cells. The group sizes following inoculation with the piroplasm, were TB = 9 and B = 4. This was because with one mice in each group problems were encountered with the intravenous injection; because of this these mice had to be rejected as it was not possible to be certain that they had received the full dose.

3.3 Results

The parasitaemias for each treatment group by days since infection are shown in Figure 3.1. The data for each day were analysed separately to determine whether the means for the two groups on that

Figure 3.1

The mean percentage parasitaemias of Balb/c mice infected with *Taenia taeniaeformis* and *Babesia* administered intravenously.


MEAN PERCENTAGE PARASITAEMIA
70
60
50
40
30
20
10
• TB group
■ B group
★ significantly different
at p=0·01
10
20
30
40
challenge
DAYS POST-BABESIA INFECTION

day were significantly different from each other. The ANOVA tables are in Appendix 3. The table for day 10, the day of peak parasitaemia for both treatment groups is given below.

Table 3.1 ANOVA table for day 10

Source of variation	SS	df	MS	F
(1) Treatment	2580.977	1	2580.977	16.7
(2) Field	151.978	2	75.99	0.5
(3) Interaction	168.399	2	84.2	0.5
Error	4326.19	28	131.1	

This table shows that the treatment effect is very significant ($P < 0.001$) but that the effect of different fields and the interaction of reading method with treatment is not.

The days on which the mean parasitaemias were significantly different are indicated in Figure 3.1.

The results show that:-

(i) The peak parasitaemia is reached on day 10 for both treatment groups.

(ii) The parasitaemia in the TB group is consistently greater than that in the B group.

(iii) The peak level is 44% for the B group and 62% for the TB group and this difference is highly significant. The levels of parasitaemia decline thereafter with a secondary peak on day 16.

(iv) The difference between the groups remains significant until day 25.

3.4 <u>The Rate of Decline of the Parasitaemia from the First Peak</u>

Regression analysis showed that the polynominal form gave the best fit as judged by the R^2 values of about 0.85 that were obtained. These values were lower in the other analyses. Analysis was done on the data for the control mice and the concurrently infected mice separately in order to get values for the parameters giving the closest fit to the specific data. Terms with significant parameters were found up to the third power for the control group and to the fourth power for the concurrently infected group. These equations are:

$$\underset{(25.7)}{PST = 60.15} - \underset{(9.2)}{11.61T} + \underset{(5.1)}{1.10T^2} - \underset{(3.9)}{0.049T^3} + \underset{(3.3)}{0.00788T^4}$$

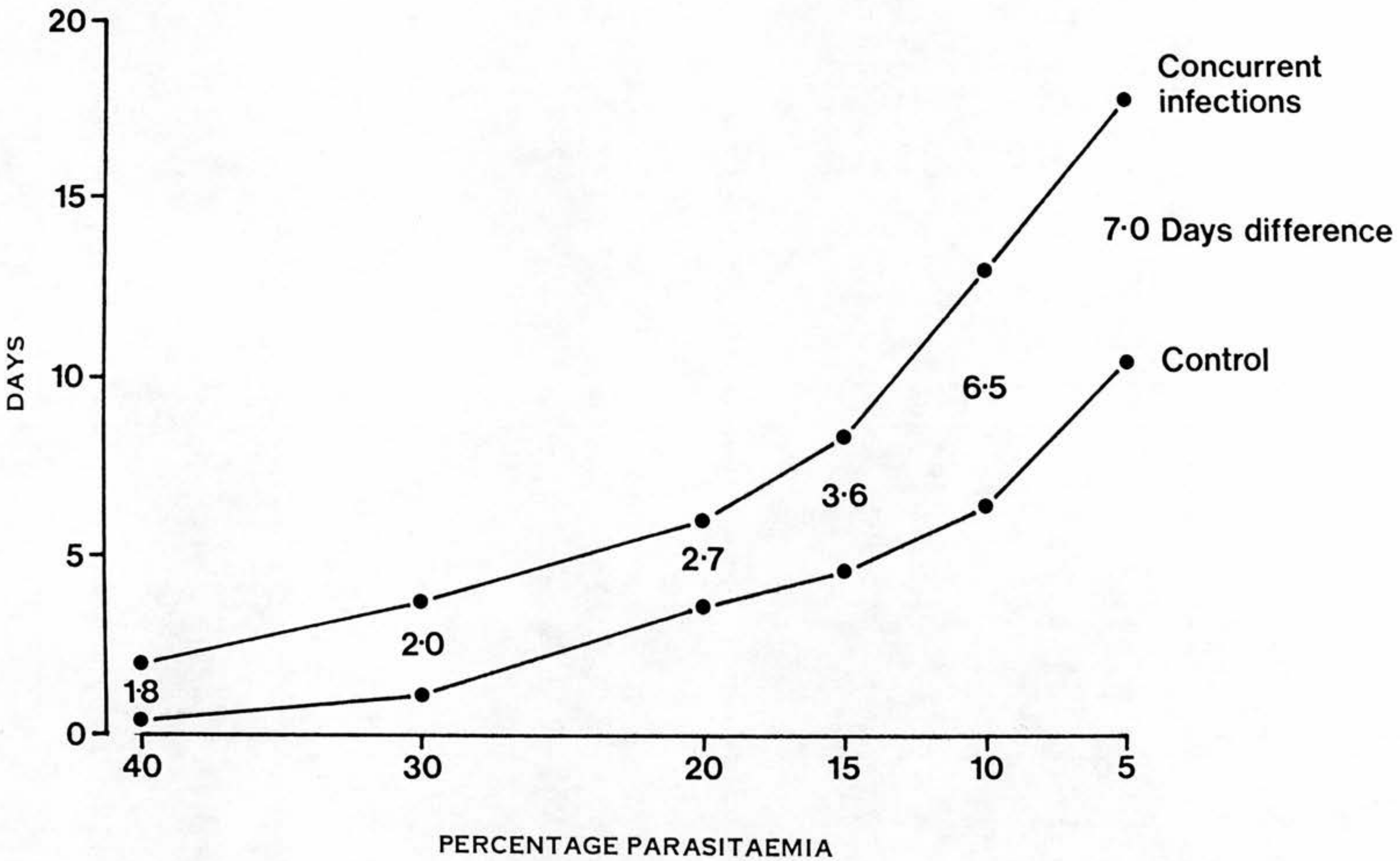
for the TB group, and

$$\underset{(15.9)}{PST = 41.498} - \underset{(8.3)}{6.905T} + \underset{(5.3)}{0.392T^2} - \underset{(4.1)}{0.0072T^3}$$

for the B group. No significant serial correlation of the error terms was found. One of the crucial assumptions for regression models is the independence of the stochastic error term. This is tested for by the use of the Durbin-Watson test for first-order correlation. The further away from 2.0 that the Durbin-Watson statistic is, the less confident one can be that there is no serial correlation in the disturbances. The estimated peak parasitaemia was 41.5% for the control group and 60.2% for the concurrently infected group, a difference of 18.7%. The equations also indicated a more rapid decline in the number of infected red blood cells in the concurrently infected group in absolute terms as indicated by the first-order parameter values against days. However, the regressions were also used to determine the number of days that the

Figure 3.2

The rate of decline in parasitaemia expressed as the number of days above a given percentage parasitaemia that the concurrently infected and *Babesia* only group remain.


DAYS
20
15
10
5
0
40
30
20
15
10
5
PERCENTAGE PARASITAEMIA
1·8
2·0
2·7
3·6
6·5
7·0 Days difference
Concurrent infections
Control

parasitaemia levels remained above particular values. These are shown in Figure 3.2. This shows that the concurrently infected mice experienced given levels of parasitaemia for longer than the control mice. The difference increased as the parasitaemia level declined indicating that the concurrently infected mice reduced their level of parasitaemia more slowly in relative terms than the control group.

A regression was also carried out on the combined data in order to determine directly the least square estimations of the differences between the groups. These are corrected for unequal replication between groups and can therefore be different from the experimental values on any given day. This regression is reported in Appendix 3 and has an R^2 of 0.82. The values for the peak parasitaemia indicated 45.5% for the control group and 59% for the concurrently infected group, a difference of 13.5%. This is less than that estimated from the separate regressions due to unequal replication leading to more emphasis being given to the higher values. Only one of the slope dummies is significant, that being β_{11}, for the parameters for T. Although significant, the difference between the groups is only 0.5 for this compared to a much larger difference of 4.7 from the separately estimated regressions. The differences in the other parameters were too small to be significant. However, this approach does also show significant differences in peak values and in the pattern of decline.

Once the average level of paraistaemia drops below 3% the chances that some of the fields will include no parasitized cells increases rapidly from 0.09 at 3% to 0.2 at 2% and 0.45 at 1%. The chances of three

separate fields having no parasitized cells increases correspondingly. The decline in parasitaemia at lower levels is therefore measured by classifying mice according to the number of positive fields they show on each day that smears were taken.

Table 3.2 Parasitaemia pattern at lower levels

Day	B Group		TB Group	
	Median No. of positive fields	Mean parasitaemia	Median No. of positive fields	Mean parasitaemia
18	3	11.6	3	16.5
21	2	2.1	3	9.9
24	1	1.1	3	10.0
25	2	2.7	3	7.0
28	0	2.3	3	3.0
30	0	0.2	2	3.0
35	0	0	1	1.7
37	0	0	0	2.1
38	0	0	0	0.3

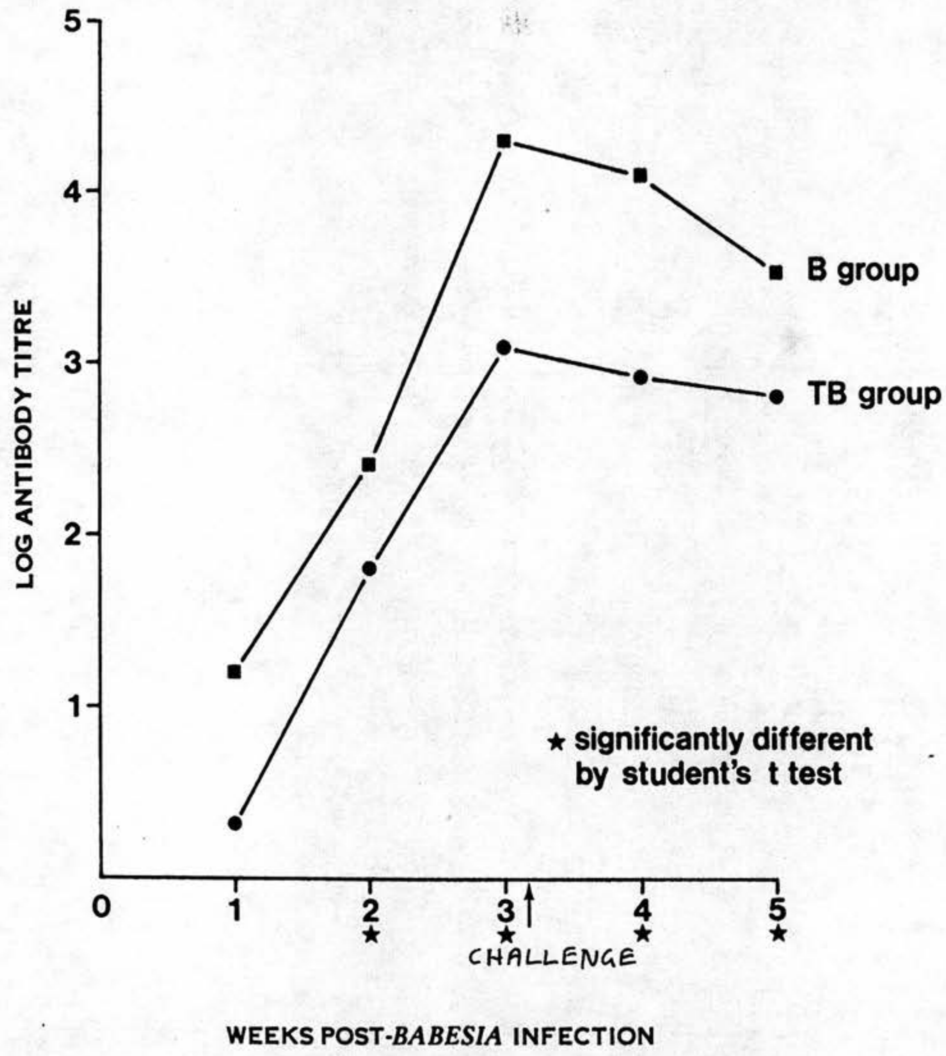
By day 30 all the control mice had fewer than three fields with parasitized cells. On the same day in the concurrently infected group 5/9 mice had three infected fields. Low levels of parasitaemia lingered in the concurrent group until the end of the experiment but the infection disappeared in the control group after day 30.

3.5 The Humoral Response to B. microti

This was assessed by the indirect fluorescent antibody technique (IFAT). The end-point titres that were observed were expressed as log values and the mean values are shown in Figure 3.3. The results

Figure 3.3

The mean antibody response to *B. microti* by mice infected with *Taenia taeniaeformis* and *Babesia* (TB) and the *Babesia* only controls (B) detected by IFAT.


LOG ANTIBODY TITRE
5
4
3
2
1
0
1
2
3
4
5
CHALLENGE
B group
TB group
★ significantly different
by student's t test
WEEKS POST-BABESIA INFECTION

show that after two weeks post infection the mice in the TB group have a significantly depressed antibody response to B. microti when compared with the mice in the B group. The challenge dose in week 3 does not appear to have caused an elevation in the antibody response. Using the hypothesis that the mean end-point titres of the concurrently infected groups may be less than or equal to those of the mice in the B group, the two treatment groups were compared using a t-test.

The degrees of freedom are:

$$(M_1 - 1) + (M_2 - 1) = 8 + 3 = 11$$

where M_1 is the TB group and M_2 is the B group.

In week 4 the sera collected from one mouse in the TB group showed no reading in spite of a clear antibody response to Babesia both before and after this bleed date. For this reason this result was rejected as there was assumed to have been a technical error on this one multi-spot slide. The degree of freedom for that week was therefore 10.

The t-values indicating the level of significance are shown in Table 3.3.

Table 3.3

Week post-Babesia infection	t-value	Level of significance
1	0.54	Not significant
2	9.91	$P < 0.001$
3	5.45	$P < 0.001$
4	2.77	$P < 0.02$
5	3.69	$P < 0.01$
6	5.62	$P < 0.001$

3.6 Spleen/Body Weight Ratio

The spleen was removed from each mouse at necropsy and weighed. These weights were then expressed as a ratio of total body weight. The purpose of this was to determine whether there were any differences in the degree of splenomegaly caused by the *Babesia* infection in the mice in the TB group when compared with the B group. The spleen/body weight ratios of *T. taeniaeformis* and *Babesia* concurrently infected mice are shown in Table 3.4. The results show that the mice in the TB group have a slightly but significantly greater degree of splenomegaly than the B group. The spleen/body weight ratios of the B group, the uninfected controls and the *T. taeniaeformis* only mice are not significantly different from each other.

3.7 The Humoral Response to T. taeniaeformis

This was assessed by an enzyme-linked immunosorbent assay (ELISA) as described in the Materials and Methods section.

3.8 Preliminary Chequer-board Titrations

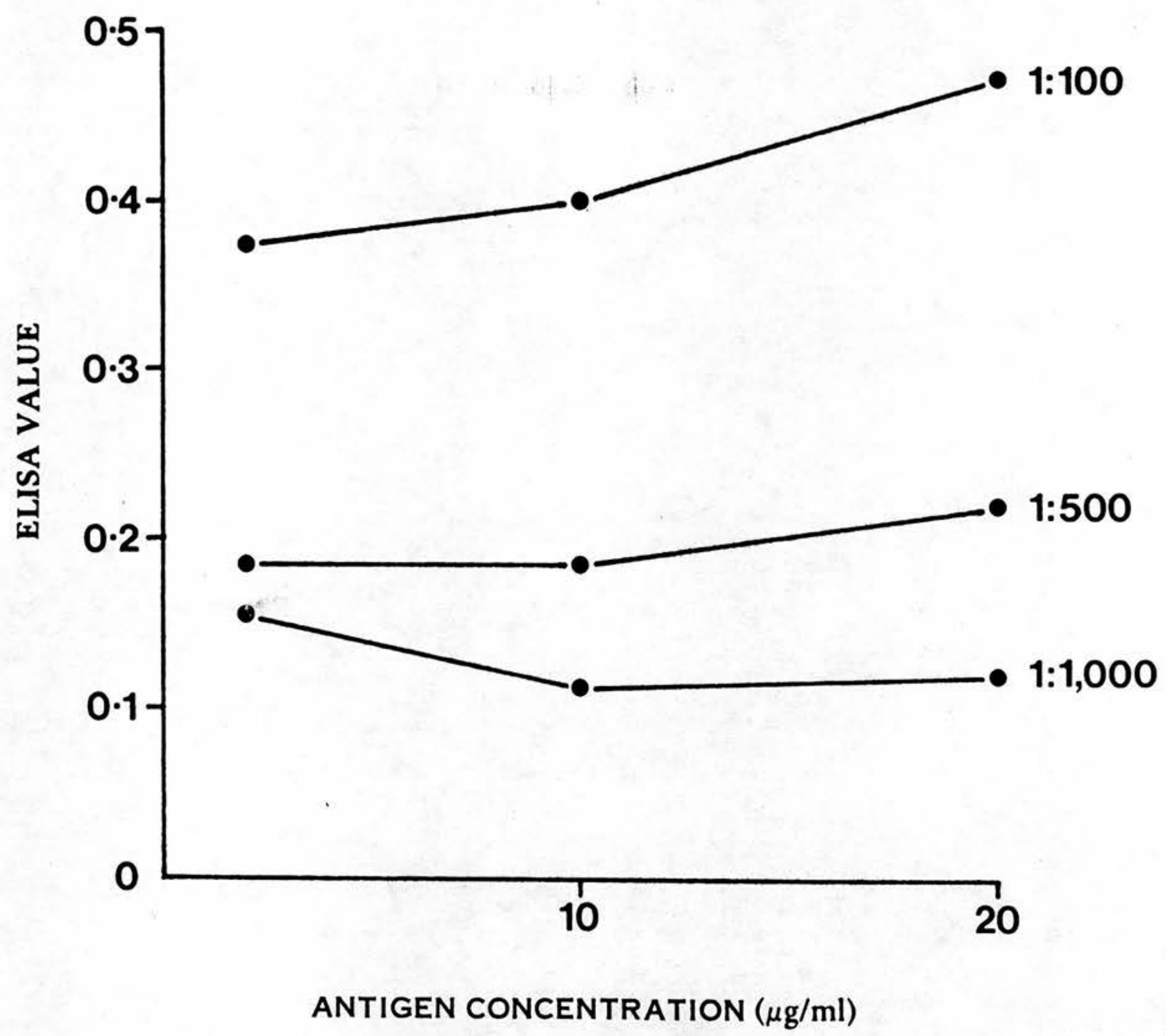
The optimal antigen-antibody dilution range was studied for the *T. taeniaeformis* excretory-secretory antigen preparation. Chequer-board titrations of various protein concentrations of the antigen against positive (12 week old infections) and negative (normal) sera were carried out. Three soluble antigen concentrations were used (1, 10 and 20 μg/ml) against three dilutions of sera (1/100, 1/500 and 1/1000). Such low dilutions of sera were considered preferable in view of the small quantities of sera obtainable from each mouse.

Table 3.4 The spleen/body weight ratios of *T. taeniaeformis* and *Babesia* concurrently infected mice

Treatment	Mean spleen weight ± SD	Mean body weight ± SD	Mean spleen/body weight ratio	t-value	Level of significance
TB	0.825 ± 0.35	43.6 ± 5.9	0.0189 ± 0.0067	2.43	$P < 0.05$
B	0.409 ± 0.23	36.6 ± 2.0	0.0112 ± 0.000		
Uninfected controls	0.23 ± 0.08	37.3 ± 1.7	0.0062 ± 0.004	1.76	Not significant
T. taeniaeformis only	0.23 ± 0.17	41.9 ± 2.3	0.0057 ± 0.006		

Figure 3.4

The absorbance values obtained by ELISA from the chequer-board titrations of various concentrations of the *Taenia taeniaeformis* excretory-secretory antigen against three dilutions of sera. The results are expressed as the differences between positive and negative sera results for each dilution.


ELISA VALUE
0·5
0·4
0·3
0·2
0·1
0
1:100
1:500
1:1,000
10
20
ANTIGEN CONCENTRATION (μg/ml)

3.8.1 Results

Figure 3.4 shows the ELISA values from the chequer-board titrations and from this the optimal antigen concentration was economy selected. The results are expressed as the differences between the positive and negative sera results for each dilution. At the sera dilution of 1:500 and antigen concentration of 20 μg/ml, the difference between the mean positive and negative sera results was 0.24. This was considered a workable difference for subsequent assays whilst having to take into account the need to use the available sera from each mouse economically. Ideally, a sera dilution of 1:100 would be used at this same antigen concentration but this would not have allowed sufficient sera for repeat assays and the IFAT studies.

3.9 Antibody Response to T. taeniaeformis

The mean ELISA values of the TB, B and T. taeniaeformis only groups are shown in Figure 3.5. There is a rise in antibody levels in the TB group following primary and challenge inoculations of B. microti and this is particularly prominent in the case of the challenge dose. This is not found in the T. taeniaeformis only group. The two metacestode infected groups are compared by means of a Student's t-test and the results are shown in Table 3.5. The means are not significantly different from each other except for a period of 1-2 weeks immediately following the inoculation of the unrelated second parasite.

Figure 3.5

The mean antibody response to *T. taeniaeformis* by mice infected concurrently with *T. taeniaeformis* and *Babesia*, and *T. taeniaeformis* only.

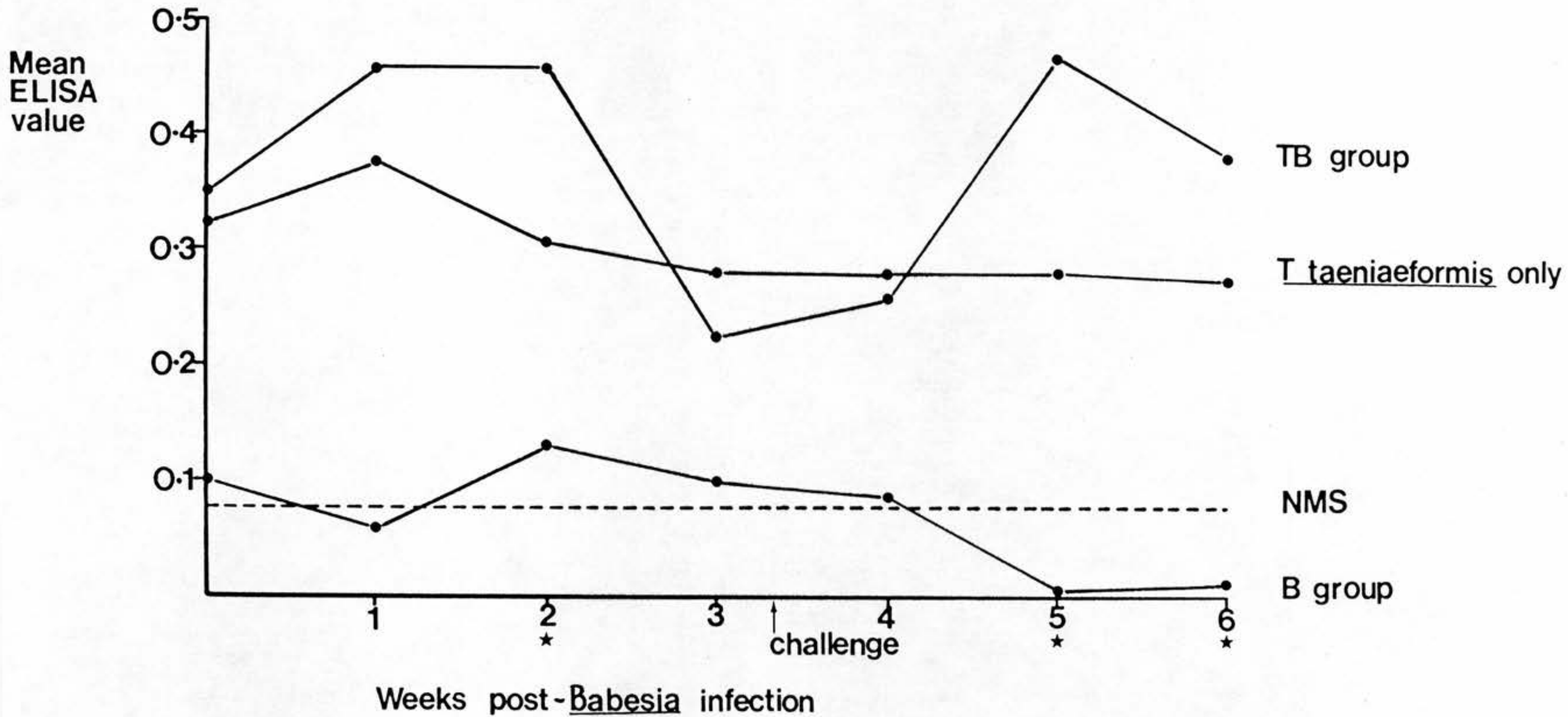

Mean
ELISA
value
0·5
0·4
0·3
0·2
0·1
1
2
3
4
5
6
challenge
Weeks post-Babesia infection
TB group
T taeniaeformis only
NMS
B group

Table 3.5 Comparison of the mean ELISA values of the TB group and the T. taeniaeformis only group by the Student's t-test

Weeks post infection with Babesia	T-value	Level of significance
0	1.51	Not significant
1	1.73	Not significant
2	6.79	Significant at $P < 0.001$
3	2.01	Not significant
4	1.65	Not significant
5	6.24	Significant at $P < 0.001$
6	5.88	Significant at $P < 0.001$

3.10 The Interaction Between Low Level Infections of T. taeniaeformis and B. microti

3.10.1 Aims

The previous experiments investigating the interaction between T. taeniaeformis and B. microti demonstrated that this metacestode is capable of causing immunosuppression. However, in this experiment the mice had large metacestode burdens which were not representative of the burdens commonly found in natural infections. An experiment was carried out in order to see if smaller numbers of metacestodes were capable of similarly increasing the host's susceptibility and reducing the humoral response to B. microti. If it could be shown that lower burdens also mediate immunosuppression then it could be argued that this phenomenon is a valuable mechanism for natural parasitic infections to evade the host immune response.

3.10.2 Experimental design

Twenty six-week-old Balb/c mice were infected by mouth with one of four different doses of viable eggs. The five mice in each

dosage group received respectively 5, 15, 45 or 200 eggs each. The latter group was included as a positive control as this was the infection level used in the previous experiment. Ten mice were set aside as Babesia only controls and inoculated, along with the other mice, with 10^6 B. microti infected red blood cells five weeks later.

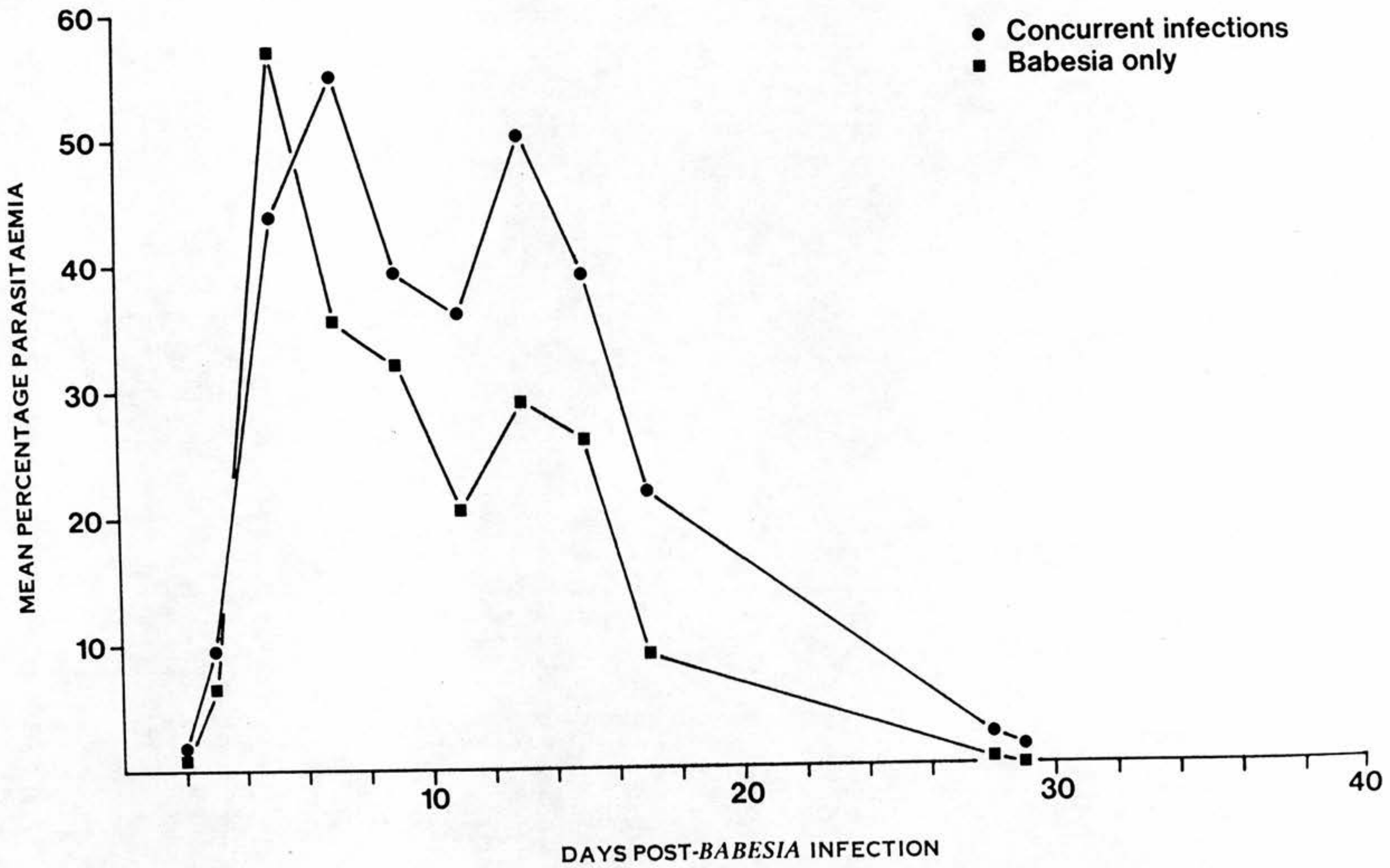
Blood smears were made and examined at frequent intervals to determine the percentage parasitaemia. Larger bleeds (up to 0.2 ml) were taken weekly from the tail for serological analysis. At necropsy at the end of the experiment, spleen weights and numbers of cysticerci in the livers were recorded. As a challenge dose of B. microti on week 9 of this experimental design had not caused recrudescence or any appreciable change in the antibody response to B. microti in the previous experiments, this step was eliminated from the plan.

3.10.3 Results

On necropsy, at the end of the experiment, no cysts were found in any of the mice in either the group that received five eggs or that which received 15 eggs. The groups that received 45 and 200 eggs had mean burdens of 6.8 ± 2.4 and 8.5 ± 3.1 cysticerci respectively, these not being significantly different by the t-test. It was decided to salvage as much information as possible from this experiment by pooling the data from the two infected groups since these contained fairly low level infections. In the previous T. taeniaeformis experiment the mean burden was 62.4 ± 15.3. Thus, the mean burden in the mice in this experiment was approximately one-tenth of that in the previous metacestode burden.

Figure 3.6

The mean percentage parasitaemias of Balb/c mice infected with low doses of *T. taeniaeformis* and *Babesia* and *Babesia* only controls.



MEAN PERCENTAGE PARASITAEMIA
60
50
40
30
20
10
Concurrent infections
Babesia only
10
20
30
40
DAYS POST-*BABESIA* INFECTION

The reasons for the low level of establishing metacestodes in this experiment are unclear but may be associated with a poor batch of eggs. Ideally, this experiment would have been repeated but time precluded this.

3.10.4 Parasitaemia results

The patterns of mean parasitaemias for the two groups following infection with B. microti are shown in Figure 3.6. The data for each day was analysed separately to determine whether the means differed significantly. The ANOVA tables are given in Appendix 3. The days on which the mean parasitaemias were significantly different are indicated by * in Figure . The peak parasitaemia in the Babesia only group was on day 5, two days earlier than that in the combined treatment group. The differences between these two peaks were not significant (Table 3.6).

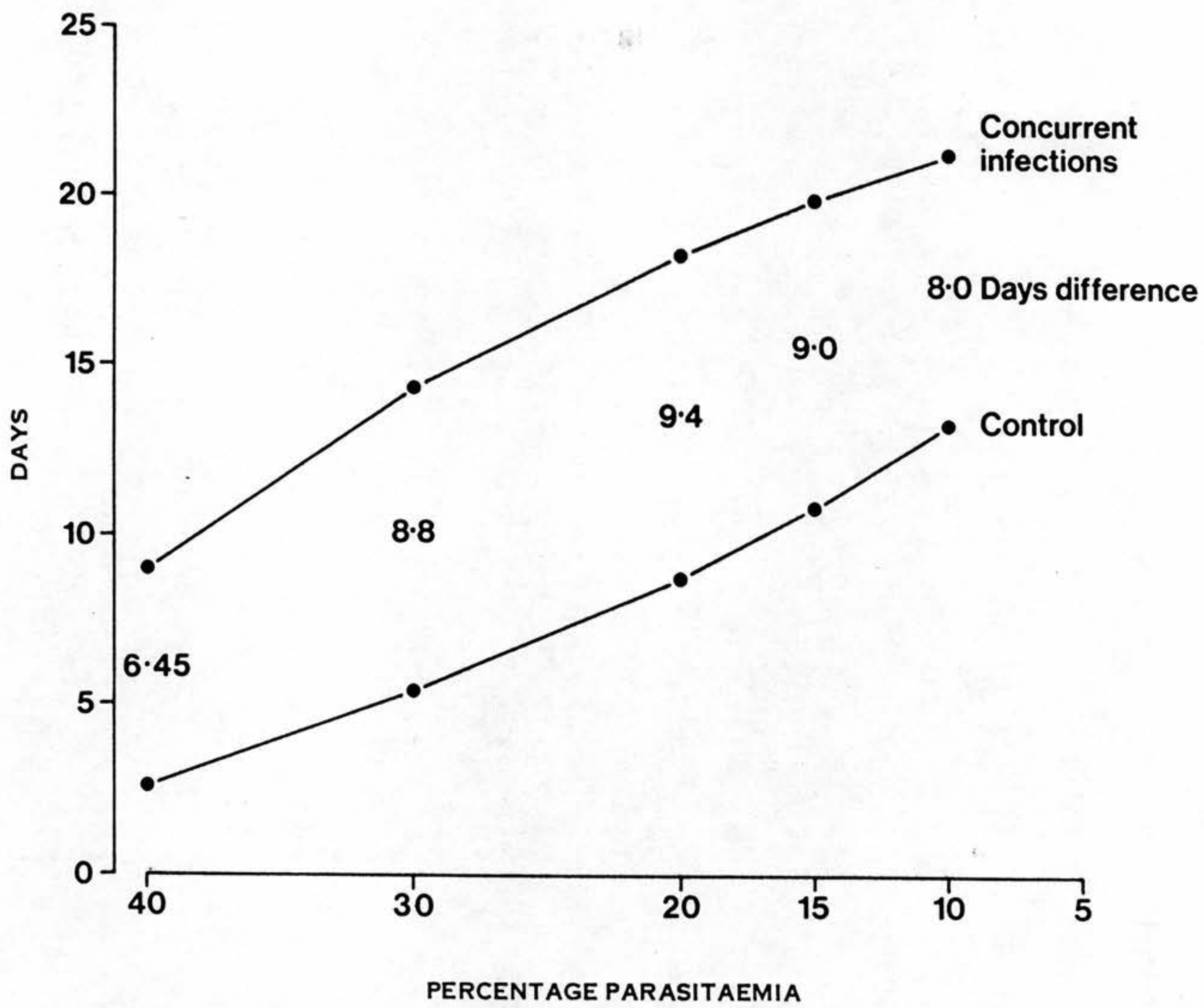
Table 3.6 ANOVA for the peak parasitaemias of the concurrent group (day 7) compared with those of the Babesia only control group (day 5)

Source of variation	SS	df	MS	F
(1) Treatment	9.86	1	9.86	0.04
(2) Field	862.35	2	431.17	2.01
(3) Interaction	271.5	2	135.74	0.63
Error	8,360.4	39	214.4	

The peak level of parasitaemia in the TB group was 55% and 57.5% for the control mice although the percentage parasitaemia in the TB group reached a similar level during the first peak it remained elevated for longer than in the B group. The secondary peak in the TB

Figure 3.7

The rate of decline in parasitaemia expressed as the number of days above a given percentage parasitaemia the *Taenia taeniaeformis* and *Babesia* group and the *Babesia* only control group remain.


DAYS
25
20
15
10
5
0
40
30
20
15
10
5
PERCENTAGE PARASITAEMIA
Concurrent infections
8·0 Days difference
9·0
9·4
8·8
6·45
Control

group was 50%, much higher than the 28% in the B group. Accordingly the mean parasitaemias in the treatment group were significantly greater than those in the control group from day 7 to day 19.

3.10.5 The pattern of decline of the parasitaemia from the first peak

Regression analysis showed that the polynominal form, $PST = a + bT + cT^2$ gave the best fit for the control group as judged by the relatively high R^2 values of 0.73 that were obtained. However, using this form for the treatment group gave a value for b that was not significant. Thus, an equation in the form $PST = a + cT^2$ was found to be the best fit in this case, with all the terms significant and an R^2 value of 0.733. The best fit equations were:-

for Group B $\quad PST = \underset{(11.02)}{50.20} - \underset{(4.36)}{4.290T} + \underset{(2.46)}{0.089T^2}$

for Group TB $\quad PST = \underset{(32.9)}{46.65} - \underset{(15.56)}{0.080T^2}$

As no significant serial correlation of the error terms was found, no correction was necessary. The estimated peak parasitaemia was 50.2% for the control group and 46.6% for the pooled treatment group. The equations also indicated a more rapid decline in the number of infected red blood cells in the control group than the concurrently infected group in absolute terms as shown by the parameter values against days only and day squared.

This is shown most clearly when the regressions were used to determine the number of days that the parasitaemia levels remained above particular values. These are shown in Figure 3.7. The concurrently infected mice experienced any given level of parasitaemia for longer than the control mice. This difference was greatest

between 15% and 20% parasitaemia, indicating that the treatment group reduced the number of infected red cells slower in relative terms than the control group.

A regression was again carried out with the combined data in order to determine directly the least squares estimations of the differences between the groups. These are corrected for unequal replication between groups and can therefore be different from the sample values on any given day. The equation chosen was:-

$$\begin{array}{c} PST = 50.87 - 4.35T + 0.108T \\ (10.72)\ (0.746)\ \ (2.9) \end{array}$$

with an R^2 value of 0.758. The values for the peak parasitaemias indicated are not significant as has been shown by the ANOVA table. The parameters that estimate the differences in decline from the peaks of the two groups are significant and this confirms the results of the regressions carried out on the data separately. This data has been corrected for serial correlation which was found to be significant. As in the previous experiment, the decline in parasitaemia at low levels was also assessed by classifying the mice according to the number of positive fields they show.

Table 3.7

Day	B Group		TB Group	
	Median number of positive fields	Mean parasitaemia	Median number of positive fields	Mean parasitaemia
28	2	0.47	3	1.5
29	1	0.19	3	1.1

3.10.6 The humoral response to *B. microti*

This was again assessed by the indirect fluorescent antibody technique (IFAT). The end-point titres were expressed as log values, as in the previous experiments. A one tailed t-test was carried out as the hypothesis was that the end-point titres of the treatment group would only be expected to be less than or equal to those of the mice infected with *Babesia* only. The t-values obtained when the mean (log) end-point titres were compared are shown in Table 3.8.

Table 3.8

Week post infection	t-value	Level of significance
1	1.66	Not significant
2	8	$P < 0.001$
3	6.98	$P < 0.001$
4	12.94	$P < 0.001$
5	6.96	$P < 0.001$

df = 12

From week 2 to week 5, the TB group of mice had a significantly depressed antibody response to *B. microti* as compared to the control group.

CHAPTER FOUR

CONCURRENT INFECTIONS OF SECONDARY HYDATIDOSIS AND *BABESIA MICROTI*

4.1 Aims

This study was designed to determine whether the low homologous antibody response to secondary hydatidosis (Section I, Chapter Four) was associated with heterologous immunosuppression and whether mice containing transplanted secondary hydatid cysts had a depressed immune response to a subsequent infection with *B. microti*.

4.2 Experimental Design

The infection schedule remained the same as in the previous studies. Ten six-week-old Balb/c mice each received four hydatid cysts which had developed in donor Balb/c mice by surgical transplantation into the peritoneal cavity and five weeks later these mice and five uninfected Balb/c mice of the same age were each inoculated intravenously with 10^6 *Babesia*-infected red blood cells. The mice were challenged intravenously on week 9 with 10^6 *Babesia*-infected red blood cells.

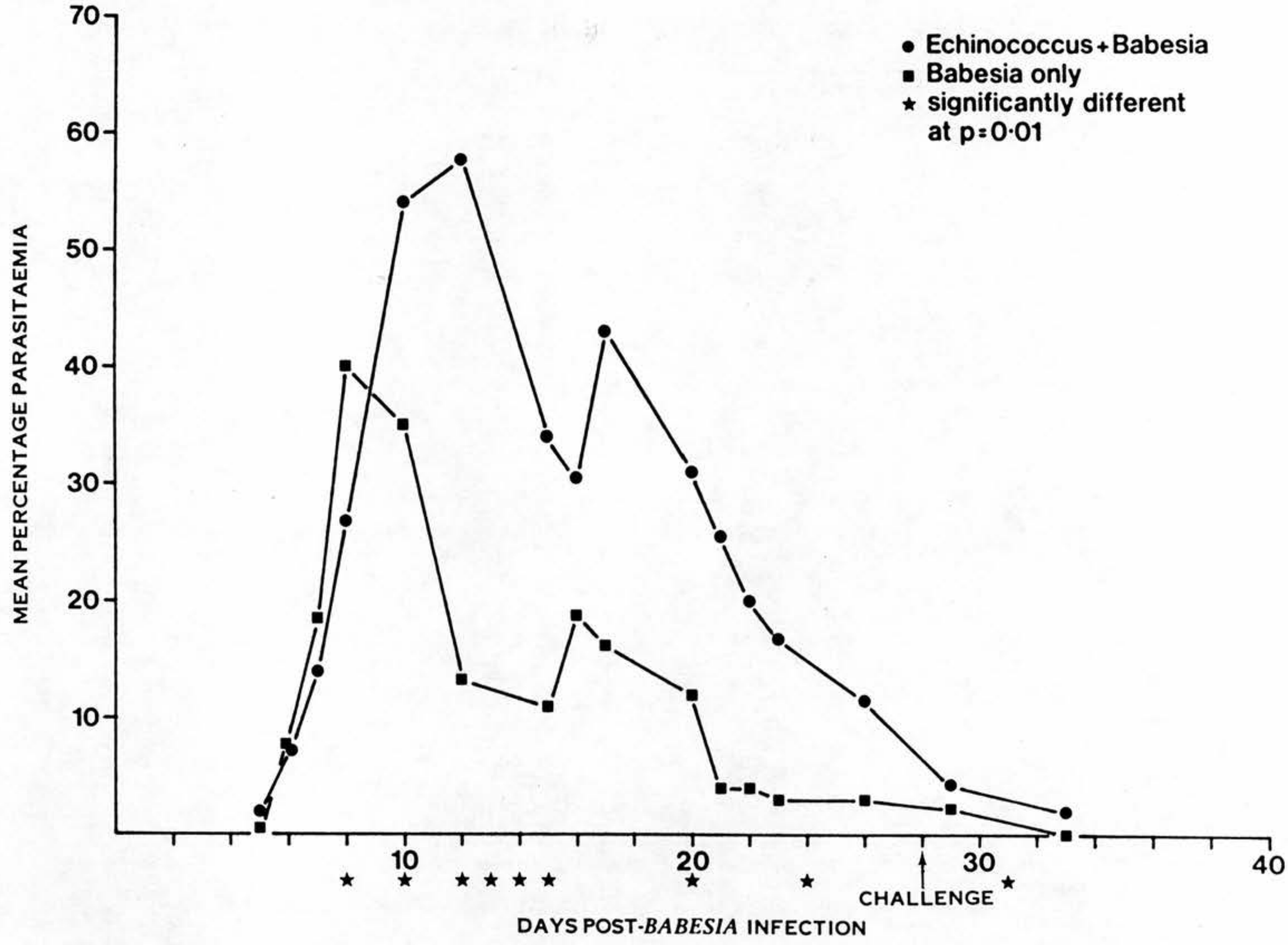
4.3 Echinococcus and Babesia Experimental Results

4.3.1 Daily average parasitaemia results

The pattern of parasitaemia for both treatment groups by days following infection with *Babesia* are shown in Figure 4.1. As usual, the data for each day was analysed separately to determine whether the means differed significantly. The ANOVA tables are in Appendix 3. The peak parasitaemia in the concurrently infected group was on day 10 but that for the *Babesia* only control group was on day 8. Table 4.1 gives the ANOVA data comparing the two peaks on successive days.

Figure 4.1

The mean percentage parasitaemia of Balb/c mice infected with secondary hydatidosis and *Babesia* and the *Babesia* only controls.



● Echinococcus + Babesia
■ Babesia only
★ significantly different
at p=0·01
MEAN PERCENTAGE PARASITAEMIA
10
20
30
40
50
60
70
10
20
30
40
CHALLENGE
DAYS POST-BABESIA INFECTION

Table 4.1 ANOVA for the peak parasitaemias in the concurrently infected group (day 10) compared with those in the Babesia-only control group (day 8)

Source of variation	SS	df	MS	F
(1) Treatment	2,961.24	1	2,961.24	16.08**
(2) Field	12.16	2	6.09	0.03
(3) Interaction	100.24	2	50.12	0.27
Error	4,972.16	27	184.15	

The days on which the mean parasitaemias were significantly different are indicated in Figure 4.1 by a symbol below the y-axis.

The mean parasitaemia was 40% for the control mice and 58.2% for the mice with concurrent infections. The TB group also retained an elevated parasitaemia for longer than that of the control group, this difference being significant from day 9 to day 31.

In both groups there was a secondary peak, this was day 15 for the B group and day 16 for the TB group. Regression analysis showed that the polynominal form $PST = a + bT + cT^2$ gave the best fit as judged by the R^2 values of 0.78 that were obtained. This analysis was again done on the data for the control mice and the concurrently infected mice separately in order to assess the values for the parameters giving the closest fit for each set of data.

Terms with significant parameters were found up to the second power for both groups. These were:-

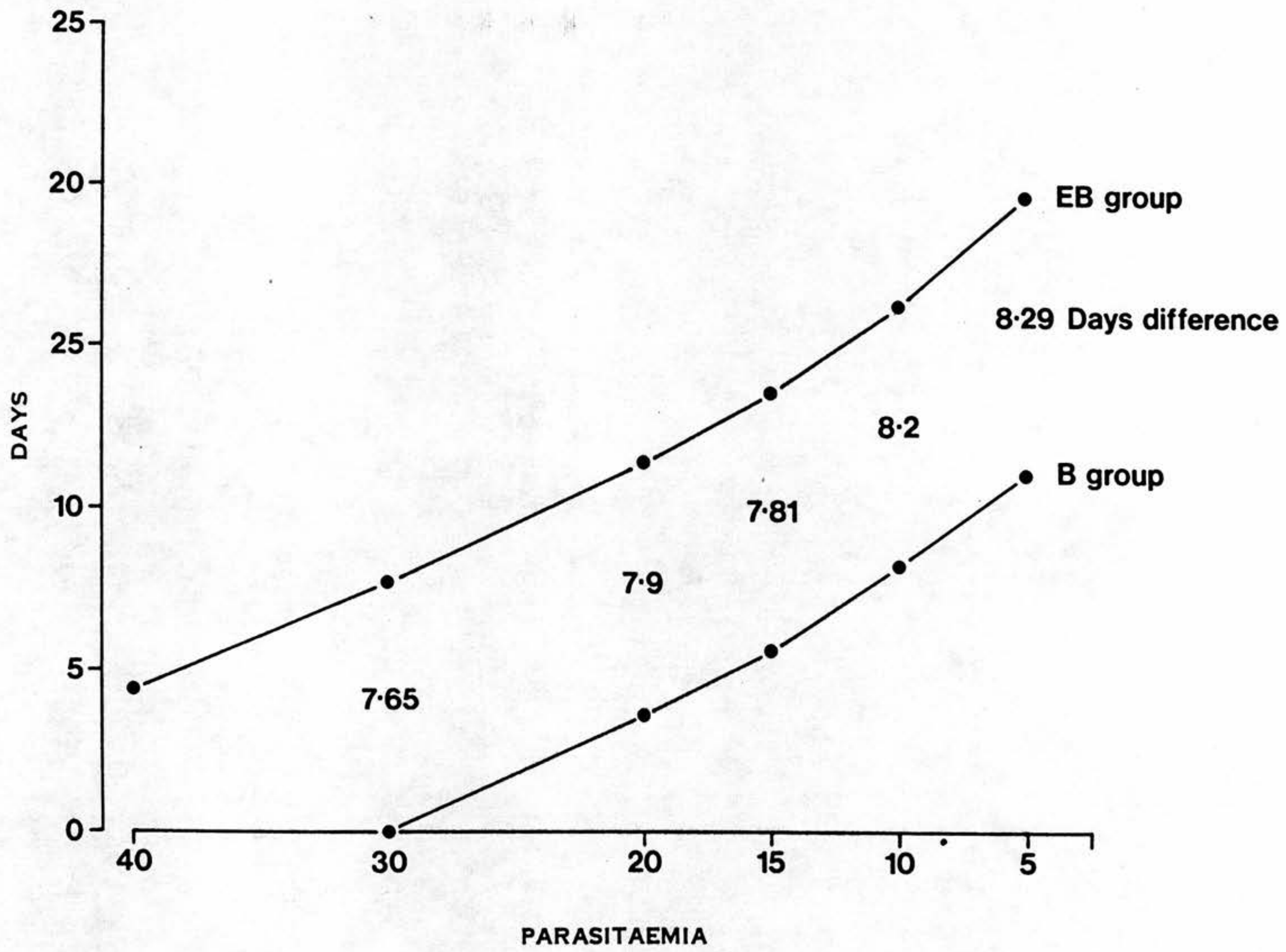
$$PST = \underset{(17.3)}{56.4} - \underset{(8.5)}{9.05T} + \underset{(4.1)}{0.25T^2}$$ for the EB group

and

$$PST = \underset{(19.8)}{30.4} - \underset{(3.8)}{6.4T} + \underset{(6.5)}{0.037T^2}$$ for the B group

Figure 4.2

The rate of decline in parasitaemia expressed as the number of days above a given percentage parasitaemia the EB group and B group remained.


DAYS
25
20
25
10
5
0
40
30
20
15
10
5
PARASITAEMIA
EB group
8·29 Days difference
8·2
7·81
7·9
7·65
B group

No significant serial correlation of the error terms was found so no corrections were needed. From these best fit equations, the estimated peak parasitaemia was 30.4% for the B group and 56.4% for the EB group. The equations also indicated that in both groups there was a similar rate of decline in the number of infected red blood cells per field. This was indicated by the parameter values against days only and by the values of the first differentials of these equations. The regressions were also used to determine the number of days that the parasitaemia levels remained above particular values. These are shown in Figure 4.2. This shows that the concurrently infected mice consistently experienced given levels of parasitaemia for longer than the control mice. This difference increased slightly at lower levels of parasitaemia indicating that the concurrently infected mice reduced the number of infected red cells more slowly than the control group.

A regression was also carried out on all the data combined in order to determine directly the least squares estimations of the differences between the groups. These are corrected for unequal replication between groups and may therefore differ from the sample values on any given day. The values for the peak parasitaemias indicated are 28.25 for the control group and 56.5 for the concurrently infected group, which are similar to those estimated from the separate regressions.

When both slope dummies DT and DT^2 are included neither is significant and there is no change in the R^2 value which remains at 0.81. However, when either is included on its own the parameter is significant indicating that there is a significant difference in

decline from the peak independently of the level of peak parasitaemia. This suggests that the immune mechanisms responsible for resolving the parasitaemias in both groups are neither identical nor proceeding at an identical rate.

As before, the decline in parasitaemia at lower levels was compared by classifying mice according to the number of positive fields observed in the smears from their blood.

Table 4.2

Day	Group B		EB Group	
	Median number of positive fields	Mean parasitaemia	Median number of positive fields	Mean parasitaemia
25	3	11.6	3	3.0
28	1	4.6	3	2.7
32	0	2.3	3	0
34	1	0.6	2	0.5
35	0	0.5	1	0.1
39	0	0	0	0

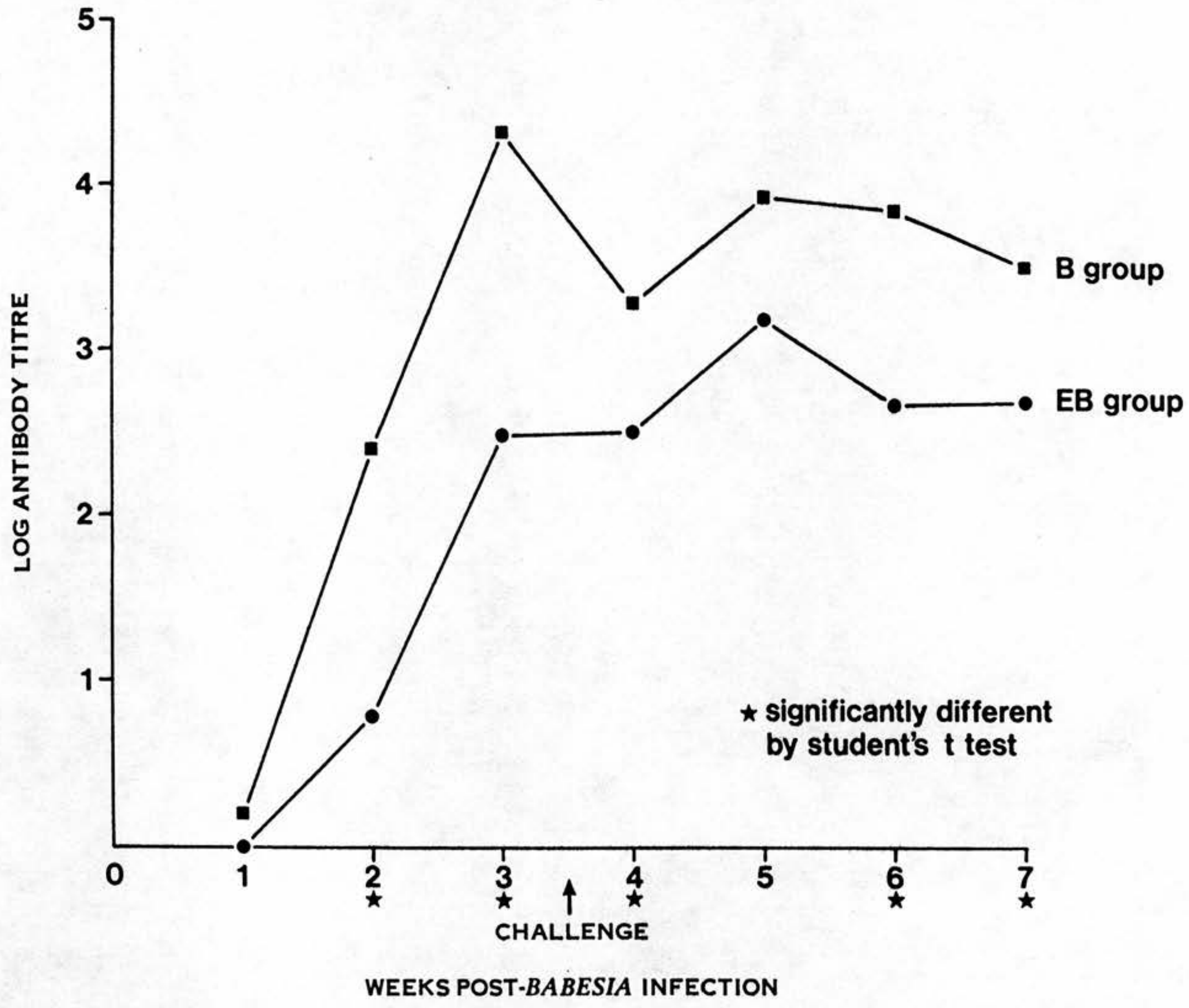
By day 32, none of the control mice had more than two fields with parasitized cells. On the same day, three infected fields were found in smears from 5/6 of the EB group. By day 39, all the control and EB group had ceased to show any parasites in their blood smears.

4.3.2 The humoral immune response to B. microti

From one week after infection with B. microti the mice infected with secondary hydatid cysts and Babesia had a consistently lower antibody response to B. microti than the control group and this was significant for each week except week 5. The challenge dose in week 4

Figure 4.3

The mean antibody response to *B. microti* by mice infected with secondary hydatids and *Babesia* (EB) and the *Babesia* only controls (B) detected by IFAT.


LOG ANTIBODY TITRE
5
4
3
2
1
0
1
2
3
4
5
6
7
B group
EB group
★ significantly different
by student's t test
CHALLENGE
WEEKS POST-*BABESIA* INFECTION

did appear to cause some elevation of antibody response during week 5 but this declined over the following two weeks in both groups. The antibody levels on week 5 declined but remained significantly different from each other.

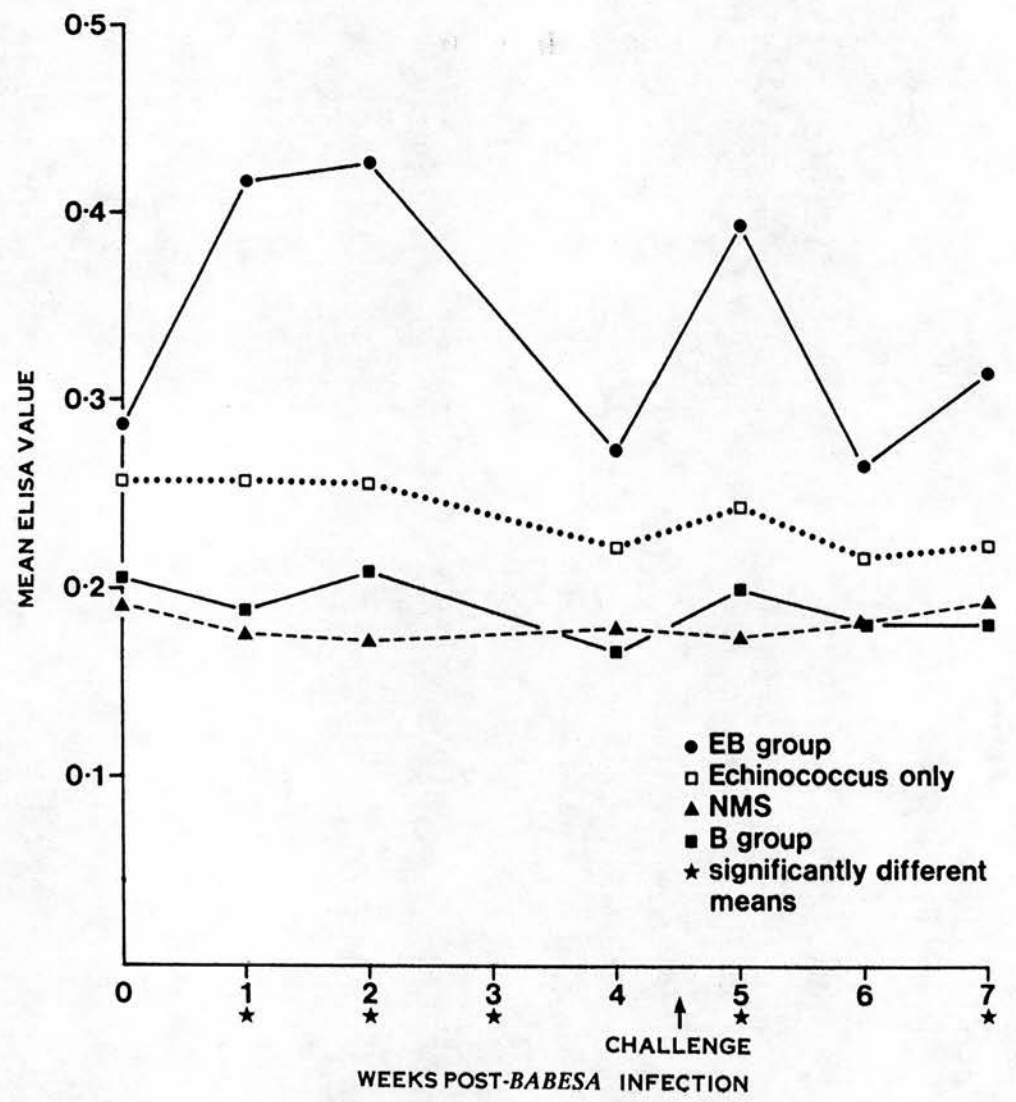
It is not clear why little or no rise in antibody level follows challenge in the T. taeniaeformis and Babesia concurrently infected mice but the same experimental method resulted in a significant increase in both treatment groups in the Echinococcus and Babesia experiments.

4.3.3 The humoral response to Echinococcus granulosus

This was assessed by ELISA. The antigen preparation used to coat the plates was excretory-secretory products collected from the in vitro culture of protoscolices aspirated sheep liver cysts. The antigen was used at a concentration of 20 µg/ml that had been determined from chequer-board titrations described in Chapter Three. The sera dilution was 1:500 and the conjugate dilution 1:1000. A blocking step of 2% FCS was included in preliminary ELISA assays with the same pool of positive and negative sera to reduce the non-specific binding causing less difference between the two control sera. However, as this was found to reduce the overall level of colour development to much the same extent, the practise was discontinued. The procedure of running the antigen preparation through an immunoadsorbent column containing insolubilised goat anti-mouse IgG removes host proteins that might cross-react with the conjugate and proved to be a more efficient means of clarifying the difference between the positive and negative control values.

Figure 4.4

The mean antibody response to secondary hydatidosis of the mice concurrently infected with secondary hydatids and *Babesia* (EB), hydatids only and the *Babesia* only controls (B).


MEAN ELISA VALUE
0·5
0·4
0·3
0·2
0·1
0
1
2
3
4
5
6
7
★
CHALLENGE
WEEKS POST-BABESA INFECTION
● EB group
□ Echinococcus only
▲ NMS
■ B group
★ significantly different means

4.3.3.1 Results: The mean corrected ELISA results with E.g PS antigen in both the Echinococcus and Babesia concurrently infected mice and the Babesia only infected mice are shown in Figure 4.4. The antibody response of the uninfected controls and of the Echinococcus only infected controls are also shown. Table 4.3 shows the t-values obtained when the mean ELISA values of the Ech B group and the Echinococcus only group were compared.

Table 4.3

Weeks post infection	t-value	Level of significance
Pre	1.59	Not significant
1	3.6	Significant $P < 0.01$
2	4.31	Significant $P < 0.01$
4	1.31	Not significant
5	3.17	Significant $P < 0.02$
6	1.7	Not significant
7	2.5	Significant $P < 0.05$

The antibody levels to E.g PS antigen in the Ech B group rose following infection with B. microti but thereafter fell until by week 4 they were not significantly greater than those in the Echinococcus only group. Following the challenge dose of B. microti, the ELISA values from the Ech B group rose again and were again significantly greater than those from the Echinococcus only group. This may be due to a short-term non-specific stimulation of the clones already responding to Echinococcus by the inoculation of an unrelated antigen; but this effect appeared to be of short duration as the antibody levels dropped and were comparable with the Echinococcus only group within four weeks in the case of the primary inoculation of Babesia and two weeks in the case of the challenge dose.

The difference in ELISA values between the B group, the control mice (NMS) and the *Echinococcus* only group is not marked. This appears to be partly due to residual non-specific binding giving elevated values with the sera from Groups B and NMS, caused by host components remaining in the antigen preparation despite immuno-affinity purification. In addition, the mice harbouring the secondary hydatid cysts appear to be producing only low levels of antibody as observed in the work described in Chapter Four. It is interesting that the non-specific stimulation from the intercurrent infection is temporarily able to overcome this immunosuppression.

CHAPTER FIVE

DISCUSSION

The results of the three metacestode species concurrent infections presented in this section have shown common features and will be discussed together.

The initial experiment involving intraperitoneal inoculation of *B. microti* into mice harbouring *T. crassiceps* metacestodes was intended to determine whether this metacestode was capable of depressing the host's immune response to a parasitic infection in the knowledge that this cestode is capable of depressing the humoral response to sheep erythrocytes (Good and Miller, 1976). The parasitaemias in the concurrently infected mice were in fact reduced compared to those in the controls, yet the circulating anti-*Babesia* antibody response was depressed in the concurrently infected group. There are several possible explanations for this, firstly the presence of a large biomass of budding metacestodes in the peritoneal cavity may block or hinder the passage of *B. microti* infected red blood cells into the circulation. However, the depression of the parasitaemia might be accounted for by other factors rather than a physical interaction. Non-specific resistance to *Babesia* can be induced by several agents such as *Cornyebacterium parvum* (Clark *et al.*, 1977), *Brucella abortus* (Herod, Clark and Allison, 1978) and even prior infection with *Trichinella spiralis* (Carlow, Wakelin and Phillips, 1981). It has been suggested that such agents stimulate the production of a macrophage soluble factor which causes intracellular death of the *Babesia* and this could also be the case with the metacestode as peritoneal burdens of *T. crassiceps* are known to be closely associated with numerous macrophages.

The lower antibody response to B. microti may have been due to metacestode mediated immunodepression or simply the result of there being fewer piroplasms present to stimulate antibody production.

This experiment therefore gave a slightly confusing result in that the depressed humoral response did not appear to effect any enhancement of the parasitaemia. Thus, it was felt necessary to verify whether this particular stabilate of B. microti was capable of showing enhancement in an immunosuppressed host, despite the fact that Cox and Wedderburn (1972) also working with the Kings strain of B. microti had shown elevated and prolonged parasitaemias in mice concurrently infected with a lymphomagenic virus. However, as the infected athymic (nude) mice had enhanced and unresolved parasitaemias, it is clear that defects in the thymus-dependent areas of the murine response do exacerbate infections with this strain of B. microti. It must be said that this fact alone does not confirm that this stabilate (TREU 1764) is capable of being influenced by a host immunosuppressed in other ways but it does indicate that mechanisms of immunosuppression acting on T-cell dependent functions can alter the normal course of the parasitaemia. Mice depleted of T lymphocytes by thymectomy, lethal irradiation and reconstitution with anti-theta serum-treated bone marrow cells have also been shown to develop severe infections of B. microti (Ruebush and Hanson, 1980).

When B. microti was later inoculated intravenously into mice infected with intact or burst T. crassiceps metacestodes, the resultant parasitaemias showed a significantly higher peak in the former group suggesting an increased susceptibility to the superimposed infection. The mice inoculated with the burst metacestodes, which

showed no cystic development in the peritoneal cavity at necropsy, did not have a greater primary peak of parasitaemia than the controls but both the experimental groups showed a slower rate of decline in parasitaemia principally due to an extended secondary peak. This latter fact is borne out by the higher number of positive fields in the blood smears at low levels of parasitaemia in these groups.

The humoral response to B. microti indicates that both live metacestodes and parenterally administered disrupted parasite material mediate the depression of antibody levels. There was only a slight increase in antibody levels following the challenge dose of B. microti, which may be due to the infected cells being rapidly destroyed by the previously activated cell-mediated mechanisms shortly after inoculation.

The spleen/body weight ratios were calculated to see whether the concurrent infections affected the normal rate at which the spleen returns to its habitual size as this process is T-lymphocyte dependent (Ruebush and Hanson, 1980) but there was no difference between the ratios in the three groups.

The antibody responses to the larval cestodes measured by ELISA, in this section, show similar levels in the concurrently infected animals as in the animals infected only with metacestodes with the exception of the period of one to two weeks immediately following inoculation of the piroplasm. This could be accounted for by B clones being stimulated by the new antigen to produce both specific antibodies and also antibodies of completely different specificities, particularly in clones previously stimulated by the

presence of the metacestodes. The underlying mechanisms of this apparent contradiction of the strict classical clonal theory are unclear but may reflect secondary disturbances of the idiotype-anti-idiotype network following exposure to antigen or be the result of non-specific T cell stimulation of bystander B cells (Terry *et al.*, 1980). This effect is short-lived and it appears as if the modulator mechanisms rapidly regain control.

The mice concurrently infected with *T. taeniaeformis* and *Babesia* show a similar but more markedly enhanced susceptibility to the secondary infection and an even slower rate of decline compared to the controls. However, in no case did the challenge dose of *Babesia* bring about any recrudescence of the parasitaemia, so despite this evidence of immunosuppression the immunity to reinfection remained solid (Clark, 1979). Once again, at low levels of parasitaemia the resolution of the infection was retarded in the TB group so whereas the immunosuppression was not sufficient to prevent the mice ultimately controlling the piroplasm it did slow down the rate of elimination of the parasites from the blood.

The humoral response shows the same marked depression of anti-*Babesia* antibodies as in the concurrent infections with *T. crassiceps* metacestodes.

In this experiment the spleen/body weight ratios showed a significantly greater degree of splenomegaly in the TB group possibly indicating some impairment of the T lymphocyte-dependent process of recovery.

The effect of secondary hydatidosis on a superimposed infection of *B. microti* showed the most markedly increased susceptibility to

the protozoal infection and the rate of decline from the higher peak of parasitaemia was significantly lower than in the control group. This again suggests that both immune mechanisms affecting the multiplication of the piroplasms and also those influencing the rate of resolution of the infection are impaired by the presence of the metacestode.

The humoral response was again depressed as with the other two metacestode infections and there was little or no increase in antibody levels following challenge with _B. microti_.

In summary, it is quite clear that all three metacestode species are capable of suppressing the immune response to a superimposed infection of _B. microti_.

The factors derived from the metacestodes which are responsible for modulating the course of _B. microti_ infections remain to be elucidated but Good _et al_. (1982) suggested that interaction between larvae and host antibody or cells may be important in stimulating the release of "antigens" responsible for immunodepression. If this is the case, the relevant agents are presumably contained in the disrupted parasite material of the BTC and B group in Chapter Two. Differential shedding of surface antigens has been described by Kemp _et al_. (1980) in schistosomiasis as being due to partial shedding of antibodies bound to different molecules on their surfaces and it is possible that similar mechanisms may exist in larval cestodes. Interaction with host immune cells or antibodies might be involved in determining both the nature of the antigens released into the host circulation and their rate of release. Such a mechanism could account for the variability in the condition of larvae within

a single mouse, the variability of antigens recognized by sera from patients with cysticercosis (Flisser et al., 1980) and the false negative serological results found in some hydatid patients (Chemtai, 1981).

Excretory-secretory products collected from the in vitro culture of T. crassiceps larvae can mediate immunosuppression (Good et al., 1982) and this material will include sloughed off membrane proteins and components of the glycocalyx as well as metabolic products.

It is not possible to say whether the three metacestode species studied here modulate the immune response of the host in the same way but the effects on parasitaemia and antibody response are similar.

Good et al. (1982) found that with T. crassiceps the depression of the IgG response was more pronounced than was depression of the IgM response to sheep red blood cells. This is not known for T. crassiceps and E. granulosus but it is probable that this could occur in view of the similarities of their parasitaemic and humoral results. The precise way in which such a depression could affect the enhancement of the Babesia parasitaemia is unclear but Phillips (1981) has proposed that protective antibodies, which may be thymus-dependent, inhibit the invasion of red cells, and control at this early stage in infection may be defective in the metacestode infected hosts. In addition, depressed antibody levels may affect other immune mechanisms including opsonization, phagocytosis, and the production of soluble factors from immune macrophages that are thought to be facilitated by antibodies. Alternatively, disruption of the hosts immune response may be a more central effect such as an increase in the numbers of

suppressor cells (Lammie and Katz, 1984) or the result of polyclonal activation leading to clonal exhaustion (Terry *et al.*, 1980), which has been postulated to be a feature of secondary echinococcosis (Cox *et al.*, 1984). These workers found enhancement of transformational response in the early stages of infection but after 42 days there was a depression in the mitotic activity in the lymphocytes which could produce a functional indifference to both the metacestode and to superimposed infections. This aspect of immunosuppression will be discussed further in Section III, where the level of immunoresponsiveness of mice infected with these three metacestodes is investigated.

Finally, this phenomenon of immunosuppression and the way in which it aids parasite evasion of the host response need not involve only one mechanism, in fact it is more likely to modulate a variety of immune responses. For example, it is thought that hydatid cysts induce a local impairment of the immune response by parasite-derived toxins (Annen *et al.*, 1980) but this process of evasion must be additional to those mediating the effects on an heterologous parasite as shown in this study. However, what role this non-specific immunosuppression of the response to *B. microti* by mice infected with larval cestodes plays in relation to the evasion of host immunity by the metacestode remains unclear, as there would seem to be no direct advantage to the metacestode but, possibly, the overall suppression of host immunity also impairs the host's ability to adversely affect the metacestode.

SECTION III

CHAPTER ONE

LYMPHOCYTE TRANSFORMATION BY LARVAL CESTODES

1.1 Introduction

Lymphocytes become transformed when stimulated by a variety of agents, including several plant lectins and certain components of bacterial cell membranes. The early work in this field carried out by Nowell (1960) showed that the addition of a small quantity of an extract of red kidney bean, Phaseolus vulgaris to human T lymphocyte cultures resulted in large, active cells which became mitotic by the third or fourth day of culture. Substances that evoke significant stimulation without an immune basis necessitating specific immunity are known collectively as mitogens. Several mitogens are known to selectively stimulate different lymphocyte sub-populations. Concanavalin A (Con A) stimulated both immature and mature thymocytes whereas the extract used by Nowell (1960) now called phytohaemagglutin (PHA) stimulated only the mature sub-population. These mitogens also activated peripheral T lymphocytes.

Blastic transformation usually involved both afferent and efferent events in the process of inducing an immune response, consisting of a "self" histocompatibility determinant and a foreign antigen. However, a single stimulus was occasionally sufficient and this was the situation when T cells were exposed to certain plant lectins or foreign histocompatibility antigens which led to effector activity in the lymphocytes without requiring any antigenic pre-sensitization of the lymphoid populations (reviewed by Katz, 1977).

Lymphocyte transformation has been widely used as a means of assessing the immune responsiveness of a host to a particular parasite or antigen both in vitro and in vivo, as the degree of cellular

proliferation can be assayed quantitatively from the level of ^{3}H-thymidine incorporation resulting from DNA synthesis. This technique has been used extensively to study the suppression of murine lymphocyte responses to mitogens *in vitro* by trypanosomes (Jayawardena, Waksman and Eardly, 1978; Mansfield, 1981; Maleckar and Kierszenbaum, 1983) by monitoring the blastic response of normal spleen cells to plant mitogens when cultured in the presence of various parasite extracts. The *in vivo* immune response of mice infected with trypanosomes had been studied by the removal and culture of lymphocytes at various times throughout infection, assaying their proliferative response to a plant mitogen. This approach has been used in the study of helminth infections by Lammie and Katz (1984) who found that *Brugia pahangi* was capable of suppressing the host's immune response by studying the *in vitro* culture of spleen cells from infected jirds and *Nippostrongylus brasiliensis* infections in rats were also monitored this way (Bloch, Towle and Mills, 1977). The analysis of the *in vitro* responsiveness of normal lymphocytes to helminth parasite extracts has been used by several groups of workers to determine the nature of the immunomodulation by certain infections. For example, by using this system Auriault, Dessaint, Mazingue, Loyens and Capron (1984) were able to show that a low molecular weight, heat stable fraction of *Schistosoma mansoni* was capable of potentiating both T- and B-lymphocyte proliferation.

Primary lymphocyte transformation has been demonstrated in the pre-establishment phase of secondary hydatidosis by Dixon *et al.* (1982). They found that protoscolices induced normal, naive cells from Balb/c mice to undergo rapid transformation to lymphoblasts

and to commence mitosis as indicated by both direct cytological observation and increased ^{3}H-thymidine (^{3}H-Tdr) incorporation. This was not thought to be dependent on antigenic priming or to occur only with lymphocytes from this mouse strain as it has been demonstrated with spleen cells from day-old mice, which precludes presensitization, and with lymphocytes derived from normal horses indicating that the response is not limited to strain or species among mammals. There is evidence that such stimulation occurs with other metacestode species, as extracts of the metacestode of _T. solium_ induce primary transformation (Willms, 1975; Sealey, Ramos, Willms and Ortiz-Ortiz, 1981). A strong blastic response has also been reported with the cyst fluid from the coenurus of _T. multiceps_ (Judson _et al_., in press). The _E. granulosus_ protoscolices - mouse lymphocyte interaction has been found to require actual contact with the living parasite for the full lymphoproliferative response to occur (Dixon _et al_., 1982). The nature and origin of these mitogenic effectors has not been identified. In the case of _E. granulosus_ the possibility that it is due to accretion of host material has been ruled out as protoscolices grown in Balb/c mice stimulate syngeneic cells (Dixon _et al_., 1982).

Conversely, there is evidence that factors circulating in the plasma of rabbits heavily infected with _T. pisiformis_ can inhibit lymphocyte reactivity in lectin-mediated transformation assays (Rickard and Williams, 1982). Also, the addition of _E. granulosus_ cyst fluid to lectin-stimulated lymphocyte cultures has a depressive effect on blast transformation (Annen _et al_., 1980). Thus, there appears to be a complex of non-specific stimulatory or inhibitory factors associated with metacestode infection but their precise role

in facilitatory evasion of the host immune response remains unclear. Indeed the complexity is highlighted by the recent findings of Cox (1984), working on the *E. granulosus*-murine lymphocyte system, that infections longer than 42 days result in a marked depression of mitotic activity.

1.2 Aims

A study was undertaken to determine whether metacestode extracts and live metacestodes of the three species previously shown to suppress the immune response to a concurrent infection with *B. microti*, were modifying the response of spleen and lymph node cells to the mitogens Concanavalin A (Con A) and phytohaemagglutinin (PHA). The work was divided into two parts:-

(a) Assessing the *in vitro* transformation of murine lymphocytes from non-immune donors by cestode extracts or living larvae.

(b) Assessing the *in vivo* effect of the cestodes by studying the ability of extracts and living larvae to cause transformation in lymphocyte subpopulations from mice carrying metacestode burdens.

This study was intended to aid the elucidation of the way in which these parasites interact with their host's immune response and induce immunosuppression.

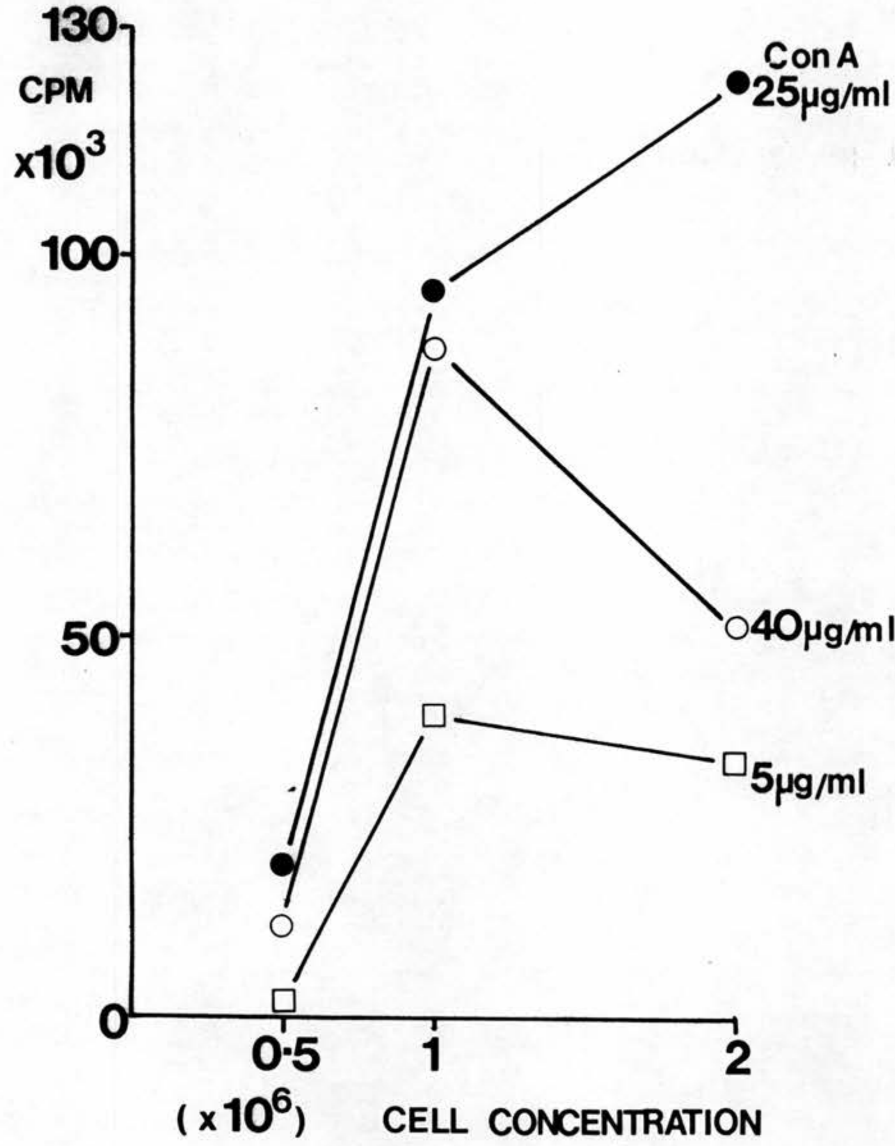
1.3 Standardisation of Lectin Dose and Cell Concentration

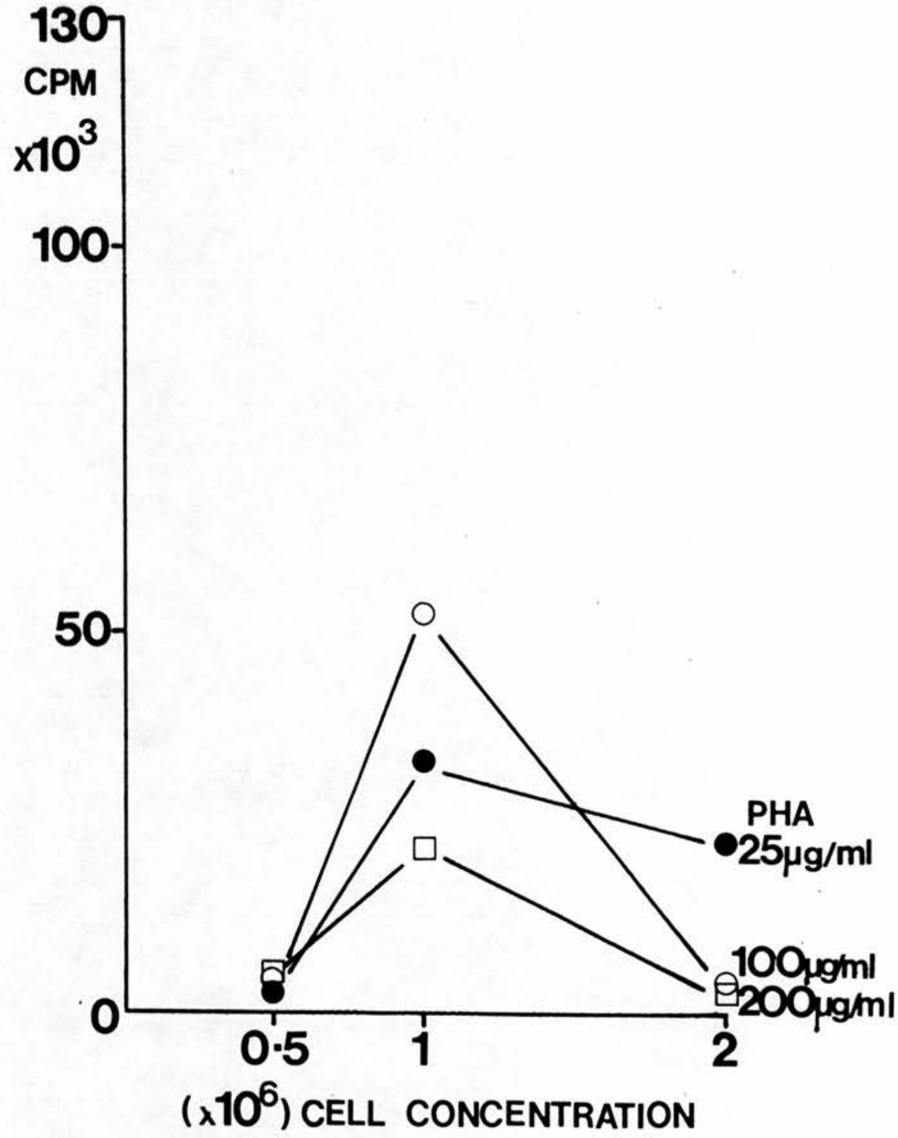
A series of experiments were conducted to define the lectin and cell concentrations giving optimal blast transformation by murine mesenteric lymph node cells.

Mesenteric lymph node cells from uninfected six-week-old Balb/c mice were dispensed into flat-bottomed microtitre plates to give

Figures 1.1 and 1.2

The mean ^{3}H-Tdr incorporation of naive MLNC at three concentrations when stimulated with three strengths of Con A or PHA. The results are expressed as counts per minute.


130
CPM
x10³
100
50
0
0·5
1
2
(x10⁶)
CELL CONCENTRATION
Con A
25μg/ml
40μg/ml
5μg/ml


130
CPM
x10³
100
50
0
0·5
1
2
(x10⁶) CELL CONCENTRATION
PHA
25μg/ml
100μg/ml
200μg/ml

5×10^5, 1×10^6 or 2×10^6 cells per ml. Con A was added to the wells to give final concentrations of 5, 25 or 40 μg/ml and PHA likewise to concentrations of 25, 100 or 400 μg/ml.

The results are summarised in Figures 1.1 and 1.2 for Con A and PHA respectively and the detailed data are given in Appendix 4. Con A gave a good response at 25 and 40 μg/ml when cells were used at 1×10^6/ml and 2×10^6/ml. The response to all three concentrations of PHA at the lowest cell concentration was negligible. The best responses were observed at a cell concentration of 1×10^6/ml when the lectin was used at 100 μg/ml.

Overall PHA was clearly a poorer mitogen in this system than Con A and for all future assays only Con A was used. The cell concentration chosen for the subsequent experiments was 2×10^6/ml and the mitogen concentration chosen was 25 μg/ml, since these concentrations gave a high level of stimulation.

1.4 Assay of Larval Cestode Extracts for Cytotoxicity

1.4.1 Aim

Surface larval cestode extracts have been prepared as described in Section I, 3.10 but in order to verify that any observed effects in subsequent lymphocyte transformation assays were not due to traces of the preparative chemicals, such as N-octyl glucoside, remaining after purification, crude and purified extracts were added to a culture of a bovine lymphosarcoma cell line. This cell line normally multiplies exponentially *in vitro* thus the effect of the crude or purified extracts on the normal pattern of growth could be monitored by ^{3}H-Tdr incorporation and cell viability studies. It

was felt that if cytotoxic effects remained in the purified extracts, these could then be ascribed to the parasite rather than to chemical contaminants.

1.4.2 Experimental design

The bovine lymphosarcoma cell line (BL20) which had been maintained in culture for 49 passages in the Protozoology Section of the Centre for Tropical Veterinary Medicine, was seeded into the 24 wells of a multi-dish plate (Costar, Nunc) at a concentration of 1 x 10^6 cells/ml in each of the wells with the exception of the peripheral wells. These were filled with 1 ml of RPMI 1640 plus media and additives only. The peripheral wells commonly give poor growth probably due to inconsistencies in the humidity and pH encountered on the outside of a plate but filling them with media only maintained the humidity for the inner test wells. This cell concentration has been found to be optimal for the normal growth of BL20 with minimal cell death (Brown, 1984). The metacestode extracts were used at the same concentration as in the previous lymphocyte transformation experiment - 25 μg/ml, all three surface extracts and the excretory-secretory products from the Echinococcus protoscolices being used in this study. For each surface extract two kinds of sample were prepared:

(a) The extract before purification by passage through a column packed with Sephadex G-25 (medium) (Pharmacia Fine Chemicals) which has a fractionation range of 1,000 to 5,000 so that molecules with a molecular weight above the upper limit of this range are totally excluded from the gel and elute in the void volume, for example

the small molecules of the solubiliser, N-octyl glucoside (N-O-G) will have been retained while the much larger proteins in the surface extracts were collected in the void volume and used in the assay.

(b) The extract after purification, as used in the previous lymphocyte transformation assay.

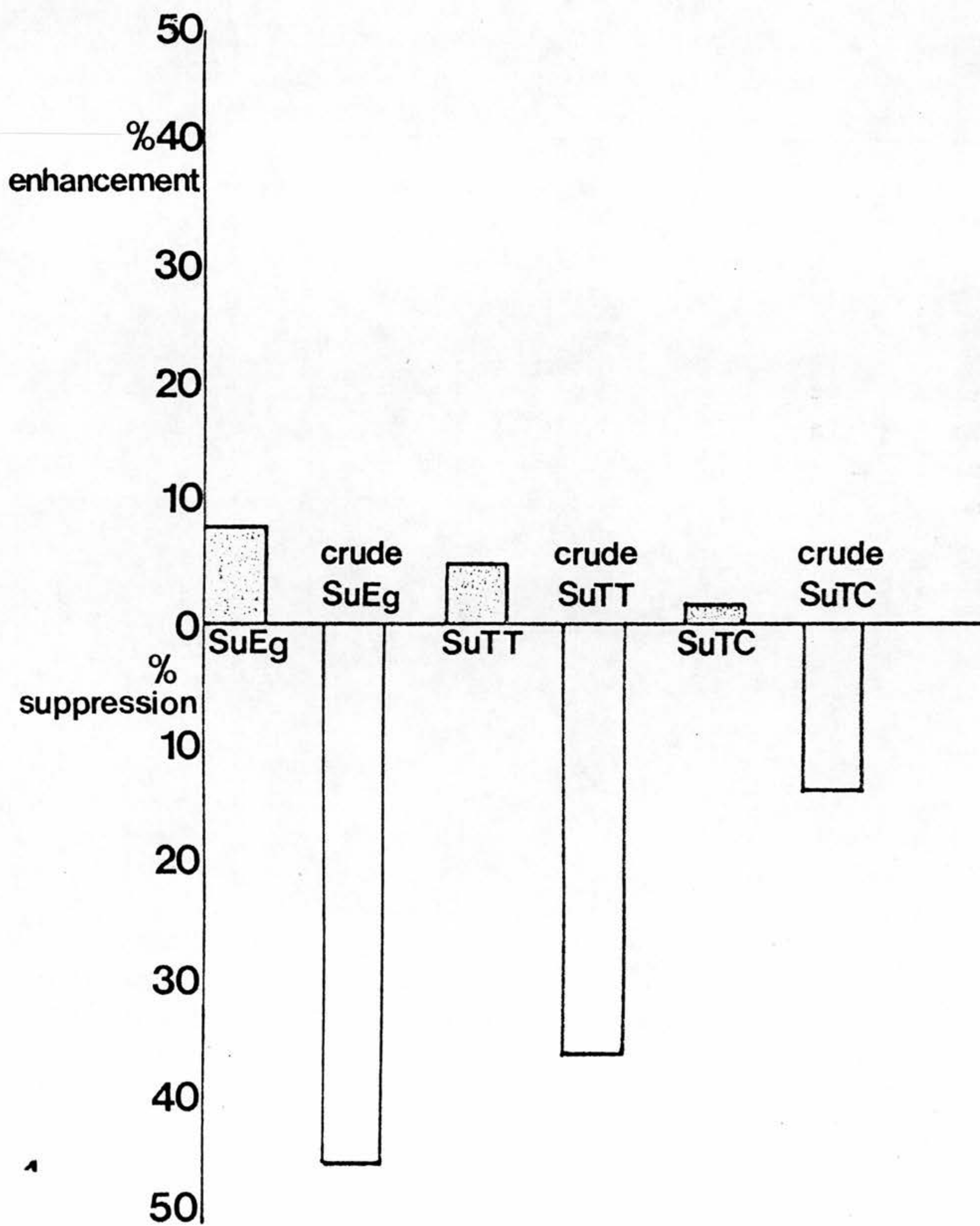
The extracts were added to the Costar wells on day 0 and the cultures maintained for three days in a 6.3% CO_2 incubator at 37°C. On day 3, the cell suspensions in each well were well mixed by pipetting up and down with a sterile Pasteur pipette and 200 µl of this suspension was removed to a 96-well microtitre plate (Nunc). These new cultures were clearly labelled as to treatment and 18.5 kBq of 3^H-methyl-thymidine was added to each well. Eighteen hours later the cells were harvested, washed onto glass fibre pads (Flow Laboratories) and the amount of incorporation assessed.

The remaining BL20 cells in the Costar plates were used to prepare cytocentrifuge smears so that the appearance of the cells could be examined and the percentage viability calculated, using the dye exclusion test.

The ^{3}H-Tdr incorporation data was expressed as the percentage enhancement or suppression of the rate of incorporation found in the unstimulated control wells. The mean counts are shown in Appendix 4 as are the t-values obtained when the means were compared with the level of incorporation in the control wells.

Figure 1.3

The mean percentage enhancement or suppression of the normal ^{3}H-Tdr incorporation of BL20 cells when crude or purified larval cestode surface extracts are added to the cultures for three days.



50
%40
enhancement
30
20
10
0
%
suppression
10
20
30
40
50
SuEg
crude
SuEg
SuTT
crude
SuTT
SuTC
crude
SuTC

1.4.3 Results

Figure 1.3 shows the mean percentage enhancement or suppression of ^{3}H-Tdr incorporation by the multiplying BL20 cells caused by each of the extracts. All the crude surface extracts caused significant suppression, whereas when these extracts had been treated by passage through a G-25 column no suppression was found. All these surface extracts gave between 1 and 8% enhancement but these results are not significantly different from the control incorporation rates.

1.4.4 BL20 cell viability

Examination of Giemsa-stained cytocentrifuge smears made on day 3 of culture showed a large proportion of dead cells from the wells that had received the crude surface extracts of the three metacestode species. The smears made from wells that had received the purified surface extracts showed a low proportion of dead cells, but fewer with a lymphoblastoid appearance.

The result from this cursory examination of cell viability was consolidated by the calculation of the percentage cell viability by means of the dye exclusion test on days 1, 2 and 3 of culture. The results are shown in Appendix Table 4.6 and are expressed as mean percentage viability. The results are also shown in Figure 1.4.

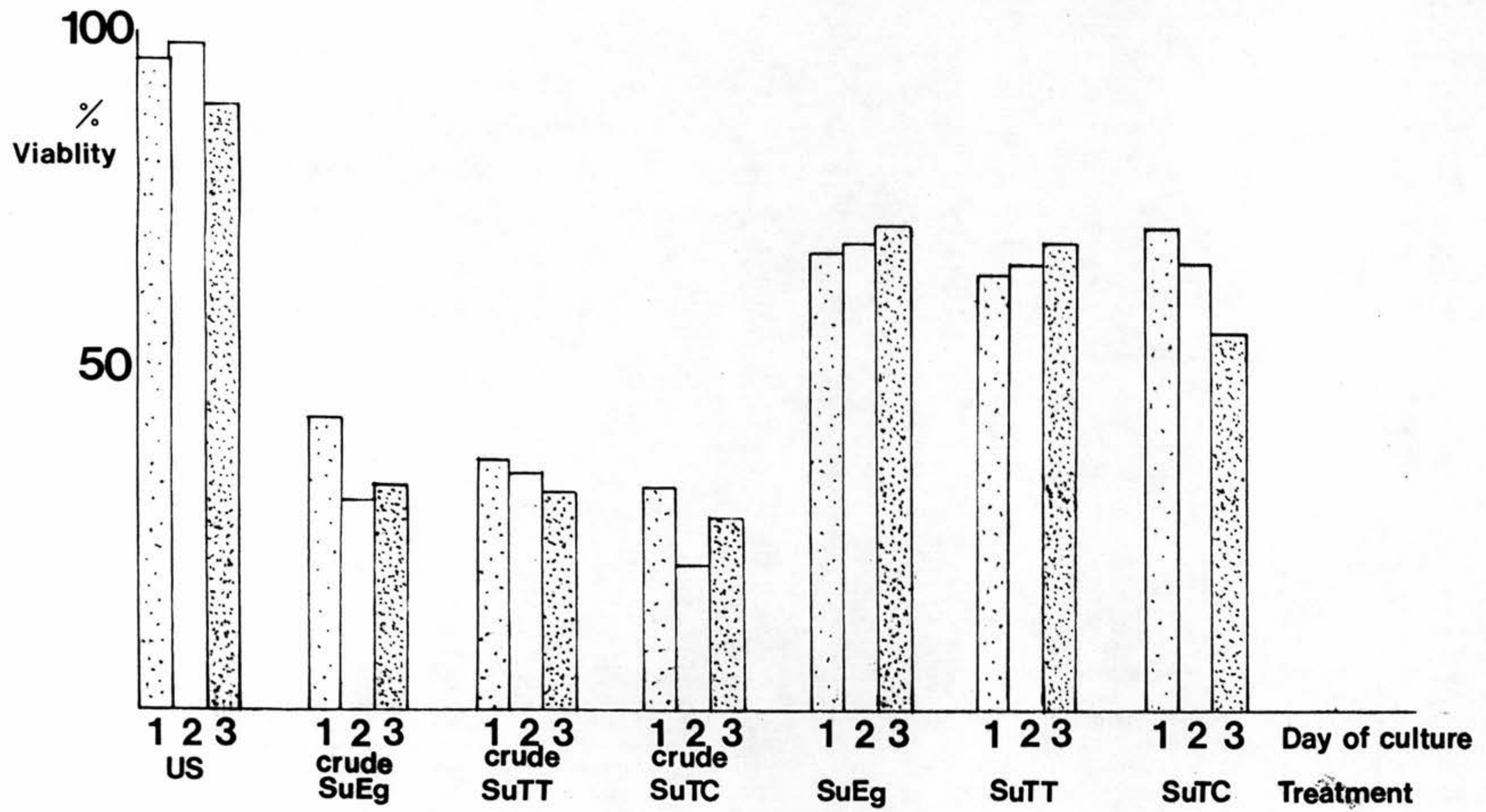
1.5 Incorporation of ^{3}H-thymidine by Naive Murine Lymphocytes Stimulated with Cestode Antigens or Con A

1.5.1 Aim

This experiment was devised to study the lymphoproliferative response of mesenteric lymph node cells (MLNC) to metacestode extracts.

Figure 1.4

The mean percentage viability of BL20 cultures on Day 1, 2 and 3 of culture when crude or purified extracts are added.


100
%
Viablity
50
1 2 3
US
1 2 3
crude
SuEg
1 2 3
crude
SuTT
1 2 3
crude
SuTC
1 2 3
SuEg
1 2 3
SuTT
1 2 3
SuTC
Day of culture
Treatment

The cells were derived from naive donors and the study therefore indicates whether any of the parasite preparations used were capable of causing primary blastic change in the absence of presensitization of the host.

1.5.2 Experimental design

MLNC were derived from six-week-old Balb/c mice and the cell suspensions made up to a concentration of 2 x 10^6/ml.

The metacestode extracts were initially used at two protein concentrations , 25 µg/ml and 0.25 µg/ml. The extracts used were:

(a) A surface extract of secondary hydatid cysts (Su Eg).

(b) A somatic extract of secondary hydatid cysts (So Eg).

(c) Excretory-secretory products of hydatid protoscolices (EgES)

(d) A surface extract of Taenia crassiceps (Su Tc)

(e) Excretory-secretory products of T. crassiceps (TcES)

(f) A surface extract of Taenia taeniaeformis (Su TT)

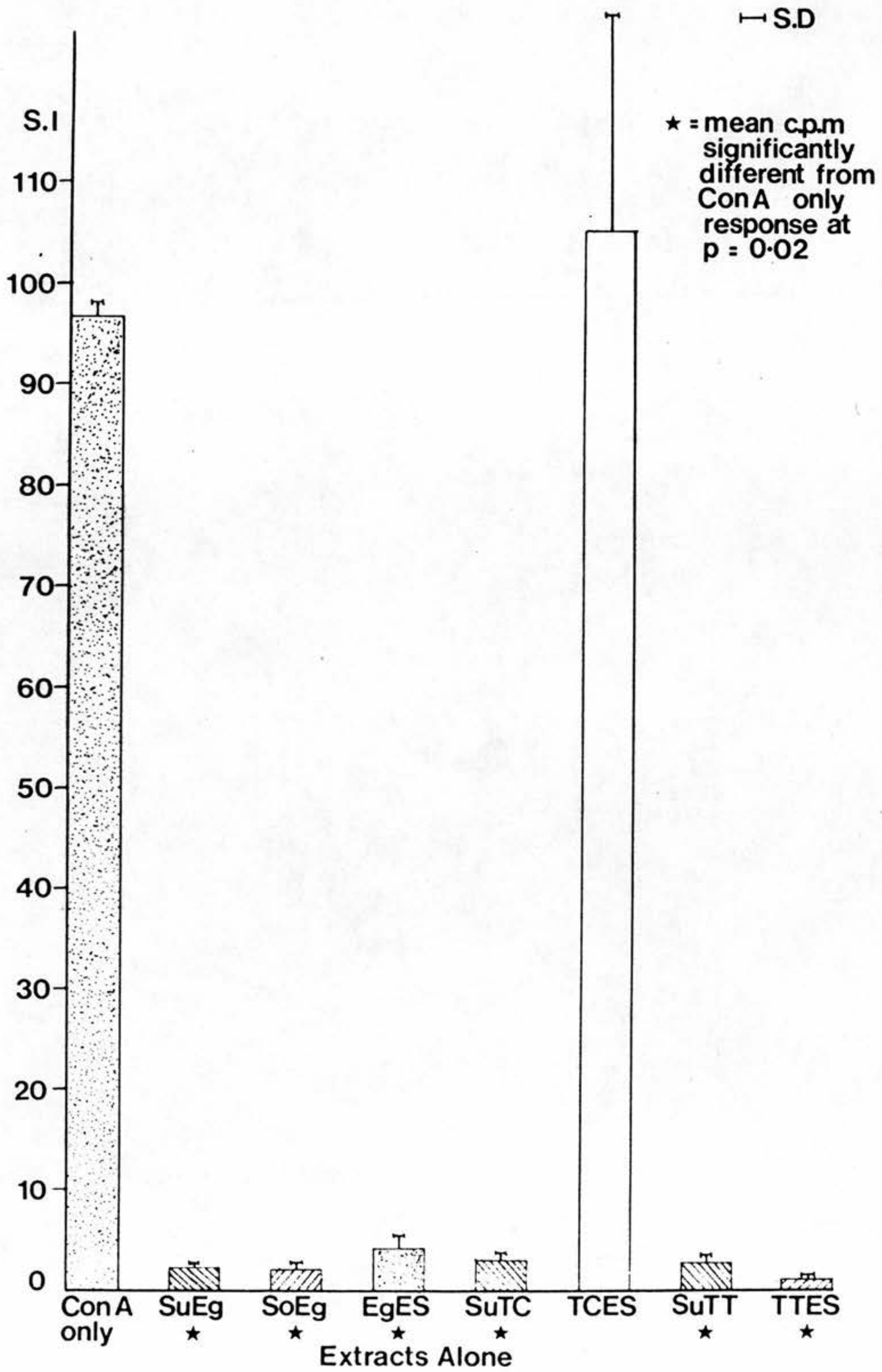
(g) Excretory-secretory products of T. taeniaeformis (TTES)

All the extracts were sterilised by passage through a 22 µm membrane filter (Millipore, U.K.) prior to addition to the cultures. The mitogen concentration used was 25 µg/ml.

The results are expressed as mean stimulation indices (SI) for the five replicates with each extract. They were compared in a Student's t test with the positive controls, which were MLNC stimulated with Con A only. The response of MLNC to parasite extracts together with Con A was similarly expressed as the percentage suppression in the mean MLNC and Con A stimulation caused by the addition of the metacestode extracts.

Figure 1.5

The mean stimulation indices by MLNC from naive Balb/c mice when cultured with metacestode extracts.



S.D
★ = mean c.p.m significantly different from Con A only response at p = 0·02
S.I
110
100
90
80
70
60
50
40
30
20
10
0
Con A only
SuEg ★
SoEg ★
EgES ★
SuTC ★
TCES
SuTT ★
TTES ★
Extracts Alone

1.5.3 Results

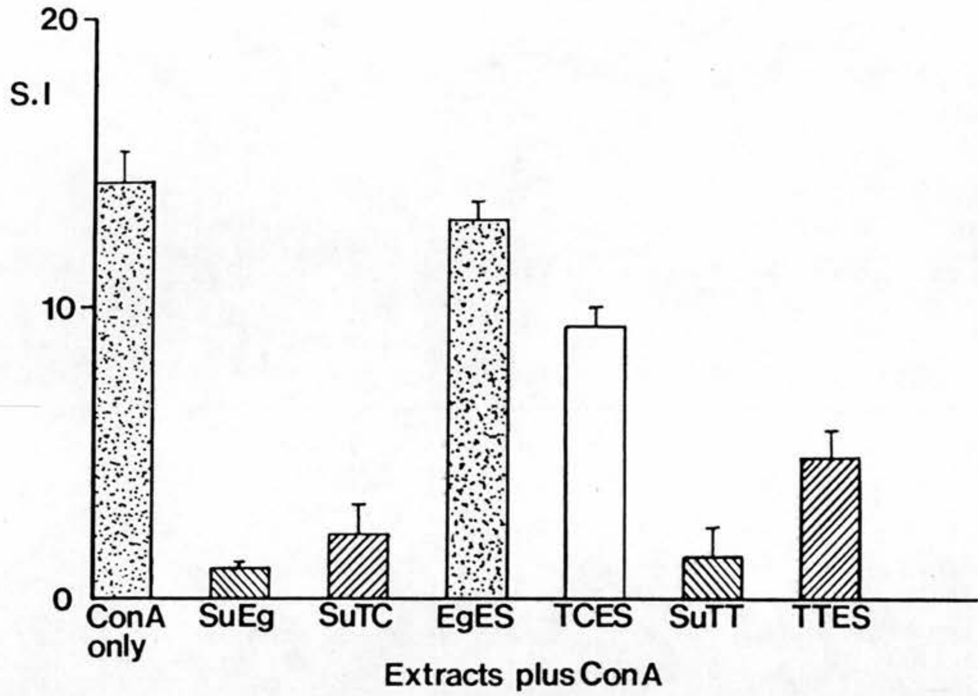
The response of the MLNC to cestode extracts at a concentration of 0.25 μg/ml were so highly variable for each series of replicates that they were not usable. It is not clear why this should be the case but as the 25 μg/ml extract gave closely grouped results within the replicate series only this data was further considered.

Figure 1.5 shows the mean stimulation indices and standard deviations when cestode extracts at 25 μg/ml were added alone to MLNC. None of the extracts except ES Tc caused any degree of stimulation and the incorporation in the wells containing the extracts was not significantly different from the response in the control wells that received no additives. These results and their respective t-values are shown in Appendix 4. The ES Tc preparation gave a high rate of ^{3}H-Tdr incorporation with a SI 9.2 higher than that for the Con A stimulated cells. Figure 1.6 shows the mean SI values obtained when the various cestode extracts were added to MLNC at the same time as Con A. The Con A positive control is shown for comparison.

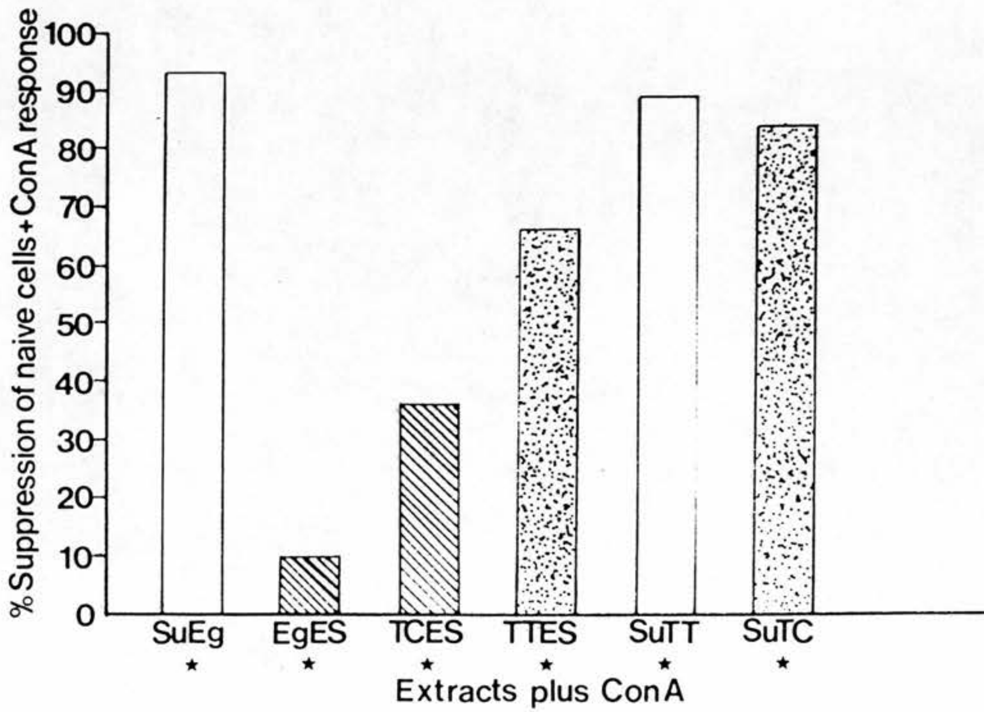
The surface extracts of all three metacestode species caused inhibition of the normal Con A response. The extent of this suppression is shown as a percentage in Figure 1.7. The excretory-secretory products of secondary hydatids, T. crassiceps and T. taeniaeformis also caused suppression of the Con A response but to a lesser degree, particularly in the case of ES Eg. The means, standard deviations and t-values of the counts per minute (cpm) data for each extract are in Appendix 4.

Figures 1.6 and 1.7

The mean stimulation indices of naive MLNC from naive Balb/c mice when cultured with metacestode extracts plus Con A. (B) The results expressed as percentage suppression of the normal Con A blastic response.



20
S.I
10
0
ConA only
SuEg
SuTC
EgES
TCES
SuTT
TTES
Extracts plus ConA
★= mean c.p.m significantly different from ConA only response at p=0·02



% Suppression of naive cells+ConA response
100
90
80
70
60
50
40
30
20
10
0
SuEg ★
EgES ★
TCES ★
TTES ★
SuTT ★
SuTC ★
Extracts plus ConA

The response to ES Tc is confusing, as the high level of lymphoproliferation found when this extract was added on its own to the culture was not consistent with the 36% suppression of the normal Con A response found when both ES Tc and Con A were added.

1.6 The Incorporation of ^{3}H-Tdr by MLNC from Naive and Infected Donors Cultured with Living Hydatid Cysts

1.6.1 Aim

The MLNC were derived from naive donors or from mice that had been infected with secondary hydatid cysts for five weeks, the same length of time as the mice in Section II, Chapter Four. Thus, it was hoped that this experiment would afford an additional evaluation of the level of immune responsiveness in these hosts in addition to their susceptibility to concurrent infections.

1.6.2 Experimental design

MLNC derived from three Balb/c mice that had been infected for five weeks with secondary hydatidosis and MLNC from three uninfected Balb/c mice of the same age were seeded at a concentration of 2 x 10^{6} cells/ml in Costar plates. The final volume of each well was 0.2 ml. The five treatments for both infected and uninfected cells were:

(a) 25 μg/ml Con A

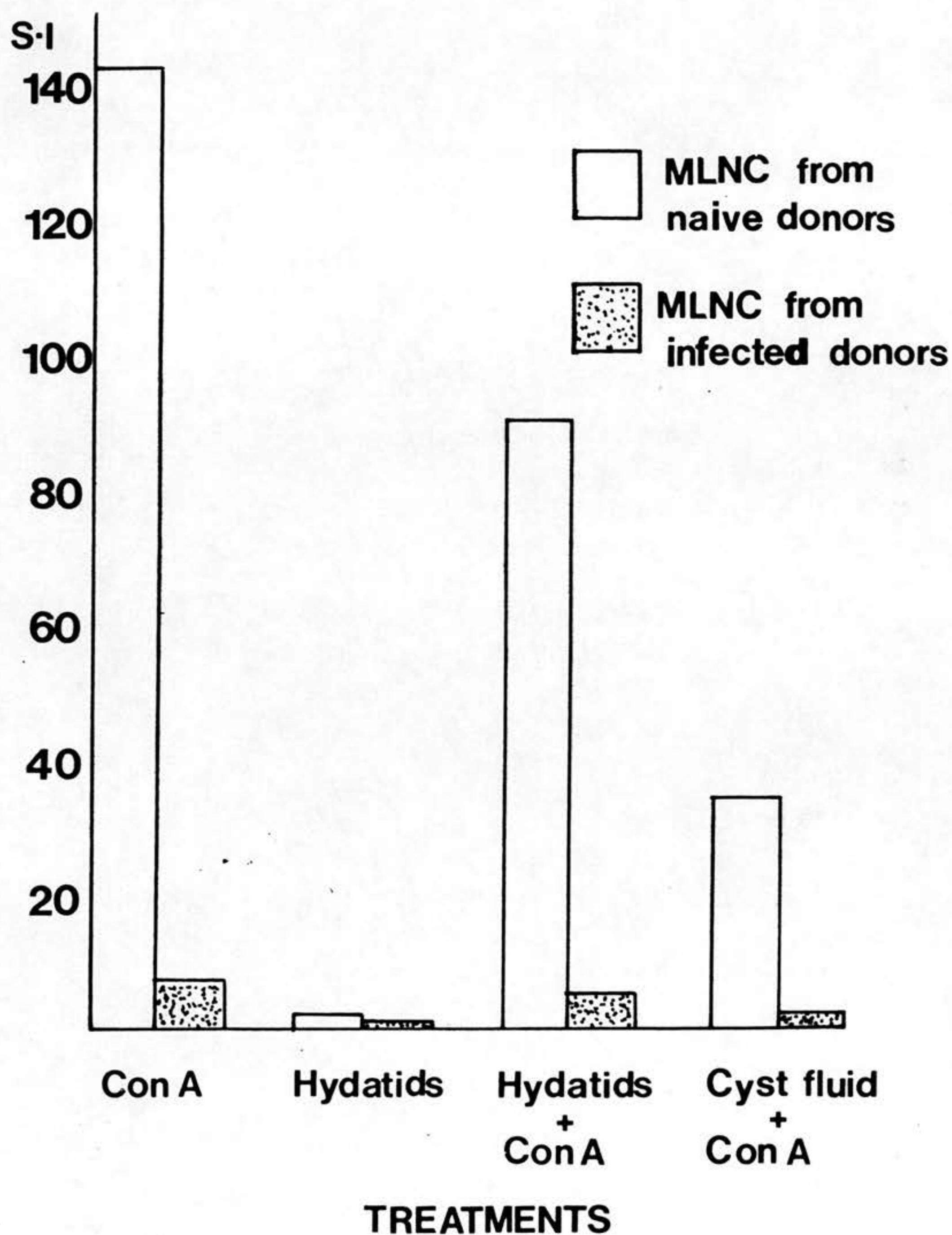
(b) A single 5-10 mm diameter secondary hydatid cyst removed from a mouse and washed in sterile medium.

(c) A similar single hydatid cyst plus 25 μg/ml Con A.

(d) Cyst fluid from a secondary hydatid cyst in a Balb/c mouse plus 25 μg/ml Con A.

Figure 1.8

The mean stimulation indices of MLNC from naive and infected Balb/c mice when cultured with live hydatid cysts or cyst fluid plus Con A.


S·I
140
120
100
80
60
40
20
MLNC from naive donors
MLNC from infected donors
Con A
Hydatids
Hydatids + Con A
Cyst fluid + Con A
TREATMENTS

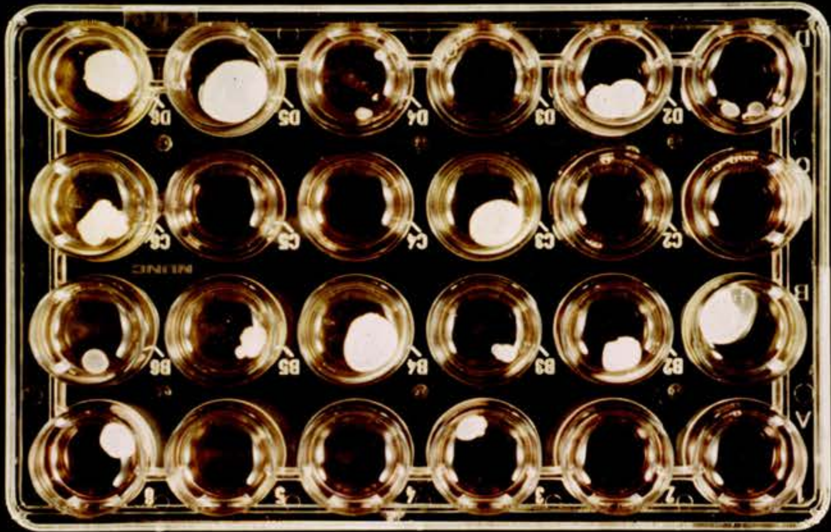
(e) Unstimulated.

On day 3 of culture, the cell suspension was well mixed by pipetting up and down with a sterile Pasteur pipette and 200 μl from each well was placed in a single well of a microtitre plate. The treatment type of each new well was clearly marked and 18.5 k Bq of ^{3}H-Tdr was added to each well. The cells were harvested 18 hours later, washed and counted in a liquid scintillation counter. The results were expressed as mean cpm and compared by Students t test against the results obtained in the positive control wells containing lymphocytes from naive mice stimulated with Con A only. This data is given in Appendix 4. The data was also expressed as stimulation indices.

1.6.3 Results

Figure 1.8 shows the mean stimulation indices of MLNC from infected and naive donors when exposed to the various treatments. The blastic response to Con A was significantly depressed in the MLNC from infected donors. The response to living hydatids only was negligible and was not significantly different from that given in the unstimulated control wells. The combination of hydatids plus Con A depressed the normal response to Con A by naive cells. The addition of cyst fluid caused a similar but greater depression of the response to Con A by the naive cells.

Plate 6 A costar plate, as used in the lymphocyte transformation assay of the effect of live hydatid cysts on the normal response of MLNC to Con A.



1.7 The ^{3}H-Tdr Incorporation by MLNC from both Infected and Naive Donors Cultured with Living Hydatid Cysts for Various Times

1.7.1 Aim

The previous experiment had shown that living hydatid cysts are capable of suppressing the normal blastic response to Con A when lymphocytes and cysts are cultured together for three days. A study was devised to see how long the period of exposure of the cells to the cysts must be for this suppression to occur.

1.7.2 Experimental design

The Costar plates were seeded with MLNC from infected or naive donors as in the previous experiment and one hydatid cyst per well was added on the first, second or third day of the culture. Con A was added to the cell suspensions on the first day in all cases except for the negative controls. The mean counts and statistical analysis are given in Appendix 4 and again the results are also expressed as stimulation indices.

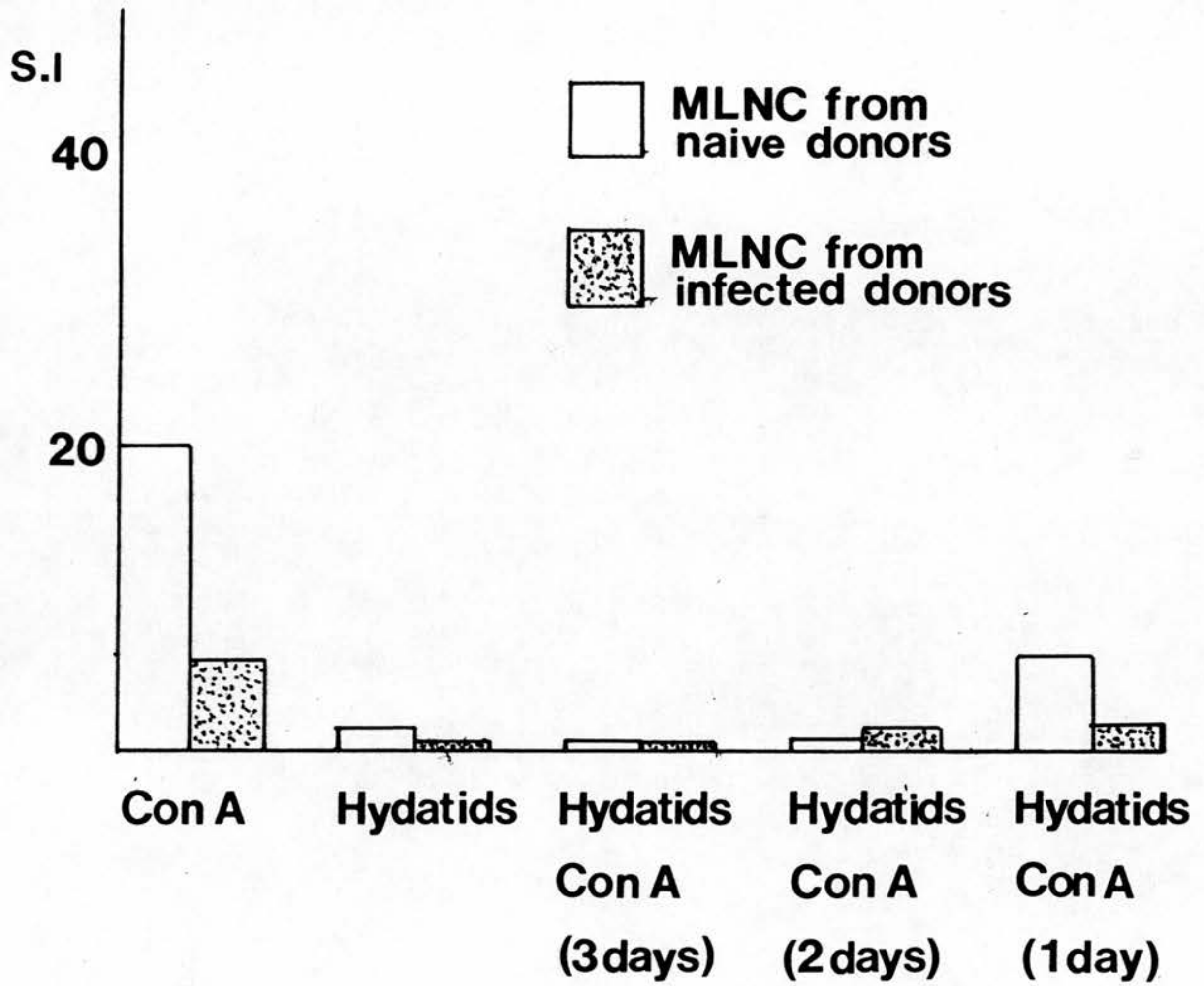
1.7.3 Results

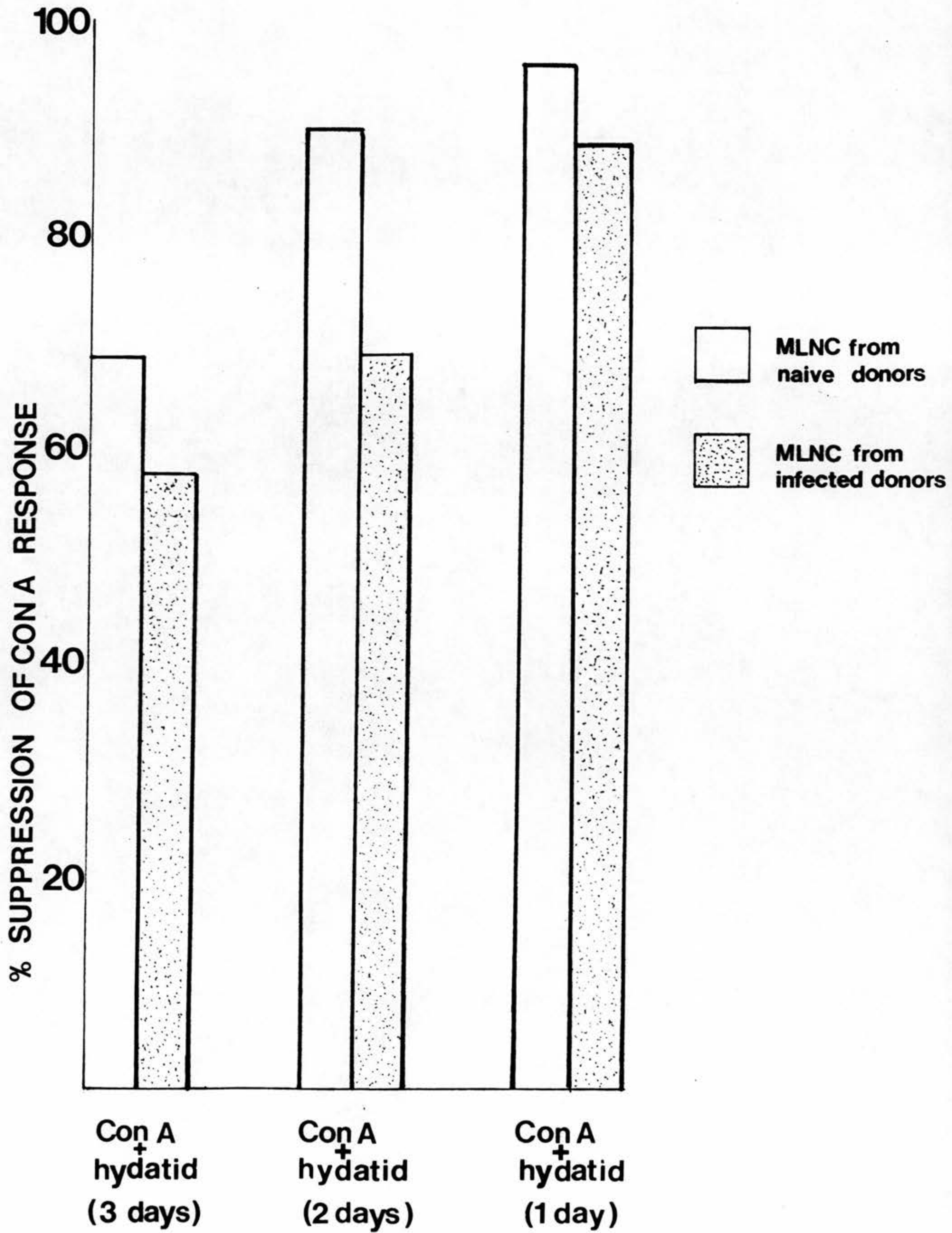
Figure 1.9 shows the mean stimulation indices for each treatment. All the Con A treated cells, irrespective of the period of exposure of MLNC to the metacestode, showed less lymphoproliferation than with the similar treatments in the previous experiment. It is not clear why this happened and when the data was expressed as the percentage suppression of the blastic response to Con A alone, only the responses of the naive cells differed markedly from those in the previous experiment. This aberrant response means that the results from this study must be regarded with caution, although there

Figure 1.9

A. The mean stimulation indices of MLNC from both naive or infected Balb/c mice when cultured for one, two or three days with live hydatid cysts plus Con A.

B. Results expressed as the percentage suppression of the normal Con A response.


S.I
40
20
MLNC from naive donors
MLNC from infected donors
Con A
Hydatids
Hydatids Con A (3days)
Hydatids Con A (2days)
Hydatids Con A (1day)



100
80
60
40
20
% SUPPRESSION OF CON A RESPONSE
MLNC from naive donors
MLNC from infected donors
Con A + hydatid (3 days)
Con A + hydatid (2 days)
Con A + hydatid (1 day)

was an overall tendency for the degree of suppression of lymphoproliferation to vary with the length of exposure of the lymphocytes to the hydatid cysts. Ideally, time permitting, this study would have been repeated to obtain a more consistent response from the naive MLNC and also to investigate the effect of even shorter periods of exposure of MLNC to hydatid cysts.

1.8 The ^{3}H-Tdr Incorporation by MLNC from Naive and Infected Donors Cultured with *Taenia crassiceps* Metacestodes

1.8.1 Aim

The purpose of this experiment was similar to that of the previous experiment, that is to compare the lymphoproliferative response in mice presensitized by five-week-old infections of *T. crassiceps* to that in naive donors when their lymphocytes were exposed in culture for varying lengths of time to *T. crassiceps* metacestodes.

1.8.2 Experimental design

MLNC cells from three Balb/c mice that had been infected for five weeks with 20 *T. crassiceps* metacestodes and MLNC cells from three uninfected Balb/c mice of the same age were seeded at a concentration of 2 x 10^{6} cells/ml in Costar plates. The final volume of each well was 200 µl. The six treatments used were:

(a) 25 µg/ml Con A only

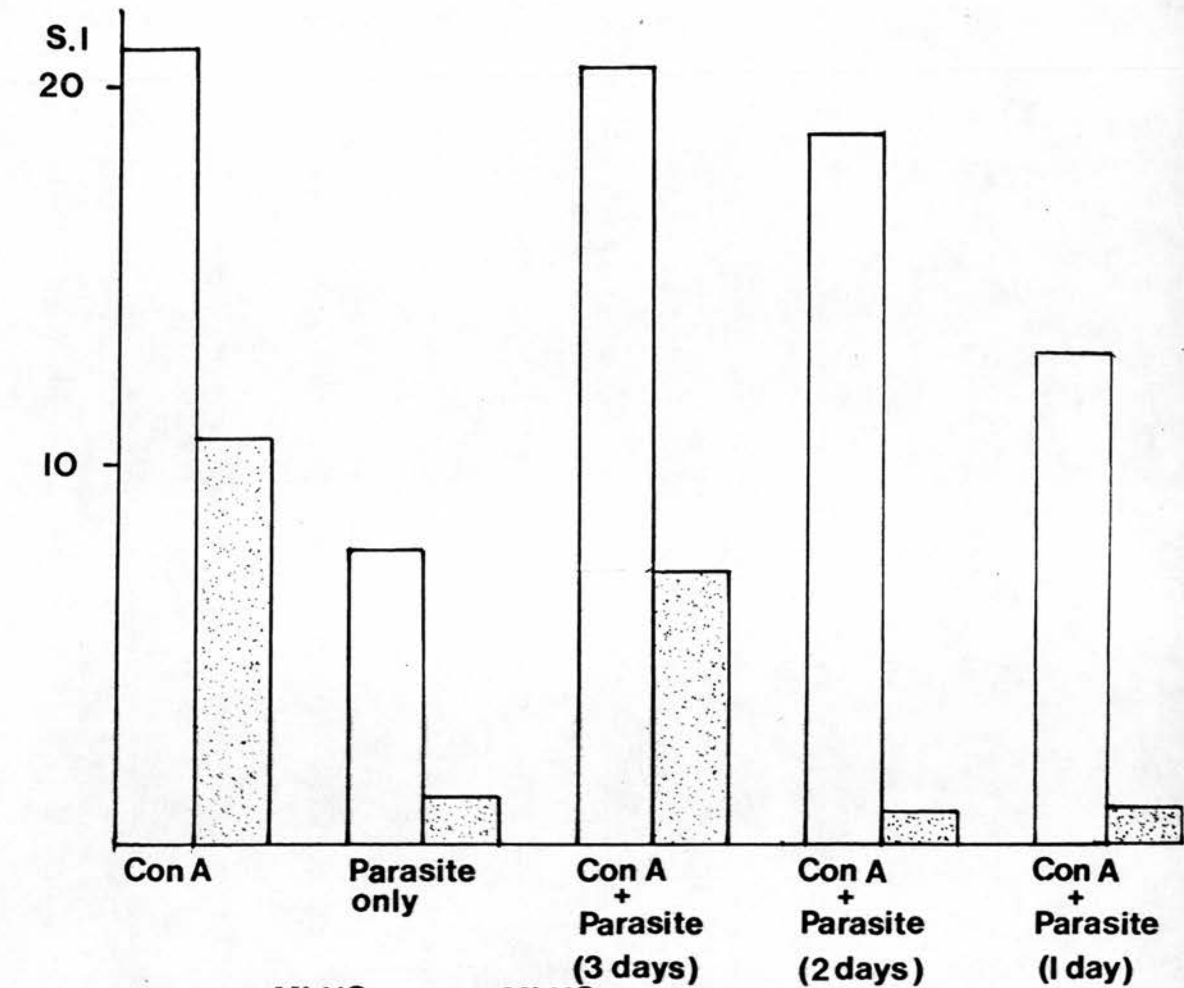
(b) 5 metacestodes 1-2 mm in diameter

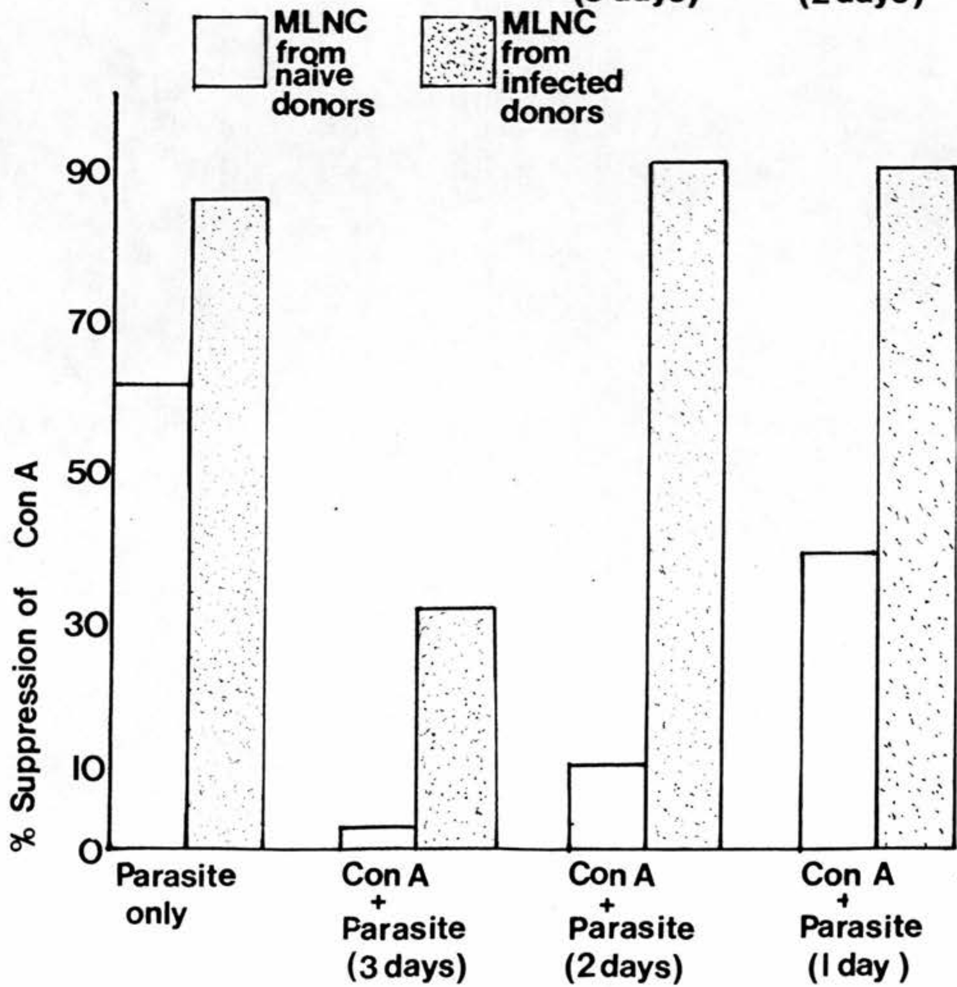
(c) 25 µg/ml Con A and 5 metacestodes added on day 0 of culture.

(d) 25 µg/ml Con A on day 0 and 5 metacestodes added on day 1.

Figure 1.10 and 1.11

The mean stimulation indices of MLNC from both naive and infected Balb/c mice when cultured for one, two or three days with live *Taenia crassiceps* metacestodes plus Con A.


S.I
20
10
Con A
Parasite only
Con A + Parasite (3 days)
Con A + Parasite (2 days)
Con A + Parasite (1 day)
MLNC from naive donors
MLNC from infected donors


% Suppression of Con A
90
70
50
30
10
0
Parasite only
Con A + Parasite (3 days)
Con A + Parasite (2 days)
Con A + Parasite (1 day)

(e) 25 μg/ml Con A on day 0 and 5 metacestodes added on day 2.

(f) Unstimulated.

The metacestodes were washed three times in sterile RPMI 1640 prior to use.

All other experimental details were as in the previous experiment. The mean counts and statistical analyses are given in Appendix 4.

1.8.3 <u>Results</u>

Figure 1.10 shows the stimulation indices for each treatment type. The response of the cells from the infected donors was significantly lower than the response of the naive cells with all the treatments. The response of naive cells to exposure to metacestodes for two or three days was lower but not significantly so than that by the positive control in which the cells were stimulated with Con A only. However, MLNC cultured with the metacestodes for one day only did have a significantly depressed lymphoproliferative response.

When these results were expressed as percentage suppression of the Con A response of either naive or infected cells as in Figure 1.11, it is clear that there was a reversal of the situation encountered with the secondary hydatid cysts as the greatest suppression response of the naive cells to Con A was found when the metacestodes were present for only one day. The MLNC from infected donors showed the greatest suppression when the parasites were present for one or two days and there was no significant difference between these two treatments. It appears that short exposures to this parasite may

have a greater suppressive effect than longer periods. This result is difficult to explain and ideally this experiment would also have been repeated had time permitted.

1.9 Discussion

These lymphocyte transformation studies provided an *in vitro* assessment of host cell-parasite interactions that may be occurring during the metacestode infections and help to elucidate possible mechanisms by which these parasites manipulate the immune response of the host.

Prior to the addition of metacestode surface extracts to MLNC it was considered necessary to verify that any mitogenic or cytotoxic properties were not due to traces of the chemicals used in the preparative procedures, such as N-octyl glucoside, remaining in the extracts. Surface extracts, both before and after purification through a G25 column were added to BL20 cell cultures and their effect on the normal growth potential was monitored by the level of ^{3}H-Tdr incorporation and the percentage of cell viability. The ^{3}H-Tdr uptake of the cells in the wells that received the crude extracts were significantly depressed compared to the unstimulated cells. This suggests that traces of N-octyl glucoside or another low-molecular component of the crude extracts can have a detrimental effect on cell growth. The purified surface extracts all showed a slight but not a significant enhancement of the ^{3}H-Tdr incorporation by the BL20 cells as compared to the unstimulated controls. This enhancement may only be a reflection of the high level of variation in response commonly observed in lymphocyte transformation assays.

The crude extracts were also shown to dramatically reduce cell viability over the three days of culture, confirming that traces of the preparative agents were toxic to BL20 cells. The viability of the cells that received purified surface extracts remained at about 70% over the three days of culture, indicating that cell death was still occurring at a greater rate than in the unstimulated control wells even after having twice passaged the extracts through a G25 column which can safely be expected to hold back all low-molecular weight traces of chemicals. Thus, these cytotoxic properties are likely to be due to parasite components alone.

It is interesting that despite a depletion of approximately 30% of the living BL20 cells in the wells receiving purified extracts, these cells showed a slight enhancement of the ^{3}H-Tdr incorporation when compared with the unstimulated control cells. Thus, this slight enhancement may be of greater significance when the lower numbers of viable cells present are taken into consideration. The implications of this are not clear but it could be that the parasite extracts induce cytotoxicity on certain cells whilst others are capable of responding blastically. This theory has basic flaws, in that BL20 lymphosarcoma cells are by their very nature of continuous multiplication and culture identical and thus would be expected to react to the addition of metacestode surface extracts in an identical manner, unless the death of a proportion of the cells liberates a cell component that alters the receptive nature of the remaining cells to the parasite so that they will respond mitogenically to the parasite components.

It is felt to be more likely that this slight enhancement is due to the large variation in levels of incorporation found in these assays particularly in view of the results of the following two experiments in which purified metacestode extracts were added to MLNC both with and without Con A.

The addition of purified metacestode extracts or products does not have any lymphostimulatory effect on mesenteric lymph node cells from naive donors with the exception of the excretory/secretory preparation from the in vitro culture of T. crassiceps (ES Tc). It is not clear why this preparation should have such a different effect from the other secretory/excretory products and this may have been a chance observation. Such stimulation, without the need for pre-sensitization of the lymphocytes has been previously demonstrated with the cyst fluid from the coenurus of Taenia multiceps (Judson et al., 1984) and protoscolices of E. granulosus (Dixon et al., 1982).

These same extracts and products of metacestodes have a depressive effect on the normal lymphoblastic response to Con A. This was true even for the ES Tc preparation and further suggests that the apparent stimulatory response when this was added to MLNC without Con A may have been a chance result, perhaps due to bacterial contamination. When the results were expressed as a percentage suppression of the Con A response, it was clear that the extracts prepared from the surface of the metacestodes caused the greatest suppression. The excretory/secretory products caused a lower degree of suppression, particularly in the case of ES Eg. The fact that surface components seemed to depress the mitotic activity of naive lymphocytes to a greater extent, correlated well with the possibility that the release

of surface antigens from *T. crassiceps* caused by their interaction with host antibody and cells may be responsible for the immunosuppression (Good *et al.*, 1982). However, Annen *et al.* (1980) found that *E. granulosus* cyst fluid caused a considerable depression of ^{3}H-Tdr incorporation in Con A stimulated rat spleen cells to a level below that in unstimulated control cells. They ascribed the effects as due to the cytotoxic properties of the cyst fluid but were unable to conclude whether they were due to a parasite-specific cytotoxin or to a non-specific reaction by parasite metabolites.

The effect of live hydatid cysts on both naive and sensitized MLNC was to depress the normal blastic response to Con A. The effect was more marked on cells derived from infected donors, possibly indicating prior *in vivo* depletion of the cells that respond to Con A followed by the toxic effects of the live parasites in this assay. It is clear that live hydatids have a potent cytotoxic effect on MLNC, although this is less than occurs with hydatid cyst fluid. This latter component of the metacestode was used in this experiment in order to confirm the cytotoxic effects of hydatid cyst fluid reported by Annen *et al.* (1980) and to afford a direct comparison of the potency of cyst fluid and live parasites. It would otherwise have been imprudent to attempt to compare the effects of the live hydatids to the effects of cyst fluid reported by Annen *et al.* (1980) due to the known variability of individual lymphocyte transformation assays even in the hands of the same operator.

The degree of suppression of the normal Con A response by live hydatids increased with the length of time that the cells were exposed to the metacestode, both in the case of naive and sensitized

MLNC. This agrees with Annen et al. (1980) who showed decreased ^{3}H-Tdr incorporation with increasing length of exposure to cyst fluid. The depression of these mitogen-induced responses by living metacestodes could possibly be due to mitogen absorption by the parasite (Maleckar and Kierszenbaum, 1984), but this seems unlikely since extracts and cyst fluid mediate similar suppression. The effect could be tested for by increasing the levels of Con A in the wells above those producing optimal responses. It is known that above a given level of Con A concentration the mitogenic response starts to diminish thus if mitogen absorption occurs the addition of otherwise excess Con A would not cause a depression in the mitogenic response as no excess mitogen would be effectively present because it would be removed by the absorption.

However, when MLNC were exposed to T. crassiceps metacestodes for varying lengths of time, the greatest degree of suppression of the Con A response was in the shorter periods of exposure. This was particularly apparent when the response of the naive cells was examined. There is no ready explanation of this and this experiment requires repetition, which unfortunately time did not allow.

There are various theories as to the basis of the immunosuppressive effects mediated by parasites and these include antigenic competition, in which it is postulated that the release of large amounts of parasite antigen causes inhibition of the immune response to unrelated new antigens (Goodwin, 1970), non-specific activation of immunoglobulin synthesis leading to inhibition of subsequent immune responses (Urquhart et al., 1973; Cox et al., 1984) and direct suppressive activity by the parasites on host cells (Annen et al., 1980;

Lammie and Katz, 1984; Maleckar and Kierszenbaum, 1983). The evidence from the lymphocyte transformation studies indicates that the latter theory may explain immunosuppression of _B. microti_ infections by larval cestodes, despite the evidence of Cox _et al_. (1984) that _E. granulosus_ protoscolices stimulated early abnormal lymphoid proliferation.

The nature of the immunosuppressive component of the metacestodes and the target cells it acts on remains to be elucidated, but these results suggest that the surface of these parasites merits investigation in addition to cyst fluid as reported by Annen _et al_. (1980). These workers concluded that the cytotoxic substances released by the parasites through the cyst wall act only locally. However, in order to explain the generalised immunosuppressive effects demonstrated by increased susceptibility to intercurrent infections a rather more pervasive modulator must be implicated. In this respect it is interesting that Chemtai (1980) has demonstrated factors in the sera of human hydatid patients that suppressed lymphoblast formation in the presence of Con A. It was far more likely that there was a complex interplay of parasite-derived factors influencing the hosts immune mechanisms to account for the impressive ability of these metacestodes, particularly hydatid, to survive in a supposedly immunologically adverse environment.

The suppressive activity of both metacestode extracts and living larvae described here are not entirely discordant with the work of Cox _et al_. (1984) as recent experiments on established secondary infections of _E. granulosus_, as opposed to their previous analysis of early infection with the protoscolices, have shown depressed mitotic

activity in lymphocytes stimulated with Con A. This has led them to postulate an early abnormal lymphoproliferation involving both T and B cells leading to depression of any response to mitogens and possibly functional indifference to the parasite. Yet, in the present study neither the metacestode extracts nor live parasites added alone or with Con A to MLNC stimulated even early lymphoproliferation.

Clearly, the *in vitro* host-parasite interaction in metacestode disease requires further investigation. Such studies could involve the physico-chemical characterization and isolation of the effectors of this response. *In vitro* studies could be carried out using lymph node cell cultures from naive or infected mice and fractions of various extracts of the surface, secreted and somatic compartments of the metacestode could be added to these to identify the effector fractions. Those fractions producing an immunosuppressive effect *in vitro* could then be tested for modulation of the immune response to an unrelated antigen such as sheep red blood cells *in vivo*.

Furthermore, the target cells of the suppressive factors could be isolated by assessing the *in vitro* response in defined cell subpopulations to the selected parasite fractions.

CHAPTER TWO

CONCLUSIONS

The work detailed in this study has shown that existing infections of either of the three metacestode species Taenia crassiceps, Taenia taeniaeformis and Echinococcus granulosus render the host more susceptible and depress their immune response to a secondary infection of Babesia microti. This was most marked in the case of mice infected with secondary hydatidosis in which the greatest enhancement of peak parasitaemia was recorded. The features of the intercurrent infections using the three metacestode species are extremely similar, since all show enhanced peak parasitaemias and a slower rate of decline from the peak when compared with the controls infected with B. microti only. It is noticeable, though, that in none of the experiments was the immunosuppression sufficient to prevent the mice controlling the piroplasm infection ultimately. B. microti-infected mice normally exhibit solid immunity to subsequent re-infection (Clark, 1979) and this still appears to be the case in the concurrently-infected animals although Good and Miller (1976) reported that infection with T. crassiceps had a more marked depressive effect on the secondary humoral response.

The extracts prepared from the surface of these three metacestode parasites caused suppression of the normal blastic response to Con A of MLNC derived either from naive or previously infected donors. The excretory/secretory products of these same parasites caused a lower degree of suppression.

The effects of living secondary hydatid cysts is also to depress the normal Con A response of both naive and sensitized MLNC and this increased with the length of time that the cells were exposed to the metacestode. Unfortunately, attempts to study the response of Con A

stimulated MLNC to living *T. taeniaeformis* metacestodes met with little success as the cell death was almost total by day 1 possibly due to the level of activity of these strobilocerci in culture. *T. taeniaeformis* induced dramatic pH changes from neutral to acid over the period of 24 hours due to their high metabolic activity. It must all be considered that these worms liberated from their cysts are not in the same form that host cells will be exposed to *in vivo*, where the cysts reside in the liver tissue.

The response of MLNC to *T. crassiceps* metacestodes yielded rather confusing results, as the degree of suppression of the Con A response increased with a decrease in the period of exposure. It was felt that discussion of this experiment would be ill-advised without repetition of the assay.

Generalised immunosuppression is known to occur in a wide range of parasitic infections and has been particularly extensively researched in malaria and trypanosomiasis so that many of the mechanisms proposed in the literature related to these diseases. It has become clear that there is no single underlying mechanism which applies to all host-parasite systems and in fact it is likely that multiple mechanisms operate within a single system.

Terry (1977) suggested that the many mechanisms proposed could be conveniently divided into two groups:-

(a) those parasitic infections where depression depends on the antigenic properties of the parasite, a situation of antigenic competition.

(b) those parasitic infections where the depression relates to other properties of the parasite.

(a) Antigen competition

It has long been known that unrelated antigens may compete with one another in that the response to an antigen may be markedly reduced if this antigen is given after exposure of the animal to an unrelated antigen. This can occur due to the stimulation of non-specific suppressor cells which can depress a wide range of responses including those to lymphocyte mitogens. Jayawardena and Waksman (1977) showed that the addition of T cells from *T. brucei* infected mice would inhibit the response of normal lymphocytes to a variety of mitogens. Other workers have claimed that such infections stimulated macrophages with a suppressor function (Wellhausen and Mansfield, 1979) although Jayawardena *et al*. (1978) failed to implicate macrophages in their system.

It is also possible that antigenic competition may take place at the surface of accessory cells such as mononuclear phagocytes as attachment of a second antigen B to sites appropriate for associative recognition could be hindered by their occupation by antigen A (Schrader and Feldmann, 1973).

(b) Mechanisms unrelated to antigenic competition

The phenomenon of antigenic competition has been reported in many parasitic diseases, but there is evidence of other mechanisms unrelated to competition which play a part in immunosuppression. One of these is B cell polyclonal activation that occurs in trypanosomiasis and malaria (Terry *et al*., 1980). The high immunoglobulin levels, particularly IgM found during these diseases appeared to be largely non-specific causing generalised immunosuppression which

constitutes an important evasion mechanism as the parasite is able to survive in the midst of the resulting "immunoconfusion". The continuous activation of B cells is likely to lead to a progressive depletion of antigen-reactive cells as they become secretor cells. Corsini *et al*. (1977) found that B cells taken from the later stages of trypanosomiasis did not produce Ig, and it was concluded that clonal exhaustion had occurred. Whether polyclonal activation occurs in helminth infections is not clear, but this is the mechanism postulated by Cox *et al*. (1984) to explain the early abnormal lymphoid proliferation stimulated by *E. granulosus* leading to depressed responsiveness of lymphocytes in six-week infections.

However, this present study has shown that both metacestode extracts and live hydatids suppress the normal Con A blastic response so the existence of polyclonal activation leading to immunodepression would seem untenable in this system.

The existence of lymphocytotoxic factors secreted by helminths has been shown in serum of *T. spiralis* infected mice to agglutinate and subsequently kill lymphocytes (Faubert and Tanner, 1975) and Annen *et al*. (1980) have found similar activity in the cyst fluid of *E. granulosus*. The secretion of such factors may well lead to local or even a generalised impairment of immune responses such as has been demonstrated during the concurrent infections of metacestodes and *B. microti*. Such factors appear to be present to a greater extent in the surface extracts prepared from the three metacestode species.

Alternatively, the parasites may secrete factors that are not directly cytotoxic for immunocytes but instead act to prevent their

activation and subsequent participation in immune responses. Capron and his co-workers (Capron *et al*., 1976; Dessaint *et al*., 1977; Mazingue *et al*., 1980) have isolated a schistosome-derived inhibition factor (SDIF) which may be peptidoglycan that suppresses a variety of lymphocyte functions by a direct inhibitory action on responder cells, rather than by stimulating suppressor cells. They reported that the factor inhibits mast cell degranulation and thus may hinder various antibody dependent cell cytotoxicity mechanisms proposed to operate against the schistosomula stage.

On the basis of the results detailed in the present study it is not clear which of these models of immunosuppression metacestode infections induce but it is likely that several mechanisms combine. It is possible that metacestodes induce lymphocytotoxic reactions locally as described by Annen *et al*. (1980) and revealed by the cytotoxic effects of metacestode preparations on BL20 cells. In addition, other or indeed the same parasite factors may induce the generalised immunosuppression indicated by the concurrent infection results possibly by the suppression of lymphocyte responses or the induction of suppressor cells by parasite antigens (Lammie and Katz, 1984). It is not possible to conclude that the mechanisms of the metacestode induced immunosuppression or indeed the target cells that the parasite influences, without a great deal of further work involving fractionation of parasite antigens and the use of separated and defined cell sub-populations.

Nevertheless, this study has revealed metacestode-induced immunosuppression both *in vivo* and *in vitro* that may well play an

important role in the evasion of the immune response of these chronic infections. The possible consequences of metacestode-associated immunosuppression in addition to the hosts increased susceptibility to infection as shown here are: that vaccination may be less optimally effective in infected animals; they may be more liable to develop spontaneous tumours and concomitant parasitism may render the host less likely to develop allergic diseases.

The study of the evasion of the immune response by larval cestodes may reveal more of the complex interactions that regulate the immune response and lead to the possibility of shifting the regulatory balance in favour of the host instead of the parasite.

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APPENDICES

Appendix Table 1

Mean percentages of initial inoculum developing into secondary hydatid cysts.

Treatment group		Mean percentage ± SD	t-value
Gerbils	2000	0.31 ± 0.23	0.44
Positive infections	4000	0.26 ± 0.2	
Gerbils	2000	0.31 ± 0.23	2.22*
Total infections	4000	0.12 ± 0.14	
Mice (equine strain)		1.5 ± 1.25	1.38
Positive infections		0.76 ± 0.47	
Mice (equine strain)		1.08 ± 1.2	1.75
Total infections		0.36 ± 0.49	
Mice (ovine strain)		1.56 ± 1.07	2.37*
Positive infections		0.47 ± 0.36	
Mice (ovine strain)		0.78 ± 1.09	1.95*
Total infections		0.1 ± 1.16	

*Significant P < 0.05

Appendix Table 2

IFAT results from *Echinococcus* and *Babesia* concurrent infections experiment

Weeks post *Babesia* infection	t-value	Level of significance
1	3.97	P < 0.001
2	31.59	P < 0.001
3	5.83	P < 0.001
4	3.53	P < 0.001
5	6.11	P < 0.001
6	4.62	P < 0.001
7	3.42	P < 0.001

Appendix Table 3.1

Nude mice experiment

Source of variation	SS	df	M	F
ANOVA table for day 6				
(1) Treatment	4.75	1	4.75	1.56
(2) Field	0.34	2	0.17	0.56
(3) Interaction	0.34	2	0.17	0.56
Error	3.66	12	0.3	
ANOVA table for day 9				
(1) Treatment	7.3	1	7.3	0.79
(2) Field	25.03	2	12.51	1.35
(3) Interaction	34.66	2	17.33	1.87
Error	110.98	12	9.25	
ANOVA table for day 11				
(1) Treatment	1360.33	1	1360.33	1.81
(2) Field	18.43	2	9.22	0.01
(3) Interaction	26.78	2	13.39	0.02
Error	9025.16	12	752.0	
ANOVA table for day 12				
(1) Treatment	1.04	1	1.04	0.05
(2) Field	25.51	2	12.76	0.59
(3) Interaction	22.15	2	11.07	0.51
Error	259.66	12	21.64	
ANOVA table for day 13				
(1) Treatment	1461.6	1	1461.6	23.2*
(2) Field	380.2	2	190.0	3.02
(3) Interaction	165.19	2	82.6	1.31
Error	756.03	12	63.0	
ANOVA table for day 15				
(1) Treatment	7626.1	1	7826.1	45.94*
(2) Field	815.57	2	407.78	2.46
(3) Interaction	18.31	2	9.15	0.06
Error	1991.86	12	165.9	

*Significantly different at the 5% level

Appendix Table 3.1 (continued)

Source of variation	SS	df	M	F
ANOVA table for day 18				
(1) Treatment	14011.0	1	14011.0	116.16*
(2) Field	252.61	2	126.31	1.05
(3) Interaction	164.7	2	82.35	0.68
Error	1447.5	12	120.63	
ANOVA table for day 20				
(1) Treatment	4062.0	1	4062.0	111.7*
(2) Field	0.69	2	0.34	0.01
(3) Interaction	1.02	2	0.51	0.01
Error	436.37	12	36.36	
ANOVA table for day 22				
(1) Treatment	2625.7	1	2625.7	29.48*
(2) Field	52.86	2	26.43	0.3
(3) Interaction	73.97	2	37.0	0.42
Error	1068.9	12	89.0	
ANOVA table for day 25				
(1) Treatment	7480.28	1	7480.28	283.28*
(2) Field	273.56	2	136.78	5.18
(3) Interaction	239.21	2	19.5	4.23
Error	316.87	12	26.41	
ANOVA table for day 27				
(1) Treatment	6452.48	1	6452.48	455.24*
(2) Field	500.0	2	250.0	17.63
(3) Interaction	558.72	2	279.36	19.71
Error	170.0	12	14.17	

*Significantly different at 5% level

Appendix Table 3.2

T. crassiceps and Babesia experiment - intravenous route

Source of variation	SS	df	M	F
ANOVA table for day 1				
(1) Treatment	25.19	2	12.59	6.62*
Error	17.08	9		
ANOVA table for day 3				
(1) Treatment	24.29	2	12.14	2.14*
Error	88.56	9		
ANOVA table for day 4				
(1) Treatment	581.5	2	290.75	2.68
Error	974.9	9	108.3	
ANOVA table for day 10				
(1) Treatment	599.0	2	299.5	11.9*
Error	225.97	9	25.10	
ANOVA for day 12				
(1) Treatment	124.4	2	62.2	2.1
Error	265.87	9	29.54	
ANOVA table for day 15				
(1) Treatment	216.36	2	108.15	12.5*
Error	77.8	9	8.64	
ANOVA table for day 18				
(1) Treatment	5.5	2	2.75	1.53
Error	16.22	9	1.8	
ANOVA table for day 22				
(1) Treatment	14.04	2	7.02	1.84
Error	34.4	9	3.8	

*Significantly different at 5% level

Appendix Table 3.3

T. taeniaeformis and Babesia experiment

Source of variation	SS	df	M	F
ANOVA table for day 4				
(1) Treatment	5.92	1	5.92	8.46*
(2) Field	2.08	2	1.04	1.49
(3) Interaction	0.93	2	0.46	0.66
Error	23.07	33	0.7	
ANOVA table for day 14				
(1) Treatment	507.12	1	507.12	8.85*
(2) Field	65.92	2	32.96	0.58
(3) Interaction	7.45	2	3.73	0.07
Error	1891.37	33	57.31	
ANOVA table for day 18				
(1) Treatment	102.58	1	102.58	2.09
(2) Field	53.89	2	26.95	0.55
(3) Interaction	2.86	2	1.43	0.03
Error	1617.42	33		
ANOVA table for day 21				
(1) Treatment	531.61	1	531.61	11.91*
(2) Field	20.95	2	10.47	0.23
(3) Interaction	8.09	2	4.05	0.09
Error	1472.98	33	44.64	
ANOVA table for day 24				
(1) Treatment	647.47	1	647.47	12.44*
(2) Field	51.16	2	25.58	0.49
(3) Interaction	5.55	2	2.77	0.05
Error	1717.46	33	52.04	
ANOVA table for day 30				
(1) Treatment	93.08	1	93.08	3.21
(2) Field	1.59	2	0.79	0.03
(3) Interaction	2.2	2	1.1	0.04
Error	956.93	33	29.0	

*Significantly different at the 5% level

Appendix Table 3.3 (continued)

Source of variation	SS	df	M	F
ANOVA table for day 35				
(1) Treatment	18.78	1	18.78	7.57*
(2) Field	0.28	2	0.14	0.06
(3) Interaction	0.12	2	0.06	0.02
Error	81.93	33	2.48	
ANOVA table for day 37				
(1) Treatment	1.56	1	1.56	1.58
(2) Field	22.0	2	11.0	0.81
(3) Interaction	9.78	2	4.89	0.36
Error	450.57	33	13.65	

Appendix Table 3.4

Taenia crassiceps and Babesia experiment - intraperitoneal route

Source of variation	SS	df	M	F
ANOVA table for day 6				
(1) Treatment	5.09	1	5.09	7.74*
(2) Field	1.51	2	0.76	1.15
(3) Interaction	1.63	2	0.82	1.24
Error	19.7	30	0.66	
ANOVA table for day 8				
(1) Treatment	405.17	1	405.17	61.58*
(2) Field	9.56	2	4.78	0.72
(3) Interaction	3.27	2	1.635	0.24
Error	197.4	30	6.58	
ANOVA table for day 15				
(1) Treatment	8.9	1	8.9	0.17
(2) Field	144.55	2	72.2	1.37
(3) Interaction	7.1	2	3.5	1.06
Error	1582.0	30	52.7	
ANOVA table for day 18				
(1) Treatment	55.5	1	55.5	0.4
(2) Field	229.7	2	114.8	0.832
(3) Interaction	24.8	2	12.42	0.04
Error	4141.0	30	138.0	
ANOVA table for day 20				
(1) Treatment	26.7	1	26.7	0.32
(2) Field	31.7	2	15.9	0.29
(3) Interaction	13.0	2	6.5	0.55
Error	1454.5	30	48.4	
ANOVA table for day 22				
(1) Treatment	23.4	1	23.4	1.21
(2) Field	28.0	2	13.9	0.72
(3) Interaction	6.0	2	3.0	0.155
Error	578.3	30	19.2	

*Significantly different at the 5% level

Appendix Table 3.4 (continued)

Source of variation	SS	df	M	F
ANOVA table for day 25				
(1) Treatment	39.9	1	39.9	4.37*
(2) Field	2.88	2	1.44	0.15
(3) Interaction	7.56	2	3.78	0.41
Error	274.1	30	9.1	
ANOVA table for day 27				
(1) Treatment	106.4	1	106.4	11.8*
(2) Field	2.73	2	1.36	0.152
(3) Interaction	0.35	2	0.17	0.019
Error	269.0	30	8.97	

*Significantly different at the 5% level

Appendix Table 3.5

Low level T. taeniaeformis and Babesia experiment

Source of variation	SS	df	M	F
ANOVA table for day 2				
(1) Treatment	0.86	1	0.86	0.36
(2) Field	1.17	2	0.58	0.26
(3) Interaction	1.96	2	0.98	0.44
Error	86.4	39	2.2	
ANOVA table for day 3				
(1) Treatment	89.56	1	89.56	2.17
(2) Field	65.4	2	32.7	0.79
(3) Interaction	60.22	2	30.1	0.73
Error	1606.22	39	41.2	
ANOVA table for day 5				
(1) Treatment	1943.2	1	1943.2	9.17*
(2) Field	65.4	2	32.7	0.154
(3) Interaction	3.06	2	1.53	0.0072
Error	8261.9	39	211.8	
ANOVA table for day 7				
(1) Treatment	3954.8	1	3954.8	13.63*
(2) Field	1296.3	2	648.2	2.2
(3) Interaction	144.86	2	72.4	0.24
Error	11314.8	39	290.0	
ANOVA table for day 9				
(1) Treatment	1508.3	1	1508.3	6.09
(2) Field	93.3	2	46.66	0.18
(3) Interaction	53.34	2	26.6	0.10
Error	9653.9	39	247.5	
ANOVA table for day 11				
(1) Treatment	3618.8	1	3618.8	49.7*
(2) Field	76.8	2	38.4	0.52
(3) Interaction	51.8	2	25.9	0.35
Error	2838.9	39	72.7	

*Significantly different at the 5% level

Appendix Table 3.5 (continued)

Source of variation	SS	df	M	F
ANOVA table for day 13				
(1) Treatment	4169.2	1	4169.2	133.99*
(2) Field	120.42	2	60.2	1.93
(3) Interaction	58.6	2	29.33	10.94
Error	1213.48	39	31.11	
ANOVA table for day 15				
(1) Treatment	1991.86	1	1991.86	30.6*
(2) Field	18.1	2	9.0	0.1
(3) Interaction	235.9	2	117.97	1.81
Error	2538.1	39	65.0	
ANOVA table for day 19				
(1) Treatment	3122.28	1	3122.28	105.5*
(2) Field	33.1	2	16.5	0.56
(3) Interaction	54.0	2	27.0	0.91
Error	1153.52	39	29.5	
ANOVA table for day 22				
(1) Treatment	106.71	1	106.71	21.39*
(2) Field	0.15	2	0.077	0.01
(3) Interaction	1.72	2	0.86	0.17
Error	194.54	39	4.98	
ANOVA table for day 25				
(1) Treatment	9.08	1	9.08	8.59
(2) Field	0.26	2	0.13	0.12
(3) Interaction	1.35	2	0.67	0.64
Error	41.2	39	1.05	

Appendix Table 4.1

The 3H-Tdr incorporation by MLNC from naive Balb/c mice when cultured with metacestode extracts with or without Con A

Treatment	With Con A	Mean* cpm ± SD	t-value when $\bar{x}$ is compared with Con A
Unstimulated	-	1826.25 ± 59	3.76
So Eg	-	2713.8 ± 352	25.3
Su Eg	-	2842.0 ± 401	26.8
ES Eg	-	4015.0 ± 703	15.03
Su Tc	-	3885.0 ± 661	14.75
ES Tc	-	129478.6 ± 9981	0.76
Su Tt	-	4390.0 ± 916	14.6
ES Tt	-	2903.0 ± 331	25.0
Su Eg	+	2207.0 ± 461	12.6
ES Eg	+	24852.5 ± 5083	3.2
Su Tc	+	4119.0 ± 648	9.0
ES Tc	+	25819.0 ± 5515	3.6
Su Tt	+	3419.0 ± 465	11.5
ES Tt	+	15884.0 ± 2086	7.2

cpm = counts per minute

*Mean ± SD of four replicates

Appendix Table 4.2

The ^{3}H-Tdr incorporation of BL20 when cultured with parasite extracts both before and after purification

Treatment	Purified (P) or Crude (C)	Mean* cpm ± SD	t-value when $\bar{x}$ compared with unstimulated controls
ES Eg	-	24795 ± 5351	2.74
Su Eg	C	530 ± 33	6.8
Su Eg	P	13542.5 ± 1343	2.47
Su Tc	C	8836 ± 554	3.8
Su Tc	P	11021 ± 641	2.4
Su Tt	C	648 ± 49	5.3
Su Tt	P	15033 ± 781	2.7

cpm = counts per minute

*Mean ± SD of four replicates

Appendix Table 4.3

The ^{3}H-Tdr incorporation of MLNC when cultured with living hydatid cysts with or without Con A

Treatment	Mean* cpm ± SD	t-value when $\bar{x}$ compared with Con A only
Unstimulated	2761 ± 1124	2.8
Parasite alone	111706 ± 9153	2.0
Parasite and Con A	4341 ± 1707	2.3

cpm = counts per minute

*Mean ± SD of four replicates

Appendix Table 4.4

The ^{3}H-Tdr incorporation of MLNC from infected and naive donors when cultured with secondary hydatid cysts

Source of MLNC	Treatment	Mean* cpm ± SD	t-value when $\bar{x}$ compared with Con A only
Naive	Unstimulated	136.5 ± 38	21.2
Naive	Parasite only	727 ± 155	18.6
Naive	Parasite (Day 0) + Con A	298 ± 163	19.4
Naive	Parasite (Day 1) + Con A	256 ± 63	20.7
Naive	Parasite (Day 2) + Con A	1642 ± 71	16.3
Infected	Unstimulated	235 ± 78.6	24.7
Infected	Parasite only	833 ± 13	25.0
Infected	Parasite (Day 0) + Con A	2319 ± 219	20.3
Infected	Parasite (Day 1) + Con A	5607 ± 136	14.6
Infected	Parasite (Day 2) + Con A	7751 ± 621	8.9

cpm = counts per minute

*Mean ± SD of four replicates

Appendix Table 4.5

The ^{3}H-Tdr incorporation of MLNC from infected and naive donors when cultured with T. crassiceps metacestodes

Source of MLNC	Treatment	Mean* cpm ± SD	t-value when $\bar{x}$ compared with Con A only
Naive	Unstimulated	533 ± 32.2	18.6
Naive	Parasite only	5204.4 ± 1844	4.91
Naive	Parasite (Day 0) + Con A	10647 ± 916	0.9
Naive	Parasite (Day 1) + Con A	9475.2 ± 815	1.977
Naive	Parasite (Day 2) + Con A	5380.8 ± 2746	2.94
Infected	Unstimulated	1076.8 ± 111	11.07
Infected	Parasite alone	1441.5 ± 162	10.7
Infected	Parasite (Day 0) + Con A	7878.4 ± 567	4.25
Infected	Parasite (Day 1) + Con A	1000 ± 89.57	11.1
Infected	Parasite (Day 2) + Con A	1162.6 ± 109	12.5

cpm = counts per minute

*Mean ± SD of four replicates

Appendix Table 4.6

Treatment	Day 1	Day 2	Day 3
Crude Su Eg	43.0 ± 19.0	31.9 ± 12.0	33.3 ± 5.0
Crude Su Tt	37.4 ± 11.0	35.1 ± 5.8	32.5 ± 6.0
Crude Su Tc	33.0 ± 5.3	21.5 ± 6.0	29.4 ± 18.0
Su Eg	67.5 ± 20.0	68.4 ± 19.0	71.7 ± 21.0
Su Tt	63.7 ± 8.4	65.3 ± 14.0	69.4 ± 6.8
Su Tc	71.0 ± 15.0	66.1 ± 19.0	56.3 ± 11.0
ES Eg	97.2 ± 18.0	91.2 ± 9.4	86.3 ± 10.3
Untreated	96.5 ± 20.0	98.5 ± 17.3	84.1 ± 18.0

Appendix 5

Names and Addresses of Commercial Companies

Amersham International Ltd., Amersham, Bucks., England, U.K.

Beckman-RIIC Ltd., High Wycombe, England, U.K.

Becton-Dickinson (U.K.) Ltd., Wembley, England, U.K.

Bio Rad Laboratories Ltd., Watford, Hertfordshire, England, U.K.

BDH British Drug House Chemicals Ltd., Poole, Dorset, U.K.

Cambridge Medical Company, Cambridge, England, U.K.

Cytospin Centrifuge Shandon Southern Products Ltd., Cheshire, U.K.

Difco Laboratories, Detroit, Michigan, U.S.A.

Dow Corning, Midland, Michigan, U.S.A.

Endecotts Ltd., London, England, U.K.

Ernst Leitz, D-6330, Wetzlar, W. Germany.

Eschmann Bros. & Walsh Ltd., Shoreham-by-Sea, Sussex, England, U.K.

Fisons Scientific Apparatus, Loughborough, England, U.K.

Flow Laboratories Ltd., Irvine, Scotland, U.K.

Gibco Europe Ltd., Paisley, Scotland, U.K.

Gilson, Villiers-Le-Bel, France

Glaxo Laboratories Ltd., Greenfield, England, U.K.

Greiner Labor Technik (Dynatech Laboratories Ltd.), Billinghurst, U.K.

Hellma (England) Ltd., Westcliff-on-Sea, England, U.K.

Hughes and Hughes Ltd., Romford, Essex, U.K.

ICI Imperial Chemical Industries Ltd., Macclesfield, England, U.K.

LKB Instruments Ltd., South Croydon, England, U.K.

Luckham Ltd., Burgess Hill, Sussex, U.K.

Microflow Ltd., Fleet, England, U.K.

Miles Laboratories Ltd., Slough, England, U.K.

Millipore Ltd., London, England, U.K.

MSE Measuring Scientific Equipment Ltd., Crawley, England, U.K.

Nordic Immunological Laboratories, Tilburg, The Netherlands.

Nuclear Enterprises Ltd., Sighthill, Edinburgh, Scotland, U.K.

Oxoid Ltd., Basingstoke, England, U.K.

Pharmacia Fine Chemicals AB, Uppsala, Sweden

Philips Analytical Department, Pye Unicam Ltd., Cambridge, U.K.

Pye-Unicam Ltd., Cambridge, U.K.

Sarstedt W. (U.K.) Ltd., Leicester, England, U.K.

Scientific Suppliers Co. Ltd., London, U.K.

Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Sterilin Ltd., Teddington, England, U.K.

Vickers Instruments Ltd., York, England, U.K.

Wellcome Reagents Ltd., London, England, U.K.

Whatman Ltd., Maidstone, Kent, England, U.K.

Plates 1 and 2 Protoscolices from equine hydatid cysts

A = invaginated

B = Evaginated

C = Dead protoscolices which have taken up the dye of a 0.2% tryphan blue in PBS solution.

Arrows indicate the calcareous corpuscles.

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Immunosuppression by larval cestodes of *Babesia microti* infections

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Parasitic helminths often survive for long periods of time in immunologically competent hosts. Presumably the parasite either fails to stimulate the host's protective responses or these mechanisms are evaded or resisted.

One means by which parasites are thought able to persist is by suppressing the host's immune response. Immunosuppression has been demonstrated in a number of parasitic infections including malaria (Wedderburn, 1974), trypanosomiasis (Clayton *et al.*, 1979) and *Nematospiroides dubius* (Behnke *et al.*, 1983).

The suppression of the immune response to apparently unrelated antigens by cestode infections was first shown by Good and Miller (1976) using *Taenia crassiceps*. Mice infected with this metacestode had depressed primary and secondary antibody responses to sheep red blood cells.

The effect of prolonged cestode infections on the hosts's response to subsequent disease may be of relevance in the field where animals are exposed to numerous pathogens. A study was therefore commenced on the interaction in mice between the larval forms of *T. crassiceps* and *Taenia taeniaeformis* and both host susceptibility and the humoral response to the intraerythrocytic protozoa *Babesia microti*.

MATERIALS AND METHODS

Experimental Design

T. CRASSICEPS AND *B. MICROTI*

This pilot study comprised three groups of six-week-old CF1 mice.

GROUP 1. Eight mice inoculated intraperitoneally with 20 intact metacestodes of less than 2 mm diameter.

GROUP 2. Eight CF1 mice inoculated with 20 metacestodes that had been burst by forcing them through an 18 g needle. The cyst fluid was washed away so that only tissues were present in the inoculum.

GROUP 3. Eight uninfected mice.

Five weeks later the mice were all inoculated intravenously with 10^6 *B. microti*-infected red cells. The subsequent parasitaemia was monitored by examining Giemsa-stained blood smears. Mice which failed to show a parasitaemia by two days after inoculation were excluded. Blood was also collected for serological assays every seven days.

When the parasitaemia had remained for several days below 5% of red cells infected, a challenge dose of 10^6 *Babesia*-infected red cells was given by the same route.

T. TAENIAEFORMIS AND *B. MICROTI*

Ten six-week-old CF1 mice were infected with 200 eggs by mouth. Five weeks later these mice and ten uninfected CF1 mice of the same age were inoculated intravenously with 10^6 *Babesia*-infected red cells. The experiment then proceeded as described above.

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Parasites

Taenia crassiceps larvae of the TOI strain were propagated in CF1 mice by intraperitoneal inoculation of 20 metacestodes. Larvae were harvested two to three months later and washed in saline and antibiotics prior to administration to the experimental mice.

Taenia taeniaeformis of a Belgian strain was maintained in cats and the proglottids collected regularly. The eggs were released and passed through at 50 μm and into a 23 μm mesh sieve. The eggs were washed three times with saline and antibiotics and the concentration assessed by a modification of the McMaster technique.

Babesia microti of the Kings strain, maintained at the C.T.V.M. was preserved as frozen stabilate in liquid nitrogen. To initiate each experiment the thawed blood was inoculated intraperitoneally into several CF1 mice. These were exsanguinated by cardiac puncture under anaesthesia six to eight days later when a parasitaemia of over 30% had been reached. This blood was mixed with heparin (50 units ml^{-1}) at a 1 : 10 dilution. The red cell count was estimated using a Neubauer haemacytometer.

Humoral Immune Response to *B. microti*

This was assessed by the indirect fluorescent antibody technique (IFAT).

Heparinized red cells from the pool used to initiate the *Babesia* parasitaemias were washed three times with phosphate buffered saline. The washed red cells were smeared across 15 well Multi-spot slides (Flow Ltd.) and air-dried. The smears were fixed in acetone for 15 minutes and, when dry, wrapped in foil in a moisture-free container to be stored at −20°C until required.

Each serum was applied to the spots for 20 minutes at room temperature before the slide was washed and treated with FITC-labelled goat anti-mouse IgG conjugate (Nordic Ltd) at 1 : 10 dilution. Finally the slides were counterstained with Eriochrome black, at a 1 : 10 dilution and examined using an Orthoplan microscope (Leitz, Wetzlar, Germany) equipped for selective incident excitation of FITC at ×675 magnification. The observed fluorescence was visually assessed by end-point titration.

Statistical Analysis

The levels of parasitaemia and humoral antibody response were compared by repeated Student's *t* tests.

RESULTS

***T. crassiceps* and *B. microti* Concurrent Infections**

The piroplasms appeared in the blood after one day in all the mice which had received an intravenous infection and the mean percentage parasitaemia rose rapidly in the mice infected with intact metacestodes to a peak on Day 6 (Fig. 1). In the mice which had received the burst metacestodes the parasitaemia rose less rapidly to lower peak on Day 9. The parasitaemia in the control mice also peaked on Day 9. The parasitaemia in the mice containing intact metacestodes remained significantly greater than the controls until Day 12. A second smaller peak, characteristic of *B. microti* infections, occurred in all the groups. It appeared somewhat greater in the group of mice which had received the burst metacestodes but this was not significant.

No recrudescence of the *Babesia* infection was observed in any of the mice after challenge on Day 28. All the mice developed a humoral response to the *Babesia* as assayed by IFAT (Fig. 2), but this was significantly lower in the mice concurrently infected with intact *T. crassiceps* metacestodes. The response in the mice which had received burst metacestodes

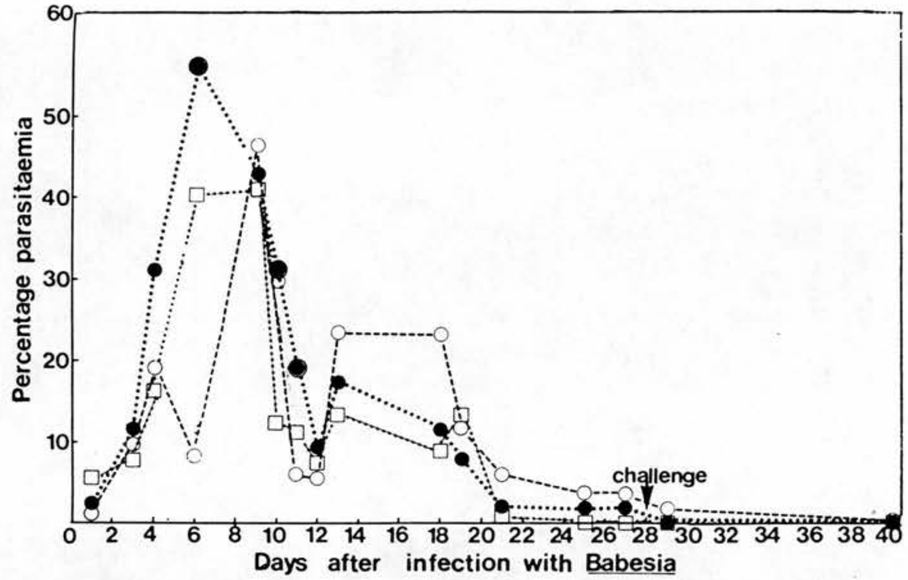

Fig. 1. The pattern of parasitaemia of *Babesia* in mice concurrently infected with intact *T. crassiceps* metacestodes (●), burst metacestodes (○) and *B. microti*-only infected mice (□). ◯ indicates means significantly greater than *Babesia*-only controls in a Students *t* test at the 5% significance level.

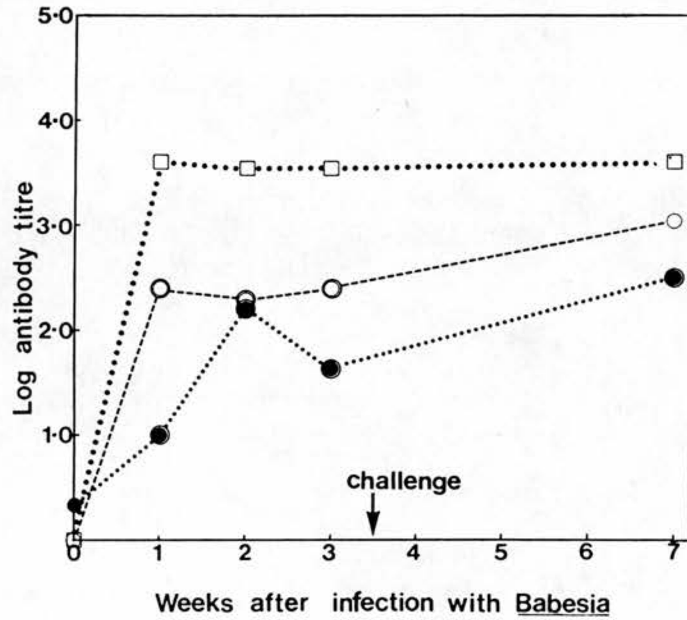

Fig. 2. Antibody titres to *B. microti* as measured by IFAT in mice concurrently infected with intact *T. crassiceps* metacestodes (●), burst metacestodes (○) and *Babesia* only (□). ◯ indicates means significantly lower than *Babesia*-only controls.

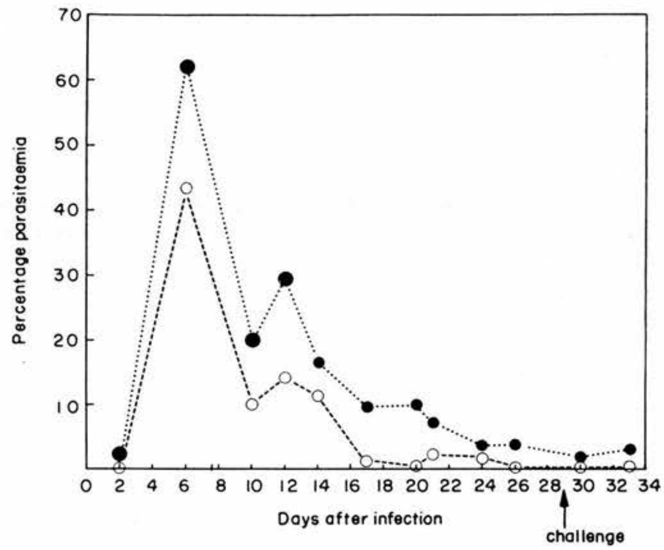

Fig. 3. The pattern of parasitaemia of *B. microti* in mice concurrently infected with *T. taeniaeformis* (●) and *Babesia*-only controls (○). ◯ indicates means significantly greater than the controls.

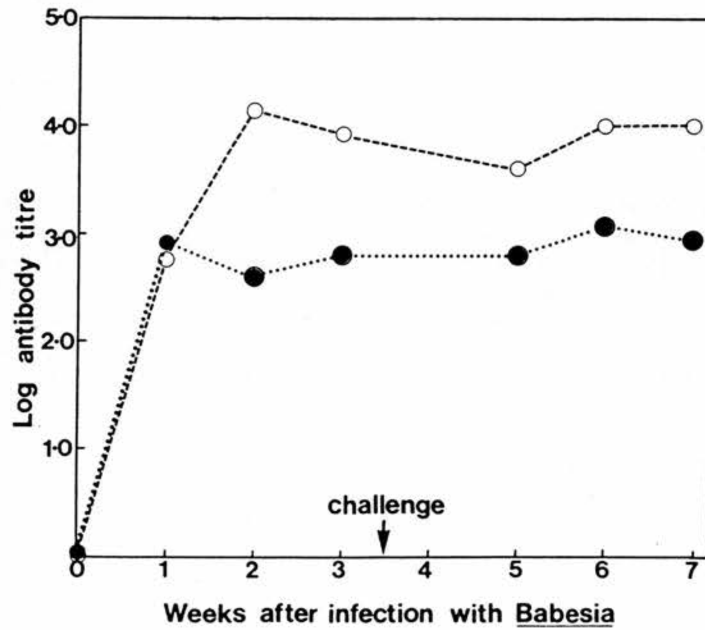

Fig. 4. Antibody titres to *B. microti* as measured by IFAT in mice concurrently infected with *T. taeniaeformis* (●) and *Babesia*-only controls (○). ◯ indicates means significantly lower than those of controls.

was intermediate but still significantly lower than that in the controls. There was little change in the antibody titres in any of the mice following challenge, those for the concurrently-infected mice remaining below those for the controls.

At necropsy the peritoneal cavity of the mice infected with intact metacestodes was packed with living larvae, whereas there was no evidence of the cestodes in the mice which had received burst metacestodes.

T. taeniaeformis and *B. microti* Concurrent Infections

Piroplasms were first detected in the blood two days after the intravenous inoculation and both groups show a rapid increase in parasitaemia that reached a peak at Day 6 (Fig. 3). The parasitaemia in the concurrently-infected mice was significantly greater than that in the controls from Day 6 to Day 12. Once again challenge did not bring about a rise in parasitaemia.

The humoral response to *Babesia* in the mice concurrently infected with *T. taeniaeformis* (Figs 3 and 4) was significantly lower from two weeks onwards.

DISCUSSION

The evolution of a complex of interacting immunological effector mechanisms by the host necessitated the acquisition of evasion mechanisms by those parasites which must survive for more than a few days in their host in order to propagate. There have been several proposed mechanisms by which such immune evasion can be achieved including modification of induction or expression of host immune responses (Ogilvie and Wilson, 1976; Mitchell, 1982).

The normal course of *B. microti* infections in mice is mild and self-limiting with a good host survival rate. The level of parasitaemia declines to less than 0·01% 35–45 days after infection but the host remains in a state of premunition and the parasitaemia returns following splenectomy (Cox and Young, 1969). This protozoan was considered to be potentially suitable for monitoring the immunosuppressive effect of these larval taeniid infections, as the chronic nature of the infection allowed sequential sera sampling over many weeks.

Existing infections with *T. crassiceps* or *T. taeniaeformis* appeared to render the mice somewhat more susceptible to infection with *B. microti* in that their peak parasitaemia was significantly higher. This was more marked with *T. taeniaeformis* but in neither case was the immunosuppression sufficient to prevent the mice controlling the piroplasm. *Babesia microti*-infected mice normally exhibit solid immunity to subsequent re-infection (Clark, 1977) and this still appears to be the case in the concurrently-infected animals although Good and Miller (1976) reported that infection with *T. crassiceps* had a more marked depressive effect on the secondary humoral response.

There were consistently lower antibody titres in the concurrently-infected mice than in the mice only infected with *Babesia*. There was only a slight increase in antibody titres in any of the experimental mice following challenge. This may be due to the infected cells being destroyed by the previously activated cell-mediated mechanisms shortly after inoculation.

There was also a significant reduction in the humoral response to *B. microti* in mice inoculated with burst metacestodes, although these failed to develop despite the presence of intact scolices in the inoculum. Excretory/secretory preparations collected from *in vitro* culture of *T. crassiceps* was found to be capable of depressing primary antibody responses to sheep red blood cells (Good *et al.*, 1982). The burst metacestodes will mainly consist of

somatic antigen components common to the intact metacestodes, but these also appear able to depress the humoral response.

The effector mechanisms principally involved in *Babesia* destruction are thought to involve macrophages and natural killer cells (Allison *et al.*, 1978). If so, a depressed antibody response, on its own, might not be expected to modify the normal course of the parasitaemia. It is probable that this immunosuppressive effect of the larval cestodes would be more deleterious to the host if it was expressed against pathogens, such as viruses, which are more influenced by humoral effector arm of the immune system. It would also be of interest to investigate the influence of such metacestode infections on the host's response to vaccination, as this is of potential importance in domestic farm animals.

The mechanisms whereby such immunosuppression is mediated are as yet unknown. There are many possible explanations including antigenic competition or the secretion of substances having a direct immunosuppressive effect on the immune system. Good *et al.* (1982) suggested that interactions between *T. crassiceps* larvae and host cells or antibodies could be responsible for immunodepression. Even if this is so, this interaction with the immune system is worthy of investigation.

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