MURINE GIARDIASIS: INTESTINAL MUCOSAL IMMUNE RESPONSES

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For Pam

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Summary and Conclusions

Declaration

I declare that this thesis was composed by myself and that unless otherwise stated, the work was performed personally.

Grant H. Munro

Abstract

An experimental model of giardiasis, namely primary infection of adult mice with Giardia muris, was established. Measures of immunity and intestinal damage were used to examine the hypothesis that T cell factors, lymphokines, were responsible for the mucosal damage. The model allowed observation of the host cellular response in relation to the size of parasite load at different times during infection. Aspects examined were the number of IgM, IgA, IgG and IgE containing plasma cells; the number of intraepithelial lymphocytes, the number of mucosal mast cells; the number of goblet cells and function of associated mucus. Intestinal damage was assessed by measurement of villus height, crypt depth, crypt cell production rate and disaccharidase activity.

The number of trophozoites rapidly increased during the first week of infection to a plateau phase lasting two to three weeks, followed by a rapid expulsion of the parasite. During primary infection there was evidence of intestinal damage with mild villus atrophy, crypt hyperplasia, increased crypt cell production rate and decreased disaccharidase activity. The number of plasma cells, intraepithelial lymphocytes, and mucosal mast cells also increased. Goblet cell numbers and the quantity of mucus were not altered during primary infection. Prolonging gut motility by loperamide treatment did not alter the time course or characteristics of infection, suggesting that motility was not involved in parasite elimination.

Concurrent graft versus host reaction and giardiasis was examined in an attempt to alter infection through the "innocent bystander" phenomenon. This unrelated tissue damage results from the immune response active during the graft versus host reaction. Concurrent early or late graft versus host reaction did not alter the infection, but additive increases in mucosal damage and numbers of intraepithelial lymphocytes and mucosal mast cells were found. This suggested that lymphokines did not directly eliminate the parasite, but may mediate the mucosal damage. The hypothesis that intestinal damage is related to immunity was tested in nude (athymic) mice, where such damage should not occur. These infected

mice showed crypt hyperplasia, increased crypt cell production rate, and decreased disaccharidase activity. Numbers of mucosal mast cells detected by an esterase stain increased, but intraepithelial lymphocytes were unchanged. These aspects of intestinal damage can occur without T cells and are therefore not mediated by lymphokines. Conflicting evidence was provided by transferring immune spleen and mesenteric lymph node cells into chronically infected nude mice. This resulted in a reduction of parasite numbers, villus atrophy and a further decrease in disaccharidase activity, but no exacerbation of other existing intestinal damage. Variable changes in intraepithelial lymphocytes and mucosal mast cells were also found. Steroid treated mice were used as another example of altered host immunity. Treatment led to recrudescence of infection in immune mice and massive trophozoite numbers, but did not result in mucosal damage. An increase in intraepithelial lymphocytes, goblet cells and variable changes in mucosal mast cells were found in mice with steroid induced recrudescent infection.

The conclusions of these studies were that the mucosal damage could not be directly attributed to the action of T cell dependent lymphokines. This is in contrast with other models of mucosal injury, such as helminth infection and graft versus host reaction, where the mucosal damage is as a result of the T cell mediated immune response. The trophozoite itself was not conclusively excluded as the causative agent of the enteropathy. An alternative hypothesis is that mucosal mast cells and their mediators are responsible for, or are major contributors to the mucosal intestinal damage during infection with G. muris.

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List of Abbreviations

B-cell	bone marrow derived lymphocyte
CCPR	crypt cell production rate
CMI	cell mediated immunity
DTH	delayed-type hypersensitivity
F1	first generation after cross breeding
GvHD	graft-versus-host disease
GvHR	graft-versus-host reaction
h	hour
H & E	haematoxylin and eosin
IEL	intraepithelial lymphocyte
i.g.	intragastric
i.p.	intraperitoneal
Lyt	T lymphocyte associated antigen (mouse)
M cell	microfold cell
MLN	mesenteric lymph node
MMC	mucosal mast cell
p	probability
SD	standard deviation
SEM	scanning electron microscope
TEM	transmission electron microscope
T lymphocyte	thymus derived lymphocyte
w.r.t.	with relation to

Units

Length		
µm	micrometre	10^{-6} m
mm	millimetre	10^{-3} m
cm	centimetre	10^{-2} m
Weight		
mg	milligram	10^{-3} g
g	gram	1 g
kg	kilogram	10^{3} g
Volume		
ml	millilitre	10^{-3} l
l	litre	1 l
Concentration		
M	molar	
Area		
mm^2	square millimetre	
Miscellaneous		
g	gravitational acceleration	
log	logarithm to the base 10	
pH	reciprocal log hydrogen ion concentration	
Symbols		
<	less than	
/	per	
=	equals	

Chapter 1

Giardiasis: The Parasite

A. Discovery.

Giardia is a single cell parasite found in the small intestine of man and many animals. It has two stages; the active parasitic trophozoite and the resistant cyst. It is probably one of the first parasitic protozoa described. Antony van Leeuwenhoek observed in 1681:

> animalcules a-moving very prettily; some of 'em a bit bigger, others a bit less, than a blood-globule, but all of one and the same make. Their bodies were somewhat longer than broad, and their belly, which was flatlike, furnisht with sundry little paws, wherewith they made such a stir in the clear medium
>
> (loc. cit. Burke, 1977).

B. Nomenclature.

Lambl gave a more accurate description in 1859. In 1888, Blanchard suggested the commemoration of Lambl by naming the protozoan found in man, Lamblia intestinalis. This nomenclature is still favoured in the USSR and Eastern Europe. The genus name, Giardia, was ascribed by Kunstler in 1882, when naming protozoa isolated from the gut of tadpoles.

Kofoid and Christiansen in 1915, proposed the binomial, Giardia lamblia, which is now commonly used for the parasite isolated from man.

Giardia species were named according to the host from which they were isolated. The result was a plethora of "species". This form of nomenclature has been carried over and is still occasionally used today for convenience and easy identification of the original host (Levine 1979). This practice, though taxonomically incorrect, does serve to underline the remaining uncertainty of specific lineage in the genus.

In 1952, Filice published a monograph on the genus Giardia and identified the presence of two mammalian and one amphibian form, designated as the species:

G. duodenalis (lamblia)
G. muris
G. agilis
(loc.cit. Meyer & Radulescu, 1979)

C. Life Cycle.

I. Trophozoite Form.

The motile flagellate parasitic form of Giardia is the trophozoite (Fig.1.1). This is a bilaterally symmetrical pear-shaped organism, 9 to 21 µm long, 5 to 15 µm wide and 2 to 4 µm thick. There are four pairs of flagellae. The ventral surface is mostly covered by a convex, ovoid disc composed of a series of microribbon structures (Friend 1966, Sheffield 1979, Holberton 1981).

There is a unique organelle, called the median body. This is composed of groups of microtubules and is thought to be a reserve of lipoprotein (Taniko & Yardley 1965, Morecki & Parker 1967). Friend (1966) suggested that the median body functioned as a supporting structure for the posterior portion of the trophozoite. The median body of G. muris is small, round and central. G. duodenalis may either have a single median body, or more commonly two. In G. duodenalis, the median body resembles the claw of a claw hammer, lying transversely across the trophozoite.

Movement is achieved by the corkscrew action of the flagellae; this is not a strongly directional force and in suspension, trophozoites appear to have little control over their movement. It is probable that surface interaction, such as "ground effect", is required for their efficient directional propulsion.

The mechanism of adhesion of trophozoites to a surface has been the cause of much discussion and various tenable theories are published. These all centre on the ventral disc apparatus. The disc is composed of overlapping microribbons and may enable reduction of the disc diameter by sliding over each other in the manner of an iris diaphragm. This would result in effective grasping of the substrate (Mueller et al 1973, Meyer & Radulecu 1979, Erlandsen et al 1979). The ventral flange of the disc is the

structure implicated in grasping or contraction during attachment (Friend 1966, Erlandsen & Chase 1974, Owen et al 1979).

Holberton (1973, 1974) suggested a novel hydrodynamic model involving the disc acting as a suction cup. The action of the central pair of flagellae passing through the posterior of the disc would produce a vortex of flow away from the exit gap. This results in reduced pressure within the disc when in opposition to a surface, thus clamping the trophozite to the substrate. Erlandsen et al (1979) described the mechanism of detachment, namely that the tail flexed and so released the suction. Further evidence for a strong suction force being involved is found in the marked impression of the disc structure left in the microvillus coat, as seen during SEM (Erlandsen & Chase 1974, Erlandsen et al 1979, Friend 1966, Owen 1979, Erlandsen in Meyer & Radulescu 1979). In TEM, the disc and ventral flange are often seen deeply embedded in the microvillus coat causing damage to these structures.

1. Metabolism.

Nutrient uptake for metabolism occurs by a process of invagination of the cell membrane and the formation of vacuoles beneath the plasmalemma. This occurs primarily on the dorsal surface, but the ventral surface is also capable of vacuole formation. Bockman & Winborn (1968) described this process by observing localization of ferritin from the medium. Friend (1966) suggested the vacuoles exuded a gelatinous or mucoid substance during encystation.

The metabolic requirements have been elucidated by the techniques of axenic culture. Lindmark (1980) described Giardia as aeroto l erant and able to respire in the presence of oxygen despite the absence of mitochondria. Glucose stimulated endogenous respiration, but other carbohydrates and Krebs cycle intermediates did not. Study of the effects of metabolic poisons lead to the conclusion that metabolic energy was produced by glycolysis, by a substrate level phosphorylation and a flavin, iron-sulphur protein mediated electron transport system. Oxidative phosphorylation by a cytochrome mediated pathway and a functional Krebs cycle were

apparently absent.

The importance of bile to Giardia was described by Bemrick (1963) who showed that ligation of the rat bile duct resulted in a failure of Giardia to establish normal infection. Twenty years later, Farthing et al (1983) were able to explain these results. Giardia have a limited ability to synthesize membrane lipids de novo and hence bile salts are needed to facilitate the uptake of membrane phospholipids from the media. Further supportive evidence for bile salt metabolism is the clinical observation of bile salt deconjugation in 82.5% of patients (Tandon et al 1977) and steatorrhea develops as a result of this. Giardia also inhibit lipase hydrolysis of fat (Meyer & Radulescu 1979), which may also contribute to steatorrhea.

Early axenic culture experiments required symbiotic coculture with yeasts, such as Candida guillermondii and Saccharomyces cereviciea (Karapetyan 1962). Pure axenic culture has now been achieved. Yeasts are found in association with chronic Giardia infections in man (Bemrick 1965), but these seem to be coincidental infections. The initial requirement of coculture was due to an inadequate supply of B vitamins in the original media.

2. Generation Time.

Generation times vary greatly with culture conditions, and even within a monoculture, slow and fast dividing strains may be selected. In vitro growth responses are commonly reported; Danciger & Meyer (1971) studied trophozoite cultures from rabbit and found an average generation time of 18.1 $\pm$ 1.6 h. Karapetyan (1962) examined trophozoites of human origin and found a generation time of 36-38 h. Other studies found 20-30 h and 30-40 h as generation times in trophozoites isolated from rabbits (cited Danciger & Meyer 1971).

Only one study has used an in vivo model; De Carnerii et al (1977) determined generation time using a single trophozoite inoculum in mice. The generation time under these "natural" conditions is much shorter, having a mean of 5 h 7 mins.

II. Cyst Form.

The resistant form of Giardia is the cyst (Fig.1.1) which is an ellipsoidal structure 8 to 12 /um long and 7 to 10 /um wide. The contents show as a fine granular cytoplasm. This is encompassed by a highly refractile, colourless, thin hyaline wall. The surface is smooth and has no distinct characteristics that might serve to identify host origin (Tombes 1979). The faecal cyst generally has four nuclei and the median bodies are clearly visible. Sheffield (1977) noted that peripheral vacuoles in the cyst seem analogous to those reported by Bockman (1968) as an endocytotic mechanism. This function is not likely to occur in the cyst stage and an alternative function suggested by Friend (1966) was that they are involved in cyst wall formation.

The cyst is rapidly destroyed on drying, but was shown by Rendtorff (1979) to remain viable for several weeks under moist conditions. Craft (1982) found cysts, stored at 4° C as a faecal homogenate in distilled water, retained some degree of infectivity for up to a year.

When ingested the cyst passes through the acidic condition of the stomach which acts as the stimulus for excystation (Bingham & Meyer 1979). Upon passing into the duodenum, the trophozoite adopts its parasitic lifestyle and divides rapidly by longitudinal fission.

III. Distribution of Trophozoites and Cysts.

Icwanczak (1968) studied the distribution of trophozoites and cysts in white mice. Trophozoites were found throughout the small intestine with maximum numbers at 10cm from the pyloric sphincter. Cyst numbers were found to plateau 30 to 35 cm from the pylorus. A more detailed study was carried out by Gillon et al (1982a). In this study trophozoites were described as inhabiting the majority of the small intestine during the first two weeks of infection. By day 35, trophozoites occupied the proximal half,and by day 42, the proximal third of the small intestine. Olveda et al (1982) studied distribution relative to infective dose and trophozoite load; using 10 cysts as inoculum, jejunum and to a lesser extent, ileum, were inhabited by trophozoites, after one week of infection. At peak

trophozoite load, after 14 days of infection, duodenum, jejunum and ileum were equally populated. Residual populations at 49 days were mainly in the jejunum. An inoculation of 10,000 cysts produces an approximately equal parasite load in duodenum, jejunum and ileum, from the first sampled time point of 4 days, up to 14 days. Thereafter, distribution and parasite load were as for the 10 cyst infection. These trophozoite numbers were obtained by simple wash through of segments of intestine. This method does not remove all trophozoites present, so the results are not as accurate as those of Gillon et al (1982a). Owen et al (1979) found trophozoites localized in the proximal 25% of small intestine and described the basal area of the villi as the preferred site. The predeliction for the proximal intestinal environment is likely to be due to the bile salts dependence, while the predeliction for the basal area of the villi is explained by this being a relatively unstirred area of the lumen. Belsovic & Faubert (1983) noted trophozoites inhabiting the proximal 25% of the small intestine between days 3 and 8 of infection, increasing the area to 40% at peak infection. Owen et al (1979) also recorded adherence of trophozoites to columner cells of Peyers Patches, but no contact with the specialized M-cells involved in transport of sampled lumenal contents to components of the lymphoid system.

IV Infection.

Infection can be achieved with one trophozoite (De Carneri et al 1977) or many (Hewlett et al 1982), though cysts rather than the trophozoite are the natural infective stage. Food soiled with trophozoites has been used as the infective medium to demonstrate the possibility of this mode of infection, so being similar to the mode of transmission of amoebiasis in South African townships (De Carneri & Trane 1978). This transmission mode is strictly limited firstly by the period during which the trophozoites remain viable and infective, and secondly by the number of trophozoites required for infection to be established.

The gastric acid barrier is known to be bacteriocidal (Gianella et al 1973). This also protects against ingestion of trophozoites

which are easily destroyed in an acid environment.

Rendorff (1954, 1979) gave human volunteers cysts in varying doses, in capsules and observed the course of infection by subsequent faecal cyst output and symptomology. One cyst did not produce an infection, whereas 10 to 25 cysts infected one third of subjects, and over 100 cysts produced infection in all subjects. The study can be criticized in that some of these prison volunteers, though initially screened for Giardia infection and questioned about past history of symptomatic infection, had, at some stage probably encountered this parasite. The observed duration of infection ranged from 5 to 41 days and suggests prior exposure to the parasite in those subjects with short infections.

Olveda et al (1982) used infective doses of 10 and 10,000 cysts in the mouse model. The smaller dose required more time to reach maximum parasite load and produced less marked histopathological changes. The types of histopathological change, trophozoite distribution, parasite load and cyst output and length of time to resolution of infection were similar in the 10 and 10,000 cyst infections. These findings were supported by Belsovic & Faubert (1983) using 10 and 100,000 cyst infective doses.

Transmission of infection in experimental systems is always by the per oral route. In nature, water supplies are invariably implicated in infection, and in epidemic outbreaks, contamination often results from filter or chlorinating plant breakdown (Lippy 1979). From 1972 to 1977, the most commonly identified pathogen in waterborne epidemic gastroenteritis in the United States was Giardia lamblia (Craun 1979). Craun (1979) noted the ineffectiveness of chlorination as a sterilizing agent against Giardia cysts. This was based on a dye exclusion criterion which remained unaltered following chlorination. Hoff (1979) suggested that this did not reflect cyst viability because infectivity was destroyed by chlorination. Bingham & Meyer (1979) further supported this view, showing that eosin exclusion tests gave consistently higher viability than could subsequently be demonstrated by in vitro excystation.

D. Epidemiology and Source of Infection.

Leningrad has been associated with Giardia infection of visitors (Craun 1979). This association was so firm that Jokipii & Jokipii (1974) were able to plan a prospective study of sixty Finnish students, recording visits to Leningrad and subsequent course of infection.

Craun (1979) listed the recent giardiasis epidemics in the United States and suggested that the main causes were due to ineffective treatement of water supplies. He recommended that all surface water should be treated by sedimentation and filtration, in addition to further treatment by chlorination.

The contaminated source has often been traced to human faeces, but outbreaks among campers in very remote areas led to investigation of a zoonosis. Jurenek (1979) compared three US outbreaks and reported on the likelihood of beavers acting as a natural reservoir host for human epidemic giardiasis. Beavers were also implicated in the 1976 epidemic at Camas, Washington. Davies & Hibler (1979) surveyed domestic and wild mammals in Colorado; this area had the highest incidence of epidemics of recent years (Craun 1979). Domestic dogs may be a source of infection, and in two instances, cats were associated with familial giardiasis. Giardia lamblia has been used to infect dogs by Hewlett et al (1982) supporting the possibility of them being natural reservoir hosts for the human disease. Regarding wild or non-domestic animals, Davies & Hibler (1979) used infection of dogs as an indicator of G. lamblia and successfully infected using isolates from beavers and cattle only. Other animals are carriers and active excretors of Giardia, but the inability to cross-infect the challenged dogs suggest that these were not acting as reservoir hosts.

Ultimately, humans themselves provide much of the contamination and infection. In secluded and "wild" areas, the long survival of cysts ensures a reservoir of infectivity in lakes and streams.

Human to human direct transmission is common in institutions and day care nurseries (Black et al 1977). Familial transmission is common (Wilcox 1975). Giardiasis is also common among homosexuals and Owen (1979) described this as a venereal disease. Giardia has

been found in vaginal smears, from an environment inhabited by another closely related flagellate protozoan, Trichomonas vaginalis.

E. Detection of Current Infection.

The incidence of giardiasis in populations is very difficult to assess and it seems likely that the majority of people are exposed to this pathogen at some time in their lives. The generally employed techniques of stool examination, duodenal aspiration and biopsy only indicate an ongoing infection. Olveda et al (1982) found that in mice, the presence of cysts was only reliable after four days and before ninety days after infection.

Chronic human infection appears common (Rendtorff 1954). Healy (1979) found prevalence rates of 4 to 22% in US children from different areas. Much depended upon local conditions and socioeconomic status of the cohort examined. Prevalence in adults falls, generally to single figure percentages. Australian adults, as assessed by selected gastrointestinal patients, showed 2.1% prevalence, as compared to 2 to 6% worldwide (Kerlin et al 1978). An earlier study of Australian children (Alp & Hislop 1969) found 33% to be infected in a group selected from various towns, and 21% as assessed from selected hospital admissions. Black et al (1977) surveyed daycare centres, examining children of six months to three and a half years old; in three daycare centres, 54%, 38% and 29% infection rates were found, compared to 2% prevalence in age matched controls not attending centres.

These previously reported studies rely upon cyst isolation and concentration from faeces as the criterion for current giardiasis. Kerlin et al (1978) selected patients at upper gastrointestinal endoscopy and examined smears of duodenal and jejunal origin, to arrive at a prevalence rate of 2.1%. This test is more reliable than cyst isolation, as some chronically infected patients do not excrete cysts in easily detectable numbers. This fact was well demonstrated by Klima et al (1977) in an electron microscope study of diagnosis; false negatives were found to occur in 50% of diagnoses. The study found similar occurrence of giardiasis in European children, 3 to 26% and Russian children, 10% to 20%.

A recent development in diagnosis has been the use of counterimmunoelectrophoresis (CIE) of faeces to detect G. lamblia antigens (Craft & Nelson 1982). This non-invasive method gave comparable results to the total number diagnosed by cyst isolation, duodenal fluid examination and duodenal biopsy in sixty-six cases.

Another highly specific method of diagnosis is by detection of anti-Giardia specific antibodies. This method will also indicate recent prior exposure to the parasite and a higher incidence rate may be found. Smith et al (1981) subjected sera of 197 unselected individuals in the Washington area to an ELISA for specific Giardia antibodies of IgG class and found 14% prevalence rate in this group. A larger control group of non-symptomatic individuals showed 12% with evidence of specific IgG against Giardia.

Roberts-Thomson & Mitchell (1981) also developed an ELISA method utilising the cross reactivity of G. muris and G. lamblia trophozoite antigens. This assay was sensitive enough to show some degree of specific anti-Giardia antibody response in almost all tested residents of the Melbourne area.

Worldwide, the prevalence of current giardiasis would appear to be of the order of 2 to 6% (Kerlin et al 1978).

F. Factors Leading to a Predisposition to Chronic Giardiasis.

Incidence is related to many factors; those of local conditions, socioeconomic status and age have been noted. Several authors have correlated blood group A with giardiasis in children (Barnes & Kay 1977, Paulson 1977, Zismann 1977). Roberts-Thomson et al (1980) found no such association in adults. Achlorhydria and hypochlorhydria render subjects susceptible to chronic giardiasis (Taniko & Yardley 1965, Paulson 1977, Knight 1980) and these patients also suffer more frequent Salmonella and Shigella infections. The "gastric bacteriocidal barrier" proposed by Gianella et al (1973) may protect normal individuals or the absence of H+ ions may result in a more favourable environment for survival of Giardia trophozoites and allow colonization of a larger proportion of the small intestine.

Protein energy malnutrition (PEM) causes a disruption of a variety of immunological responses and results in a predisposition to chronic giardiasis. PEM is also a cause of pancreatic disease and hypochlorhydria, all features associated with giardiasis. The presence of Giardia also exacerbates the PEM condition (Knight 1980).

Immunoglobulin deficiencies also predispose to chronic giardiasis (Brown et al 1972, Hoskins et al 1967, Webster 1980). Webster (1980) noted that common variable hypogammaglobulinaemic patients are prone to gastrointestinal complications, one third of these adults have diarrhoea, of whom half have detectable giardiasis. Adult onset of hypogammaglobulinaemia is commonly accompanied by achlorhydria. Hoskins et al (1967) suggested that giardiasis exacerbates hypogammaglobulinaemia, as does PEM, though many chronically infected patients have normal or elevated levels of serum immunoglobulins (see later).

G. Clinical Features.

I. Symptoms.

The general clinical features of patients presenting with giardiasis include steatorrhea with frequent large, soft, pale or yellow, offensive stools and flatus; abdominal distension and pain, weight loss, lassitude, anorexia and nausea. A more diarrheoic stool containing mucus may also occur at times. Symptoms generally develop following a prepatent period of two weeks and the average duration of illness is about six weeks.

II. Malabsorption.

In human giardiasis, many reports of clinical laboratory analysis indicate malabsorption of D-xylose, vitamin B_{12} and of fat (Blenkinsopp et al 1978, Desai & Kalro 1983, Hartong et al 1979, Poley & Rosenfield 1982, Ridley & Ridley 1976, Riis 1975, Sheehy & Holley 1975, Tandon et al 1974, Wright et al 1977b, Wright 1979, Vinayak et al 1978).

III. Morphological Changes.

In several studies, the degree of malabsorption appears to be directly related to the morphological changes seen in the small intestine (Erlandsen & Chase 1974, Ridley & Ridley 1976, Saha & Ghosh 1977, Tandon et al 1974, Tewari & Tandon 1974, Wright & Tomkins 1977, 1978, Wright 1979, 1980).

In mice, morphological changes in the small intestine vary with the course of infection. In the human clinical condition, some degree of morphological change in gut architecture is apparent, though the range extends from macroscopically normal gut to subtotal villus atropy. The crypts of Lieberkuhn show some degree of hyperplasia and the mitotic index of crypt cells is raised, indicating an increased crypt cell production rate. Wright & Tomkins (1978) recorded a decrease in surface area of villi in human giardiasis with malabsorption.

These clinical manifestations were also observed in the mouse model of giardiasis in which the time course of morphological change has been observed (Roberts-Thompson et al 1976b, MacDonald & Ferguson 1978, Ferguson et al 1980a,b, Gillon et al 1982a).

The morphological changes will be described more fully in chapter 2 when their relationship to the pathology and its causes will be discussed.

IV. Other Symptoms Associated with Giardiasis.

Other symptoms have been described in direct association with giariasis and their resolution noted on treatment of the enteric infection.

Goobar (1977) recorded joint pain as a symptom in children, and polyarthropathy and urticaria were described by Farthing et al (1983a). Basset et al (1978) described cases of peripheral neuropathy in two patients with giardiasis and malabsorption. Malabsorption itself is a recognised cause of peripheral neuropathy in other conditions. Webster (1958) described urticaria in six patients and this condition resolved upon treatment of the giardiasis. Lactose intolerance was recorded by Wolfe (1978) and Burke (1977) found giardiasis to mimic coeliac disease in some

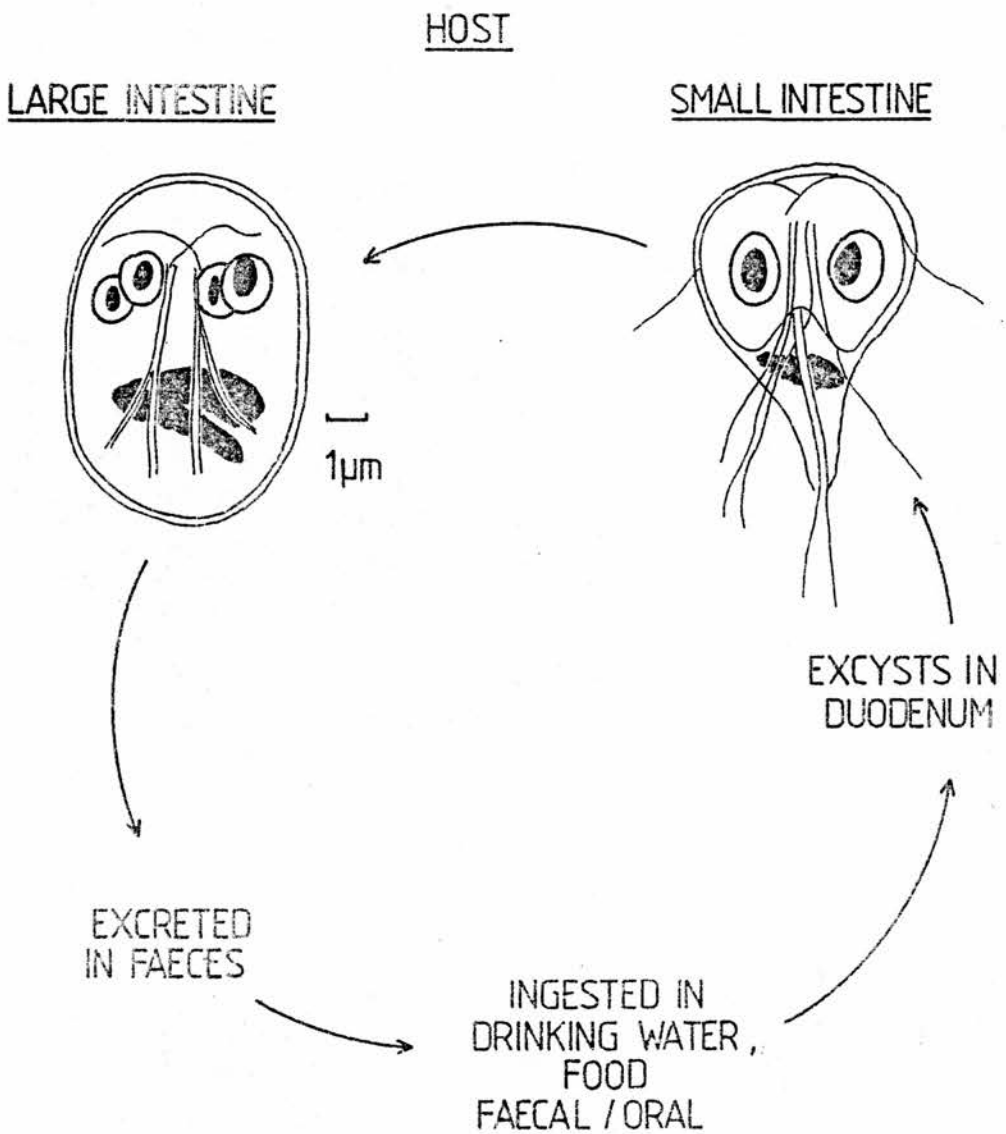
cases. Sheehy & Holley (1975) suggested that giardiasis induced malabsorption in cases of pancreatitis, where the pancreatitis in itself was not sufficient to result in malabsorption. Chronic cholecystitis and duodenitis have also been associated with giardiasis (Soto & Dreiling 1977), as has bronchial asthma (Brea et al 1979).

H. The Mouse Giardiasis Model.

The study of human giardiasis in patients gives no information on the time course of infection, cause of pathogenicity and development of the appropriate and effective immune response.

The use of resistant strains of mice which rapidly develop an immune response against Giardia provide an excellent model for investigation of these features. Susceptable mouse strains which maintain chronic infections may be used as models for the chronic clinical disease. This allows further investigation of the pathology and pathogenic processes involved. These are examined in the next chapter.

Figure 1.1



Giardia muris Life Cycle

Chapter 2

Giardiasis: The Host

Giardia is primarily a luminal dwelling parasite, though reports of tissue invasion are found (Brandborg et al 1967). These are probably descriptions of chance migrations through an already damaged and incomplete epithelial layer, rather than active invasion of the host tissues.

Mice acquire significant and lasting resistance to G. muris after only three days contact with the parasite (Belsovic & Faubert 1983). The mediators of immunity to Giardia, both at the primary infection control phase and at subsequent challenge can act at many levels and this section will describe evidence of immune reactions in mice and indicate similarities to the human disease.

A. Non-specific Protective Responses.

The gut contents are constantly being moved due to a repeated progression of peristaltic waves of various frequencies and propagative characteristics. McHerron et al (1983) describe the increased occurrence of propagated repetitive bursts of action potentials (pRABP) and delay in the appearence of the migrating myoelectric complex which is thought to help clear bacteria from the small intestine.

The human disease also shows the characteristics of increased gut activity, accounting for the symptoms of nausea and fullness. Alone, motility will not have a significant effect. However, if the parasites are displaced from the basal villus zone and presented in the lumen by some method, they are then amenable to motility mediated clearance. This could be achieved by mucus trapping of trophozoites; increased mucus is found in mouse and human stools during infection and others report increased goblet cell numbers (Taniko & Yardley 1965, Saha & Ghosh 1977), increased secretions (Burke 1977) and increased mucopolysaccharide fuzzy coat (Barbieri et al 1970), to the extent that Poley & Rosenfield (1982) suggested it was acting as a pseudomembrane (see later).

The function of increased mucus production has been implicated in the "rapid expulsion" phenomenon of secondary challenge clearance of the nematode, Nippostrongylus brasiliensis from the rat small intestine (Miller et al 1981). The mucus trapping of nematodes is a

mucosal mast cell and IgE mediated phenomenon and has a T cell dependent component (Levy & Frondoza 1983). This effect is relevant to the findings of Roberts-Thomson et al (1976a), who examined concomitant infections of Giardia and the nematode, Trichinella spiralis. They described non-specific clearance of Giardia at the same time as expulsion of T. spiralis. Thus the nematode expulsion mechanism also caused expulsion of the Giardia trophozoites as a bystander effect; hence no immunological memory to Giardia is expressed on subsequent challenge. The nematodes may also have altered the intestinal environment. The actual cause of the non-specific expulsion of Giardia was not further investigated and remains unresolved.

The work of Brett & Cox (1982) also indicated that the explusion of Giardia was immunologically specific, being independent of that of Spironucleus muris, another protozoan parasite which, though preferentially inhabiting the ileum, showed a great degree of cross-over of habitats with Giardia.

B. Human Giardiasis: Specific Immune Responses.

A great deal of research has centred on humoral response during Giardia infection. Investigators have obtained data, sometimes conflicting, from many different sources.

I. Luminal Immunoglobulins.

Local mucosal immunoglobulins, such as IgA and IgM are involved in the immune response to Giardia, and are capable of specific identification of targets and trigger other events in the immune response. Immunoglobulin classes are found in the lumen to varying degrees. IgA is secreted into the intestine in two ways; either directly by the intestinal epithelium or indirectly in the bile. IgA is able to mediate killer cell activity (ADCC) in the gut and activate the alternative pathway of complement fixation.

IgM also occurs in the lumen and is transported by the same active transport mechanism as IgA, but to a much lesser degree. IgM initiates complement fixation by the classical pathway.

Jones & Brown (1974) found no difference in total luminal

levels of IgA or IgM in human chronic giardiasis, but IgG was raised by a factor of five compared to normal control values. Perhaps this indicated gross damage in the intestine and was more a measure of leakage, rather than an active immune response in the lumen.

Webster (1980) noted that when both achlorhydria and decreased intestinal antibody were present this predisposed to chronic giardiasis. An alternative idea is that giardiasis suppresses production of immunoglobulin. Zinneman & Kaplan (1972) and Popovic et al (1974) found decreased levels of IgA in duodenal aspirate during human chronic giardiasis. Wright (1980) also found reduced IgA and IgM mucosal responses in human giardiasis.

II.1.Serum Immunoglobulins: Total Antibody Levels.

Serum immunoglobulins have been variously reported as decreased, normal or increased. In the human chronic disease, total IgA, IgM, and IgG are normal in children (Hartong et al 1979), but these classes may be decreased in adults. Severely ill patients were found to have little IgD, and slight increases in IgM and IgA (Jokipii & Jokipii, 1982). Others found serum IgG, IgA, IgM and IgE similar to controls (Jones & Brown 1974) and this is supported by Naik et al (1979) who found no change in IgA, IgM or IgG. Gupta et al (1980) however found raised levels of IgA, IgM and IgG in 30 Indian patients compared to healthy control subjects. Three weeks after treatment, IgM and IgG levels remained high and IgA levels were further elevated. No relationship was found with pathology, malabsorption or duration of symptoms. Geller et al (1978) found no difference in IgE levels when Brazilian controls were compared with chronic giardiasis patients. Walker et al (1984) record an increase in serum IgE during giardiasis of one patient. This case was a bone marrow transplant recipient and the situation must be considered most abnormal and the result unusual.

Webster (1980) also noted that though hypogammaglobulinaemia predisposed to giardiasis, the majority of patients had normal serum immunoglobulin levels.

II.2.Serum Immunoglobulins: Giardia Specific Antibody.

Using cyst or trophozoite preparations as antigen, more specific and informative results of antibody levels have been obtained. Ridley & Ridley (1976) described increased anti-Giardia antibody in serum and noted a crude correlation between antibody titre and the severity of histological lesions in the jejunum. Wright et al (1977a) also found anti-Giardia antibody present in the sera of giardiasis patients with malabsorption. These two studies used immunofluorescence as the detection method and cysts as the antigen. Vinayak et al (1978) also used cysts as antigen, but detected positive sera with a double diffusion precipitin test. These results show the presence of specific antibodies of indeterminant class, and possibly increased levels when associated with malabsorption. The poor keeping quality and variable reproducability of the test antigen was noted in all three papers.

Using unfixed trophozoites as the antigenic target, IgG specific for Giardia has been detected by ELISA in over 80% of symptomatic patients (Smith et al 1981). Five of seven patients with recurrent infection had high titres of IgG, so this class of antibody appears not to protect the patient against establishment of Giardia infection and is not involved in the immune clearance of the protozoan. IgG does not occur naturally in the lumen to any degree, so this might be expected. This same study found increased Giardia specific IgG in 14% of controls in the Washington area, agreeing with the epidemiologic evidence of occurrence and suggesting that giardiasis is endemic in this area. Roberts-Thomson & Anders (1981) also described high specific antibody IgG titres in current giardiasis as measured by a solid phase radioimmunoassay. The antigen used in this study was trophozoites of G. muris from athymic nude mice. Cross-reactivity with the antigens of G.lamblia was confirmed by immunoprecipitation of G. muris antigens by sera of infected patients. IgG levels fell rapidly following treatment. Giardia specific IgA is also elevated during infection and this does not fall so rapidly following cure. The antibody levels of both isotypes were very similar in treated patients and controls suggesting that all people in the Melbourne area encountered Giardia

at some time.

III. Immunoglobulin Containing Cells.

As noted by Kraft (1979), a distinction must be made between the serum and secreted immunoglobulins, as only those able to gain access to the major mass of parasites in the gut lumen will have the ability to influence the course of this enteric infection.

Care must also be taken in analysis of immunoglobulin containing cell numbers in the lamina propria. An increase in the number of cells containing an immunoglobulin isotype does not necessarily indicate increased immunoglobulin production, and the immunoglobulin produced is not necessarily directed against parasitic antigens, but may be produced in response to increased antigenic stimulus due to the disrupted integrity of the enterocyte surface of the gut.

Four studies of the number of immunoglobulin containing cells have been carried out on giardiasis patients. Popovic et al (1974) describe low counts of pyroninophilic cells before metronidazole treatment, with a rise to normal control levels following treatment. Immunoglobulin containing cells in the small intestinal lamina propria were also detected using an immunofluorescent technique with antibodies directed against IgA, IgM and IgG. The total of these counts indicated numbers of cells slightly lower than control values and no increase of numbers following treatment. This disparity may reflect the difficulty of counting plasma cells using the methyl green pyronin staining method. When immunoglobulin classes were considered individually, numbers of IgA containing cells were significantly lower than controls before treatment and rose to values below, but not significantly below, control values, after treatment. Cells bearing IgM were increased in number in pre-treatment samples, falling to varying degrees following treatment, but remaining higher than control values. The concentration of sIgA in duodenal aspirate paralleled the serum IgA levels and changes in IgA containing cells. The sIgA level remained significantly below control levels following treatment. This may well be a reflection of the known predisposition to giardiasis of IgA deficient subjects. IgM

concentrations in duodenal aspirate also paralleled the IgM containing cell numbers, and showed increased levels before and after treatment, and remained significantly higher than control values. Ridley & Ridley (1976) also described increased IgM cell numbers, concluding that this was due to an early immune response by the patient and IgG and IgA cell numbers would increase later. This was not confirmed by a follow up study. Thompson et al (1977) described increases in IgA, IgM and IgG in thirteen patients. The increased cell numbers followed no general pattern, increasing in IgM alone, IgA alone, IgM and IgA, IgA and IgG, IgM, IgA and IgG, in different patients. A rough correlation was suggested between an independent or associated rise in IgA and/or IgM without raised IgG and the occurrence of mild or moderate lesions. When IgG cell numbers were raised, more severe mucosal changes were found. This paralleled the findings from studies of serum immunoglobulin levels, as previously described. No correlation was found between duration of illness and the number of plasma cells containing the various antibody isotypes. Blenkinsopp et al (1978) found increased IgA, IgM and IgG bearing cells in a study of 40 patients. Total cell numbers were increased by a factor of three before treatment, as compared to post treatment figures. Unfortunately, no normal control values were obtained or stated in this work, so no major conclusions may be drawn regarding Giardia infection nor the status of the patients. Jokipii & Jokipii (1982) repeated the general findings of increased IgA and IgM containing cells and suggested that these increases were due to increased antigenic stimulus because of the damaged gut. The results of Gillon et al (1982b) differed from the previous result in describing no change in cell numbers containing IgA or IgM. Following treatment, however, the previously noted rise in IgA containing cells was recorded. IgE and IgD containing cells increased during infection and fell following successful treatment. IgE containing cells were also described as increased in number by Kraft (1979), though this was not reflected in serum levels of IgE, which remained normal. IgE produced locally in the lamina propria may be immediately bound to the FcE receptor of mucosal mast cells (MMC) and so not reach the serum.

Alternatively, the increased IgE bearing cells may not have been plasma cells, but MMC; double staining with a specific esterase substrate would clarify this uncertainty. Gillon et al (1982b) also suggested, as have others, that Giardia may have the ability to suppress local IgA production and that IgE may have an important role in the elimination of giardiasis (see later).

Studies of chronically ill patients with giardiasis provide interesting and perhaps diagnostically useful information, but it is derived from a situation where the infection has not been naturally eliminated. The results can only indicate an inappropriate or ineffective immune response by the host. The use of experimental models is vital to allow dissection of the appropriate and efficient immune response to Giardia.

C. Animal Models of Giardiasis.

Investigation of Giardia infection in the mouse model has followed several routes. The first involved animals with genetically based immunological defects; examples are the athymic (nude) mice, the mast cell deficient w/w^f mice, and IgE deficient SLJ/L mice.

The second approach has been the investigation of differences in susceptability of immunologically normal inbred strains. Examples of this are the resistant BALB/c and CBA mice, and the C3H/He strain which supported a chronic infection.

The third approach examined the influence of the animal's physiological status on the infection; variables examined were age, sex and pregnancy.

The final approach involved deliberate modification of the experimental animals by steroid treatment, irradiation, thymectomy or concurrent infection with other parasites.

The results of these various approaches are selected and grouped for presentation, as was done earlier for the similar results from human giardiasis studies.

I. Serum Immunoglobulins.

The use of mouse strains deficient in some component of their immune response allowed mimicking of chronic disease and accurate assessment of the relative importance of the different arms of the immune system.

Kunstyr et al (1977) investigated the immune response to Spironucleus muris, a gut protozoan closely related to Giardia, in athymic nude mice and thymectomised, lethally irradiated, bone marrow reconstituted mice (B mice). Both groups maintained chronic infections. Nude mice, and their heterozygote littermate controls which cleared the infection naturally, both showed elevated IgG in their serum. This supports the findings of elevated IgG in the chronic human disease as being an ineffective or inappropriate response.

Owen (1980b) described the development of the antibody response; firstly IgM is elevated, then IgA and IgG. Andre et al (1983) examined the effects of pesticides in the diet on non-reaginic antibody responses. Though the pesticides Lindane and Carboryl significantly increased systemic non-reaginic antibodies to Giardia, the duration of infection was increased, so the spontaneous elimination of Giardia is independent of this response.

Anders et al (1982) compared the resistant BALB/c and susceptible C3H/He mouse strains and found Giardia specific IgA and IgG in serum of both strains; levels in C3H/He mice were even further elevated in late infection. Intestinal secretory IgA was elevated in both strains, albeit ten days later in C3H/He when compared to BALB/c. This difference may be due to strain differences in generating an immune response. The authors suggested that the immune response of the susceptible C3H/He mice was directed against a different antigen (or epitope) to that seen by the BALB/c mice, so resulting in an inappropriate, and ineffective, immune response.

The effect of an antibody response is therefore dependent upon the ability of the isotype to gain access to the target, that is to enter the lumen, and upon the antigenic determinant to which the antibody is targeted on the trophozoite.

The reproducible finding of elevated IgG, both specific and

total is possibly due to gut damage allowing influx of greater amounts of antigen, both of Giardia and normally ingested origin, from the lumen. Specific IgG is also produced due to both accidental invasion and active uptake (see later) of trophozoites, which are then presented to the systemic immune system.

An effect of systemic IgG is to enhance the low level of spontaneous monocyte cytotoxicity for Giardia trophozoites (Smith et al 1982, 1983), and to opsonise trophozoites, so increasing their phagocytosis by peritoneal macrophages (Radulescu & Meyer 1981). Macrophages beneath the basal lamina propria of mice extended pseudopodia into the epithelium and trapped invading trophozoites, enclosing them in phagolysosomes (Owen et al 1981).

Anders et al (1982) described the equivalently elevated IgA and IgG serum levels in both resistent BALB/c and chronically susceptible C3H/He mice. This chronic infection model was initially described by Underdown et al (1981), and may reflect the macrophage activation defect of C3H/He J mice, though neither group specified whether the J or N strain was examined. Mice of the latter strain have normal macrophage function.

II. Secreted Immunoglobulins.

Immunoglobulins are found in a variety of secretions, one of which is milk. The first milk delivered following parturition is the colostrum. This is a particularly rich source of components of the maternal immune system, passively transferring humoral and cellular immunity to the neonate.

As described below,the immunity seen in the neonate model is different to that of the adult giardiasis model in that the former show no immune memory response to a later challenge infection.

Stevens & Frank (1978) examined the ability of lactating female mice to transfer passive immunity in milk to their offspring. As pregnancy proceeds, the immune females gradually began to excrete cysts as the rem nant enteric population of Giardia trophozoites expanded. At the time of birth high cyst output was found and this was maintained to the day of natural or premature forced weaning. Following this, a gradual decline to zero cyst output was found.

The loss of protection in the gut and appearance of factors capable of passively transferring protection in milk may be linked to the enteromammeric association of IgA. sIgA is more commonly associated with conferring protection against micro-organisms such as Escherichia coli, Vibrio cholerae, Salmonella and Shi gella, in the adult gut by preventing the attachment of these micro-organisms to mucosal membranes. The attachment is necessary for these bacterial infections to establish in the gut. The amount of IgA in human breast milk is of the order of 0.25 to 0.5 g per day (Hanson et al 1981). This significant level of immunoglobulin is secreted into the milk by the same active transport system as is found in the gut epithelium. The production of such quantities of IgA takes place locally. Committed IgA-producing lymphoid cells, have been exposed to the antigens of pathogens in the gut, by the antigen presenting cells in the Peyers patches and have a breast homing capacity. In pigs, 90% of sIgA in the milk is produced locally in the breast. In the same way, the lactating mouse loses some capacity for immune response in the gut, allowing pathogen multiplication. There is also evidence of uptake of IgA dimers from the circulation by attachment to secretory component and subsequent active transport to the breast milk.

At a BSI workshop a report of unpublished results indicated that pregnant animals produce a vast excess of secretory piece which is readily found in serum. This binds to free serum dimeric IgA and renders it unable to bind to the active transport site and is therefore functionally isolated from its luminal antigenic targets. By these methods, factors capable of mediating immunity in the gut are lost.

IgA in the colostrum is transferred to the neonate within phagocytic cells and in non-cellular globules (Mestecky et al 1981). IgM or IgG may also be transferred with IgA by this mode. The phagocytic cells are macrophages and PMN. These incorporate the immunoglobulins by pinocytosis. Human colostrum contains approximately 3 million cells per ml, of which 30 to 47% are macrophages, 40 to 60% are PMN, 5 to 9% are lymphocytes, with some 4% colostral and epithelial cells. Lactoferrin, peroxidase and

lysosyme are also present, both free and within cells. Plasma cells are not present, nor can a specific plasma cell proliferative or differentiation response be elicited from colostral lymphocytes. The immunoglobulin containing cells and non-cellular globules can produce IgA haemolytic plaques when assayed against LPS coated SRBC (Mestecky et al 1981). This action may result in the passive transfer of immunity phenomenon described by Stevens & Frank (1978) in mice. Though this group stated that the action of the cellular component of colostrum had not been excluded.

Andrews & Hewlett (1981) further examined this mouse model and supported the theory of specific antibody mediating the protection in neonates. This group assayed for specific anti-Giardia immunoglobulin classes present in milk and found that IgA was the main specific component, with little IgG specific activity and no IgM directed against Giardia antigens. The transfer of passive immunity is only effective if present within 3 to 5 days of Giardia inoculation and has no effect on an established infection. This suggests that the immune reaction is either mediated early in the establishment of infection, perhaps against some antigen expressed during excystation or division, or that the limited amount of specific anti-Giardia antibody can only act effectively against low numbers of trophozoites. Support for the former theory may be given by Anders et al (1982) in the susceptible C3H/He mouse model for chronic giardiasis. Impressive passive immunity is conferred upon the young of this strain by the milk of infected C3H/He mothers. These mothers, though unable to clear their own primary infection despite high titres of specific IgA and IgG, are capable of exhibiting normal immunological memory and resistance to secondary challenge, comparable to the resistant BALB/c, strain upon secondary challenge. This suggests that specific sIgA protects against a challenge of Giardia providing that the challenge is not overwhelming.

The mode of clearance in the neonate, without development of immunological memory is therefore quite different from the adult immune response to infection where an efficient and lasting memory response is found. Roberts-Thomson & Mitchell (1978) supported this

in demonstrating that immunity cannot be passively transferred to adult nude mice with immune serum.

Thus, it would appear that a humoral reponse of varying degree is elicited by Giardia infection, and this may be effective as a mode of protection by a variety of mechanisms, discussed later in this chapter. The antibodies occur both in serum and in the secretions of the mammary gland and the small intestine.

III. Cellular Immunity.

1. Thymus Independent Cellular Immunity.

Phagocytic cells, monocytes and granulocytes also express immune responses to Giardia under appropriate conditions. These functions are present in nude (athymic) mice and neonatally thymectomised mice, yet these animals sustain chronic, sometimes lethal infections of Giardia. Nude mice may clear the infection very gradually over a six month period and thereafter exhibit a limited memory response, such that subsequent challenge results in an attenuated infection the course of which resembles a primary infection in resistant mouse strains (Stevens et al 1978). This has not been found by other groups (Roberts-Thomson & Mitchell 1978). Owen et al (1980, 1981) demonstrated an increased number of activated macrophages in the infected nude mouse model, suggesting that this acted as a compensatory immune response. In turn, these macrophages acted to present antigen to lymphocytes. The now stimulated lymphocytes produced immunoglobulin and this resulted in some degree of secondary immune response. Alternatively, nude mice develop a limited T cell like activity with advancing age, and by the time a primary infection has been cleared, the apparent memory response to secondary challenge may be due to expansion of this T cell like clone. As the immunity is not complete nor rapid it seems likely that this is not the case and the immunity is due to a thymus-independent mechanism.

Keast & Chesterman (1972) suggested that disturbance of the macrophage ability to initiate an immune response to Hexamita (Spironucleus) resulted in chronic susceptibility and death in the

C57BL mouse model. Owen et al (1981) also reported phagocytosis of G. muris trophozoites by macrophages in Peyer's patch epithelium in mice. This is carried out by M-cells sampling the luminal medium. Another cell type with phagocytic and lysosomal function is the Paneth cell located in the crypts. Erlandsen & Chase (1972) reported phagocytosis and intracellular digestion of Hexamita (Spironucleus) trophozoites after seeing large numbers of trophozoites contained in several vacuoles within Paneth cells. Giardia is somewhat larger than Spironucleus and, as Spironucleus inhabits the lumen of crypts (Flatt et al 1978, Brett & Cox 1982) whereas Giardia are found on the villi, it is unlikely that this form of "digestive immunity" (Giannella et al 1973) is effective in Giardia infection.

2. Evidence of Thymus Dependence of Immunity.

The relationship between the lack of a thymus and chronic, often lethal infections of Hexamita (Spironucleus) and Giardia was recorded by Boorman et al (1973a). This group carried out thymus transplants in nude and neonatally thymectomised mice and described a reduced number of flagellates in thymus recipients. Kunstyr (1977) further descibed chronic Spironucleus infection of nude mice and suggested antiflagellate drug treatment to prolong the life of these animals.

Roberts-Thomson & Mitchell (1978) transferred anti-Giardia immunity to nude mice by lymphoid cell transfer from immune heterozygote littermates. Cells from heterozygotes, previously exposed to Giardia infection resulted in a more rapid expulsion infection in nude mice than did lymphoid cells from naive heterozygotes. Immune serum from heterozygote donors had no influence on the nude mouse giardiasis, with or without naive or immune lymphoid cell transfer.

Further investigations of the thymus dependency of the immune response to Giardia have been accomplished by approach from other routes. Perturbing the immune system by neonatal thymectomy (Vasudera et al 1982, Ganguly et al 1982), irradiation (Aggarwal et al 1980) and the application of steroids (Aggarwal et al 1980, Nair

et al 1981) all produced chronic infection, though of varying duration.

3. Intraepithelial Lymphocytes (IEL).

Most intraepithelial lymphocytes (IEL) carry T-cell markers and the number of these cells is reduced in nude and thymectomised mice. IEL numbers are increased in both human giardiasis (Webster 1980, Gillon et al 1982b) and in the mouse model of giardiasis (Macdonald & Ferguson 1978, Owen et al 1979, Kraft 1979, Ferguson et al 1980 a,b, Gillon et al 1982a). Using SEM, several of these authors (Owen et al 1979, Kraft 1979) have noted cells in the gut lumen at the time of maximum parasite expulsion in Giardia and Spironucleus infection of mice. These cells are often attached to the dorsum or flagellae of the protozoan and have been further investigated and characterised by Heyworth et al (1982, 1983) and Ganguly et al (1981, 1983). The cells are generally T-lymphocytes and carry the cytotoxic/suppressor marker, Lyt 2, as do IEL (Schrader et al 1983). Lymphocytes are known to be found in the lumen either by active migration or due to gross damage of the gut allowing leakage and sloughing of enterocytes and IEL into the lumen during other diseases.

IEL have a limited NK activity, about 2% in normal control mice (Boorland & Mowat, 1983) and Targan et al (1979) described isolation of NK and pre-NK cells from epithelium of human colonic origin.

Tagliabue et al (1981, 1982) isolated lymphocytes from the epithelium and other sources and described the K cell activity (ADCC) of these cells. This demonstrated the ability of lymphoid cells in the epithelium to function as effectors of immunity against invading pathogens and their ability to limit uptake of antigen to which the body is already primed.

4. Delayed Type Hypersensitivity (DTH).

Only Anders et al (1982) have attempted to assay for specific DTH to Giardia. This was carried out in the resistant BALB/c and susceptible C3H/He mouse models. The assay involved intradermal

injection of the test antigen to the left ear and control treatment to the right. This was followed by i.p. injection of 5-fluorodeoxyuridine and ^{125}I labelled 5-iodo-2-deoxyuridine. The mice were left for 36 hours then sacrificed and the ears cut off at the hairline. Counts were expressed as the left to right ear ratio. Following cyclophosphamide treatment and parenteral subcutaneous immunization with trophozoite antigens in CFA, both strains respond equally well upon radiometric ear challenge, showing positive DTH. However, no T-cell response could be elicited using sonicated trophozoites as the challenge antigen in the assay at two weeks of infection, when parasite numbers are high and at ten weeks when the infection had been eliminated. Assay at ten weeks would avoid effects of antigenic competition. If cyclophosphamide had been used prior to the assay during infection, to deplete the suppressor cell population, a DTH response might have been elicited in the ear. Furthermore, T-cells in the gut show distinct homing and preference for this site and are primed for antigens encountered at this organ. It may be argued therefore, that one should not expect to find a DTH response to an enteric antigen, in the ear. Recirculation of T cells may result in a response later, following resolution of infection, but not at times of peak immune response to the parasite, when all primed cells involved will be retained in the gut by the presence of specific antigen. This experiment supports the theory of selective and independent mucosal and systemic DTH responses.

5. Mucosal Mast Cells (MMC) and IgE.

The involvement of MMC in giardiasis has only recently been suggested. Roberts-Thomson et al (1981) found persistant giardiasis in w/w+ mice which are mast cell deficient. SJL/J mice also exhibit prolonged Giardia infection. This strain is well known for its low IgE production, probably due to its unusual sensitivity to histamine & serotonin, which can cause its death following mast cell degranulation (Mitchell et al 1982).

Gillon et al (1982b) suggested that Type 1 hypersensitivity at the mucosal level, though not directly involved in the parasite elimination, would increase capillary and mucosal permeability, so

facilitating transfer of primed lymphocytes and circulating immunoglobulin to the mucosal surface and lumen. Serum IgE is normally unchanged in giardiasis, though disease states associated with IgE, such as eczema, urticaria, bronchial asthma and food allergy have been exacerbated by giardiasis. Serum IgE need not reflect IgE production at the mucosal site.

6. Goblet Cells and Mucus.

Although not strictly immune cells, goblet cells and the mucus they produce are involved during immune responses to some parasites. Quantitative and qualitative changes in mucus production and release from goblet cells on stimulation by factors released from MMC during antigen stimulation may be related to strain susceptibility.

7. Cross Protection.

The non-specific release of mucus may explain the suppression of giardiasis during the intestinal phase of Trichinosis, descibed by Roberts-Thomson et al (1976a). Increased mucus production, intestinal secretion from the mucosa into the lumen and increased gut motility at this time may all contribute to a non-specific temporary expulsion and limiting of Giardia numbers. The suppression is transient and has no influence on the normal development of the specific anti-Giardia immune response. Other factors that may cause this non-specific suppression are competition for essential nutrients, secretion of toxic products by the nematode and changes in gut characteristics, such as altered pH and changes in digestive enzyme concentration. T. spiralis also causes a reduction in villus length, modifying and reducing the available habitat for Giardia infection. The small intestinal inflammatory response against T. spiralis mediated by T-lymphocytes may involve non-specific cytotoxic factors, such as the various oxygen radical combinations, which would damage Giardia as an "innocent bystander" effect.

Bruce & Wakelin (1977) examined the non-immune mediated early expulsion of Trichuris muris, a large bowel and caecum inhabiting nematode, simultaneously during the immune mediated expulsion phase

of T. spiralis from the small intestine. Conversly, when T. muris is expelled before T. spiralis the simultaneous expulsion described above is not found. This may be interpreted by invoking mucus trapping as the non-specific effector of immunity. A normal T-cell and IgE mediated MMC specific immune response localised in the small intestine is directed against T. spiralis and mucus production increases, resulting in nematode expulsion by mucus trapping. This mucus then passes down the intestine carrying the nematodes, specific IgE and various secretions due to increased gut permeability. The lumenal transport of mediators initially responsible for stimulating the mucus secretion along with all effects related to Type 1 hypersensitivity in the intestine move from the resistant region upstream in the gut to the level of the large bowel and the T. muris infection. The path of mucus trapping and worm expulsion is reinitiated by stimulation of goblet cell mucus release in the large bowel. This explains the simultaneous non-specific expulsion of T. muris initiated by T. spiralis immune expulsion. Considering the converse experimental protocol, in T. muris immune expulsion, the gut contents will not contact the region of T. spiralis infection, no such non-specific expulsion would be expected by this hypothesis, and experimentally, none is found.

D. Pathogenicity of Giardia.

Giardia infection has been shown to result in numerous pathological states, as previously discussed. The hypotheses for the development of these intestinal changes will now be discussed.

I. Mechanical Blockade.

Due to the vast numbers of trophozoites inhabiting the infected gut, several authors have suggested mechanical blockade as a cause of malabsorption (Palumbo et al 1962, Tandon et al 1974, Erlandsen & Chase 1974). The SEM work from many sources shows that though many trophozoites are present, they do not appreciably occlude the absorptive surface of the gut. Other workers have described confluent sheets of trophozoites on villi. A rough calculation using parasite numbers from a very heavily infected mouse gives a

ratio of five trophozoites per epithelial cell. These numbers indicate that mechanical blockade is a possible cause of malabsorption.

II. Competition.

Competition for essential nutrients has also been suggested, but the actual competing biomass of the parasites appears insignificant in comparison to that of the host. Giardia does not compete with the host for vitamin B_{12} (A. Cummins, Pers.Comm.), although malabsorption of this vitamin is commonly reported.

III. Disruption of Mucosal Structure.

The mucoid fuzzy coat in which the microvilli are embedded is a structure seen by TEM; its origin is unknown. The microvillus brush border and fuzzy coat are the locus for the enzyme systems of active absorption and transport of nutrients. The presence of many trophozoites causes mechanical damage and disruption of both structures is commonly reported (Tandon et al 1974, Erlandsen & Chase 1974, Saha & Ghosh 1977, Balzas & Szatloczky 1978, Wright 1980). The reduction in the fuzzy coat is not a consistent finding and Barbieri et al (1970) and Poley & Rosenfield (1982) described increased thickness of this structure. Increased numbers of goblet cells and increased secretory activity are also described. The thickened fuzzy coat was suggested as acting as a mucoid pseudomembrane (Poley & Rosenfield, 1982), so representing a functional mechanical barrier to entry of nutrients.

Anand et al (1980) found that the free diffusion of potassium was unaffected in rat giardiasis and this result was repeated for free diffusion of tetracycline, so suggesting that no functional mechanical barrier exists.

IV. Toxins.

Soluble substances are excreted or secreted by trophozoites in culture (Nash et al 1983) and the presence of a toxin has been suggested (Taniko & Yardley 1965, Ackers 1980). The toxin may be an excreted metabolic byproduct, such as ethanol or acetate which are

capable of denaturing proteins and would be in close proximity to the enzyme systems necessary for the active absorption processes. There have been no reports of specific antibodies against excreted or secreted products being produced during normal infections.

V. Increased Enterocyte Transit Rate.

An alternative theory involves the active transport capabilities of enterocytes changing with maturity of the cell. The characteristics of the malabsorption syndrome may be accounted for by rapid transit of enterocytes up villi and premature sloughing before the full complement of enzyme functions has developed. The increased crypt cell production rate (CCPR) has been well documented (MacDonald & Ferguson 1976, Ferguson et al 1980a,b).

VI. Concomitant Infections.

The previous hypotheses have identified the Giardia trophozoite directly as the originator of intestinal changes, but the association of this protozoan with yeasts and bacteria during infection has been previously noted and discussed. Though large numbers of giardiasis cases have concomitant infection with these organisms, they show no definitive association nor relation to the malabsorption or severity of pathologic state. Boorman et al (1973b) related post weaning death in mice to the aerobic enteric bacterial infection concomitant with Hexamita (Spironucleus) muris infection. The bacteria in this case probably exacerbated the gut damage or, as their culture from lymph nodes and spleen was also described, were involved in a generalized invasion of the mouse body and toxaemia resulted in death.

The Giardia infection model used by many workers throughout the world was obtained from the original model developed by Roberts-Thomson et al (1976b). The trophozoites and cysts have been shown to contain endosymbiont coliforms, surrounded by a cell wall and plasma membrane. These bacteria are known to divide within Giardia and are therefore viable infective agents (Owen 1980a).

There remains the possibility that Giardia acts as a vector for viral infection, carrying latent infective forms in the same way as

the endosymbiont bacteria.

VII. Bacterial Colonization.

The suggestion that bile salt deconjugation by bacteria leads to the malabsorption syndrome was disproved by Tandon et al (1977) and Desai & Kalro (1983) who described malabsorption in many patients with no detectable bacterial colonization. The former authors suggested that Giardia trophozoites have the ability to deconjugate bile salts themselves.

Further evidence of the non-involvement of bacterial overgrowth as a causative agent of malabsorption was that treatment of the bacterial infection with tetracycline did not improve the malabsorption (Desai & Kalro 1983). However, treatment with the antiflagellate drug, Metronidazole, did resolve the malabsorption (Wright 1979).

VIII. Innocent Bystander Effect.

An alternative hypothesis of the pathogenesis centres not upon the parasite directly, but upon the host's immune response to the parasite. The cytotoxic factors released during the immune response may damage the gut as an "innocent bystander". This effect has been described in the thymus dependent hypersensitivity that leads to partial villus atrophy during N. brasiliensis infection of the mouse (Ferguson & Jarrett 1975), and in the graft versus host reaction (GvHR) in mice (Mowat & Ferguson 1981).

The number of cells in the gut capable of producing cytotoxic factors increases during giardiasis. In particular, the rise in the IEL numbers correlates well with infection and decreases following resolution of infection as a result of the immune response or antiflagellate drug treatment. As most IEL bear T-cell markers and occur in reduced numbers in athymic and neonatally thymectomised mice which are susceptible to chronic giardiasis, the thymus dependency of a competent and effective immune response to Giardia is well supported. Roberts-Thomson & Mitchell (1978) further supported the hypothesis of the T-cell mediated immune response resulting in gut damage, by recording the increased gut damage in

Giardia infected nude mice previously reconstituted with 10^8 immune lymphoid cells derived from spleen and MLN of heterozygote littermates immune to Giardia. Transfer of immune serum had no effect on the gut morphology during infection, independently or in association with cell transfer in groups of eight to ten mice. Nude mice reconstituted with $4x10^8$ spleen and MLN cells from naive heterozygote donors show a massive increase in IEL numbers after six weeks of infection, at a time when the infection is being cleared (Gillon & Ferguson 1984).

Wright & Thomkins (1977) examined IEL numbers in giardiasis patients with and without malabsorption and described no significant difference between controls and patients without malabsorption. When malabsorption is present, increased IEL numbers are found. This may support the hypothesis of IELs being involved in the pathogenesis, but alternatively may reflect increased antigenic uptake due to the damaged and leaky gut of patients with malabsorption resulting in immunological stimulation. The malabsorption may also be accounted for by immaturity of the enterocyte population and further antigenic uptake occurs as immature epithelial cells absorb macromolecules by pinocytosis to a greater degree than mature cells.

IX. Other Factors Involved in the Immune Response.

Inconsistencies in the thymus dependency hypothesis of immune mediated elimination and resultant pathology are, as previously discussed, that nude mice may show gradual resolution of infection and exhibit a limited, but effective memory response. Further, in children presenting with Di George syndrome, exhibiting thymic aplasia and consequent severe T-cell deficiency, giardiasis was not in evidence (Webster 1980). This lack of infection may well be due to the exceptional hygienic care taken with these rare cases.

Gillon et al (1982b) advocated the involvement of IgE and the MMC in the local immune response to Giardia. The occasional direct association of systemic allergic conditions, such as urticaria and bronchial asthma, with giardiasis may be the result of either an amplification or a failure of suppression of the local mucosal IgE

response in atopic subjects. The resultant Type 1 hypersensitivity at the mucosal site results in increased capilliary and mucosal permeability (King & Miller 1984) and may facilitate transfer of primed lymphocytes and specific immunoglobulins from the circulation and gut associated lymphoid tissue to the lumen. However, as noted previously, passive transfer of potassium and tetracycline in Giardia infected rats was not affected (Anand et al 1980) indicating no such change in the integrity of the gut. Jones & Brown (1974) provide evidence to dispute this from chronic giardiasis patients, showing an increase by a factor of five in lumenal IgG concentration, indicating a leaky gut in these cases.

Mucus release and trapping of trophozoites during MMC degranulation in conjunction with increased peristaltic activity at this time would also aid trophozoite expulsion.

The chronic giardiasis mouse models of mast cell deficient, w/w+ mice (Roberts-Thomson et al 1981) and of IgE deficient, SJL/L mice (Mitchell et al 1982) previously described lend further support to involvement of MMC and Type 1 hypersensitivity in the immune response to Giardia.

The susceptible C3H/He mouse was being investigated for evidence of MMC deficiency that would render it unable to mount an intestinal anaphylactic response (Mitchell et al 1982). In support of this theory, this strain is unusually unresponsive to the histamine sensitising factor of Bordetella pertussis.

Thesis Aims.

The ultimate objective of the work described in this thesis was to characterise changes in the small intestine and investigate their pathogenic origin during Giardia muris infection. I first needed to establish a model, using G. muris in the mouse, that would be suitable for study of the immune response to this parasite. Requirements of the model were that it should rapidly develop an effective immune response, and subsequent immunity to G. muris must be readily demonstrable by rapid control of a challenge infection. Using this model, changes in the gut were to be characterised and their pathogenic origin investigated, with particular reference to the role of mucosal cell mediated immunity. I approached the subject by a series of related experiments.

A. Development of the infection and local immune response to G. muris was to be followed by observing changes in gut structures and components of the immune system associated with immunity in the gut.
B. As well as the specific immune response, non-specific factors that may be involved in the expulsion of G. muris were to be examined. Examples of these factors are motility, goblet cells and mucus production. Additional methods of investigation to be used at various times in the study were SEM and TEM of infected mouse gut.
C. Investigation of the possible protective effect of a concurrent, but unrelated cell mediated immune reaction in the gut by induction of a graft versus host reaction (GvHR) in infected mice. This would indicate if there was non-specific protection against G. muris infection by antigen specific T cells active in the GvHR.
D. An attempt was to be made to transfer to naive mice the demonstrable immunity to a G. muris challenge infection, found in previously infected, immune mice.
E. The immune response and pathogenicity of infection were to be further investigated in immunocompromised mice susceptible to chronic infection. These studies were to use nude (athymic) mice and BALB/c mice treated with steroids.

Chapter 3

Materials and Methods

A. Animals.

Mice bred in the Western General Hospital Animal Unit were used throughout the experiments, with the exception of Nude (athymic) mice which were obtained from Bornholpgard, Denmark.

The inbred strains, CBA/CA (H-2k/k) and BALB/c (H-2d/d) were originally obtained from Jackson Laboratories, USA, in 1974. These strains were used as pure bred animals and as the F1 progeny of female CBA and male BALB/c matings.

All animals used were between 6 and 8 weeks of age at the initiation of experimental protocols.

B. Diet.

Animals were fed Spratts Number 1 standard rodent diet and had free access to water.

Where animals were dosed with Loperamide (Imodium (R), Janssen) in drinking water, a consumption rate of 6 ml of water per mouse per day was assumed.

C. Anaesthesia.

Procedures requiring immobilized animals were carried out under light ether anaesthesia.

D. Killing of Animals.

Mice were killed by cervical dislocation.

E. Body Weight.

Animals were weighed using an Oertling TD30 single pan balance.

F. Spleen Weight.

The spleen was dissected out and freed of surrounding tissue. It was sealed in a Universal container and subsequently weighed on a torsion balance (White Electrical Instrument Company).

G. Removal of Tissues.

All tissues taken for histology were removed as soon as possible after death. Samples of gut were taken avoiding Peyers

patches. Jejunum was taken at 8 cm from the pylorus; ileum was taken at 5 cm proximal to the ileocaecal junction. Specimens of 5-10 mm length were placed on card, opened longitudinally and gently spread using mounted needles, villus surface uppermost. Tissues were handled only by the edges. The card and tissue were then immersed in fixative.

H. Fixatives and Histology.

I. Haematoxylin and Eosin (H & E).

For conventional histology, tissues were fixed in 10% buffered formalin, processed and embedded in paraffin wax blocks. Sections of 4 to 5 μm were cut and stained by the standard H & E procedure.

II. Chloroacetate Esterase (CAE).

The chloroacetate esterase staining method required a short tissue fixation time of 24 hours in 10% buffered formalin. The CAE staining procedure was by a standard method, as described in Bancroft & Stevens (1982) and listed below. The stain is specific for mast cells and polymorphonuclear leucocytes (PMN); positive cells show bright red staining of cytoplasmic granules.

Staining Schedule.

Reagents.

Substrate:

Napthol AS-D Chloroacetate 30 mg

Dimethylformide 3 ml

Buffer:

0.1 M Michaelis buffer pH 7.0, 6.8

Pararosanilin-HCl stock:

Pararosanilin-HCl 2 mg

2 M HCl 50 ml

Sodium Nitrite solution:
Sodium Nitrite 400 mg
Distilled Water 10 ml

Hexazotised Pararosanilin solution:
Pararosanilin stock 1.2 ml
Fresh sodium nitrite solution 1.2 ml

Incubating Medium.
To 2.4 ml fresh hexazotised pararosanilin add 90 ml of buffer; adjust to pH 6.3 with HCl. Immediately add 3 ml substrate. Filter and use clear pink filtrate immediately.

Method.
1. Sections to water.
2. Incubate in medium for 30 mins. at room temperature.
3. Rinse in water.
4. Stain nuclei lightly in Mayers haematoxylin.
5. Blue up, rinse.
6. Dehydrate, clear and mount in DPX.

Results.
Chloroacetate esterase activity red/pink.
Nuclei blue.

III. Astra-Blue/Safranine (AB/S).

Tissues taken for the demonstration of mucosal mast cells were stained by the astra-blue/Safranine (BDH Ltd) method at pH 0.3 after fixation in Carnoys fixative (Strobel et al 1981). Positive cells show blue staining of cytoplasmic granules.

IV. Peroxidase-Anti-Peroxidase (PAP).

Reagents used in the PAP staining were obtained from Uniscience, and were products of Litton Bionetics. These suppliers did not produce an IgE detecting reagent, so the IgE reagents were obtained from Miles Scientific.

The PAP staining technique required a 24 hr tissue fixation in Mercuric Buffered Formalin (B5). The staining technique and antisera dilutions involved are descibed below. Positive cells show accumulation of brown peroxidase activity.

Many combinations of reagent dilution, temperature, and duration of incubation were tested, and these optimised for each step as noted in Table 3.1.

Staining Schedule.

1. Sections in paraplast and put through three, five minute washes, from xylene to water.
2. Wash for five minutes in tap water; rinse in methanol.
3. Wash in 0.5% H_2O_2 in methanol (fresh) for 30 minutes to block endogenous peroxidase.
4. Wash in water; wash in TBS for five minutes. Place in TBS preheated to 37°C for ten minutes.
5. Put into trypsin preheated to 37°C for 25 minutes (except λ light chain sections: 18 minutes).
6. Into TBS, gentle agitation, fresh TBS for five minutes.
7. Dry slide around sections, treat with non-immune blocking serum for 10 minutes to block non-specific binding.
   1:4 NGS/TBS for K and λ light chains and IgM, IgG, IgA.
   1:4 NRS/TBS for IgE.
8. Drain off. Treat with primary antibody diluted in NGS/TBS, (NRS/TBS for IgE) in moist box at 4°C, overnight. See Table 3.1 for dilutions.
9. Drain and wash four times, five minutes each, with TBS.
10. Treat with second antibody. Incubate in moist box for one hour at room temperature.
11. Drain and rinse three times, five minutes each,

with TBS.

12. Treat with PAP in moist box for one hour at room temperature.
13. Drain and rinse three times, five minutes each, with TBS.
14. Treat with DAB solution for 10 minutes.
15. Wash in tap water for five minutes. Stain nuclei with Mayers haematoxylin (diluted to 50%) for 3 to 5 minutes.
16. Blue up in Li_2CO_3, wash for five minutes, dehydrate, clear and mount in DPX.

Control.
Omit primary antibody, incubate in NGS/TBS or NRS/TBS.

Results.
Immunoglobulin sites stain with red/brown granules.
Nuclei stain blue.

Table 3.1

PAP Reagent Dilutions and Incubations.

PAP to detect	Reagent	Dilution	Temperature	Duration of Incubation
K light chain	R ∝ M	1/300	4° C	Overnight
	G ∝ R	1/40	Room temp	60 mins
	R:PAP	1/80	Room temp	60 mins
λ light chain	R ∝ M	1/800	4° C	Overnight
	G ∝ R	1/40	Room temp	60 mins
	R:PAP	1/80	Room temp	60 mins
IgM	R ∝ M	1/100	4° C	Overnight
	G ∝ R	1/40	Room temp	60 mins
	R:PAP	1/80	Room temp	60 mins
IgG	R ∝ M	1/100	4° C	Overnight
	G ∝ R	1/40	Room temp	60 mins
	R:PAP	1/80	Room temp	60 mins
IgA	R ∝ M	1/600	4° C	Overnight
	G ∝ R	1/40	Room temp	60 mins
	R:PAP	1/80	Room temp	60 mins
IgE	G ∝ M	1/1000	4° C	Overnight
	R ∝ G	1/40	Room temp	60 mins
	G:PAP	1/80	Room temp	60 mins

M = Mouse

R = Rabbit

G = Goat

x:PAP = species peroxidase-anti-peroxidase complex.

V. Periodic Acid Schiff (PAS).

Demonstration of mucus in the gut lumen and in goblet cells was by PAS staining following tissue fixation in Baker's formol calcium fixative. A comparison was made of both PAS and Alcian-blue stains, both standard methods described in Bancroft & Stevens (1982). The PAS stain gave preferable results and was adopted for goblet cell counts. PAS positive cells show dense purple staining.

I. Microscopy.

A Leitz Ortholux II microscope with x10 or x12.5 eyepieces was used to examine all histological specimens.

J. Cell Counts.

I. Intraepithelial Lymphocytes (IEL).

IEL counts were made on H & E sections by the method of Ferguson & Murray (1971). A minimum of 500 epithelial cells and their associated lymphoid cells were counted at x1000 and x1250. Results were expressed as IEL per 100 epithelial cells.

II. Mucosal Mast Cells (MMC).

These cells were counted using a 10 x 10 grid square eyepiece graticule fitted to the Leitz x10 eyepiece. The graticule was aligned with the base of the crypts, above the muscularis mucosa, at a magnification of x1000. Counts of MMC were made within this field, along with a score, to the nearest 10%, of grid area covered by tissue. The grid was then moved up the thickness of the mucosa and this procedure repeated until the villus tip was included. At least ten such grids were scored per specimen.

Cell counts were calculated and expressed as MMC per 500μm tissue unit, or 500μm^2 tissue unit area. The 500μm tissue unit is the length measured along the muscularis mucosa. The 500μm^2 area tissue unit is an area measured over the total thickness of the tissue.

III. Peroxidase-Anti-Peroxidase (PAP) Plasma Cells.

The method used for PAP plasma cell counts was as described for MMC counts, above.

IV. Goblet Cells.

Goblet cells were scored as for MMC and PAP plasma cell counts, but at the lower magnification of x400. Counts were expessed as cells per 500 μm^2 tissue unit and also as cells per villus. Goblet cells present on the surface of twenty perpendicularly sectioned, complete villi, were counted and the average number on one villus calculated.

K. Microdissection Technique.

I. Specimens.

Following the method of Clarke (1970), groups of animals were injected intraperitoneally with colchicine (BDH Ltd) in saline, at a dose of 7.5 mg/Kg. Individuals were killed at intervals over a 1 to 2 hour period. Intestinal segments of 2 cm length were removed for the microdissection technique. The gut was placed on card and opened with the villus surface uppermost, placed in Clarke's fixative and gently shaken. This prevented matting of villi, and enabled easy and accurate cutting of sections later in the procedure. Specimens were fixed in Clarke's fixative for 24 hours and were transferred to 75% ethanol.

II. Staining.

The tissue was rehydrated, prepared and stained by a modified Feulgen reaction, as follows:-

Storage		75% ethanol
10	mins	50% ethanol
10	mins	tap water
6-8	mins	1 M HCl at 60° C (hydrolysis stage)
10	mins	tap water, with three changes of water
20-30	mins	Schiff Reagent (Difco)

Specimens could then be stored in distilled water for several days, or returned to 75% Ethanol and rehydrated as required.

III. Microdissection.

The stained tissue was placed in a petri dish and viewed by a x32 Zeiss Steriomicroscope, 4B. The muscularis mucosa was removed

with fine forceps. Viewing from the villus surface, thin sections of single rows of villi were cut using a fine cataract knife. Ten to fifteen such sections were cut and mounted on a glass microscope slide in 45% acetic acid, and a glass coverslip placed on top.

IV. Measurement.

Villus height and crypt depth were measured on a Wild M20 Microscope with a calibrated eyepiece graticule. Measurements were made at x100 and x400 respectively. At least ten villi and ten crypts were measured, sampling from several different sections of the specimen.

V. Metaphase Accumulation.

Excess acetic acid was absorbed from the mounted sections, which were then gently squashed using pressure from an orange stick directly over each section. Villus structure was disrupted to leave the more robust crypts intact, but disassociated from the lamina propria. Accumulated metaphase cells were counted at x400 in 20 crypts, again sampling from different sections of the specimen.

The crypt cell production rate (CCPR) per hour was derived from the gradient of the regression line calculated from accumulated metaphase numbers found at times of specimen fixation in the group.

L. Preparation of Cell Suspension from Lymphoid Organs.

Spleen or mesenteric lymph nodes were removed immediately after the animals were killed and placed in RPMI 1640 (Flow Labs) and freed of surrounding tissue. The organs were then placed on a 60 gauge wire mesh and cut into small pieces with fine scissors. The tissue was then gently passed through the mesh using a 5 ml syringe plunger. The resultant suspension was drawn up and expressed gently using the 5 ml syringe, to break up cell clumps. The suspension was left to stand for 2 or 3 minutes to allow debris to settle, and the supernatant was decanted into a clean Universal. This was spun at 400 g for seven minutes, and washed three times in fresh RPMI 1640. Counts of viable cells were made by suspension in trypan blue and total cell counts by suspension in white cell diluting fluid. A Neubauer Haemocytometer was used to determine cell numbers. Cell viability was always better than 70%, and generally about 80%.

M. Graft Versus Host Reaction (GvHR).

I. Induction of Graft Versus Host Reaction.

GvHR was induced by transferring adult parental BALB/c spleen cells. The cells were injected i.p. into adult female CBA x BALB/c F1 recipients.

Recipients received 6 to 8 x 10^6 parental spleen cells in 0.5 ml RPMI, i.p.; cells were prepared as described above.

Experience in this laboratory showed no difference between transfer of an equivalent number of F1 spleen cells and medium (RPMI 1640) alone as the control procedure, so medium alone was adopted as the standard control.

II. GvHR Assay.

The Spleen Index (Simonsen 1962) was calculated as a measure of the GvHR. A spleen index greater than 1.00 indicated splenomegaly associated with GvHR. Mice were weighed prior to killing; the spleen removed and weighed. The relative spleen weight was then calculated for each mouse and expressed as mg spleen per 10 g body weight.

The Spleen Index (SI) was calculated as follows:-

$$\frac{\text{mean relative spleen weight GvHR mice}}{\text{mean relative spleen weight control mice}} = \text{SI}$$

N. Assay of Epithelial Cell Enzymes.

A specimen of gut about 5 mm in length was removed and cleaned of surrounding tissue and luminal contents. The wet weight of the sample was recorded and samples then stored at -20°C. The brush border disaccharidase enzyme activity of sucrase, lactase, maltase and trehalase were assayed by the method of Dalqvist (1964) and results expressed as μ.mol. of substrate hydrolysed per minute per gram wet weight of tissue, at 37°C.

O. Tissue Preparation for Scanning Electron Microscopy (SEM).

Samples of gut, of 1-2 cm, were taken, opened, and pinned out, villus surface uppermost, on flat wax dishes filled with 2.5%

gluteraldehyde in cacodylate buffer (pH 7.3) fixative. Tissues were fixed for at least 24 hours at 4°C. Tissues were then washed in fresh cacodylate buffer for 1 hour, followed by washing in distilled water. Processing then progressed through graded acetone steps to 100% acetone. This was followed by critical point drying in CO_2 and mounting on aluminium stubs. Specimens were coated with gold in a sp utter coater and examination was made using an ISI 60 Scanning Electron Microscope. Tissue preparation was carried out by Ms. J. Tocher, of the Teaching and Research Unit, Western General Hospital.

P. Giardia muris.

I. Cyst Isolation.

Infected mice were placed individually in clean plastic cage boxes and faecal output collected over a two hour period.

The collected faeces were then soaked in 5 ml distilled water for 30 minutes, then ground up to a smooth suspension, using a glass rod. This suspension was carefully layered on 5 ml 1 M Sucrose (BDH) and centrifuged at 400 g for 15 minutes. Cysts were collected at the faecal/sucrose interface and were drawn off by Pasteur pipette. The collected suspension was made up to 10 ml with distilled water and centrifuged at 550 g for 10 minutes to pellet the cysts. The supernatant was discarded and the pellet resuspended in distilled water to a volume of 2 ml. Cysts were stained by addition of Lugols iodine and counted in a Neubauer haemocytometer. Cyst counts were expressed as cyst output per hour per mouse, and group means taken.

II. Trophozoite Isolation.

Infected mice were killed and the small intestine dissected out and freed of surrounding tissue. The intestine was then everted by threading onto an inert metal spiral and the ends tied off. The spiral and gut were then clamped to an industrial vibrator (Vibromax, Chemap AG, Switzerland), and subjected to maximum vibration for 10 minutes in 100 ml cold saline, containing 2% mucolytic n-acetylcysteine (Airbron(R), Duncan, Flockhart and Co. Ltd.). A 10 ml sample of the resultant suspension was centrifuged at 400 g for 15 minutes and the pellet resuspended in 1 ml saline.

Trophozoite counts were made using a Neubauer haemocytometer and expressed as total trophozoites per mouse intestine, and group means calculated.

III. Giardia muris Infection.

Infections were initiated by dosing mice with cysts or trophozoites. Intra-gastric intubation of 1500-2000 cysts, or 3000-4000 trophozoites in 0.2 ml medium was sufficient to establish infection and was used as the standard infective dose. These methods were used to maintain a stock infection. Groups were infected every two to three weeks with cysts or trophozoites obtained from the previously infected group. Trophozoite initiated infections were used at a time when the parasite was not encysting. The time course and characteristics of infection, as assesed by trophozoite numbers, remained unchanged at this time.

Q. Giardia Culture Medium.

The medium used in the short term culture experiments was modified Diamond's Medium TPS-1. The formula, listed below, was obtained from the School of Tropical Medicine and Hygiene, London.

Basic Medium (90 ml).

Trypticase BBL	1.00 g
Panmed	2.00 g
Glucose	0.50 g
L-cysteine HCl	0.10 g
Ascorbic acid	0.02 g
NaCl	0.50 g
KH_2PO_4 (anhyd.)	0.06 g
K_2HPO_4	0.10 g
glass distilled water	90.00 ml

1. Dissolve ingredients one by one.
2. Adjust to pH 7.0 and filter.
3. Filter sterilise.
4. Complete medium by adding 10 ml inactivated

horse serum, 2.5 ml of Wellcome vitamin mixture NCTC 107 and 0.05 to 0.1 ml of an antibiotic mixture containing 200 mg/ml streptomycin sulphate and 100 mg/ml Ampicillin (+ 0.1 ml of a 40 mg/ml Gentamycin solution).

5. Store at -20°C and return to 37°C for 24 hours before use.

R. Result Presentation and Statistical Analysis.

Results are presented as the mean ± one standard deviation (SD). The Students t-test for unpaired samples was used to detect significant differences between groups.

Crypt cell production rates were calculated from the regression slopes fitted by the method of least residual squares. Data analysis of the regression lines was by Covariance analysis.

S. List of Fixatives.

1. 10% buffered formalin (1000 ml)

formalin (40% formaldehyde)	100.0 ml
distilled water	900.0 ml
$NaH_2PO_4 \times 2H_2O$	4.0 g
Na_2HPO_4	6.5 g

2. Clarke's fixative (1000 ml)

ethanol (96%)	750.0 ml
glacial acetic acid	250.0 ml

3. Carnoys fixative (1000 ml)

ethanol (96%)	600.0 ml
chloroform	300.0 ml
glacial acetic acid	100.0 ml

(store in a dark, airtight container)

4. Bakers formol calcium (1000 ml)

formalin (40% formaldehyde)	100.0 ml
calcium chloride	20.0 g
distilled water	900.0 ml

5. B5: buffered formol sublimate (1000 ml)

formalin (40% formaldehyde)	100.0 ml
mercuric chloride	12.0 g
sodium acetate (anhyd.)	2.5 g
distilled water	900.0 ml

Chapter 4

Infection Characteristics

Introduction.

As described earlier, mouse strains may be broadly separated into those resistant, and those susceptible to chronic infection by G. muris.

Previous work in this laboratory has investigated G. muris infection mainly in the CBA mouse strain. The aim of this preliminary work was to compare the established CBA model with infection in the BALB/c mouse strain. This was to lead on to experiments comparing infection of male and female mice and observation of the time course of a secondary challenge infection of immune mice.

The time course of infection was followed by faecal cyst output which has been shown to parallel intestinal trophozoite numbers (Gillon et al 1982a). Assay of cyst numbers was chosen in preference to trophozoite counts as it does not require sacrifice of animals.

The cyst counts are expressed as the $\log_{10}$ value per hour, during a two hour faecal collection. This was to normalise the otherwise positively skewed distribution.

This preliminary work was to establish techniques and select the strain and sex of mouse to be used in subsequent experiments. Five features of the infection time course were examined:-

a. The prepatent period. This is the time between initial infection and faecal cysts being detected.
b. The rise in faecal cyst output to a plateau value.
c. The level of faecal cyst output during the plateau phase of infection.
d. The time when faecal cyst output begins to drop rapidly.
e. The time when faecal cyst output is no longer detectable.

The final model required a short, clear cut infection with a sharp end point. This would allow investigation of the immune components involved in clearing the parasite infection.

Each experiment will have a short introduction describing the

protocol. All animals were 6 to 8 weeks old, except in experiment 5, investigating primary and secondary infections, where animals were age matched and about 12 weeks old.

Experiment 1.
Comparison of Giardia muris infection in BALB/c and CBA female mice (Fig. 4.1).

Materials and Methods.

Female BALB/c and CBA mice were infected with 2,000 cysts in 0.2 ml water, by i.g. intubation.

Results.

The results, expressed as $\log_{10}$ number of faecal cysts passed per hour, showed that both mouse strains have a similar prepatent period, when no faecal cysts are detected. The female CBA showed a higher cyst excretion on day 6 of infection, but a similar parasite load during the plateau phase. The BALB/c female eliminated the infection very rapidly between days 26 and 28, whereas the CBA female showed a later explusion, between days 33 and 36.

Experiment 2.
Comparison of Giardia muris infection in male and female BALB/c mice (Fig. 4.2).

Materials and Methods.

Male and female BALB/c mice were infected with 2,000 cysts in 0.2 ml water, by i.g. intubation.

Results.

The results show that the prepatent period and maximum cyst excretion are similar in male and female infections. Cyst excretion by female mice fell significantly compared to that of males from day 19 onwards, indicating termination of the infection. In contrast, cyst excretion by male mice continued until day 34, after which numbers fell.

Experiment 3.

Comparison of infections initiated by cysts or trophozoites in adult female BALB/c mice (Fig. 4.3).

Materials and Methods.

Adult female BALB/c mice were infected with either 2,000 cysts or 2,000 trophozoites in 0.2 ml water or saline respectively, i.g.

Results.

The detection of faecal cysts was earlier (day 4) in the trophozoite infected mice, than in the cyst infected mice (day 7). There was no significant difference at the plateau phase of infection, but the time to zero cyst output was later (day 30) in the cyst infected group, compared to day 21 in the trophozoite infected group.

Experiment 4.

Comparison of infections initiated by cysts or trophozoites in adult female CBA mice (Fig. 4.4).

Materials and Methods.

Adult female CBA mice were infected, as in experiment 3, with 2,000 cysts or 2,000 trophozoites.

No samples were collected early in infection, so no difference was detected in the prepatent period, although one might expect to find a divergence in this case, as was seen in experiment 3 (see later).

Results.

The cyst infection in this case produced an increased cyst excretion compared to the trophozoite infection. Resolution of infection was achieved at the same time in both groups, but on day 33, the cyst excretion of the trophozoite infection was significantly reduced ($p<0.05$) compared to that of the cyst infection.

Experiment 5.

Comparison of primary and secondary infections in female BALB/c mice (Fig. 4.5).

Materials and Methods.

Adult female BALB/c mice at 6 weeks of age were infected with 2,000 cysts as previously described. The time course of infection was followed by faecal cyst output, and animals were shown to recover, as expected, from the infection. Two to three weeks after the last detected faecal cysts were observed, these animals were challenged with a secondary infective dose of 2,000 Giardia muris cysts in 0.2 ml water, i.g. A group of age and sex matched controls were also infected at this time to show the primary infection time course.

Results.

The results show a shorter prepatent period in the secondary infection, cysts being detected by day 3. The number of faecal cysts excreted per hour during the plateau phase is very significantly reduced in secondary infection, compared to a primary infection. The plateau phase of a secondary infection is also reduced in duration compared to that of the primary infection. The fall in faecal cyst output to zero at resolution of infection is faster in the secondary infection compared to the primary infection.

Summary and Conclusions.

The infection time course in mice of the same strain, age and sex shows some variation in the time when faecal cysts can no longer be detected. Throughout these studies a difference of up to one week was found between short and long infection time courses in comparable animals. This stressed the importance of appropriate control infections in each experiment of the study.

The results from this series of experiments show that the BALB/c strain has a short acute infection with a discrete end point.

The BALB/c and CBA mouse strains carry equivalent parasite loads, as assesed by the plateau phase of infection.

The more rapid resolution of infection in female mice is in agreement with many other results from a variety of experimental systems, indicating a more efficient anti-Giardia response in non-reproducing female animals compared to equivalent males.

The second challenge infection (Fig. 4.5) shows cysts detected earlier than expected from a cyst infection. This is due to the immune response, possibly sIgA or mucus (see later) inhibiting establishment of trophozoites in sites in the gut. The trophozoites are conveyed to the large intestine with the intestinal contents and here encyst, to pass out with the faeces and be detected. This is a rapid expression of immunological memory and limits the number of infective forms, so a lower parasite load results. It is probable that numbers of infective forms are limited, and other effectors of immunity are also involved in limiting the growth of the parasite load. These factors may be directly cytotoxic against the trophozoites or inhibit division of the parasite or prevent its establishing infection in the gut.

The short plateau and rapid explusion are due to recruitment, expansion and rapid expression of immunity; a typical memory response. This experiment confirmed the observation that stock mice had not previously encountered G. muris. Breeding pairs were injected with 2.5 mg of cortisone acetate, sc, when withdrawn from the colony, to check for G. muris infection. No contamination of BALB/c or CBA stock was ever found.

Cyst initiated infections require excystment of trophozoites, activation of quiescent metabolic pathways and accumulation of protein sufficient to enable division to occur. In contrast, the use of trophozoites as the infective agent produces an earlier appearance of cysts in faeces because infection is by an already multiplying population of parasites. Trophozoites are not the usual agent for transmission of Giardia infection. Prior to these studies, difficulty in maintaining the infection had been encountered due to the parasite not encysting. When this occured, the infection was transfered by i.g. dosing with trophozoites. The current work proved to be useful later in the study when the parasite again ceased normal encystment and trophozoite initiated

infections had to be used.

The parasite load in BALB/c mice infected with trophozoites rose above the cyst infection load, whereas in the CBA strain, the reverse situation occurred. This may be due to differences in stomach and intestinal volumes. The 0.2 ml infective dose would result in gastric emptying. This was demonstrated using a carmine particulate dye. Animals were sacrificed immediately after dosing and dye was be seen up to 13-14 cm along the small intestinal length. The BALB/c experiment (Fig. 4.3) suggests that more trophozoites were left in suitable sites for infection than cysts, which might be cleared from the small intestine before being able to establish. The CBA experiment (Fig. 4.4) would suggest the reverse, more cysts surviving to produce infective trophozoites. A primary infection shows trophozoites, at peak parasite load, inhabiting 70% of the small intestinal length (see chapter 8), indicates that gastric emptying distributes the infective cysts over some distance of the small intestine and the infection of less ideal habitats occurs.

The trophozoite is more susceptible to damage during isolation, and possibly the sudden change from donor animals (BALB/c) gut environment to CBA gut resulted in some loss of infective trophozoites. The time taken to generate an immune response against G. muris after appearance of faecal cysts is similar in trophozoite and cyst infected groups. This would suggest that the immune response is directed against a trophozoite associated antigen. The rate of elimination is similar in both cyst and trophozoite infections, so the mode of expulsion by the immune response is the same.

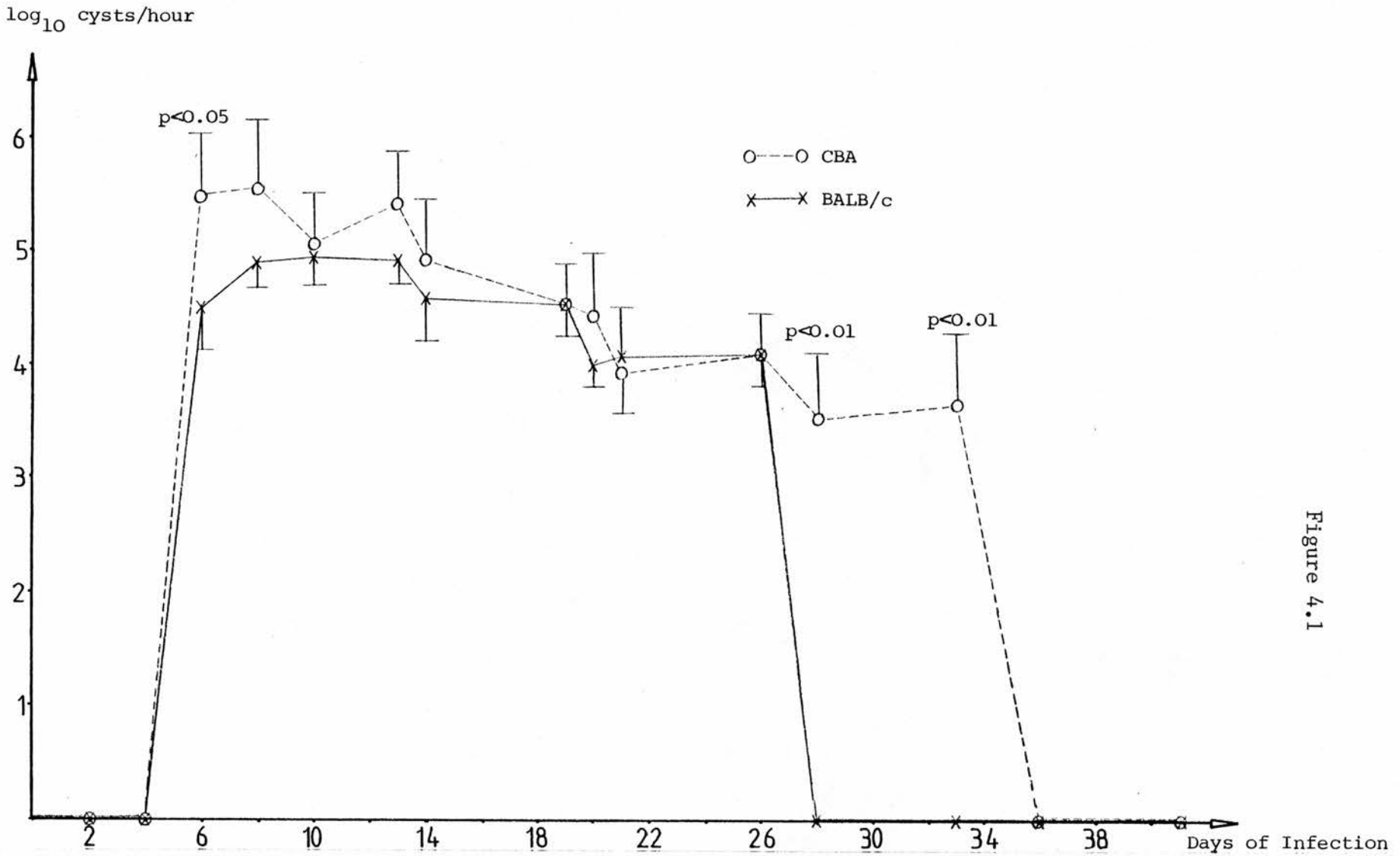


Figure 4.1

Progress of primary Giardia muris infections in adult female CBA and BALB/c mice. Infection on day 0 was by i.g. intubation of 2,000 cysts in 0.2ml water. Marks represent means ± 1 SD of the number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

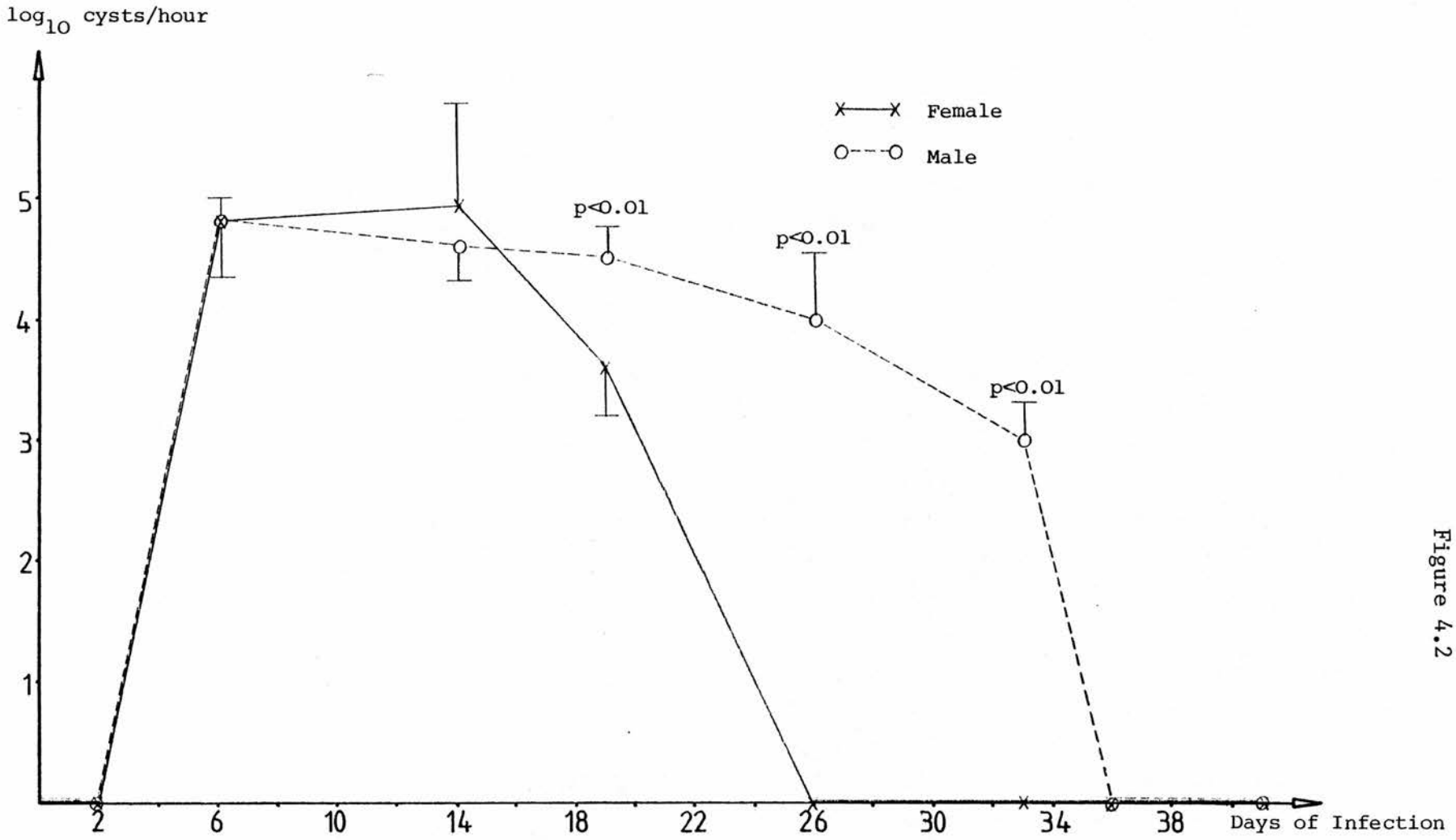


Figure 4.2

Progress of primary Giardia muris infections in adult male and female BALB/c mice. Infection on day 0, by i.g. intubation of 2,000 cysts in 0.2 ml water. Marks represent means ± 1 SD of the number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

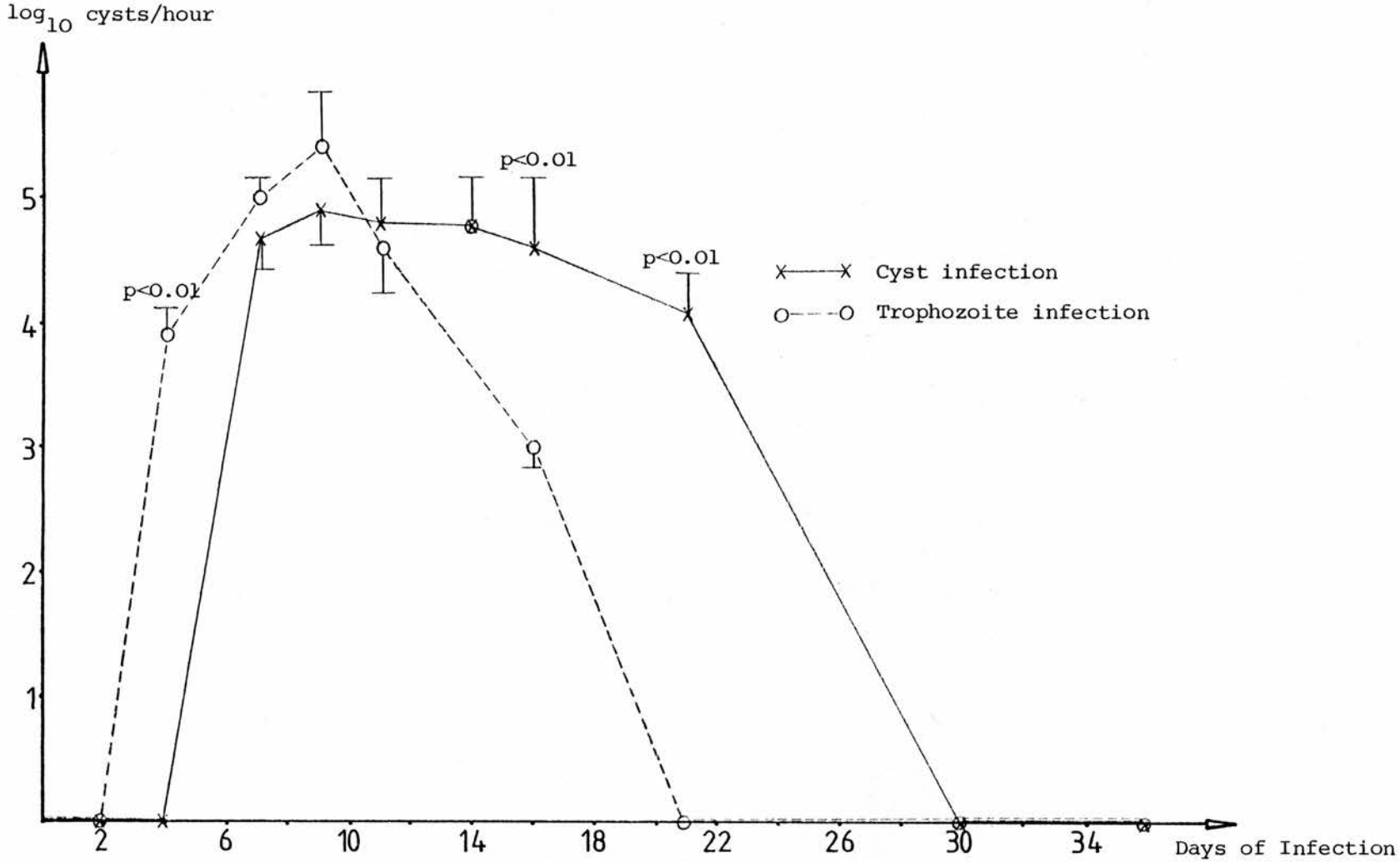


Figure 4.3

Progress of primary Giardia muris infections in adult female BALB/c mice. Infections initiated on day 0 by 2,000 cysts or 2,000 trophozoites in 0.2ml water, i.g. Marks represent means ± 1 SD of number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

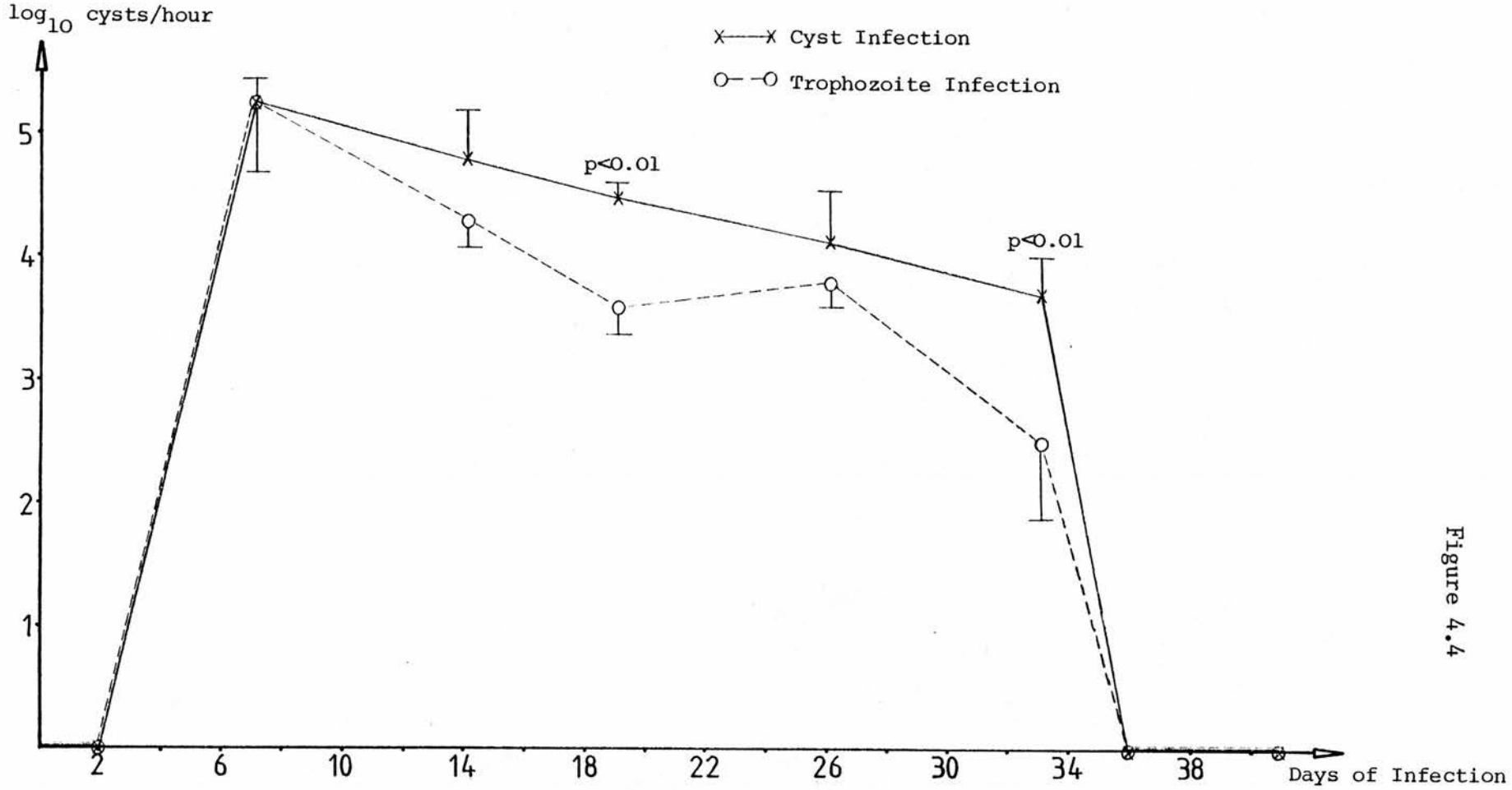


Figure 4.4

Progress of primary Giardia muris infections in adult female CBA mice. Infections initiated on day 0 by 2,000 cysts or 2,000 trophozoites, in 0.2ml water, i.g. Marks represent means ± 1 SD of the number of cysts passed per hour during a 2 hour faecal collection (six mice per group).

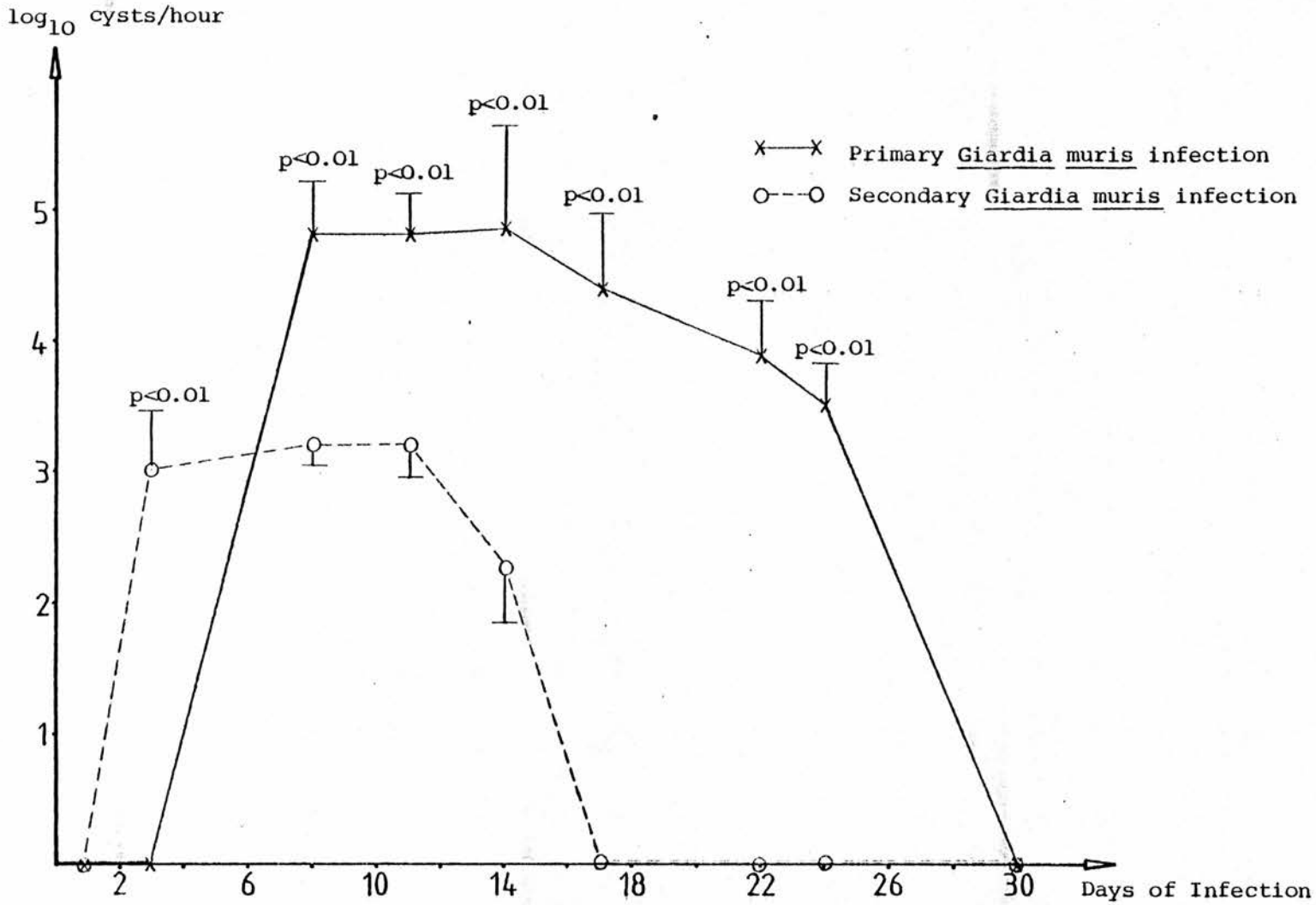


Figure 4.5

Progress of primary and secondary Giardia muris infections in age matched adult female BALB/c mice. Infections initiated on day 0 by 2,000 cysts in 0.2ml water, i.g. Marks represent means ± 1 SD of the number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

Chapter 5

Histology of a Primary Infection

Introduction.

The aim of this study was to examine the role of cellular immunity, specifically that of lymphocytes, in the mouse during a primary infection of G. muris.

As discussed in chapter 2, the few reports in the literature concerned with lymphoid cell numbers in the rodent models have centered on the increase in IEL numbers.

Both IEL and plasma cell numbers in jejunal biopsy specimens have been reported from human giardiasis research, but these counts are not representative of a normal, naturally resolved infection.

This study examined the lymphoid cellular changes at times of particular interest during the infection. These were:

a. Before infection: to obtain control values for the cell types examined in the adult female BALB/c mouse.
b. During the plateau phase of infection, when trophozoite numbers are maximum.
c. At the time when faecal cyst count begins to fall rapidly, indicating the onset of resolution of the infection.
d. When faecal cyst output is approaching, or has reached zero.

These time points coincided with changes in mucosal architecture and function during a primary infection of adult female BALB/c mice. These results are described in chapter 11, but a summary is useful here. Crypt hyperplasia is very significant on days 11 and 25, but villus height is unchanged. The activity of the disaccharidase enzymes, lactase, sucrase, maltase and trehalase are all very significantly reduced on days 11 and 25.

The results of this experiment are not only descriptive, but allow examination of the relationship between the lymphoid cells and parasite numbers, mucosal changes, time of parasite expulsion and the inter-relationship between different cell types.

Materials and Methods.

Animals used were female BALB/c mice, 6 to 8 weeks old.

Specimens of gut were taken immediately on sacrifice and placed on card as previously described, and immersed in buffered formol sublimate (B5) fixative, if tissue was to be processed for PAP and H & E.

Results.

The PAP staining for IgM, IgG and IgA was occasionally of weak intensity, despite a great deal of work being done to optimise the processing conditions. The IgE-PAP stain was always of good intensity. The positive staining of cells containing each immunoglobulin class was seen to vary from specimen to specimen during the random blind cell counts. Upon regrouping the results, it was found that the more intensely staining cells were present in samples from infected animals, suggesting an increased immunoglobulin content in individual cells, as well as an increase in cell numbers.

Course of Infection.

The course of infection was followed by faecal cyst output (Fig. 5.1). After a prepatent period of 4 days the infection rose to a plateau phase beginning on day 5 and slowly fell until day 33, when a rapid decrease followed, indicating termination of infection.

Intraepithelial Lymphocytes (IEL).

The IEL counts rose from the control baseline of 13.6 to a mean of 18.4 on day 25, which was significant (Fig. 5.2).

Plasma Cell Counts.

As an assay of total plasma cells present in the specimens, PAP stains were used to detect K and λ light chains of the immunoglobulins. The normal ratio of K to λ in the mouse is about 25:1, though some strain variation may occur.

Cells Containing Immunoglobulins with K Light Chains.

Numbers of plasma cells containing K light chains (Fig. 5.3) rose significantly ($p<0.05$) above control values (76.5) by day 11

(182.0) and remained significantly elevated ($p<0.05$) on day 25 (165.5) and day 32 (156.3, $p<0.05$).

Cells Containing Immunoglobulins with λ Light Chains.

Numbers of plasma cells containing λ light chains (Fig. 5.4) also rose significantly ($p<0.05$) by day 11 (23.2), however values for day 25 (13.8) and day 32 (12.5) did not significantly differ from the control value (9.0).

Cells Containing IgM.

The number of plasma cells containing IgM (Fig. 5.5) appeared raised by day 11 (5.7), and remained elevated on day 25 (3.5) and day 32 (5.8) compared to control numbers (0.8). Due to a great degree of variation in the experimental groups, these differences were not significant.

Cells Containing IgG.

The number of plasma cells containing IgG (Fig. 5.6) was significantly ($p<0.05$) raised on day 11 (14.3) of infection but fell to control values (3.2) by day 25 (5.7) and day 32 (6.7).

Cells Containing IgA.

A highly significant rise ($p<0.01$) in plasma cells containing IgA (Fig. 5.7) was found when comparing numbers on day 11 (142.2) and day 25 (125.2) of infection to control values (52.0). The mean numbers found on day 32 (87.8) were similar to those of control specimens, but with great variation in the infected group values (39 to 175).

Cells Containing IgE.

The number of plasma cells containing IgE (Fig. 5.8) was significantly increased ($p<0.05$) on day 25 (19.0) of infection, compared to control values (5.2). Again, a high degree of variability was seen within each group, and so there was no significant increase on day 32 (16.3).

The IgE containing cells were not increased due to confusion with staining of the limited amounts of cytoplasmic and surface IgE associated

with MMC. The IgE-PAP stain was scored for intracellular IgE rather than surface IgE. The staining of positively scored cells was always very intense, indicating large amounts of IgE and finally, the cells' morphology and, in particular, their distribution in the lamina propria of the villi made them quite distinct from the MMC.

Summary and Conclusions.

G. muris infection followed the typical time course for infection in adult female BALB/c mice, as previously described.

There was a rise in IEL numbers before explusion of the parasite, as has been described by MacDonald & Ferguson (1978), Owen et al (1979), Ferguson et al (1980a,b), Gillon et al (1982a). The role of the increased IEL numbers is unknown and is discussed in the final chapter.

The total number of immunoglobulin containing cells was increased by days 11 and 25 of infection. This increase can be broken down to isotype specific changes.

There was an increase in IgM containing cell numbers throughout infection, but due to the variability within groups, these increases were not significant. The increase in IgM containing cells was compatible with a developing immune response.

The rise in IgG containing plasma cells paralleled the establishment of infection. Damage to the epithelium occurs at this time, as indicated by decreased disaccharidase activity. An increase in antigen influx through the leaky epithelium may have resulted in this increase in IgG cell numbers. However, when parasite numbers fell, a parallel fall in the number of these cells was noted, suggesting that IgG was directly involved in the anti-parasite response, rather than in response to increased antigenic influx through the damaged epithelium.

The rise in IgA containing cells was consistent with an immune response at a mucosal surface. Increased sIgA in the gut lumen has been described in the mouse model of giardiasis (Anders et al 1982). The reduction in IgA cell numbers on day 32 seems premature, but the range of values detained from specimens was great (39-175) suggesting that some animals had eliminated the infection at this

stage. IgA cell numbers were often described as falling rapidly upon treatment of giardiasis in patients.

The number of IgE containing plasma cells also rose, though later than the other immunoglobulin cell types. The stimulus which resulted in an increase in IgE cells may not have been the same as that which stimulated the IgM, IgG or IgA cell increases. The latter occur by day 11, and the former by day 25. Alternatively, the IgE containing cells may take longer to develop in an immune response in the gut. It has been suggested (Durkin et al 1981) that IgE bearing cells arise in the Peyers patches, either de novo or by switching from precursors carrying IgM or IgA. The increase in IgE containing cells suggests that a Type 1 hypersensitivity reaction is involved in the immune response to G. muris in the mouse.

It has been shown that both cellular and humoral effectors of the mucosal immune system are involved in the immune response to G. muris in this model.

The course and nature of the immune response are discussed further in the final chapter.

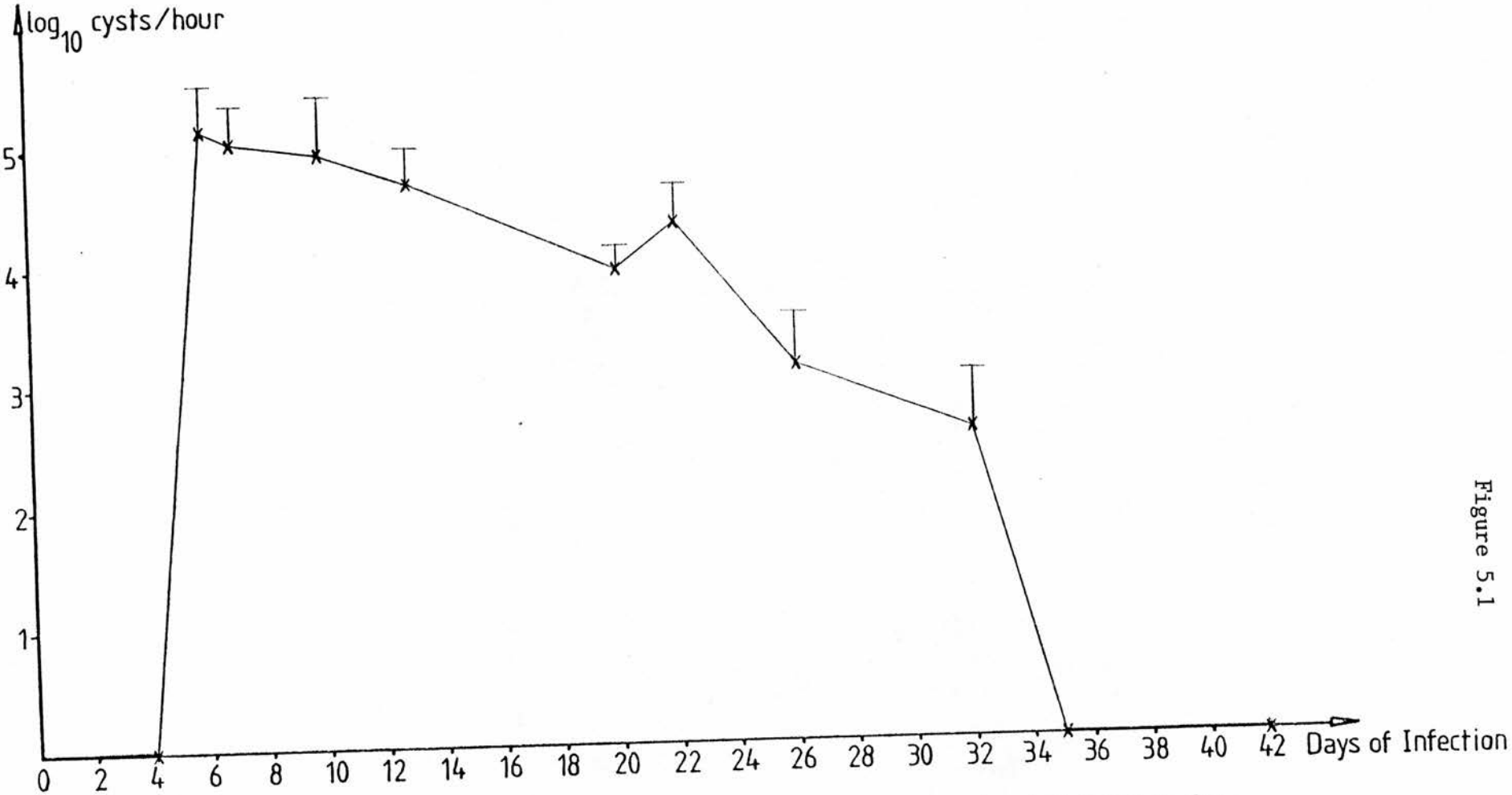


Figure 5.1

Progress of primary Giardia muris infection in adult BALB/c mice. Infection initiated on day 0 with 2,000 cysts, ig. Marks represent means ± 1 SD of number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

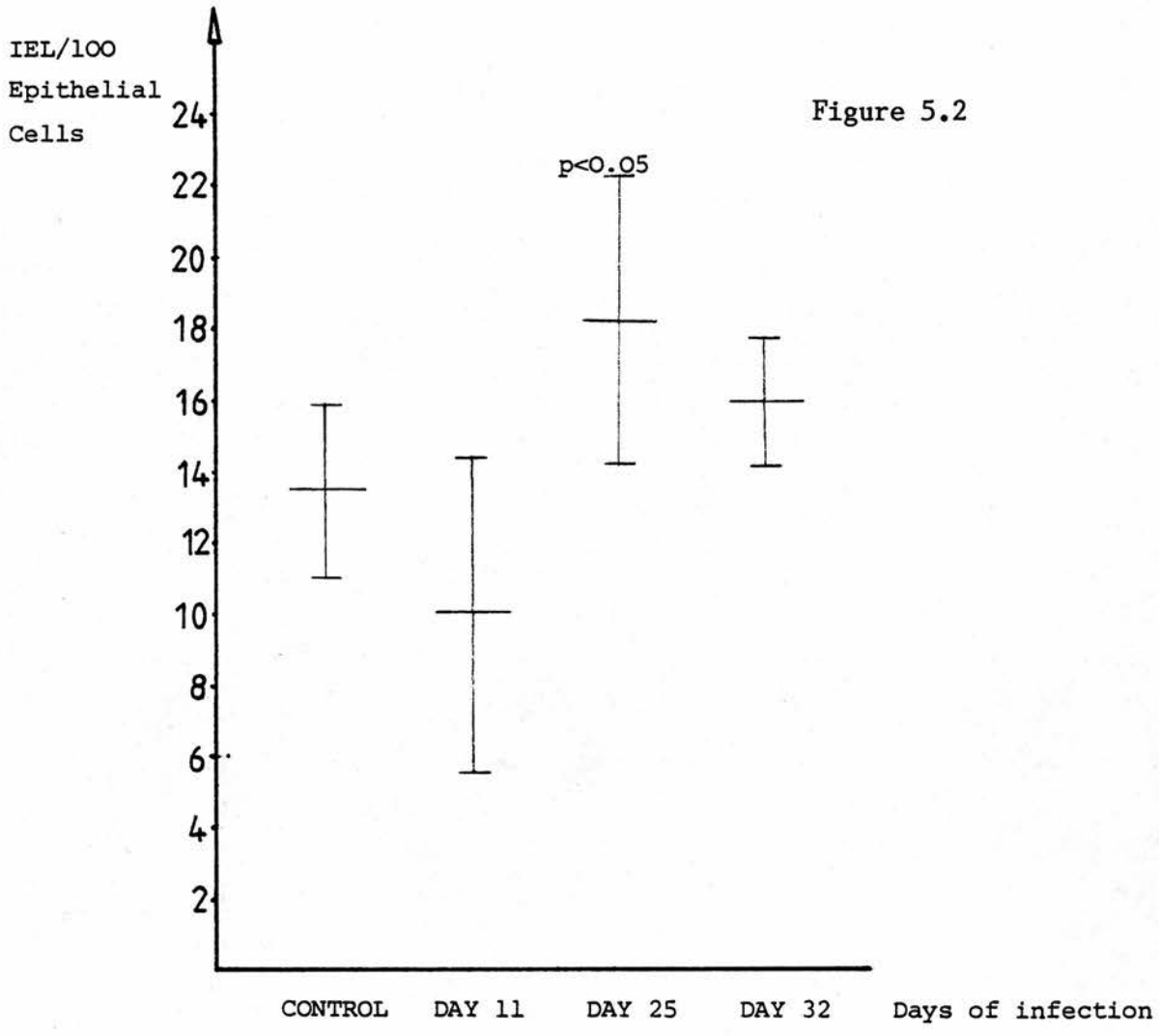


Figure 5.2

Numbers of IEL per 100 epithelial cells during a primary G. muris infection of adult female BALB/c mice. Results are of mean ± 1 SD (six mice per group).

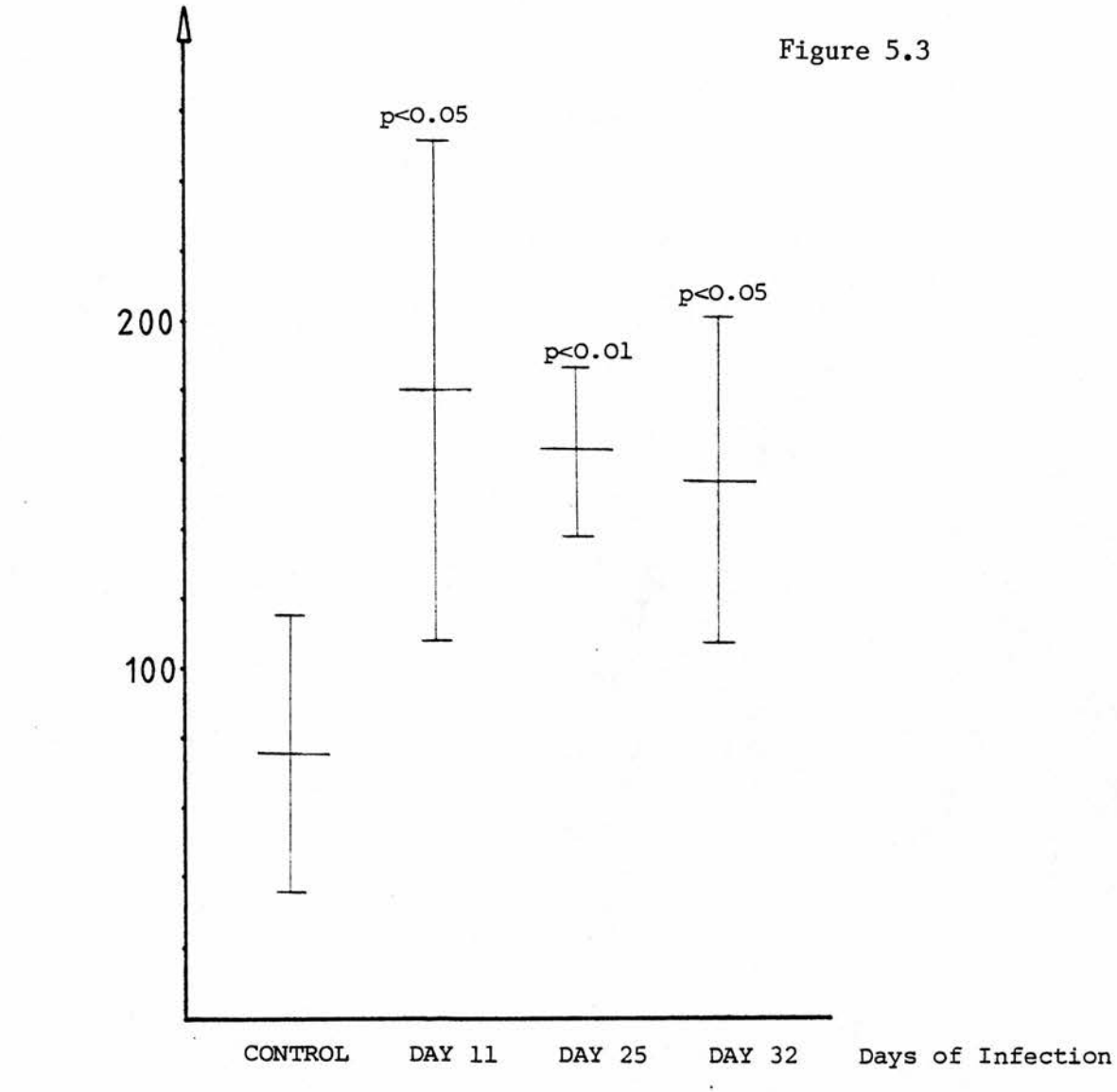


Figure 5.3

Numbers of K light chain positive cells detected by PAP staining during a primary G. muris infection of adult female BALB/c mice. Results are of mean cell count per 100 µm muscularis mucosa, ± 1 SD (six mice per group).

λ Light Chain Positive Cells per 100 μm muscularis mucosa.

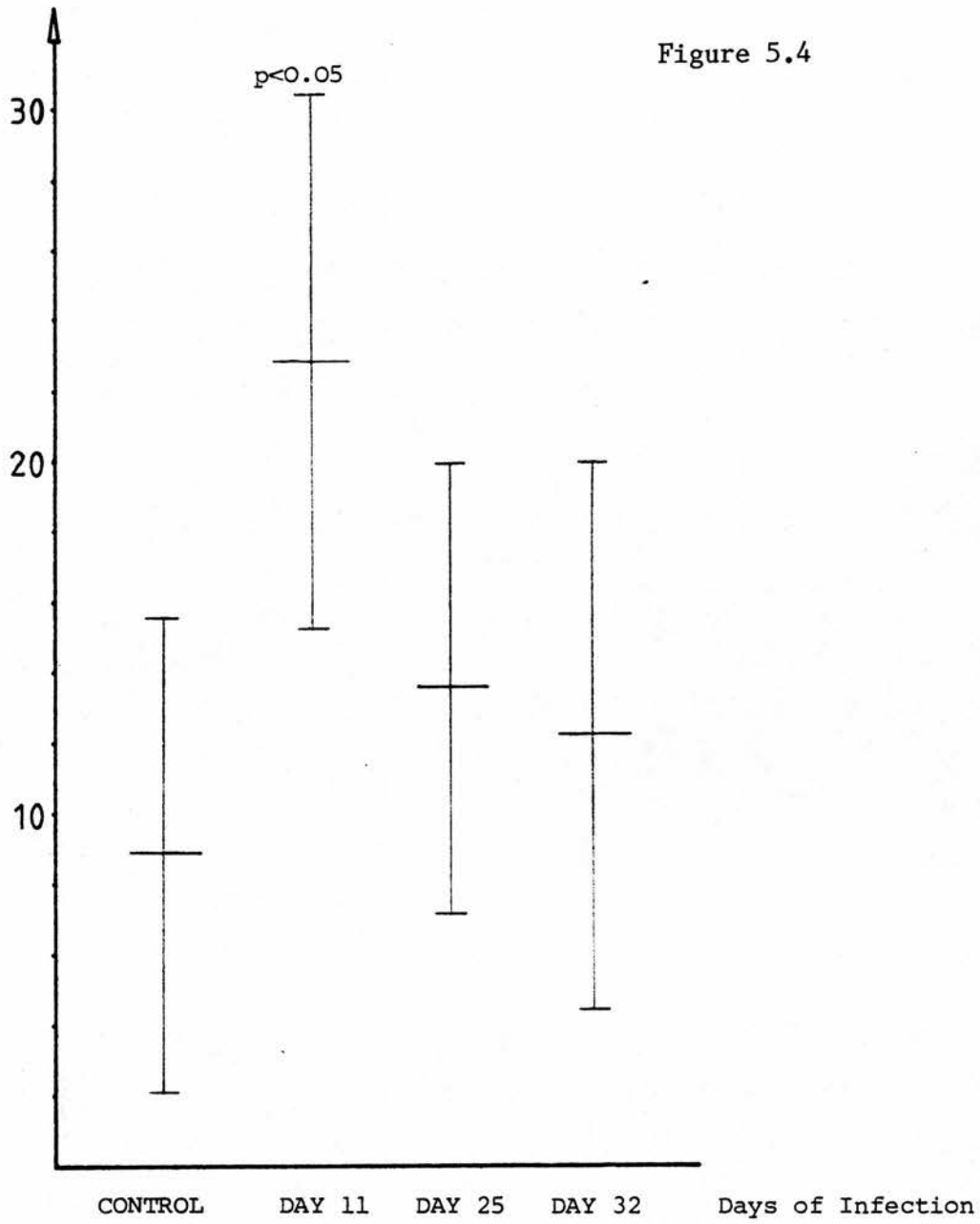


Figure 5.4

Numbers of λ light chain positive cells detected by PAP staining during a primary G. muris infection of adult female BALB/c mice. Results are of mean cell count per 100 μm muscularis mucosa, ± 1 SD (six mice per group).

IgM positive cells per 100 µm muscularis mucosa

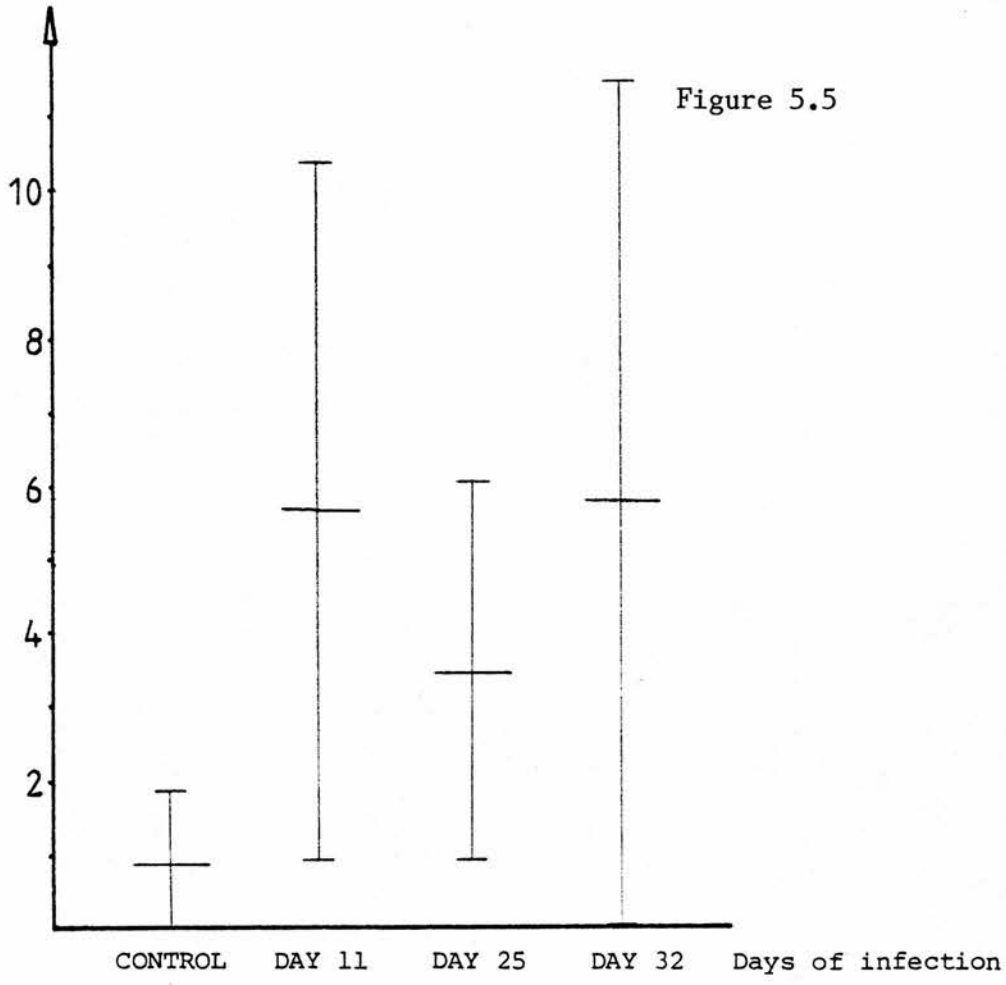


Figure 5.5

Numbers of IgM positive cells detected by PAP staining during a primary G. muris infection of adult female BALB/c mice. Results are of mean cell count per 100 µm muscularis mucosa, ± 1 SD (six mice per group).

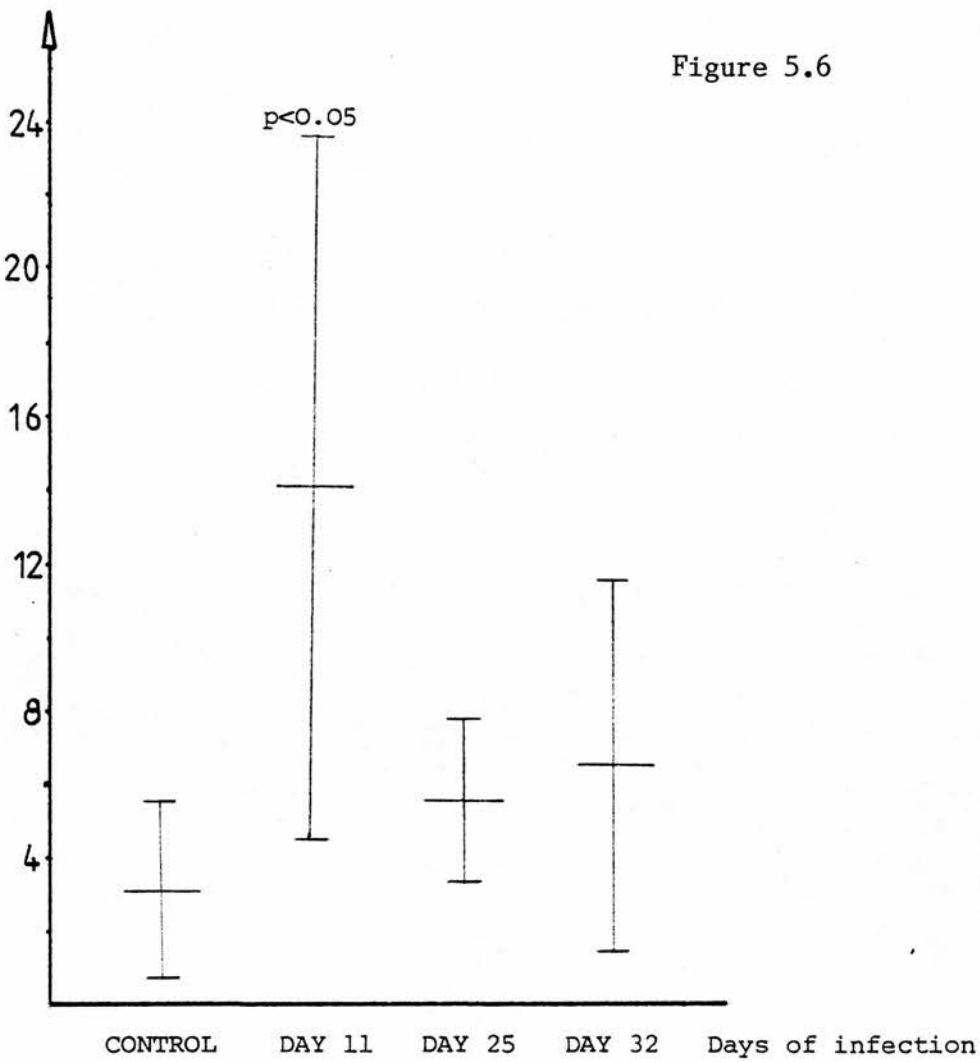


Figure 5.6

Numbers of IgG positive cells detected by PAP staining during a primary G. muris infection of adult female BALB/c mice. Results are of mean cell count per 100 µm muscularis mucosa, ± 1 SD (six mice per group).

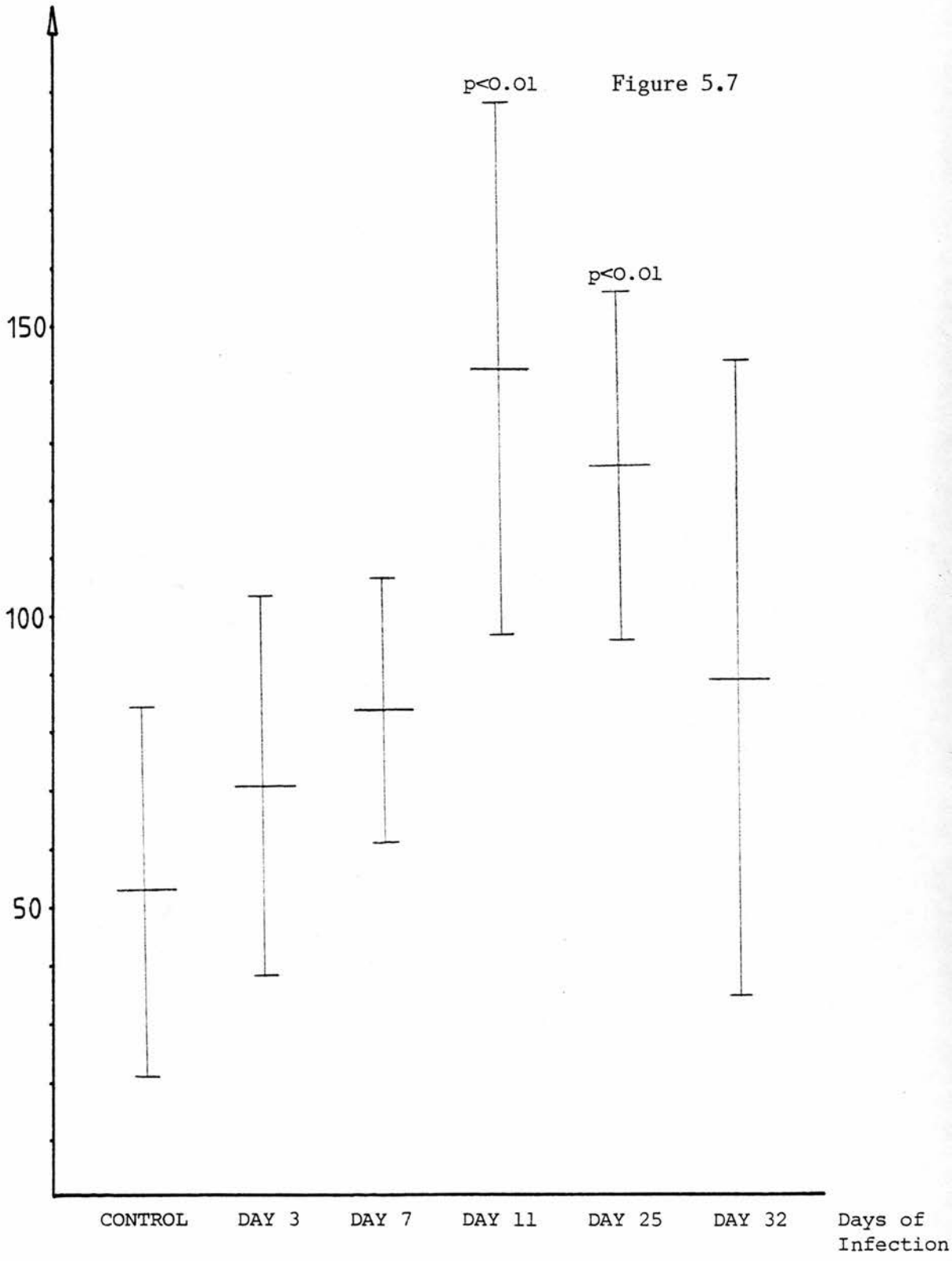


Figure 5.7

Numbers of IgA positive cells detected by PAP staining during a primary G. muris infection of adult female BALB/c mice. Results are of mean cell count per 100 µm muscularis mucosa, ± 1 SD (six mice per group).

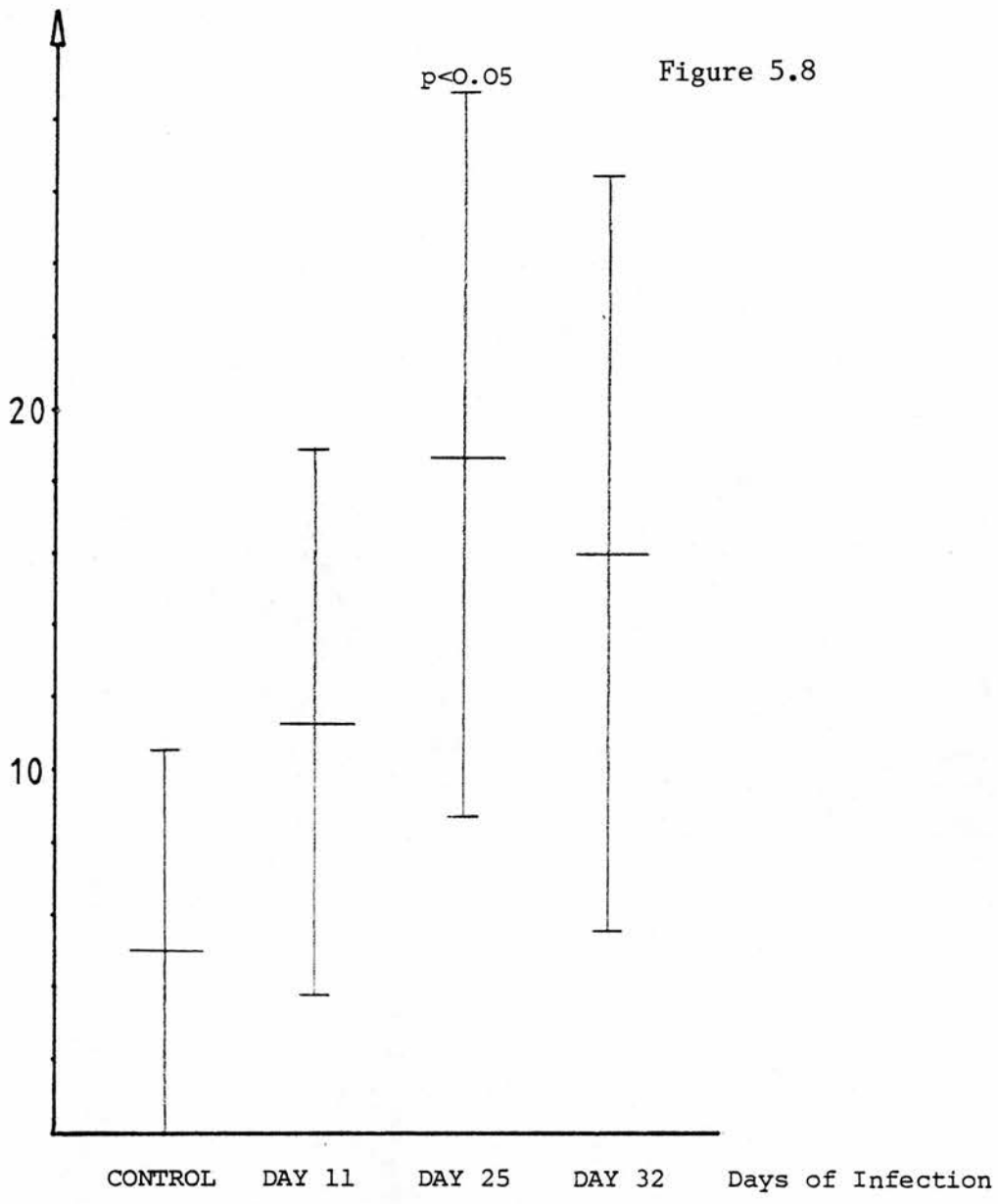


Figure 5.8

Numbers of IgE positive cells detected by PAP staining during a primary G. muris infection of adult female BALB/c mice. Results are of mean cell count per 100 µm muscularis mucosa, ± 1 SD (six mice per group).

Chapter 6

Giardia muris Culture

Introduction.

At a time when the G. muris infection, for some unknown reason, was not producing a faecal cyst output as expected, an attempt was made to culture the trophozoites isolated from mouse jejunum.

The culture of trophozoites isolated from several species has been described by various authors (see Chapter 1). The culture of G. muris from the mouse has not yet been established. The purpose of these preliminary experiments was to assess the potential of short term survival of trophozoites rather than long term culture.

Materials and Methods.

G. muris trophozoites were isolated from mouse small intestine, as previously described. The isolation and washes were in Hank's Balanced Salt Solution (HBSS). This was because many established culture media were based on the standard HBSS formula. Handling and disturbance of the trophozoites was kept to a minimum.

The culture medium was a Modified Diamonds Medium (MDM), as used by the London School of Tropical Medicine and Hygiene. The formula of this medium was listed in chapter 3. Further modifications were the addition of naive mouse bile, collected by puncturing the gall bladder of the mouse into a non-heparinized haematocrit tube. The bile flowed into the tube by capilliary action and was later retrieved and pooled. Bile was diluted by placing 0.035 ml into a Millipore sealed filter unit (0.2 μm pore size) and 10 ml of MDM was then passed through this filter, directly into sterile siliconized glass culture tubes. These were capped and stored at 37°C for several days to check that no bacterial contamination was present.

The second modification to MDM was the addition of hydrocortisone which, when applied to previously infected apparently immune mice, resulted in the development of an overt recrudescent infection from an undetected remnant trophozoite population harboured by the mice (chapter 12). The hydrocortisone concentration was 10^{-12} M; this was adjudged to reflect the expected peak serum concentrations in treated mice. The hydrocortisone preparation used was the soluble hydrocortisone sodium succinate.

The culture tubes were made up in duplicate and 10 ml aliquots of MDM with appropriate modifications placed in each and incubated at 37°C.

Culture 1.

Long Incubation Periods.

Materials and Methods.

Culture tubes were inoculated to give a concentration of about 6000 trophozoites per ml; cultures were incubated for 24 hours and 48 hours.

Prior to withdrawing samples for trophozoite counts, tubes were placed in a 4°C cold room for 30 minutes to cool, causing reduced trophozoite activity and enabling their removal from the culture tube walls by a short application to a Vibromixer. Trophozoite numbers per ml. were established by haemocytometer counts and their viability assessed by observing flagella movement and by the trophozoites' general appearance.

Results.

The mean number of trophozoites per ml for each duplicate culture (Table 6.1) showed that no multiplication of trophozoites had occurred in any of the three media, both at 24 h and 48 h of incubation. At both time points, all trophozoites seen were totally inactive and the bodies appeared vacuolated and swollen. An interesting observation was that numbers of bacteria present in MDM containing naive mouse bile were very much higher than numbers found in the other media. This was true of both culture tubes containing bile.

It was decided that a second trial incubation should be attempted and more frequent observations made to establish the survival time of trophozoites in MDM.

Culture 2.

Short Incubation Periods.

Materials and Methods.

The media used in this experiment were prepared as before; an additional duplicate culture set was that of the trophozoite isolation medium, Hank's BSS. Trophozoites were isolated from mice injected with 2.5 mg cortisone acetate, four days prior to killing. This resulted in an increased trophozoite yield. The concentration of trophozoites in the culture medium was increased to 40,000 per ml; it was hoped that this might result in a higher probability of trophozoites surviving in culture. The final procedural modification was to cool culture tubes for only five minutes at 0° C to cause release of trophozoites from the glass tube wall, but not disturb the media temperature to such a great extent. Samples of media and trophozoites were taken in duplicate as before, but after 60, 120 and 180 minutes of incubation.

Results.

The results (Table 6.2) were expressed as the mean of two duplicate culture tubes in each group. There was no increase in trophozoite numbers over the 180 minute incubation. This was expected for three reasons; firstly, few dividing trophozoites were seen in the original inoculum samples. Secondly, the period of incubation was less than the possible doubling time of the trophozoite and finally, there is generally a lag in multiplication of most micro-organisms when culture medium is changed. The trophozoites seen in the initial inoculum were all active and apparently viable. The viability of trophozoites isolated in this way was confirmed by other experiments in which mice were successfully infected by i.g. trophozoite intubation. Within 60 minutes of incubation in MDM, regardless of further modifications, the majority of trophozoites were dead. At 120 minutes of incubation in these media, all trophozoites viewed were dead. This result was paralleled in the Hank's BSS culture.

Summary and Conclusions.

Under the incubation conditions used, MDM in either its original form or modified as described is not suitable for the maintenance nor the multiplication of G. muris trophozoites.

The deficiency in the protocol may be as simple as incorrect pH or oxygen tension, but the successful culture of G. muris still eludes several very competent and otherwise successful research groups. These preliminary experiments were designed not to establish long term culture, but to examine short term viability and survival of trophozoites. The results were disappointing, even in respect of this simple aim, and following discussion with Dr. E. Meyer, who has notable success in Giardia culture, this line of experimentation was abandoned.

Under successful culture conditions this method could be used to examine substances that may effects the trophozoite. In this study, interest in adding bile to the culture medium was due to the report of Bemrick (1963), when it was noted that ligation of the bile duct in the rat prevented subsequent Giardia infection. Farthing et al (1983) used the successful G. duodenalis culture model to identify a limited ability of this Giardia form to synthesise membrane lipids de novo, and that bile salts facilitated uptake of preferred membrane phospholipids from the media.

The second modification to the medium was the addition of hydrocortisone. It has been shown that the injection of steroids to previously infected but apparently immune mice, stimulates the recrudescence of a normally undetected latent trophozoite population (Nair et al, 1981). This recrudescent infection declines rapidly on cessation of steroid treatment, as described in chapter 12. The addition of hydrocortisone to the medium was to test the hypothesis that the steroid might directly stimulate the metabolic activity and multiplication of the trophozoites. This would result in the recrudescent infection, and subsequent rapid resolution of infection in the absence of the steroid treatment.

A second interesting application of culture techniques is currently under investigation by Meyer & co-workers; this is the examination of trophozoites isolated from different sources and

examination of substances released into media by these trophozoites, that may explain the differing pathogenicity of these strains. More importantly, attempts are being made to isolate components of the Giardia that present essential antigenic targets, capable, when presented in an appropriate form, of stimulating the development of an effective immune response to a subsequent challenge by viable Giardia parasites.

Table 6.1.
Long Incubation Periods.

	Mean trophozoites/ml		Bacteria	
	24 hours	48 hours	24 hours	48 hours
Modified Diamonds Medium (MDM)	1.1×10^3	0.7×10^3	+	++
MDM and Bile	1.0×10^3	0.65×10^3	++++	++++
MDM and Hydrocortisone	0.55×10^3	0.55×10^3	+	++

Table 6.2.
Short Incubation Periods.

	Duration of Incubation			
	0	60	120	180
Medium	Mean Trophozoites/ml $\times 10^3$			
MDM Alone	40	20	20	40
MDM and Bile	40	10	20	30
MDM and Hydrocortisone	40	10	15	15
Hank's BSS	40	10	20	20

Chapter 7

Loperamide HCl Treatment and *Giardia muris* Infection

Introduction.

The primary aim of these experiments was to modify the mouse giardiasis model in a non-immunological way to achieve increased numbers of parasites in the host's gut. This was to investigate whether large numbers of trophozoites resulted in more tissue damage, either directly due to the parasite, or indirectly through an exaggerated immune response. The results would also indicate whether the guts activity was involved in the elimination of G. muris at the end of infection.

The hypothesis to be tested was that decreased motility would result in increased trophozoite numbers. If successful, the model would be used to investigate the immune response against large numbers of trophozoites.

The method employed to alter the gut's motility i.g. treatment with Loperamide HCl. This drug increases the capacitance of the gut by inducing a change in motor function of the intestinal musculature. This results in delayed passage of lumenal contents through the intestine and so more fluid absorption by the gut. (Kachel et al 1983, Ruppin et al 1983, Schiller et al 1984). The influence of loperamide on the intestinal morphology, cell kinetics and IEL numbers was also investigated.

Materials and Methods.

Loperamide HCl was administered as the syrup presentation marketed by Janssen Pharmaceutical Ltd., as Imodium (R).

Establishing the Dose of Loperamide.

Adult female BALB/c mice were treated with a range of doses, calculated around the basis of the recommended dosage regime for children. Treatment was by daily i.g. intubation. The range of doses tested was:

(all as 0.2 ml volume)

0.00 (H_2O control)

0.04 mg Loperamide HCl

0.20 mg Loperamide HCl

0.40 mg Loperamide HCl

Animals were weighed daily and individual 24 hour faecal collections made. The amounts of food and water consumed by each group was also recorded.

Results.

Body weight, food and water consumption did not alter significantly, but wet weight faecal output was significantly reduced in each group receiving loperamide HCl. (Table 7.1)

Subsequent experiments incorporated loperamide in the drinking water and, assuming 6 ml per mouse per day, a daily dose of 0.12 mg loperamide HCl was achieved. This mode of drug application influenced faecal output as noted before, reducing daily faecal output compared to the control group.

Table 7.1

Loperamide HCl (mg/0.2 ml)	Mean Wet Faecal Output/Day (g)	p Value w.r.t. control
0.00	4.30 $\pm$ 0.29	
0.04	3.74 $\pm$ 0.35	<0.05
0.20	3.34 $\pm$ 0.03	<0.01
0.40	2.65 $\pm$ 0.66	<0.01

Experiment 1

Cyst Initiated Infection and Loperamide Treatment.

This experiment investigated the course of infection initiated by 2,000 cysts i.g., in control and loperamide treated adult female BALB/c mice. As can be seen (Fig. 7.1), no significant change was noted in the infection characteristics.

Experiment 2

Trophozoite Initiated Infection and Loperamide Treatment

This experiment investigated the influence of loperamide treatment on an infection initiated by 2,000 trophozoites. A more compliant gut might accommodate a larger percentage of the infective

inoculum and so produce an infection time course which rises more rapidly to peak parasite load. As can be seen (Fig. 7.2), a significant difference was detected in the rise to plateau in cyst output, and a significant delay in expulsion was noted at day 21 in the loperamide treated group in this experiment.

Experiment 3

Large Inoculum Trophozoite Initiated Infection and Loperamide Treatment.

Based upon the previous result, it was decided that a larger initial infective dose might better show any alteration in the inoculum accommodated in loperamide treated gut. Animals were infected with 15,000 trophozoites i.g. This would also act as a repeat experiment to verify the delayed expulsion seen in the loperamide treated group, which, though significant, was due to the persistance of infection at high cyst output levels in four of six mice, two having no detectable faecal cyst output at this time point.

The results showed (Fig. 7.3) that there was no significant difference in the infection time course characteristics of control and loperamide treated mice. The delayed resolution of infection seen in experiment three was not found in this experiment.

The administration of loperamide had no significant effect on intestinal architecture (Fig. 7.4) and kinetics, and caused no change in the numbers of IEL (Fig. 7.5) present in non infected adult female BALB/c mice.

Summary and Conclusions

Treatment of mice with loperamide HCl, by direct i.g. intubation or in drinking water resulted in decreased wet weight faecal output. The prepatent period, rise to plateau and plateau level of infection in loperamide treated and control mice were similar.

Resolution of the infection was also unchanged. The one result of delayed expulsion may be considered as aberrant as two of the six mice had cleared the infection at this time, and the delay was not

seen again in the similar third experiment.

Alteration of gut motility by loperamide treatment does not increase cyst excretion, and by inference trophozoite load and does not alter the course of infection compared to that seen in control mice.

Mucosal architecture, cell kinetics and IEL numbers are not altered during loperamide treatment.

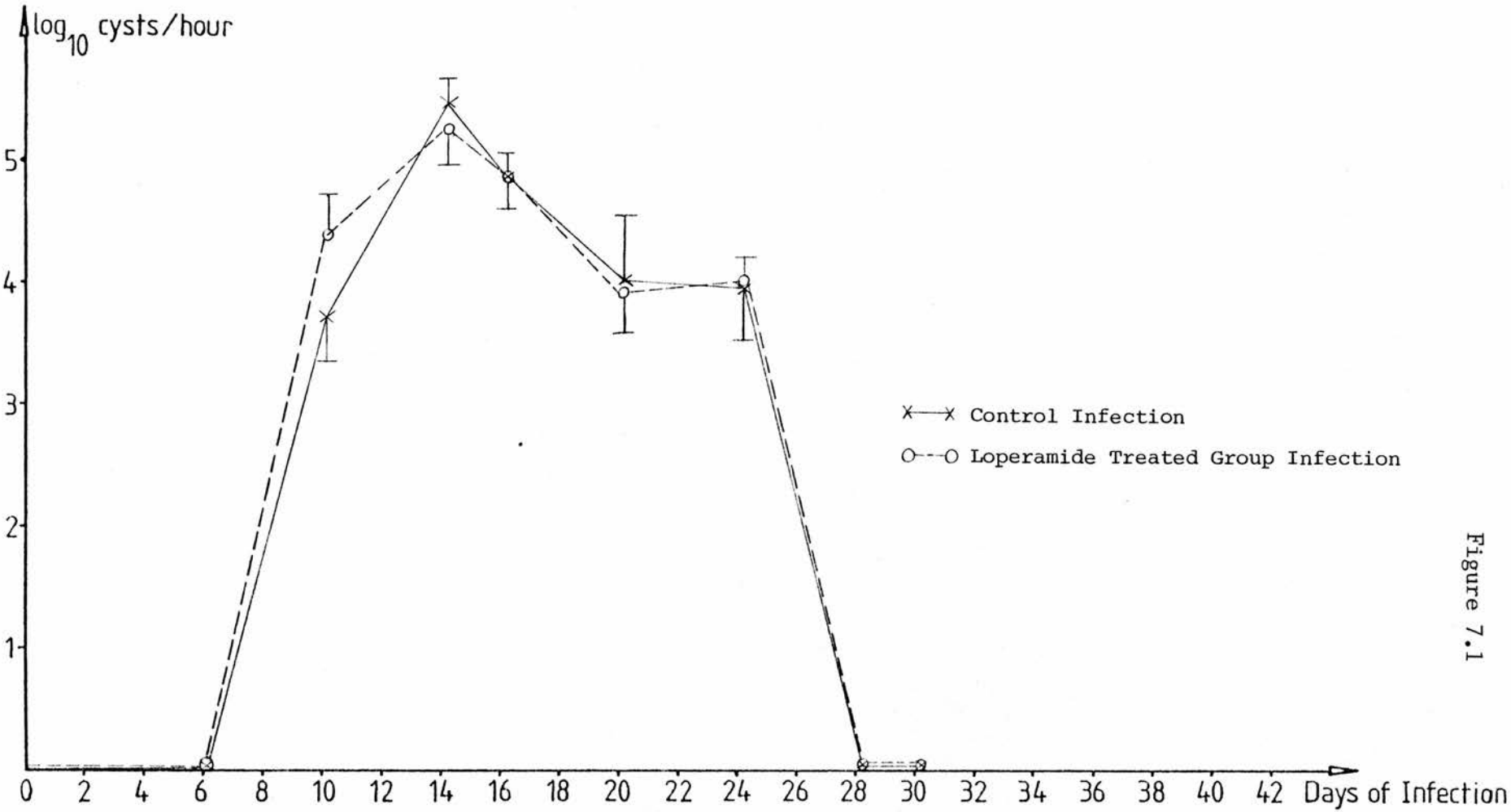


Figure 7.1

Progress of primary Giardia muris infections in adult female BALB/c mice. Infections initiated on day 0 with 2,000 cysts, ig, in control and loperamide treated mice. Loperamide treated mice received 0.12mg loperamide HCl per day through their drinking water, from 6 days prior to infection. Marks represent means ± 1 SD of number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

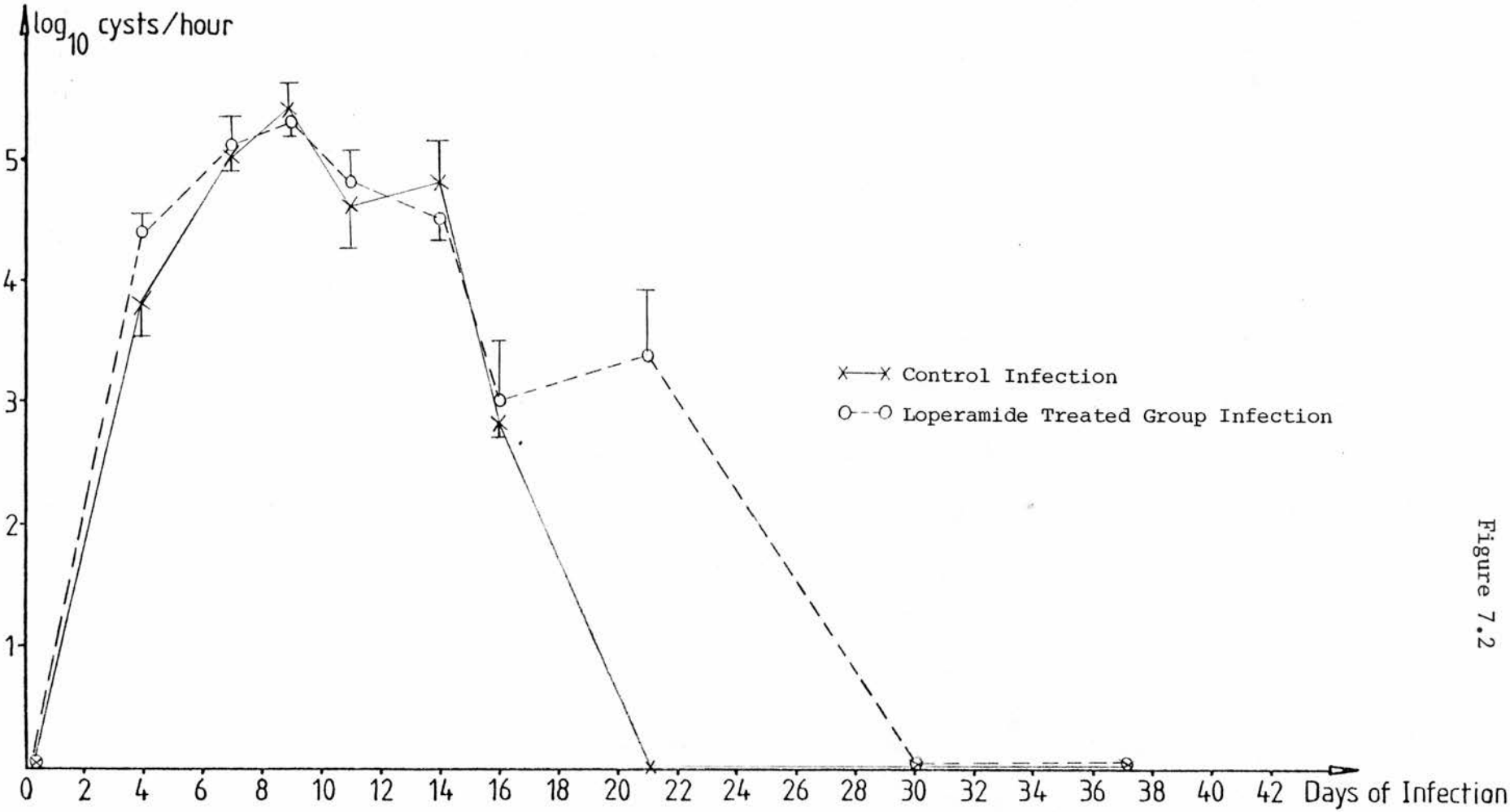


Figure 7.2

Progress of primary Giardia muris infections in adult female BALB/c mice. Infections initiated on day 0 with 2,000 trophozoites, ig, in control and loperamide treated mice. Loperamide treated mice received 0.12mg loperamide HCl per day through their drinking water, from 4 days prior to infection. Marks represent means ± 1 SD, of number of cysts, passed per hour during a 2 hour faecal collection (six mice per group).

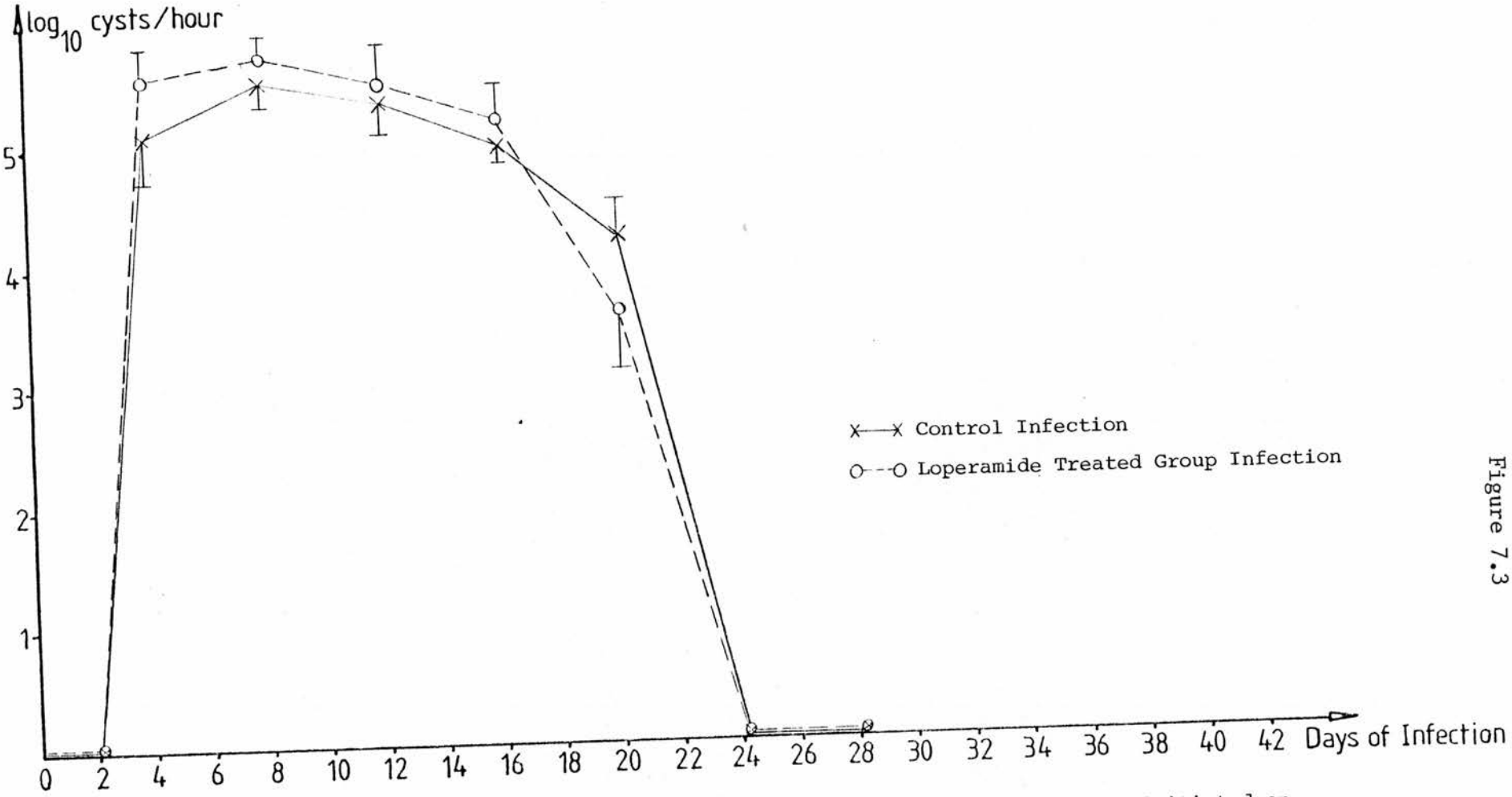


Figure 7.3

Progress of primary Giardia muris infections in adult female BALB/c mice. Infections initiated on day 0 with 15,000 trophozoites, ig, in control and loperamide treated mice. Loperamide treated mice received 0.12ml loperamide HCl per day through drinking water, from 7 days prior to infection. Marks represent means ± SD of number of cysts passed per hour, during a 2 hour faecal collection (six mice

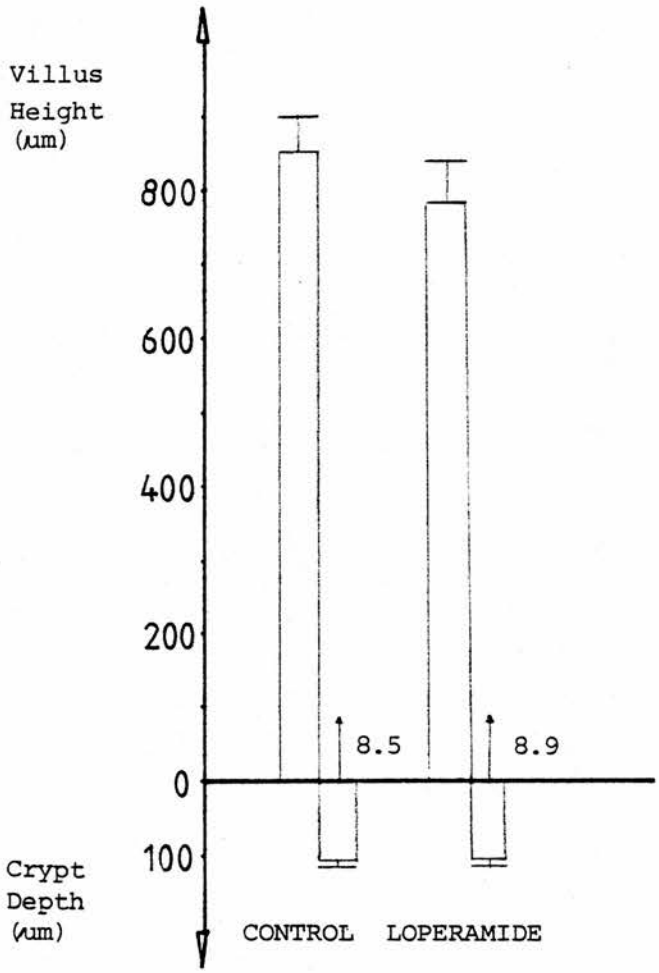


Figure 7.4

Control versus Loperamide Dosed Mice

Effect of Loperamide HCl at 0.12mg per day on mucosal architecture of adult female BALB/c mice. Villus height, crypt depth and CCPR after 14 days of this dose delivered through drinking water. Bars represent means ± 1 SD for villus height and crypt depth, and arrows show CCPR (six mice per group).

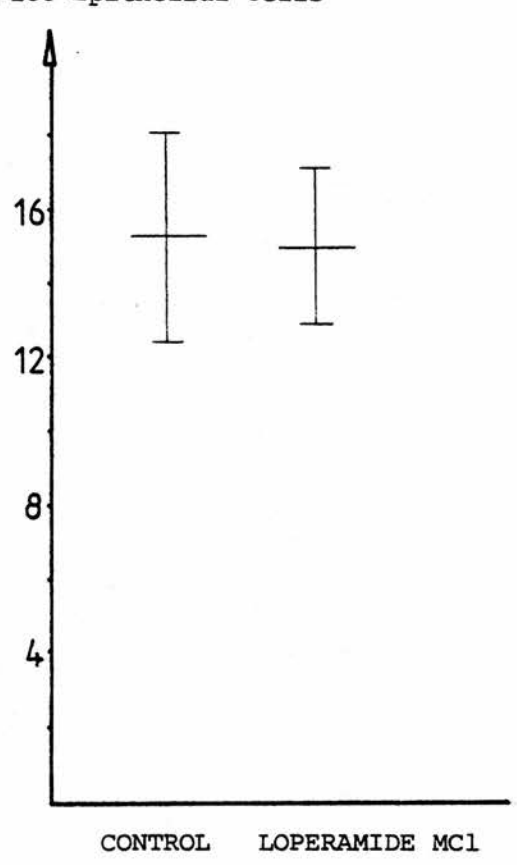


Figure 7.5

Control versus Loperamide Dosed Mice

Effect of Loperamide HCl at 0.12mg per day on IEL counts of adult female BALB/c mice. This dose delivered through drinking water for 14 days. Results are of mean IEL count ± 1 SD (six mice per group).

Chapter 8

The Role of Goblet Cells and Mucus in *G. muris* Infection

Introduction.

The aim of this investigation was to examine the role of goblet cells and mucus during G. muris infection.

Increased faecal mucus was noticed during the cyst isolation procedure and it seemed likely that this was involved in the host's response to G. muris infection.

Goblet cells and mucus have not previously been associated with intestinal protozoan infections. Changes in the mucopolysaccharide fuzzy coat in which the microvilli are embedded have been associated with giardiasis (described in Chapter 2). Their involvment in the response to both primary and challenge infections of the rat by the nematode, Nippostrongylus brasiliensis, have been well documented (Lee & Ogilvie 1979, Levy & Frondoza 1983).

Interest in goblet cell numbers and mucus involvement during giardiasis was stimulated by the work of Miller et al (1981) on the infection of the rat by the nematode, Nippostrongylus brasiliensis. Of particular interest was the suppression, by steroid treatment, of the rapid expulsion phenomenon during secondary challenge by this nematode in immune rats.

It had been necessary to investigate the role of goblet cells and mucus during infection by several methods. Some of the results presented here have been obtained from experiments described fully in other chapters. For this reason, some results are considered with only a brief description of the original experiment.

Experiment 1

Goblet Cell Numbers During Primary G. muris Infection.

Materials and Methods.

These specimens were takenduring the primary G. muris infection described in Chapter 5. This was an infection of normal, adult female BALB/c mice, initiated by 2000 cysts i.g.

Results.

The number of goblet cells (Fig. 8.1) did not significantly vary from the control value at any time during infection.

Experiment 2

SEM of Infection in Nude and Heterozygote Mice.

Introduction.

The aim of this study was to examine the intestinal morphology by SEM during chronic infection of nude (athymic) mice and the acute infection of heterozygote littermates.

Materials and Methods.

This examination was done on gut taken from mice during the primary infection phase of the experiment described in Chapter 11.

Results.

The SEM of small intestine from both heterozygote and nude mice, taken ten days after primary infection is shown on Plates 8.1 and 8.2, respectively. Both show dehydrated strands of mucus closely associated with many trophozoites. A similar finding was present at 24 days after infection in both groups of mice (Plates 8.3 and 8.4).

At higher magnifications, trophozoites are seen enmeshed in mucus (Plate 8.5), while in another field free luminal cells are also present (Plate 8.6).

Experiment 3a

Steroid Induced Recrudescent Infection.

Introduction.

The aim of this series of experiments was to investigate steroid induced recrudescence of giardiasis and subsequent reaction to steroid withdrawal.

Materials and Methods.

Adult female BALB/c mice were immunized by infection as before and treated with daily s.c. injection of 2.0 mg hydrocortisone sodium succinate. This treatment continued for five days and then animals were killed in groups of six on the following days:

A. Control (Previously infected immune mice, before commencement of any treatment)

B. Steroid treatment:
   Day 1
   Day 5 (Peak recrudescent infection attained)

C. Post-Steroid treatment:
   Day 1
   Day 2
   Day 3

Specimens of gut were taken for counting goblet cells and histologically examined for evidence of mucus trapping of trophozoites in the lumen.

Specimens of jejunum were taken in Baker's Formol calcium fixative; the gut was gently washed through with about 2 ml of fixative to expel macroscopic lumenal contents and the 2 cm specimen placed, unopened in the fixative.

Results

Using a fixed field technique, there was a significantly increased number of goblet cells after five days of steroid treatment, compared to the untreated control. This increase was not sustained during steroid withdrawal (Fig. 8.2). Similar findings were also present when the cell count was expressed per villus unit, with an increased goblet cellcount on day five of steroid treatment (Fig. 8.3). No obvious mucus trapping of trophozoites was observed in specimens from any group.

Experiment 3b

Steroid Induced Recrudescent Infection.

Introduction.

This experiment was designed to parallel the protocol of Experiment 3a, but included an additional control group of naive uninfected animals. This control group was also treated with steroids.

Materials and Methods.

Adult female BALB/c mice were infected and immunised as previously described. A group of age and sex matched uninfected controls were obtained.

Groups of six mice were sacrificed from naive and immune groups before treatment, after five daily steroid treatments, and three days after the last steroid treatment.

Due to difficulties encountered in performing cell counts in the previous "intact tube" method of fixing and sectioning the gut, the specimens were opened and placed on card for this experiment.

Results

No significant change was observed in the numbers of mature goblet cells per villus in any group (Fig. 8.4). This result is discussed later in this chapter.

The ambiguity of these results prompted an approach to the question of mucus involvement from a different direction.

Experiment 4

Trophozoite Distribution and Ease of Displacement.

Introduction.

This experiment was designed to determine whether G. muris trophozoites were being excluded from a safe, preferred intervillus site by mucus during the period after steroid treatment was withdrawn, and parasite numbers were in decline.

Trophozoite Distribution.

Materials and Methods.

Firstly, the area of the small intestine holding the highest parasite load was determined by taking 2 cm specimens of gut, opened and placed in 5 ml of Hank's BSS at 4°C. Specimens were taken distal to the pylorus at 1, 7, 15, 25 and 30 cm. The specimens were vigorously shaken forty times in the HBSS and trophozoite samples counted from each by haemocytometer. Six mice were used in each group. Groups compared were a primary infection at peak faecal cyst output, and a steroid induced recrudescent infection of 5 days

duration.

Results

Results are expressed as the trophozoites isolated per sample point as a percentage of the total number of trophozoites isolated from all samples of each mouse gut (Fig. 8.5).

A very significant ($p<0.01$) increase in the proportion of trophozoites present at 1 cm distal to the pylorus was found in the recrudescent infection.

A significant ($p<0.01$) increase in the percentage of trophozoites found at 15 cm distal to the pylorus was found in the primary infection group.

Displacement of Trophozoites.

Introduction.

The second phase of this experiment compared the ease of displacement of trophozoites during a primary infection at the time of peak faecal cyst output, and one day after cessation of five daily treatments of hydrocortisone sodium succinate resulting in a recrudescent infection.

Materials and Methods.

A length of small intestine from 5 cm to 20 cm distal to the pylorus was chosen as the sample to be taken from each mouse gut. This resulted in an appropriate overlap of parasite habitation ranges in primary and steroid induced recrudescent infections. The gut was cut open and placed in HBSS at 4° C and initially "swirled". The gut was then removed and placed in a second tube of HBSS at 4°C and shaken twice. This procedure was repeated for each sample point in fresh HBSS and trophozoite numbers in each sample tube were counted by haemocytometer. The results are expressed as a cumulative percentage of the total trophozoite count for each mouse.

Results

The graph showing ease of displacement of trophozoites (Fig. 8.6) indicated that a significantly ($p<0.05$) higher proportion of

trophozoites present in the gut during a steroid induced recrudescent infection are displaced by two vigorous shakes.

Experiment 5

SEM and TEM of Steroid Induced Recrudescent Infection.

Introduction.

The question of mucus involvement was still considered to be unresolved. A further approach to this investigation was examination of gut samples at the time of rapid decline in parasite numbers following cessation of steroid treatment, by Scanning and Transmission Electron Microscopy (SEM and TEM). This work was planned in collaboration with Dr. V. Crucioli of the WGH Pathology Department.

Materials and Methods.

Immune adult female BALB/c mice were prepared as previously described and treated with 2.0 mg hydrocortisone sodium succinate, s.c., daily for eight days. Mice were killed and tissues taken for SEM and TEM, fixed and prepared by routine methods. The remainder of the intestine was vibrated to obtain trophozoite counts. Sacrifice was carried out 24 hours after the first steroid treatment; after day 6 of treatment, when parasite load was very high; and at 24 hours and 48 hours after the last steroid treatment, when parasite numbers were falling rapidly.

Dr. Crucioli examined these samples by SEM and TEM.

Results

The trophozoite counts responded to steroid treatment and its withdrawal as predicted (Fig. 8.7).

The SEM results showed many trophozoites and moderate accumulations of mucus; the appearance of these pictures was paralleled by the previous nude mouse study.

The TEM work showed two interesting features. Firstly, many of the trophozoites present in the steroid induced recrudescence showed greatly increased vertical section height (Plates 8.8, 8.10 and 8.11) compared to the primary infection trophozoites (Plates 8.7 and

8.9). The recrudescent trophozoites also appear more granular, presumably due to increased amounts of rough endoplasmic reticulum (Plate 8.8, 8.10 and 8.11). Their cytoplasm also contains many more vacuoles and tubules, which extend throughout the body (Plates 8.8 and 8.12), rather than being typically at the level of the dorsal surface, as in primary infection trophozoites (Plate 8.7). These features suggested that trophozoites from a steroid induced recrudescent infection are in a highly active metabolic state.

The second point of interest was the occurrence of numbers of lymphocyte-like cells in the gut lumen with the trophozoites at the time of parasite expulsion after steroid treatment ceased (Plates 8.13, 8.14 and 8.15). No definite evidence of attachment of these cells to trophozoites was recorded by Dr. Crucioli. The cells would appear not to originate from contamination by blood, as no erythrocytes were observed.

Summary and Conclusions.

There was a rapid increase in the number of cysts and trophozoites upon commencement of steroid treatment and rapid fall upon withdrawl of treatments. The detectable cyst output falls to zero prior to trophozoite numbers falling to undetectable levels.

The number of goblet cells was found to be increased at the peak of recrudescent infection, but when steroid treatment was withdrawn and the infection was resolving, no significant change was noted. Again, as with the MMC, it is likely that goblet cell numbers detected by PAS staining do not fully reflect the in vivo situation, as release of mucus during handling and preparation of the gut occurs despite the use of a fixative specifically for the preservation of mucus. This may explain the lack of a significant increase in the second goblet cell experiment (Fig. 8.4), when the gut was opened and fixed flat on card. In the intact tube fixed gut specimens, some mucus was preserved in the lumen, but no particular association with trophozoites was noted.

The action of the steroid may be quantitative, inhibiting production of goblet cells and/or mucus, or qualitative, altering the characteristics of the mucus. The possible qualitative change

due to steroid treatment is founded upon the known influence of endogenous steroid hormones upon mucus. This effect is well known and used diagnostically to determine the phase of oestrus of various animals, by observing the characteristic ferning patterns found after drying vaginal mucus smears on microscope slides. A less viscous mucus would be less able to entrap trophozoites and exclude them from the relatively protected basal villus sites they prefer to occupy. If present in the lumen, trophozoites would be rapidly expelled by peristalsis. The trophozoites of a steroid induced recrudescent infection occupy a site more proximal in the jejunum than do those of a primary infection. One explanation of this may be related to the comparatively large volume of the infective dose (0.2 ml) used to initiate the primary infection. This large volume causes gastric emptying, so the cyst or trophozoite infective agents are distributed widely along the small intestine. This effect of gastric emptying with 0.2 ml was confirmed by observation of the small intestinal transit of the particulate red carmine dye after i.g. intubation.

Alternatively, the luminal environment may be altered during a primary infection and the differences found were due to pH, bile salt concentrations or nutrient availability. The recrudescent infection develops from a small, microscopically undetectable reservoir of trophozoites that probably inhabit the region of small intestine about 5 cm distal to the pylorus. This is the region immediately distal to the ampulla of Vater, where the common bile duct enters the small intestine. The high bile concentration in this region present favourable conditions for the trophozoites, which have a known requirement for bile. Some earlier reports of giardiasis also describe Giardia trophozoites inhabiting the bile duct, so this site may also act as a reservoir for latent infection.

The ease of displacement of trophozoites in primary and recrudescent infections showed a significantly easier displacement of trophozoites present in the recrudescent infection. This indicated that at the time of rapid expulsion of the recrudescent infection, a proportionately higher number of trophozoites are, for some reason, more amenable to physical displacement. This may be as

a result of various factors, which are discussed in the final chapter.

The SEM and TEM showed two features potentially capable of mediating trophozoite expulsion. Firstly, mucus: one of the problems inherent in convential SEM is that specimens are dehydrated, so the relationship of hydrated semi-solid mucus to the trophozoites is, to some degree, lost. The position of remaining dehydrated strands indicated an association of mucus and trophozoites. The second possible mediators of immunity were the lymphoid cells present in the lumen and seen by TEM. Other workers, both using SEM and TEM, have noted cells in the lumen at the time of Giardia expulsion in primary infection. Heyworth et al (1982, 1983) characterised these cells by immunofluorescence, showing them to be T lymphocytes bearing the Lyt 2 marker of cytotoxic/suppressor cells. The presence of these cells could cause trophozoite expulsion by direct cytotoxic activity or by attaching to the flagellae. The hydrodynamic model of trophoizoite attachement relies upon the flagellar action causing a negative pressure under the ventral disc, when interacting with a surface. By interrupting flagellar action, the trophozoites cannot attach to the villus surface and may be lost to the lumen and expelled.

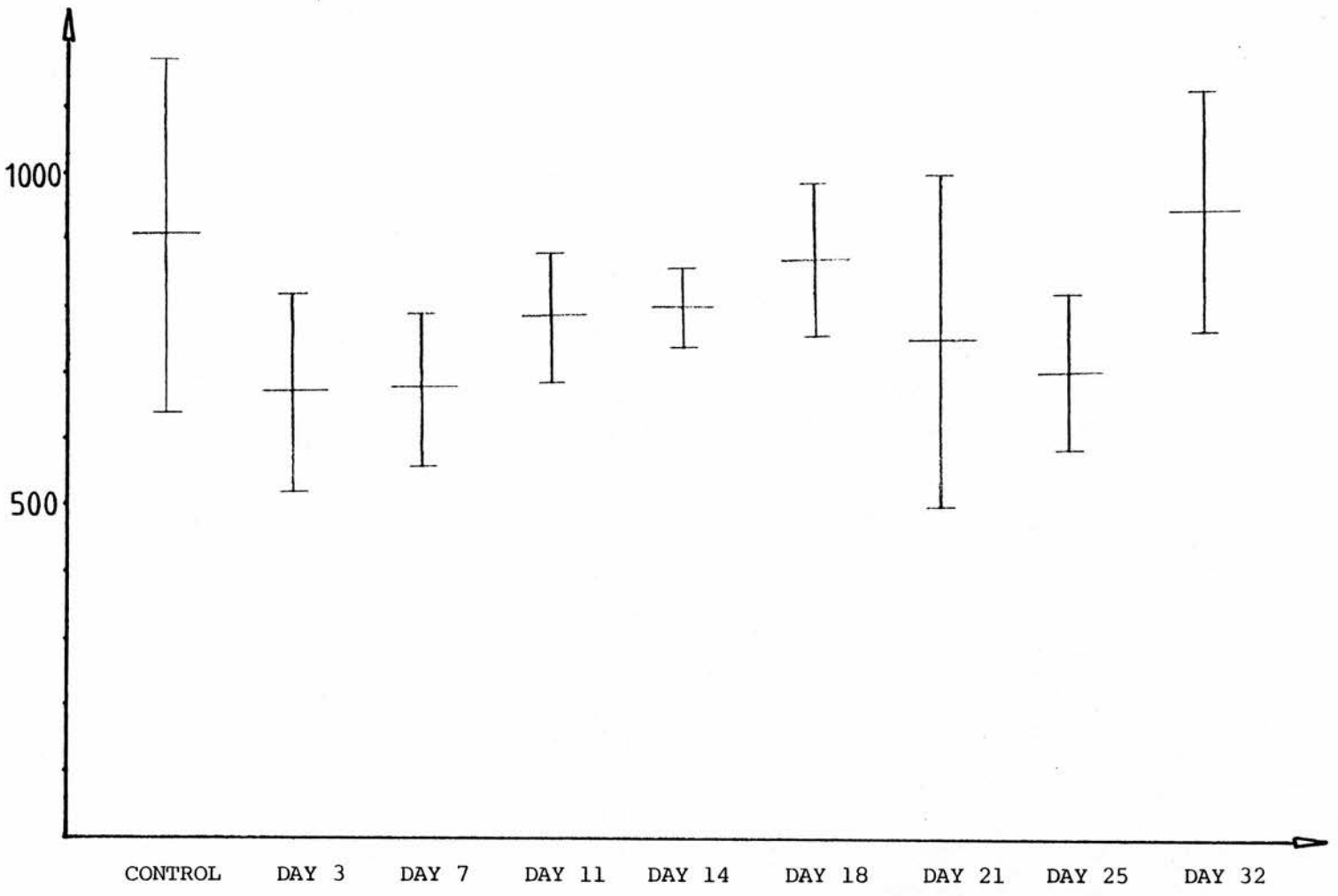


Figure 8.1

Numbers of mature Goblet Cells per 500 µm muscularis mucosa during a primary G. muris infection in adult female BALB/c mice. Results are of mean ± 1 SD (six mice per group).

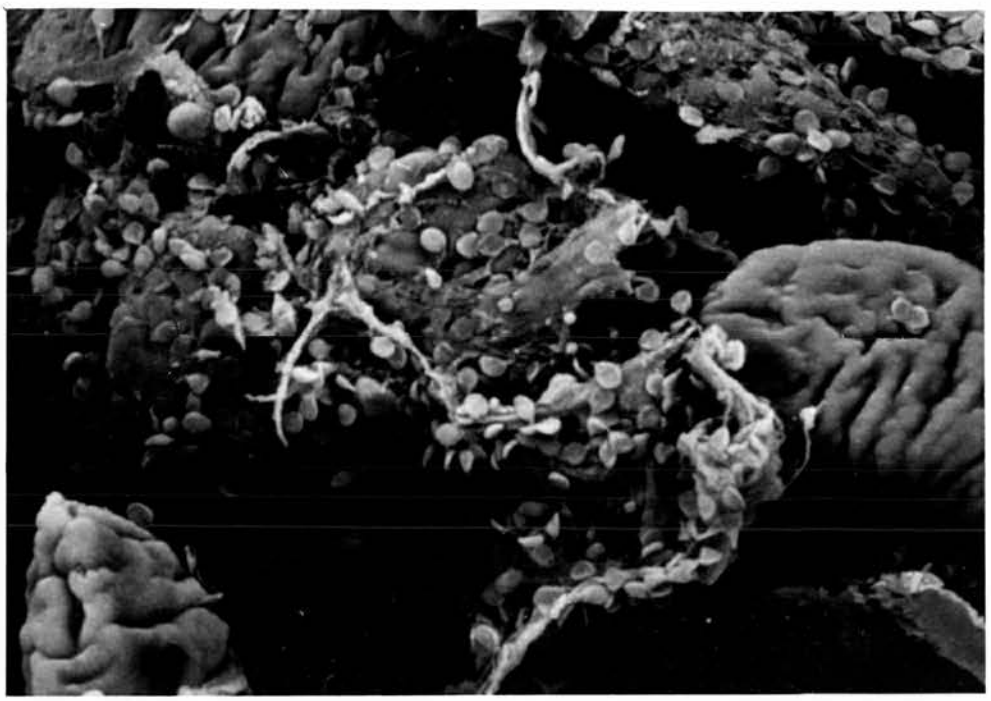


Plate 8.1: Trophozoites and mucus in the gut lumen of a heterozygote mouse after 10 days infection (x200).

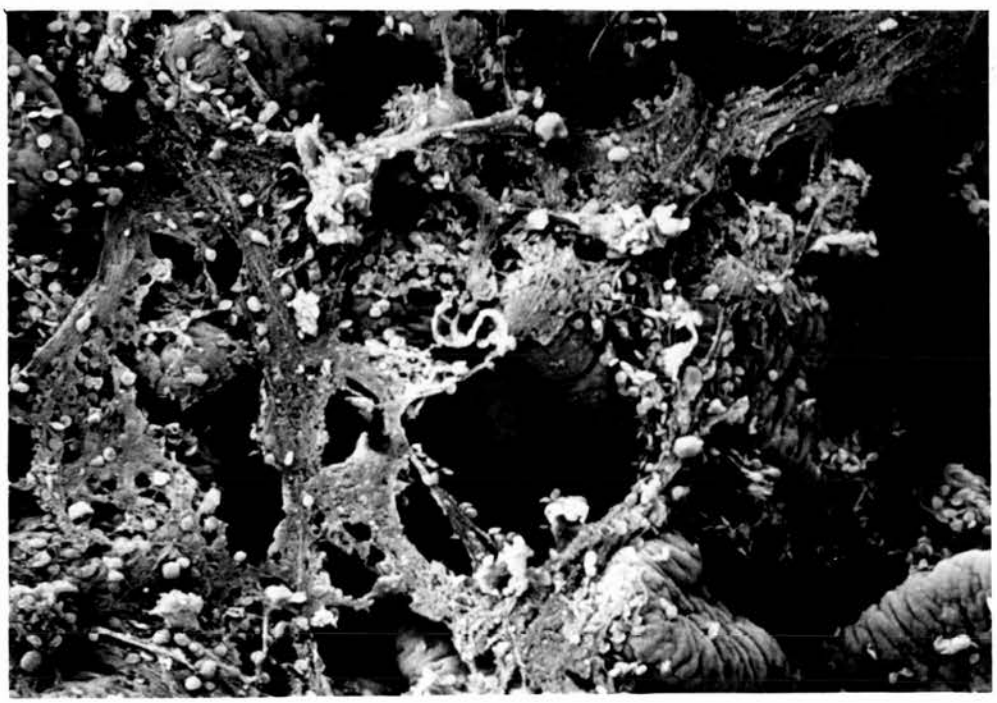


Plate 8.2: Trophozoites and mucus in the gut lumen of a nude (athymic) mouse after 10 days infection (x150).

Plate 8.3: Trophozoites associated with mucus in the gut lumen of a nude (athymic) mouse after 10 days infection (x500).

Plate 8.4: Trophozoites associated with mucus in the gut lumen of a nude (athymic) mouse after 24 days infection (x700).

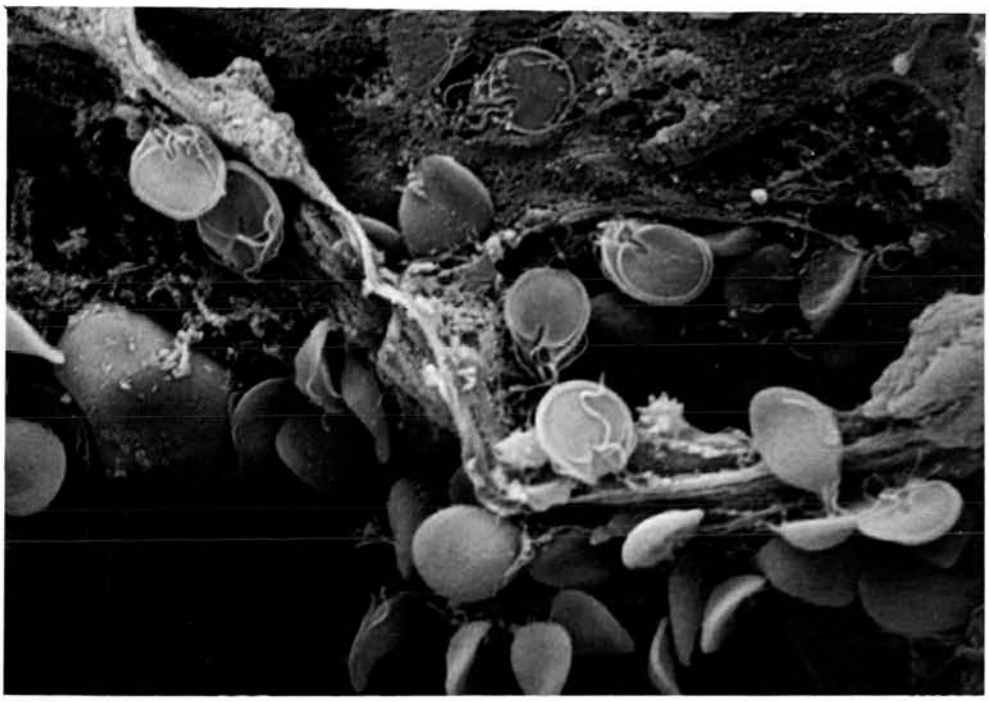


Plate 8.5: Trophozoites and strands of mucus in the gut lumen of a heterozygote mouse after 10 days infection (x1,100).

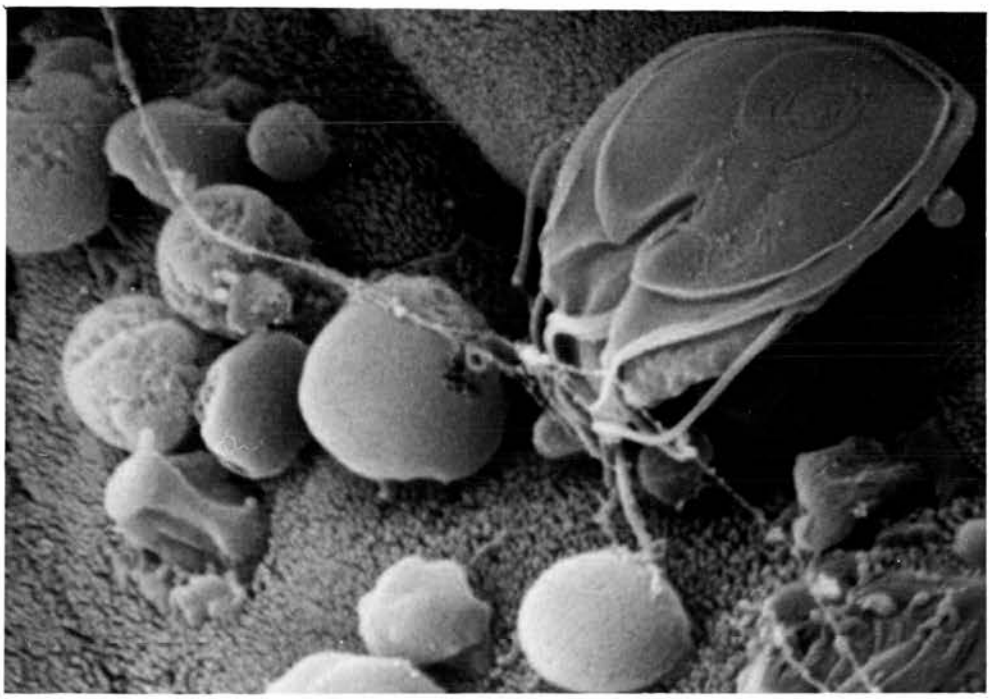
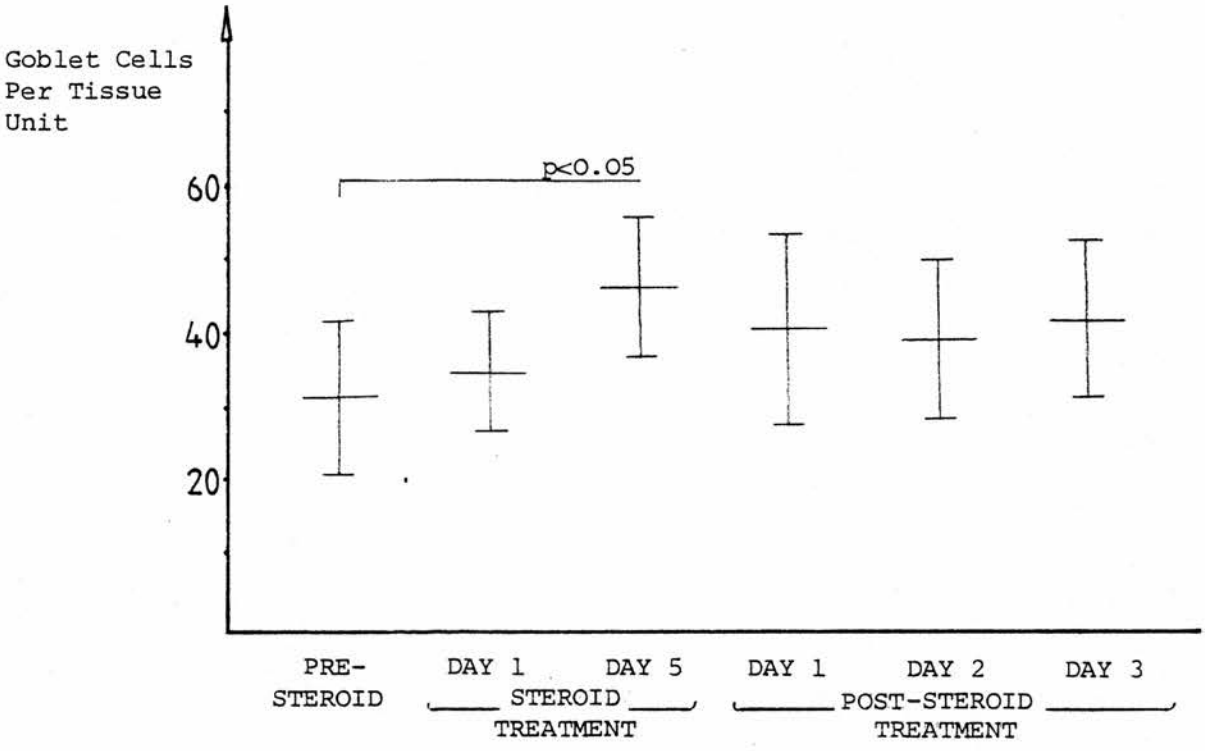


Plate 8.6: Trophozoite and cells in the gut lumen of a heterozygote mouse after 10 days infection (x3,700).

Figure 8.2



G. muris Infection and Steroid Treatment: Influence upon Goblet Cell Numbers

Influence of daily 2.0mg hydrocortisone sodium succinate injections (7 days treatment) and subsequent cessation, upon goblet cell numbers per tissue unit grid area at x400, in previously infected, immune adult female BALB/c mice. Gut fixed unopened. Marks represent means ± lSD (six mice per group).

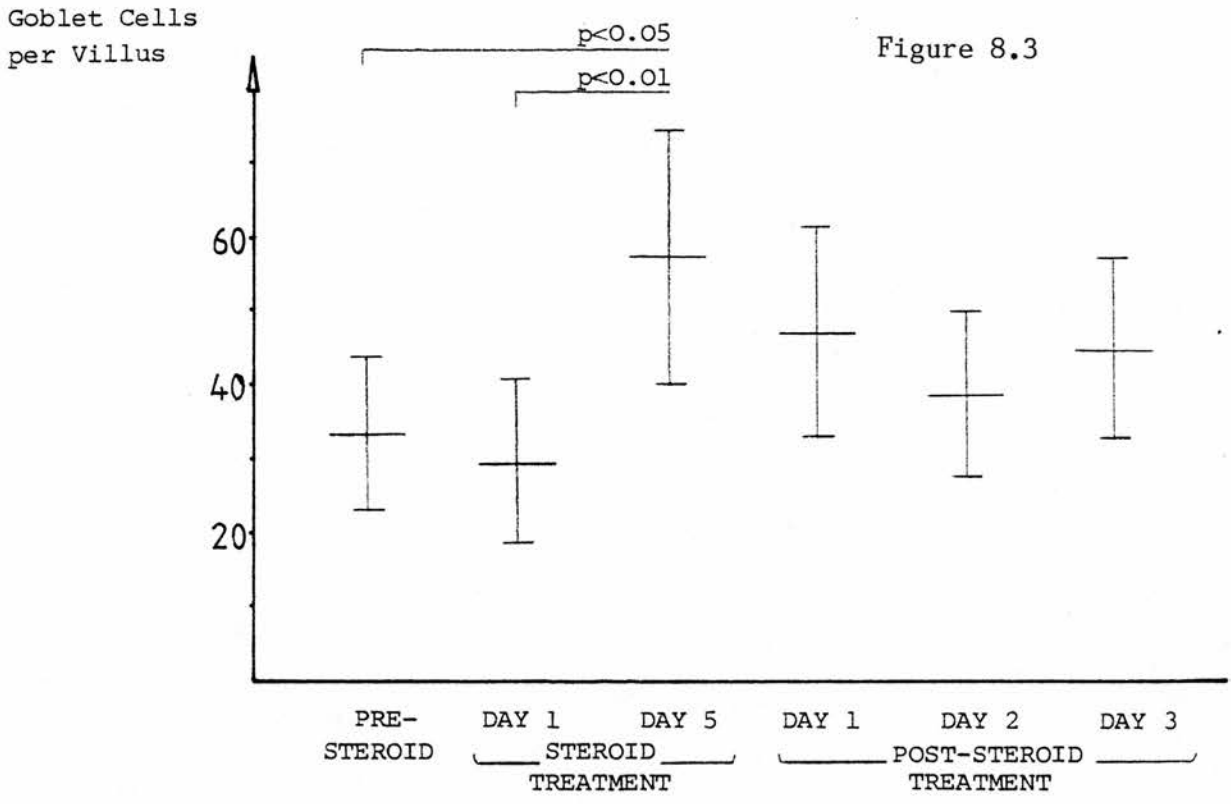
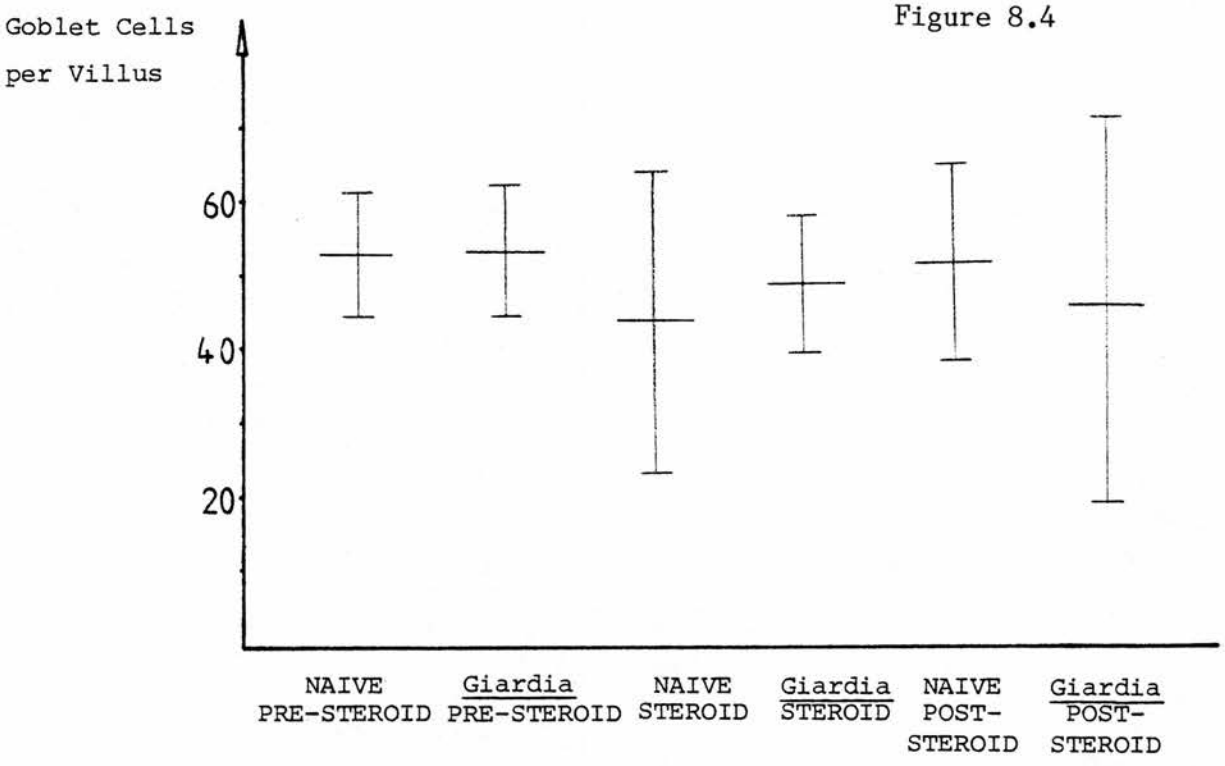


Figure 8.3

G. muris Infection and Steroid Treatment: Influence upon Goblet Cell Numbers

Influence of daily 2.0mg hydrocortisone sodium succinate injections (7 days treatment) and subsequent cessation, upon goblet cell numbers per villus, in previously infected, immune adult female BALB/c mice. Gut fixed unopened. Marks represent means $\pm$ 1 SD (six mice per group).

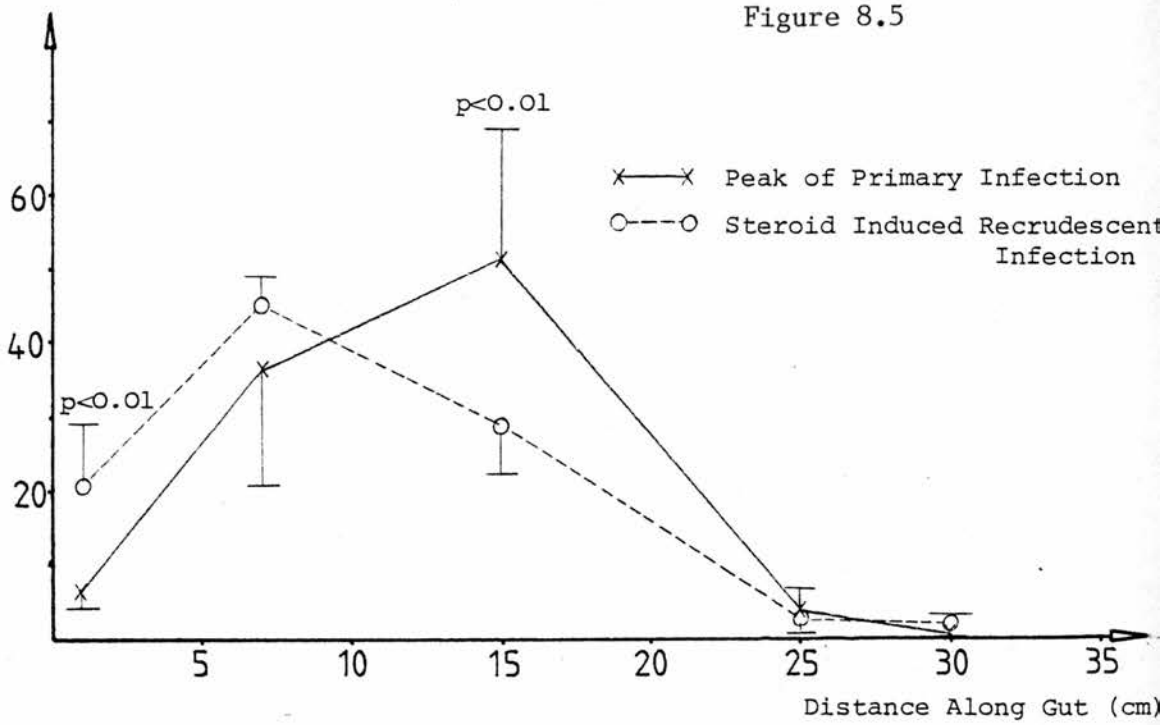
Figure 8.4



G. muris Infection and Steroid Treatment: Influence upon Goblet Cell Numbers

Influence of daily 2.0mg hydrocortisone sodium succinate injections (7 days treatment) upon goblet cell numbers per villus in previously infected, immun adult female BALB/c mice and age matched naive controls. Gut opened and fixed flat on card. Marks represent means ± 1 Sd (six mice per group).

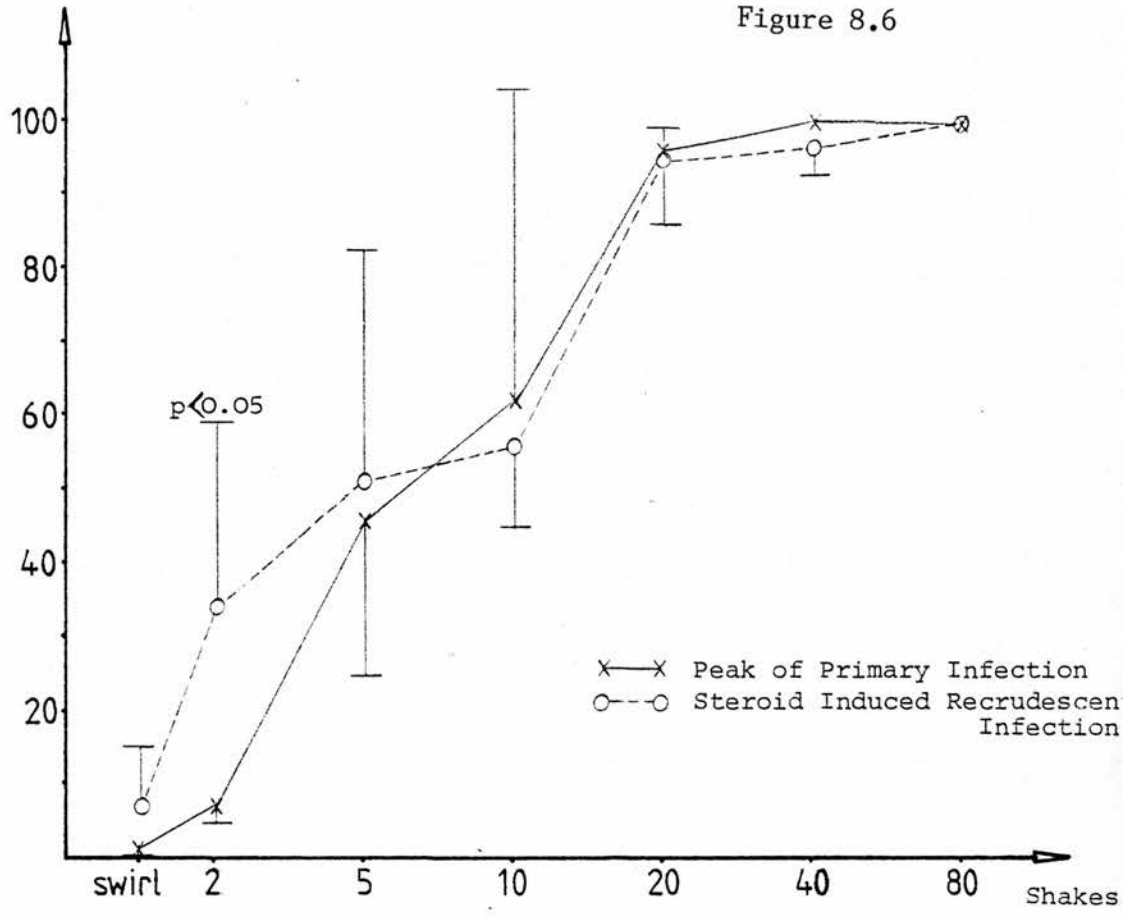
Figure 8.5



G. muris Trophozoite Distribution in Primary and Steroid Induced Recrudescent Infections

Comparison of trophozoite distribution in the small intestine of adult female BALB/c mice. Primary infection sampled on day 14 after infection; steroid induced recrudescent infection sampled 24 hours after the last of five daily s.c. 2.0mg hydrocortisone sodium succinate injections. Marks represent means ± 1 SD of the precentage of the total number of trophozoites isolated at each sample point, for each mouse (six mice per group).

Figure 8.6



G. muris Primary and Steroid Induced Recrudescent Infections: Ease of Trophozoite Displacement

Ease of displacement of trophozoites from an opened section of gut (5 to 20cm distal to pylorus) from mice at peak of primary infection and from mice 24 hours after the last of 5 daily s.c. 2.0mg hydrocortisone sodium succinate injections. Marks represent means ± 1 SD of percentage of total for each mouse (six mice per group).

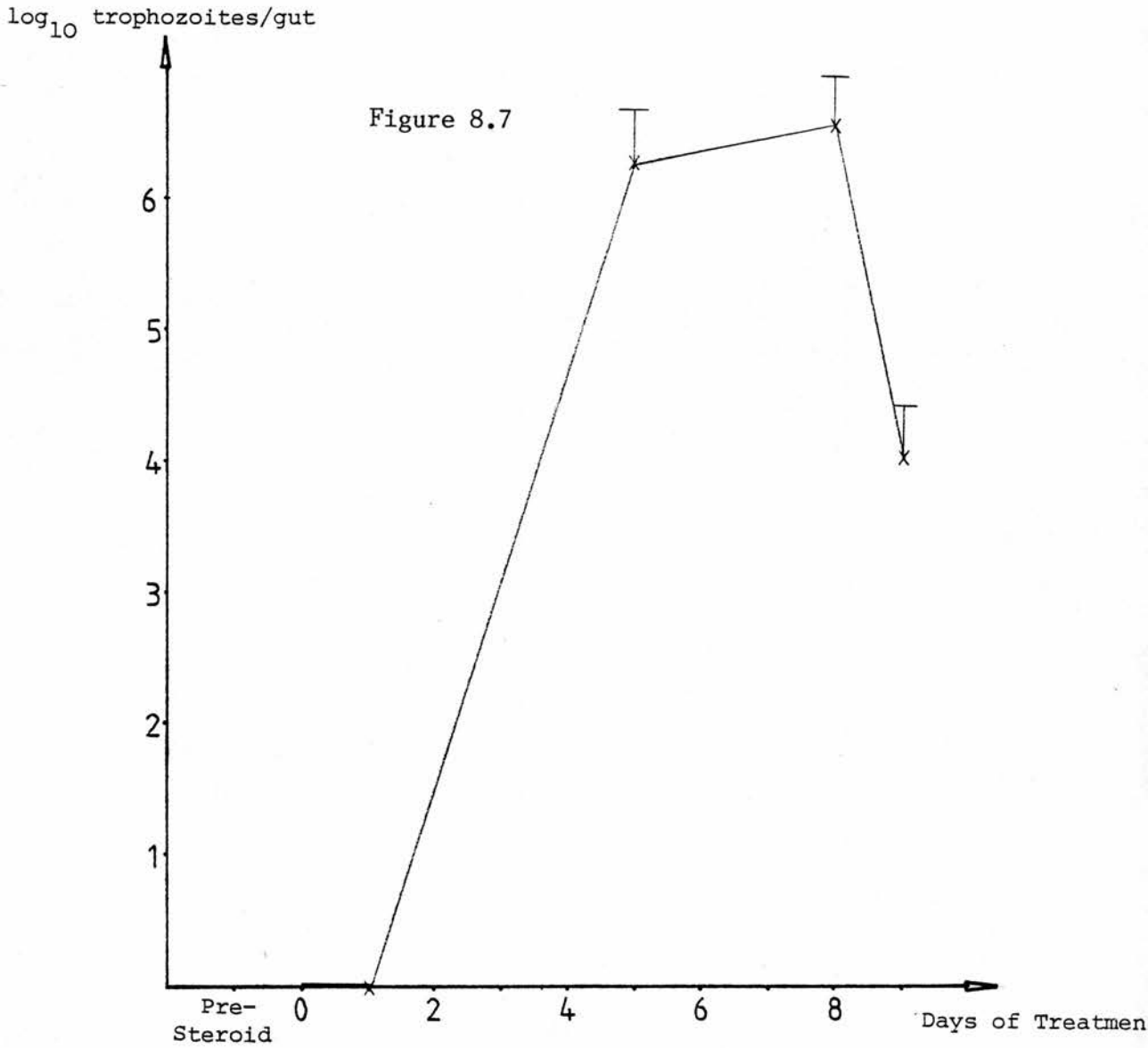


Figure 8.7

Steroid Induced Recrudescence of G. muris Infection

Time course of trophozoite numbers during development of steroid induced recrudescent infection. Hydrocortisone sodium succinate (2.0mg) treatment by 8 daily s.c. injections. Marks represent means ± 1 SD of number of trophozoites per mouse small intestine (six mice per group).

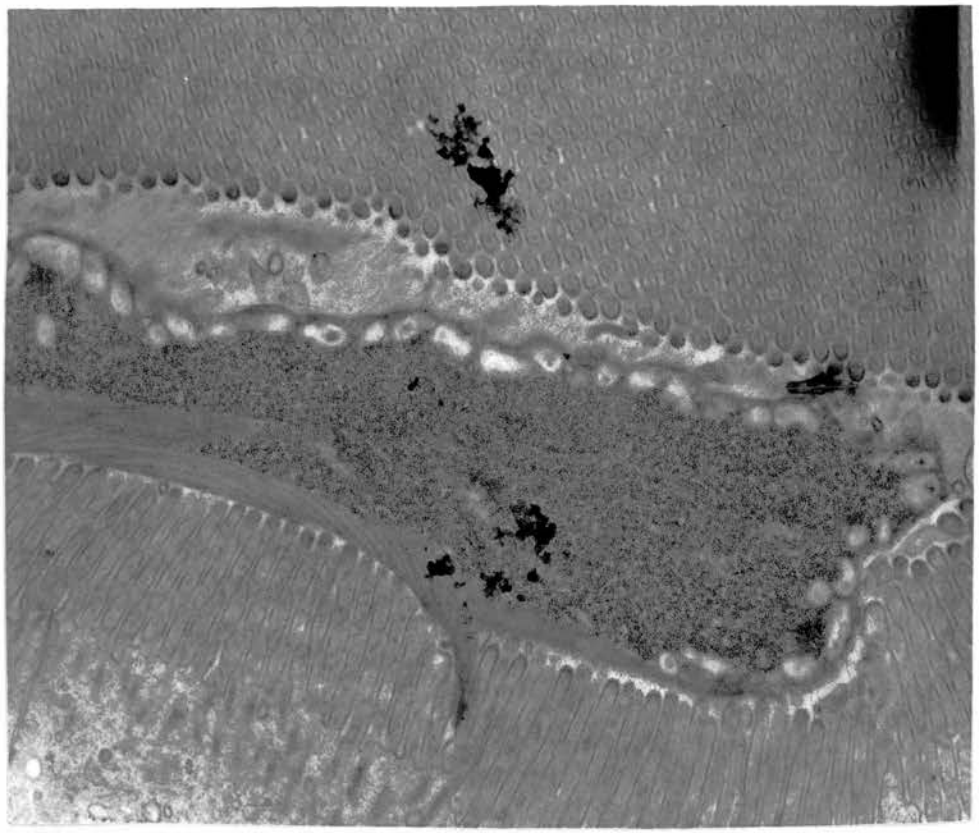


Plate 8.7: Trophozoite in the gut lumen of a BALB/c mouse, during a normal primary infection. The ventral disc is forced between the enterocyte microvilli (x11,000).

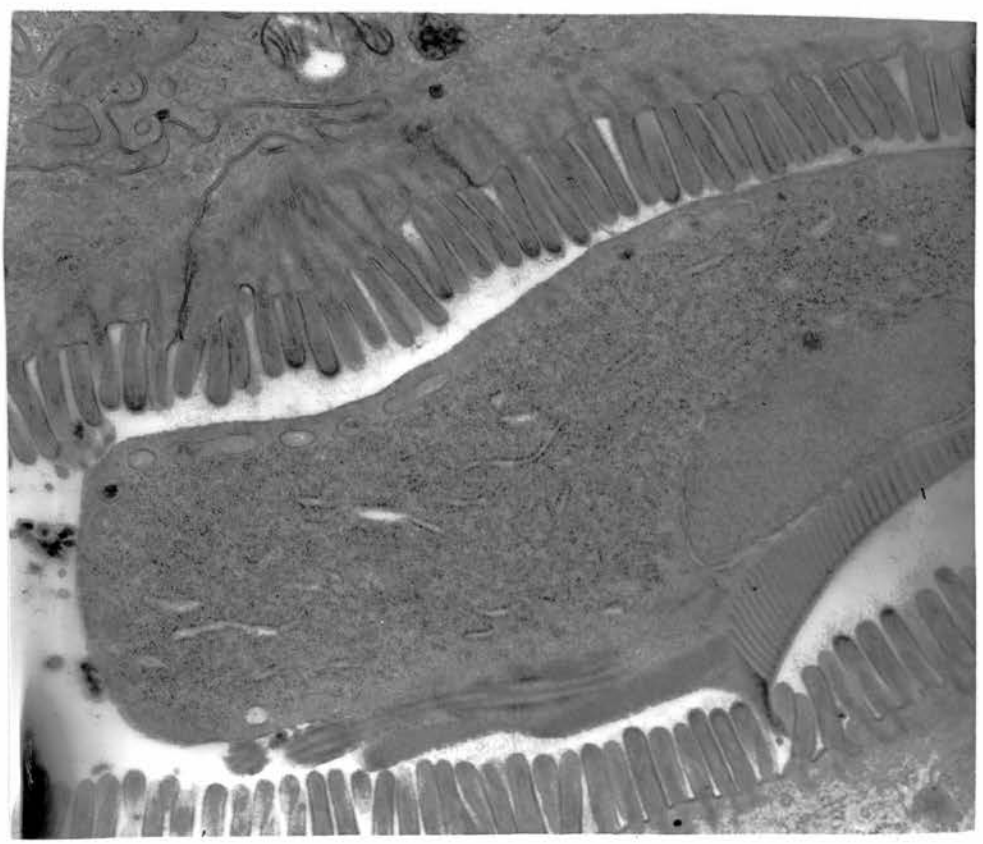


Plate 8.8: Steroid induced recrudescent trophozoite with increased vertical thickness, increased RER and extensive tubule/vacuole network throughout body (x11,000).

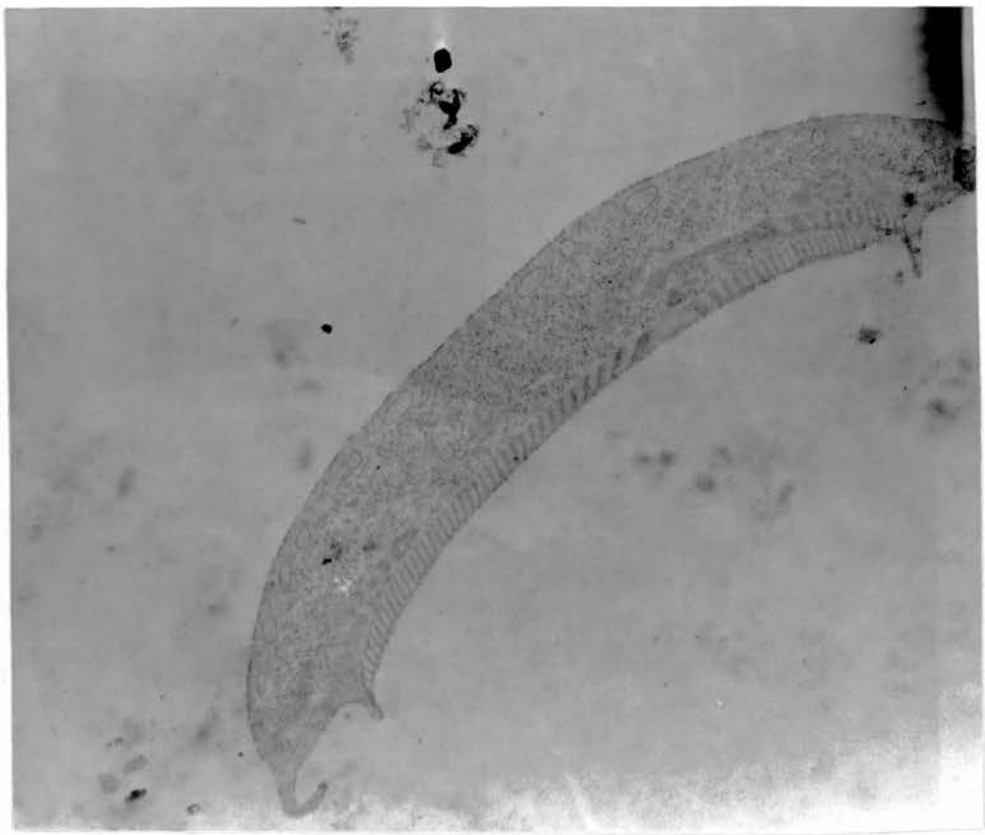


Plate 8.9: Transverse vertical section of trophozoite from a normal primary infection (x7,000).

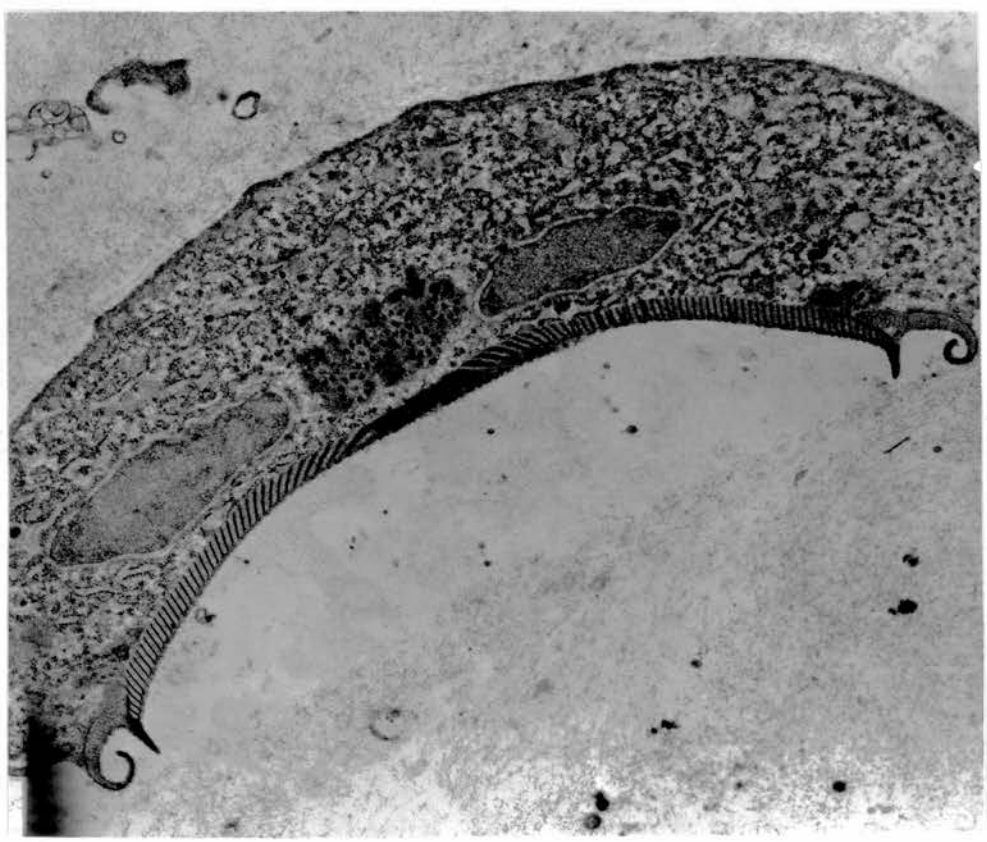


Plate 8.10: Transverse vertical section of trophozoite from a steroid induced recrudescent infection. Shows increased RER and extensive tubule network (x10,000).

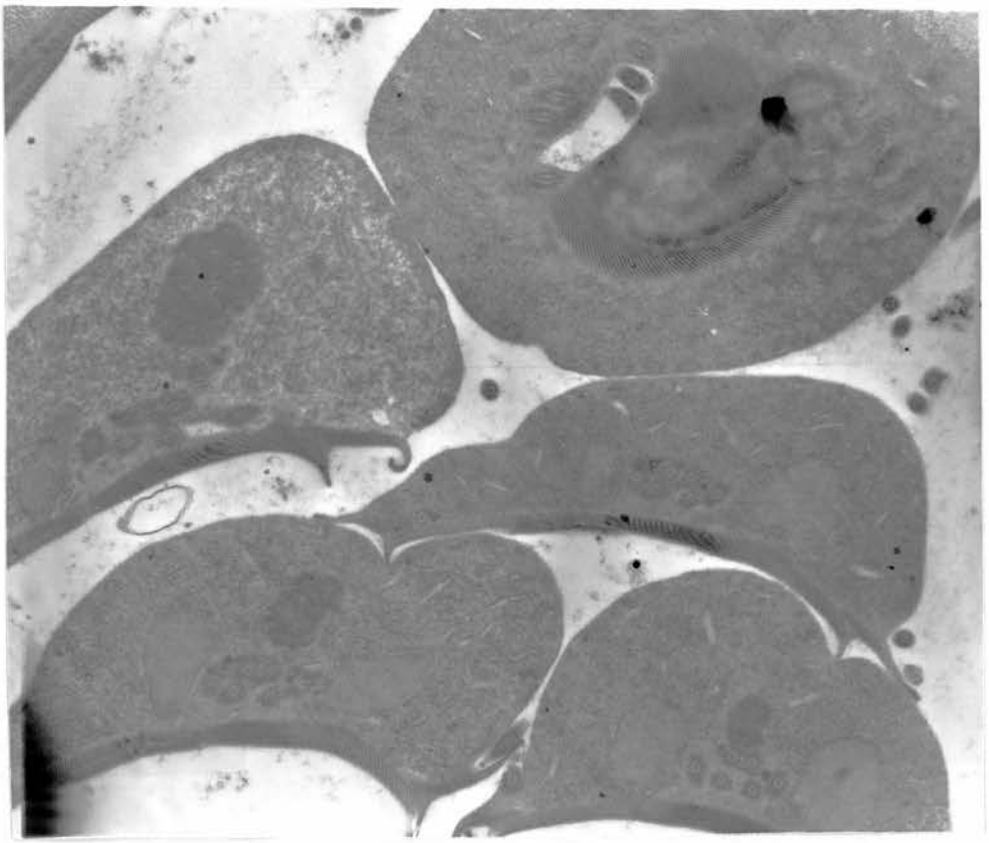


Plate 8.11: Trophozoites from a steroid induced recrudescent infection showing increased thickness, RER and tubule network (x4,000).

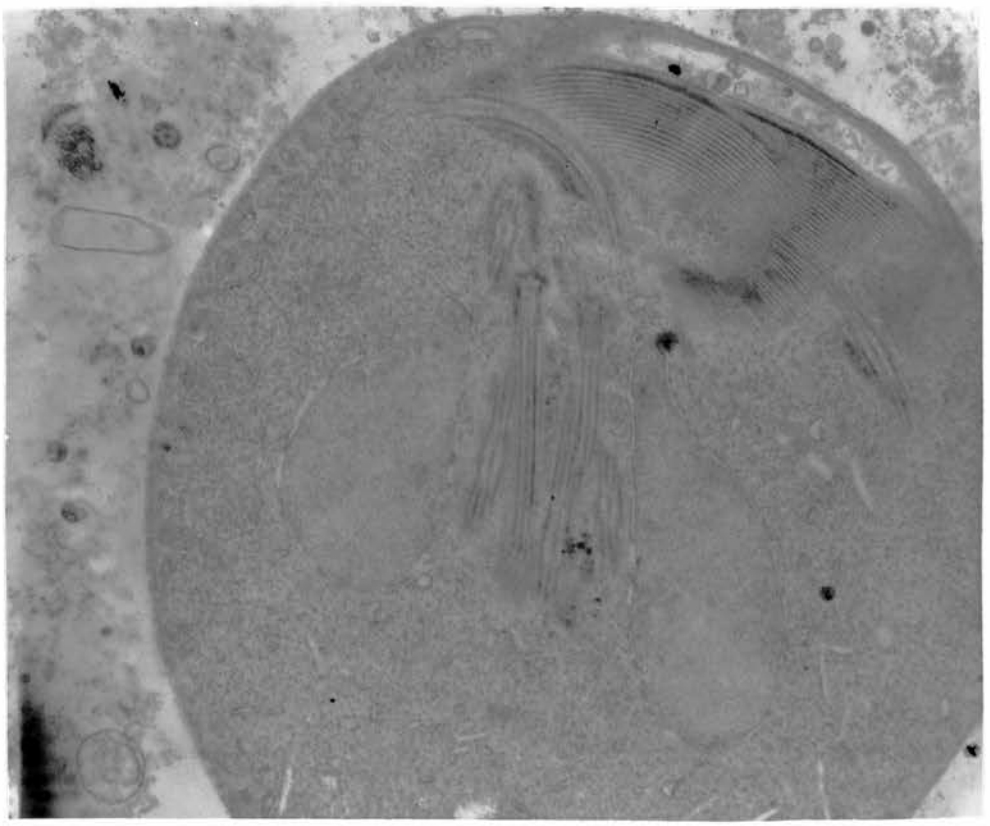


Plate 8.12: Lateral section of trophozoite showing nuclei, peripheral vacuoles and part of ventral disc microribbon structure (x7,000).

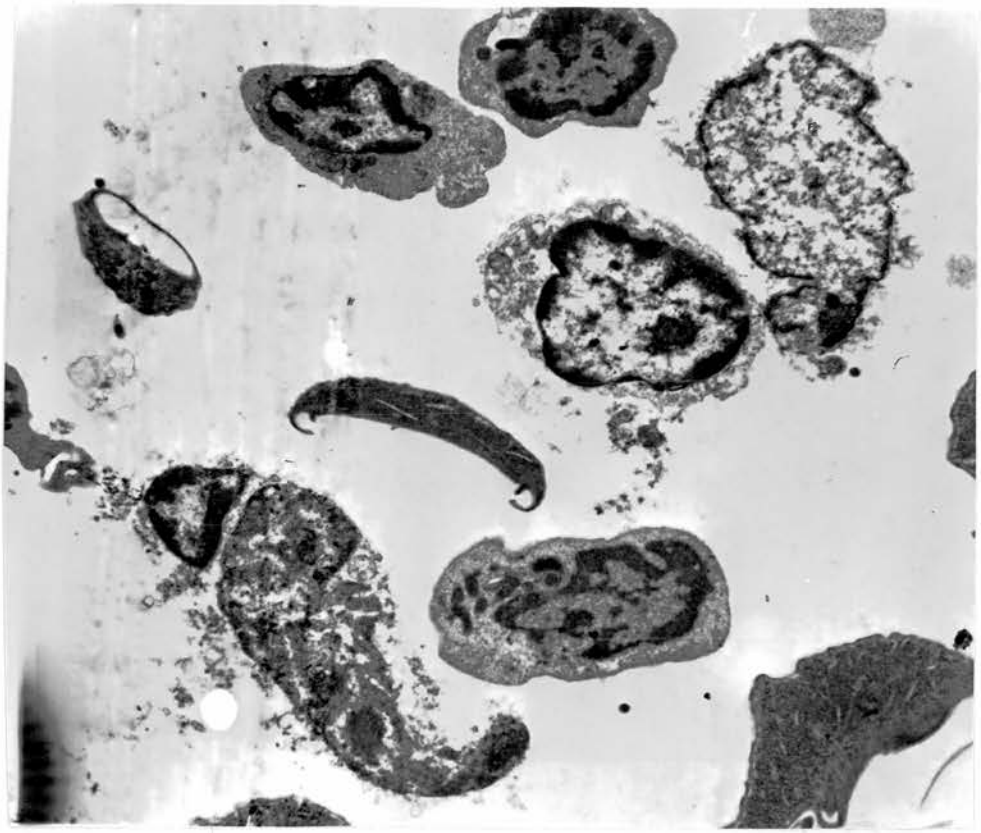


Plate 8.13: Trophozoites and cells in the gut lumen at the time of parasite elimination following withdrawal of steroid treatment (x3,000).

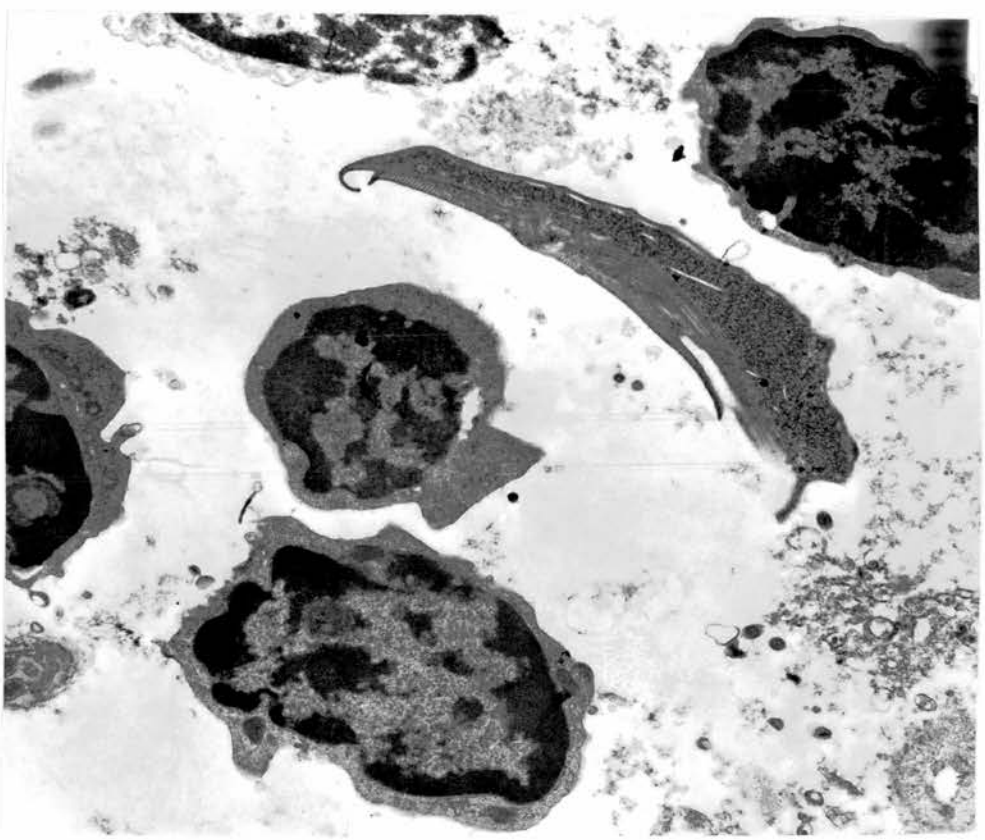


Plate 8.14: Trophozoites and cells in the gut lumen at the time of parasite elimination following withdrawal of steroid treatment (x5,000).

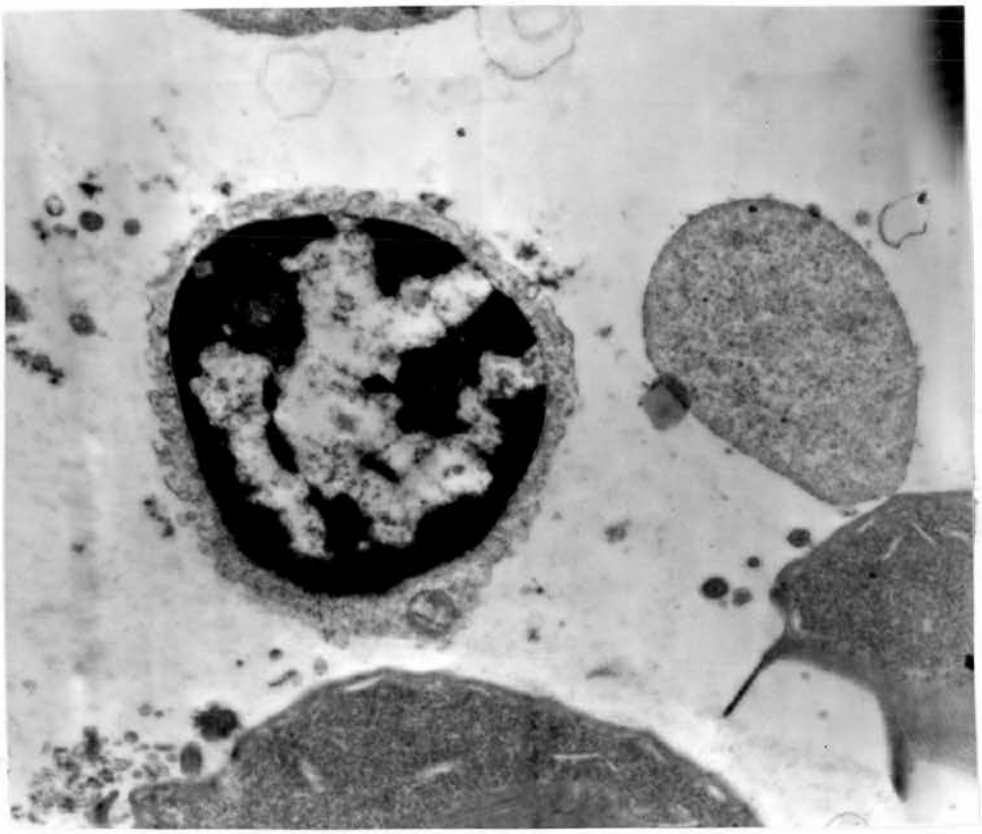


Plate 8.15: Detail of a lymphocyte in the lumen of the gut during parasite elimination following withdrawal of steroid treatment (x15,000).

Chapter 9

Graft Versus Host Reaction and *Giardia muris* Infection

Introduction.

The aim of following the G. muris infection time course in mice with concurrent graft versus host reaction (GvHR) was to test the hypothesis that the "innocent bystander" effect seen in the latter would influence the parasite infection. The innocent bystander effect was a suggested reason for the premature non-specific expulsion of G. muris during the expulsion phase of T. spiralis (Roberts-Thomson et al 1976a).

The use of the GvHR would investigate this theory from a different approach.

The GvHR model used has not been mentioned previously in this thesis, so a description is appropriate. This GvHR model results in a syndrome of pathological alterations which develop following transfer of parental strain lymphocytes to F1 hybrid recipients which are genetically tolerant to parental strain cells, but the grafted parental T lymphocytes react against the allogeneic histocompatibility antigens of the F1 host. This reaction was first described by Barnes & Loutit (1954) in a model involving unsuccessful attempts to reconstitute lethally irradiated mice with allogeneic spleen cells.

The reaction (GvHR) and disease (GvHD) are due to alloreactive T cells in the transferred cell population (Cantor & Asofsky 1972, Kerckhaert et al 1973). In non-irradiated F1 recipients, GvHD may be broadly divided into two forms: the first shows stimulatory pathological symptoms, including persistent lymphoid hyperplasia, mainly of B lymphocytes, which leads to hypergammaglobulinaemia and the production of autoantibodies. The second form of GvHD is characterized by suppressive pathological symptoms. It is this first stimulatory GvHR that was generated and studied in the following experiments.

The small intestine is an important target organ and diarrhoea and wasting often result in GvHR. The small intestinal architecture is altered, showing villus atrophy and crypt hyperplasia (Reilly & Kirsner 1965, Elson et al 1977). An infiltration of lymphocytes, mast cells and other mononuclear cells into the mucosa has also been described (Guy-Grand et al 1978, Mowat & Ferguson 1981). An

expansion of cytotoxic cell numbers (Cerottini et al 1977, Singh et al 1972) and increased cell mediated cytotoxicity during GvHR has been invoked as the possible effector of the tissue damage observed. An "Innocent Bystander" phenomenon has been described, whereby tissues, syngeneic with the alloreactive injected cells, such as skin (Billingham & Streilen 1968, Streilen 1972) and kidney (Elkins & Guttman 1968), are also damaged. Elson et al (1977) described a model involving innocent bystander tissue damage of foetal small intestinal grafts. Production of an enteropathic lymphokine has been suggested as a mediator of tissue damage (Elson et al 1977, MacDonald & Ferguson 1977, Mowat & Ferguson 1981). Local CMI responses in the small intestine may act both as effectors of immunity against pathogenic organisms and also act as the mediators of intestinal damage (Ferguson & Parrot 1973, Mowat & Ferguson 1981).

Stimulation of the crypt cell production rate (CCPR) is the earliest noted response of the mucosa during GvHR (MacDonald & Ferguson 1977). This is also associated with a rise in the numbers of intraepithelial lymphocytes (IEL) (MacDonald & Ferguson 1976, 1977).

Experimental Design.

Female (CBA x BALB/c) F1 adult mice were injected with 8x10 CBA spleen cells in 0.5 ml RPMI 1640, i.p. to elicit the GvHR. Control animals received 0.2 ml RPMI 1640 i.p. alone, at the same time. Giardia muris infection was initiated by i.g. intubation of 1,500 cysts in 0.2 ml water. Each experiment was designed to optimise the effects of the developing GvHR upon the G. muris infection, and a short protocol explanation precedes each experiment.

Experiment 1.

Early GvHR and Trophozoite Numbers During Infection.

Materials and Methods.

This experiment was designed after consideration of neonatal GvHR work carried out in this lab. Maximum damage of the gut was

assumed to occur approximately one week after GvHR induction. The logarithmic growth phase in G. muris trophozoite numbers occurs until day 4 or 5 after infection. This was planned to coincide with the time of maximum change to the gut during the GvHR. The GvHR was initiated in F1 mice on day 0. On day 4 the mice were infected with 1500 cysts i.g.

Mice were weighed at sacrifice, the Spleen Index calculated, and pieces of jejunum were taken for routine H & E histology and microdissection.

Trophozoite counts were chosen as the assay of infection because the normally predictable faecal cyst output was becoming unreliable at this time. The course of infection, when followed by trophozoite numbers, was shown to be unaltered compared to previous infections when the normal cyst output was found. Shortly after this time no faecal cysts could be detected and infections were initiated by i.g. passage of trophozoites. A period of absence of faecal cysts in the G. muris model has occurred three times in this laboratory and has also been found by other workers. The infection was maintained for several months by using the trophozoites as the infective agent. After this time, the cysts spontaneously reappeared. There was no change in the animal housing conditions, handling or food that would account for the change in cyst output. The hypothesis that the G. muris model had specifically adapted to the BALB/c host and was not therefore being expelled normally, thus leading to a lack of encystment, was tested by infection of other mouse strains. These infections did not result in faecal cyst output. The reason for this change in the parasites life cycle remains unknown.

Results.

Evidence of GvHR.

The Spleen Index showed a significant splenomegaly of 1.35 in both GvHR groups, at day 11. There was a significant increase in the mean number of IEL from a control of 18.1 to 23.1 in the GvHR group (Fig. 9.1).

The numbers of IEL in the G. muris infected group also

significantly rose above control levels, to 22.2.

Trophozoite Numbers.

The number of trophozoites of G. muris isolated from the intestines of animals at sacrifice were not significantly different in control infections ($1.0 \pm 0.7 \times 10^6$) compared to infections with concurrent GvHR ($1.6 \pm 0.7 \times 10^6$).

Mucosal Architecture (Fig. 9.2)

Microdissection and measurement of the mucosal architecture and cell kinetics revealed no significant alteration in villus height in any group. Crypt depth significantly increased in the group with GvHR and concurrent G. muris infection (146.3 μm) when compared to control (118.8 μm), GvHR alone (128.0 μm) and G. muris infection alone (132.3 μm). The CCPR, although higher in groups with G. muris infections than in controls, were not significantly altered in any group.

Experiment 2

Early GvHR and Faecal Cyst Output During Infection.

Introduction.

This experiment was designed to repeat the previous experiment at a time when G. muris cysts could be reliably detected in faeces, and so assess any influence of the GvHR upon the G. muris infection time course.

Materials and Methods.

Both GvHR induction and G. muris infection were initiated on the same day, using (CBA x BALB/c) F1 adult female mice and protocols exactly as previously descibed.

Results.

A Spleen Index of 1.44 was achieved in the GvHR and concurrent G. muris infection group with respect to the G. muris infection alone group.

The time course as described by the faecal cyst output (Fig.

9.3), shows a lower level of infection in the GvHR group, this being on the fifteenth day of both GvHR and G. muris infection.

Experiment 3.

Late GvHR and G. muris Infection.

Introduction.

The aim of this experiment was to investigate the time course of infection when peak trophozoite numbers coincided with late GvHR.

Materials and Methods.

The GvHR was initiated and infection carried out on day 19 of the GvHR; both procedures as previously described.

Earlier work suggested that the mucosal mast cell was involved in the GvHR and in the immune response to G. muris. To examine this further, an assay of MMC numbers was included in this experiment.

Results.

The Spleen Index of the GvHR group in this experiment was 1.92, indicating significant GvHR.

The time course of G. muris infection, as described by faecal cyst counts (Fig. 9.4) showed a significantly increased faecal cyst output in the GvHR animals at 12 days of infection.

Day 16 of infection (day 35 of GvHR) shows a highly significant fall in faecal cyst output in the control group, whereas the GvHR group infection remained at the plateau phase of the infection. The experiment was terminated at this time to take specimens for mucosal mast cell counts.

Mucosal Mast Cell (MMC) Study.

Materials and Methods.

The Chloroacetate Esterase (CAE) stain was used to detect MMC. This stain was chosen in preferance to the Astra Blue/Safranin (AB/S) stain at pH 0.3, as the latter gave very poor positive staining on mouse tissue.

Results. (Fig. 9.5).

The results show a significant increase in MMC numbers on day 6 of G. muris infection. No significant increase in MMC was seen on day 25 of GvHR. Infection at day 6 and concurrent GvHR at day 25 show a significant increase in MMC. G. muris infection alone at day 16, and GvHR alone at day 35, show no significant increase, but GvHR and concurrent infection resulted in a significant increase in MMC.

Summary and Conclusions.

These experiments were designed to examine the possibility of an ongoing GvHR influencing the time course of G. muris infection due to cytotoxic events occurring during the former. The experiments were essentially preliminary studies to determine whether any change of the infection characteristics could be detected and be attributed to the concurrence of the conditions. The design was therefore based upon previous work by myself and colleagues, and both experimental conditions were altered to favour the known characteristics of each model. This allowed rapid screening of various possible conditions as a compromise to what would have been a very large study if all combinations of each experimental time course were fully investigated.

The GvHR was successfully produced in each experiment and concurrent G. muris infection time courses compared to control infections of untreated F1 mice. The control infections showed faecal cyst counts and time courses similar to the established BALB/c model.

The trophozoite and cyst counts showed that early GvHR has no influence on the G. muris infection and there is no evidence of host protection by the innocent bystander effect.

The increased cyst count in the late GvHR experiment suggest a degree of immunodeficiency during the late phase of GvHR.

The additive effect of GvHR and infection on IEL and MMC numbers showed that these cell types are not directly nor non-specifically protective against the G. muris infection. These changes, along with the similar additive effect of the two conditions on increased crypt length, are fully discussed in the final chapter.

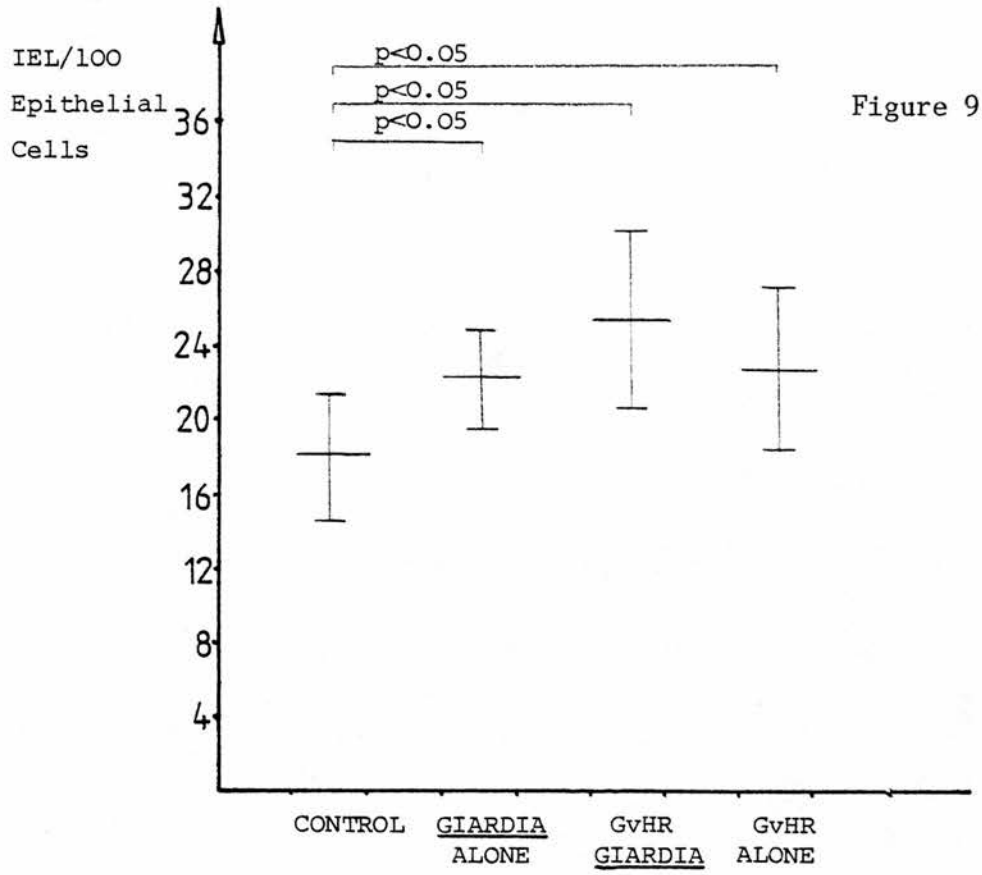
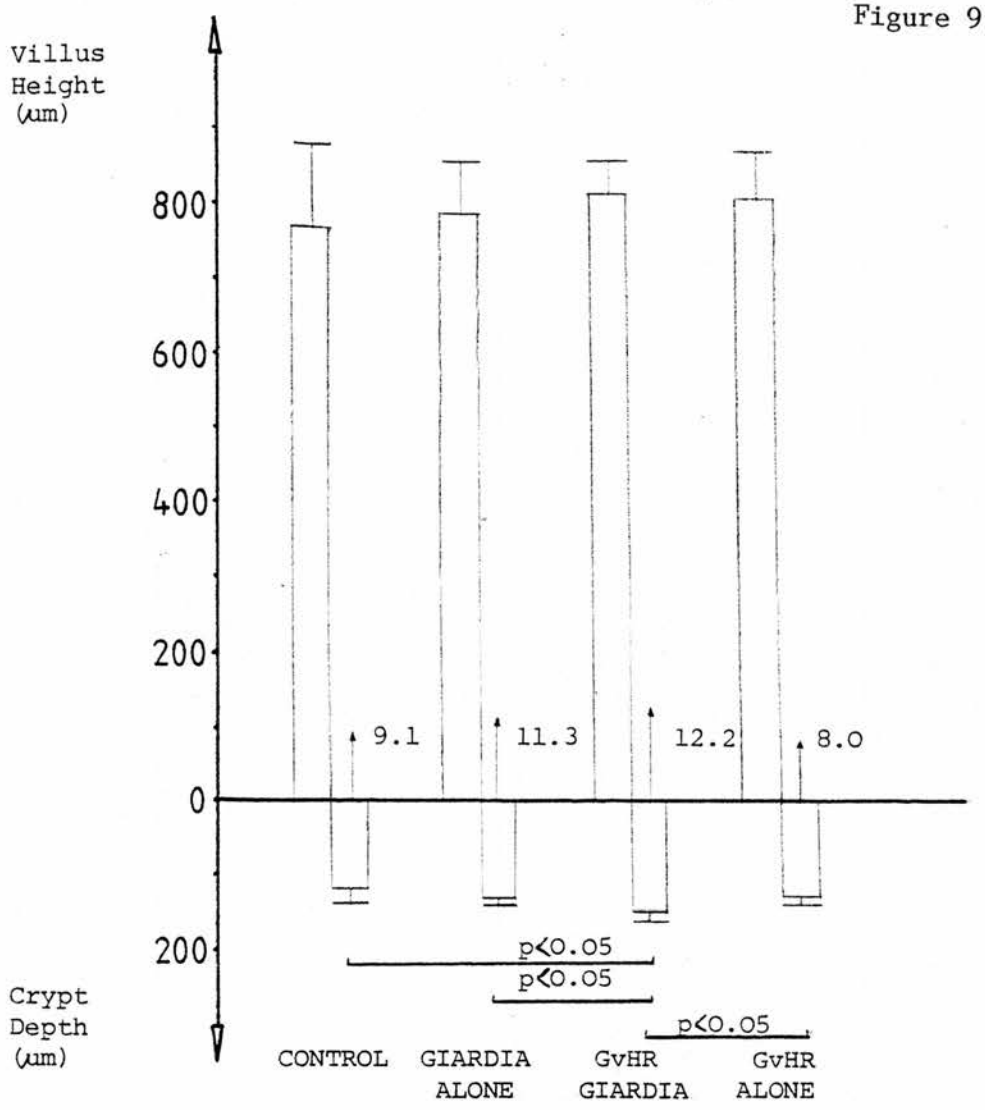


Figure 9.1

Control versus GvHR, Giardia muris infection, and Concurrent GvHR and G. muris infection.

Effect of the above treatments on Intraepithelial lymphocyte (IEL) counts. GvHR initiated 11 days previously by transfer of 8×10^7 parental Spleen cells to adult (CBA x BALB/c) F_1 mice. G. muris infection initiated 7 days previously by 1,500 cysts in 0.2ml, ig. Results are of mean IEL count $\pm$ 1 SD (six mice per group).

Figure 9.2



Control versus Graft versus Host Reaction (GvHR), Giardia muris Infection and Concurrent GvHR with G. muris Infection

Effect on jejunal mucosa of GvHR 11 days after initiation, or G.muris after 7 days of infection, or both treatments being concurrent. Bars represent means ± 1 SD for villus height and crypt depth, and arrows show CCPR (six mice per group). GvHR initiated by transfer of 8×10^7 parental Spleen cells to adult (CBA x BALB/c) F_1 mice. G. muris infection initiated by 1,500 cysts in 0.2ml water, ig.

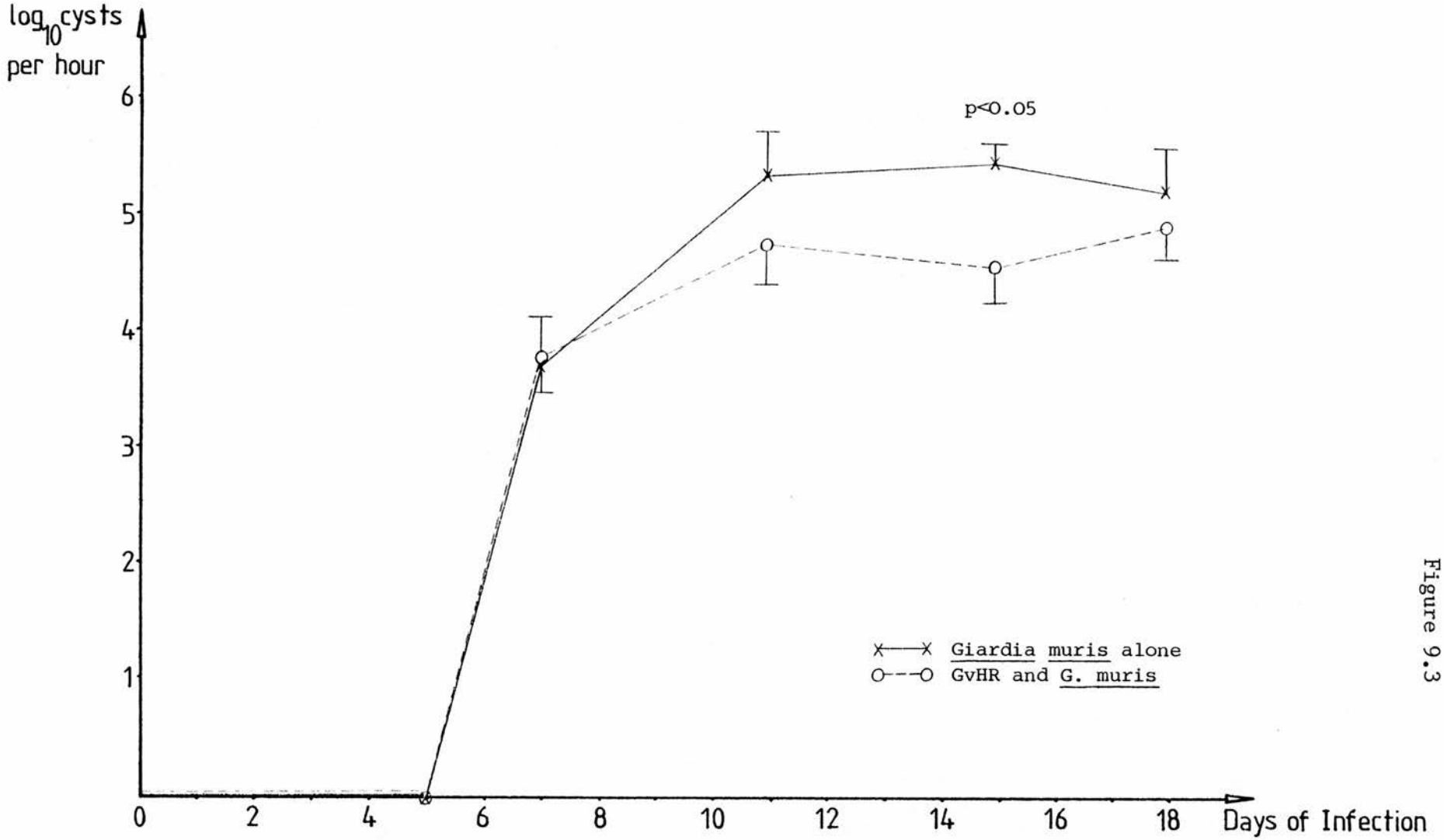


Figure 9.3

Progress of Giardia muris infection in (CBA x BALB/c) F_1 adult mice, with and without GvHR induced on day 0, by injection of 8×10^7 parental Spleen cells, ip. Marks represent means $\pm$ 1 SD of number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

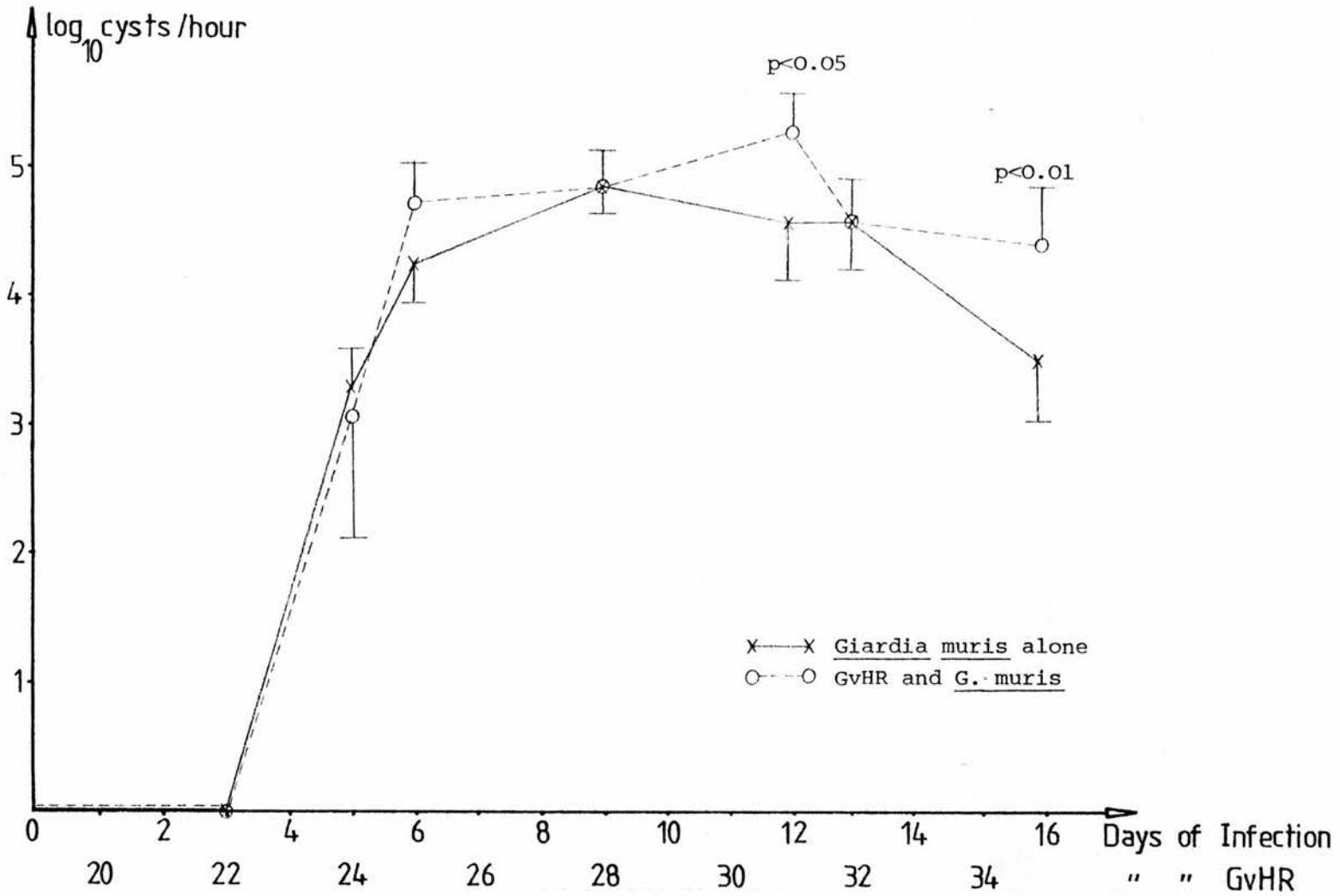


Figure 9.4

Progress of Giardia muris infection in (CBA x BALB/c) F_1 adult mice, with and without GvHR induced on day -19 compared to the day of infection by 1,500 cysts of G. muris in 0.2ml, ig. GvHR induction was by 8×10^7 parental Spleen cells, ip. Marks represent means $\pm$ 1 SD of the number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

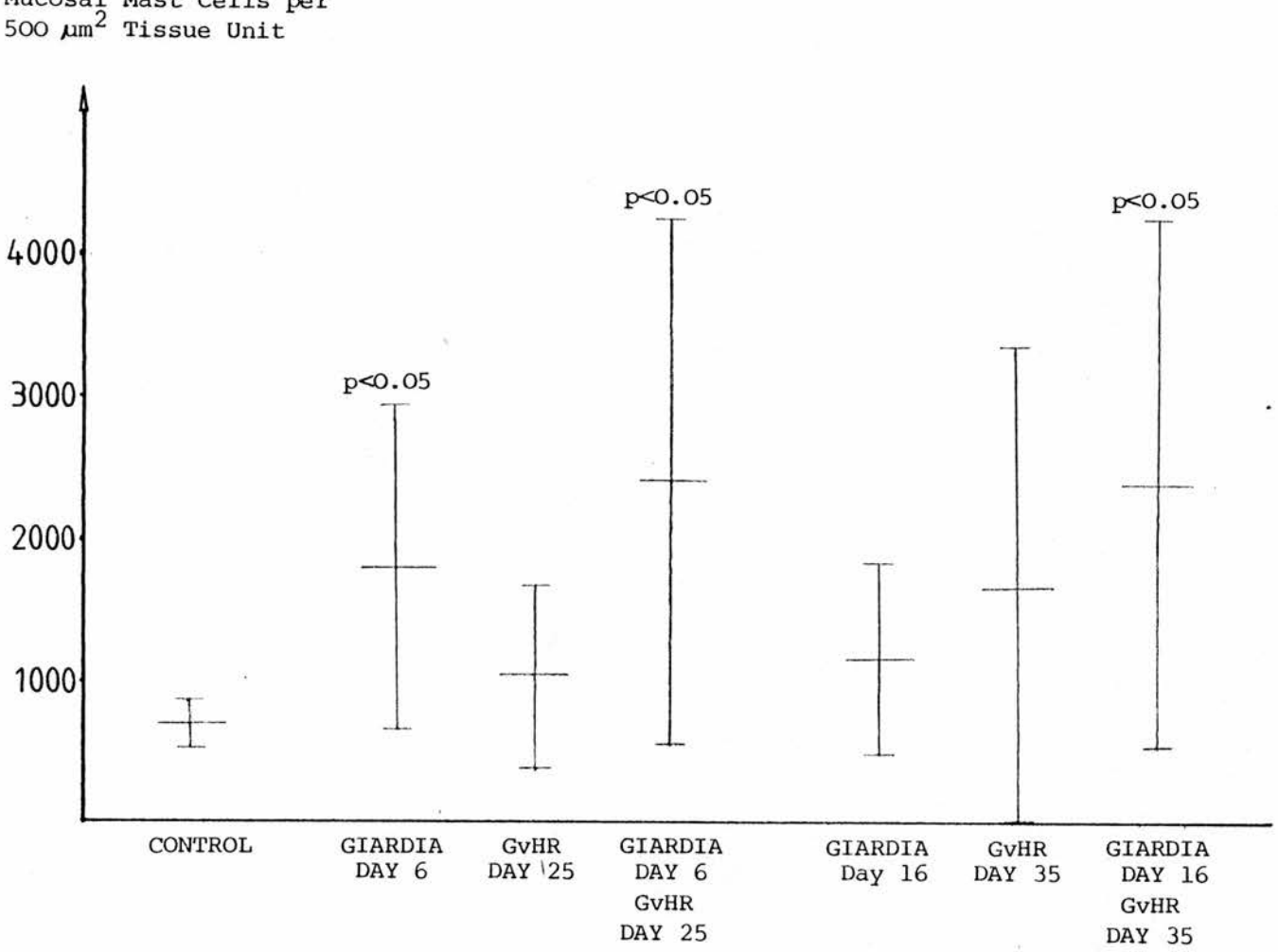


Figure 9.5

Control versus GvHR, Giardia muris infection, and concurrent GvHR and G. muris infection

Effect of the above treatments on Mucosal Mast Cell numbers as detected by Chloroacetate Esterase positive staining at the noted times after initiation of GvHR or G. muris infection. Results are of mean MMC counts ± 1 SD, expressed per 500 μm^2 Tissue Unit (six mice per group).

Chapter 10

Cell Transfer Studies

Introduction.

The aim of this experiment was to transfer immunity to G. muris to naive adult female BALB/c mice with spleen and mesenteric lymph node (MLN) cells from immune donors.

G. muris infection of adult female BALB/c mice results in an active immune mediated clearance of the parasite and development of easily demonstrated immunity to subsequent challenge.

Roberts-Thomson & Mitchell (1978) have transfered this immunity by transfer of 10^8 spleen and MLN cells to naive nude (athymic) mice from naive or immune heterozygote donors. This resulted in a more rapid resolution of infection in nude mice receiving immune cells, compared to naive cells. This demonstrated the thymus dependency of an efficient immune response against G. muris. An increase in villus damage was also found in the nude mice which received immune cells prior to infection. This showed that the thymus dependent immune mediated tissue damage resulted in villus atrophy. Transfer of a single large volume (0.4 ml) of serum had no influence on the immunity that developed following transfer of cells. It was therefore of interest to repeat this work in an immunologocally intact, rather than athymic nude mouse model.

Immunization of Donor Mice.

Materials and Methods.

Adult female BALB/c mice were infected with 2,000 cysts i.g. The infection was seen to develop and resolve as expected. The mice were reinfected six weeks after the first infection was started, again with 2,000 cysts i.g. The expected secondary response was seen by following faecal cyst output and the mice were rested for one week after the last faecal cysts were detected.

Cell Transfer Preparations

Donors were sacrificed and their spleens and MLN removed. Single cell suspensions were made of spleen and MLN, as previously described.

Each cell suspension was then injected i.p. into recipient mouse groups, with a control group receiving cell free RPMI 1640

inoculation. All groups were rested for one week before G. muris infection was initiated by i.g. intubation of 2,000 cysts. Subsequent infection time courses were followed by faecal cyst counts.

Results

The transfer of 14.3×10^6 spleen cells from immune mice did not affect the prepatent period, rise in parasite numbers or peak parasite load, as assessed by faecal cyst output (Fig. 10.1). A highly significant decrease in cyst output was noted on day 20 in the spleen cell recipient group, but numbers were similar to the control primary infection at day 24 and no cysts were seen in either group on day 28, so this may be considered as a spurious result.

The 3.7×10^6 MLN cell recipients (Fig. 10.2) showed increased cyst output compared to controls on day 10, but the characteristics of the infection thereafter were indistinguishable from those of the control group.

Summary and Conclusions

Neither the spleen nor the MLN cell suspensions transferred any of the qualities expected of a secondary immune response limiting a challenge G. muris infection. As previously demonstrated, such a challenge infection shows a reduced period between initiation of infection and the detection of faecal cysts; also, a reduced peak cyst output, and an infection of significantly reduced duration are expected. The significant differences found were transient and not compatible with the transfer of this secondary immune response.

Because of these negative results in this immunologically intact mouse model, the overall aim of investigating the role of T cells in the immune response and the changes in gut histology during G. muris infection could not be pursued.

The nude mouse model of chronic giardiasis described by Roberts-Thomson and Mitchell (1978) was used to continue the investigation of the thymus dependent immune response to G. muris.

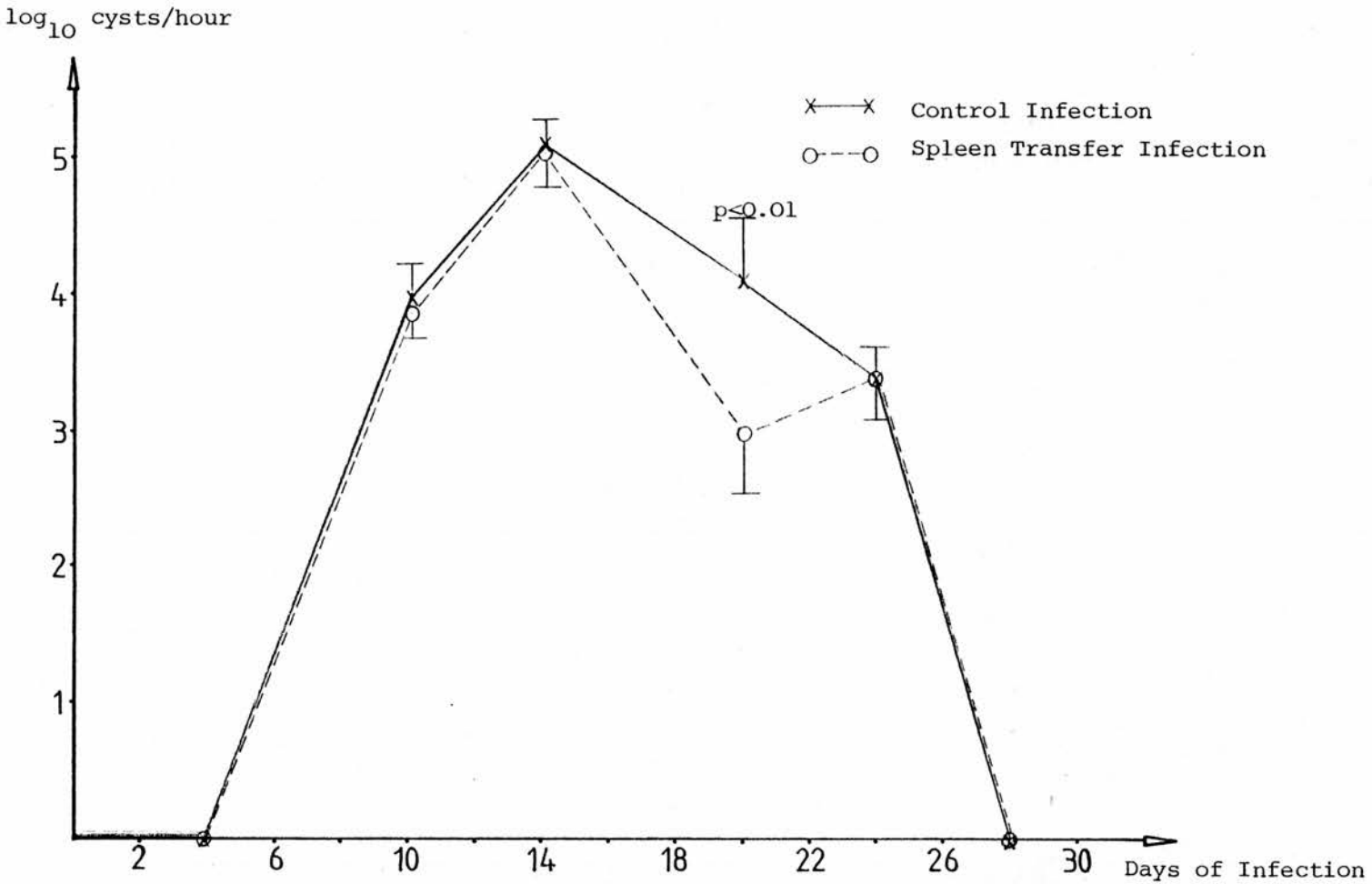


Figure 10.1

Progress of primary Giardia muris infections in adult female BALB/c mice. Infections initiated on day 0 with 2,000 cysts i.g. Control mice received 0.3ml RPMI 1640 i.p. on day 17; Spleen transfer mice received 1.43×10^7 spleen cells i.p. from G. muris immune adult female BALB/c donor mice, in 0.3ml RPMI 1640, on day -7. Marks represent means $\pm$ 1 SD of number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

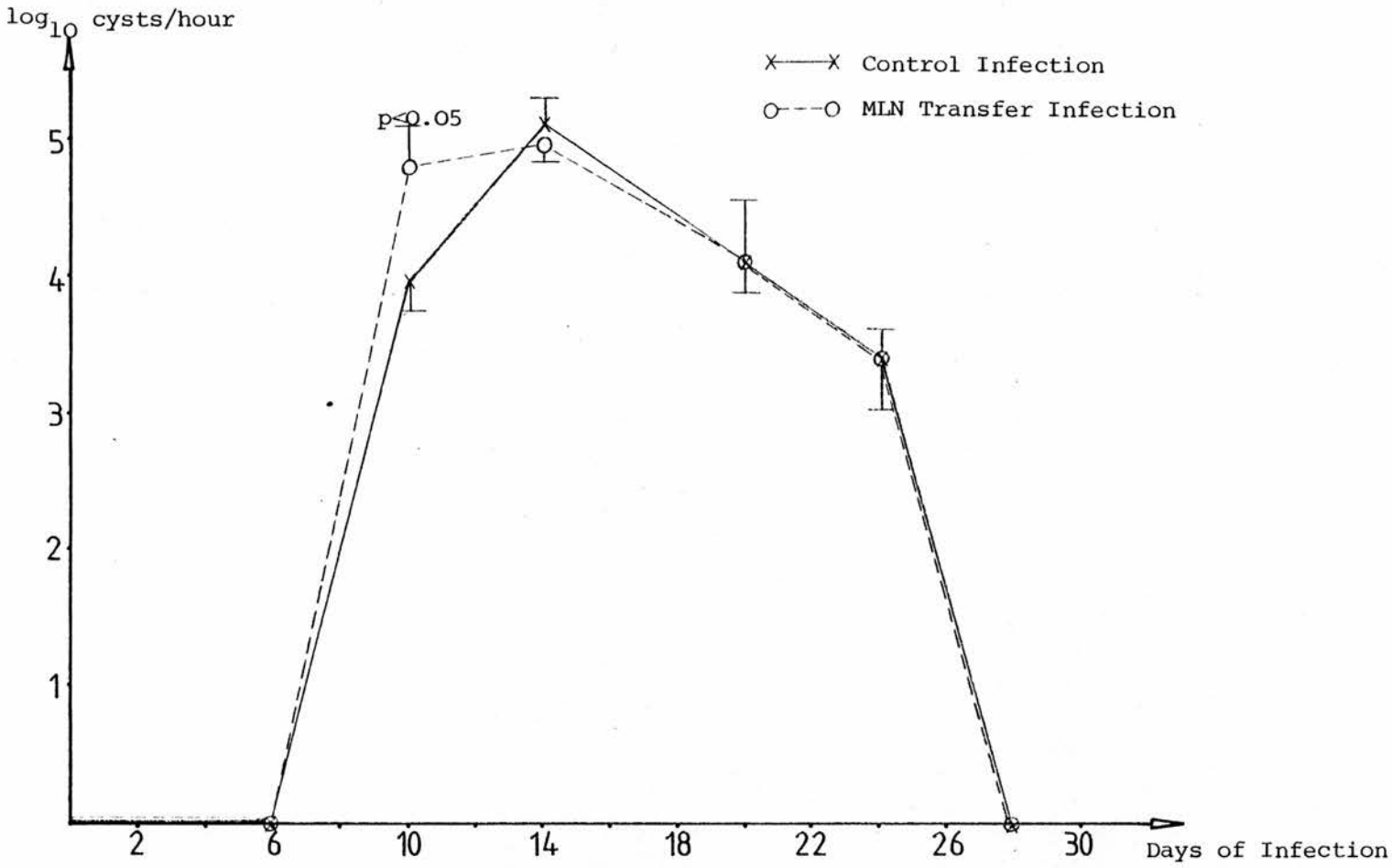


Figure 10.2

Progress of primary Giardia muris infections in adult female BALB/c mice. Infection initiated on day 0 with 2,000 cysts i.g. Control mice received 0.3ml RPMI 1640 i.p. on day -7; MLN transfer mice received 3.67×10^6 MLN cells in 0.3ml RPMI 1640 i.p., from G. muris immune adult female BALB/c donor mice, on day -7. Marks represent means $\pm$ 1 SD of number of cysts passed per hour, during a 2 hour faecal collection (Six mice per group).

Chapter 11

G. muris Infection of Nude (Athymic) Mice

Introduction.

The aim of this experiment was to compare the course of infection and resultant changes in gut morphology in the nude (athymic) mouse (chronic giardiasis model) and in immunologically intact heterozygote littermates (acute giardiasis model). Deficiencies in the nude mouse that result in chronic infection were to be investigated.

This initial phase of the work was to be followed by an attempt to transfer anti-G. muris immunity to chronically infected nude mice by transfer of cells isolated from immune heterozygote littermate donors. The role of the resultant immune response against G. muris in eliminating the parasite and resulting in changes to the gut morphology was to be observed.

The few studies of G. muris infection in nude mice have concentrated on the infection time course (Stevens et al 1978) and its lethality (Boorman et al 1973a) to the mice. The one exception to this is the report of Roberts-Thomson & Mitchell (1978) where the transfer of anti-G. muris immunity to nude mouse recipients by transfer of cells from immune heterozygote donors was accomplished, prior to the recipients' infection.

Materials and Methods.

Adult female nude (nu/nu) mice and adult female heterozygote (nu/+) littermates, of BALB/c background, were obtained from Bornholpgard, Denmark. These mice were kept under clean conditions in an incubator. They were isolated from other animals in the unit.

One additional precaution that differed from the normal experimental protocol was to isolate the G. muris trophozoites for infection in RPMI 1640 supplemented with penicillin and streptomycin (Difco) to 2% (stock solution 5000 U and 5000 IU, respectively). This was done to reduce the likelihood of passage of bacteria to the nude mice, which would exacerbate the pathogenicity of the infection and result in increased mortality of the animals (Boorman et al 1973b).

The study may be divided into three main phases and their aims:

1. Primary infection - how does the nude infection differ from the heterozygote infection?
2. Does the nude infection gradually resolve, as described by Stevens et al (1978), or remain at high levels, as described by Roberts-Thomson & Mitchell (1978)?

3a. Can immunity be transferred by immune lymphoid cells during chronic infection?

b. Does the immune response that results from transfer of lymphoid cells induce changes in the gut?

Results.

Infection Time Course.

As may be seen in Fig. 11.1, the G. muris infection in the heterozygote mice resulted in a time course of faecal cyst excretion similar to that found in adult female BALB/c mice from our own Animal Unit stocks and had resolved by day 25.

The infection in nude mice progresses initially in parallel with that of the heterozygote infection but, on day 9, a significantly ($p<0.05$) increased faecal cyst output was found. The nude infection faecal cyst output remains very high for the duration of the experiment (98 days).

Mucosal Architecture.

Villus Height.

The heterozygote infection (Fig. 11.2) showed a significant ($p<0.05$) reduction in villus height on day 17. This reduction was transient and villus height returned to normal control levels by day 24 of infection.

The villus height of the nude mice did not show any significant variation during infection (Fig. 11.3).

Crypt Depth.

The heterozygote infection shows a very significant increase ($p<0.01$) in crypt length by day 3 of infection and this increase is maintained to day 24 of infection.

Crypt depth in the nude infection is increased to a highly significant ($p<0.01$) degree by day 10 and, as with the heterozygote infection, this significant increase is maintained to day 24 of infection.

Crypt Cell Production Rate (CCPR).

The CCPR is increased in the heterozygote infection by day 10, and remains elevated to day 24. This is paralleled in the nude infection, again with increased CCPR by day 10 and remaining elevated to day 24.

Mucosal Function.

As an indicator of mucosal damage, an assay of four disaccharidase activity levels was performed.

Lactase.

Lactase activity in the heterozygote infection (Fig. 11.4) fell significantly ($p<0.01$) by day 3 and remained at this low level throughout the infection. This result was repeated in the nude mouse infection (Fig. 11.5), lactase activity being significantly ($p<0.01$) depressed by day 3 of infection and remaining depressed to day 24.

Sucrase.

Sucrase activity in the heterozygote infection (Fig. 11.6) is depressed significantly ($p<0.01$) by day 3 of infection, and again remains very significantly lowered to day 24.

The nude infection (Fig. 11.7) again parallels these results, sucrose activity very significantly decreased by day 3 and remaining at this level to day 24.

Maltase.

Maltase activity levels in the heterozygote infection (Fig. 11.8) are significantly ($p<0.01$) decreased by day 3, and remain significantly decreased to day 24.

Again, maltase levels in the nude infection (Fig. 11.9) parallels the heterozygote infection reults, being significantly decreased by day 3 and remaining so to day 24.

Trehalase.

Trehalase activity levels in both heterozygote (Fig. 11.10) and nude mouse (Fig. 11.11) infections differ from those of the other disaccharidase activities, in that no significant alteration was found in either group on day 3, but on day 10, in both groups, significant ($p<0.01$) reductions in trehalase activity were found. These decreased activity levels remained very significant in both groups to day 24 of infection.

Cellular Changes.

Intraepithelial Lymphocytes (IEL).

During the heterozygote infection (Fig. 11.12) the numbers of IEL became significantly increased ($p<0.05$) by day 24 of infection.

The infection in nude mice (Fig. 11.13) did not result in any change in IEL numbers on day 10 or day 24 of infection.

The numbers of IEL in uninfected nude mice are significantly ($p<0.05$) lower than those found in age and sex matched uninfected heterozygote littermates.

Chloroacetate Esterase (CAE) Positive Cells.

During the heterozygote infection (Fig. 11.14) numbers of CAE positive cells (MMC) significantly increase ($p<0.05$) by day 10, but decrease to levels not significantly different from control by day 24.

The nude mouse infection (Fig. 11.15) shows a significant ($p<0.05$) increase in CAE positive cell numbers by day 10, and this decreases, but remains significantly elevated by day 24 of infection.

At this point of the experiment, the nude mice received lymphoid cells transfers as detailed below.

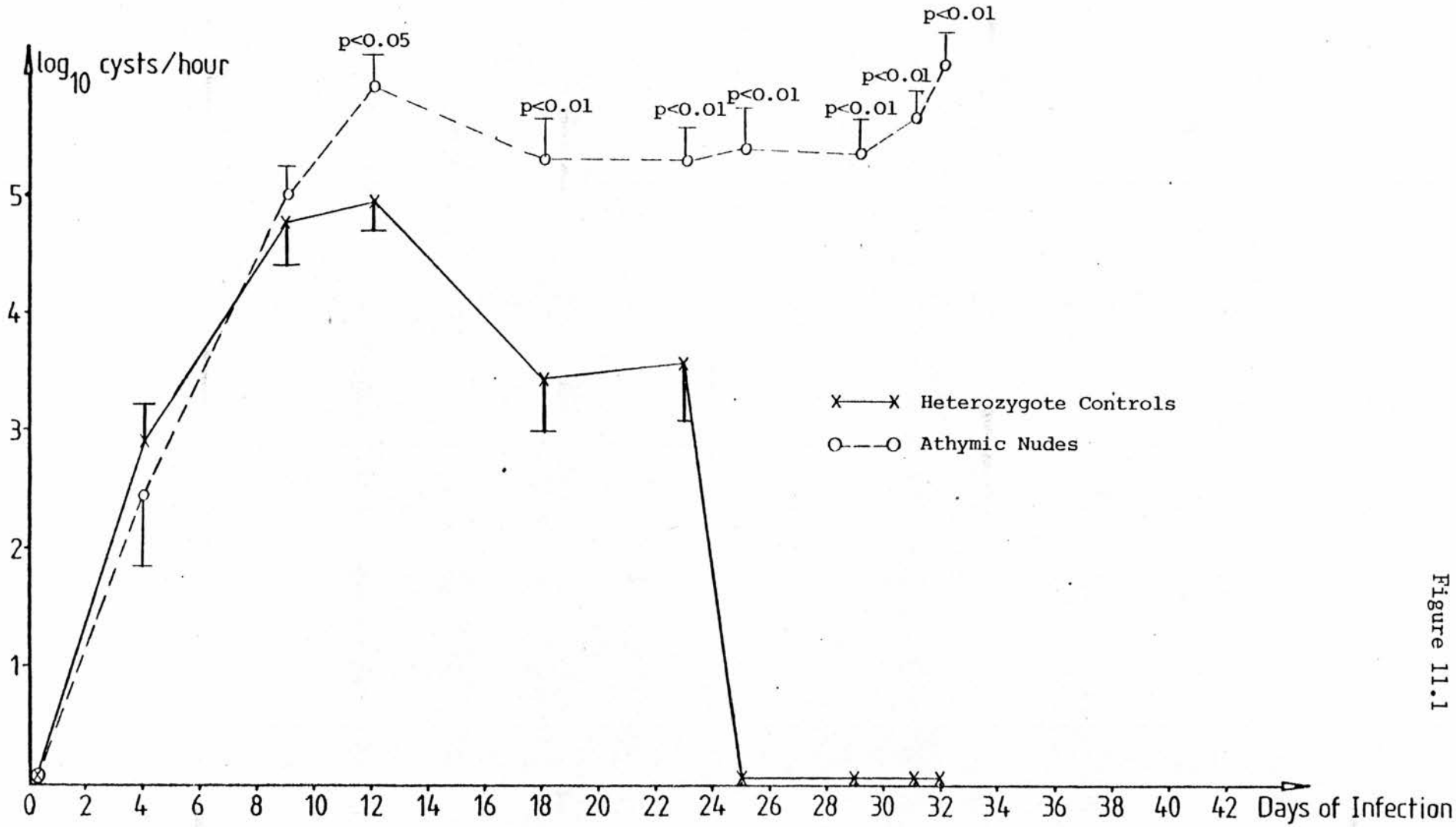


Figure 11.1

Course of primary G. muris infection in athymic nude mice and heterozygote littermate controls. Infection initiated on day 0 with 3000 trophozoites in 0.2ml i.g. Marks represent ± 1 SD of number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

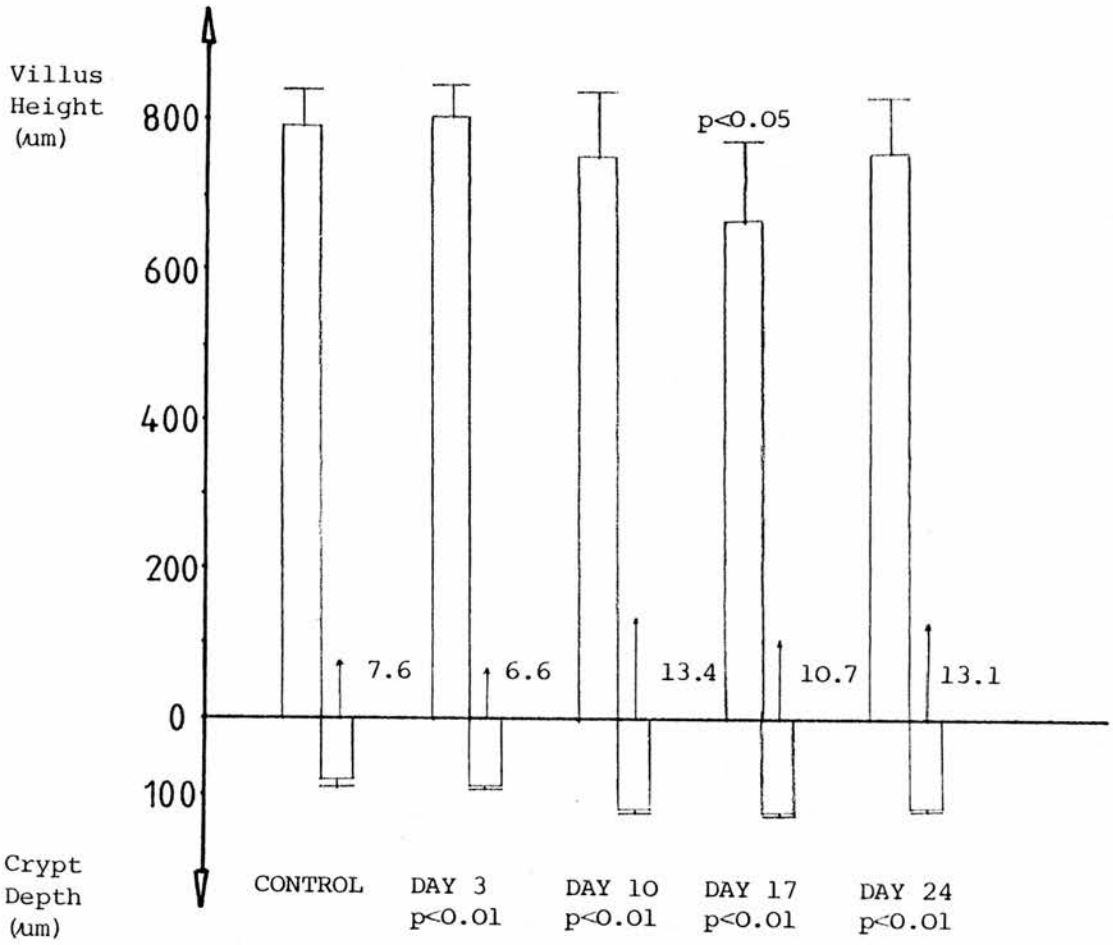


Figure 11.2

G. muris Infection in Heterozygote (nu/+) Mice

Effect of G. muris infection on mucosal architecture of adult female heterozygote nude mice, BALB/c background. Bars represent means ± 1 SD for villus height and crypt depth, and arrows show CCPR (six mice per group).

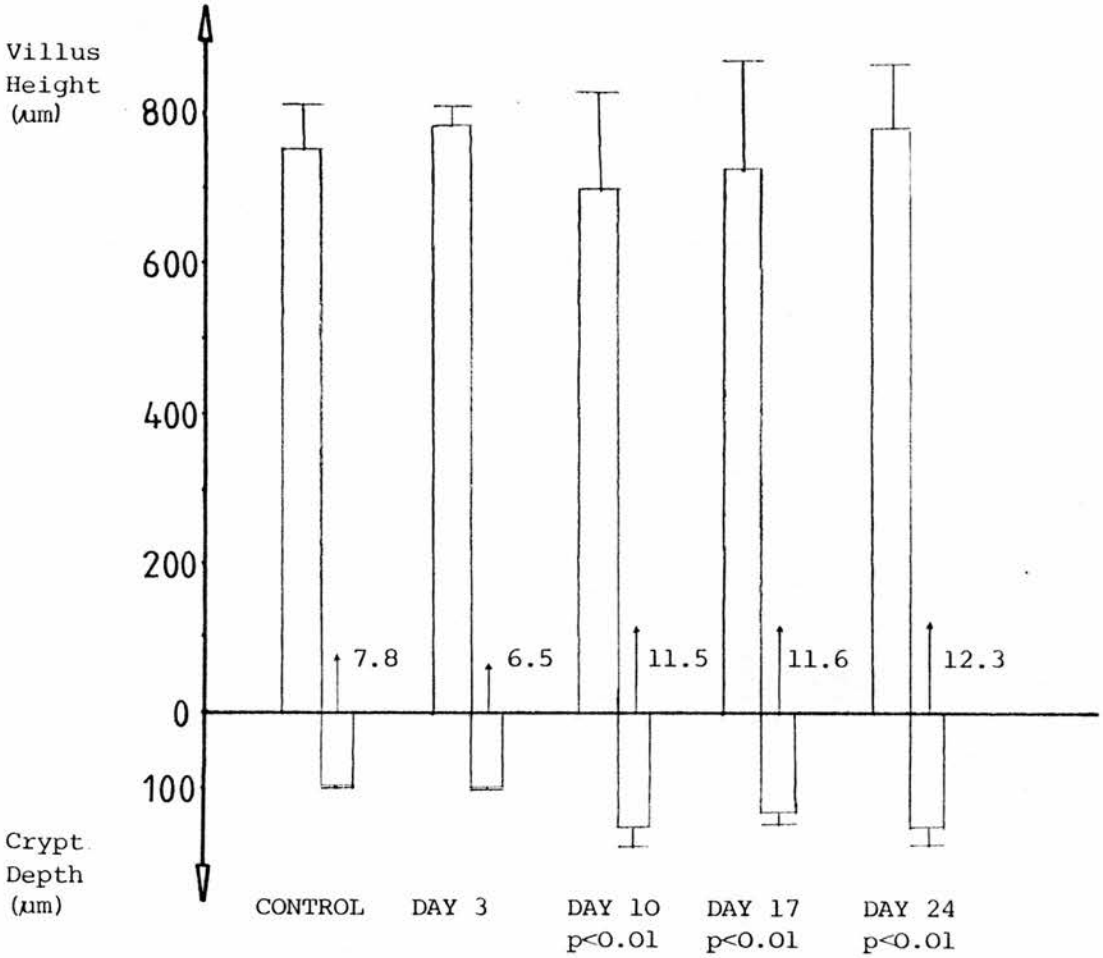


Figure 11.3

G. muris Infection in Nude (nu/nu) Mice

Effect of G. muris infection on mucosal architecture of adult female nude (athymic) mice, BALB/c background. Bars represent means ± 1 SD for villus height and crypt depth, and arrows show CCPR (six mice per group).

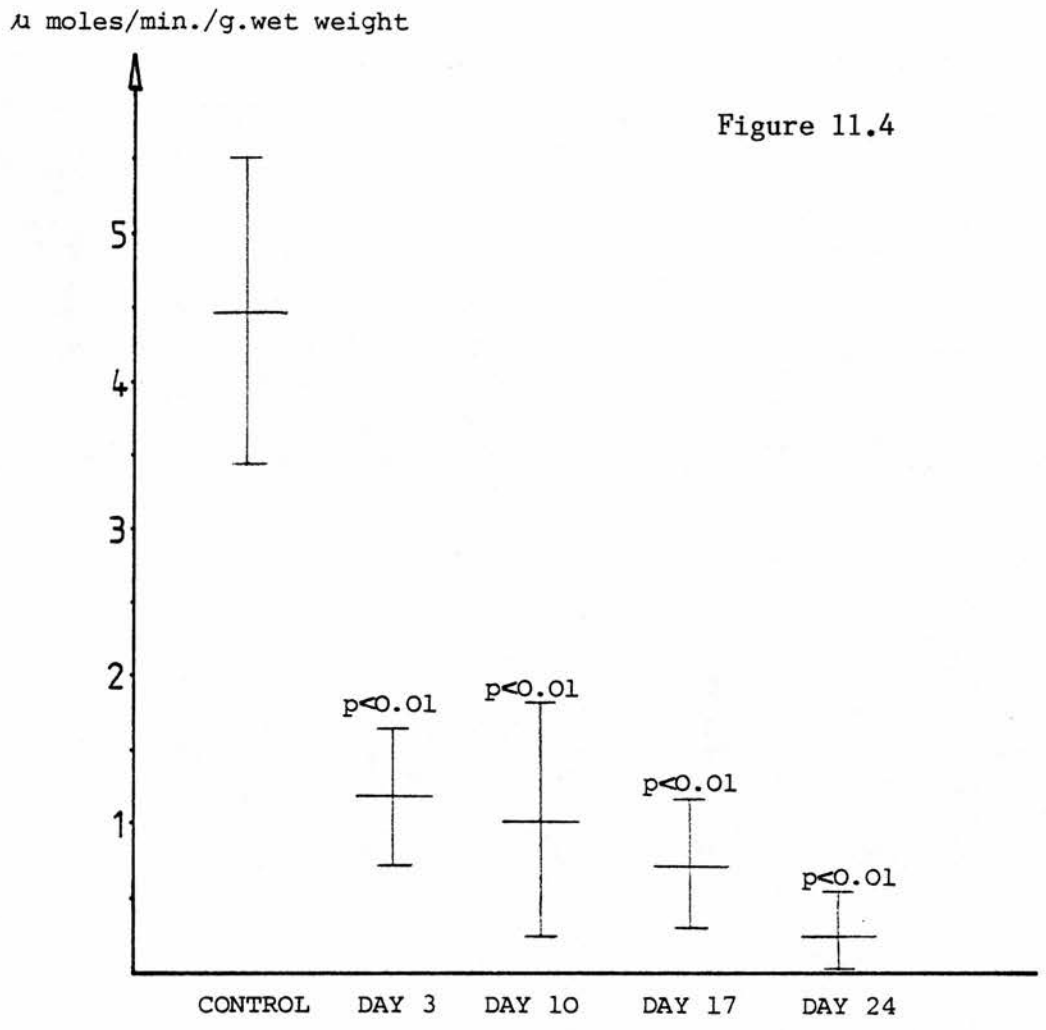
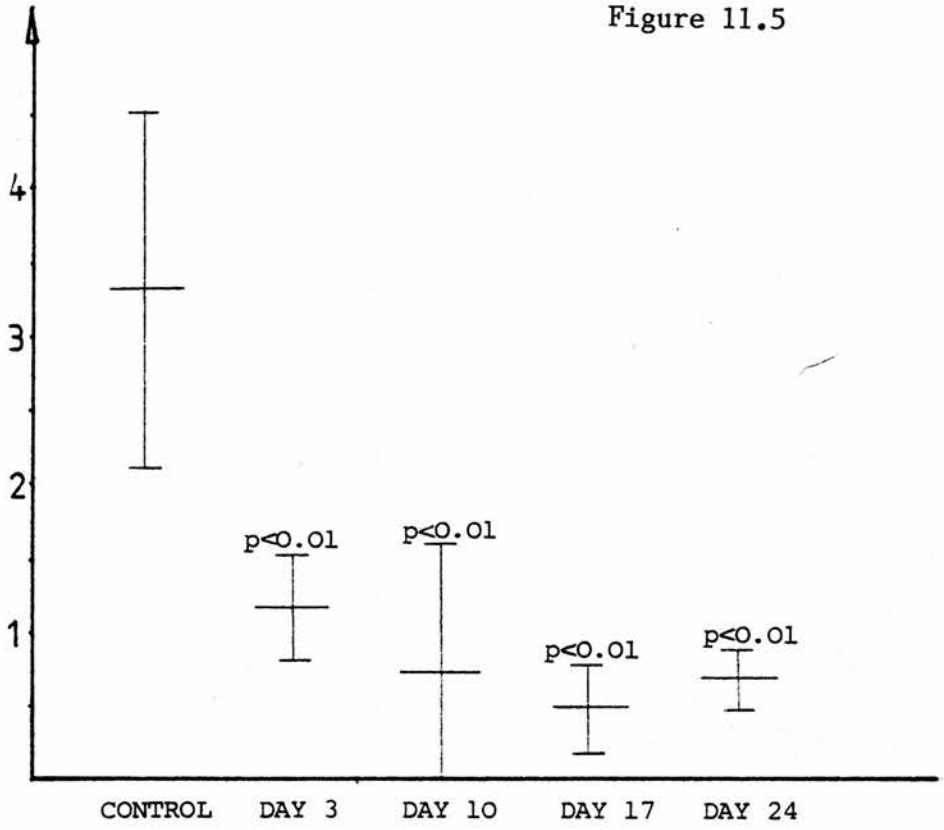


Figure 11.4

G. muris Infection in Heterozygote (nu/+) Mice

Effect of G. muris infection on lactase activity in adult female heterozygote nude mice, BALB/c background. Bars represent means ± 1 SD (six mice per group).

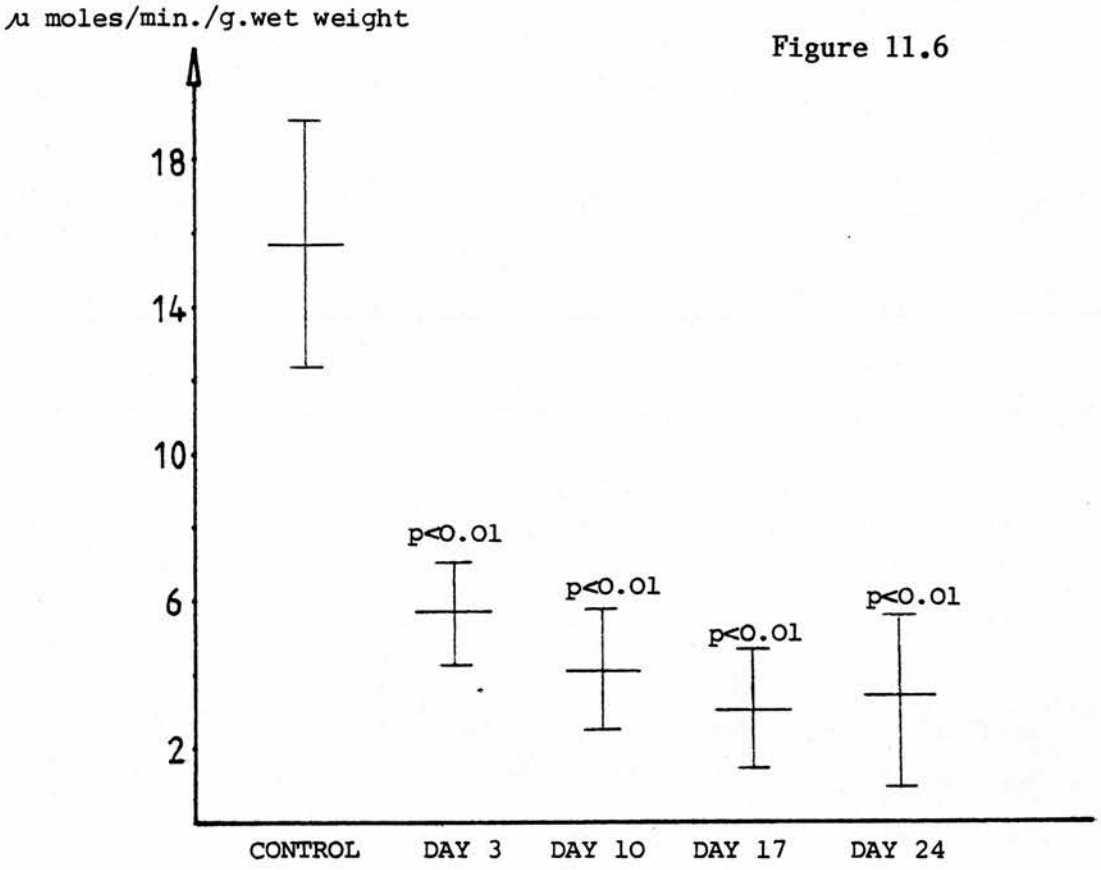
Figure 11.5



G. muris Infection in Nude (nu/nu) Mice

Effect of G. muris infection on lactase activity in adult female nude (athymic) mice, BALB/c background. Bars represent means ± 1 SD (six mice per group).

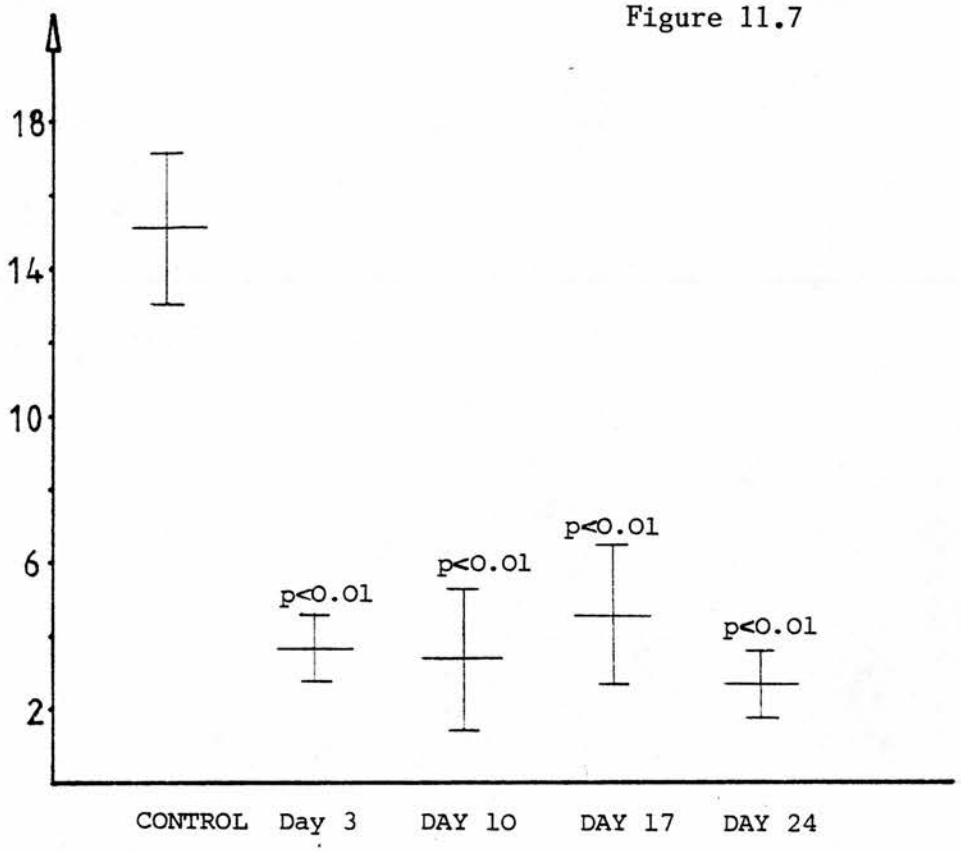
Figure 11.6



G. muris Infection in Heterozygote (nu/+) Mice

Effect of G. muris infection on Sucrase activity in adult female heterozygote nude mice, BALB/c background. Bars represent means ± 1 SD (six mice per group).

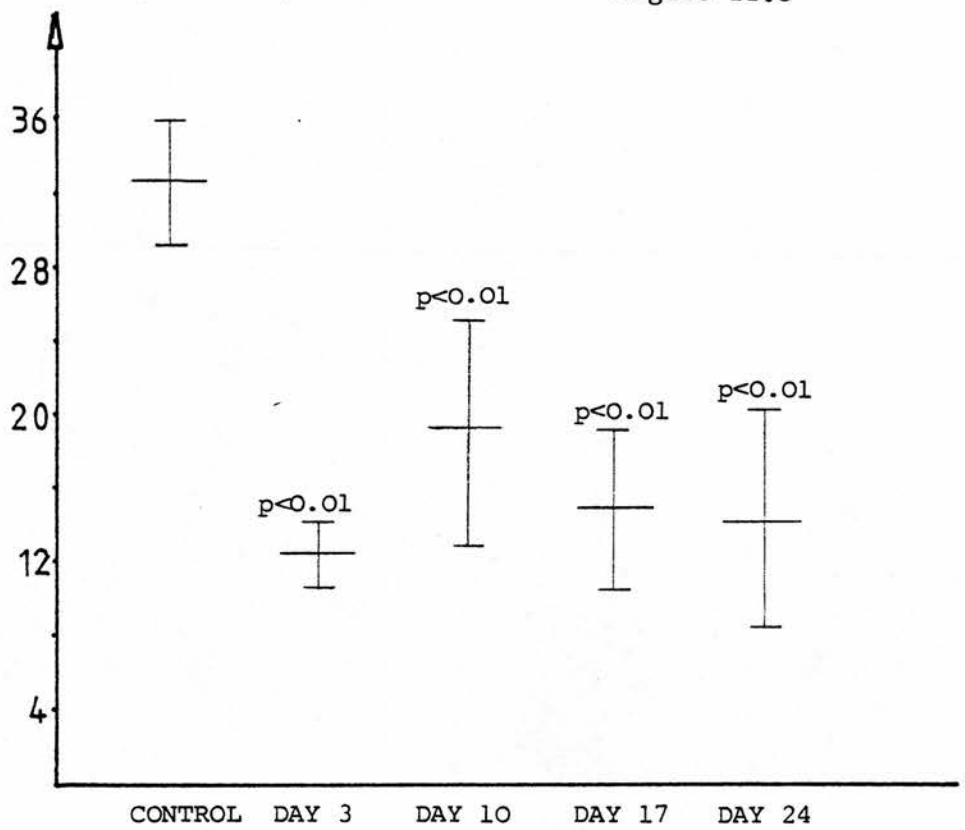
Figure 11.7



G. muris Infection in Nude (nu/nu) Mice

Effect of G. muris infection on Sucrase activity in adult female nude (athymic) mice, BALB/c background. Bars represent means ± 1 SD (six mice per group).

Figure 11.8

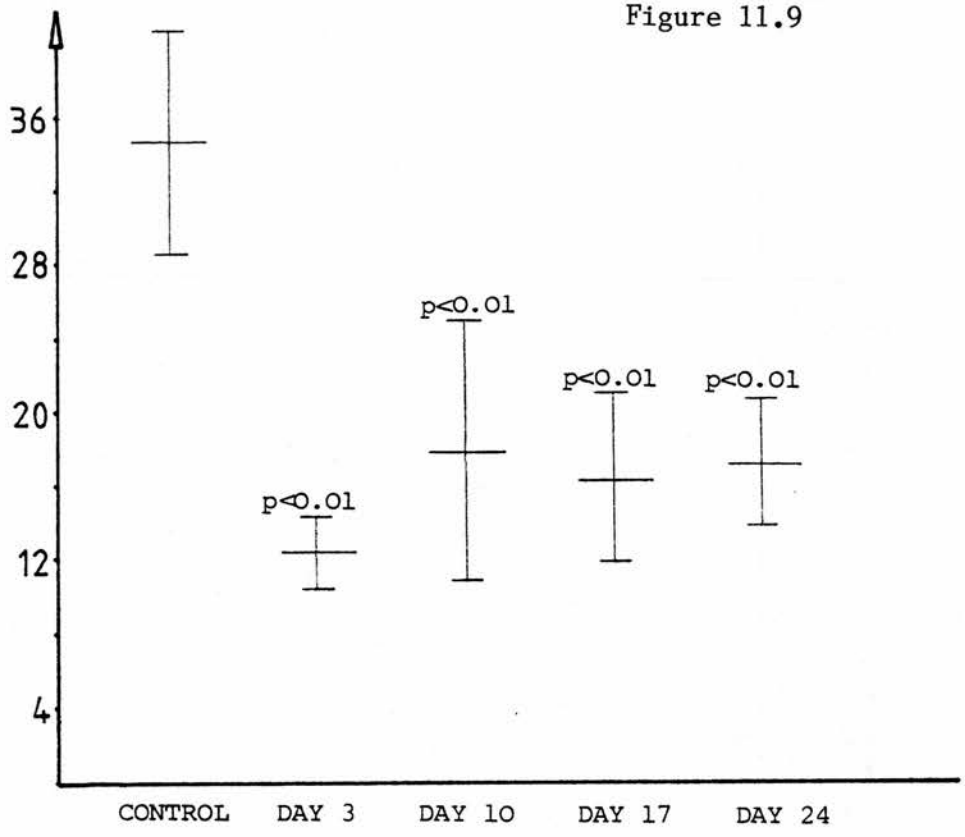


G. muris Infection in Heterozygote (nu/+)Mice

Effect of G. muris infection on Maltase activity in adult female heterozygote nude mice, BALB/c background. Bars represent means ± 1 SD (six mice per group).

μ moles/min./g.wet weight

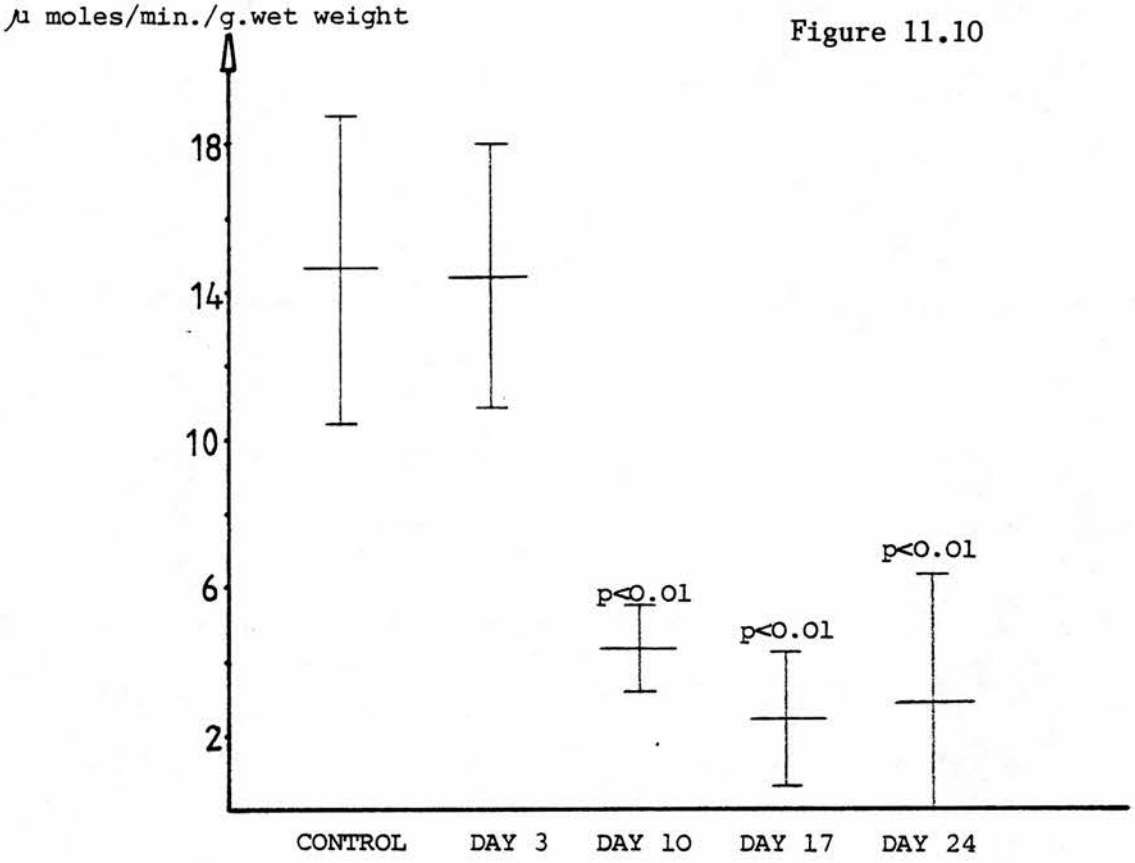
Figure 11.9



G. muris Infection in Nude (nu/nu) Mice

Effect of G. muris infection on Maltase activity in adult female nude (athymic) Mice, BALB/c background. Bars represent means ± 1 SD (six mice per group).

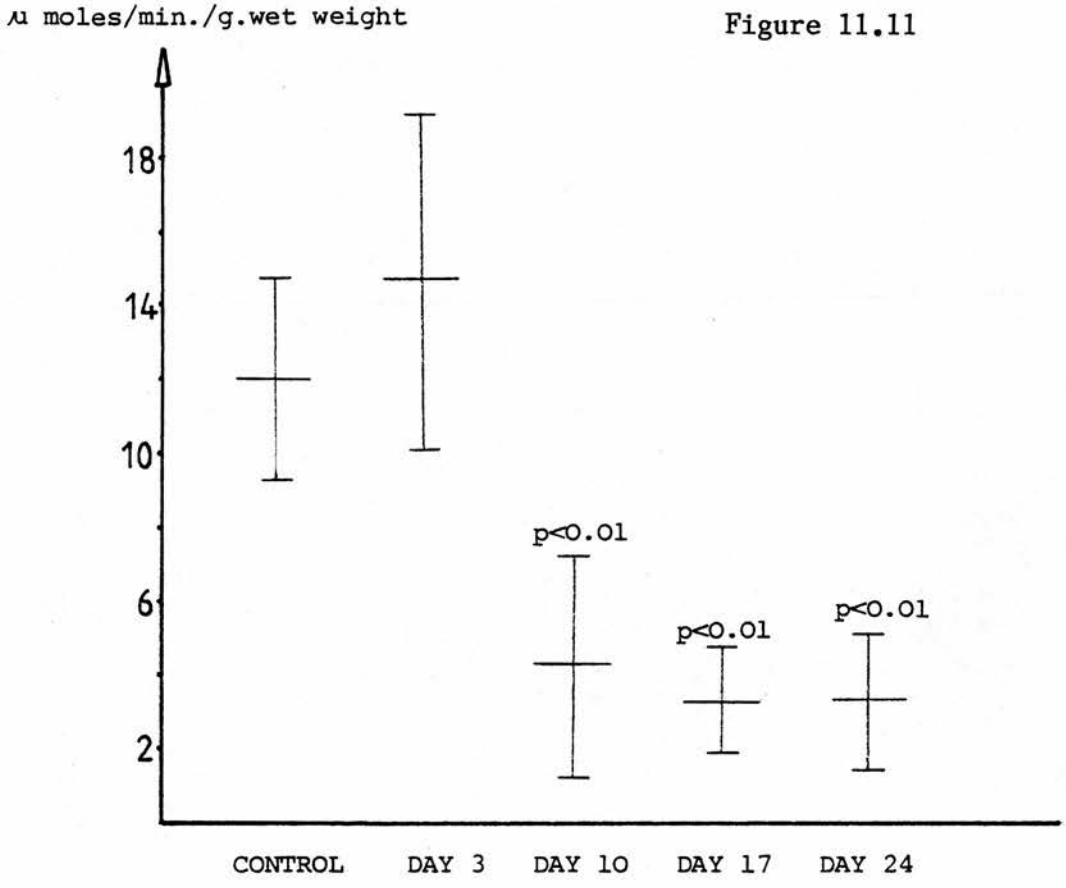
Figure 11.10



G. muris Infection in Heterozygote (nu/+) Mice

Effect of G. muris infection on Trehalase activity in adult female heterozygote nude mice, BALB/c background. Bars represent means ± 1 SD (six mice per group).

Figure 11.11



G. muris Infection in Nude (nu/nu) Mice

Effect of G. muris infection on Trehalase activity in adult female nude (athymic) mice, BALB/c background. Bars represent means ± 1 SD (six mice per group).

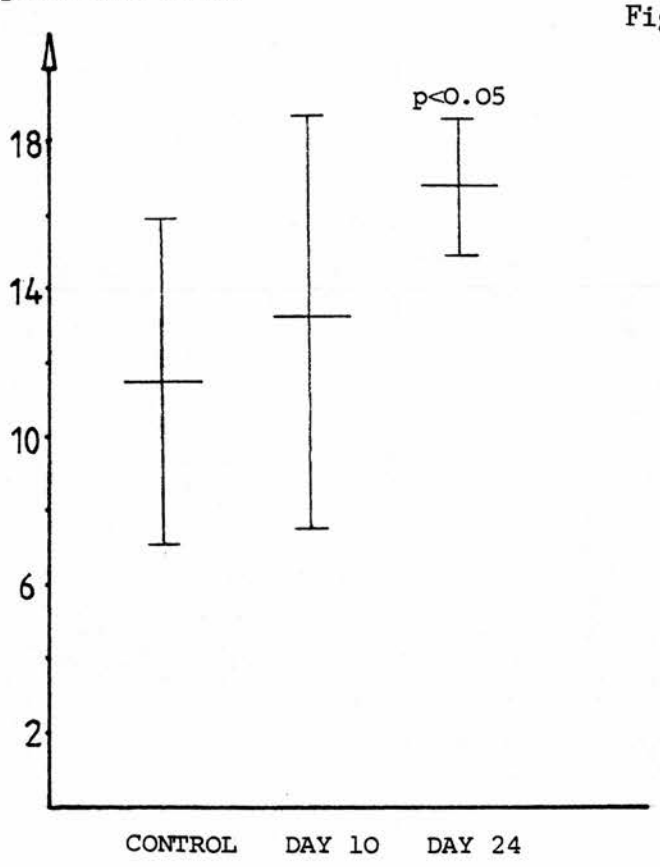
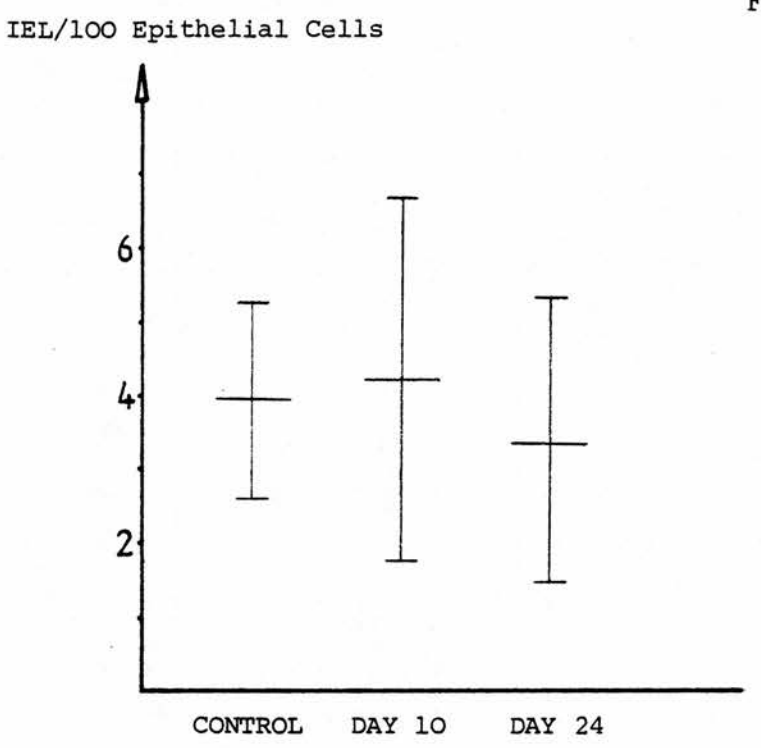


Figure 11.12

G. muris Infection in Heterozygote (nu/+) Mice

Effect of G. muris infection on numbers of IEL per 100 epithelial cells in adult female heterozygote nude mice, BALB/c background. Results are of means ± 1 SD (six mice per group).

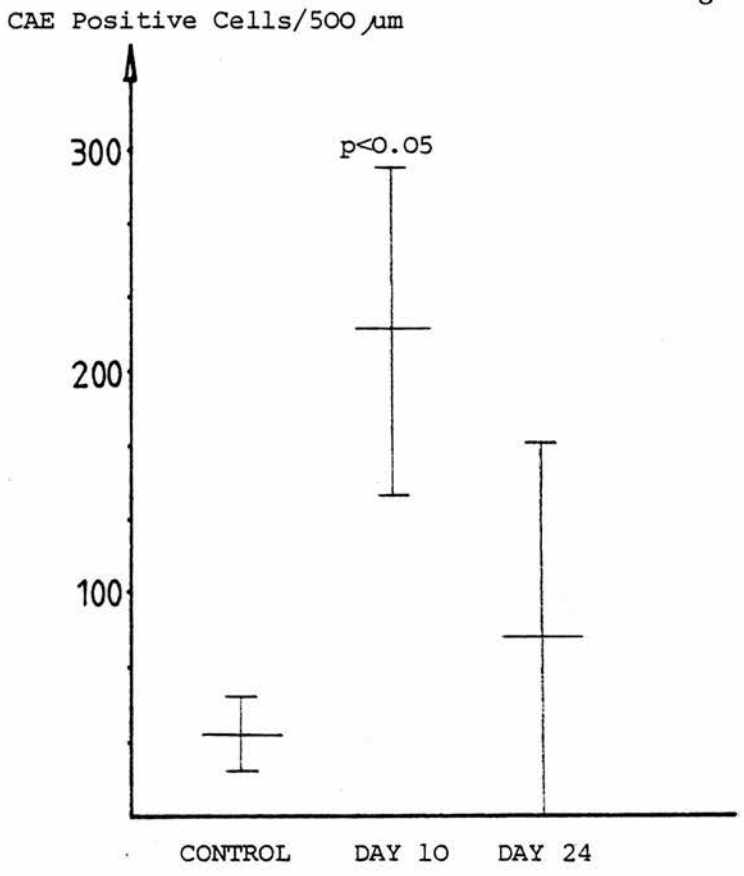
Figure 11.13



G. muris Infection in Nude (nu/nu) Mice

Effect of G. muris infection on numbers of IEL per 100 epithelial cells in adult female nude (athymic) mice, BALB/c background. Results are of means ± 1 SD (six mice per group).

Figure 11.14



G. muris Infection in Heterozygote (nu/+) Mice

Effect of G. muris infection on the number of CAE positive cells (MMC) in adult female heterozygote nude mice, BALB/c background. Bars represent means ± 1 SD (six mice per group).

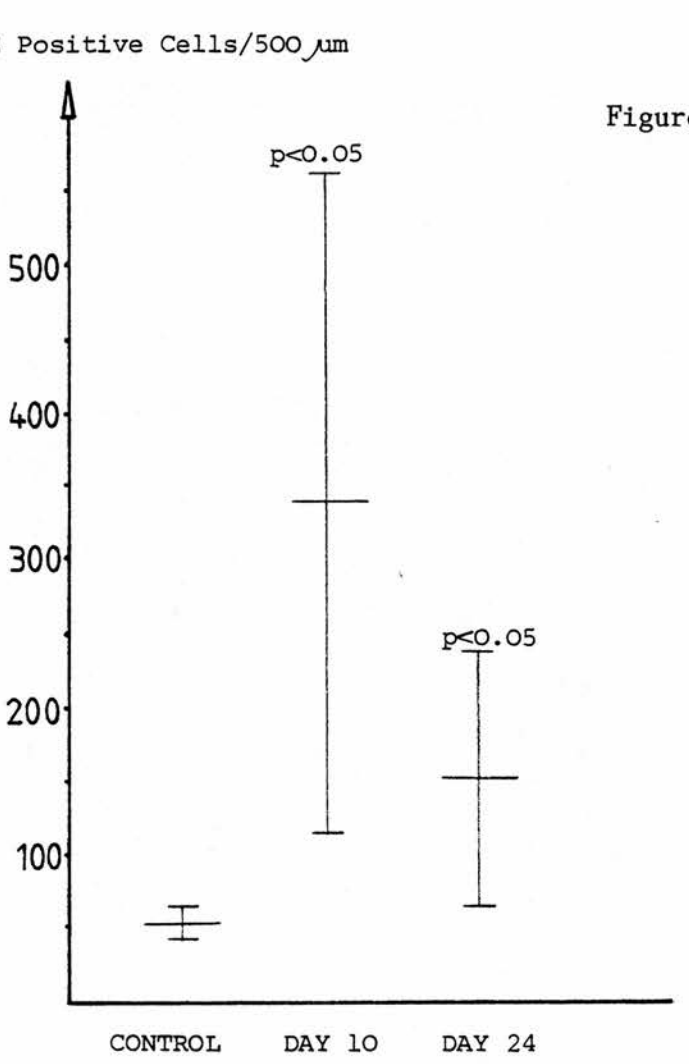


Figure 11.15

G. muris Infection in Nude (nu/nu) Mice

Effect of G. muris infection on the number of CAE positive cells (MMC) in adult female nude (athymic) mice, BALB/c background. Bars represent means ± 1 SD (six mice per group).

Transfer of Immunity.

Introduction.

The aim of this part of the experiment was to investigate changes in the gut which might result in elimination of the parasite, and evaluate changes in the gut morphology thought to be associated with this response. By day 33 of infection, the heterozygote mice had eliminated the G. muris trophozoites and were considered to be immune to this parasite, as had been shown to occur in adult female BALB/c mice in previous experiments. No significant mortality had occurred in the chronically infected nude mouse group and remaining numbers were sufficient to attempt a transfer of immunity with cells from immune heterozygote donors, and include appropriate control groups.

Materials and Methods.

The chronically infected nude mice were divided into three groups at random. The number of remaining immune heterozygote littermates available as donors limited the number of nude mice in the group which received immune cells.

The three nude mouse groups were:

a. No cell transfer: chronically infected mice receiving 0.3 ml RPMI 1640 i.p.
b. Naive cell transfer: chronically infected mice receiving 7.5×10^7 Spleen & MLN cells from uninfected heterozygote littermate donors.
c. Immune cell transfer: chronically infected mice receiving 7.5×10^7 Spleen & MLN cells from previously infected, immune heterozygote littermate donors.

The course of infection in these three groups was then followed by faecal cyst output. At the time when a decrease in the faecal cyst output of mice receiving lymphoid cells from immune heterozygote donors was noted, mice were killed to examine the gut, as was done earlier in the infection.

The group of infected nude mice which received lymphoid cells from immune heterozygote donors was limited to six animals due to the number of donors available. To allow a reasonable statistical

comparison, all six mice in this group were sacrificed, along with six mice from the other two groups, on day 55 after cell transfer (day 88 of infection), when there was a consistent divergence of faecal cyst output between the groups (see later). The remaining six mice in each group receiving medium alone or lymphoid cells from naive heterozygote donors were sacrificed on day 65 after cell transfer (day 98 of infection).

Results.

Infection Time Courses Following Cell Transfers.

Chronically infected nude mice receiving medium alone (Fig. 11.16) showed some undulation of faecal cyst output, but no consistent change in infection was noted.

Mice receiving cells from naive heterozygote donors showed parallel variations of faecal cyst output compared to mice receiving medium alone, indicating that the cyst isolation procedure and counting were the origin of this variation. A trend towards lower cyst output in mice receiving cells was noticed, but this was only transiently significant ($p<0.05$), on day 55 after cell transfer, day 88 of infection.

Mice receiving cells from immune heterozygote donors (Fig. 11.17) show a trend toward lower faecal cyst output after cell transfer. This decrease becomes transiently significant ($p<0.05$) on day 16 after transfer and consistently significant on day 26 and later, after cell transfer. A sudden and dramatic decrease in faecal cyst output in some mice which received immune cells was noted on day 43 after transfer, but this was only seen in three of the six recipient mice. Although the decrease in faecal cyst output was significant, the mean value for the group remained high.

Comparison of the faecal cyst output of mice receiving naive cells or immune cells from heterozygote littermates (Fig. 11.18) shows again the trend toward lower cyst output in mice receiving immune cells. This decrease becomes transiently significant ($p<0.05$) on day 19, day 43, day 49 after cell transfer, compared to the faecal cyst output of mice which received naive heterozygote cells.

Mucosal Architecture. (Fig. 11.19).

Villus Height.

At the time when a consistent and significant reduction in faecal cyst output was noted in the group of infected nude mice which received cells from immune heterozygote donors, a villus height which is significantly ($p<0.05$) reduced compared to the other two infected groups (no cell transfer and naive cell transfer) was found. This reduction was not significant when compared to the age matched uninfected nude control group.

On day 65 after cell transfer, day 98 of infection, there was no significant difference between villus heights of infected mice which received medium alone, or naive cells, and age matched control values.

Crypt Depth.

Infected groups show significant ($p<0.05$) or highly significant ($p<0.01$) increases in crypt depth, compared to the age matched controls at both day 55 and day 65 after immune or naive cell transfer. The exception was the no cell transfer group, on day 65 after receiving medium alone (day 98 of infection), which was not significantly different to the age matched control value. No significant variation between experimental groups was found.

Crypt Cell Production Rate (CCPR).

All infected groups, regardless of treatment, showed increased CCPR with relation to the age matched uninfected control value.

Mucosal Function.

Lactase.

The lactase activity level (Fig. 11.20) was very significantly reduced in infected groups with the exception of the group receiving medium alone on day 33 of infection. This group had a particularly large standard deviation and this resulted in the reduced activity value being non significant. All values of infected groups were comparable to that of day 24 of infection.

The lactase activity level of the age matched control group (22 weeks old) shows the expected reduction with age compared to the previous control value of younger mice (7 weeks old).

Sucrase.

All infected groups sucrase activity values (Fig. 11.21) were very significantly reduced ($p<0.01$), regardless of the status of transferred cells, compared to age matched control values.

In addition, the sucrase activity of mice receiving immune cells was very significantly ($p<0.01$) reduced compared to the value of mice receiving cells from naive heterozygote donors.

Maltase.

All infected groups showed highly significant ($p<0.01$) reductions in maltase activity values (Fig. 11.22) on day 55 after cell transfer, compared to the age matched controls. Mice receiving cells from immune heterozygote donors also had significantly ($p<0.05$) decreased maltase activity on day 55 after cell transfer (day 88 of infection), compared to that of infected mice receiving medium alone at the time of cell transfer.

Trehalase.

All infected groups had very significantly ($p<0.01$) decreased trehalase activity levels (Fig. 11.23) compared to age matched controls.

Cellular Changes.

Intraepithelial Lymphocytes (IEL).

The numbers of IEL per 100 epithelial cells (Fig. 11.24) in infected groups showed no significant variation from control values, regardless of treatment at the time of cell transfer. Very large variations were recorded within all groups. The age matched control group also showed a trend towards higher numbers of IEL compared to the control value of mice at 6 weeks of age; this increase was not significant.

Chloroacetate Esterase (CAE) Positive Cells.

The numbers of CAE positive cells (MMC) (Fig. 11.25) showed a trend towards increased values in infected groups, but due to large variations within each group, no significant difference from the control value was found. The value obtained for CAE positive cells

on day 24 of infection is included for comparison, this showed a significant increase ($p<0.05$) compared to control values at that time.

Summary and Conclusions.

The nude heterozygote mouse of BALB/c background showed an infection course typical of that previously described for adult female BALB/c mice. The homozygote nude mouse maintained a chronic infection with high faecal cyst output during the 98 day study. The heterozygote infection resulted in transient villus height reduction and consistent crypt hyperplasia and increased CCPR. This indicated gut damage during infection, resulting in a more rapid turnover of cells; cell loss and cell production generally balanced, so little change to villus height was noted.

The same altered characteristics were noted during infection of the nude mouse. These changes in the nude mouse gut were not expected, based upon the hypothesis that the gut damage was as a result of the thymus dependent immune response against G. muris.

The disaccharidase activity levels in both heterozygote and homozygote mice were greatly reduced during infection, reiterating that the gut was damaged and its function impared.

The IEL of the heterozygotes rose, as predicted, whereas the IEL of the homozygote nude mice remained severely depressed, at a time when the gut was damaged.

The cell transfer was planned at the end of the initial phase of the experiment when, due to very low mortality, many more animals remained than had been expected. The infected nude mice had remained in apparently good condition, being alert and active, with no sign of diarrhoea or intestinal disturbance. At sacrifice the gut occasionally appeared thin and translucent, and occasionally inflated to some degree with gas; in either case, individuals so affected appeared otherwise healthy.

Experiments investigating transfer of immunity would ideally involve cell transfer prior to infection and subsequent infection with limited numbers of parasites. This would allow cells to become resident in preferred sites, establishing in a microenvironment where they would be capable of receiving appropriate signals and responding to these signals as they would in the intact donor. Use

of limited numbers of the infective agent would allow transferred immunity to be expressed and effective.

Because the number of cells transferred was possibly below optimum, and cells were transferred i.p., during chronic infection, it was thought that the immune response that could develop after lymphoid cell transfer would be relatively ineffective against the massive antigenic load of chronic infection in the nude mouse. The immune response in the nude mouse would also be limited by the relative absence of host lymphoid cells that can be recruited to enhance the response.

Surprisingly then, the infection did seem to be controlled to some degree by transfer of immune cells, but unfortunately in only half of the recipients, and after some considerable period of time (55 days). At the time of this reduction in parasite load, the villus height of mice receiving immune cells was significantly reduced compared to chronically infected mice of the same age. This may indicate some thymus dependent local mucosal CMI mediated damage of the intestine due to the hosts' secondary immune response to the parasite. Roberts-Thomson and Mitchell (1978) also described increased gut damage during infection when immune cells were transfered to nude mice prior to infection.

Disaccharidase levels remain reduced in infected nude mice, but sucrase and maltase show significant further reductions in activity in mice receiving immune cells compared to naive cells or medium. This further supports the villus height evidence of increased damage to the gut in this group.

The IEL numbers were very variable within groups, and no conclusions may be drawn other than noting an apparent, though again variable, increase in numbers of IEL in nude mice, with age.

CAE positive cells were also very variable within groups though a trend towards persistence of elevated numbers was noted.

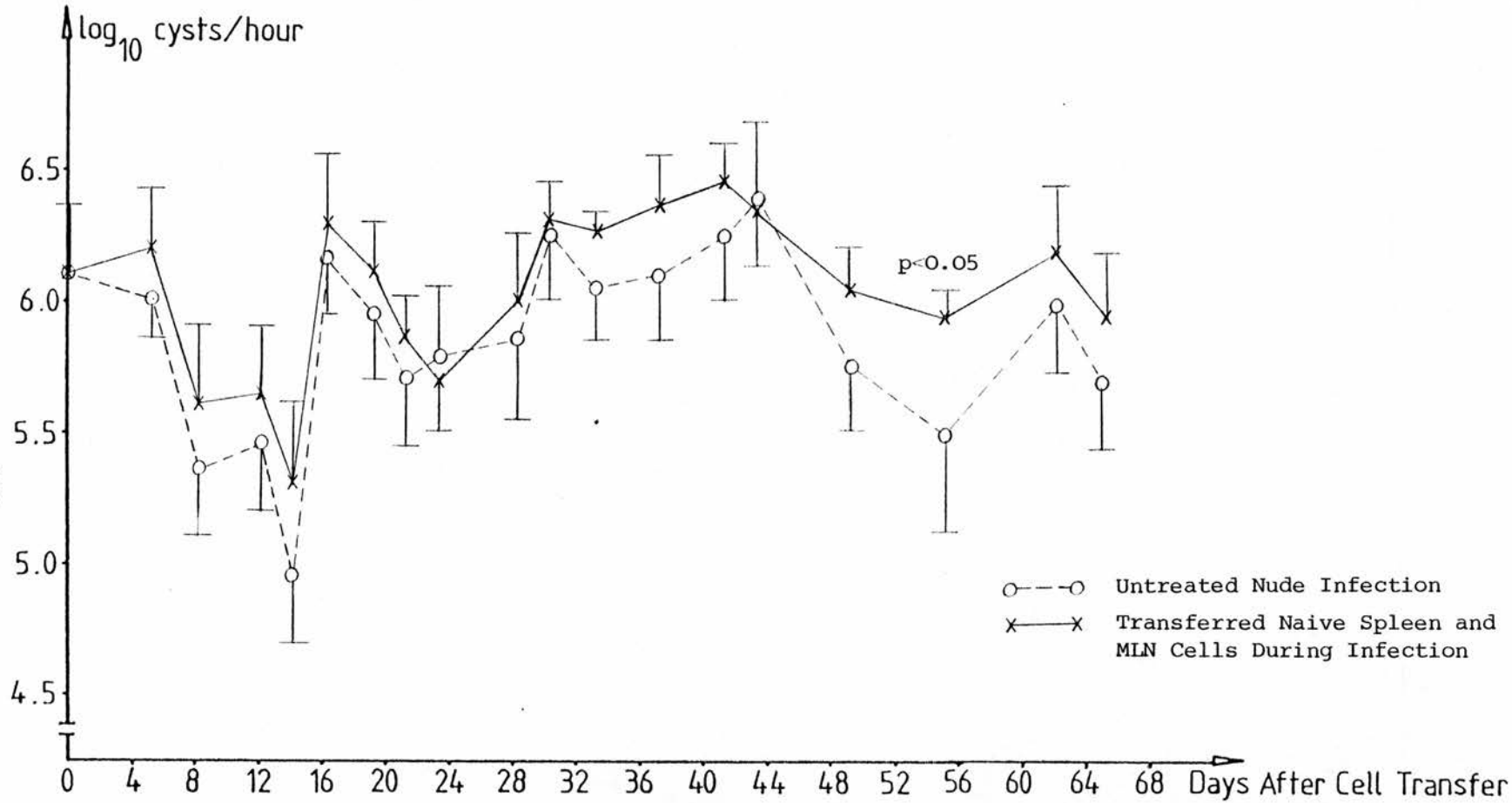


Figure 11.16

Course of chronic G. muris infection in untreated nude mice and nude mice receiving 7.5×10^7 Spleen and MLN cells from naive heterozygote littermate donors, on day 33 of primary infection. Marks represent means $\pm$ 1 SD of number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

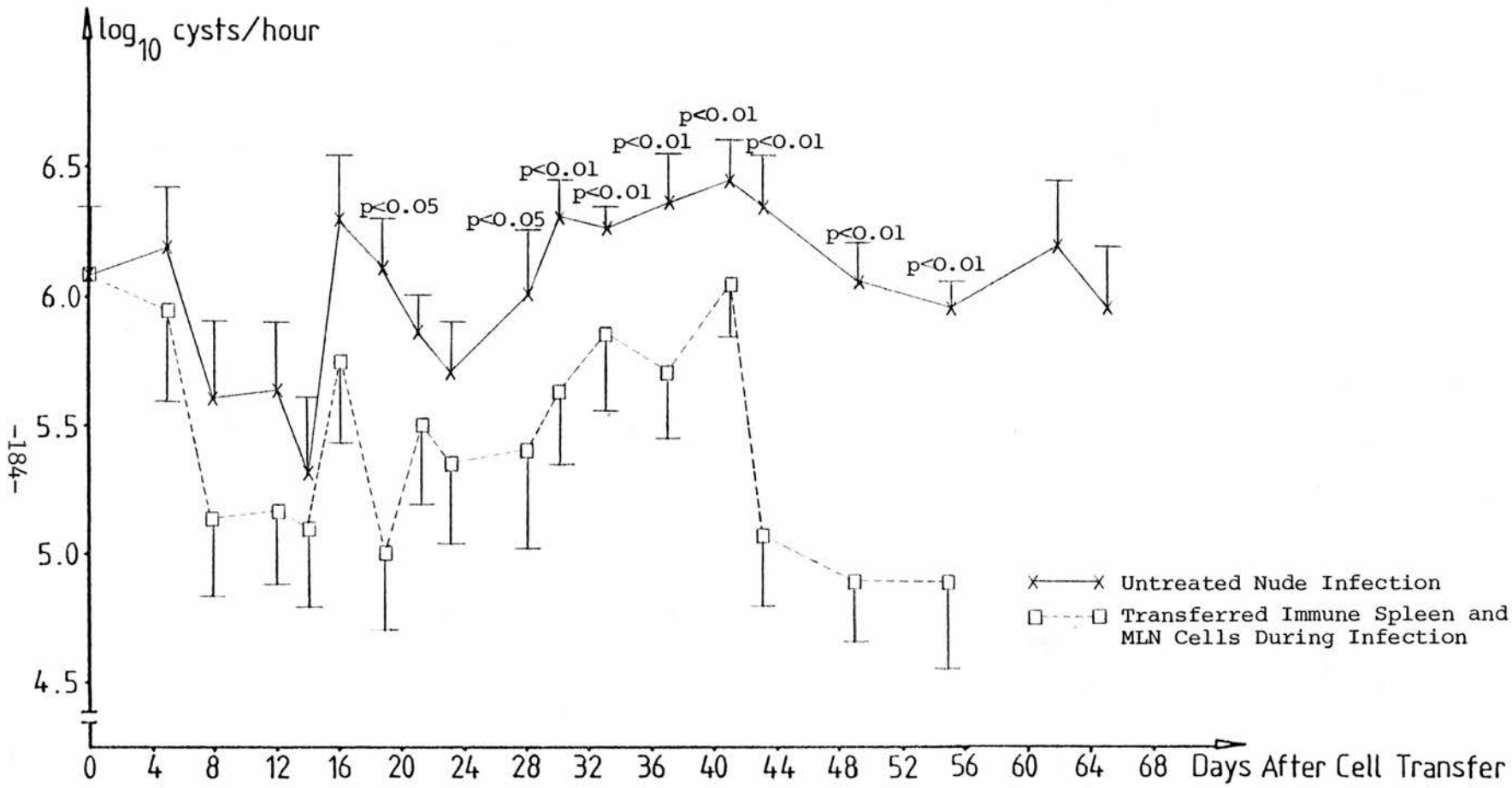


Figure 11.17

Course of chronic G. muris infection in untreated nude mice and nude mice receiving 7.5×10^7 Spleen and MLN cells from immune heterozygote littermate donors, on day 33 of primary infection. Marks represent means $\pm$ 1 SD of number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

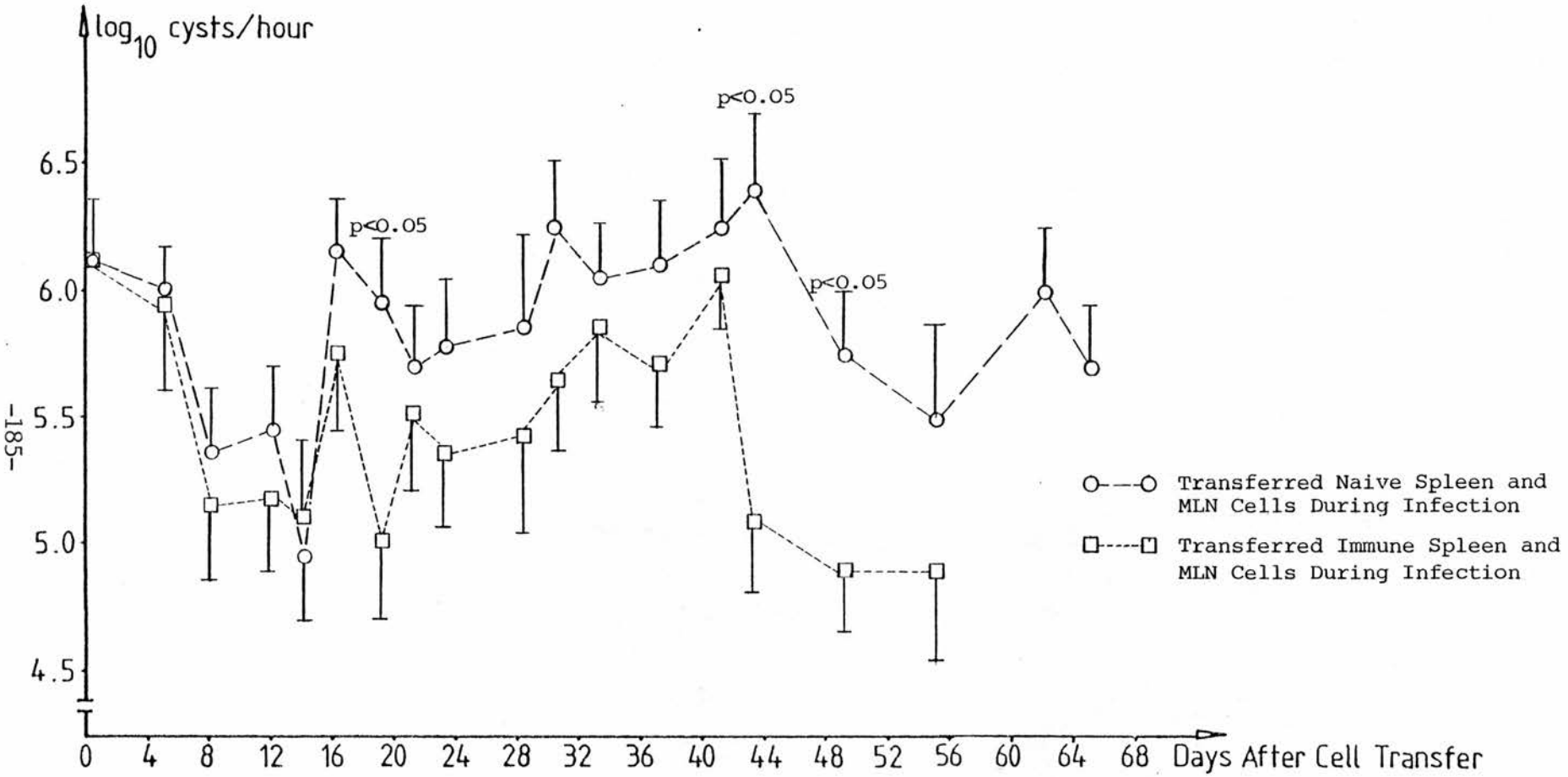
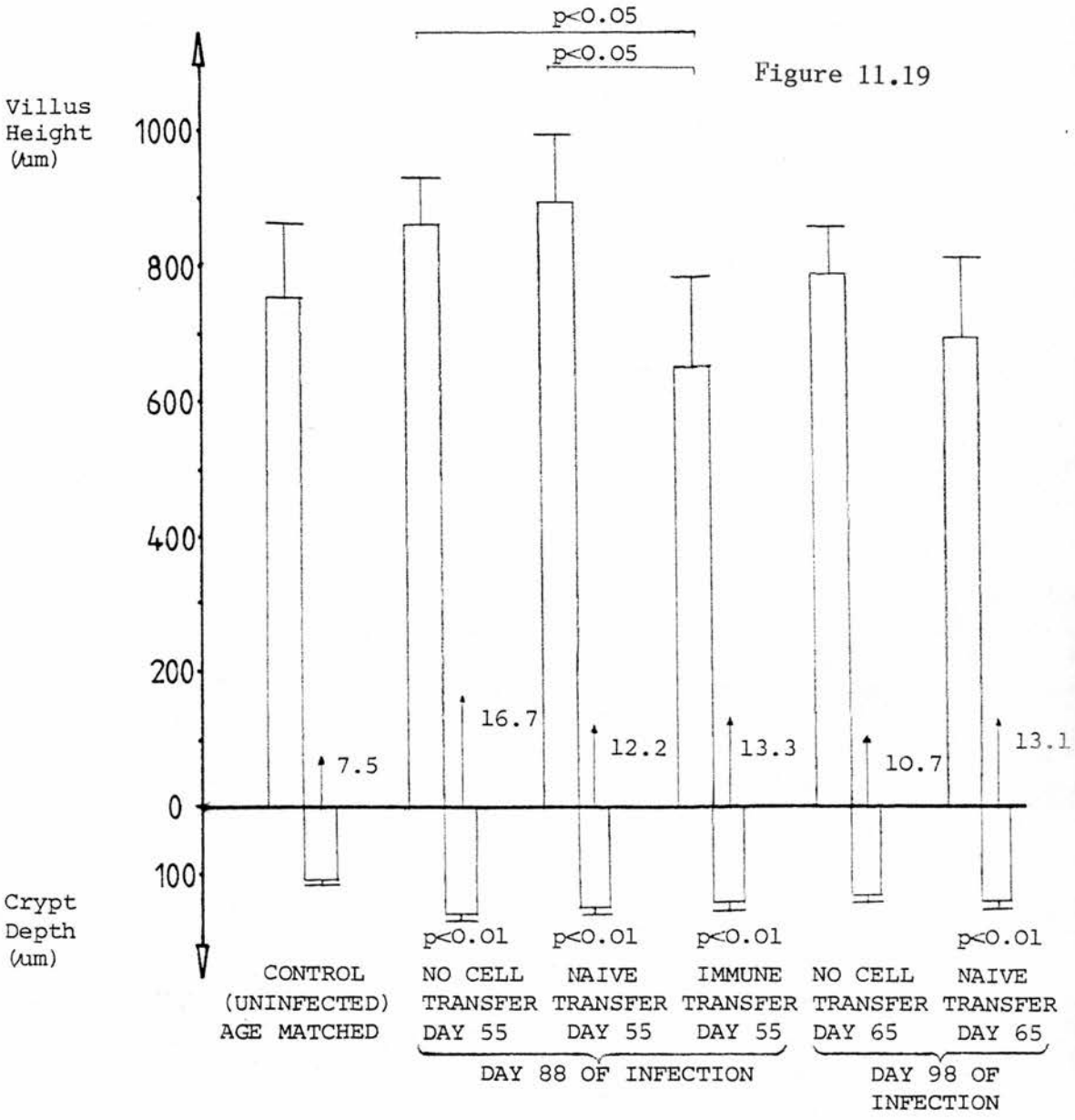


Figure 11.18

Course of chronic G. muris infection in nude mice receiving 7.5 x 10^7 Spleen and MLN cells from either naive or immune heterozygote littermate donors, on day 33 of primary infection. Marks represent means ± 1 SD of number of cysts passed per hour, during a 2 hour faecal collection (six mice per group).

Figure 11.19



G. muris Infection in Reconstituted Nude (nu/nu) Mice

Effect of G. muris infection on mucosal architecture of adult female nude (athymic) mice, BALB/c background, which received on day 33 of primary infection 0.3ml RPMI 1640 (No Cell Transfer group), or 7.5×10^7 Spleen and MLN Cells from either uninfected (Naive Transfer) or previously infected and immunologically cured (Immune Transfer) heterozygote littermate donors. Bars represent means $\pm$ 1 SD for villus height and cyrpt depth and arrows show CCPR (six mice per group).

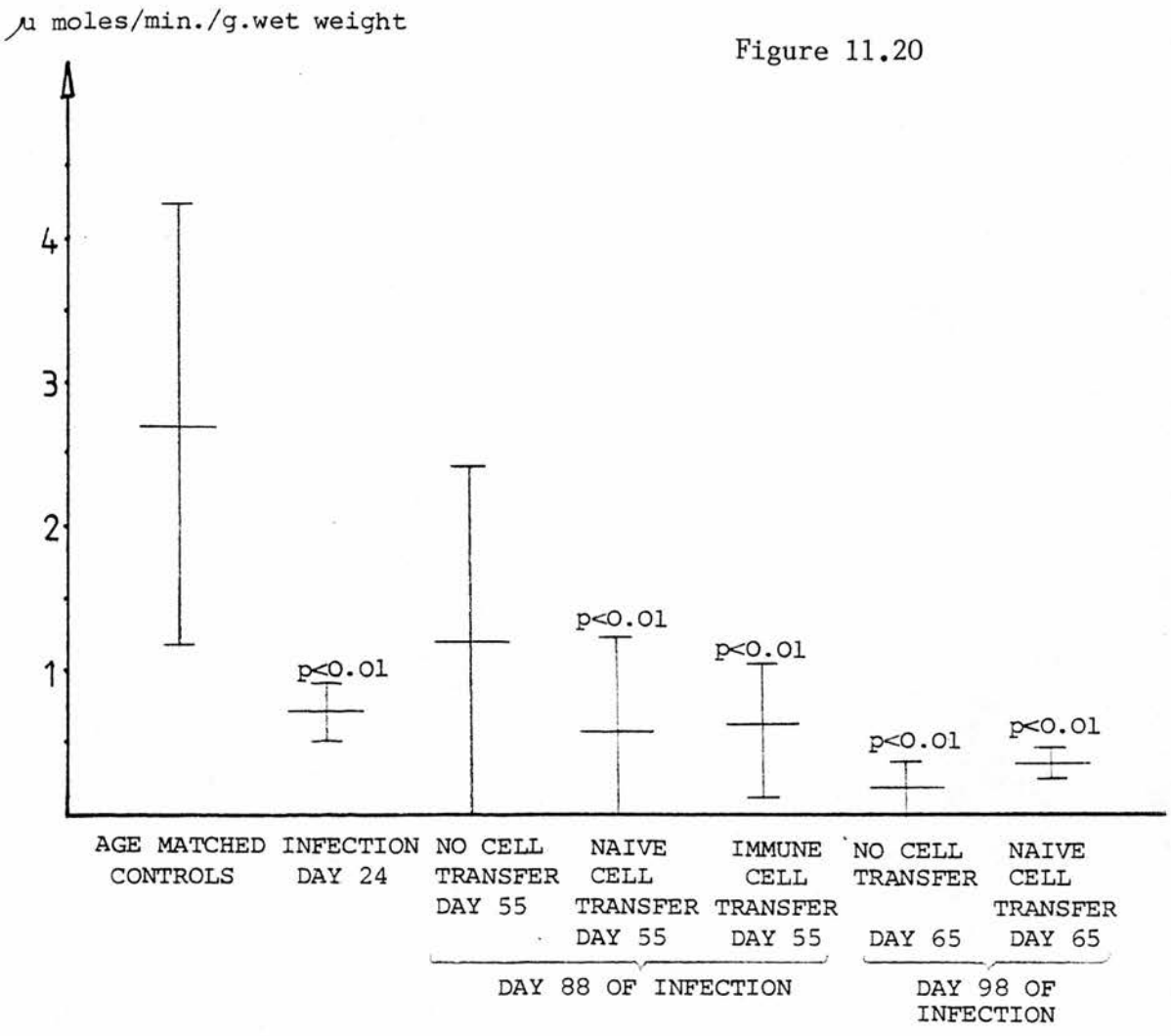


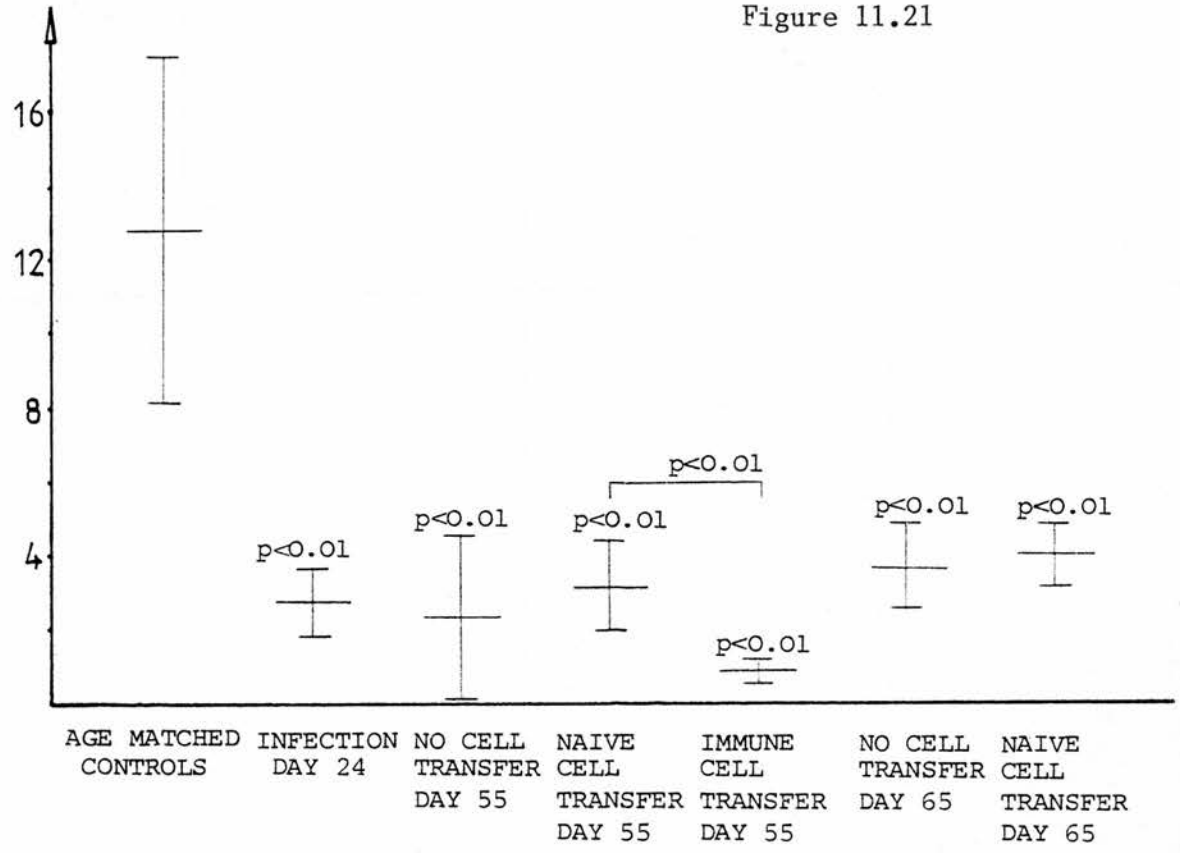
Figure 11.20

G. muris Infection in Reconstituted Nude (Nu/nu) Mice

Effect of G. muris infection on Lactase activity of adult female nude (athymic) mice, BALB/c background, which received on day 33 of primary infection 0.3ml RPMI 1640 (No Cell Transfer group) or 7.5×10^7 Spleen and MLN cells from either uninfected (Naive Transfer) or previously infected and immunologically cured (Immune Transfer) heterozygote littermate donors. Bars represent means $\pm$ 1 SD (six mice per group).

μ moles/min./g.wet weight

Figure 11.21



G. muris Infection in Reconstituted Nude (nu/nu) Mice

Effect of G. muris infection on Sucrase activity of adult female nude (athymic) mice, BALB/c background which received on day 33 of primary infection 0.3ml RPMI 1640 (No Cell Transfer group), or 7.5×10^7 Spleen and MLN cells from either uninfected (Naive Transfer) or previously infected and immunologically cured (Immune Transfer) heterozygote littermate donors. Bars represent means $\pm$ 1 SD (six mice per group).

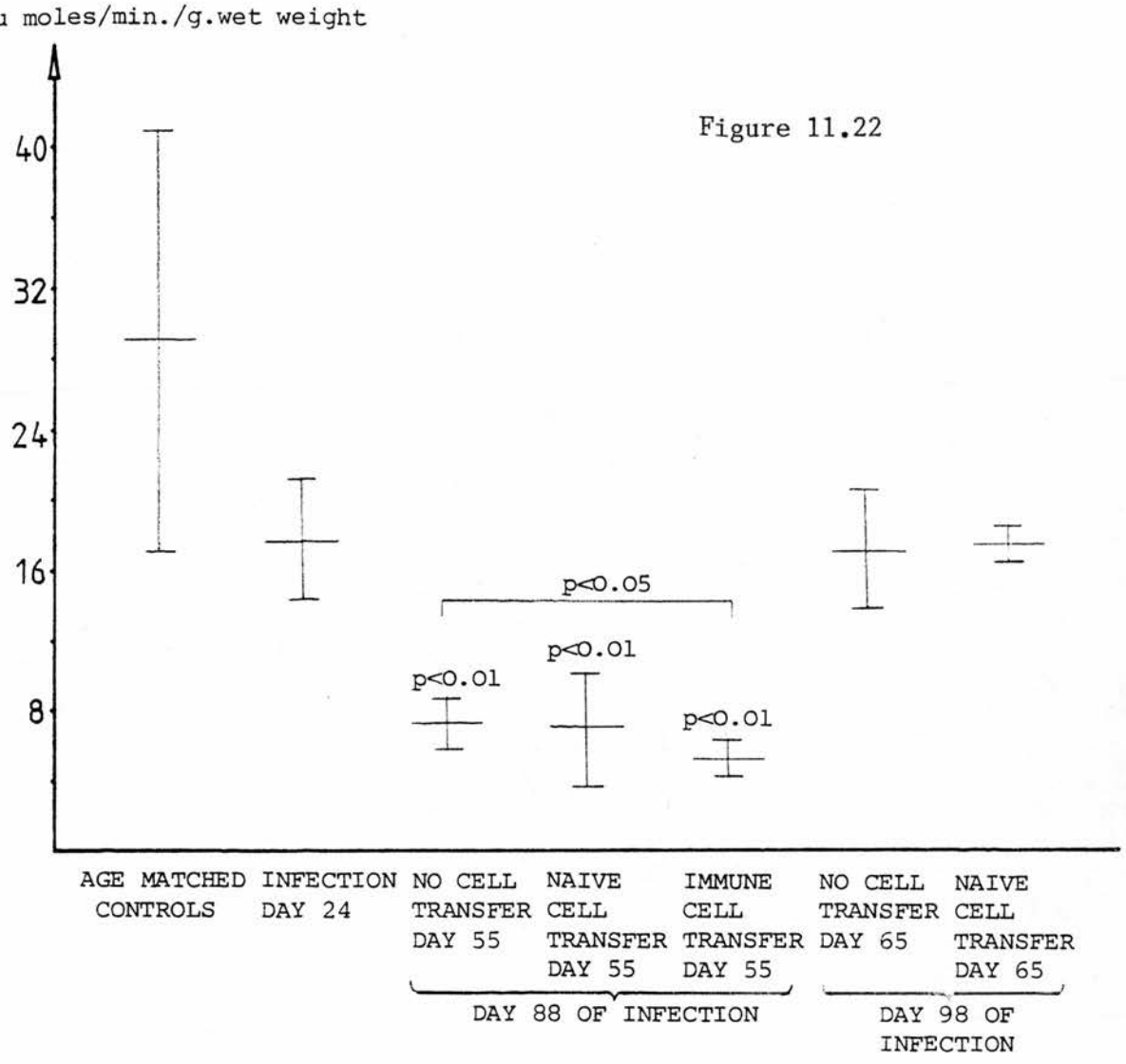
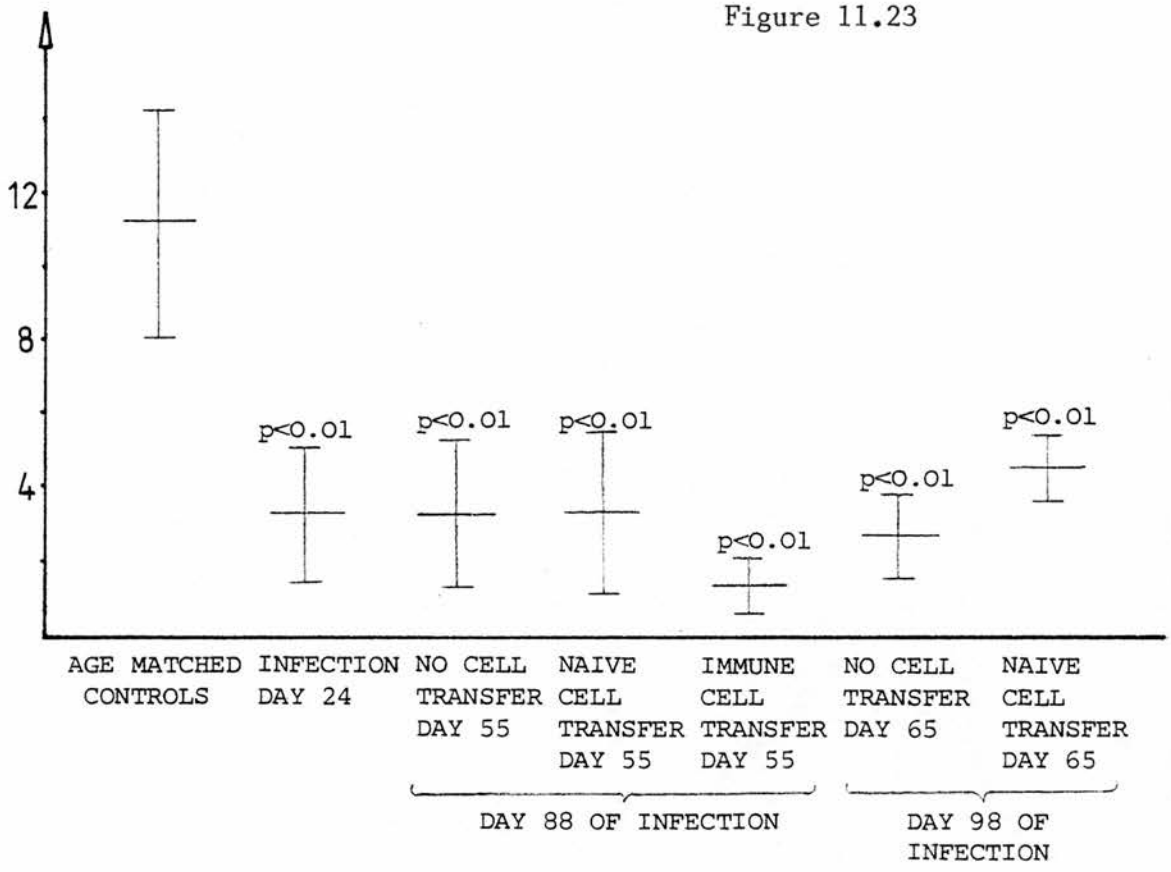


Figure 11.22

G. muris Infection in Reconstituted Nude (nu/nu) Mice

Effect of G. muris infection on Maltase activity of adult female nude (athymic) mice, BALB/c background, which received on day 33 of primary infection 0.3ml RPMI 1640 (No Cell Transfer group) or 7.5×10^7 Spleen and MLN cells from either uninfected (Naive Transfer or previously infected and immunologically cured (Immune Transfer) heterozygote littermate donors. Bars represent means $\pm$ 1 SD (six mice per group).

Figure 11.23



G. muris Infection in Reconstituted Nude (nu/nu) Mice

Effect of G. muris infection on Trehalase activity of adult female nude (athymic) mice, BALB/c background, which received on day 33 of primary infection 0.3ml RPMI 1640 (No Cell Transfer group) or 7.5 x 10^7 Spleen and MLN cells from either uninfected (Naive Transfer) or previously infected and immunologically cured (Immune Transfer) heterozygote littermate donors. Bars represent means ± 1 SD (six mice per group).

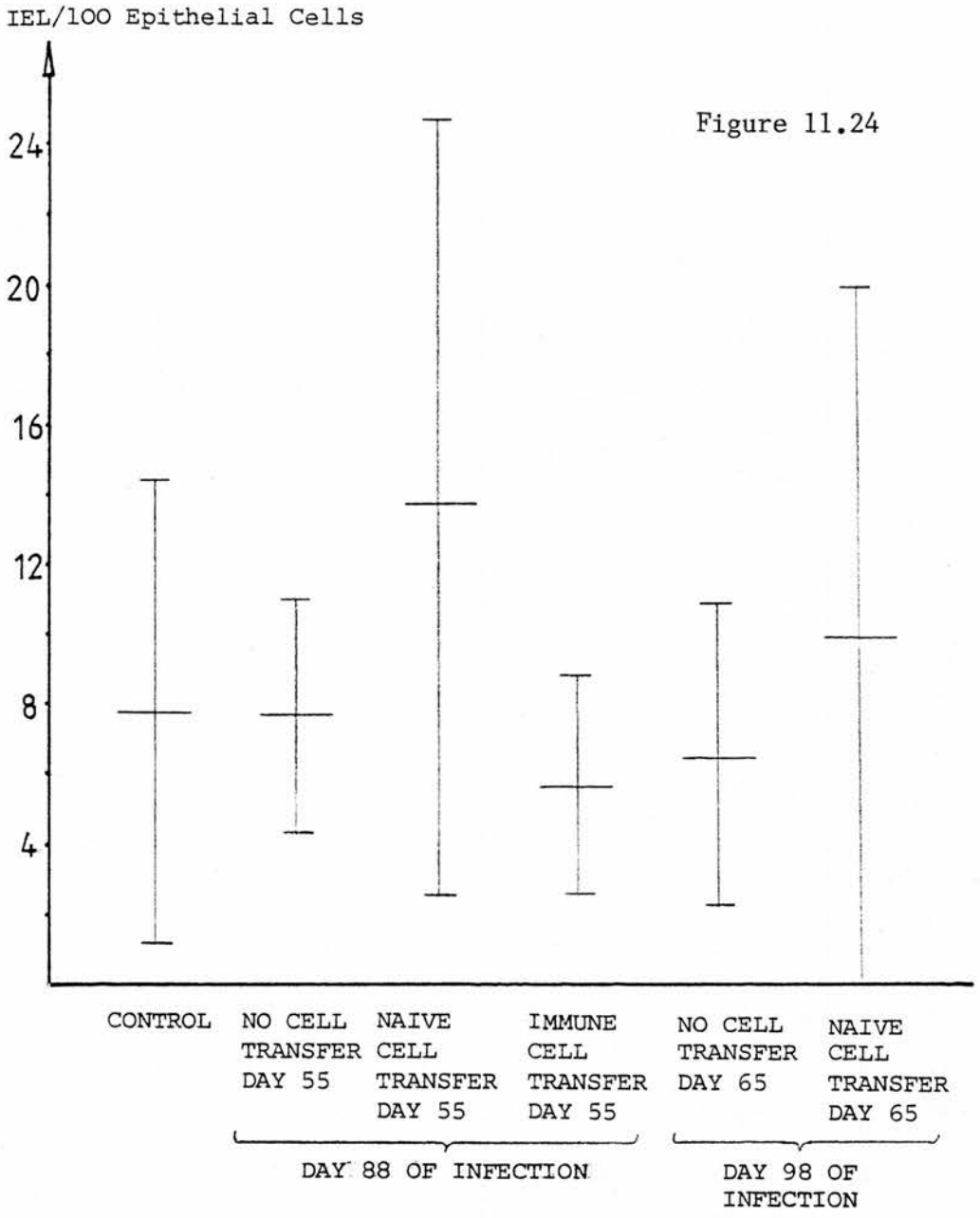


Figure 11.24

G. muris Infection in Reconstituted Nude (nu/nu) Mice

Effect of G. muris infection on numbers of IEL per 100 epithelial cells in adult female nude (athymic) mice, which received on day 33 of primary infection 0.3ml RPMI 1640 (No Cell Transfer group) or 7.5×10^7 Spleen and MLN cells from either uninfected (Naive Transfer) or previously infected and immunologically cured (Immune Transfer) heterozygote littermate donors. Bars represent means $\pm$ 1 SD (six mice per group).

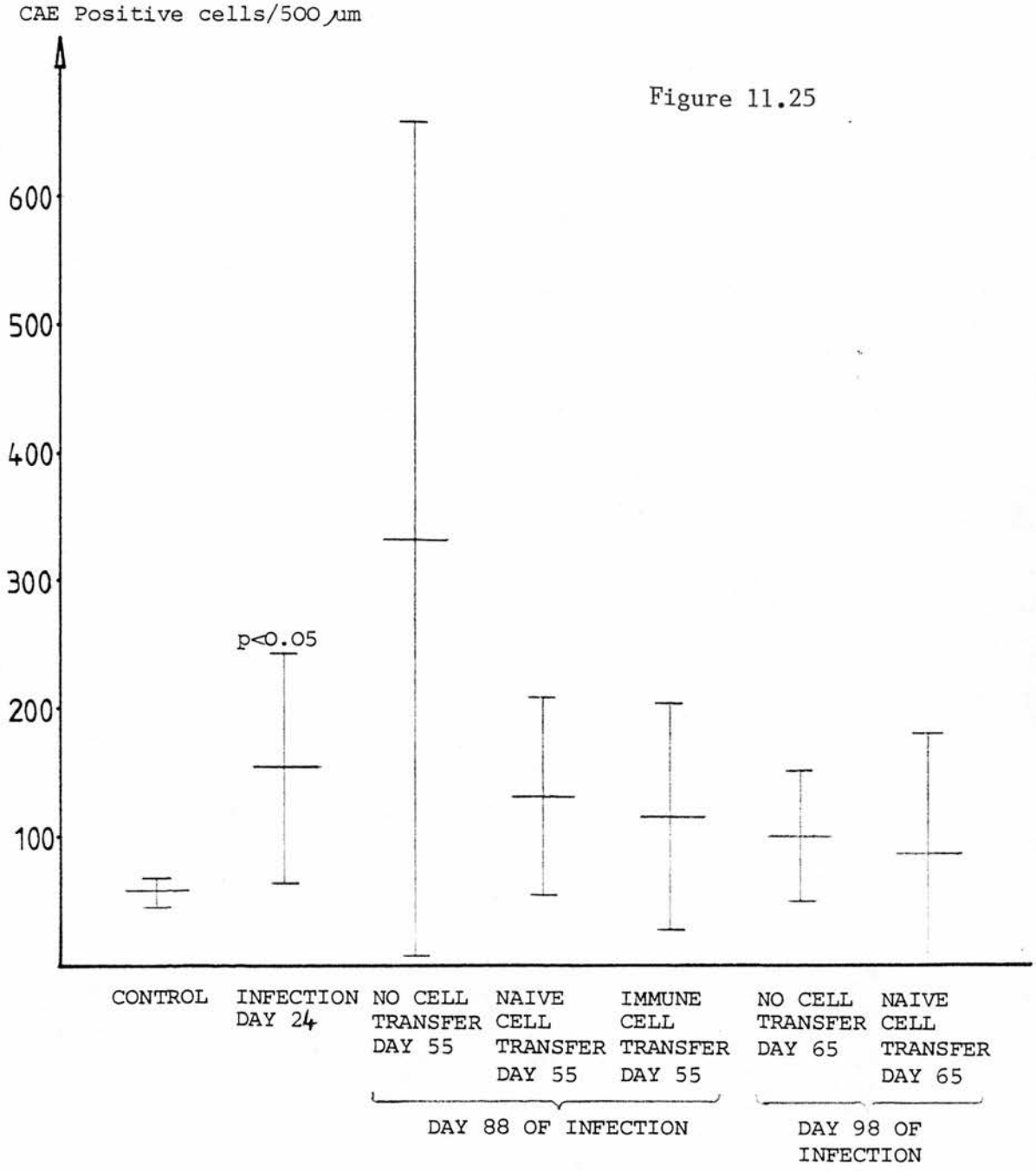


Figure 11.25

G. muris Infection in Reconstituted Nude (nu/nu) Mice

Effect of G. muris infection on number of CAE positive (MMC) cells of adult female nude (athymic) Mice, BALB/c background, which received on day 33 of primary infection 0.3ml RPMI 1640 (No Cell Transfer group) or 7.5×10^7 Spleen and MLN cells from either uninfected (Naive Transfer) or previously infected and immunologically cured (Immune Transfer) heterozygote littermate donors. Bars represent means $\pm$ 1 SD (six mice per group).

Chapter 12

G. muris Infection and Steroid Treatment

Introduction.

The aim of this series of experiments was to investigate the changes brought about by steroid treatment of apparently G. muris free, immune mice, that allow the development of a recrudescent infection.

The recrudescence of G. muris infection after steroid treatment was described by Nair et al (1981). It was found that a single s.c. injection of 2.5 mg cortisone acetate one day prior to infection of CBA mice resulted in higher trophozoite counts and prolonged infection compared to the infection in untreated CBA mice. Higher numbers of trophozoites were found ten days after infection of mice treated with the steroid at both one, and two weeks prior to infection. It was shown that this single dose of cortisone acetate induced recrudescence of occult G. muris infection in the immune CBA mice, within 48 hours of treatment.

The dose of 2.5 mg cortisone acetate administered subcutaneously to each mouse would have profound and lasting effects on the immune system, suppressing various responses for different lengths of time (Dracott & Smith 1979a,b). This study was to investigate the parameters previously studied in primary infections and assess any changes in these that may allow the development of the recrudescent infection.

Materials.

Animals.

The mice used in the first experiment were adult male CBA strain, about 30 weeks old. These mice had been infected at 6 weeks of age and were not excreting cysts before treatment.

All experiments subsequent to this were in adult female BALB/c mice, infected at 5 to 6 weeks of age and left for a minimum of 6 weeks to allow immunity to develop and the infection to resolve.

Steroids.

The glucocorticosteroids used in treatments were cortisone acetate (Cortistab (R) [aqueous suspension], Boots) and hydrocortisone sodium succinate.

Methods.

The majority of methods used in this investigation have been previously described, but those that are new are described in the appropriate experimental protocol sections.

Experiment 1.

Cortisone Acetate Stimulated Recrudescent Infection.

This experiment was designed to repeat the work described by Nair et al (1981) and establish the time course of the detectable recrudescent infection following subcutaneous injection of 2.5 mg cortisone acetate to each mouse (Fig. 12.1). Faecal cysts became detectable 24 hours after steroid treatment and rose rapidly until day 4 when the experiment was terminated.

This preliminary experiment showed the very rapid response of the recrudescent infection following steroid treatment. This experiment relied upon the depot effect of the acetate presentation of the steroid, so was not flexible enough to enable more detailed studies. To overcome this problem, the subsequent experiments were designed to investigate the use of hydrocortisone sodium succinate, which results in an immediate systemic corticosteroid effect, which is of short duration.

Experiment 2.

Pathology of Steroid Induced Recrudescent Infection.

This experiment was designed to compare the pathological changes found in the recrudescent infection with those known to occur during a primary infection. The results would indicate whether hydrocortisone had any effect on the gut of uninfected mice. The postnatal development of the gut can be accelerated by steroid treatment (Goland & Forster 1974, Henning 1981).

G. muris infection has been found to result in prolonged or permanent increases in jejunal villus height, disaccharidase activity levels and IEL numbers, from weeks three and four, to at least week ten of infection in CBA mice (Gillon et al 1982a). This

experiment would examine these changes in the BALB/c mouse strain.

The steroid induced recrudescent infection involves over ten times the number of trophozoites compared to a primary infection. The resultant changes in the gut bearing this massive parasite load were to be examined and compared to the control groups (see below).

The hydrocortisone dose equivalent to the cortisone dose used previously was calculated as 2.0 mg per mouse, and this was administered subcutaneously, as before. The experiment required four groups of age matched animals, as follows:

A. Naive mice receiving daily saline s.c. injections.
B. Naive mice receiving daily hydrocortisone sodium succinate s.c. injection.
C. Immune mice receiving daily saline s.c. injections.
D. Immune mice receiving daily hydrocortisone sodium succinate s.c. injections.

Mice were treated for five days, during which the progression of the recrudescent infection was verified by the observation of faecal cysts. All mice were also examined for the presence of trophozoites at sacrifice by smearing specimens of jejunum in a drop of saline on a microscope slide and scanning the preparation at x100.

Animals were treated with colchicine and samples taken from jejunum (10 cm distal to the pylorus) and ileum (5 cm proximal to the ileocaecal junction) for microdissection, IEL counts, MMC counts and disaccharidase estimation.

Results.

Mucosal Architecture.

The jejunal mucosal architecture (Fig. 12.2) showed that the villi of previously infected, immune mice were very significantly lengthened ($p<0.01$) compared to the saline treated control group; G. muris infection results in a prolonged lengthening of villi.

Steroid treatment and recrudescent infection of five days duration resulted in significantly ($p<0.01$) increased villus length compared to naive, saline treated controls. The crypt depth was not

altered in any group, nor was the CCPR changed in any group.

The ileal mucosal architecture (Fig. 12.3) also showed that steroid treatment and recrudescent infection result in a highly significant ($p<0.01$) increase in villus height compared to naive mice receiving hydrocortisone and a significant increase ($p<0.05$) compared to previously infected immune mice receiving saline treatments.

The crypt depth in the group receiving hydrocortisone and resultant recrudescent infection is significantly ($p<0.05$) increased compared to naive mice receiving saline. The CCPR is not altered in any group.

Mucosal Function.

Jejunum.

G. muris infection resulted in significantly ($p<0.05$ and $p<0.01$) prolonged reduction in jejunal lactase activity levels (Fig. 12.4), which was not further exacerbated by the steroid induced recrudescent infection of five days duration.

G. muris infection also resulted in significant ($p<0.05$) or very significant ($p<0.01$) prolonged elevations of jejunal sucrase activity levels (Fig. 12.5), which are not further altered by the steroid induced recrudescent infection of five days duration.

Ileum.

There was no significant alteration in ileal lactase levels in any group (Fig. 12.6). However, G. muris infection resulted in significant ($p<0.05$) prolonged elevations in ileal sucrase activity levels. These were very variable, but the trend towards increased activity was significant. The steroid induced recrudescent infection of five days duration did not significantly alter this elevated sucrase activity.

Cellular Changes.

Intraepithelial Lymphocytes (IEL).

The recrudescent G. muris infection stimulated by five days of steroid treatment resulted in significantly increased numbers of IEL

in jejunal specimens (Fig. 12.8). IEL numbers in the ileum of mice bearing a recrudescent infection after five days of steroid treatment (Fig. 12.9) were significantly ($p<0.05$) increased compared to similar previously infected, immune mice receiving control saline treatments, but not significantly different to the values of the two naive control groups.

The results for jejunal mucosal architecture, kinetics, function, and numbers of IEL during the period after expulsion of G. muris by the BALB/c mouse are similar to those of Gillon et al (1982) regarding giardiasis in the CBA mouse. However, these measurements in the ileal mucosa mirror the jejunal changes, although they were less marked, and so are at variance with the above published results. This may stem from a species difference in small intestinal characteristics of morphology or response to giardiasis.

Mucosal Mast Cells (MMC).

The number of jejunal MMC was not significantly altered in any group (Fig. 12.10).

The number of ileal MMC (Fig. 12.11) did show a significant ($p<0.01$) difference between the high value from naive mice receiving steroid treatments and the lower value of mice bearing the steroid induced recrudescent infection of 5 days duration. These results are discussed later in this chapter.

Experiment 3.

Limited Duration Treatments of Rapid Acting Steroid and Recrudescent Infection.

BALB/c female mice were infected with a standard infective dose of 2,000 cysts i.g., at six weeks of age. Infection was confirmed in each animal by faecal cyst detection and animals allowed to develop immunity to G. muris and eliminate the infection.

The steroid was administered to groups of twelve mice under the following regimes:

A. Continuous Control: daily saline injections from the first day of experiment (day 0 on Figs.).
B. One Treatment: one hydrocortisone injection on the first day of experiment.
C. Five Treatments: five, daily hydrocortisone injections from the first day of experiment.
D. Continuous Treatment: daily hydrocortisone injections from the first day of experiment.

Results.

Faecal Cyst Counts.

The daily saline injections were included to parallel the stress induced in the experimental groups. This stress will increase output of endogenous corticosteroids by the adrenals and this is known to be an important factor in some experimental models. The saline treatment had no influence in this experiment, no cysts being detected during the trial period (Fig. 12.12). The application of one hydrocortisone injection resulted in a transient faecal cyst output one day after treatment in two of the six animals examined. The faecal cyst count had returned to zero by day three after the single treatment

Daily injection of hydrocortisone for five days resulted in detectable faecal cysts by the fourth day of treatment, and which was sustained until day 6, 1 day after the last hydrocortisone treatment. However, by day 8, three days after the last treatment, faecal cyst counts had returned to zero.

Continuous daily treatment with hydrocortisone resulted in detectable faecal cysts by day four of treatment and numbers rose to a plateau for the duration of the steroid treatment.

Trophozoite Counts.

Mice were killed and jejunal specimens taken on days 0, 7 and 14. Following this, the remaining lenghts of gut were vibrated to obtain trophozoite counts.

Trophozoite counts (Fig. 12.13) reflect those of the cyst counts; no detectable trophozoites in any control group; few found on day 7 in the single steroid treatment group, and in this group only found in one of six mice sacrificed at this time. The groups receiving five days of treatment and continuous treatments showed high numbers of trophozoites at day 7 and day 14.

An interesting feature of the group which received five daily treatments of hydrocortisone was noted after treatment ceased. Faecal cyst output fell rapidly, and no cysts were found on day 8 (Fig. 12.12). The trophozoite count from these mice showed that a considerable number of parasites were present in the gut on day 14, when faecal cyst output was zero. This is a similar situation to that of the periods when G. muris does not encyst, but the characteristics of the infection are otherwise unchanged. Both situations are so far unexplained.

The rapid decline in faecal cyst numbers upon cessation of hydrocortisone sodium succinate treatment, and parallel decline in trophozoites in this group, stimulated interest in this post-steroid treatment period.

Experiment 4.

Steroid Treatment and Primary Infection: I.

The increased metabolic activity apparent in trophozoites from steroid treated mice prompted a theory that the steroid might have a direct stimulatory effect upon the trophozoites. This theory was tested by injecting naive mice with 2.5 mg cortisone acetate s.c., at weekly intervals, from one week prior to infection i.g. with either 2,000 trophozoites or 2,000 cysts of G. muris.

Results.

The infections initiated by cysts (Fig. 12.14) show that the faecal cyst output of cortisone acetate treated mice is significantly elevated compared to the normal primary infection.

This pattern was repeated in the faecal cyst counts of the groups infected with trophozoites (Fig. 12.15), the steroid treated mice showing significant increases in faecal cyst output.

Had the sole effect of the steroids been to stimulate the G. muris trophozoites to be metabolically active and so multiply rapidly, the immune response would develop to control and eliminate the infection. The work of Dracott & Smith (1979a,b) suggested generalized and lasting immunosuppression with single treatments of this amount of steroid. A final experiment was performed to detect the suppression of the immune response to G. muris.

Experiment 5.

Steroid Treatment and Primary Infection: II.

This experiment was designed to parallel the previous experiment over the initial period of the normal primary infection, to the resolution of this infection.

Again, animals were treated with weekly s.c. injections of 2.5 mg/mouse, cortisone acetate, from one week prior to infection and the final steroid injection was given on day 58 of infection.

Results.

The faecal cyst output results (Fig. 12.16) again show significant increases from day 14 onwards in the groups receiving cortisone acetate. The normal untreated primary infection resolved on day 35, but the steroid treated groups maintained high faecal cyst output. The remaining steroid treated group showed no trend towards resolving the infection, even when steroid treatment was withdrawn, up to day 101 of infection, approximately six weeks after the last steroid treatment. By day 165 of infection, 107 days after the last steroid treatment, the infection had resolved.

Summary and Conclusions.

A recrudescence of G. muris infection in previously infected, apparently immune mice can result from the use of a depot steroid, cortisone acetate, or a steroid which attains maximum serum concentration about 100 minutes after injection, hydrocortisone sodium succinate.

The recrudescent infection showed some of the characteristics associated with a primary infection, such as increased villus

height, increased crypt depth and altered mucosal function. Some of these changes appear to be long lasting or permanent, as they are present in mice previously infected but which are immune and apparently free of any G. muris infection.

Many of the changes in the jejunal morphology and function are paralleled in the ileum. G. muris is primarily a jejunal parasite, though trophozoites do occur in the ileum. The changes may simply be of an organ specific nature, therefore the whole small intestine is affected, rather than strictly local changes. A more likely explanation is due to trophozoites, cysts and parasite antigens passing through the ileum; as an immune response is active against these antigenic targets in the jejunum, an equivalent immune response is stimulated by these same antigens in the ileum, and this results in similar changes in this area of the small intestine.

An IEL increase was also noted in the recrudescent infection, as occurs in a primary infection.

The number of MMC in the jejunum also increased during recrudescent infection, but a significant decrease in these cell numbers was found in the ileum of these animals. This may be attributed to a variety of causes; if MMC are involved, an increase in their number may not be detected due to degranulation, and subsequent staining is therefore poor or absent. Secondly, the steroid treatment is thought to result in degranulation of MMC as a mode of its immunosuppression, though this was not noted in the appropriate control group. Finally, the AB/S stain does not work as well in mouse tissue as it does in human or rat tissue. This poor positive staining of granules leads to counting errors. As can be seen, large SD are present in all jejunal and ileal MMC groups (Figs. 12.10 and 12.11) indicating considerable variability within the groups. The use of the rapidly acting hydrocortisone sodium succinate shows that the mediators of immunity against the recrudescent infection are very steroid resisitant and are merely suppressed during high steroid concentrations. Cysts and trophozoites show rapid rises upon commencement of steroid treatment and rapid falls upon withdrawl of treatments. The detectable cyst output falls to zero prior to trophozoite numbers falling to

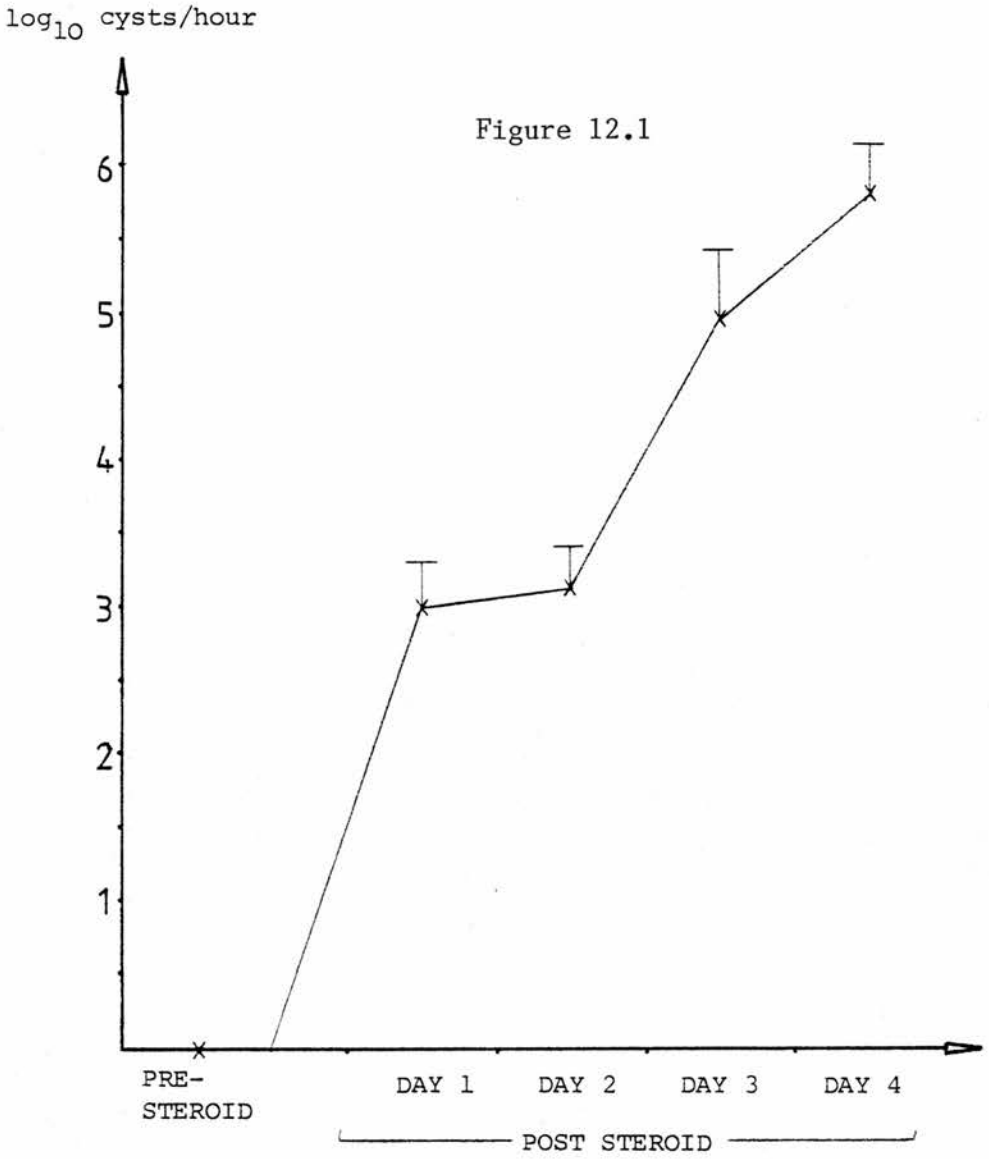
undetectable levels.

The primary infections comparing steroid treated and untreated naive mice show that the steroids create a more favourable environment for trophozoite habitation allowing a higher parasite load to be maintained, as reflected in faecal cyst output.

Alternatively, the steroids may act directly on the trophozoites resulting in increased metabolic activity and increased rate of division; higher trophozoite numbers result, occupying all favourable sites, so an increased overflow of unsuccessful trophozoites enter the lumen, to encyst during passage through the intestine and increased faecal cyst output is noted.

The continuous steroid treatment during primary infection results in an immunosuppression of great duration, but upon withdrawal of the steroid treatment, an effective anti-Giardia immune reponse eventually develops and the infection is resolved.

Figure 12.1



Effect of Cortisone Acetate upon Immune Mice

Effect of 2.5mg Cortisone Acetate, s.c., upon faecal cyst output (G. muris) in previously infected, immune adult male CBA mice. Marks represent means ± 1 SD of faecal cyst output per hour (six mice per group).

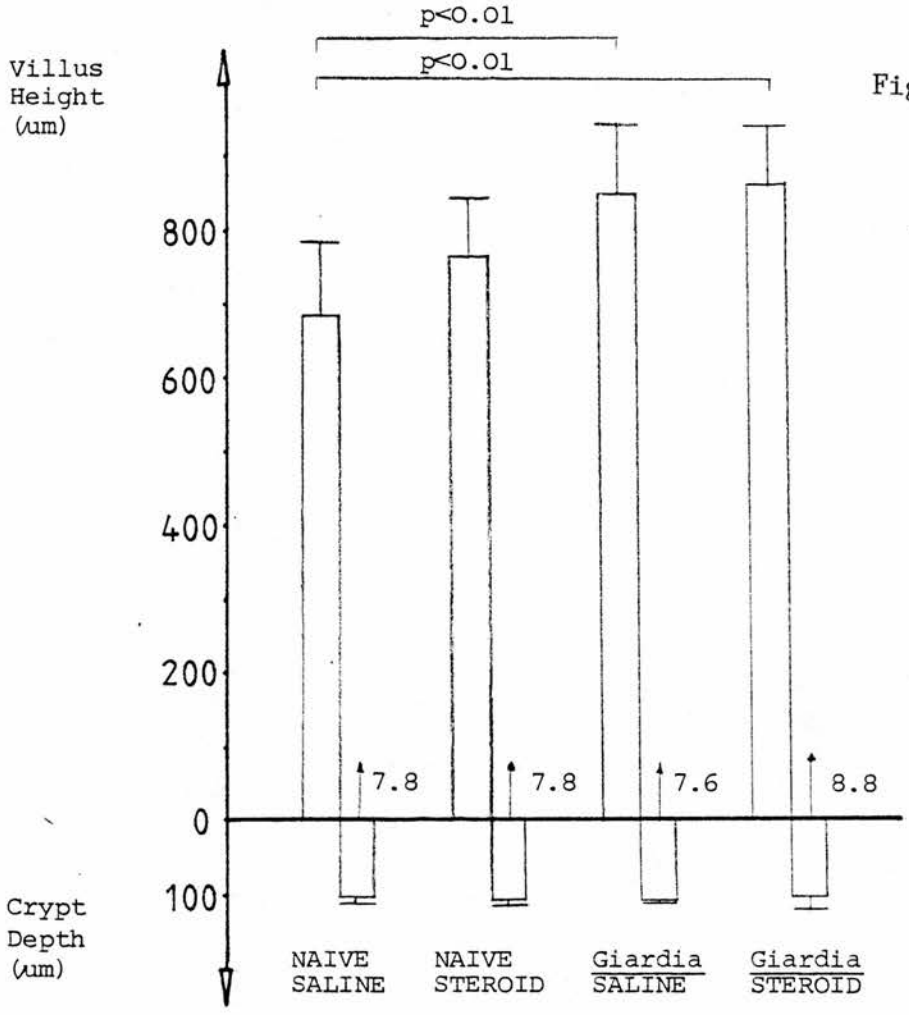
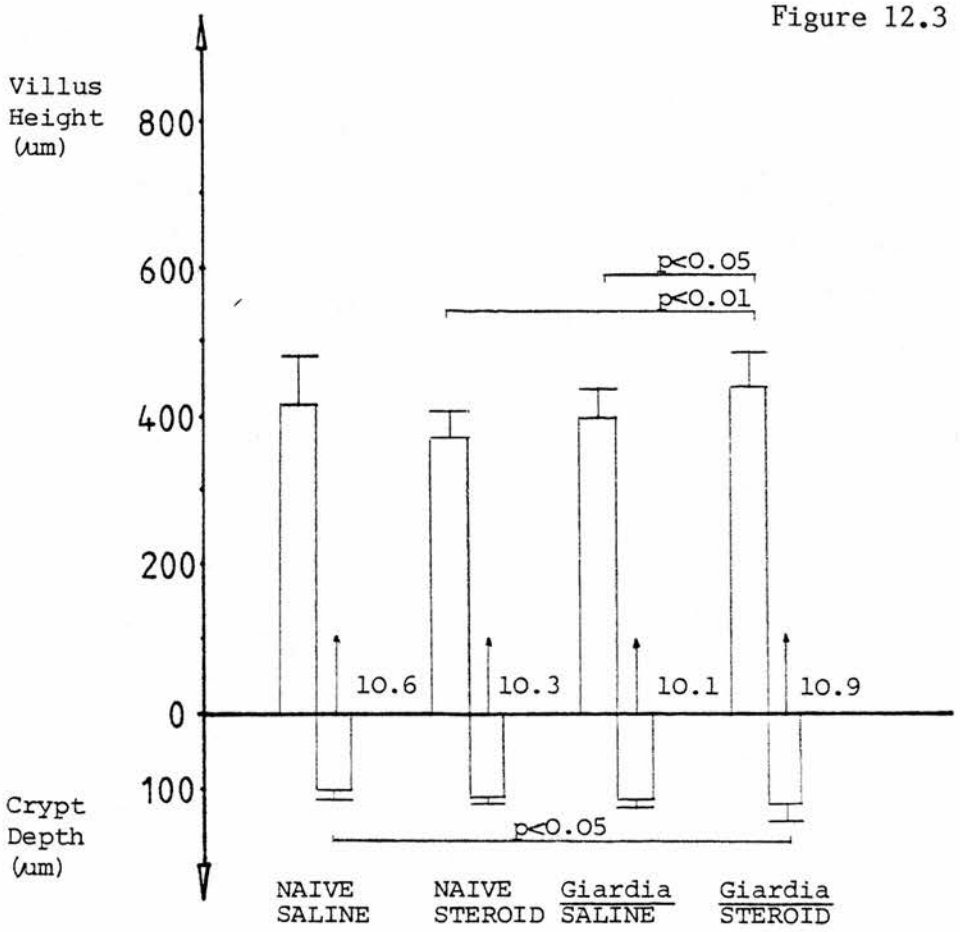


Figure 12.2

Influence of Steroid Induced Recrudescent Infection on Jejunal Mucosal Architecture and Kinetics

Influence of 2.0mg hydrocortisone sodium succinate s.c. injection on 5 consecutive days in naive adult female BALB/c mice, and comparable, previously infected, immune mice. Saline injected controls included in both groups. Bars represent means ± 1 SD for villus height and crypt depth and arrows show CCPR. Eight mice in naive/saline, naive/steroid and Giardia/saline groups; 16 mice in Giardia/steroid group.

Figure 12.3



Influence of Steroid Induced Recrudescent Infection on Ileal Mucosal Architecture and Kinetics

Influence of 2.0mg hydrocortisone sodium succinate s.c. injection on 5 consecutive days in naive adult female BALB/c mice and comparable, previously infected, immune mice. Saline injected controls included in both groups. Bars represent means ± 1 SD for villus height and crypt depth and arrows show CCPR. Eight mice in naive/saline, naive/steroid and Giardia/saline groups; 16 mice in Giardia/steroid group.

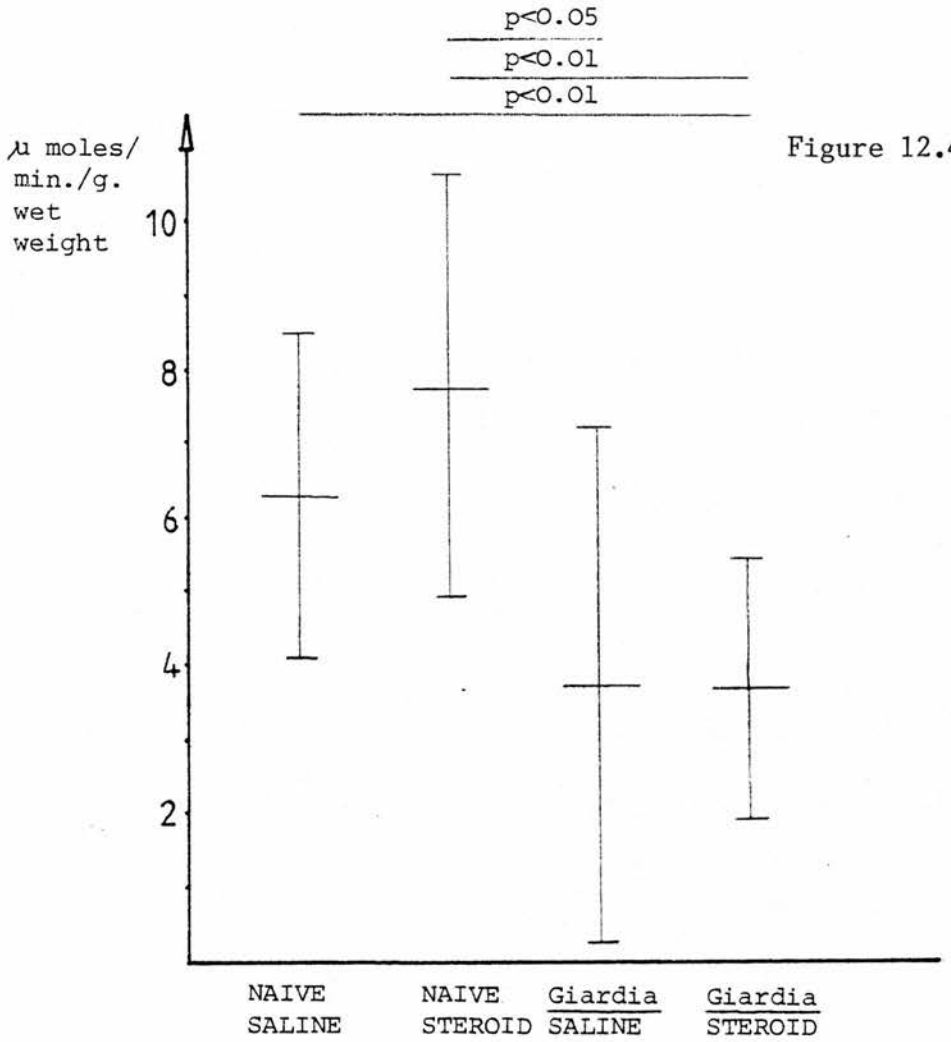


Figure 12.4

Influence of Steroid Induced Recrudescent Infection on Jejunal Lactase Activity Levels

Influence of 2.0mg hydrocortisone sodium succinate s.c. injection on 5 consecutive days in naive adult female BALB/c mice and comparable, previously infected, immune mice. Saline injected controls included in both groups. Bars represent means ± 1 SD for Lactase activity levels. Eight mice in naive/saline, naive/steroid and Giardia/saline groups; 16 mice in Giardia/steroid group.

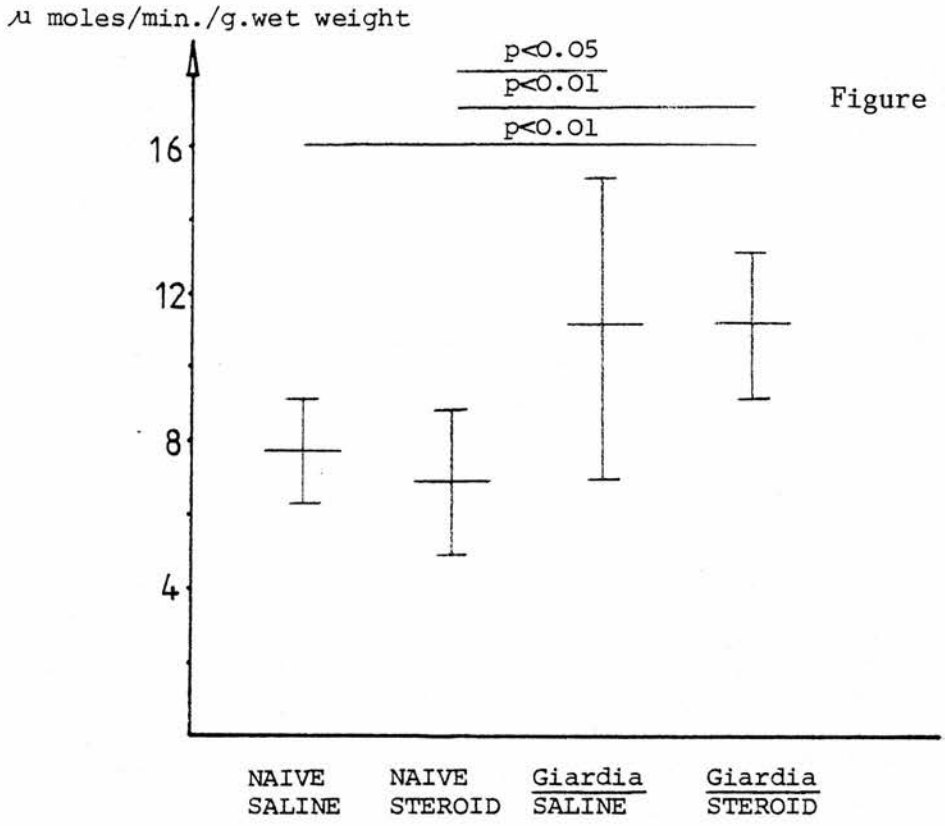


Figure 12.5

Influence of Steroid Induced Recrudescent Infection on Jejunal Sucrase Activity Levels

Influence of 2.0mg hydrocortisone sodium succinate s.c. injection on 5 consecutive days in naive adult female BALB/c mice, and comparable, previously infected, immune mice. Saline injected controls included in both groups. Bars represent means ± 1 SD Sucrase activity levels. Eight mice in naive/saline, Naive/steroid, and Giardia/saline groups; 16 mice in Giardia/steroid group.

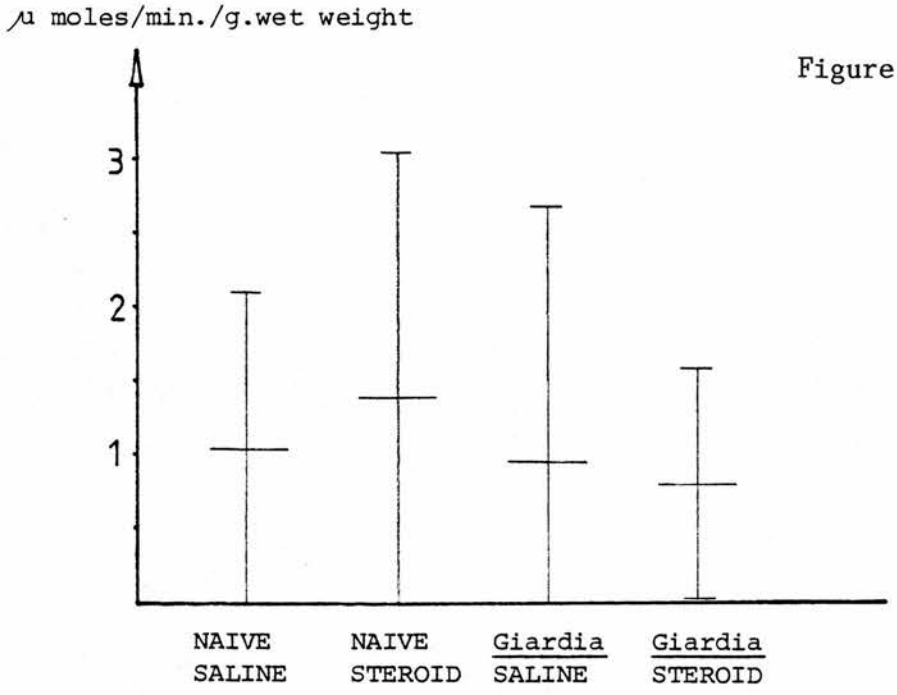


Figure 12.6

Influence of Steroid Induced Recrudescent Infection on Ileal Lactase Acitivty Levels

Influence of 2.0mg hydrocortisone sodium succinate s.c. injection on 5 consecutive days in naive adult female BALB/c mice and comparable, previously infected, immune mice. Saline injected controls included in both groups. Bars represent means ± 1 SD lactase activity levels. Eight mice in naive/saline, naive/steroid and Giardia/saline groups; 16 mice in Giardia/steroid group.

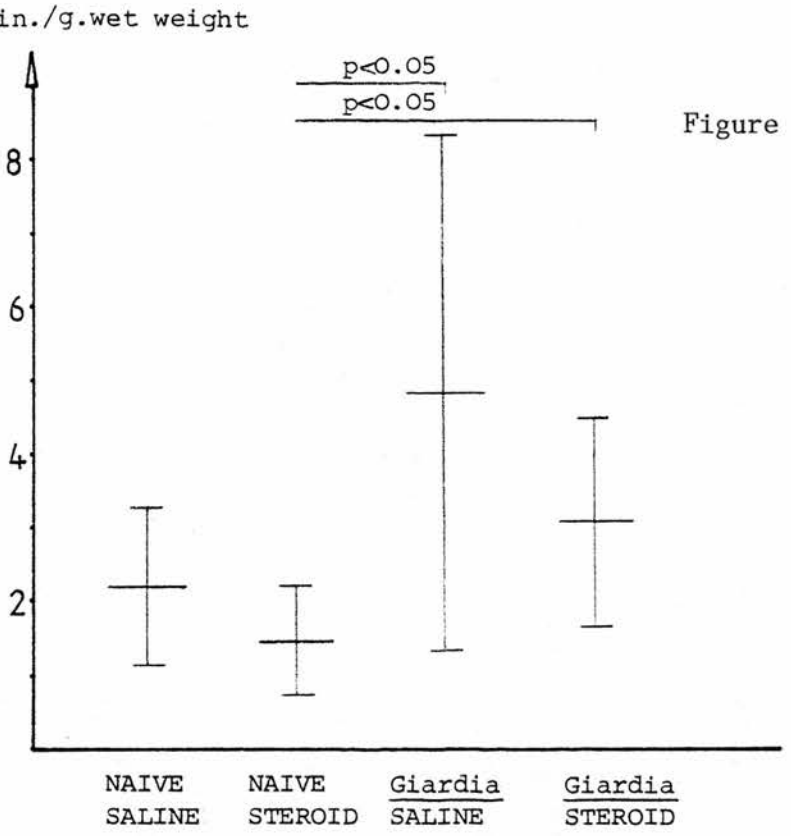
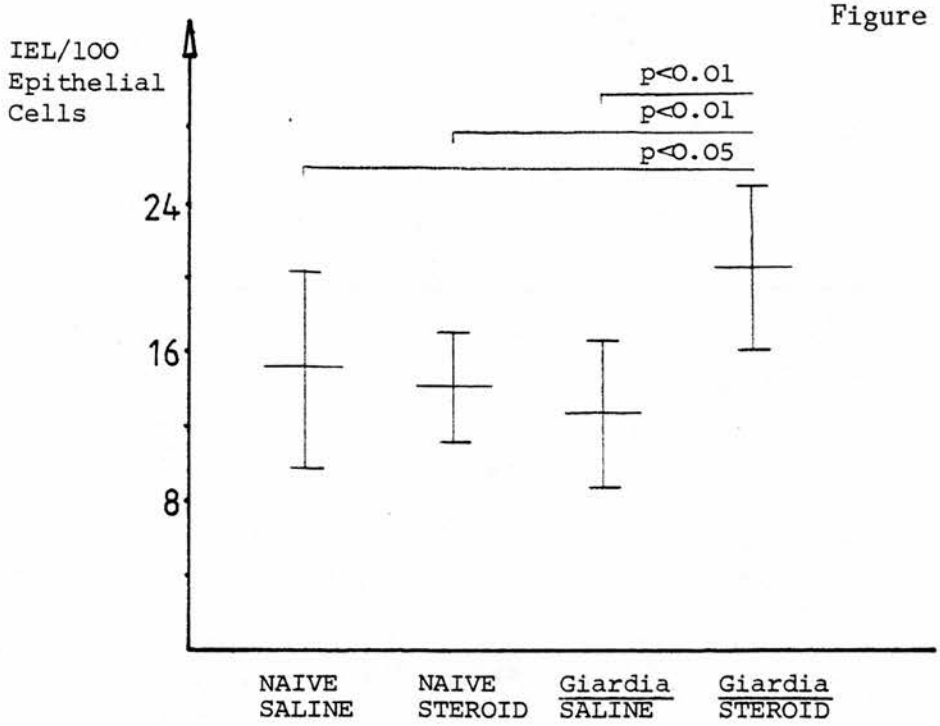


Figure 12.7

Influence of Steroid Induced Recrudescent Infection on Ileal Sucrase Activity levels

Influence of 2.0mg hydrocortisone sodium succinate s.c. injection on 5 consecutive days in naive adult female BALB/c mice, and comparable, previously infected, immune mice. Saline injected controls included in both groups. Bars represent means ± 1 SD for sucrase activity levels. Eight mice in naive/saline, naive/steroid and Giardia/saline groups; 16 mice in Giardia/steroid group.

Figure 12.8



Influence of Steroid Induced Recrudescent Infection on Jejunal IEL Numbers

Influence of 2,0mg hydrocortisone sodium succinate s.c. injection on 5 consecutive days in naive adult female BALB/c mice, and comparable, previously infected, immune mice. Saline injected controls included in both groups. Bars represent means ± 1 SD for IEL per 100 epithelial cells. Eight mice in naive/saline, naive/steroid and Giardia/saline groups; 16 mice in Giardia/steroid group.

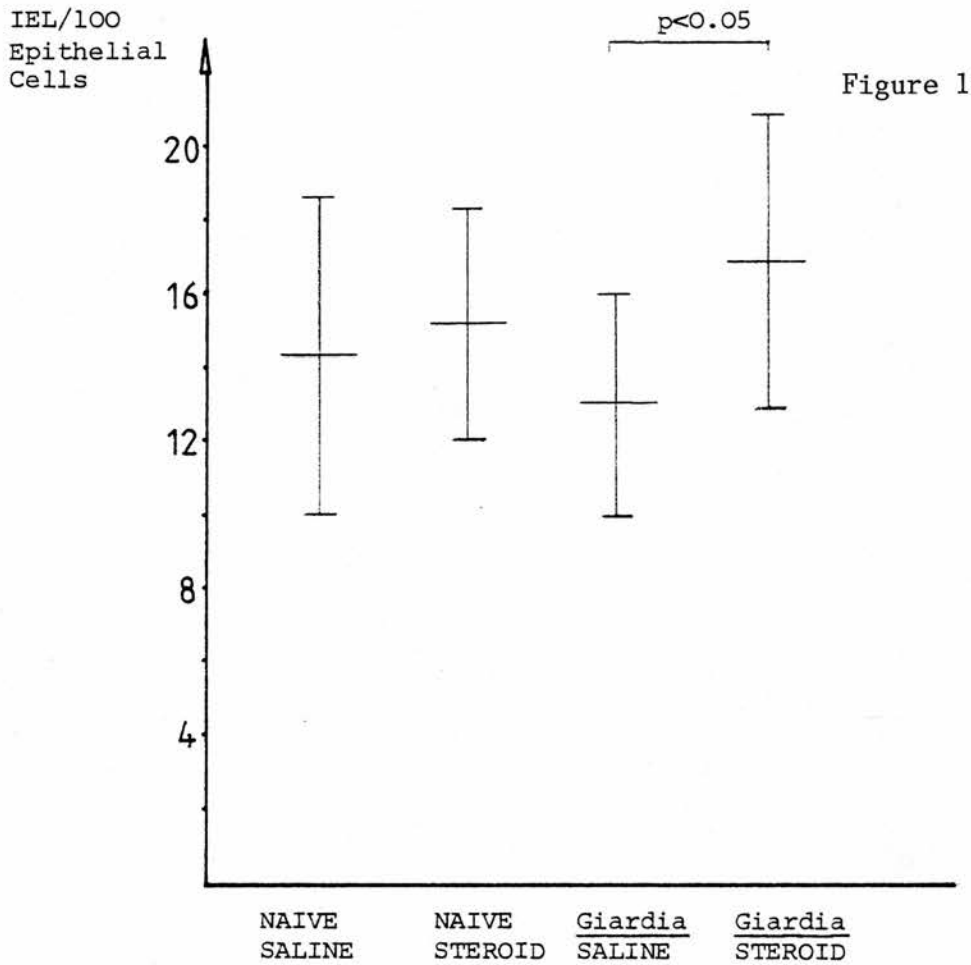
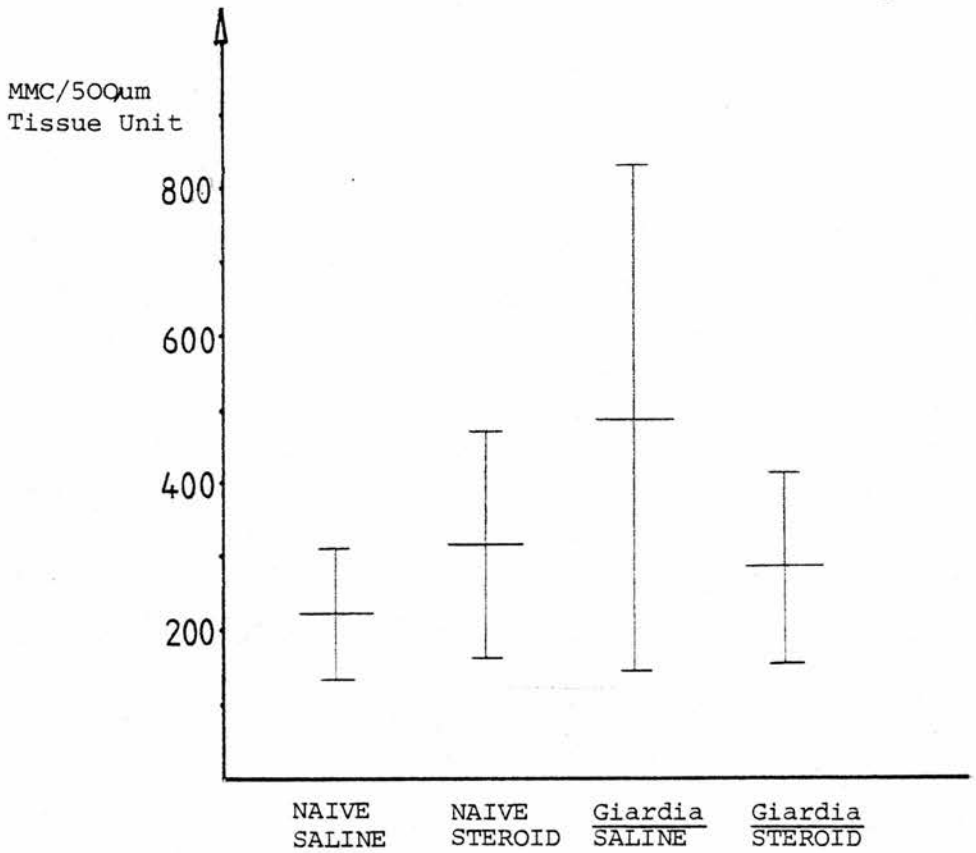


Figure 12.9

Influence of Steroid Induced Recrudescent Infection on Ileal IEL Numbers

Influence of 2,0mg hydrocortisone sodium succinate s.c. injection on 5 consecutive days in naive adult female BALB/c mice, and comparable, previously infected, immune mice. Saline injected controls included in both groups. Bars represent means ± 1 SD of IEL per 100 epithelial cells. Eight mice in naive/saline, naive/steroid and Giardia/saline groups; 16 mice in Giardia/steroid group.

Figure 12.10

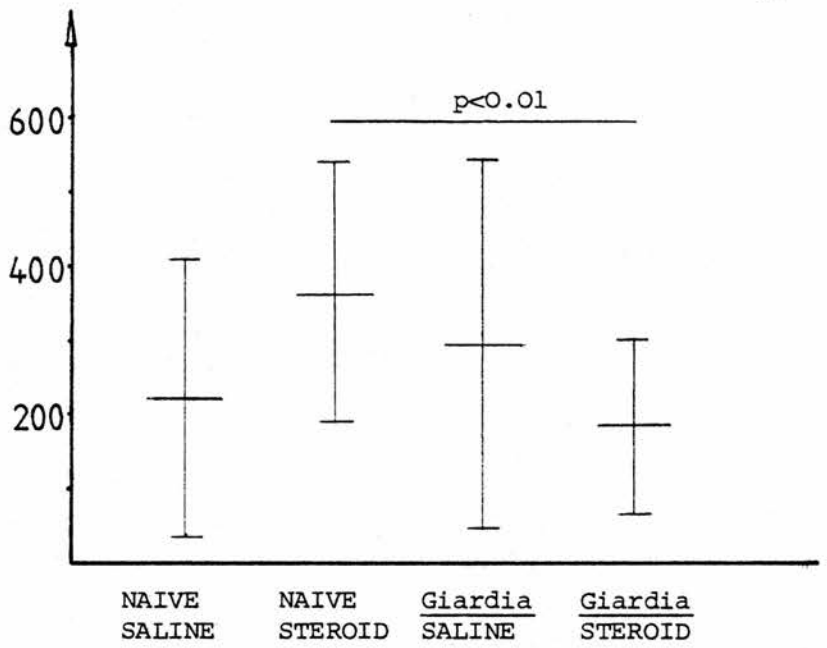


Influence of Steroid Induced Recrudescent Infection on Jejunal Mucosal Mast Cell Numbers

Influence of 2.0mg hydrocortisone sodium succinate s.c. injection on 5 consecutive days in naive adult female BALB/c mice, and comparable, previously infected, immune mice. Saline injected controls included in both groups. Bars represent means ± 1SD of MMC per 500 µm tissue unit. Eight mice in naive/saline, naive/steroid and Giardia/saline groupd; 16 mice in Giardia/steroid group.

MMC/500 µm Tissue Unit

Figure 12.11



Influence of Steroid Induced Recrudescent Infection on Ileal Musocal Mast Cell Numbers

Influence of 2.0 mg hydrocortisone sodium succinate s.c. injection on 5 consecutive days in naive adult female BALB/c mice and comparable, previously infected, immune mice. Saline injected controls included in both groups. Bars represent means ± 1 SD for MMC numbers per 500 µm tissue unit. Eight mice in naive/saline, naive/steroid and Giardia/saline groups; 16 mice in Giardia/steroid group.

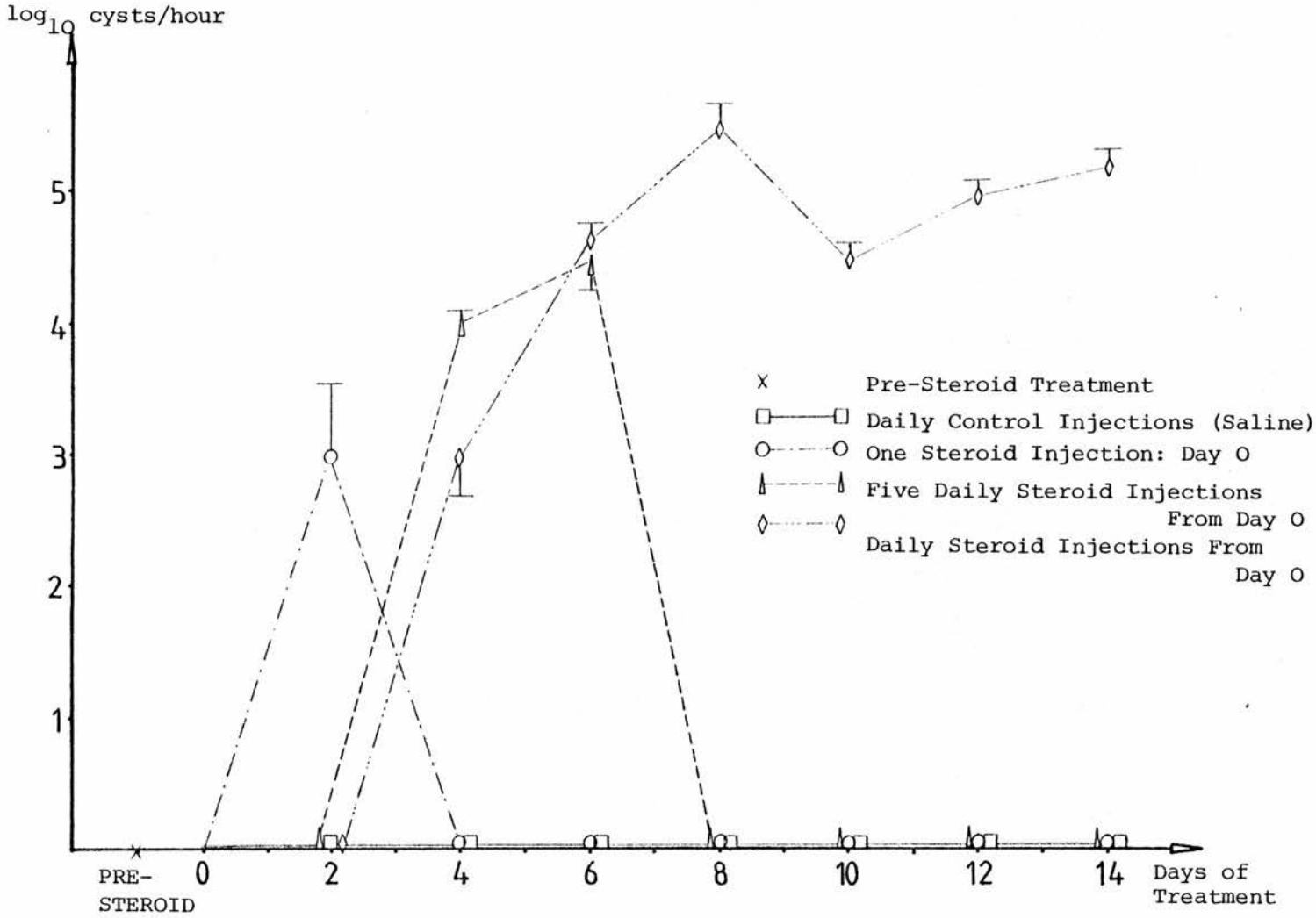
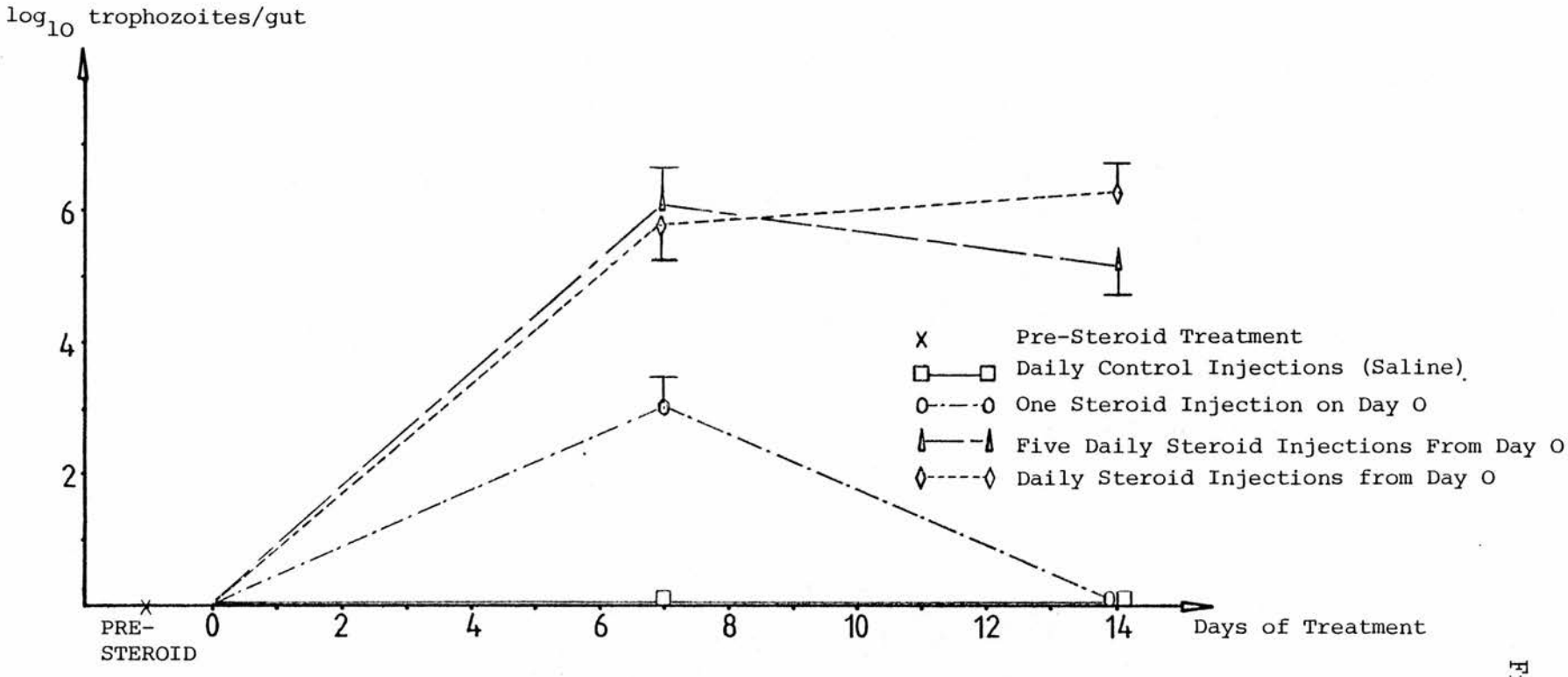


Figure 12.12

Steroid Induced Recrudescence of G. muris Infection

Effect of various durations (see key) of daily s.c. 2.0mg hydrocortisone sodium succinate injections upon the time course of recrudescent infections in immune adult female BALB/c mice. Marks represent means ± 1 SD of faecal cyst output per hour (six mice per group).

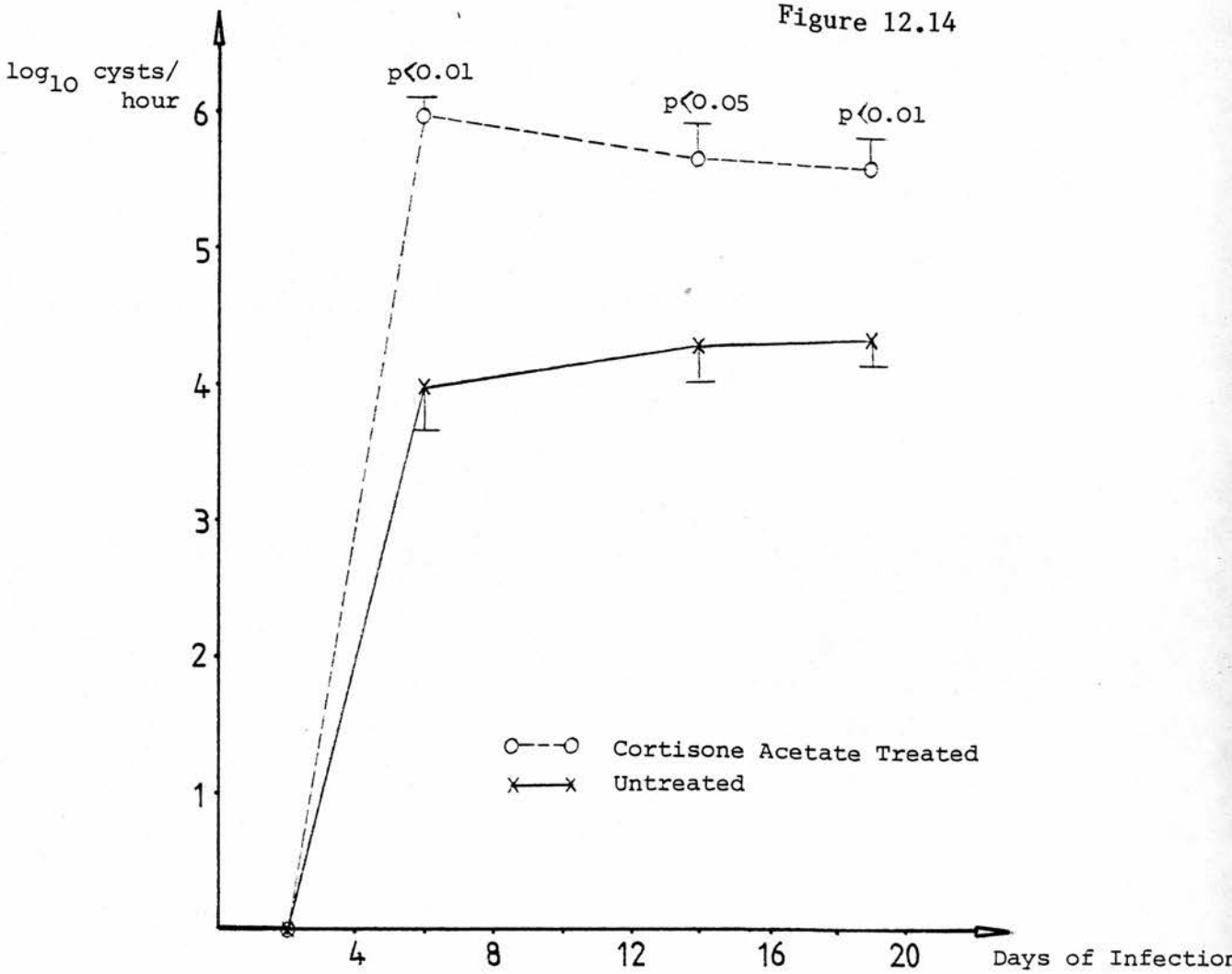


Steroid Induced Recrudescence of G. muris Infection

Effect of various durations (see key) of daily s.c. 2.0mg hydrocortisone sodium succinate injections upon the time course of recrudescent infections in immune adult female BALB/c mice. Marks represent means ± lSD of number of trophozoites per mouse gut (six mice per group).

Figure 12.13

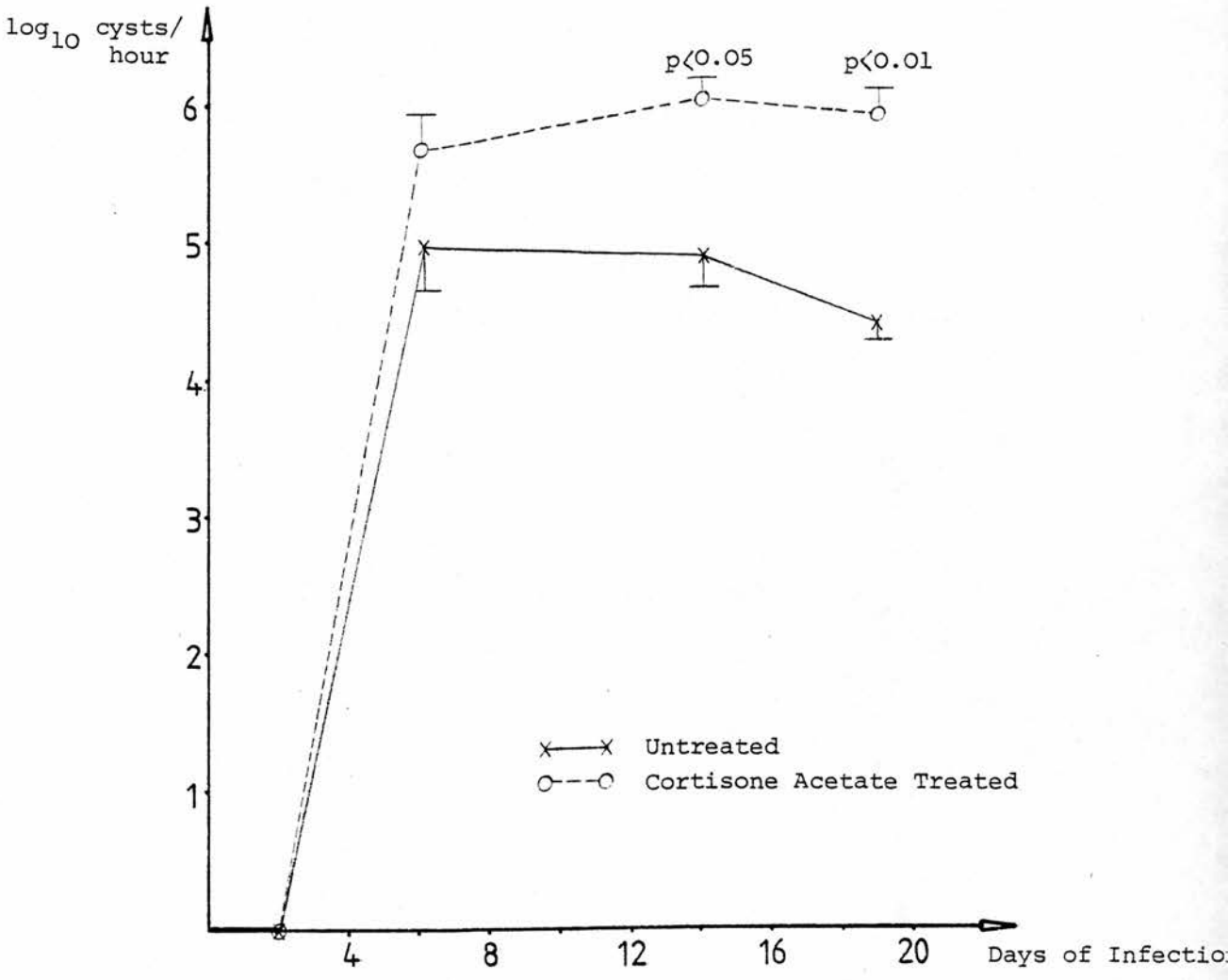
Figure 12.14



Primary G. muris Infections With and Without Steroid Treatment

Development of G. muris primary infection after i.g. intubation of 2,000 cysts, in untreated adult female BALB/c mice and mice treated with weekly s.c. injection of 2.5mg cortisone acetate. Marks represent means ± 1 SD of faecal cyst output (six mice per group).

Figure 12.15



Primary G. muris Infections With and Without Steroid Treatment

Development of G. muris primary infection after i.g. intubation of 2,000 trophozoites, in untreated adult female BALB/c mice and mice treated with weekly s.c. injection of 2.5.mg cortisone acetate. Marks represent means ± 1 SD of faecal cyst output per hour (six mice per group).

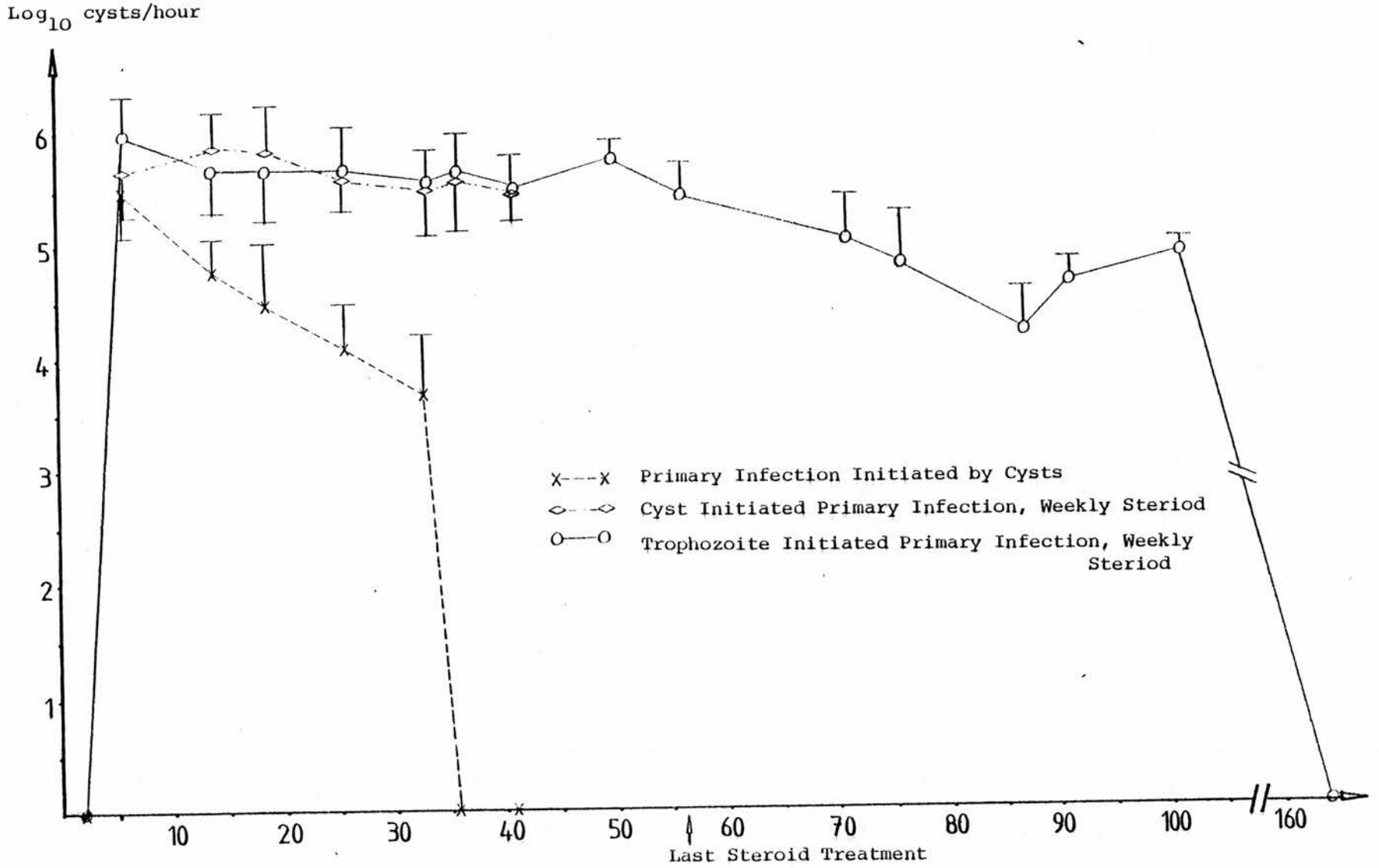


Figure 12.16

Primary G. muris Infections With and Without Steroid Treatment

Time course of G. muris primary infections in adult female BALB/c mice infected by 2,000 cysts or 2,000 trophozoites i.g., with and without weekly s.c. injection of 2.5mg cortisone acetate.

Chapter 13
Discussion

Introduction.

In the work described in this thesis, I established an animal model of giardiasis and examined immune responses, in particular cellular immunity, to the parasite, G. muris.

Giardia is a rapidly dividing, lumen dwelling, protozoan parasite, and this presents an antigenic load which changes from day to day of infection. The trophozoite may be seen as a target which consists of many antigenic determinants which are associated with the tissues of the parasite and probably with secreted products, although this has not yet been confirmed. A range of immune responses are elicited, some directed against important epitopes relevant to the trophozoites survival, others to irrelevant parasite antigens. The immune response may also be directed against coincident enteric antigens, such as secondary bacterial infection of the small intestine.

When this work was stared, the initial hypothesis was that the immune response, probably through the action of lymphokines produced by T cells, caused much, if not all of the tissue damage. The fact that the immune response depends upon antigen being present, meant that the parasite load had to be quantified to enable interpretation of any findings. To investigate the origin of mucosal damage it was essential to create situations where large numbers of parasites were present without the immune response. Even when it has been possible to measure virtually all facets of both parasite and immune cell numbers during a primary infection, interpretation of the results to determine the cause of the enteropathy has been difficult.

This work in the mouse model highlighted the problems of interpretation associated with similar work on human giardiasis tissue. The stage of infection and of developing immune response in the patient are generally unknown. Since some patients with human giardiasis have chronic infections, this implies that they have a deficient immune response. This deficiency may be in developing the appropriate immune response, in expressing the appropriate parts of the immune response to eliminate the parasite or by the inappropriate targeting of the immune response to important parasite antigens.

In order to select a convenient mouse strain for the animal model, male and female BALB/c and CBA mice were infected with G. muris cysts or trophozoites. Infection of female BALB/c mice was found to be of shortest duration, with clearcut elimination of the parasite. In this strain, a primary infection had a well defined prepatent period from the initiation of infection until day three or four, followed by a rapid rise of one or two days duration, to the plateau of peak faecal cyst output. The length of this plateau varied between three and four weeks in different experiments and ended with a rapid elimination of the parasite to undetectable levels. When immune BALB/c mice were given a challenge infection of G. muris, the time course of infection showed an effective memory response which rapidly limited the level of infection and expelled the parasite.

In subsequent experiments, the changes in mucosal structure and function, and alterations in the components of the mucosal immune system were examined and considered in the context of the rise, plateau and fall of parasite numbers.

Despite an extensive literature, there are some striking gaps in the basic descriptions of mucosal immunity and of mucosal lymphoid cells in mice with giardiasis. No one had previously reported the changes in plasma cells, MMC and IEL during a primary G. muris infection, and I found that the information generated from this study gave useful insights into the various immune processes which were occurring in the gut.

Various methods were employed to increase trophozoite numbers during infection, to examine the resultant immune response and damage to the mucosa. Treatment of mice with loperamide to alter the intestinal motility neither increased parasite numbers, nor altered the course of infection.

The steroid induced recrudescent infection involved ten times the number of trophozoites compared to a normal primary infection. This method was used to examine the roles of the trophozoite and the immune response in generating the changes to the gut structure and function.

The infection of nude, athymic mice resulted in an increase in trophozoite numbers, similar to that described above. This experiment gave two types of information: firstly, it examined the thymus dependence of the effective immune response against G. muris. Secondly, it assessed the role of the trophozoite in generating the changes in gut structure and function. Transfer of naive or immune heterozygote cells to infected nude mice was used to further examine the thymus dependence of the immune response and assess the thymus dependence of the mucosal changes during infection.

A hypothesis had previously been put forward, that CMI was able, via non-antigen specific means, to render the gut hostile to parasites (Ferguson & MacDonald 1977). An ongoing CMI reaction in the gut during GvHR was used to examine the influence of this unrelated immune response on the infection. The GvHR immune reaction and resultant innocent bystander damage had no direct influence on the course of G. muris infection.

The role of goblet cells and mucus during giardiasis was assessed in both the primary and steroid induced recrudescent infections. Goblet cell numbers were counted by histological methods. The luminal environment was observed by SEM and the distribution and ease of displacement of trophozoites compared in primary and steroid induced recrudescent infections.

The sections of this chapter will bring together features of the G. muris infection in the various situations studied, and highlight the patterns of change in both epithelium and lymphoid cells of the mucosa. The role of each component of the immune system in the G. muris infection will be suggested, and published evidence in support or contradiction of each conclusion described.

I will present evidence to support a hypothesis that the activity of the MMC is necessary for an effective immune response against G. muris and that this activity plays a major role in generating the mucosal damage found during this infection.

Mucosal Architecture.

Villus Height and Brush Border Enzymes.

Measurement of the villus height was one of the assays used to indicate mucosal damage during these studies. Due to the variability of the villus length, large standard deviations often accompany this morphological measurement and significant changes were seldom found.

The decrease in the activities of the brush border enzymes, lactase, sucrase, maltase and trehalase, were used as indicators of mucosal damage during primary infection. These enzymes were chosen as an assay of mucosal damage because the method has been extensively used in this laboratory and the activities are regularily determined as indicators of mucosal function in both experimental and clinical situations.

Gillon et al (1982a), investigated G. muris primary infection in the CBA mouse, and reported a decrease in jejunal villus height during the first week of infection. During the primary infections reported in this thesis, either no change, or a small, transient decrease in villus height was found at the peak of infection. A prolonged increase of jejunal villus height was found six weeks after resolution of primary infection in the BALB/c mouse. This was similar to the result of Gillon et al (1982a) following elimination of the parasite in the CBA mouse.

The above group found a more pronounced and consistent decrease in disaccharidase activities throughout acute infection of CBA mice. In my work, the disaccharidase activities were found to be consistently reduced during primary infection. This indicates that mucosal damage may occur at a cellular level, but not be reflected in the gross morphology of the villus height.

The disaccharidase results from a prior, resolved infection showed that prolonged changes of decreased lactase and increased sucrase activity occured. This prolonged alteration of disaccharidase activity was also found by Gillon et al (1982a) following resolution of infection in the CBA mouse.

The prolonged increase in villus height and changes in brush border enzyme activities after resolution of a primary G. muris

infection, may be seen as an overcompensation and new "setting" of the cell kinetics following mucosal damage during the primary infection.

These observations were similar to those of Castro (1982), regarding permanent structural transformations of epithelial cell membranes of rats infected with T. spiralis. This assay involved binding of wheat germ agglutinin specifically to n-acetylglucosamine and sialic acid. The author suggested the presence of some type of "paracrine" system, which involved the release of substances, such as lymphokines, that would affect certain epithelial stem cells.

The above theory implies that the mucosal changes are directly controlled by immune elements in the mucosa. Alternatively, the trophozoite may be the initiator of these changes, by its being capable of damaging enterocytes, either by physical contact disrupting the mucopolysaccharide fuzzy coat and microvilli, or by the release of toxic substances. To investigate the theory that the trophozoite was directly causing the damage to the brush border and the transient villus shortening, two methods were used to increase the number of trophozoites present during infection. Firstly, I used the steroid induced recrudescent infection, which involved a ten fold increase in the number of trophozoites compared to a normal primary infection. The jejunal villus height of mice bearing this massive recrudescent infection was not different from the control villus height. Also, the ileal villus height was increased compared to the control ileal villus height. Disaccharidase activities in the jejunum and ileum of mice bearing the massive recrudescent infection, and previously infected mice, which had numbers of trophozoites that could not be detected, were equivalent. Therefore, despite the vastly increased numbers of trophozoites, no change in disaccharidase activities or villus height were found. This may be interpreted in several ways. Firstly, it may be that the trophozoite does not cause any of the changes examined here, and these must be attributed to the immune response, which in this situation is suppressed by the steroid treatment. One aspect of this suppression is the action of the steroid which results in

stabilizing the membranes of the MMC granules and preventing their fusion to the cell membrane, therefore degranulation does not occur (Martindale 1977). The steroid treatment also prevents regeneration of histamine in connective tissue mast cell granules and may have a similar effect on MMC granule contents. Secondly, the trophozoite may normally cause the damage, but the steroid treatment results in prevention or repair of the damage. Again, this would rely upon the general stabilizing of membranes attributed to steroids. Brush border membranes protected in this way would maintain their disaccharidase activities during the steroid induced recrudescent infection. Finally, some form of blocking factor, for example IgA antibodies, may be present in these immune mice and prevent mucosal damage by the trophozoite or an associated toxin. This might be tested by examining mucosal damage during a challenge infection of immune mice, or by passive transfer of large numbers of trophozoites to the gut of immune mice.

The second method employed to increase the number of trophozoites was the infection of nude mice. This produced a chronic infection with ten times the number of trophozoites compared to a normal primary infection. The presence of massive trophozoite numbers in untreated nude mice did not result in a decrease in villus height, as would be expected if villus damage was directly due to the trophozoites or to a toxin secreted by them. The control infection of heterozygote mice showed a transient decrease in villus height on day 17 of infection.

However, infection of nude mice resulted in a reduction of disaccharidase activies equivalent to those found in the infected heterozygote littermates. This would seem to support the hypothesis that the trophozoite contributes to the damage of the enterocyte brush border and the enzyme systems located there. In contradiction of this hypothesis, the number of trophozoites in the nude mouse gut was ten times greater than that found in the normal control infection, but the decreases in disaccharidase activies were equivalent. As an explanation of the similar decreases of disaccharidase activities in the nude and heterozygote mouse

infections, these could indicate that the trophozoite is responsible for the brush border damage. This hypothesis requires that the brush border damage is already maximal under the conditions imposed by the trophozoite load present during a normal primary infection, therefore the increased trophozoite numbers in the nude infection cannot further reduce the disaccharidase activities. Alternatively, the brush border damage may be caused by a non-T dependent factor (see later).

A very important observation was that the transfer of immune lymphoid cells to these infected nude mice did result in a decreased villus height. This was found 55 days after the immune cell transfer, at the time when faecal cyst output fell in this group. The result was similar to that of Roberts-Thomson & Mitchell (1978), who transferred immune heterozygote lymphoid cells to nude mouse recipients, prior to infection. Increased mucosal damage was found to occur upon subsequent infection. The above work, along with that of Stevens et al (1978), demonstrates the ability of nude mice, reconstituted with heterozygote cells, to develop an effective thymus dependent immune response against G. muris.

A particularly interesting feature noted at this time in the nude mouse infection, was the further drop in sucrase and maltase activities of the infected mice which received spleen and MLN cells from immune heterozygote donors.

The results confirm that there is a T cell dependent component to the mucosal damage, and that it is responsible for the decrease in villus height, and can therefore contribute to the alteration of brush border enzyme activities. Therefore the changes in the brush border enzyme activities may originate from both the trophozoite and the host, during primary infection.

The hypothesis that MMC mediators were the stimulus for villus shortening, through the action of a proteinase (see later), is supported by the results of the nude mouse infection. Alternatively, the results could equally be interpreted as supporting the direct action of cytotoxic T cells. However, the pathogenic MMC hypothesis is favoured due to this cell types greater

cytotoxic potential.

Untreated nude mice have been shown to have as many MMC as do their heterozygote littermates, but that the accumulation of these at the site of infection is under the control of long lived recirculating T cells, and this is therefore absent in the nude mouse (Mayrhofer & Bazin 1981). Haig et al (1982) have shown that the final differentiation of MMC is under the control of factors produced by T cells. The MMC detected in nude mice, using histological stains, may be a precursor MMC with limited activity.

It has been shown that the mast cell plays an important role in enabling cellular events to develop. For example, Askenase et al (1983) examined the deficient elicitation of delayed type hypersensitivity responses (DTH) in the ear and footpad of mast cell deficient mice. The DTH was directed against sheep red blood cells and picryl chloride in the W/W^{v} and Sl/Sl^{d} mouse strains. It was shown that the defect which results in the inability of these mice to mount a DTH response was the lack of mast cells and not due to a lack of T-effector cells.

The production of IgE is also under strict T cell control, so MMC in nude mice would be relatively inactive, as they cannot become primed to specific antigens. Transfer of primed T cells from immune heterozygote donors would result in a proliferation of IgE producing cells in the gut associated lymphoid tissues (GALT) of the nude mice. MMC would accumulate in the mucosa, become primed to the trophozoite antigens and so become fully active. Specific antigen binding results in MMC degranulation and resultant villus shortening by the proteinase action.

Nude mice only developed gross villus damage after transfer of immune heterozygote lymphocytes. Prior to this transfer the MMC would have had deficient function because of a lack of T cell influence. The transfer of immune heterozygote cells to infected nude mice would allow MMC degranulation and so exacerbate the mucosal damage. Further reduction in the disaccharidase activities confirmed the increased mucosal damage.

Crypt Depth and Crypt Cell Production Rate (CCPR).

The crypts main function is the production of epithelial cells. The crypt depth was measured because this indicates a response by the crypt, either to the mucosal requirement for replacement epithelial cells due to gut damage, or through the direct action of lymphokines on the crypt stem cells.

The second cytokinetic parameter measured was the CCPR. The increase in the CCPR and lengthening of crypts are two facets of the mucosal response that result in increased epithelial cell production. As measures of the mucosal requirement to replace enterocytes, these were, therefore, indirect assays of mucosal damage.

The measurement of crypt depth is an accurate measure of gut changes and significant differences are often found because the standard deviation is normally small. In contrast, the measurement of villus height often has a large standard deviation, and this results in less frequent finding of a significant difference. The statistical comparison of the CCPR is a particularly rigorous test and significant differences are very rarely found. For this reason, these results were expressed simply as increased, or similar to control values.

An increase in both crypt depth and CCPR was consistently found to occur by the end of the first week of a normal primary G. muris infection.

The GvHR study showed that the predicted increases in crypt depth and CCPR occured in mice with GvHR. In addition, the mice with GvHR and concurrent G. muris infection showed further increases in crypt depth and CCPR.

There are several possible mechanisms that would stimulate these responses.

Firstly, the trophozoite damage of the epithelial cells and brush border may release the crypt stem cells from the inhibitory influence of a factor produced by villus tip cells. May et al (1981) described a factor isolated from villus cells that specifically inhibited crypt cell division and DNA synthesis. This factor must be produced by mature enterocytes near the tip of the

villus and represents a self regulating feedback mechanism, which would react to the enterocyte damage.

Secondly, a mitogenic factor from activated T cells may stimulate the crypt depth and CCPR responses. MacDonald & Ferguson (1977) and Mowat & Ferguson (1981) proposed that lymphokines released by alloreactive T cells were responsible for the initial mucosal damage during GvHR in the mouse.

A third possibility is the action of some other mitogenic factor associated with the immune response. By this hypothesis, MMC may be directly involved in the regulation of cell production in the gut through the presence of serotonin. This substance is released by mast cells, has been shown to stimulate an increase in both crypt depth and CCPR in rat jejunum (Tutton 1974).

The results from the GvHR and concomitant giardiasis experiment support the existence of a lymphokine or similar product that independently mediates the crypt depth and CCPR response in each of these experimental conditions.

In those infections where villus height decreased, an increase in crypt depth and CCPR always occurred. Also, wherever a decrease in disaccharidase activity was found, crypt depth and CCPR were always increased. The evidence that the villus height reduction was T dependent is described above, therefore there was either a T dependent effect on the crypts, or the crypt depth and CCPR responses are indirectly mediated through feedback of the T cell action on the villi. Specimens with normal villus height, but decreased disaccharidase activities support the hypothesis of a link between epithelial cell damage (of the enterocyte brush border) and resultant feedback from these villus cells stimulating the crypt proliferative response. This association was found during normal primary infections throughout the study.

The hypothesis is further supported by the results from the steroid induced recrudescent infections. During these infections the crypt depth and CCPR in the jejunum and ileum did not change compared to the controls. The disaccharidase activities in these areas of the gut also remained unchanged.

The final evidence in support of this link between decreased disaccharidase activities and the crypt cell proliferative response was obtained from the results of the infection of nude mice. As described earlier, it was originally suggested that the mucosal changes found during G. muris infection were thymus dependent. However, during the infection of nude mice, the crypt depth and CCPR both increased, and the disaccharidase activities decreased, in parallel with, and to the same degree as, the changes found during the control infection of heterozygote littermates. Therefore, the lymphokine hypothesis described earlier may explain the pathogenesis in the GvHR model, but is not supported as the mediator of damage in this giardiasis model.

When spleen and MLN cells were transferred from immune heterozygote donors to chronically infected nude mice, the disaccharidase activities were further reduced, but the crypt depth and CCPR remained at their previous elevated levels. At this time, the villus height was found to have decreased. The reduction of the gross villus structure would result in a loss of the total of brush border enzymes and so explain the further decrease in disaccharidase activities at this time. Therefore, when interpreting these results there was the complicating factor of a T dependent change (the decreased villus height) exacerbating a T independent change (the reduction of disaccharidase activities).

The idea that T lymphocytes influence the epithelium has been supported by studies of other parasite models. Castro (1982) examined N. brasiliensis infection of rats and proposed that there existed a permanent influence by immune elements in the lamina propria, which acted upon epithelial stem cells in the crypts. This influence was initiated during the initial inflammatory response and regulated and maintained the mucosal structure and function.

In the work described above, immune cells were described as regulating and maintaining the mucosal structure and function. The converse has been shown, where primed cells, transferred to naive recipients, induced premature or increased damage upon subsequent challenge with the priming antigen. An example of this, the nude mouse model of G. muris infection, was described earlier.

Manson-Smith et al (1979) examined the gut of thymectomised, irradiated, bone marrow reconstituted mice infected with T. spiralis. This group found that the villus height reduction, crypt hyperplasia and nematode elimination were linked, and dependent upon the degree of T cell depletion. Mice that were completely thymus deficient did not eliminate the parasite and did not develop the villus atrophy or crypt hyperplasia associated with this response. However, following transfer of $25x10^6$ MLN cells from primed donors, villus height decreased and crypt depth increased, compared to infected mice that had not received MLN cells. This work indicated that the mediators of the mucosal damage were the gut homing T cells transferred in the MLN cell suspension.

Ferguson & Jarrett (1975) described the lack of mucosal damage during N. brasiliensis infection of T cell depleted rats (B rats). As discussed earlier, the MMC hyperplasia and accumulation is thymus dependent, and the results of the work described above could equally support the hypothesis of MMC mediated damage, as the response of these cells was thymus dependent.

The results described above have shown that the villus atrophy and crypt hyperplasia associated with nematode infections is undoubtedly thymus dependent. In contrast, I have shown that during giardiasis, the crypt hyperplasia and damage to the villus cell brush border membrane is thymus independent, but that the reduction in gross villus height is thymus dependent.

The initial stimulus in the gut which results in mucosal change, is the parasite. However, the trophozoite is not solely responsible for the gut damage, as two experimental procedures resulted in greatly increased numbers of trophozoites, but no increase in mucosal damage, compared to that of a normal primary infection.

The G. muris infection produced prolonged villus hypertrophy and altered disaccharidase activity following elimination of the acute infection, even though the crypt depth and CCPR returned to normal values. During a steroid induced recrudescent infection the villus height and disaccharidase activity were not further altered,

nor were the values for crypt depth and CCPR changed. Steroid treatment has been shown to prematurely induce digestive enzyme production in suckling rat intestine (Goland & Forstner 1974). However, the stimulation of disaccharidase enzymes in adult animals by steroid treatment has not been described (Henning 1981). The results of the present study supported this finding. Damage to the gut and resultant changes in mucosal architecture were therefore not found when the mouse is immunosuppressed, despite the massive recrudescent infection. It would be of interest to examine the gut function, architecture and kinetics at the time of rapid resolution of the steroid induced recrudescence infection, shortly after treatment is withdrawn.

An alternative theory is that the steroid protects the gut in some way and that this is the reason for no damage being found. Giardia trophozoites have been shown to excrete or secrete immunogenic components into culture medium (Mitchell et al 1982, Nash et al 1983). It has not been reported if these substances have any cytotoxic effect on the gut. Jonas et al (1978) described the secretion of a disaccharidase liberating factor by bacteria. The extracts were obtained from blind loops of rat intestine containing Bacteriodes, Clostridium or Streptococcus. A maltase releasing factor was isolated, which also removed lactase, sucrase and alkaline phosphatase from rat epithelium. The factor had an elastase-like substrate specificity. It is possible that an excretory or secretory product of Giardia trophozoites has a similar activity, and this may explain the disaccharidase changes found during the nude mouse infection, without a change in villus height. G. muris trophozoites and cysts are known to carry endosymbiont bacteria which have been seen to divide within the protozoan (Nemanic et al 1979). It may be these bacteria, and possibly toxins or factors produced by them, that alter the pathogenicity of the infection. This effect is known to occur with the amoebic infection by Entamoeba histolytica. Prolonged axenic culture of this organism results in a loss of symbiotic bacteria and a resultant loss of pathogenicity. This is normally counteracted by frequent passage of the pathogen through an animal host.

Cellular Changes.

Immunoglobulin Containing Cells.

Specific antibodies against trophozoite antigens have been described by several groups and there are several different ways in which specific antibodies could contribute to immunity against Giardia.

Specific IgG and sIgA anti-Giardia antibodies have been shown to enhance adherence of both neutrophils and macrophages, and to enhance phagocytosis by the macrophages (Kaplan et al 1985). Radulescu & Meyer (1981) and Smith et al (1983) have shown increased antibody dependent cell mediated cytotoxicity (ADCC) against G. lamblia trophozoites, by rabbit peritoneal exudate cells and human blood peripheral neutrophils, respectively. Tagliabue et al (1983, 1984) have shown that murine lymphocytes from the gut associated lymphoid tissues (GALT), along with sIgA, are able to perform ADCC against bacterial targets, so this mode of killing may also operate against the trophozoites. It has been known for a number of years that sIgA is involved in the resistance against pathogenic intestinal bacteria. Williams & Gibbons (1972) described the inhibition of bacterial attachment to epithelial surfaces by binding of specific sIgA and Fubara & Freter (1973) further described this feature using Vibrio cholera infection of the mouse.

Specific anti-Giardia antibodies may also result in complement mediated damage, as both IgG and IgM stimulate the classical complement fixation reaction and IgA, IgG and IgE all stimulate the alternative complement pathway (Fundenberg et al 1978).

Mucus is known to contain IgM and IgA and specific anti-Giardia antibodies of these classes have been found by several groups (Anders et al 1982, Mitchell et al 1982, Loftness et al 1984). Such antibodies are often directed towards antigenic targets on the dorsum, flagella and ventral disk of the trophozoite. Binding of antibodies in mucus to these surface antigens of the trophozoite would result in effective mucus trapping of the trophozoite and allow their subsequent expulsion by peristalsis.

There are several descriptions of specific anti-G. muris sIgA in colostrum and milk of immune mice, protecting neonates from

infection (Stevens & Frank 1978, Andrews & Hewlett 1981, Kaplan et al 1985). In the mouse model, sIgA may protect neonates against G. muris infection by several methods. Firstly, binding of antibody to the ventral disk could inhibit trophozoite attachment to enterocytes. Secondly, binding to trophozoite antigens might result in agglutination of the parasites. Binding of antibodies to flagella may inhibit trophozoite motility, and more importantly prevent production of the suction force necessary for the trophozoites attachment to surfaces. Finally, the antibody would stimulate the ADCC reported above. These effects of sIgA could all be transmitted in colostrum, along with numerous effector cells from the maternal immune system. Because of the immune components of the colostrum, it is capable of passively protecting the neonate from G. muris infection. This "immune response" in the lumen of the neonatal gut is entirely supplied by the maternal immune system, and so explains the lack of immunological memory shown by neonates after weaning, in contrast to good immunological protection before weaning.

My work, described in chapter five, is the first complete study of intestinal mucosal immunoglobulin containing cells during G. muris infection of the mouse. The changes in the numbers of these cells were examined in a primary infection. The number of cells containing the immunoglobulin isotypes IgM, IgG, IgA and IgE increased with the development of the immune response against G. muris. These cellular changes paralleled some of the the changes known to occur in serum immunoglobulins in the mouse model of giardiasis (Owen 1980b, Anders et al 1982).

An early rise in IgM containing cells, followed by an increase in IgA containing cells would be anticipated during the development of an immune response in the gut, and these were found to be increased during giardiasis. IgM and IgA are the immunoglobulins associated with secretions and are actively transported, via secretory component, across the epithelium.

The IgG containing cells were increased at the same time as IgM and IgA containing cells, but fell rapidly thereafter. This transient increase may be part of a brief accumulation of systemic B

cells to an inflamed site, rather than its being part of the mucosal immune response. IgG is not actively transported to the lumen of the gut. Despite this, there is evidence of an increased luminal level during human giardiasis (Jones & Brown 1974). This was thought to reflect the "leaky" nature of the gut epithelium at that time, due to mucosal damage.

At this time, the leaky gut would also allow proteins and other large molecules to more readily pass both into, and out of, the gut lumen. Thus, an alternative explanation is that the increase in IgG containing cells reflects a generally increased antigenic stimulus from this influx of luminal contents.

Whatever the reason, if some of the IgG is specifically directed against trophozoite antigens, this creates the potential for transient immune complex injury, as well as protective IgG mediated immunity.

The rise in IgE containing cells was later than those noted above. This increase occurred around the time of trophozoite expulsion. There were several possible reasons for the later rise in IgE containing cells. The kinetics of this cell types response in the gut has received less attention than that of IgA plasma cells, and is mostly concerned with the IgE response of the rat during N. brasiliensis infection.

The time that the IgE containing cell increase was found, may be the time in the developing immune response when the increase in IgE containing cells occurs. Durkin et al (1981) has shown that in rats, the IgE bearing cells arose at the end of plasma cell ontogeny, by switching of precursors bearing IgM or IgA. They originated in the gut associated lymphoid tissues (GALT), particularily in the Peyer's patches. Many Peyer's patch lymphocytes bear both IgE and IgA on their surface. Also, the T-helper and T-suppressor control of IgE plasma cells may be more complex than that of the other immunoglobulin containing cells. Through its role in the sensitization of MMC to specific antigens, IgE may be directly involved in the expulsion of the parasite (discussed later).

Although, as mentioned above, the changes in plasma cells of

different immunoglobulin classes were similar to patterns of anti-Giardia antibodies reported by others, my results could be due to the influx of the non-Giardia antigen through the leaky epithelium. For this reason, further plasma cell assays using the mouse model will need to be done, and should involve specific staining of plasma cells containing immunoglobulins directed against trophozoite antigens.

The degree of involvement of specific anti-Giardia antibodies in resolving a normal primary infection is uncertain. The C3H/He mouse strain maintains a chronic primary infection while having similar specific IgA and IgG antibody levels to those found in the resistant BALB/c mice during infection. However, the specific anti-Giardia IgA and IgG in the serum of infected BALB/c mice recognizes a complex of four acidic protein antigens, of approximate molecular weight 32000, better than does the IgA and IgG found in the serum of infected C3H/He mice. Unfortunatly the susceptability to chronic infection is not directly linked to the lack of antibody responsivness to these antigens, as these features do not strictly segregate as would be predicted in (BALB/C x C3H/He) F2 mice (Erlich et al 1983).

Intraepithelial Lymphocytes (IEL).

IEL are a morphologically and functionally heretogeneous population of cells (Ernst et al 1985). Counts of IEL accurately reflect an ongoing CMI reaction in the mucosa (Mowat & Ferguson 1981) and various functional assays suggest that they could contribute to immunity or enteropathy. They have been shown to possess qualities of effector T(DTH) cells, monocyte/macrophage-like function, cytotoxicity (ADCC and NK) and MMC-like activity (Ernst et al 1985). Aspects of cellular events in the epithelium and lumen relevant to all of these functions have been described by different workers, but the results are very variable. This has led to great difficulty in the interpretation of the role of IEL in the gut.

Various cell types have been identified in the lumen of mouse gut during giardiasis. Among these were lymphocytes bearing the cytotoxic/suppressor marker, Lyt 2 (Heyworth et al 1982, 1983,

Ganguly et al 1981, Ganguly & Mahajan 1983), as do IEL (Schrader et al 1982). Smith et al (1982) demonstrated the spontaneous killing of trophozoites by monocytes, and ADCC by monocytes and peripheral blood granulocytes has also been described (Smith et al 1983). Isolated IEL show little of this activity, either due to a limiting dilution effect of necessary accessory cells (Dillon & MacDonald 1984) or due to the isolation procedure disrupting the ability of IEL to perform in the assays. A variable percentage of IEL have numbers of azurophilic granules in their cytoplasm. These granules have been shown to exhibit some limited proteinase activity, which is similar to that of the MMC granules (J. Huntly, pers. comm.). The staining characteristics of IEL granules suggest that their contents are similar to those of MMC granules, but their specific functional capabilities are unknown. Tagliabue et al (1981) showed that many, but not all, granulated IEL in the mouse had strong natural killer (NK) activity. Tagliabue et al (1982) also showed that the mast cell deficient W/W^{v} mouse, which is susceptable to chronic giardiasis, had normal NK activity. This may either indicate that NK activity is not important in the immune response to G. muris, or that MMC are required to enable cells with NK activity to gain effective access to the luminal trophozoite targets.

As part of the general examination of the mucosa in my experiments, I carried out counts of IEL during the two pathological conditions of G. muris infection and GvHR. Primary G. muris infection resulted in an increase in IEL numbers by day 17 and this remained elevated for at least three weeks of infection, at the time when the trophozoites were being, or had been expelled. During GvHR, IEL numbers were raised by day 11, but the presence these IEL did not alter the course of concurrent G. muris infection.

G. muris infection of nude mice did not result in an increase in the low numbers of IEL present in these athymic animals. When reconstituted with spleen and MLN cells from heterozygote donors, the IEL numbers became very variable, some increasing greatly and others remaining low in number. There was no relationship between the IEL numbers and the experimental procedures of each group. Thus it was not possible to link IEL with the decline in parasite numbers

in those mice which received immune heterozygote cells.

During steroid induced recrudescent infection, the number of trophozoites increased, but no change in mucosal architecture (villus and crypt lenghts), kinetics (CCPR) or function (disaccharidase activities) were found, compared to control values. An interesting observation was that the number of IEL increased, showing that this response was steroid resistant, but by itself did not control the infection.

As with the immunoglobulin containing cells, it is not known to what extent, if any, the IEL response is specifically directed to the parasite, or to other luminal antigens. The actual purpose of the IEL response during giardiasis remains unknown. There is the possibility that the IEL are part of the "immune elements" described earlier (Castro 1982) involved in the maintenance of mucosal structure and function. This heterogeneous population of cells requires a great deal of further investigation to establish the roles they play in the mucosal immune system.

Mucosal Mast Cells (MMC).

I propose that the MMC is of great importance in both the effective immune response to expell the trophozoite and in the pathogenesis of the infection.

As described earlier, Askenase et al (1983) demonstrated that the mast cell was involved in enabling cellular events to develop. This was shown by examining the defective elicitation of DTH responses in the W/W^{v} and Sl/Sl^{d} mast cell deficient mouse strains.

Additional evidence of an important role for mast cells in the immune response to G. muris, is the fact that the mast cell deficient mouse strain W^{f}/W^{f} develops chronic giardiasis (Roberts-Thomson et al 1981). The mast cell deficient mouse strains have been shown to possess normal B and T cell responses.

Furthermore, MMC may be indirectly implicated in the chronic giardiasis of the SLJ/J mouse strain, for this strain is known to be a very low producer of IgE. IgE normally binds to the FcE receptor on the mast cell surface, and in doing so sensitizes the mast cell to specific antigens. Binding of these antigens and specific IgE on

the mast cell results in its degranulation. Thus, the IgE deficiency would result in the inability of mast cells to be activated by this route.

Further evidence supporting this hypothesis comes from another chronically susceptible strain, the C3H/He mouse. Development of the IgE response in this strain is also abnormal, in that they are particularly insensitive to the IgE stimulating factor of Bordetella pertusis. Because of this, the mast cells are unlikely to degranulate through the normal specific antigen-IgE binding process.

In my study of a primary infection, I found that there was an increased number of MMC from day six of primary infection.

I suggest that the activity of the MMC is necessary for an effective mucosal immune response against G. muris and that this activity contributes to the tissue damage found during infection. My hypothesis is that during a normal primary G. muris infection of the mouse, proteinases released by the MMC loosen the intercellular bonds and result in premature sloughing of enterocytes. There is an associated decrease in brush border enzyme activity because these enzymes develop as the enterocytes mature and move up the villus. The premature loss of cells results in the changed activity levels. There is also increased cell production to compensate for the loss of enterocytes, so cells on the villi are immature. The compensatory increase in CCPR and crypt depth results in maintenance of the normal villus height. This indicates the link between the immune response to the trophozoites and the regulation of enterocyte kinetics and villus damage.

As mentioned in an earlier section, the control of crypt stem cell proliferation can also be linked to the MMC. Serotonin is released from MMC and is known to stimulate CCPR and increase crypt depth in the rat jejunum (Tutton 1974).

Rat mucosal mast cell protease II (RMCP II) is released by MMC in the rat and is thought to damage basement membrane and intercellular bonds. There is evidence of an equivalent proteinase from the MMC of mice. This enzyme activity may act in the same way as the enteropathic lymphokine proposed by Mowat & Ferguson (1981), and could explain mucosal damage during GvHR in the mouse.

Therefore, the mast cell, rather than direct T cell mediators, is the final agent in the chain of events which results in the mucosal changes.

The hypothesis that the MMC and their granule contents cause the mucosal changes found during giardiasis could be tested in the chronically susceptible mast cell deficient strains. I predict that the normal changes to the mucosal architecture, kinetics and function found during primary infection would be absent in the mast cell deficient strains infection. There has been no report in the literature of the extent of mucosal damage during infection of these mice.

This MMC hypothesis is supported by the results of experiments investigating the steroid induced recrudescent infection. In these, villus height, crypt depth and CCPR of mice bearing a massive recrudescent infection did not change from the values of the control immune mice, which had undetectable trophozoite numbers. An explanation as to why no mucosal damage occured during the steroid induced recrudescent infection may be that the MMC activity was suppressed at that time. Steroids are known to stabilize the mast cell granule membranes so that the contents cannot be released upon stimulation (Martindale 1977), and it has also been suggested that steroids suppress mast cells by virtue of their inhibitory action on the function of histidine decarboxylase. This is the enzyme that converts histidine to the active mediator, histamine. This was shown in connective tissue mast cells, but since MMC are known to contain only very small quantities of histamine, the mode of action of steroids on these cells is less likely to be by histidine decarboxylase.

The mediators of Type 1 hypersensitivity, released by MMC, are demonstrably capable of damaging cells and tissues through the innocent bystander phenomenon, and the MMC must be seriously considered as the origin of the cytotoxic mediators which result in mucosal damage. The numbers of MMC rise in both giardiasis and GvHR, at a time when the gut is damaged. Recent work in this laboratory investigating the rat model of GvHR, has shown that the proteinase RMCP II increases in gut homogenates as the GvHR

progresses, indicating increased MMC activity (personal observation). As described earlier, RMCP II is thought to have specificity for the basement membrane and intercellular bonds of the epithelium and its action could result in the premature sloughing of enterocytes. One useful function of this apparently deleterious response will be to enable or aid diapedesis of primed effector cells from the mucosa to the lumen of the gut, and so gain access to the trophozoite targets. Increased transudation of serum and secretions, as a result of increased capillary permeability and leakage through the damaged epithelium, has been fully demonstrated in the N. brasiliensis infection of rats, and shown to be MMC related (King & Miller 1984). The finding of increased luminal IgG during giardiasis supports the occurence of this phenomenon in Giardia infection (Jones & Brown 1974). As previously described, specific immunoglobulins which have leaked into the lumen can then bind to the antigenic targets, stimulating ADCC, complement fixation, opsonisation and mucus trapping.

Erlich et al (1983) provided further support of the MMC involvement from a different approach. This group treated the normally resistant BALB/c mouse strain with cyproheptadine prior to and during G. muris infection. This is a drug with antihistamine and antiserotonin action. The result was a prolonged G. muris infection of cyproheptadine treated mice. When drug treatment was withdrawn, the infection rapidly resolved, indicating that the immune response against G. muris had developed, but was suppressed.

This MMC hypothesis may also be applied to the GvHR experimental condition, with concurrent giardiasis. MMC numbers increased during GvHR, and when concurrent with G. muris infection, showed a further increase, but because of large standard deviations this was not statistically significant. The gut damage, as assessed by crypt depth, was also further exacerbated when the conditions are concurrent. The more extensive information, generally higher numbers and better staining characteristics of MMC in the rat, would enable testing of this hypothesis in a rat giardiasis model. This model would also allow measurement of RMCP II from serum or gut homogenates, as a direct assay of MMC activity.

Goblet Cells.

Mucus can influence the G. muris infection in two ways; firstly, it can act as a physical buffer and prevent the trophozoites from inhabiting preferred intervillus sites and from attaching to enterocytes. Secondly, mucus is known to contain IgM and IgA. Specific anti-Giardia antibodies of these classes have been found by several groups (Anders et al 1982, Mitchell et al 1982, Loftness et al 1984). These antibodies are often directed towards antigenic targets on the dorsum, flagella and ventral disk of the trophozoite. Binding of the antibodies and antigens would result in effective mucus trapping of the trophozoite and their subsequent expulsion by peristalsis.

Lee & Ogilvie (in Levy & Frondoza 1983) demonstrated the importance of specific antibodies in the process of mucus trapping. They preincubated T. spiralis in immune serum prior to in vitro incubation with mucus. This resulted in increased mucus trapping of the nematodes and suggested some non-specific affinity of immunoglobulins and mucus.

Two relevent stimuli that result in mucus release are the degranulation of MMC and the presense of IgE-antigen complexes. There is also evidence from the mast cell deficient W/W^{V} mouse strain, which release normal amounts of mucus, that IgE can bind to the goblet cell and so directly stimulate mucus release upon antigen binding (Levy & Frondoza 1983).

The goblet cell counts from experiments in this thesis may be lower than actual in vivo numbers, due to loss of cell contents during processing and resultant lack of positive stain.

A direct assay of mucus production would be a more reliable measure of the involvement of this response during infection. Various methods were employed to examine the direct action of mucus and despite the problems, some interesting data was obtained.

The number of goblet cells remained similar to the control value during a normal primary infection. During steroid induced recrudescent infection there was an increase in the number of goblet cells by day 5 of treatment. At this time, there were ten times the number of trophozoites compared to a primary infection. This increase of goblet cells may be a protective response stimulated by

the mechanical irritation of the many trophozoites. This form of irritation is known to stimulate mucus release from goblet cells (Forstner 1978). Alternatively, the increase may be a steroid resistant aspect of the immune response to a secondary challenge infection of G. muris.

It is useful to compare this limited increase in the number of goblet cells with that found to occur in another parasite model, N. brasiliensis in the rat, where a two to four times increase has been described (Levy & Frondoza 1983). This suggests that the goblet cell response during G. muris infection is not as important as that present during the nematode infection.

The SEM results showed trophozoites closely associated with mucus during primary infection of both normal heterozygote and athymic nude mice. Nude mice appear to produce as much mucus during chronic infection as do heterozygote mice during an acute, immunologically resolved infection. Therefore, if mucus played a substantial role in eliminating the trophozoites, it must be dependent upon the additional presence of T dependent factors, such as the specific anti-Giardia immunoglobulin content.

However, the C3H/He mouse strain is susceptible to chronic infection, but has been shown to produce specific IgA against G. muris to the same, or higher levels than do the resistant BALB/c mice. As previously described, some of the immunoglobulins produced by the BALB/c mice are directed toward different antigenic targets than are those of the C3H/He mouse immunoglobulins (Mitchell et al 1982, Erlich et al 1983). However, binding of antibodies contained in mucus to trophozoite antigens would still be expected to result in mucus trapping, so it would appear that this is not an important response during primary infection.

If mucus trapping was important in the expulsion of a normal primary infection, the treatment of mice with loperamide, to influence the motility of the gut, might be expected to result in less efficient expulsion, and so delayed resolution of the infection. This was not found in the series of loperamide experiments, again suggesting that mucus trapping does not play a major role in control of a primary infection.

When the C3H/He chronic infection is resolved by drug

treatment, immunity to subsequent challenge infection is comparable to that of immune BALB/c mice. The immune C3H/He resistance is also passively transferred to neonates in colostrum and milk. These results suggest that immunoglobulins are more important in protection against challenge infections than in the expulsion of a primary infection. Specific anti-G. muris IgG and sIgA that passively transfer protection to neonates have been found in colostrum and milk, as described earlier.

Mucus may also be more important during the response to a challenge infection, as it is in the rat and N. brasiliensis infection model. Miller et al (1981) described the mucus trapping of a challenge worm infection within two hours of intubation, leading to rapid expulsion of the nematodes.

When steroid treatment was withdrawn, parasite numbers fell rapidly. It was shown that a higher proportion of trophozoites were easily displaced from the gut at this time in a steroid induced recrudescent infection, than in a normal primary infection. This might be explained by the return of the ability of mucus containing specific antibodies to trap the trophozoites, and render them more amenable to displacement.

The close association of mucus and trophozoites was also seen during SEM examination of the gut from mice bearing a steroid induced recrudescent infection, at a time when the infection was being rapidly eliminated following withdrawal of treatment.

Small changes in the levels of endogenous steroids are known to alter the quality of mucus. The comparatively massive doses of steroid used to stimulate recrudescent infection would, therefore, be expected to have profound effects on the mucus composition. The experiments which involved pretreatment of naive mice with steroids and continuing treatment during primary infection showed that a more favorable environment for trophozoite habitation was produced and a higher parasite load was supported. Nair et al (1981) found increased numbers of trophozoites following a single steroid treatment, one or two weeks prior to G. muris infection of CBA mice. This single dose also resulted in delayed resolution of the infection.

I have shown that a primary G. muris infection of the BALB/c mouse resulted in an immune response that eliminated the parasite from the gut. This immune response involved a proliferation of plasma cells containing IgM, IgG, IgA and IgE. MMC were also involved in the immune response against G. muris and the number of IEL was shown to increase during infection. Evidence has been presented for the independence of the mechanism that results in decreased villus height, which appears to be thymus dependent, and that which regulates the production of cells by the crypts, which is thymus independent. The decrease of disaccharidase activity levels is linked to the response of the crypt cells and can occur independently of decreased villus height. By chronic infection of nude (athymic) mice and transfer of immune heterozygote cells to these mice, I showed that the reduction in villus height, associated with infection, was thymus dependent and as a result of the host's immune response. The mucosal changes found during infection were therefore not solely due to the trophozoite. A quite different method made use of steroid treatment to temporarily suppress the immune response and this allowed a recrudescent infection to develop. Mucosal damage did not occur while the mice were steroid immunosuppressed, despite massive trophozoite numbers. Although goblet cells and mucus were not directly involved in the primary immune response to G. muris, they may play a role in the expulsion of the steroid induced recrudescent infection.

These experiments have demonstrated that the immune response of mice to the intestinal protozoan, G. muris, is complex. The response involves both thymus dependent and independent mechanisms, and antigen specific, as well as non-specific factors. The initial hypothesis on which the work was based suggested that lymphokines caused the mucosal changes seen during primary giardiasis. This was shown to be an oversimplification, and finally to be incorrect. It appears that mucosal mast cells are likely to be the central agent in causing the enteropathy of giardiasis.

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