Studies on the Host-Parasite Relationship in Trichomoniasis

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C O N T E N T S

SUMMARY

An investigation to elucidate the pathogenic mechanisms of *Trichomonas vaginalis*, the flagellate protozoan of man responsible for the sexually transmitted disease trichomonal vaginitis was undertaken. The research was undertaken in collaboration with Dr M.J. North and Dr B.C. Lockwood of the University of Stirling.

The project involved the use of models of the trichomonad infection including an *in vivo* the subcutaneous mouse model, an intravaginal mouse model developed as part of this project, an *in vitro* model of the interaction of *T. vaginalis* and mammalian cells, again developed for this project.

As virulence is believed to attenuate with prolonged cultivation *in vitro* a collection of fresh isolates was obtained from patients at Glasgow Royal Infirmary genito-urinary clinic. This collection of isolates was then compared for their effect in each of the models: growth kinetics in axenic culture were measured as generation time is thought to correlate with virulence of isolates; the cytotoxicity of isolates towards mammalian cells was measured and compared to growth rate of subcutaneous lesions in mice and infectivity to mice intravaginally. It was found that some correlation did occur between effects in the subcutaneous and intravaginal mouse models and the effect on mammalian cell lines, but no correlation occurred between effect in these models and growth kinetics of isolates in axenic culture. Some biochemical properties of the collection of isolates were also investigated by our co-workers at Stirling University, these included a comparison of ornithine decarboxylase activity and a comparison of proteinase activity in the isolates. Again, no correlation was found between these activities and the effects of isolates in the models used.

The models used to compare isolates were used to test the effects of a number of inhibitors. Firstly, the effect of leupeptin, an inhibitor of cysteine proteinase activity, was examined in each of the models. Cysteine proteinases are known to be present in large amounts in *T. vaginalis*, although their role in the biochemistry of the parasite is unknown. Cysteine proteinases have been implicated in pathogenicity of some other parasitic protozoa e.g. *Entamoeba histolytica* and for these reasons a potential role of cysteine proteinases in trichomonad pathogenicity was investigated.

At concentrations which had only a slight inhibitory effect on growth in axenic culture, leupeptin totally prevented trichomonad cytotoxicity towards mammalian cells *in vitro*; an effect on the subcutaneous mouse model was observed although leupeptin proved highly toxic to mice. Numerous variations in method were tested to achieve maximum effect of leupeptin e.g. multiple dosing, route, times of administration and pretreatment, however the maximum effect achieved was a delay in growth of subcutaneous lesions, rather than an inhibition of lesion growth. The effect of leupeptin on intravaginal infections in mice was investigated, mice infected intravaginally with *T. vaginalis* were treated in the same way as achieved maximum effect against the subcutaneous infection, however, no effect on infectivity was observed. These results therefore indicate that cysteine proteinases are involved in pathogenicity of *T. vaginalis* although they are by no means the only factor and may play a minor role in the host-parasite relationship. This was also indicated by the work carried out at Stirling showing that proteinase activity of the various isolates did not appear to correlate with virulence as measured by any of our models.

The same model systems were then used to investigate the effects

of difluoromethylornithine (DFMO). This is a specific inhibitor of ornithine decarboxylase, an enzyme involved in the synthesis of polyamines, most commonly putrescine, spermidine and spermine which are known to be required for trichomonad growth although their function has not yet been precisely defined. A link between polyamines and trichomonad infection has been suggested by the presence of putrescine in the vaginal fluid of trichomoniasis patients. DFMO has excited interest as a potential drug against polyamine biosynthesis and so preventing growth of infectious organisms and tumours, and it has been shown to be effective against certain protozoan infections, especially trypanosomiasis.

Again at concentrations which had only a slight inhibitory effect on growth of trichomonads in axenic culture, DFMO totally prevented *T. vaginalis* cytotoxicity toward mammalian cell lines *in vitro*; an effect on the subcutaneous mouse model was also observed but again this was a delay in lesion growth until after treatment had stopped rather than a total inhibition of lesion growth; again this dose regime appeared to have no effect on intravaginal infectivity. It can be concluded therefore that polyamines do play some role in pathogenicity, although again this may be minor. The work carried out at Stirling University also showed that no correlation occurs between levels of ornithine decarboxylase activity of isolates and virulence in our models.

The third inhibitor investigated was D,L-propargylglycine which is a specific irreversible inhibitor of homocysteine desulphurase and methionine-γ-lyase and inhibits production of methanethiol. Methanethiol has been found to be important in some bacteria and higher plants in which its ecological function is to repel parasites and pathogenic fungi. As *T. vaginalis* is known to produce methanethiol and propargylglycine inhibits the enzymes involved in its production I

decided to investigate the potential role methanethiol might play particularly in establishing an intravaginal infection where many types of bacteria may interact with trichomonads.

In axenic culture trichomonad growth is not inhibited at 10^{-5}M D,L-propargylglycine although cells grown at this concentration had no homocysteine desulphurase activity. On the interaction of trichomonads and mammalian cells *in vitro* this concentration had no effect. For the *in vivo* models, trichomonads were grown prior to the infection in medium containing 10^{-5}M propargylglycine, then infected both subcutaneously and intravaginally. Using the subcutaneous model no difference in rate of abscess growth was observed although results for the intravaginal model indicated that trichomonads grown in the presence of propargylglycine have a lower infectivity for mice intravaginally. Therefore, propargylglycine had no effect, at the concentrations tested, in any of the models except for the intravaginal model. This may indicate that methanethiol is important in establishing an infection by having some effect on bacteria which may be detrimental to the trichomonads.

The effects of these inhibitors not only reveals the importance of these enzymes in the host-parasite interaction but also allows the comparison of models used and emphasizes some of the advantages and disadvantages of each. The effects of these inhibitors cannot be detected in axenic culture as although the compounds inhibit trichomonad enzymes they are not lethal to the trichomonads in culture, and a more appropriate model of the trichomonad infection *in vitro* is the interaction between trichomonads and mammalian cells. The effects of both leupeptin and DFMO are apparent using both this model and the subcutaneous model although perhaps a more appropriate *in vivo* model of the trichomonad host-parasite relationship is the

intravaginal infection which is the only model in which the effect of propargylglycine can be detected. The results, therefore, emphasize that the different models are probably measures of different parameters of the trichomonad infection.

The *in vitro* model of the trichomonad/mammalian cell interaction has the advantage of being more versatile and this model was used to further investigate the mechanisms of trichomonad pathogenicity. Mammalian cell monolayers were exposed to spent trichomonad culture medium, concentrated spent medium and various trichomonad lysates, all of which appeared to have some detrimental effects on the mammalian cells. Finally, the effects of trichomonads on mammalian cells within a mixed culture were tested when actual contact was prevented by a membrane barrier. The presence of the barrier totally prevented the effect on the mammalian cells and indicated that either the trichomonads must come into contact with mammalian cells to damage them or whatever toxic substance is produced by them cannot pass through a membrane of 0.45uM pore size.

Metronidazole (Flagyl) is the 5-nitroimidazole routinely used for the treatment of tricho moniasis. Since its introduction 25 years ago an increasing number of reports have been made of trichomoniasis patients failing to respond to treatment and an *in vitro* test was devised to determine whether this was due to trichomonad resistance to metronidazole or to some other factor. However, little work had been carried out using *in vivo* models for screening for metronidazole resistance. It was decided, therefore, to use the subcutaneous mouse model and our intravaginal model and compare metronidazole sensitivity of isolates, as detected by these models, to the results for an *in vitro* test.

Isolates of *T. vaginalis* were obtained from clinicians treating trichomoniasis patients who proved difficult to cure effectively.

Results obtained suggested a strong correlation between isolate sensitivity in each of the models tested. The systems developed for testing metronidazole sensitivity in these models can now be used for screening new anti-trichomonal compounds.

This project has, therefore, achieved the development of a mammalian cell model *in vitro*, although these had previously been used, my own model has modifications which make it more efficient than those used by previous workers. We also developed a completely new, simple and straightforward, intravaginal mouse model which will be of great value to other trichomonad researchers both for studying the host-parasite relationship and for chemotherapeutic screenings. We have shown that all our models can be used to detect differences in trichomonad isolates but that these models are probably measuring different parameters of the trichomonad infection. The project has revealed that cysteine proteinases and polyamines are both involved in the host-parasite relationship although their roles may be minor and actual contact is also important. Methanethiol may have a role in establishing an intravaginal infection by repelling bacteria detrimental to the trichomonads, and results of metronidazole sensitivity tests have confirmed reports that metronidazole resistance exists. *In vivo* model systems were developed suitable for screening for new anti-trichomonad compounds with metronidazole as a comparison.

1.0 INTRODUCTION

1.1. *TRICHOMONAS VAGINALIS* AND OTHER TRICHOMONADS - THE PARASITES

1.1.1. Classification

Trichomonas vaginalis is a pathogenic protozoan parasite which inhabits the urogenital tract of man and causes the sexually-transmitted disease trichomoniasis or trichomonal vaginitis.

The classification of this organism and other trichomonads is at present as follows:

Phylum:	Protozoa
Subphylum:	Sarcomastigophora
Superclass:	Mastigophora
Class:	Zoomastigophorea
Order:	Trichomonadida

Honigberg (1963) divided the order *Trichomonadida* into 4 families. One of these, the *Trichomonadidae* was further subdivided into the subfamilies: *Trichomonadinae*, containing the genus *Trichomonas* (including *Trichomonas vaginalis*, *T. tenax* from the oral cavity of man, and *T. gallinae* from fowl and pigeons); the *Pentratrichomonadinae* containing the genus *Pentatrichomonas* (including *Pentatrichomonas hominis* from the digestive tract of man; and the *Tritrichomonadinae*, including the genus *Tritrichomonas* (including *Tritrichomonas foetus*, from the urogenital tract of cattle).

The order *Trichomonadida* comprises single celled organisms whose locomotor organelles are flagella. Reproduction is asexual by binary fission. Members of the *Trichomonadida* have 4-6 flagella, of which one is trailing and may be attached to form an undulating membrane. Trichomonads have an axostyle and parabasal body (Honiberg, 1978). True cyst stages appear not to occur. *Trichomonas vaginalis* itself has 4 anterior flagella and a short undulating membrane which extends

approximately half way down the body of the cell. It has no trailing flagellum, the fifth recurrent flagellum terminating with the undulating membrane. The capitulum of the axostyle is moderately extended and the parabasal body is rod-shaped.

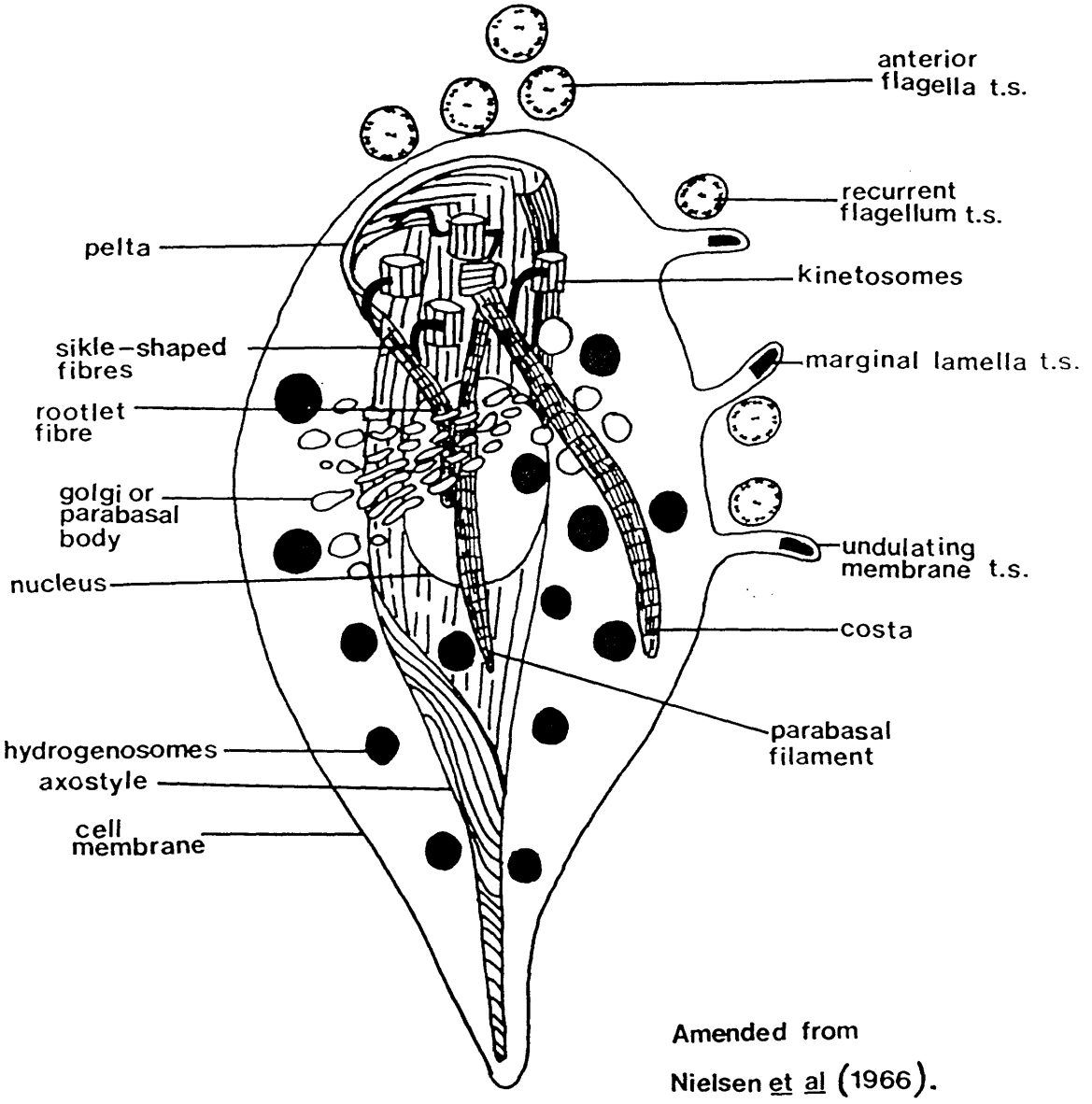
Other species of the order *Trichomonadida* were also investigated in this study, namely *Tritrichomonas foetus*, which is a parasite of the urogenital tract of cattle and causes septic abortion (Honigberg, 1978). This has 3 anterior flagella, one posterior flagellum passing into a long trailing falgellum. It has a well-developed undulating membrane running the full length of the body and a thick axostyle sharply pointed at the posterior end. The parabasal body is rod-shaped and very long.

Pentratrichamonas hominis, the apparently non-pathogenic inhabitant of the intestine of man was also studied. This has five anterior flagella, four in one group and one solitary flagellum beating in independent rhythm. The posterior flagellum passes into a long trailing flagellum. The undulating membrane is well-developed and extends to the termination of the body. The axostyle is of medium width and the capitulum of the axostyle is widened by lateral membranes. The parabasal body is composed of one or several granules surrounded by an elliptical or spherical zone.

Figure 1 shows a diagrammatic representation of *Trichomonas vaginalis*. The majority of my studies concerned this parasite with other trichomonads being used only in some comparative studies. Consequently, I shall concentrate on *T. vaginalis* and human trichomoniasis in my introduction, but shall compare with other species and diseases where appropriate.

Figure 1: Diagrammatic representation of *Trichomonas vaginalis* based on Electromicrographs.

Derived from M.H. Nielsen *et al.* (1966).



Amended from

Nielsen et al (1966).

1.1.2. The structure of *Trichomonas vaginalis*

1.1.2.1. Light microscopy

Honigberg and King (1964) described the morphological details of *T. vaginalis* using phase contrast light microscopy. They found that the shape of the cell is variable, actively swimming forms being ellipsoidal or ovoidal, sometimes spherical. All strains have the capacity to form pseudopodia-like extensions which are said to be used in feeding and for attachment to stationary objects. The protozoan has 4 flagella originating in an anterior basal granule complex from which the undulating membrane and costa also arise. The free edge of the membrane consists of the accessory filament and the recurrent fifth flagellum. The posterior end of the costa is usually obscured by the terminal segment of the undulating membrane. Two rows of paracostal granules (hydrogenosomes) are visible in haematoxylin-stained preparations and in living organisms viewed in a phase-contrast system. The axostyle has a widened capitulum of about one third of its total length extending anteriorly into a small crescent-shaped pelta. The trunk of the axostyle is a thin rod passing through the centre of the organism and projecting from the posterior surface. The parabasal body can often be seen as a v-shape. The ellipsoidal or ovoidal nucleus is situated near the anterior end of the body, often containing a small, spherical nucleolus in a chromatin-free area. There is no cytostome and food particles are ingested in the posterior region by fine pseudopodia-like processes.

1.1.2.2. Electron miscroscopy

The first published studies of the fine structure of *T. vaginalis* were carried out by Inoki *et al.* (1960). They found that *T. vaginalis* possessed a double-layered nuclear membrane with pores, a lamellar Golgi apparatus and flagella with 9 peripheral double fibrils and one

central fibril. Ludvik *et al.* (1961, in Jirovec & Petru, 1968) studied ultrathin sections and total preparations shadowed with beryllium and chrome. They found four anterior flagella of equal length (15-22μm) consisting of 10-11 fibrils forming a bundle surrounded by a flexible sheath, 9 fibrils being arranged in a circle and one or two fibrils being central. The undulating membrane was 1-1.6μm thick, the exterior border being formed by a fine marginal fibril. In the middle of the undulating membrane is the fifth, recurrent flagellum. This ends in the first half of the cell body along with the undulating membrane; it is never free and trailing.

The flagella and undulating membrane arise in the group of five basal granules situated in the terminal part of the axostylar capitulum. Anteriorly the axostyle forms a thick capitulum. The parabasal body (5-6μm long, 1um wide) lies in the anterior part of the cell body mostly dorsal to the nucleus. Most cytoplasmic, osmiophilic granules are crowded around the spindle or drop-shaped nucleus and along the axostyle. Finer granules occur especialy along the costa beneath the undulating membrane.

Nielsen *et al.* (1966) carried out extensive studies on *T. vaginalis* ultrastructure (see Figure 1). They found that the nuclear membrane is an ordinary 3 layered membrane about 7 mm wide. Most nuclei contain several large electron dense granules, either nucleoli or clusters of chromatic material. The parabasal body is a Golgi zone located near the anterior pole opposite the axostyle, measuring about 0.5-1.5um and composed of flattened, layered cisternae. Vesicles that appear to arise from individual cisternae by constriction or budding are present, all have a triple-layered membrane 7-10μm wide. The sheath of the axostyle consists of a monolayer of 50-55 tubular fibres. Four of the basal granules (kinetosomes) have parallel long

axes and are distributed radially around the fifth granule to which the recurrent flagellum is attached. Each *T. vaginalis* cell has at least two flagellar fibres (costae) and a parabasal filament attached to the kinetosomes by fibres. The costa is longer and wider than the filament. It is located near to the attachment of the undulating membrane. The parabasal filament lies nearer to the centre of the cell. The recurrent flagellum is attached to the undulating membrane along its entire length. The endoplasmic reticulum is found frequently as a corona around the nucleus and is always abundant in the cytoplasm inside the capitulum of the axostyle. Free ribosomes are also distributed throughout the cytoplasm.

1.1.3. Biochemistry and Cell Biology

The general biochemistry of *Trichomonas* has been studied in some detail and reviewed by Honigberg (1978). However, I shall only discuss the biochemistry of trichomonads that has some relevance to this work, namely polyamines and proteinases.

1.1.3.1. Proteinases

Proteinases have been found in all parasitic protozoa studied, which include *Plasmodium* species (Aissi *et al.*, 1983; Dejkriengtraikhul & Wilairat, 1983), *Entamoeba histolytica* (Goldberg *et al.*, 1982; Avila *et al.*, 1985), *Leishmania mexicana* (Coombs, 1982), trypanosomes (North *et al.*, 1983) and *Trichomonas* species (Coombs & North, 1983). This was reviewed by North (1982).

In 1979 McLaughlin and Muller reported that a low-molecular weight proteinase purified from *Tritrichomonas foetus* appeared to be of the cysteine type and the sole major proteinase present. Coombs (1982) detected relatively high levels of activity in *Trichomonas vaginalis* and these were found to be due to multiple forms of cysteine proteinases (Coombs & North, 1983). Subsequently, Lockwood *et al.*

(1984) detected at least 4 proteolytic activities in the lysates of each of *T. vaginalis*, *T. foetus* and *T. batrachorum* (a non-pathogenic trichomonad isolated from the large intestine of the leopard frog, *Rana pipiens*). Electrophoretic studies using polyacrylamide tube gels containing denatured haemoglobin showed that the two parasites of the urogenital system, *Trichomonas vaginalis* and *Tritrichomonas foetus* were more similar to each other than to *Trichomitus batrachorum* in terms of the complexity of the proteinase band patterns observed. The enzymes were sensitive to the proteinase inhibitors TLCK, TPCK, iodoacetic acid, leupeptin and antipain, and they required DDT for optimal activity suggesting that they were cysteine proteinases (Lockwood *et al.*, 1987).

Inhibitors of proteinases are known to exist in plasma (Andersson *et al.*, 1979). Lushbaugh *et al.* (1981) demonstrated that cytotoxic activity of lysate from *Entamoeba histolytica* was dependent upon removal of serum from the tissue culture assay system, and identified the factors in non-immune sera responsible for producing *in vitro* inhibition of amoebal toxin cytotoxicity towards Hela cells. The high molecular weight inhibitory fraction they found was identified predominantly as α-2-macroglobulin and a low molecular weight inhibitory fraction was identified as predominantly α-1-antiprotease. The characteristics of the serum inhibitors suggested that the amoebal toxin has proteinase activity. As yet little is known about inhibition of trichomonad proteinases by serum factors, although these are possibly relevant to my studies especially those involving the interaction of trichomonads and mammalian cells *in vitro*.

Lushbaugh *et al.* (1984) clarified the proteinase nature of *E. histolytica* cytotoxin and also confirmed that a correlation existed between cytotoxicity and proteinase activity (Lushbaugh & Pittman,

1982). The evidence for a link between trichomonad proteinases and cytotoxicity will be discussed in a later section. The known production of proteinases by trichomonads and the link between cytotoxicity and proteinase activity in *E. histolytica* encouraged my investigation into the possible role of proteinases in the trichomonad host-parasite relationship.

1.1.3.2. Polyamines

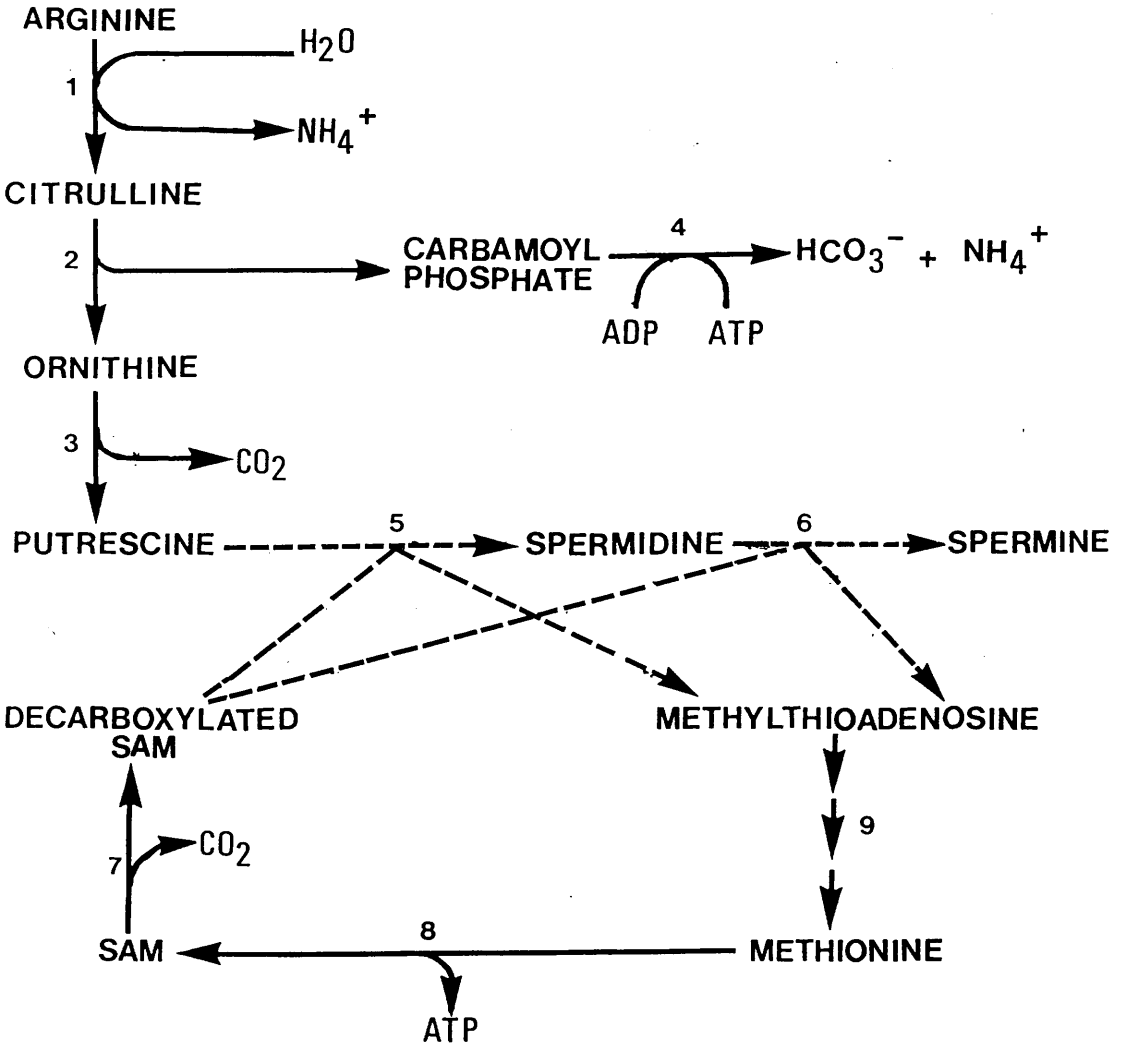
Because of its central role in the urea cycle and the biosynthesis of polyamines, the enzymology of arginine catabolism has been extensively studied in animals. Less attention has been paid to the catabolism of this amino acid in microorganisms despite its possible importance as a precursor of polyamines.

Linstead and Cranshaw (1983) observed that when *Trichomonas vaginalis* grew in defined and semi-defined media, arginine was rapidly depleted from the medium. At the same time there was an increase in the amount of ornithine in the medium. The three enzymes of the arginine dihydrolase pathway were all detected in extracts of *T. vaginalis* (see Figure 2). The occurrence of this pathway of arginine catabolism is unusual in a eukaryotic organism, although relatively common in prokaryotes. Linstead and Cranshaw suggested that the major role of the pathway would be to produce ornithine for biosynthesis of polyamines; the observed presence of the first enzyme of the polyamine biosynthetic pathway, ornithine decarboxylase, lent support to this possibility. The importance of the arginine catabolism pathway as a source of ATP is unknown. In laboratory culture glucose is utilized more rapidly than arginine (Mack & Muller, 1978) and glycolysis and pyruvate catabolism are usually considered the major source of ATP in *T. vaginalis*. However, arginine is present at very high levels in the extracellular fluid of mammals (Currie *et al.*, 1979) and may make a

Figure 2: Polyamine metabolism in *Trichomonas vaginalis*. Pathways that are present (--->) and not known (- - ->). Key to enzymes: (1) arginine deaminase; (2) ornithine carbomoyl transferase; (3) ornithine decarboxylase; (4) carbonate kinase; (5) putrescine aminopropyltransferase or spemidine synthetase; (6) spermidine aminopropyltransferase or spermine symthetase; (7) SAM decarboxylase; (8) SAM synthetase; (9) conversion of methyladenosine to methionine.

(Figure courtesy of Dr K.W. Thong).

SAM - s-adenosyl methionine.


ARGININE
H_2O
1
NH_4^+
CITRULLINE
2
CARBAMOYL PHOSPHATE
4
HCO_3^- + NH_4^+
ADP
ATP
ORNITHINE
3
CO_2
PUTRESCINE
5
SPERMIDINE
6
SPERMINE
DECARBOXYLATED SAM
METHYLTHIOADENOSINE
9
7
CO_2
SAM
8
METHIONINE
ATP

significant contribution to the energy balance of *Trichomonas vaginalis* in its natural habitat.

The polyamine content of *Trichomonas vaginalis* does not conform to any generally accepted pattern found in prokaryotes or other eukaryotes (White *et al.*, 1983). The parasite contains much more putrescine, and has a higher putrescine/spermidine ratio than mammalian tissues or other protozoa (Raina & Janne, 1975; Tabor & Tabor, 1984). Also, spermine appears to be present in *T. vaginalis* at typical mammalian levels. White *et al.* (1983) also confirmed the presence of ornithine decarboxylase in *T. vaginalis*.

Chen *et al.* (1982) and Sanderson *et al.* (1983) both reported putrescine and cadaverine occurring in vaginal washings from patients with trichomoniasis. It was also reported that putrescine was secreted by *T. vaginalis* in large amounts (Sanderson *et al.*, 1983).

North *et al.* (1986) determined the polyamine content and ornithine decarboxylase activity for three species of trichomonad, *T. vaginalis*, *Tritrichomonas foetus* and *Trichomitus batrachorum*. In all 3 species the major polyamine was putrescine, the product of the ornithine decarboxylase reaction. Treatment of *Trichomonas vaginalis* with α-difluoromethylornithine (DFMO), a specific irreversible inhibitor of ornithine decarboxylase, reduced the level of intracellular ornithine decarboxylase activity yet growth was only slightly affected. Although the amount of putrescine was reduced there were still high concentrations of polyamine present. This suggests that there is an alternative source of polyamines. Radioactively labelled putrescine is taken up from the growth medium by *T. vaginalis* (North *et al.*, 1986) and so extracellular putresciene could provide this source.

As *T. vaginalis* growing in culture does not require the high ornithine decarboxylase activity normally present in the cells, this

raises doubts about the need for polyamine biosynthesis *in vitro*. *In vivo* an alternative source of polyamines may not be available and so inhibition of ornithine decarboxylase could have an effect if polyamines are essential.

As polyamines are present in all eukaryotes and are thought to have many functions in the synthesis and structure of macromolecules (Pegg & Jacobs, 1983), this has led to investigations of polyamines as potential targets for antimetabolic chemotherapy (McCann *et al.*, 1981). A major advance in the use of ornithine decarboxylase as a chemothereapeutic target was the development of the inhibitor already mentioned, DFMO (Metcalf *et al.*, 1978). DFMO slows or completely blocks cell division in mammalian cell cultures, preferentially acting on rapidly dividing tumour cells or transformed cell lines as opposed to cell lines derived from normal tissues (Mamont *et al.*, 1978; Luk *et al.*, 1980). DFMO underwent trials in humans as an antitumour agent (Janne *et al.*, 1978). Studies with DFMO on a short term, rapidly fatal *Trypanosoma brucei brucei* infection in the laboratory demonstrated its curative property on its own (Bacchi *et al.*, 1980) or in combination with the antitumour agent, bleomycin (McCann *et al.*, 1981; Bacchi *et al.*, 1982) and the effects *in vivo* of DFMO on the metabolism and morphology on this parasite were investigated by Bacchi *et al.* (1983). DFMO also suppresses laboratory infections of *T. b. rhodesiense* (McCann *et al.*, 1981), *T. congolense* (Karbe *et al.*, 1982) and of *Eimeria tenella* (Hanson *et al.*, 1982).

Sjoerdsma and Schechtor (1984) described clinical observations in patients with African trypanosomiasis successfully treated with oral doses of DFMO, as well as reviewing the effects of DFMO on various protozoan parasites. They suggested that the efficacy of DFMO as a growth inhibitor appeared to be related directly to the rapidity of

replication of the particular cellular system and the degree of its depedency on ornithine as a putrescine source. These factors seem to explain the susceptibility of African trypanosomes.

At the start of my studies there were no publications containing information on the effect of DFMO on trichomonads. The finding of high levels of polyamines in the vaginal washings of patients suffering from trichomonasis suggested a possible link between polyamines and survival of trichomonads in the urinogenital tract and the high levels of polyamines secreted by trichomonads indicated a possible role in pathogenicity. The susceptibility of African trypanosomes to an inhibitor of polyamine production and its suitability for use in humans led to my investigation of the effects of DFMO on trichomonads *in vivo* and *in vitro*.

1.2. HUMAN TRICHOMONIASIS - THE DISEASE

1.2.1. Epidemiology

1.2.1.1. Geographic Distribution

Human trichomoniasis is endemic in many and possibly all major populations of the world. Reports of incidence in populations have come from several European countries including the U.K. (Whittington, 1957). It is not possible from the data available to know if the disease occurs more frequently in some countries than others, but it appears, however, that climate or genetic make-up plays little part in determining infection rates.

The average frequency of infection with *Trichomonas vaginalis* is about 10% in normal populations (2-15%, Kuceva, 1957) but can be as high as 25%, as Nagesha *et al.* (1970) reported from India. Incidence varies with the age of the population, as the maximum infection rate occurs during the years of highest sexual activity (Mascall, 1954). Many reports of trichomoniasis incidence within populations are based

on data taken from patients attending urinogenital clinics or gynaecological patients and so it is concluded that these are unlikely to reflect the situation in the population as a whole. Zrubek and Szymanski (1985) studied the incidence of *T. vaginalis* in factory workers in Poland, and results for the mostly married, multiparous women of reproductive age, showed the presence of *T. vaginalis* in only 4% of them. This is much less than other authors have reported and they concluded that the majority of studies, which are based on gynaecological patients, overestimate the incidence of trichomoniasis in the population as a whole.

1.2.1.2. Distribution within the Population

Transmission of *T. vaginalis* by sexual intercourse is indicated by the distribution of infection within the population, for example, Petru (1964, in Jirovec & Petru, 1968) found only 23.7% of the married women he examined to be positive for *T. vaginalis* whilst 48.2% of the divorced women were positive.

Burch *et al.* (1959) found prevalence to be greatest in women aged 30-49 years. Herbst *et al.* (1960) studied prevalence of *T. vaginalis* amongst prison inmates and showed that prevalence of infection amongst prisoners varied with age group: prevalence was lower in the 51-79 year age group than in the 17-50 age group.

Prevalence of *T. vaginalis* in the male population has been more difficult to determine since the infection is mostly latent or subclinical and therefore does not bring the male to consult a doctor or clinic in the majority of cases. In the past, *T. vaginalis* infections of the human male were considered rare, but reports have become more numerous especially in connection with non-specific urethritis (N.S.U.). Whilst investigating the cause of recurrence of trichomonal vaginitis, Karnaky (1938) found trichomonads in 38 out of

150 husbands of infected women; they were found in the prostate, urethra or preputial sac. Lancely and McEntegart (1953) inoculated volunteers intra-urethrally and found that 3 out of 5 developed urethritis from which the protozoa were recovered for different periods. Only one patient complained of symptoms and these were trivial. The infection appeared to be self-limiting and the period during which the protozoa could be found varied from 4-94 days.

Subsequently, Robinson *et al.* (1965) reported that 15% of the female adult population they examined carried trichomonads whereas the corresponding male segment of the population showed an infection rate of less than 5%. The authors, however, commented upon the difficulty in identifying the organism in males and so their estimation of prevalence in the male population may be low.

Infections with *T. vaginalis* are not limited to adults. Early workers suggested that female babies may bcome infected from their mothers at birth. It is thought that *T. vaginalis* can settle only in an oestrogenized environment. Breast-fed babies are under the influence of their mother's oestrogen for several weeks after birth. Doderlein's *Lactobacillus* is present in the vagina of newborn girls and glycogen is abundant in the epithelial cells, representing the same environment as in healthy, adult women. Trichomonads are acquired during birth if the mother is infected with the parasites, although another possible cause of infection in newborn babies is highly unhygienic conditions. There are no records of duration of infection in newborns as infected babies are treated immediately and can be cured relatively quickly. *T. vaginalis* is very rare in girls and virgins, but the incidence of infection rapidly increases after girls become sexually active (Peter, 1957).

It is obvious from the evidence presented that *T. vaginalis* infections in women are most highly prevalent during the period of

high oestrogen levels in the woman's body i.e. from puberty to the menopause. However, this is, of course, the most highly sexually active period of a woman's life and therefore risk of infection is considerably greater during these years due to lifestyle alone. It is, therefore, unknown whether an oestrogenized environment is necessary for trichomonal infections. The incidence of trichomonad infection in newborn babies would indicate the necessity of oestrogen, although the longevity of such infections is entirely unknown, and the fact that post-menopausal women can sustain infections would indicate the opposite.

1.2.1.3. Transmission

It is now accepted that *T. vaginalis* is spread primarily as a sexually transmitted disease although controversy still remains as to the extent to which non-venereal transmission can occur. At the First International Symposium on Trichomonad Infections, Rheims, May 1957, it was agreed that human trichomoniasis is usually transmitted by sexual intercourse.

The possibility of transmission of *T. vaginalis* by other means than sexual intercourse has been suggested by several authors. There are many instances of female babies becoming infected from their mothers at birth. Other possible routes of transmission have also been investigated although despite the possibilities of non-sexual routes of transmission, the current evidence is that the vast majority of cases of trichomoniosis arise from parasite transmission through sexual contact (Caterall & Nicoll, 1960; Robinson *et al.*, 1965).

The influence of the trichomonad infection on the vaginal epithelium will be detailed in a later section (1.5.2), however, the symptoms produced by this interaction will be discussed here. Inflammatory changes occur in the vaginal epithelium, these resemble

in appearance mucous membranes observed in precancerous states (Kazanowska, 1962), and warnings about the possibility of false diagnosis were made by Bechtold and Reicher (1952). Occasionally, the presence of both cancer and trichomonads is observed (Bertini & Hornstein, 1970). Extreme cytological disturbances, however, are seen in a relatively small number of trichomoniasis cases and in most instances a cervical smear shows only minor morphological changes. In many cases there may be a complete absence of clinical symptoms.

T. vaginalis, however, does not inhabit the vagina exclusively, but can also colonize the urinary tract (Kean & Weld, 1955). Kean suggested a relationship between *T. vaginalis* and recurrent cystitis caused by *Escherichia coli* and other bacteria; the bacteria possibly being carried to the bladder by trichomonads. *T. vaginalis* can survive in the female urethra, paraurethral glands and in mucous crypts but causes less damage than in the vagina. Evidence of trichomonads in the urine has been provided by many authors and, as mentioned above, it is thought by some that relapses have their origin in urethral infection.

1.2.2. Diagnosis

Diagnosis of *T. vaginalis* infection cannot be based on clinical symptoms alone, especially in chronic or latent cases. Microscopical examination must be carried out, and when negative complemented by cultivation, this is the method routinely used in genito urinary clinics. The necessity of cultivation, a time consuming and expensive process has been questioned but has recently been confirmed (Philip *et al.*, 1987).

Trichomonads are most abundant two days following menstruation. In a weak infection the number of flagellates is low and the preparation can easily be mistakenly considered negative. If no flagellates can be found in the vagina, the urethra, paraurethral

ducts and greater vestibular glands are examined. Careful records are made of clinical findings in the vagina, cervix and external genitals of amount, colour, smell and appearance of vaginal secretion.

Microscopical examination may be confused by motile leucocytes attached to spirochaetes, flagellated bacteria, sperm or ciliated cells; these can be mistaken for trichomonads. Coutts *et al.* (1959), therefore, recommended the use of unstained dry smears observed under dark field microscopy to show clearly visible flagella. Starzyk *et al.* (1958) recommended the addition of 0.1% safranin, and Holtorff (1957, in Honigberg, 1978) facilitated finding *T. vaginalis* in native preparations by adding brilliant cresyl blue. With these strains only dead flagellates are stained intensively, red or blue respectively. Living flagellates remain unstained in contrast to leucocytes, epithelial cells etc. in preparations.

The medium used for cultivation of *T. vaginalis* for diagnostic purposes in clinical laboratories is usually one of Johnson's CPLM, Vt-bouillon (Magara) and Feinberg's. Cultivation has been found to increase the number of positive cases detected by at least 10%, although instances occur where a negative result is obtained in culture even when the microscopical finding was positive. Consequently the use of both methods is recommended.

More recent investigators e.g. Fouts and Kraus (1980) have confirmed that the patient's clinical symptoms are not sufficient for the diagnosis of trichomoniasis in women. 56% of the infected women studied had discharge and 18% dysuria, however, these symptoms were equally common amongst women attending the genito-urinary clinic but who did not have trichomoniasis.

Amsel *et al.* (1983) described the difficulties in diagnosis of yeast and trichomonad vaginitis. These can be confused with the

vaginitis caused by small Gram-negative rod-like bacteria, *Gardnerella vaginalis*, which are normally present in the vagina; their pathogenicity is dependent upon their number. Diagnosis of vaginitis due to this bacteria is based largely on the presence of 'clue cells' (epithelial cells covered with bacteria in vaginal smears). Symptoms of *G. vaginitis* are similar to vaginitis caused by *Candida* spp, the gonococcus, and *T. vaginalis*.

The existence of serotypes among isolates of *T. vaginalis* was first demonstrated many years ago although application of serological methods for diagnosis have not been fully explored. Kuberski (1978) described an indirect haemagglutination (IHA) test using a polysaccharide antigen extracted from cultured *T. vaginalis*. Antibodies were demonstrated in 97% of culture-positive women and in 90% of male patients from whom *T. vaginalis* was isolated. Matthews and Healy (1983) examined the methods of indirect haemagglutination (IHA) and gel diffusion (GD) to evaluate their relative usefulness for diagnostic and epidemiological purposes. The IHA test is rapid and well suited for screening large numbers of serum specimens. The GD assay is very simple to perform and requires little or no specialized equipment.

Recently, there has been renewed interest in diagnosis of *T. vaginalis* infection by detection of *T. vaginalis* antigen in urine and vaginal secretions. Yule and Ackers (1985) and Yule *et al.* (1987) applied an enzyme immunoassay technique to this task and Torian *et al.* (1985) produced a monoclonal antibody specific for the nucleus of all the *T. vaginalis* strains they tested, which they believed could be useful for the diagnosis of trichomoniasis by immunofluorescence.

Chen *et al.* (1982) attempted to assess correlations between the content of the diamines, putrescine and cadaverine, in vaginal fluid and the clinical manifestations of vaginitis, and developed a rapid

procedure for the determination of diamines. The diamine content of vaginal fluid specimens after therapy with metronidazole was correlated with clinical response to treatment. Unfortunately, the detection of increased diamine content in vaginal fluids does not allow laboratory differentiation between non-specific vaginitis (n.s.v.) and vaginitis due to *T. vaginalis*, so diagnosing infections with *T. vaginalis* would depend upon simultaneous wet mount examination or culture. Both conditions, however, can be treated successfully with metronidazole. Also, it was pointed out that the high predictive value of a negative test result is helpful in excluding n.s.v. or trichomonal vaginitis.

1.2.3. Treatment

In the 1940s and 1950s the only treatment available for trichomoniasis was the local application of trichomonacidal, bacteriocidal and mycocidal substances e.g. 'Triflocid' and 'Fluocid' (Spofa), 'Anathuose' (Guillaumin), 'Tricolpon' (Organon) and 'Viozol' (Ciba). Although these treatments appeared to cure the infection of the vagina, the occurrence of relapses was high. Treatment methods for the male were even less satisfactory. After the introduction of antibiotics, various combinations were given either by mouth or locally applied in an attempt to treat trichomoniasis, but no permanent cure was achieved (Greene, 1952).

The availability of *in vitro* and *in vivo* systems for evaluating potential antitrichomonal agents enabled the discovery that azomycin (2-nitroimidazole), isolated from a *Streptomyces* species, had weak trichomonacidal activity. Over 200 chemicals related to azomycin were then synthesized and screened for activity. In terms of chemotherapeutic index, metronidazole (1-hydroxyethyl-2-methyl-5-nitroimidazole) emerged as the most promising compound. The efficacy

of the drug was tested by Durel *et al.* (1959) and found to be very good. Within a few years, it was in use for the treatment of trichomoniasis all over the world. The secondary effects of metronidazole are minimal although rarely there are side effects. Cure rates of 95% or better can be expected from regimens of 200mg three times daily for 7 days or a single dose of 2g by mouth.

During the years of its widespread use for treatment of *T. vaginalis* infections, metronidazole has earned a reputation for being remarkably safe. It is generally acknowledged that it should not be given to women during the first 3 months of pregnancy or during lactation since it freely passes the placental barrier and is excreted in milk. However, no case of damage to the foetus or breast-fed child has been reported and experience indicates that the drug may be safely adminstered to children.

In the past few years, a number of investigators have reported cases of refractory vaginal trichomoniasis associated with isolates of *T. vaginalis* that were relatively resistant to metronidazole (Meingassner & Thurner, 1979). Although the oncogenic potential of metronidazole for patients who are being treated with standard doses of the drug appears to be absent or weak, the long term effects of larger doses of metronidazole such as those used in some patients with refractory vaginal trichomoniasis (Lossick *et al.*, 1986) are not known. The rarity of reports of *T. vaginalis* infections that resist treatment with metronidazole suggests a low incidence although available data indicate the problem is widespread. No recommended alternative therapy is available at the moment so these cases are generally incurable. Strains of *T. vaginalis* that resist treatment by metronidazole have been shown to resist, to various degrees, other 5-nitromidazoles *in vitro* and *in vivo* (Meingassner & Thurner, 1979).

Although cross-resistance exists the data do suggest that some of the other 5-nitroimidazoles may be relatively more effective against trichomonad strains resistant to metronidazole and further investigation is needed. The mechanisms of resistance will be discussed later.

Recently there has been more interest in the possibility of developing a vaccine active against trichomoniasis. In the late 1970s, a systemic vaccine for immunotherapy of human urogenital trichomoniasis was introduced by the Swiss firm, Solco Basle Ltd. It consists of eight inactivated strains of *Lactobacillus acidophilus* isolated from trichomoniasis patients. It is claimed that the vaccination leads to the elimination of *T. vaginalis* and other pathogens, and induces normalization of the vaginal flora, including reappearance of normal Lactobacilli (Andrial & Paric, 1985). It is also claimed that such treatment confers protection for 1-2 years against future infection by *T. vaginalis* in approximately 90% of patients. However, there is some controversy over the mechanism of action of this vaccine (Gombosova *et al.*, 1985).

1.3. CHEMOTHERAPY

1.3.1. Mode of Action of 5-Nitroimidazoles

Anaerobic prokaryotes and eukaryotes appear to have comparable mechanism for the metabolism of metronidazole and respond to the drug in a similar manner (Coombs, 1976; Ings *et al.*, 1974; Lindmark & Muller, 1976; Muller *et al.*, 1976, 1977). It is primarily anaerobic and photosynthetic organisms which are affected by this group of 5-nitroimidazoles, however there are reports of effectiveness against *Trypanosoma cruzi* (Raether & Winkelman, 1976) and *Trypanosoma evansi* (Maudour & Rahman, 1976). Evidently the specificity of metronidazole for anaerobic prokaryotes (Coombs, 1976), photosynthetic organisms

(Edwards *et al.*, 1974) and anerobic protozoa (Ings *et al.*, 1974; Muller *et al.*, 1976) depends upon the ability of these organisms to reduce the nitro group of the compounds (Coombs, 1976; Muller & Lindmark, 1978).

Under aerobic and anaerobic conditions, uptake of metronidazole by sensitive organisms is dependent upon drug concentration. In trichomonads, uptake kinetics indicate that diffusion rather than active tansport was responsible for the compounds entry into cells (Ings *et al.*, 1974; Muller & Lindmark, 1978). The concentration gradient responsible for the uptake of metronidazole is produced by the ability of sensitive cells to modify the drug by reducing its nitro group and thus lowering its intracellular concentration (Muller *et al.*, 1976).

In 1972, O'Brien and Morris found that ferredoxin-linked reactions participate in metronidazole reduction in *Clostridium*, this was confirmed by other authors (Coombs, 1976). Muller (1981) found that, in trichomonads, the site of metronidazole reduction is the hydrogenosome. A ferredoxin has been isolated from trichomonad hydrogenosomes which functions was an electron carrier from pyruvate:ferredoxin oxidoreductase and stimulates metronidazole reduction by this enzyme (Marczak *et al.*, 1983; Gorrell *et al.*, 1984; Yarlett *et al.*, 1985). Pyruvate:ferredoxin oxidoreductase functions in the presence or absence of O_2 (Cerkasov *et al.*, 1978; Muller & Lindmark, 1978). Under anaerobiosis electrons are transferred to a terminal oxidase which is responsible for the respiration of hydrogenosomes. Anaerobic conditions favour the transport of electrons to metronidazole, whilst O_2 competes with the drug for electrons (Muller, 1981).

It is assumed that an intermediate of the reduction kills the organisms, thus toxicity of 5-nitroimidazoles for susceptible

organisms decreases in the presence of O_2.

In experiments involving ^{14}C-metronidazole in *Trichomonas vaginalis* (Ings *et al.*, 1974) and *T. foetus* (Muller *et al.*, 1976), label was observed to be bound to proteins and to DNA. Further experiments with ^{14}C-labelled adenine, thymidine and uridine revealed that nitromidazoles interfered with the nucleic acid synthesis (Ings *et al.*, 1974). Fine structural studies by Nielsen (1976) on the effects of metronidazole upon *T. vaginalis* and that of Liss *et al.* (1972) on morphological changes caused by tinidazole suggest that protein synthesis and perhaps other synthetic processes are affected. According to Nielsen (1976) the number of polysomes decreased whilst the number of monosomes increased in flagellates exposed to metronidazole, and there was also a reduction of perinuclear endoplasmic reticulum. In tinidazole-treated trichomonads, Liss *et al.* (1972) found large aggregates of ribosomes. There are no reports of the ability of *T. vaginalis* to repair damaged DNA, although there have been suggestions that DNA is a primary target of 5-nitroimidazoles (Knight *et al.*, 1978). Controversy exists over the exact mechanism of action of metronidazole and there have been reports that 5-nitroimidazoles such as metronidazole did (Knight *et al.*, 1978) and did not (La Russo *et al.*, 1978) cause DNA strand breakage *in vitro*.

The first of a series of metronidazole reduction products, nitro radical anion, nitroso- and hydroxylamine derivatives, have been detected *in vivo* in *T. foetus* (Moreno *et al.*, 1984) and in *T. vaginalis* (Lloyd & Pedersen, 1985). Chapman *et al.* (1985) showed that metronidazole radical anions can be generated within intact hydrogenosomes isolated from *T. vaginalis*. These free radicals may damage the radical-generating system and the hydrogenosome membrane and are capable of outflow from isolated organelles. Experiments

showed that a similar process may occur *in vivo*. Whether the cytotoxic action of metronidazole is mediated within the hydrogenosomes as suggested by its irreversible inhibitory effect on H_2 evolution (Lloyd & Kristensen, 1985) or at some other site such as the nucleus which has been previously suggested (Edwards, 1985) remains to be elucidated.

1.3.2. Resistance to 5-nitroimidazoles

Since the introduction of metronidazole and related compounds as the universal treatment for trichomoniasis, controversy has arisen over whether there exist true differences between strains in their sensitivity to metronidazole, whether strains isolated from patients that are retractory to treatment with this drug are more resistant to it *in vitro* and in experimentally-infected laboratory animals; whether resistance to 5-nitroimidazoles can be increased experimentally *in vitro* or *in vivo* (laboratory rodents), and whether, since 1960, there has been an increase in resistance to metronidazole among strains in human populations that have been treated with this drug.

In 1976 Korner and Jensen estimated the minimum lethal concentration (M.L.C.) of metronidazole for *T. vaginalis* strains to range from 0.06 to 8.0ugml^{-1}. However, variations have been great between published reports of M.L.Cs due perhaps to differences in media used and numbers of protozoa tested. Many workers have found an M.L.C. (anaerobic) not exceeding 1ugml^{-1} for most strains (Durel *et al.*, 1966; McFadzean, 1969). Although it is clear that there do exist significant differences in sensitivity *in vitro* and *in vivo* in individual strains to metronidazole (De Carneri, 1966; Honigberg & Livingston, 1966), there is a lack of clear evidence to correlate these differences with instances of poor response by patients to treatmen with this drug.

A major insight was provided by the finding of Meingassner *et al.* (1979) that clinical isolates of *T. vaginalis* from a patient refractory to metronidazole were as sensitive as normal lines to metronidazole when tested *in vitro* anaerobically but were highly resistant if tested in the presence of oxygen. Subsequently, there have been several reports of lines of *T. vaginalis* resistant to 5-nitroimidazoles when tested under aerobic conditions *in vitro* (Lossick *et al.*,1986; Lumsden *et al.*, 1988; Robertson *et al.*, 1988).

Resistance of some strains can be enhanced *in vitro* by cultivation in the presence of gradually increasing doses of 5-nitroimidzole (De Carneri, 1966). Resistance can also be increased by serial subcutaneous passages in mice receiving stepwise increments of 5-nitroimidazoles (Bernazet & Guillaume, 1971; De Carneri, 1966; De Carneri & Trane, 1971; Durel *et al.*, 1966). Both *in vitro* and *in vivo* changes were retained in the course of numerous transfers in culture media without drugs (De Carneri & Trane, 1971; Honigberg & Livingston, 1966). Similar results have also been found in experiments with *T. vaginalis* (Meingassner & Mieth, 1976).

It has been reported that resistance to metronidazole decreases with prolonged *in vitro* cultivation of *T. vaginalis* by serial transfers in culture media (De Carneri, 1966; Honigberg & Livingston, 1966), therefore meaningful assays of drug resistance, like those of pathogenicity must be done with fresh isolates.

Benazet and Guillaume (1971) observed a high degree of cross-resistance to 5-nitroimidrazoles and felt that if clinical resistance of *T. vaginalis* to metronidazole does increase it does not seem that nimorazole will provide a solution to the problem. Ray *et al.* (1984) tested the MLCs of many nitroimidazoles including secnidazole, tinidazole, and ornidazole, for both metronidazole-resistant and metronidazole-sensitive strains of *T. vaginalis* and concluded that the

metronidazole-resistant strain was also resistant to all other nitroimidazoles tested except for a 5-nitroimidazole derivative, G010213, which they suggested warrants further investigation as a possible treatment of drug-resistant trichomoniasis.

The mechanisms involved in metronidazole-resistance of trichomonads are as yet unclear. *In vitro*, trichomonads can develop either anaerobic or aerobic resistance to metronidazole. The anaerobic resistance is accompanied by enzyme deletions inactivating the hydrogenosomal pathway of pyruvate metabolism responsible for reduction of metronidazole to a cytotoxic agent (Cerkasovova *et al.*, 1976). Both *T. vaginalis* and *T. foetus* can develop this type of resistance *in vitro* when exposed to increasing concentrations of drug in anerobic culture. The resistant organisms showed MLCs of up to 1000μgml^{-1} for metronidazole, and multiply readily in anaerobic culture at 100μgml^{-1} of the drug. The lack of activity of the pyruvate:ferredoxin oxidoreductase (PFO) is a principal feature of the resistant strains both in *T. foetus* and in *T. vaginalis*. Hydrogenase activity is also lost or decreased greatly. Acetate and H_2 are not produced due to the inactivity of the hydrogenosomal enzymes. The lack of hydrogenosomal pyruvate metabolism is substituted by enhancement of cytoplasmic pathways, alcoholic fermentation in *T. foetus* and lactate fermentation in *T. vaginalis*.

The aerobic resistance to metronidazole has been reported in clinical isolates of *T. vaginalis* from patients refractory to standard treatment in several European countries and the USA. Aerobically resistant organisms cannot multiply in the presence of metronidazole, but tolerate elevated concentrations of the drug, provided that O_2 is available at a certain low partial pressure. *In vitro*, this type of resistance is detectable in aerobic systems only, and the MLC values

rarely exceed 100μgml^{-1} metronidazole. The aerobically resistant trichomonads possess pyruvate:ferredoxin oxidoreductase activities similar to those of sensitive strains (Meingassner *et al.*, 1979; Muller *et al.*, 1980). The presence of a functional PFO is consistent with metronidazole susceptibility of these organisms under aerobiosis. Recent findings indicate that these trichomonads are deficient in their ability to remove O_2 from their surrounding medium, having decreased affinity for oxygen. Lloyd and Pedersen (1985) also suggested that a resistant strain had defective O_2-scavenging systems. Thus the residual partial pressure of O_2 may be sufficient to reoxidize the nitroradical anion of metronidazole released in the process of the drug reductive activation and assumed to be an effector of cytotoxicity (Tachezy *et al.*, 1985).

1.4. *IN VITRO* CULTIVATION AND PRESERVATION OF ISOLATES

1.4.1 The Effects of *in vitro* Cultivation on the Virulence of Isolates

Honigberg (1961) observed that strains of both *T. vaginalis* and *T. gallinae* become attenuated in their virulence when maintained in culture, subsequently there have been several reports confirming that attenuation occurs with *T. vaginalis* but is more pronounced with *T. gallinae*. In 1964 Stabler *et al.* performed an extensive study on the effects of certain laboratory procedures on virulence of a strain of *T. gallinae* for pigeons. They found that the pathogenicity levels of trichomonads appear to be genetically controlled. The reversibility of the apparent loss of virulence by bird-to-bird passage indicated, however, that the internal environment of the host is necessary for the maintenance of virulence. It was evident that some characteristics of the parasites which play an important part in their virulence while maintained indefinitely when the parasite is in normal hosts, are

gradually lost during frequent division of the flagellates *in vitro*. When the parasite is maintained at subzero temperatures, these virulence factors are not diminished. They also found that a highly virulent strain of *T. gallinae* became avirulent very quickly when isolated from infected domestic pigeons with the aid of penicillin and dihydrostreptomycin. Although comparable attenuation also occursduring cultivation following isolation in the absence of antibiotics, this virulence loss took about twice as long to become apparent as when these antibiotics were used. The authors postulated that extranuclear nucleic acids may be affected by the antibiotics although they had no experimental evidence for this. The authors also reported that attenuation of pathogenicity never occurred during serial pigeon-to-pigeon passages.

Lindgren and Ivey (1964) carried out a similar series of experiments on the effects of cultivation and freezing on the virulence of *T. vaginalis* for mice (via the subcutaneous route) and found very similar results to those for *T. gallinae*.

Kulda (1965) compared changes in the virulence of a clone of *T. gallinae* maintained *in vitro* and *in vivo* and confirmed the results of earlier workers that pathogenicity was attenuated only through subpassage *in vitro* and was maintained if trichomonads were subpassaged *in vivo*. In 1970, Dwyer and Honigberg found that *in vitro* cultivation of a highly virulent strain of *Histomonas meleagridis* a similar parasite to *T. gallinae* which killed turkeys and chickens within 9-10 days on average, was accompanied by gradual attenuation of pathogenicity. Original virulence levels could be restored for up to 15 weeks by chicken-chicken passages. No decrease in virulence occurred when the histomonads were maintained in liquid nitrogen (-196^{o}C) for 20 weeks from the time of their isolation. Ivey (1975) gave guidelines for virulence preservation of recent isolates of *T.*

vaginalis. The vast majoroity of these studies on the virulence of *T. vaginalis* included measuring virulence by methods other than intravaginal growth. It is not known how relevant the experiments are to the growth of *T. vaginalis* in the normal environment.

1.4.2. *In vitro* Drug Sensitivity Testing

Most *in vitro* drug senstivity testing has involved metronidazole and other 5-nitroimidazoles, although many other potential trichomonacides have been investigated in this way. Unfortunately, for a long time there was no standard method of determining *in vitro* sensitivity to metronidazole and this has made it impossible to compare reports from various authors. Authors have used various incubation periods, although usually 24 or 48 hours (Meingassner & Thurner, 1979); and many different culture media with varying amounts of serum. Crucially, different culture volumes and gaseous conditions have been applied such that trichomonads have been grown under anaerobic conditions or in the presence of different concentrations of oxygen. Another important variable has also been the method of assessing results. Some authors have used direct counts to calculate different percentage inhibitory levels arithmetically (Michaels *et al.*, 1962); whereas others have determined the minimum inhibitory concentration (MIC) or minimum lethal concentration (MLC), the lowest concentration at which no motile parasites were observed at the end of the incubation. More recently this has involved microtitre plates and the use of an inverted microscope (Clackson & Coombs, 1983).

Many authors have now adopted the method described by Meingassner and Thurner (1979). This involves determining MIC (or MLC) using multiwell plates and an inverted microscope after 24 hours incubation at 37^{o}C under both aerobic and anaerobic conditions.

More recently there have been attempts to improve the assessment

of results of *in vitro* during sensitivity tests. For example, Kreiger *et al.* (1985) discussed the use of a time-kill technique for susceptibility testing of *T. vaginalis*; Mason (1985) describes testing the sensitivity of *T. vaginalis* using a bromocresol purple indicator and Escario *et al.* (1985) measured the amount of lactic acid released by *T. vaginalis* into the culture medium. These last authors used as end-points the minimum cytocidal concentration after 24 hours, i.e. the lowest concentration blocking lactic acid release; and the 50% inhibitory concentration (C.I.50), the lowest concentration which reduced lactic acid release to 50% of the amount released in control cultures. Meyers and Chang (1979) described a bioautography method for monitoring growth and inhibition zones of *T. vaginalis* on agar plates using phenolphthalein monophosphate as an enhancer made deep red by hydrolysis. This method was later adapted by Latter and Walters (1985) for liquid phase cultures of *T. vaginalis* in microtiter plates, absorbance being measured on a Flow Multiscan MC. The major aim of these developments was to devise a rapid yet accurate and sensitive method that could handle many samples very efficiently. Such a method would be ideal for drug screening.

1.5. THE HOST-PARASITE RELATIONSHIP

1.5.1. The influence of *T. vaginalis* on the host vaginal epithelium

Experimental inoculation of trichomonads into the human vagina has been attempted by several investigators for the purpose of ascertaining their pathogenicity. A definite pathogenicity has been attributed to this organism since the experimental inoculation of bacteria-free strains into the human vagina by Asami and Nakamura (1955) who found that all patients successfully implanted with *T. vaginalis* developed symptoms or signs of vaginitis of various intensities. Asami and Nakamura (1955) demonstrated a decrease in the

amount of glycogen in vaginal epithelial cells following the implantation of *T. vaginalis*. Since it is well known that Doderlein's bacilli require much glycogen for their multiplication, growth of these bacilli may be inhibited in this condition of the vagina with a consequent decrease in the acidity. Thus, it is suggested that the vigorous consumption of glycogen by trichomonads facilitates the invasion of the vagina by abnormal bacteria, resulting in vaginitis through synergistic action of trichomonads and bacteria.

In cases of trichomonad infestation, vaginal smears exhibit a characteristic pattern. There is a marked increase in the number of cornified cells of the superficial, squamous type. Some of the cells have irregular outlines and are covered with smudges of greyish mucus. Cytoplasm is often dense and intensely acidophilic. Nuclei are deeply stained in well preserved cells, whilst in degenerating cells they are faintly stained and have a pinkish appearance. A distinctive and frequently observed feature is the presence of a perinuclear halo. Marked keratinization of the cells is occasionally noted. The parabasal cells are often prominent and appear singly or in small clusters. They show some variation in size and a more pronounced vacuolization of the cytoplasm than seen in normal cells (Papanicolou & Wolinska, 1955). These authors also noticed that in some *T. vaginalis* positive smears the exfoliated cells exhibited marked nuclear atypia, consisting chiefly of an enlargement, irregularity in form and hyperchromasia of the nuclei. They declared that these changes when pronounced may lead to a false interpretation of the cells as malignant. Nuclear atypia in association with *Trichomonas* may be observed not only in the superficial, intermediate and parabasal squamous cells, but also in cells of endocervical origin. However, extreme cytological aberrations are seen in a relatively small number

of *Trichomonas* infestations. In most instances the smears show only minor morphological changes and in many cases there may be a complete absence of clinical symptoms.

Dylewski (1973) carried out histochemical studies on acid mucopolysaccharides in the mucus membrane of the vaginal portion of the cervix uteri in cases of trichomoniasis. Twelve sections were taken from the vaginal portion of the cervix uteri in women with symptoms of trichomonal vaginitis and twelve sections from women without pathological changes in the vagina and these were stained. In the women with trichomonal vaginitis, an increase in the intensity of colour reactions characteristic of acid mucopolysaccharides was found in all elements of the mucus membrane. This was marked most clearly within the thickened basement membrane. Only in the portions with inflammatory infiltrations were the colour reactions negative or only weakly positive. In studies using PAS staining specific for neutral mucopolysaccharides, a decrease in the intensity of colour reactions was observed within the stratified squamous epithelium and particularly within its surface and medial layers. A rise in the level of acid mucopolysaccharides seems to be a defence reaction against the activity of *T. vaginalis* accompanied by a bacterial flora.

An electron microscopy study of *T. vaginalis* interaction with the vaginal epithelium was carried out in 1975 by Nielsen and Nielsen. They used vaginal biopsies from patients with trichomoniasis to demonstrate cell contact between cells of *T. vaginalis* and the superficial layer of the vaginal epithelium. The trichomonads were gathered in small cell clusters on the stratified squamous epithelium but covered only a small area of the surface. These authors found no evidence to show that trichomonad cells can penetrate into deeper cell layers of the stratified squamous epithelium, whereas other authors who have investigated the natural infection have occasionally noted

invasive growth of the trichomonads although only in epithelium which had already been affected by necrobiosis (Kessel & Gafford, 1935). Thus, it seems that *T. vaginalis* and *T. gallinarum* (Lee, 1972) are unable to invade normal tissue of the natural host.

T. vaginalis, however, has been found inside non-phagocytosing cells of cell cultures (Farris & Honigberg, 1970), of experimentally infected mice (Brugerolle *et al.*, 1974; Frost *et al.*, 1961) and occasionally of the vaginal epithelium (Frost *et al.*, 1961). Nielsen and Nielsen (1975) observed cells of *T. vaginalis* which were invading squamous epithelial cells, but as only the superficial layer of the epithelium and the partly desquamated cells were attacked, they believed that the strains of *T. vaginalis* examined were able to attack only necrotic cells. This was also observed in cell cultures infected with strains of *T. vaginalis* of moderate and low pathogenicity, whereas strains with high pathogenicity were thought to penetrate normal phagocytic cells (Farris & Honigberg, 1970).

Nielsen and Nielsen (1975) showed that the trichomonad undoubtedly damages the vaginal epithelium since epithelial lesions were located mostly beneath large clusters of trichomonads where a coherent layer of these cells was closely attached to the epithelium. However, the clusters of *T. vaginalis* covered only a small part of the mucosa and it is, therefore, unlikely that this damage alone was responsible for the severe vaginitis found in many patients, especially as the local inflammatory reaction of the mucus membrane was generally the same whether or not *T. vaginalis* cells were present on the epithelial surface. Thus, they concluded that the pathological changes due to the *T. vaginalis* infections were mainly due to toxic substances which were acting on the epithelium independent of contact with *T. vaginalis* cells, and which presumably could just as well have

been formed by the free trichomonads. Nevertheless, the results of this study confirmed that trichomonads adhere closely to and apply a strong tension onto the underlying epithelium. In this way, *T. vaginalis* might mechanically damage the limiting membranes of the epithelial cells and give rise to a deformation of the target cells and Farris and Honigberg (1970) observed that the number of glycogen granules in T. vaginalis infecting a human vagina was much higher than in *T. vaginalis* in axenic cultures. The glycogen content of *T. vaginalis* from experimentally infected mice was also found to be high (Brugerolle *et al.*, 1974).

Accumulation of glycogen in these cells was observed to be accompanied by glycogen depletion of the neighbouring host cells. This applies to the human vaginal mucosa where the amount of glycogen was shown to vary inversely with the number of *T. vaginalis* cells in the vaginal discharge (Asami & Nakamura, 1955). Their findings, therefore, indicate that the absence of glycogen in the vaginal epithelium is independent of direct cell contact with the trichomonads, and that the interaction between cells of *T. vaginalis* and the vaginal epithelium takes place primarily at a distance, probably by means of substances released into the vaginal fluid, and only secondarily by a direct cell contact mechanism.

A similar electron microscopic investigation was carried out by Garcia-Tamayo *et al.* (1978) who investigated the ultrastructure and histochemistry of trichomonads in the vaginal exudate. The results of Garcia-Tamayo and his co-workers confirmed the studies of Nielsen and Nielsen (1975) that groups of trichomonads frequently adhered to desquamated epithelial cells. Phagocytosis of fragmented desquamated cells by trichomonads was frequently observed. Acid phosphatase and other hydrolytic enzymes were proved to be important in digestion of engulfed cells, debris and bacteria. Previous studies had demonstrated

the presence of hydrolases in *T. vaginalis* (Muller, 1973). Phagocytosis of bacteria by trichomonads in axenic cultures and the presence of mycoplasmas within phagosomes of trichomonads in human vaginal medium (Nielsen & Nielsen, 1975) is in agreement with Garcia-Tamayo's findings. These authors suggested that the phagocytosis of desquamated epithelial cells by trichomonads is central to the production of vaginal and cervical inflammatory lesions.

1.5.2. The Interaction of *Trichomonas vaginalis* and the Vaginal Microflora in Infected Women

Knowledge of microbial associations of *T. vaginalis* is important in planning treatment; in understanding the environmental factors favouring the establishment and maintenance of the urogenital trichomonad, and to provide insight into the environmental factors that may affect pathogenicity.

Little investigation has so far been carried out into the association between trichomonads and viruses, but many workers have reported on bacterial associations. For example, Trussell (1947) reported that an association between *T. vaginalis* and gonococci is relatively frequent although later authors have disputed this (Gordon *et al.*, 1966; Zwierz, 1969). In the majority of cases, *T. vaginalis* is associated with Grade II microbial flora, which consists of many bacterial forms with the exclusion of Doderlein's bacillus. Yeast and fungi may be present and the vaginal pH (5-6) is higher than normal (4-5). Cocci, especially streptococci, and mycoplasma are frequently also present with *T. vaginalis* and diptheroids also are reasonably common. Robinson and Mirchandani (1965) found that *Micrococcaceae* are abundant in virtually all vaginal states; *Streptococcaceae* are fairly common vaginal organisms which are invariably present in symptomatic trichomoniasis; *Enterobacteriaceae* are found in the vagina of 25-50%

of patients irrespective of the presence of other microorganisms, and *Lactobacillaceae* decrease in active trichomoniasis but not in Candidiasis. Teras *et al.* (1966), however, indicated that the microflora associated with *T. vaginalis* varied widely. They observed that most of the microorganisms did not differ from these in non-infected patients and no changes in microflora were noted after trichomonads were eliminated by treatment. He found no correlation between the severity of the trichomoniasis and the constitution of microbial associates of *T. vaginalis* in males or females.

Also in 1966, Gordon *et al.* reported on the bacterial flora present in abnormalities of the female genital tract. Their study was in agreement with generally accepted views that aciduric Doderlein flora of the healthy vagina is largely replaced by a mixed flora in the presence of vaginitis. The low incidence of Grade I (Doderlein) flora in smears showing cytological evidence of severe inflammation was associated, on culture, with a much more varied aerobic and anaerobic flora. In particular, significantly high incidences of mycoplasma, aerobic streptococci and of *Haemophilus vaginalis* were seen in the severe inflammatory category, whilst obligatory anaerobic streptococci, bacteroides species and members of the *Enterobacteriaceae* were isolated more frequently in the presence of severe inflammation. A similar pattern of variation was observed in the frequency of occurrence of these microorganisms correlating with the degree of *T. vaginalis* infestation. It is generally accepted that vaginal lactobacilli bear a more or less inverse relationship to the degree of trichomonal infestation of the vagina, and this is illustrated by Gordon's findings that only 6.4% of smears containing numerous trichomonads were classified as Grade I. 59% of heavily infested exudates yielded these organisms. The incidence of aerobic

streptococci in specimens containing large numbers of trichomonads was approximately five times that observed in non-infested specimens. It seems likely that the more frequent occurrence of these microorganisms reflects a more favourable environment for their proliferation. It is well documented that the growth of mycoplasmas in the vagina is greatly favoured by a shift of vaginal pH from the normal acid range (4.0-4.5) towards alkalinity, while streptococci and coliforms grow well only above pH 6. Such conditions pertain in trichomonal infection, and also, tissue necrosis associated with the inflammatory reaction favours the growth of most of these bacteria, especially the microaerophilic and anaerobic species. It is probable, therefore, that the presence of these organisms, normally commensals of the female genital tract, is entirely secondary to the protozoal infestation.

1.6. EXPERIMENTAL MODELS OF THE TRICHOMONAD-MAMMAL INTERACTION

1.6.1. *In vitro* Effect on Mammalian Cell Lines

The first reports on the effects of *T. vaginalis* on cell cultures was made by Hogue (1943) who suggested that trichomonads injure chick and mammalian culture cells not by mechanical means but by 'toxins' produced by the flagellates. In 1957 Kotcher and Hoogasian, using a *T. vaginalis* strain of undetermined virulence in primary chick embryo explants and human cell cultures, concluded that the parasites inflicted damage upon vertebrate cells by mechanical means as they were unable to produce corresponding changes by exposing cell cultures to cell-free filtrates of trichomonad cultures. Christian *et al.* (1963) also felt that the pathological changes they observed in Hela cells exposed to *T. vaginalis* were caused by mechanical means, whilst Honigberg and Ewalt (1963) , using primary chick liver cultures and Hela cells, suggested that the relatively pathogenic strain of *T. vaginalis* they used inflicted injury on vertebrate cells both by

coming in contact with them and by some of the substances produced either by the parasites alone or in combination with the degenerating tissue culture cells.

Extensive studies of the behaviour of trichomonads in chick liver cell cultures were carried out by Farris and Honigberg (1970) using *T. vaginalis* and Kulda and Honigberg (1969) using *T. foetus*. Farris and Honigberg (1970) compared the degenerative effects of a mildly pathogenic strain of *T. vaginalis* and a more virulent strain. The pathogenic trichomonads, amoeboid in the presence of cell cultures, had a pronounced tendency for large areas of their bodies to adhere to the macrophages, fibroblasts and epithelial cells. The parasites caused severe injury to the cells to which they were applied, including abnormal cytoplasmic and nuclear changes. The typically non-amoeboid, smaller, mild strain flagellates settled upon the chick cells much less frequently and typically did not establish intimate contact with those upon which they came to rest. Thus, direct contact between the mild strain and the culture cells appeared to play a much less important role in their interaction.

In these studies it was found that relatively large numbers of macrophages contained trichomonads within their cytoplasm. The highest phagocytic activity occurred with both the mild and virulent strains during early infection; and the activity was greater in cultures exposed to the mild strain. With both strains, the rate of phagocytosis declined during the experiments, the decline being more precipitous in infections involving the virulent strain. Intracellular trichomonads of the pathogenic strain appeared normal, but most of these of the mild strain showed signs of degeneration. The number of fibroblasts and epithelial cells harbouring trichomonads within their cytoplasm were low, but significantly higher in cultures inoculated with the virulent strain than the mild strain, and it appeared likely that the

mild trichomonads could enter only degenerated cells of this type. Exposure of chick liver cell cultures to cell-free filtrates of the virulent strains caused significantly less extensive cytopathological changes than those resulting in the presence of living parasites. Filtrates of the mild strain caused almost no damage.

Division of fibroblasts was inhibited by both parasite strains, the inhibition being much greater in cultures exposed to the virulent flagellates. A significant but reversible inhibition resulted also from exposure of the fibroblasts to filtrates of the pathogenic strain; filtrates of the mild strain were least inhibitory. The development, appearance and extent of lesions differed greatly between cell cultures infected with virulent and mild strain trichomonads. Only a few lesion-like cell-free areas were found in cultures exposed to the filtrates of the pathogenic strain and none in those treated with similar filtrates of the mild one.

Another extensive study on *T. vaginalis*/mammalian cell interactions was carried out by Sharma and Honigberg (1966) who were concerned with cytochemical observations. Nucleic acids, polysaccharides, lipids and proteins of trypsin-dispersed chick liver cell cultures infected with a relatively pathogenic strain of *T. vaginalis* were studied. The authors noted a loss of cytoplasmic RNA in the chick cells and a depletion of glycogen stored in epithelial cells in the experimental cultures. A large accumulation of lipids in the fibroblasts and epithelial cells occurred and significant loss of lipids was seen in phagocytes with trichomonads closely applied to their external surfaces. In general, therefore, the cytochemistry of chick liver cells was substantially affected by the presence of trichomonads.

In 1973 Grys and Hernik examined the haemolysis of human and

rabbit erythrocytes by *T. vaginalis*. They found a difference in intensity of haemolysis between strains of *T. vaginalis*, although all strains could lyse both human and rabbit erythrocytes. The filtrate of some strains of *T. vaginalis* also produced haemolysis. Also Heath (1981) studied the effects of trichomonads on rabbit kidney tubule cell monolayers. Destruction of the monolayer was observed below adherent clumps of parasites suggesting that contact between parasites and epithelial cells was an important factor in pathogenesis.

The controversy over whether *T. vaginalis* exerts its pathogenic effect by mechanical means or by some kind of cytotoxin, has existed therefore since Hogue (1943) first suggested that cytotoxicity was caused by 'toxins'. Since then, many authors have arrived at the same conclusion: Kulda and Honigberg (1969), Honigberg *et al.* (1964), and Farris and Honigberg (1970), although those authors did suggest that mechanical effects also play some part. That any cytotoxin is produced has been disputed by other authors including Kotcher and Hoogasian (1957), Christian *et al.* (1963), and more recently Kreiger *et al.* (1982, 1985).

The controversy arising from these early investigations has not yet been solved, although recently many investigators have attempted to provide an answer. Chinese Hamster ovary cells were used to investigate pathogenic effects of *T. vaginalis* on mammalian cells by Kreiger *et al.* (1982). Filtrates of *T. vaginalis* cultures had no effect on the monolayer. *T. vaginalis* sonicates also had no effect whereas cytochalasin D ($1 ug ml^{-1}$), a microfilament inhibitor, reduced *T. vaginalis* destruction of the Chinese Hamster ovary monolayer by 66% although *T. vaginalis* motility was unchanged. They concluded that *T. vaginalis* killed target Chinese Hamster ovary cells by direct contact employing a mechanism that depends on *T. vaginalis* microfilament function and that there was no evidence for a secreted, diffusible or

cell-free cytotoxin.

Recently Alderete and Pearlman (1984) also suggested that the absence of cytotoxicity in their experiments, in which fixed trichomonads or concentrated fractions of culture filtrate were used against Chinese Hamster ovary cell monolayers, implicated contact dependent mechanisms. Although they conceded that lack of an effect of filtrate on cells could be explained by a very low level of toxin, masking of toxic moieties by medium components, or a selective effect by parasite products on host cells during certain stages in the cell cycle. Subsequently, Alderete and Garza (1985) investigated the nature of adherence of *T. vaginalis* cells to host cells using monolayer cultures of Hela cells. They observed *T. vaginalis* adherence to Hela cells via the side opposite the undulating membrane and midway between the flagella and axostyle was involved in attachment. It is not yet known whether specialized organelles are involved, although the consistent orientation of the adherent parasites as observed by Alderete and Garza (1985) does suggest that the relevant trichomonad surface components may segregate to a location on the parasite, perhaps one that is most accessible to host cell membrane sites. The apparent requirement for *T. vaginalis* metabolism for the pathogenicity to occur underlined the complexity of the interaction between host cells and trichomonads. Treatment of parasites with metronidazole or iodoacetate before incubation with cells prevented the interaction. The interaction was also found to be temperature-dependent, occurring less at lower temperatures. The data, therefore, are consistent with the concept that energy-dependent membrane fluidity and cytoskeletal functions may be necessary for trichomonal pathogenicity towards host cells. Cytochalasin B and colchicine microtubule inhibitors, although used at levels which did not affect viability or motility, could

produce non-specific membrane perturbations and toxicity unrelated to adherence events.

The rapid, saturable trichomonad adherence to cells that was observed and the results of competition experiments indicated that receptor reactions were involved in attachment. The possible role of trichomonad surface proteins in cell parasitism was indicated by experiments showing the ability of trypsinized organisms to reattach by protein synthesis. Alderete and Garza (1985) also showed that *T. vaginalis* appeared to parasitize epithelial cells to a greater extent than fibroblast cells. There was significant attachment to fibroblasts, but this did not lead to correspondingly high cytotoxicity levels, thus the data indicate a refractoriness of fibroblasts to contact-dependent killing by *T. vaginalis*.

Kreiger *et al.* (1985) observed that trichomonads formed clumps when in culture with Chinese Hamster ovary cells. These clumps then adhered to cell culture monolayers and monolayer destruction occurred only in areas in contact with protozoa. Neighbouring cells not in contact with protozoa remained intact. The authors proposed that motile *T. vaginalis* may move away from epithelium they have already damaged, thus explaining the clinical observation by Nielsen and Nielsen (1975) that cytopathology is observed in epithelium apparently free of protozoa. As the number of trichomonads in the mixed culture was increased whilst the number of target cells was held constant, the fraction of target cells killed was, according to these authors, directly proportional to the probability of their making direct contact with trichomonads. If target cell death were mediated by diffusible substances, such linear proportionality would not be expected. Filtrates of trichomonad cultures or of viable trichomonads actively destroying Chinese Hamster ovary cells had no effect on Chinese Hamster ovary cell monolayers. Also, filtrates did not

increase the rate of monolayer disr u tion by low numbers of intact viable trichomonads. Trichomonad sonic extracts also had no effect on target cells. These findings are in accordance with those of Alderete and Pearlman (1984). The microfilament inhibitor cytochalasin D inhibited trichomonad killing of Chinese Hamster ovary monolayers by 80%. In contrast, the microtubule inhibitor, vinblastine, caused only 17% inhibition of trichomonad destruction of Chinese Hamster ovary cell monolayers. The authors therefore concluded that *T. vaginalis* kills target cells by direct contact without phagocytosis, this event requires intact trichomonad microfilament function; microtubule function appears not to be essential.

Recently Pindak *et al.* (1986) surveyed growth of *T. vaginalis* in tissue cultures in order to identify the mammalian tissue cultures which were most suitable for investigation of cytopathogenicity of *T. vaginalis*. Fifteen different cell cultures were studied, all maintained under conditions which simulated the dependency of *T. vaginalis* on host tissue; the medium used allowed satisfactory growth of the organism only in the presence of live tissue cultures. The results showed that satisfactory cultivation of *T. vaginalis* is not limited to any particular type of mammalian cell culture, the choice of tissue culture depends on the objectives of the investigator. Nielsen and Nielsen (1975) found clusters of protozoa overlying mucosal microulcerations in biopsies from four of eleven women with trichomoniasis. Since trichomonads were not present in every area of epithelial disruption, the authors concluded that most cytopathology resulted from cell-free cytotoxins and that contact dependent destruction of epithelial cells was a secondary phenomenon. A neuraminidase has been implicated in the pathogenesis of *T. foetus* (Honigberg, 1979), and this and other protozoan parasites are known to

produce exoproteinases during growth and multiplication (Honigberg, 1979; Coombs, 1982; McLaughlin & Faubert, 1977; McLaughlin & Muller, 1979; Lockwood *et al.*, 1987). Pindak *et al.* (1986) demonstrated the presence of a substance which they believed to be a likely cause of the cytopathogenicity of *T. vaginalis* towards mammalian cell cultures. The presence of this substance in the culture fluid of *T. vaginalis* was first indicated by the failure of McCoy and Hela cells to form monolayers when added to two-day old cultures with high densities of trichomonads and in which the original cell monolayers had disintegrated. As attempts to duplicate such results with supernatants from similar cultures were only marginally successful, the authors concentrated the active ingredient. It was possible to obtain concentrates of the 'cell-detaching factor (CDF) which could be diluted several times and still have a demonstrable effect. Exposure to a temperature of $60^{o}C$ for 1 hour resulted in inactivation of the latter. The preparation was inactivated during controlled exposure to acidic conditions and acid sensitivity was also demonstrated under conditions of culture. The authors concluded that CDF is a metabolic product of *T. vaginalis* rather than an internal component; a lysate of the organism was inactive, whereas expected activity was present in the supernatant. They reported that the molecular weight is likely to be in excess of 10^5 kd as after ultrafiltration through a membrane with that cut off point, there was activity in the retentate but not the filtrate.

In summary, for many years it has been known that *T. vaginalis* produces degenerative changes in experimental epithelial cell cultures. Cell free filtrates have been found to produce less dramatic degenerative changes and not all authors have been able to observe these changes. Pathogenic trichomonad species have been shown to produce these effects in contrast to commensal trichomonads, for

example, *Pentatrichomonas hominis*, and *T. tenax* and differences between the effects and virulent strains of the same species have been observed. A difference in macrophage response to mild and pathogenic strains has also been shown. Extensive cytochemical changes have been detailed although the mechanisms involved in destruction of epithelial monolayers have not been elucidated. Controversy still exists today over the existence of an exotoxin produced by trichomonads. Some authors are still attempting to prove that damage of mammalian cells is mechanical via contact, a popular method being the use of microtubule inhibitors to prevent attachment. Other authors are attempting to isolate and concentrate trichomonad cytotoxins.

1.6.2. *In vivo*

Attempts have been made to grow *T. vaginalis* in experimental animals many times. The value of a reliable *in vivo* mammalian model for studying various aspects of host-parasite interactions, such as to evaluate inherent pathogenicity levels and to assay effects of antitrichomonal drugs, being realised early. Many different species of animal have been tested and many sites of trichomonad inoculation tried (Trussell & McNutt, 1941). It was found, however, that *T. vaginalis* would grow only in human and monkey vaginas and *T. foetus* was infectious only for cattle and dogs intravaginally. In 1955, Kean and Weld reported the growth of trichomonads in the anterior chamber of the rabbit eye. Although growth also occurred in the rat, the rabbit was considered more favourable because of the larger size eye and greater volume of aqueous humour. Pathological changes in the eye began several days post-infection when the cornea became opaque, and fluid removed from the anterior chamber appeared turbid. At that time parasites were present in greater numbers. A moderate inflammatory exudate was present in the aqueous humour and sections of the eye

disclosed numerous eosinophilic leutocytes and proliferation of the endothelium with the beginning of vascularization of the cornea. Transmission and serial passage of *T. vaginalis* in the anterior chamber of the rabbit eye were reported to be possible.

The intramuscular route is another method of *T. vaginalis* infection which has been attempted and found unsuitable for routine use although Schnitzer *et al.* (1950) recommended its use in mice. Ivey and Hall (1964) isolated strains of *T. vaginalis* from symptomatic and asymptomatic patients and injected these into groups of mice via the intraperitoneal, subcutaneous and intramuscular routes. No relationship was found between the human host status and the virulence of the strain when injected intraperitoneally. There seemed to be a possible relationship when mice were infected subcutaneously in that the majority of strains isolated from symptomatic patients exhibited more marked virulence for mice than did any of the strains isolated from asymptomatic persons. In contrast, the intramuscular route seemed of no value in assessing pathogenicity towards humans.

Most studies on intraperitoneal injections of *T. vaginalis* into experimental animals have been carried out using mice although Teras (1954) observed chronic inflammatory process in guinea pigs inoculated intraperitoneally with axenic cultures of *T. vaginalis*. Some of the earliest reports of intraperitoneal infections in mice came from the Estonian group (Teras, 1954; Roigas, 1961) who stated that the pathogenicity levels of *T. vaginalis* for mice, as estimated by the intraperitoneal assay, could be correlated with the manifestations of trichomoniasis these strains caused in male and female patients. A similar opinion was expressed by Bogovsky and Teras (1958). The data presented by Teras (1954), however, did not show an absolute correlation.

As mentioned previously, Ivey and Hall (1964) reported that the intraperitoneal mouse assay was unsatisfactory for estimating inherent pathogenicity levels of *T. vaginalis* strains, and Reardon *et al.* (1961) could differentiate clearly only between a strain isolated from a severe case of vaginitis and one from a mild case; pathogenicity towards mice differed among 11 strains obtained from patients with moderate symptoms. These authors also found differences in susceptibility between different mouse strains.

Gobert *et al.* (1969) studied *T. vaginalis* infection of the peritoneal cavity at macroscopic level and found that this typically involves peritonitis represented by ascites and abscesses or necrotic lesions. The first visceral organs to be attacked in trichomonad infection of the peritoneal cavity were reported to be either the liver (Teras & Roigas, 1966) or the pancreas (Gobert *et al.*, 1970).

The ultrastructural study of liver abscesses included by intraperitoneal injection of *T. vaginalis* in the mouse was undertaken by Brugerolle *et al.* (1974), who found that *T. vaginalis*, when injected into the peritoneal cavity of a mouse, grew within the liver and peritoneum as well as in contact with the pancreas and intestine. Within the liver, parasites appeared in the sinusoids and were associated with leukocytes such as neutrophils, lymphocytes and macrophages. They adhered to the cell membrane of hepatic cells which were lacking in glycogen but with increased numbers of lipidic granules in the cytoplasm. The host-cell cytoplasm was phagocytosed, becoming vacuolated on its way to complete obstruction. It was thought that hepatocytes and leukocytes were probably destroyed by the toxicity of lytic secretions from the trichomonad cell, and such secretion may also have led to the destruction of macrophages once parasites had been phagocytosed. The trichomonad cell became amoeboid and pseudopods showed numerous microfilaments. The cell membrane of

the trichomonad was sometimes covered by a thick, fuzzy coat. The Golgi apparatus was quite well developed and cytoplasm was rich in glycogen particles.

Inoki and Yamada (1953) obtained fatal infections of mice if they were treated intravenously with a suspension of washed erythrocytes from young chickens before intraperitoneal inoculation with bacteria-free cultures of *T. vaginalis*. The reasons for the increase in susceptibility were not explained. This finding was later supported by the work of Gobert *et al.* (1969). He showed that susceptibility was also increased by intravenous injection of human cells and trichomonads themselves and by oral administration of certain immunosuppressing drugs e.g. azathioprine. It would appear that in all cases the changes in host response were caused by suppression of the mouse immune system.

Laan (1966) studying intraperitoneal infections of mice with *T. vaginalis*, noted that among other physiological changes, pathogenicity levels were lowered by prolonged *in vitro* cultivation (over 4 months) of *T. vaginalis* strains. He also suggested that drug treatment of patients could reduce pathogenecity levels of trichomonad strains. In view of these results, Laan concluded that only the antigenic constitution of *T. vaginalis* was a 'stable' attribute, with virulence and other physiological characteristics being changeable. Budilova and Kulda (1978) showed a marked increase in virulence of *T. foetus* after treating host mice with intraperitoneal injections of ferric ammonium citrate and other iron-containing compounds prior to intraperitoneal inoculation of trichomonads. The results showed that an increase in virulence depends upon a continuous supply of an iron-compound in the initial three days of infection. The effect is probably related to the parasites requirement for iron and it is likely that the enhancing

effect of ferric ammonium citrate depends on this nutritional need rather than impairment of the host's cellular defence.

Two more recent studies have concentrated on a comparison of various mouse models. Dohnalova and Kulda (1975) assayed 19 strains of *T. foetus* using mice inoculating subcutaneously and intraperitoneally. The quantitative subcutaneous assay revealed that the strains tested could be grouped according to virulence. The intraperitoneal infection developed typically to the 'all or nothing' rule. Either the mice died 4-10 days after inoculation or the parasites were eliminated within a few days. Apparently the critical factor was the capability of individual strains to multiply in the peritoneal cavity. Virulent strains multiplied intensively in the peritoneal exudate without invading tissues, the avirulent strains did not multiply under the same conditions.

Brasseur and Savel (1982) evaluated virulence of trichomonad isolates in tissue cultures and compared these results to the effects of some isolates inoculated in mice. The pathogenicity levels of 25 isolates and 4 clones of *T. vaginalis* were evaluated on the observation of rapidity and intensity of cytopathogenic effect on MacCoy cell cultures. The authors assigned virulence indices to isolates in both of the models used and found that values obtained from *in vivo* methods were almost all less than those obtained using *in vitro* methods. Six isolates which showed a high index *in vitro* had an index of zero *in vivo*. However, the authors reported that statistical analysis of the results with only 19 isolates and four clones which had effects both *in vitro* and *in vivo* revealed a correlation between the two techniques of evaluation of virulence.

In summary, a reliable *in vivo* model is needed to study aspects of the host-parasite interaction, to elucidate inherent virulence levels of trichomonads and to assay effects of anti-trichomonal drugs.

Many routes e.g. intra-orbital and intramuscular were found unsuitable for routine use. Most studies on intraperitoneal infections of *T. vaginalis* have involved mice, although some investigators have had success with the guinea-pig. A correlation has been found between virulence levels of trichomonads for mice intraperitoneally and symptoms in male and female patients, however this is not absolute. The symptoms for the experimental mouse injected intraperitoneally with *T. vaginalis* include peritonitis, collection of ascites and abscesses or necrotic lesions spread throughout the peritoneum and into the liver and pancreas. Suppression of the host-immune response in infected animals leads to fatality. Using the intraperitoneal mouse model attenuation of virulence with prolonged *in vitro* cultivation was discovered. Ferric ammonium citrate introduced into the peritoneal cavity enhances virulence, this is thought to provide the nutritional requirement of *T. vaginalis* for iron. The intraperitoneal mouse assay follows the 'all-or-nothing' rule for detecting pathogenicity levels in trichomonad isolates as mice frequently die. This is a less sensitive method than detecting virulence in tissue culture cells, however, a correlation between results gained from the two methods does occur.

These methods have been used relatively little during recent years and the two *in vivo* models now favoured are those involving growth of the parasites subcutaneously and intravaginally.

1.6.2.1. Growth Subcutaneously

The first report of subcutaneous inoculations of trichomonads into a mammal was by Trussell and McNutt (1941), who inoculated cultures of *T. vaginalis* and *T. foetus* subcutan eously into guinea pigs but found no visible lesion even one month post-infection. Most later reports have all been of subcutaneous inoculation into

laboratory mice, including that of Schnitzer *et al.* (1950) who noted the formation of abscesses in mice injected with axenic cultures of *T. vaginalis*.

In 1961 Honigberg devised 'the subcutaneous mouse assay' for evaluating inherent pathogenicity buds of strains of various species of trichomonads including *T. vaginalis*. The assay involved subcutaneous inoculation of $8\text{-}9 \times 10^5$ trichomonads in an agar-containing medium into both flanks of 6-8 week old C57/BL6J mice, male or female. He established by measurements that the protruding parts of lesions corresponded to half a sphere; thus the volumes of lesions were estimated from the following formula:

$$\text{volume} = \frac{4/3\,\pi\, L/2 \times W/2 \times H}{2}$$

$$= 0.5236 \times L \times W \times H$$

where L is the length of lesions; W, the width and H, the height. Honigberg maintained that all necessary information about relative pathogenicity could be obtained on the basis of mean volumes of 6 day lesions. A statistical comparison of the mean volumes of subcutaneous lesions produced in mice by the several strains of *T. vaginalis* and *T. gallinae* tested revealed that these volumes, taken to express the degree of pathogenicity of the strains to the experimental hosts, faithfully reflected the relative virulence of the strains to the natural hosts.

The presence of agar in the inoculation medium of both *T. vaginalis* and *T. gallinae* enhanced the development of subcutaneous lesions. There was some experimental evidence e.g. absence of growth of lesions with inoculation media alone, indicating that the effects of agar were not confined to its action as an irritant upon host tissues but that this substance must have had some influence upon the

physiology of the parasites, rendering them more pathogenic. The substitution of methyl cellulose for agar in the medium enhanced the virulence of the parasites to a less degree although the abscesses produced were still quite large. Smaller lesions resulted from inocula that contain mucin in place of agar. Abscesses produced by the parasites in the presence of gelatin were no larger than those resulting from inoculation of trichomonads grown on media which included no viscous substances.

Various reports have been made of modifications of this basic assay including suggestions of alternative animal models. For example, Kulda (1965) found that with *T. gallinae* of the 8 mouse strains tested, Balb/c were the most suitable for the subcutaneous assay. Newton *et al.* (1960) made a comparative study of the growth of *T. vaginalis* subcutaneously in germ-free and conventional guinea pigs. On the basis of their results, the authors declared that, with a pathogenic strain of *T. vaginalis* the germ-free guinea pigs were more susceptible to the parasite than the conventional animals. The authors suggested that the failure of the parasite to prosper in the conventional animals was due not to the lack of nutritional essentials but rather to some tissue response or other defense mechanism not present, or underdeveloped in the germ-free animals. They concluded that the constant presence of a flora stimulated the development and maintenance of certain, nonspecific aspects of the conventional animal's defense mechanism. They considered that the failure of the germ-free animals to have mother's milk during the first few days of life, as did the conventional animals, may also have been a factor.

Kuczynska *et al.* (1984) compared the virulence of clones of two *T. vaginalis* strains using the subcutaneous assay and reported that, although for research purposes cloned populations should be employed

whenever possible, non-cloned strains produce reliable results and are suitable for use in routine virulence assays.

That differences in the mean volumes of subcutaneous lesions reflect differences in the progression of pathologic processes was demonstrated by Frost and Honigberg (1962). They reported that the outcome of the infection was determined by many factors including the rates of multiplication and death of the parasites, influx and death of polymorphonuclear leukocytes, damage to host tissues and spreading of the lesion. They reported that with a pathogenic strain of *Trichomonas vaginalis* the first massive multiplication of the flagellates occurred throughout the entire area of inoculum between the 16th and 32rd hour following their introduction into the host. Neutrophils migrating from the surrounding tissues formed a mantle against the inner margin of the lesion. These solidly packed organisms lined up against the wall, moved the first leukocyte mantle inward, and formed the first trichomonad mantle. More leukocytes were attracted and the process repeated iself. This same orderly progression occurred in all the other trichomonad strains examined, varying only in details of pattern, time intervals, and severity of response. The histopathological processes of the various strains coincided with the gross assessment of their relative levels of pathogenicity as revealed through measurements of the volumes of subcutaneous lesions (Honigberg, 1961). The peculiar palisading of the flagellates against the inner margin of the inoculation pocket resembled the arrangement of the parasites on the pharyngeal epithelium of pigeons infected experimentally with a highly pathogenic strain of *T. gallinae* (Perez *et al.*, 1961). Also Reardon *et al.* (1961) described a similar situation in liver lesions of mice injected intraperitoneally with a pathogenic strain of *T. vaginalis*. According to these workers there was always a band of parasites at the forward

edge of the necrotic lesion as it progressed through the liver. Just behind this band of flagellates was a zone of infiltrating inflammatory cells, principally polymorphonuclear leukocytes. Trichomonad breakouts through the wall of the lesion in the early stages of the lesion appeared to be associated with actively growing flagellates and dying leucocytes. The first break through the wall of a lesion of a highly pathogenic strain of *T. vaginalis* was noted on the 4th day, at about the end of the formation of the 2nd leukocytic mantle and the beginning of the formation of the 2nd trichomonad mantle. Since lysis of the wall appears to be one of the basic mechanisms of spread, it is possible that enzymes derived in part from degenerating leukocytes of the mantle account for this action. In addition, however, there is evidence that some substances produced by the parasites themselves may contribute to the breakdown of the wall (Honigberg & Ewalt, 1963). Near the end of the 14th day the wall becomes thick and fibrotic. Further breakthroughs of the parasites out of the abscess must be affected by lysis of this dense wall.

Leukocytes play an important role in trichomonad pathogenicity. Their breakdown not only appears to be related to the lysis of host tissues,but may actually provide nourishment for the parasites. Phagocytes are extremely active, however, in ingesting trichomonads within the abscess as well as out in the tissues. Numerous well preserved flagellates of a *T. gallinae* strain were observed within polymorphonuclear leukocytes. The ingestion of trichomonads by polymorphonuclear leukocytes in both natural and experimental infections has been reported by many authors including Frost *et al.* (1961). Thus, Frost and Honigberg (1962) found it unsurprising that this occurrence was very common in their preparations. Trichomonad parasites in non-phagocytic cells of the hosts, however, were not seen

in this study. The ingestion of leukocytes by trichomonads has been reported by several workers, but was not observed in this study.

Frost *et al.* (1961) examined intracellular *T. vaginalis* and *T. gallinae* in natural and experimental infections. Histopathological examination of subcutaneous lesions produced in C57/61 mice by axenic cultures of *T. gallinae* revealed host phagocytes apparently engulfing the flagellates. These included multinucleate giant cells and mononucleate macrophages. These intracellular terms usually appeared healthy, and at times several trichomonads are found in a single host cell. A tissue biopsy of the cervix uteri of a 30 year old patient in the 4th month of pregnancy revealed *T. vaginalis* within non-phagocytic human epithelial cells (Frost *et al.*, 1961).

Teras (1963) discovered that the growth rate of freshly isolated *T. vaginalis* strains in axenic cultures was inversely proportional to pathogenicity levels in mice, and according to Laan (1966), fresh isolates of highly pathogenic parasites had slower metabolism in axenic cultures than the less virulent trichomonads. Similar conclusions with regard to the relationships of pathogenicity of *T. vaginalis* strains for patients and mice and generation times in axenic cultures could be drawn from the results of Newton *et al.* (1960), and Kulda *et al.* (1970). Kulda *et al.* (1970) had an alternative explanation of this apparent inverse correlation of pathogenicity and growth rate *in vitro*. The results indicated that the slowly growing parasites were isolated from women suffering from symptomatic diseases of the urogenital organs. Not in all instances could these diseases be related to the presence of trichomonads e.g. ad enocarcinoma of endometrial vaginitis, there is much pus and caseous material in the lower parts of the urogenital tract. The flagellates flourish in such an environment, which is rich in various nutrients. Kulda *et al.* (1970) suggested that trichomonads which had lived for long periods in

such a nutrient-rich environment had become highly dependent upon the factors present in such an environment. Their low growth rate *in vitro* could be due to the absence of such factors and therefore not reflect the parasite's inherent pathogenicity.

In 1961 Honigberg used his subcutaneous mouse assay to detect differences in virulence between strains and compared this with the pathogenicity of strains in humans. Since then several other workers have performed experiments with the same aim. Dohnalova and Kulda (1975) assayed 19 strains of *T. foetus* using laboratory mice inoculated subcutaneously or intraperitoneally. The quantitative subcutaneous assay on CBA/J inbred mice revealed several groups of significantly different virulence. Convincing results with regard to a positive correlation between pathogenicity of *T. vaginalis* strains for mice and for patients were subsequently published (Honigberg *et al.*, 1966; Kulda *et al.*, 1970). Kulda and Petru (1966) however, although they found differences in pathogenicity for mice among 15 strains of *T. vaginalis*, were unable to obtain any correlation between the pathogenicity levels of the strains for animals and their effects upon the patients from whom the parasites were isolated. Possible explanations for this lack of correlation were thought to be previous infection by trichomonads, the effects of co-existing microorganisms and changes in pathogenicity due to cultivation. Brasseur and Savel (1982) compared methods of testing virulence and found that although a correlation was observed between host symptoms and virulence in the subcutaneous mouse assay, a more sensitive method was to use trichomonads in culture with mammalian cells. Alderete's (1983) results are also at variance with accumulated information concerning the dependability of the subcutaneous mouse assay. He used 5 strains of *T. vaginalis* and 1 of *T. foetus* and claimed that all these strains

were equally virulent for mice when assayed by his procedure even though 1 strain had been isolated from a patient showing no cytopathological abnormalities and another came from a very acute case of vaginitis. Despite its limitations and the uncertainty of its dependability as a measure of virulence towards humans, the subcutaneous mouse assay has been extensively used for assigning virulence values to isolates of *T. vaginalis*, for checking attenuation of virulence with prolonged *in vitro* culture, to detect differences in virulence between strains and also to test trichomonacidal activity of various compounds e.g. Meingassner and Lindmark (1977).

1.6.2.2. Growth Intravaginally

A) Experimental Infections in Humans

Experimental inoculation of trichomonads into the human vagina was attempted by several investigators for the purpose of ascertaining their pathogenicity e.g. Feo *et al.*, 1941). A definite pathogenicity has been attributed to the organism since the experimental inoculation of bacteria-free strains into the human vagina was carried out by Trussell (1947). Trussell (1947) concluded that 'intravaginal inoculation of parasite-free women with pure cultures of *T. vaginalis* may result in moderate pathologic alterations of the vaginal discharge and mucosa and less frequently in the production of symptoms'. He also believed that *T. vaginalis* cause vaginitis regardless of the bacterial flora initially present. Asami and Nakamura (1955) inoculated 9 volunteers with bacteria-free *T. vaginalis* into the vagina. Seven of them became infected and developed vaginitis of varying intensity. It has been considered that abnormal vaginal flora may favour the establishment of the infection, although the experiments of these authors suggested that this is not so. The role of vaginal glycogen in the maintenance of an acid environment which inhibits the invasion and

growth of pathogenic bacteria is well recognized (Ayre *et al.*, 1950). In cases of vaginal trichomoniasis deficiency of glycogen in the vaginal mucosa has been observed by several authors (e.g. Ayre *et al.*, 1950). Asami and Nakamura's experiments demonstrated a decrease in the amount of glycogen in vaginal epithelial cells following the introduction of the parasites. It is known that Doderlein's bacilli require much glycogen for their multiplication, so growth of these bacilli would be inhibited by this reduction in the glycogen content of the vaginal wall. Thus it is suggested that the vigorous consumption of glycogen by trichomonads leads to the presence of fewer Lactobacilli and consequently a less acid vaginal pH which facilitates the invasion of the vagina by abnormal bacteria. This results in vaginitis through synergistic action of trichomonads and bacteria.

B) Intravaginal Infections of *Trichomonas vaginalis* in Laboratory Animals

(i) Monkeys

Only a few attempts have been made to infect monkeys with trichomonads, in all cases the flagellates were introduced into the vagina. The first reported attempt was made by Hegner (1928) who cultured trichomonads from the intestine of monkeys and injected them into the vaginas of 6 experimental monkeys. On the second day after inoculation washings from the vaginas of 5 of these monkeys contained trichomonads. However, only two monkeys retained trichomonads in the vagina for 5 days, although one monkey infection lasted at least 15 days and another at least 21 days. Monkeys which had resisted infection by trichomonads from the intestine were then inoculated with trichomonads isolated from monkey vaginas. Only one of the 4 monkeys became infected, the infection lasting approximately 17 days. The author suggested that the two locations harboured the same trichomonad

species and that naturally-occurring vaginal infections in monkeys may arise from faecal contamination.

Johnson *et al.* (1950) carried out an extensive study of Rhesus monkeys infected with an axenic culture of a recently isolated *T. vaginalis* strain. The authors attempted to discover, using the monkey as a model, why exacerbations of trichomoniasis occur in humans around the time of menstruation, when symptoms are usually most severe. None of their monkeys, however, although harbouring parasites, developed signs of vaginitis. The period between inoculation of parasites and first demonstration of successful implantation of trichomonads varied from 2-23 days and the duration of infection ranged from 51-479 days. It seemed that young animals were refractory to infection with *T. vaginalis* and success in implanting the trichomonads was not likely until after the onset of the first menses. Abrupt fluctuations in the number of trichomonads in infected vaginas occurred. Parasite populations tended to subside during midcycle even to the point where parasites could not be found. The duration of these subpatent periods varied between 9-37 days. In the period just before and just after menstrual flow, sudden increases in number of parasites occurred. In many instances the population was observed to change over 48 hours from a condition in which none or very few trichomonads could be found to one in which very many were present. These periods of high population lasted only a few days. It was usual for two peaks to be reached in each cycle, one just after the period and one just before onset of the next flow. No correlation of abundance of trichomonads wih the amount of glycogen in stained vaginal smears was observed, nor with time of ovulation. Hence, Johnson and co-workers, although observing similar fluctuations in trichomonad numbers during the monkey menstrual cycle as occurs in humans, could not, from their results give a reason for this.

Cuckler *et al.* (1955) used three monkeys with 3 year old intravaginal infections of *T. vaginalis* for testing the effect of orally administered aminitrazole. Unfortunately no details of the course of infection before drug treatment were given.

(ii) Hamsters

A particular problem with using hamsters as a model for intravaginal infection of *T. vaginalis* is the existence of a naturally-occurring trichomonad which is thought to be of intestinal origin but which is often found contaminating the hamster's vagina. Kradolfer (1954) found that among several hundred females of varying ages and litters examined, cases of a 'natural' infection by trichomonads were found in samples from the vagina and also in the faeces. Of 500 tests made at each site, 11 positive findings were observed from the vagina and 8 positive smears from the faeces. The possibility of the presence of this organism should therefore be taken into account in any experiments involving hamsters. There has been a recent case of it complicating the model developed for drug testing (Latter *et al.*, 1986).

Hamsters can be infected with *T. foetus*. From the results of a study involving 149 hamsters (Kradolfer, 1954), the degree of infection, as measured by wet smears, approached its maximum on the 19th day and it was noted that the maximum of 84% was slightly higher than the infection rate of *T. foetus* obtained with rabbits as reported by McDonald and Tatum (1948). A greater number of hamsters showed an infection which persisted for long periods. For example, 20 hamsters were still positive on the 85th day, and four animals kept for more than 105 days still demonstrated typical trichomoniasis at this time. The number of trichomonads found in smears from an individual animal fluctuated greatly. Periods of exceptionally high or low parasite

numbers lasting for several days and longer were observed. This phenomenon was originally thought to be attributable to the female oestrus cycle described in detail in section 1.6.2.3., but upon further study the possible correlation was not confirmed and it was concluded that other factors were involved.

Passage from hamster to hamster of this trichomonad strain was possible. The possible occurrence of natural transmission was also investigated. Infected and non-infected female hamsters were housed together in the same cages. After two months exposure, one of the three animals initially non-infected had become positive for trichomonads.

Trichomonads were found in the vagina and cervix, but never in the horns of the uterus. Histological examination revealed three types of parasitic localization. Most of the parasites were situated in the central portion of the vagina, surrounded by leukocyte and epithelial cells; scattered parasites lay in the small lumina of the lateral crypts; relatively few parasites were detected adhering to the epithelial wall, with occasional sections showing an organism embedded between epithelial cells (Kradolfer, 1954).

By repeated intravaginal inoculations of *T. vaginalis*, Uhlenhuth and Schoenherr (1955, in Honigberg, 1978) were able to establish lasting infections in golden hamsters, providing they were at least 6 weeks old. Infections could be terminated by invasion of the vagina by *Pentatrichomonas ardindelteili*, a natural inhabitant of the gut, which evidently overgrew *T. vaginalis*. It was possible to passage intravaginally growing *T. vaginalis* through hamsters. However, in view of the likelihood of invasion by *Pentatrichomonas* species from the gut experimental infections must be most carefully monitored by microscopic examination and cultivation in order to ensure that the

correct species is being studied.

In 1969 Actor *et al.* used hamsters which they infected intravaginally with *T. foetus* in experiments to develop metronidazole-resistance in the flagellate. After infection of young, virgin, female hamsters with *T. foetus* intravaginally the animals were subsequently treated with sub-optimum doses of metronidazole. Trichomonads were then isolated from the hamster vaginas and compared with the parent strain using an *in vitro* test to measure metronidazole sensitivity. The minimum inhibitory concentration for the resistant isolate was found to be approximately ten times greater than that for the parent strain.

(iii) Guinea Pigs

Newton *et al.* (1960) were unable to establish *T. vaginalis* infections in the vaginas of guinea pigs untreated with hormones. However, later authors were able to establish *T. vaginalis* infections in guinea pig vaginas. A long series of reports were given by a group of investigators at the Medical School in Bialystok, Poland (e.g. Kazanowska, 1966; Kazanowska & Kuczynska, 1966; Kazanowska *et al.*, 1973). The first and basic report was made by Soszka *et al.* (1962) which dealt with cytological, histological and histochemical observations on the vaginal epithelium. Guinea pigs of three groups (sexually immature, sexually mature and spayed) were inoculated intravaginally with axenic cultures of *T. vaginalis*. Manifestations of inflammation appeared between 24 hours and 75 hours post-infection in all groups; the animals had copious purulent vaginal discharge and their vulvas appeared swollen. Loss of appetite and general debility were also observed. In cytological smears, besides varying numbers of leukocytes and trichomonads, there were present squamous epithelial cells with enlarged and deformed nuclei. Squamous epithelium covered

in muc ous was noted in preparations in which changes in arrangement and structure of cell layers were also present. Variable numbers of leukocytes were found to have infiltrated the stroma. On the basis of their observations, the authors concluded that 'prolonged *T. vaginalis* infection alters the metabolism of the vaginal epithelial cells, which may give rise to precancer and cancer states'. Pathological changes of the uterine mucosa of intravaginally infected guinea pigs were described by Kazanowska (1962). From the results of infections of 85 guinea pigs she suggested the possibility that disturbances in metabolism of the uterine mucosa cells in trichomoniasis constitute the source of certain pathological conditions in pregnancy and of infertility in animals and humans. Skryzpiec (1975) reported extensive cyto- and histochemical studies of the vaginal portion of the uterus of guinea pigs infected with *T. vaginalis*. Similar findings were reported by Ginel (1962) with respect to the vaginal mucosa of pregnant guinea pigs infected with *T. vaginalis*. As the infection progressed, animals aborted usually single, macerated foetuses.

By using anti-*T. vaginalis* rabbit serum conjugated with isothiocyanate, Soszka *et al.* (1965) observed strong fluorescence of trichomonads and a weaker one of the squamous epithelial cells in vaginal smears. In sections the district fluorescence was found in the entire epithelium. The authors suggested that fluorescent antibody methods could be employed for differentiation between *Trichomonas*-caused inflammation and neoplasia in the absence of trichomonad no fluorescence was seen in preparations treated with antitrichomonal conjugate.

Kazanowska (1966) reported that pathologic changes similar to, although less extensive than those observed in guinea pigs infected intravaginally with *T. vaginalis* were present in animals receiving intraperitoneal inoculations of this parasite. This finding and the

observed accumulation of flagellates around blood vessels suggested to the author that the protozoa can spread via the circulatory system; however, there is no convincing evidence that in human infections this mode of spreading is possible.

In a related investigation Soszka *et al.* (1973) examined, by histological and cytological methods, the effects of *T. vaginalis* upon the urinary system of male and female guinea pigs. Parasites were introduced either via the urethra or surgically into the bladder and renal pelvis. Variable numbers of leukocytes and desquamated epithelial cells were found in the urine sediments of both sexes. Virtually no pathological changes were noted in the mucosa of the urethra or bladder of males. In females, however, a few days post-infection, there was a piling up of epithelial cells in these organs. Pathological changes correlated with histochemical alterations in the urinary system of female guinea pigs (Kaznowska *et al.*, 1973).

(iv) Rats

Success in establishing lasting infections of *T. vaginalis* in the vaginas of rats was achieved by Cavier and Mossion (1956a,b). They used spayed rats in which permanent oestrus had been induced and maintained by a series of subcutaneous inoculations of oestradiol benzoate. Subsequently, Combscot *et al.* (1959) repeated and amplified these experiments. By using massive dosages of oestrogen, they were able to induce permanent oestrus and establish *T. vaginalis* also in intact rats. Asami (1956) castrated his rats and implanted a 30-40mg pellet of oestradiol subcutaneously to induce and prolong the oestrus condition. Trichomonads began to appear in the vaginal discharge 3-5 days after their inoculation, and subsequently an increase in population was seen in most animals. After the infection was established, it persisted as long as the rats were kept in oestrus

condition. The infection rate of the rat vagina achieved by Asami was 60%, although he admitted that it was not always this high. In a few animals, trichomonads disappeared spontaneously early in the infection. However, in rats in which the infection had become established, the flagellates usually persisted for more than one month. The infection was sufficiently stable and persistent to permit adequate post-treatment observations and evaluation of therapy.

Michaels *et al.* (1962) reported that successful intravaginal infection of rats with *T. vaginalis* may depend upon the strain of both the parasite and the rats employed. Fresh parasite isolates appeared more suitable for infection. According to these workers, a single injection of a long lasting oestradiol preparation (oestradiol cyclopentyl propionate) could be used instead of the numerous doses of oestradiol benzoate required to maintain the rats in permanent oestrus. Chappaz (1964) also established *T. vaginalis* in spayed rats treated with oestrogen. Meingassner and Mieth (1976) used spayed, oestrogen-treated rats for establishment of intravaginal infection with *T. vaginalis*. However, he found more reliable results if the introduction of *T. vaginalis* was preceded by inoculation of *Candida albicans* into the vagina of the rat. Nedvedora and Kulda (1978) using Meingassner's (1976) method found that trichomonads readily established in the vagina of rats concurrently with a *Candida albicans* infection. They reported that the infection rate was relatively low (30%), however, if only trichomonads were inoculated, and the infection was typically short-lived (3-10 days). In this same study, it was reported that inflammatory reaction was absent in most infected animals, although vaginal discharge containing trichomonads and an abundance of polymorphonuclear leukocytes appeared in three rats. These rats showed thickening of the vaginal epithelium and leukocyte

infiltration in epithelial and subepithelial tissues of the vagina. In one rat, moderate erosion of the epithelium was present and host tissue was covered with a layer of adhering amoeboid trichomonads. No pathological charges were observed in the other infected rats.

(v) Mice

There have been relatively few reports of attempts to infect mice intravaginally with trichomonads. Cappuccinelli *et al.* (1974) were able to establish a *T. vaginalis* strain isolated from a case of symptomatic trichomoniasis in oestradiol-treated mice of two strains. The best results were obtained with two successive instillations of trichomonads. Evidently hormone treatment was needed only to establish the infection, thereafter parasites survived in the absence of exogenous oestrogen. In a quarter of the attempts, the authors succeeded in transmitting infection from one female to another by allowing a male to mate first with an infected then with an uninfected, oestradiol pre-treated mouse. Infections were also established in a low percentage of untreated mice. Irradiation did not render mice more susceptible to infection; evidently immunosuppression did not affect natural resistance of mice to infection with *T. vaginalis*.

Meingassner and Mieth (1976) used an intravaginal mouse model to determine the trichomonacidal activity of 5-nitromidazole derivations in mice. He used NMRI mice pretreated once with 40mgkg bodyweight^{-1} oestradiolundecylate in 0.2ml sesame oil 3 days prior to infection. The oestrogen was administered in two equal doses subcutaneously and intraperitoneally. The parasite inoculum was 0.05ml CACH-medium containing approximately 10^5 *T. vaginalis* and *C. albicans*, added to the concentrated flagellates in a ratio of 1:80 volume/volume immediately before infection of animals. The vaginal cavity was sealed

with a plug of sponge to avoid loss of the inoculum. The results indicated that all compounds tested were systemically active against *T. vaginalis* in mice infected intravaginally. Interestingly, the compounds were less efficaceous against subcutaneously infected mice.

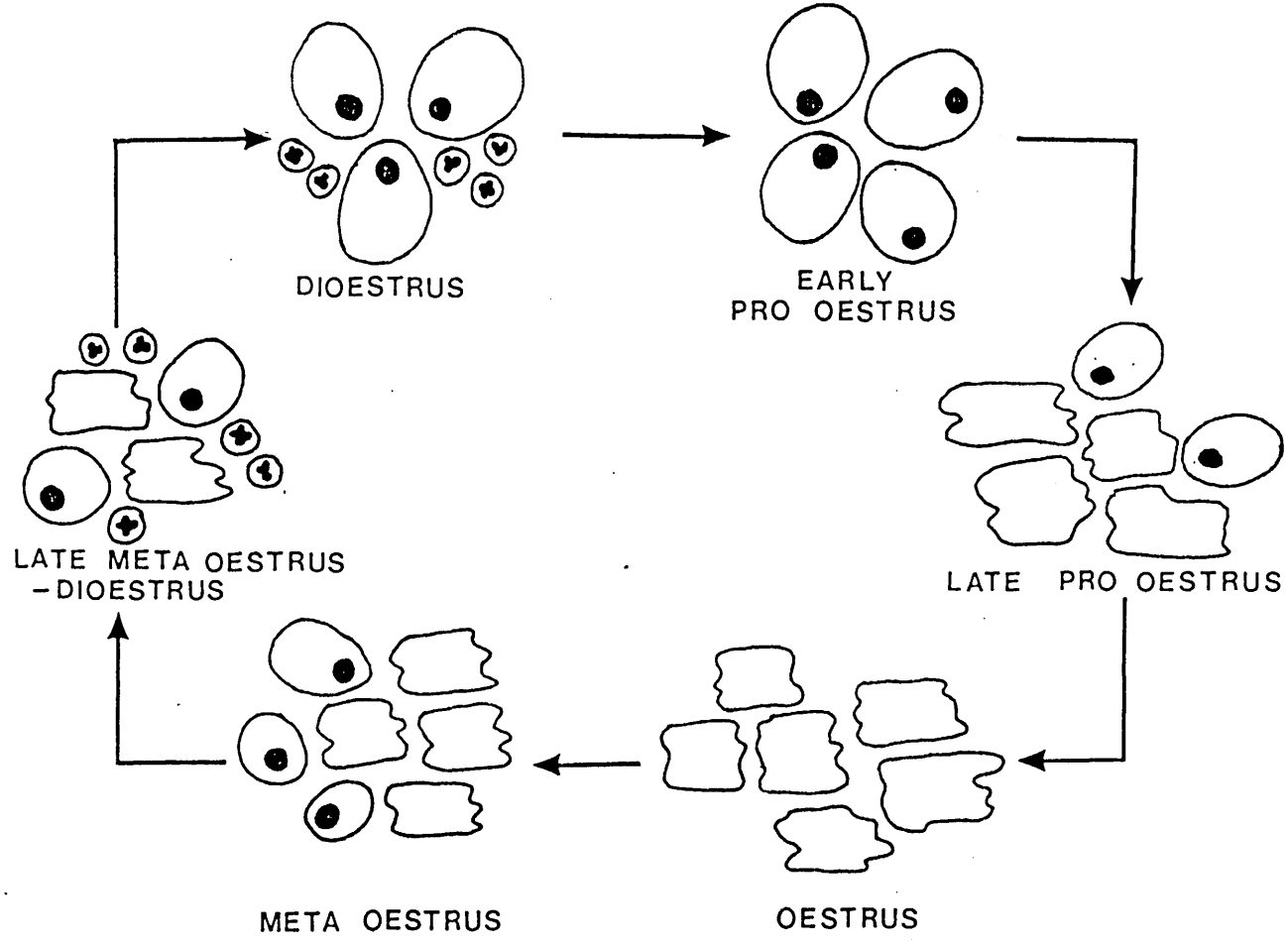
1.6.2.3. Physiology and Bacteriology of the Vagina of Laboratory Animals

External morphological changes occur during the oestrus cycle of the mouse, but these may be subtle. With many rodents there is very little or no discharge during 'heat'. The degree to which congestion and reddening occur in the vulva is also variable. From a survey of 355 random cycles (Allen, 1922), 50% of the mice showed well-marked external signs, whereas 24% showed the vulva and vagina in an apparently 'resting' condition although oestrus was shown to be present by the cell contents of the vaginal fluid. A few animals evidenced continued external signs of 'heat during metaoestrus and dioestrus. Allen (1922) described the classical phases of the mouse oestrus cycle as follows:

Dioestrus - a period of relative quiescence. In the mouse during the dioestrus the internal vulva is very inconspicuous and the orifice of the vagina is usually tightly closed. The small amount of fluid present is visc ous and stringy. The smear (Figure 3) shows epithelial cells, usually only a few, in various stages of nuclear degeneration and cytoplasmic shrinkage, and always some polymorphonuclear leukocytes.

Proœstrus - period of augmented growth and congestion. During proestrus, if exernal signs are marked, the vulva is pink or red and swollen, the vagina gapes open, the fluid in its lumen is serous and the smear shows only nucleated epithelial cells.

Figure 3: Diagrammatic representation of the cells present in vaginal smears of mice at various stages of the oestrus cycle.  , round-nucleated cells;  , square, cornified cells;  , polymorphonuclear leukocytes. (Based on information from Allen, 1922).


DIOESTRUS
EARLY
PRO OESTRUS
LATE PRO OESTRUS
OESTRUS
META OESTRUS
LATE META OESTRUS
-DIOESTRUS

Oestrus - period of sexual excitement, the climax of prooestrus conditions. During the oestrus period the vulva may still be swollen, the orifice of the vagina open and a dull, white colour, the vaginal mucosa is almost dry and vaginal smears show only cornified, non-nucleated, red eosin-staining cell remains. In animals where external signs are absent or slight, the finding of only cornified cells in the smear makes a diagnosis of 'heat' possible (Figure 3).

Metaoestrus I - period of return to dioestrus condition. In the first half of metaoestrus the vulva has usually lost most of its swelling, the orifice of the vagina still gapes open and is whitish, occasionally showing small granulations, the lumen is still dry and the smear shows red cornified elements of the previous periods, always very numerous and now bunched or caked.

Meta oestrus II - evidenced by a normal vulva and tightly or partly closed vaginal opening. The vaginal contents change as the period progresses from a pasty or viscous to a fluid consistency, and the smear shows polymorphonuclear leukocytes among the red horny elements. A few polymorphs and many cornified cells is an early metaoestrus II stage and a heavy leukocytic infiltration and decrease in number of cornified cells is a late one. This stage then merges into dioestrus with a decrease in leukocytes and the appearance in the smear of small numbers of nucleated epithelial cells (Figure 3).

Allen (1922) also gave a description of the histology of the normal mouse vagina. During dioestrus the epithelium is low (3-7 layers), has no clear cut basement membrane and is freely infiltrated by polymorphonuclear leukocytes. At the end of this interval, a basement membrane begins to be evident, mitoses became more frequent, new layers of cells are added and leukocytosis ceases. At prooestrus the growth process reaches a maximum which thickens the epithelium to 10-13 layers. A distinct basement membrane is present in most places.

The outer 3-5 layers begin to degenerate, these two areas become clearly separated by formation of a granular layer. This is converted to a cornified layer which is not superficial but underlies 3-5 layers of nucleated cells. As prooestrus merges into oestrus, these superficial layers disappear until the cornified layer becomes superficial at which time oestrus is evident. By this time continued growth has piled up the epithelium to a thickness of 12/13 cell layers under the stratum granulosum which first formed at about the 8th layer. As the meta oestrus I period sets in, the cornified layer begins to be delaminated and the lumen of the vagina is filled with fragments or masses of cornified, epithelial cells. Leucocytes filter in from the stroma and collect in the superficial layes of the germinal epithelium. After they have accumulated in considerable numbers here, they pass on to the cornified masses in the lumen, stage metaoestrus II, and in a day's time may completely digest them. Their continued action in the superficial part of the germinal epithelium reduces these layers to the appearance of a reticulum containing large clumps of leucocytes. This process gradually declines until the vaginal epithelium returns to a typical dioestrus condition. Growth activity seems practically at a standstill in the early dioestrus stage. Toward the end of this interval the growth curve begins to rise until it attains its maximum at late stage prooestrus, after which it gradually falls during oestrus and metaoestrus I to its minimum in the late metaoestrus II and early dioestrus stages.

The duration of the cycle and its various stages shows great variability. Of 563 cycles studied, Allen (1922) found that the average duration was between 4 and 6 days. The dioestrus interval shows greater variation than any other stage, lasting from less than one day to 14 days, although its duration is usually from 1-3 days. In

several cases of an extremely long lasting dioestrus interval, the smears at times show considerable amounts of cornified cells, but the presence of leucocytes makes evident the diagnosis of dioestrus. Prooestrus may be less than one day, because leucocytes may not entirely disappear from the superficial vaginal epithelium until after growth in the deep layers is well under way. Oestrus usually lasts 1-2 days but in several cases unbroken oestrus smears have continued for 9 days and 4 days of heat are not uncommon. Metaoestrus I and II stages usually last a day each and show little variation.

While variation of cycle length is great in the same individual animal it is still greater in different strains. Findings indicated that a genetic factor is accountable for some of the variation in cycle length among different strains of mice. Although it proved difficult to generalize on the variation with age of the individual, first and second cycles following puberty were usually longer than the average during later life. Allen (1922) concluded that in many particulars oestrus changes in the mouse are similar to other rodents.

The presence of glycogen in the vagina has been demonstrated in many mammals including man, the monkey, the guinea pig and the mouse. Several workers have reported cyclical changes in the glycogen of the vagina which may be correlated with the oestrus or menstrual cycles. Balmain *et al.* (1956) demonstrated that glycogen is formed in considerable amounts in the vagina of mice after keratinization has commenced, the largest amount of glycogen being present during the metaoestrus phase followed by dioestrus, prooestrus and very little being present during oestrus itself. These results were observed in intact mice undergoing normal oestrus cycles and also in ovariectomized mice artificially stimulated with oestrogen. Several workers speculated on function of the glycogen in epithelial tissues and it has been suggested that the glycogen is concerned with the

metabolism of mitosis and also with the synthesis of keratin. Gregoire and Guiness (1968) investigated cyclic and preovulatory changes in the glycogen content of the genital tract of female hamsters. They found that the greatest amount of uterine glycogen occurred on the first day of the cycle and decreased significantly during the remaining days. The greatest glycogen content occurred in cervical tissue and this also was maximal on day one, the day preceding ovulation; a rapid decrease followed on subsequent days. Glycogen was restricted to the glandular portion of the cervix, scattered as cytoplasmic granules. The vagina of the hamster consists of two anatomically different sections, the upper two-thirds being composed of convoluted epithelial tissue whilst the lower one third consists of stratified squamous epithelium. The greatest amount of glycogen appears in the upper portion of the vagina during the first day of the cycle, and is present in quantities similar to those in cervical tissue. The lower vaginal tissue contains less glycogen. In the upper vagina, glycogen is restricted to the convoluted epithelial tissue; in the lower vagina some glycogen is present in the intermediate and basal layer of the epitheliumn. Ovariectomy resulted in a decrease in glycogen levels of the entire genital tract.

Larsen *et al.* (1978) studied the bacterial flora of the female rat genital tract. The most commonly isolated organisms were x- and non-haemolytic streptococci, *Pasteurella pneumotropica* and diptheroid bacilli. The group of rats studied was relatively homogenous with respect to species comprising the microflora of the urinogenital tract. More than half the rats had 5 or 6 species making up their flora and all rats had at least three species. More isolates were obtained from the vagina than the cervix. pH values of rat vagina ranged from 6.12-7.62, the mean being 6.95.

Comparison of the genital flora in rats with data available regarding the flora in the human female genital tract shows some similarities. A comparable frequency in the isolation of diptheroids has been observed in humans. A similarity was also observed in the frequency of isolation of *Staphylococcus epidermidis*. Larsen *et al.* (1978) reported 48% frequency of isolation in rats and studies of humans showed 30-60% occurrence (Hurley *et al.*, 1974). Differences between rats and humans included low frequency of lactobacilli in rats, while reported frequency in humans approached 100% (Hurley *et al.*, 1974). The presence of lactobacilli is associated with acidic pH so the relatively high pH values observed in the rats studied may be related to the observed low frequency of lactobacilli. The high frequency of gram negative rods in rats, notably *Proteus* species, is of interest. In the human vagina *Proteus* species is usually isolated from less than 10% of vaginal cultures (Hurley *et al.*, 1974). The reasons for this difference may be a fundamental difference in the microenvironments provided by the two host species. Alternatively the association of rats with faecal material in their cages may contribute to the higher percentage of rats carrying *Proteus* species. Another difference between rat vaginal flora and human vaginal flora was the finding of *Pasteurella pneumotropica* in 68% of rats studied. This organism has not been isolated from the human vagina.

It has been known for many years that human vaginal microflora is altered by the onset of puberty, pregnancy and the menopause (Cruickshank & Sharman, 1934a,b). Larsen *et al.* (1977) went on to study the relationship of vaginal cytology to alterations of the vaginal microflora of rats during the oestrus cycle. The study demonstrated that quantitatively the density of vaginal bacterial colonization in the female rat varies profoundly and cyclically during the oestrus cycle. The changes in bacterial numbers in the animal

model studied amounted to several orders of magnitude and occurred over the time span of one day. The relationship between host cells and the dynamics of the vaginal bacterial population was examined. It appeared that if the vaginal bacterial population is influenced by vaginal cellular composition, the cells most likely involved are cornified, squamous epithelial cells, enhancing colonization, and leucocytes diminishing colonization. Other factors which may be important in the cyclic fluctuation of vaginal counts include pH, redox potential, presence of mucin, vaginal blood supply, and the presence and quantity of antibacterial substances.

1.6.2.4. The Role of Hormones in the Infection of the Vagina by *Trichomonas vaginalis*

There is much evidence that clinical recurrence and exacerbation of trichomonal vaginitis is often associated with the menses and pregnancy (Brown, 1972; Trussell, 1947). Some earlier workers were led to believe that ovarian hormones were directly responsible for flare-ups, being necessary or at least stimulating for *T. vaginalis* growth. On the basis of clinical observations some workers (Jirovec & Peter, 1950) believed that in females the effects of normal and elevated oestrogen levels might favour *T. vaginalis* infections. Many workers, however, did not support this view. According to Chappaz (1964), a moderate oestrogen deficiency favours human trichomoniasis, however, complete absence of oestrogen impairs the establishment of trichomonad infection. Netter and Lambert (1957) denied any correlation between endocrine conditions of patients and frequency, as well as course, of *T. vaginalis* infections. Unfavourable effects of high oestrogen levels on *T. vaginalis* were implied in reports of some investigators including Doring (1957). Moricard-Saulnier (1957) and Durel *et al.* (1966) who found local treatment with oestrogens to have beneficial

effects upon infections of *T. vaginalis*. These effects were attributed to changes in the vagina and were not thought to be due to direct action on the parasites. One of the indirect effects mentioned was an increase in the glycogen content of the vagina. Bacterial flora and pH are other factors that may be important in the establishment of *T. vaginalis* in the urogenital tracts of women; however, little is known about relationships between hormonal levels and these factors.

The possible role of steroid hormones in experimental infections of laboratory animals has been investigated by several workers. Caviar and Mossion (1956a,b) found success in establishing lasting vaginal infections with *T. vaginalis* in spayed rats in which permanent oestrus had been induced by a series of subcutaneous inoculations of oestradiol benzoate; the oestrogen treatment was thought to be crucial. Combescot *et al.* (1959) repeated and amplified these experiments and by employing massive dosages of oestradiol were able to induce permanent oestrus and to establish *T. vaginalis* also in non-spayed rats. Although Cavier and Mossion (1956a) reported that high dosages of the hormone were detrimental to infections, Combescot and co-workers observed no ill effects even when massive dosages of oestradiol were administered to rats. Combescot *et al.* (1957) found that oestrus could be interrupted and *T. vaginalis* infection effectively terminated by injecting rats with progesterone, testosterone and deoxycorticosterone. Larsen *et al.* (1977) investigated the role of oestrogen in controlling the genital microflora of female rats. They found that glycogen is deposited in the vaginal epithelium in response to oestrogenic hormones and this glycogen provides a substrate that promotes growth of lactobacilli, which in turn produce lactate by degradation of the tissue glycogen. The acid thus produced is believed to restrict vaginal flora to

'aciduric' species. Some evidence indicated that the effects of oestrogen on vaginal epithelium, however, results in acidification of the vaginal epithelium environment independent of the presence of lactobacilli. The vaginal tract of rats is free of appreciable glycogen and has a pH near neutrality yet bacterial counts have been shown to vary with stage of the oestrus cycle. The highest bacterial counts consistently observed during the oestrus phase of cycle and minimal counts occurred during dioestrus. After ovariectomy, bacterial counts diminished and remained relatively stable at low levels, although there were still daily fluctuations. There was no identifiable pattern formed, in contrast to non-spayed rats. Vaginal bacterial counts in control rats were not as sensitive to the effect of oestradiol as were those in ovariectomized rats. Progesterone antagonized the oestrogen action in sustaining high bacterial counts. The authors suggested that the secretion of progesterone which follows oestrogen secretion accounts for the rapid decline of bacterial counts after oestrus. Ovariectomy does not absolutely deprive the rat of oestrogen since the adrenal gland may synthesize some oestrogen. Several effects of oestrogen are known that could directly influence the vaginal microflora, including effects on vaginal cytology, mucus secretion and vascularity. Low *in vivo* concentrations of hormones in intact animals and the time lag between steroid administration and effect in ovariectomized rats, suggest that the effects of hormones on bacterial counts is mediated rather than direct. The time lag between oestrogen administration and increase in bacterial counts corresponds with the time required to produce vaginal cornification. Oestrogen secretion in intact rats substantially precedes vaginal cornification by about one day. Vaginal cornification is associated with peak vaginal bacterial counts.

Kornblut (in Honigberg, 1978) used the subcutaneous mouse assay

to test the effects of oestradiol benzoate and progesterone on development of subcutaneous abscesses in animals inoculated with a relatively pathogenic strain of *T. vaginalis*. Mean volumes of 6 day subcutaneous lesions in mice treated with 0.2ug dosages of oestradiol benzoate were significantly smaller than in control mice. When more oestradiol was given, even smaller abscesses were noted. With progesterone, only the lightest dose, 7.5mg, was enough to reduce the size of abscesses. The author concluded that incidence and periods of exacerbation of urogenital trichomoniasis in many women may depend upon a balance of the ovarian hormones that affect the conditions prevailing in the lower urogenital passages rendering them suitable for supporting the parasites.

That the direct effect of steroid hormones upon *T. vaginalis* is typically injurious to the parasite is evident from *in vitro* studies of Glebski (1969). Oestradiol benzoate was found to inhibit the growth of trichomonads, the effects being directly proportional to the concentration of the hormone. Inhibition was manifested in reduction of the number of flagellates shortened life-span of the populations, increase in generation time and decrease in cell size. Different effects were noted with progesterone, which inhibited division, but caused size increase of individual cells. Nicol *et al.* (1966) showed that oestrogen is a stimulant of the reticulo-endothelial systems (RES) and body defence. RES activity varies at different stages of the oestrus cycle and of pregnancy in both the rat and the mouse. The stages where activity is greatest correspond with those in the human subject when the output of oestrogen is increased. Therefore, although oestrogen may cause changes in the host which are necessary for the establishment of a trichomonad infection, this hormone may also aid the body's defences against the infection. The controversial role of

oestrogen in the nature of the trichomonad host-parasite relationship has recently been summarized by Sugarman and Mummaw (1988).

In summary, many experimental intravaginal infections of *T. vaginalis* have been attempted including some in humans. Although experimental infections in humans were obtained the rate was less than 100%. Laboratory animals have also been tested for intravaginal infectivity for trichomonads. Reasonable success was achieved with several, including monkeys, guinea pigs, rats and mice. Hamsters were unsuitable due to the presence of a natural intestinal trichomonad which 'overgrew' in the vagina preventing establishment of *T. vaginalis* infections. In mice, the development of the intravaginal model has advanced in some cases to enable a drug testing system.

The physiology and bacteriology of the vaginas of laboratory animals have been described. The oestrus cycle of various animals, the histology and bacteriology of the normal rodent vagina and the effects of hormones on trichomonal infections have all been investigated, and much information is now available on the microenvironment of the rodent vagina.

1.7. THE AIMS OF THE PROJECT

The overall aim of this project was to investigate the mechanisms by which *Trichomonas vaginalis* proves pathogenic towards its host by comparing fresh isolates of the parasite. In this way it was hoped to find mechanisms essential to the parasite which could be suitable for chemotherapeutic attack. Aims encompassed by the project where to develop a useable intravaginal model to aid subsequent research into the mechanisms of this disease and its cure, and to investigate the use of various models of the infection for testing existing and novel antitrichomonal compounds.

2.0 MATERIALS AND METHODS

2.1. PARASITES

2.1.1. Parasites and their Cultivation

Some of the trichomonad lines used in this project were laboratory strains which had been cultured axenically *in vitro* for many years. These included *Pentatrichomonas hominis* (A.T.C. 30098), the non-pathogenic trichomonad that inhabits the intestinal tract of man, and was obtained from Dr A. Yule (London School of Hygiene and Tropical Medicine); *Trichomitus batrachorum*, the non-pathogenic trichomonad isolated (Coombs, 1976) from the leopard frog, *Rana pipiens*; *Tritrichomonas foetus* (Pfizer strain, clone F2). I cloned this line from the parent strain which was originally obtained from Dr D. Linstead of the Wellcome Research Laboratories, Beckenham, Kent, and *Trichomonas vaginalis* G3, a clone isolated in our laboratory from a line originally obtained in 1973 from the Wellcome Research Laboratories. Other lines of *T. vaginalis* used had also been grown in axenic cultures for extensive periods. These were *T. vaginalis* Boston, *T. vaginalis* Albany, *T. vaginalis* IR78 (obtained from Dr J.G. Meingassner, Sandoz Research Institue, Austria). All other *T. vaginalis* lines used were freshly isolated from patients at Glasgow Royal Infirmary genito-urinary clinic or from other clinics around Britain; all were isolated from female patients except for *T. vaginalis* 6950 male (obtained from Dr V.C. Latter, Wellcome Research Laboratories, Beckenham, Kent, U.K.).

All *T. vaginalis* isolates were cultured routinely in 25ml universals of modified Diamond's medium (Diamond, 1957): 1 litre - 20g BBL trypticase, 10g yeast extract (Difco), 5g Maltose, 1g ascorbic acid, 1g potassium chloride, 1g potassium hydrogen carbonate, 1g potassium dihydrogen phosphate, 0.5g di-potassium hydrogen phosphate, 0.1g iron sulphate, 1000ml distilled water, adjust pH to 6.3-6.4 using 1M HCl, autoclave at 17 p.s.i. for 15 minutes. The medium was

supplemented with 10% heat-inactivated horse serum with the addition of 1mg benzyl penicillin ml^{-1} and 1mg streptomycin sulphate ml^{-1}. This medium will be referred to as MDM. Cultures were routinely incubated at 37°C with air as the gas phase. The cultures were sub-passaged daily or two-daily depending upon their density. Density was measured by counting trichomonad cells using a haemocytometer.

T. foetus was cultured in the same way as *T. vaginalis* isolates.

Pentatrichomonas hominis was cultured in the same way except for the addition of 10mg ml^{-1} galactose to the medium and 10% heat-inactivated foetal calf serum instead of horse serum. *Trichomitus batrachorum* in MDM with 10% horse serum, was incubated at 25°C.

Cultures were checked daily by microscopical examination for bacterial and yeast contamination.

Conditions of cultivation differing from these general conditions were used as stated. Anaerobic incubation of trichomonad cultures was achieved through the use of an anaerobic incubator (Whiteley Anaerobic Cabinet Mark II).

2.1.2. Cloning

Cloning of all isolates was carried out by micromanipulation. A trichomonad culture was adjusted to an appropriate low density and a small drop of this placed on a sterile cover slip on a sterile microscope slide. The drop was then examined, using a laminar flow hood to avoid contamination, at x200 magnification using phase contrast microscopy to ensure that only one trichomonad was present in the drop. If no trichomonad was present, another drop was placed on the cover slip and examined; if more than one trichomonad was observed the cover slip was discarded. When a drop of culture was found to contain only one cell, the coverslip was removed from the slide with sterile forceps and placed into a universal containing 10ml MDM (made

anaerobic in an anaerobic incubator) at 37°C. The culture was then incubated anaerobically at 37°C and checked periodically for up to 10 days for growth of the cloned cell.

2.1.3. Cryopreservation

Trichomonads were stored as 'stabilates' in liquid nitrogen for long periods. A high density trichomonad culture was taken and added to the same volume of MDM supplemented to 10% (v/v) with the cyropreservant, dimethylsulphoxide (DMSO), which had been sterilized by autoclaving. The mixture was then dispensed in 1ml aliquots into stabiliate tubes and these were wrapped in cotton wool, placed in a polystyrene box and floated overnight on liquid nitrogen. The next day the tubes were removed from the box and immersed in liquid nitrogen. To recover the cultures, the tubes were removed from liquid nitrogen, thawed in a 37°C waterbath, and 1ml of the stabilate added to 15ml of fresh MDM.

2.1.4. Harvesting Parasites for Enzyme Analysis

Trichomonads were grown in cultures of 25ml MDM in universals until a density of approximately $5x10^5$ ml^{-1} was reached. These were then centrifuged using an MSE Chilspin bench centrifuge at 3000rpm for 10 minutes, and washed using 25ml sucrose (0.25M). This procedure was repeated and the trichomonads were finally pelleted in 1.5ml plastic capped tubes and stored at -70°C until used.

2.2. COLLECTION OF FRESH ISOLATES

With the help of Dr I.B. Tait of the Department of Genitourinary Medicine, Royal Infirmary, Glasgow, it was arranged that any patient appearing to have a trichomonad infection would have a vaginal swab taken for the clinic's diagnostic purposes and at the same time a second swab would be taken and placed in culture medium for our

laboratory.

The clinic was initially supplied with universals containing 10ml MDM with 20 units Fungizone ml^{-1}, and also with tubes containing either the following other media: GTM (Diamond, 1957); TYM (Diamond, 1957), or Johnson's CPLM (Johnson *et al.*, 1945), each supplemented with the same serum and antibodies as MDM and also with Fungizone. After intial collections, however, it was found that no one particular medium showed any greater ability to support trichomonad growth than any other i.e. if trichomonads were present in MDM then they were also present in the other medium supplied, and vice versa. Therefore, after the initial few weeks the clinic was supplied with universals containing MDM.

On receiving the isolates, cultures were immediately sub-passaged into MDM, both the original culture and subculture were checked daily, microscopically, daily for trichomonads and to ensure they were axenic. If cultures had not become axenic after two subpassages they were discarded.

Axenic cultures obtained were cloned, stabilated and injected subcutaneously and intravaginally into mice.

2.3. STUDIES ON THE INTERACTION OF TRICHOMONADS WITH MAMMALIAN CELLS

2.3.1. Mammalian Cell Lines and their Cultivation

a) Murine macrophage-like cell line P388D (Flow Laboratories), which were grown in HOMEM medium (Berens, Brun & Krasser, 1976), supplemented with 10% (v/v) heat-inactivated foetal calf serum with 1mg benzyl penicillin ml^{-1}, 1mg streptomycin sulphate ml^{-1} and 20 units Fungizone ml^{-1}.

b) Mouse myeloma cell line P3-X63-Ag8-653 (Flow Laboratories), which were also grown in HOMEM medium supplemented with serum and antibiotics as above.

c) Primary chick embryo cells (Gibco), which were grown in HBME (Gibco) with 10% foetal calf serum and antibiotics as above.

d) Chinese hamster ovary cells CHOK1 (Flow Laboratories), which were grown in Ham's F12 (Flow Laboratories) with foetal calf serum (10%, heat inactivated).

e) Hela cell line (Flow Laboratories), which were grown in EMEM (Flow Laboratories), supplemented as above.

All cells were incubated routinely in 500ml tissue culture flasks at 37°C in an atmosphere of 5% CO_2, 95% air. The cells grew attached to the flasks's surface. All cells could be detached by mechanical shaking except Hela and CHO cells which were removed by trypsinizing. Cultures were sub-passaged weekly.

To preserve mammalian cells in liquid nitrogen, a high density culture was taken and mixed with the same volume of foetal calf serum (heat-inactivated) containing 10% sterile DMSO. 1ml aliquots of this mixture were dispensed into stabilate tubes (Flow Laboratories) and floated overnight on liquid nitrogen as were the trichomonad stabilates (see Section 2.1.3) before being stored in the liquid nitrogen.

2.3.2. Trichomonad Interactions with Mammalian Cell Lines

2.3.2.1. Interaction of Living Trichomonads with Mammalian Cells

Mammalian cell cultures were grown in flasks to a density of approximately 10^6 ml^{-1} in 5% CO_2, 95% air at 37°C in the appropriate medium. Mammalian cells were then detached from the surface of the flask by shaking (macrophage and myeloma cells) or trypsinization (chick embryo, Chinese hamster ovary and Hela cells). The mammalian cells were then resuspended to a density of approximately 10^5 ml^{-1} and dispensed into either Leighton tubes (2ml) or tissue culture wells (0.5ml) and incubated overnight.

Trichomonads were grown to a density of between 10^5 ml^{-1} and $5x10^5$ ml^{-1} then resuspended in MDM to appropriate densities to give varying ratios of trichomonads to mammalian cells and added to the mammalian cell cultures (0.8ml to Leighton tubes and 0.2ml to tissue culture wells).

For each experiment, controls were set up containing the same volume of mammalian cell culture medium as in the experiment but with no mammalian cells, to which the same volume of trichomonad suspension was added. Other controls contained mammalian cells to which the appropriate volume of MDM without trichomonads was added.

If an inhibitor or drug was to be tested then this was added immediately prior to addition of the trichomonads. Mixed cultures were then incubated in 5% CO_2, 95% air at 37^oC for up to one week.

Experiments performed in Leighton tubes were monitored twice daily by gently pipetting off the medium and counting the number of trichomonads present in this using an improved Neubauer haemocytometer. The medium was then replaced by fresh mammalian cell culture medium, the tubes sealed and the cells detached from the surface by mechanical shaking. The number of attached mammalian cells which were present was then counted also using a haemocytometer.

Experiments performed in tissue culture wells were monitored by observing the monolayer twice daily using a Leitz inverted microscope. The times in hours for each of the cell types to completely disappear were recorded. For convenience of presentation of the results, the time in hours for all trichomonads in monoculture to disappear was labelled 'A'; the time in hours for all mammalian cells in mixed culture to disappear was labelled 'B'; the time in hours for all trichomonads in mixed culture to disappear was labelled 'C'; and the time for all mammalian cells in monoculture to disappear was labelled 'D'.

2.3.2.2. Interaction of Trichomonad Spent Medium with Mammalian Cells

Trichomonads were grown in monoculture in the same mammalian cell medium/MDM mixture normally used for interactions of trichomonads and mammalian cells. Sufficient trichomonads were added initially so that they survived and multiplied and when a density of approximately $5x10^5$ ml^{-1} was reached the culture was centrifuged using an MSE Chilspin bench centrifuge at 3000 rpm for 10 minutes. The supernatant was removed and checked using phase contrast microscopy to ensure that no motile trichomonads were present. Aliquots of 0.5, 0.4, 0.3, 02 and 0.1ml of the supernatant were added to wells containing 0.5ml medium covering a mammalian cell monolayer and the effects on the mammalian cells monitored twice daily using a Leitz inverted microscope.

This method was also used with media in which trichomonads and mammalian cells had interacted for various times, again the cells were all removed before the medium was used.

2.3.2.3. Cell-Detaching Factor

Polyethylene glycol concentrates from culture filtrates, containing the 'cell detaching factor' were made and tested for effects on mammalian cells as described above for trichomonad spent medium. The method used was that of Pindak *et al.* (1986) and involved Hela cells being grown to monolayers in flasks containing 10ml EMEM with the usual supplements. 4ml trichomonad culture at a density of approximately 10^4 ml^{-1} was added to this. Control cultures received 4ml MDM only. These cultures were incubated for 3 days at 37°C, culture fluids were then pooled, clarified by centrifugation (3000 rpm) at room temperature, adjusted to pH 7.3 with 0.1N NaOH and filtered through a 0.45um membrane. Polythylene glycol (PEG) 8000 (Sigma) was added to a concentration of 10g to 100ml of culture fluid

and the mixture was then left overnight at 4°C. The suspension was subsequently centrifuged for 45 minutes at 2,800g and the supernatant discarded. The sediment was suspended in EMEM to 0.1 of the original volume. The resulting clear fluid was filter sterilized and assayed for activity on mammalian cells as described above for concentrated interaction medium.

2.3.2.4. Physical Separation of Cell Types

For experiments involving the physical separation of the two cell types, mammalian cells were cultured and dispensed into tissue culture wells in the normal way. Prior to addition of the trichomonad culture, however, a Millipore chamber was placed in each well. This consisted of a cylinder (12mm in diameter), closed at the bottom with a membrane of 0.45μm pore size, supported by three small legs (Millipore: Millicell-HA inserts). 0.2ml of the trichomonad culture at the appropriate density was then placed within the cylinder. Control wells contained no millipore well. The cells were monitored twice daily by observing the cell monolayer around the cylinder using a Leitz inverted microscope and by removing very small amounts of medium from within the cylinder and counting the number of trichomonads present using a haemocytometer slide. Finally, at the end of the experiment, the cylinder was removed and the whole monolayer observed using a Leitz inverted microscope.

2.4. STUDIES ON THE EFFECTS OF OF TRICHOMONADS *IN VIVO* IN ANIMAL MODELS

2.4.1. The Subcutaneous Mouse Assay

The subcutaneous mouse assay was performed using a modification of the method by Honigberg *et al.* (1966).

The general method used was as follows: Balb/c mice were shaved

on the right flank and inoculated subcutaneously with 0.2ml trichomonad suspension containing $5x10^6$ cells ml^{-1}. The cells were suspended in MDM (with horse serum but no antibiotics) supplemented with 0.16% (w/v) bactoagar (Difco). Lesions were measured daily, using a micrometer to determine length, height and width. Lesion volumes were calculated using the calculations described by Honigberg *et al.* (1966). They found that the volume of the protruding portion of the lesion represented one half of a spheroid. Thus the formula,

$$\frac{4/3 \pi l/2 \times w/2 \times h}{2}$$

where l = length, w = width andh = height, or 0.5236 x l x w x h could be employed.

The presence and number of live trichomonads in abscesses was also determined. Trichomonads were inoculated subcutaneously into a number of Balb/c mice. When the abscesses had reached certain volumes (see Figure 10) the mice were sacrificed, abscesses removed and the interior scraped into 5ml warm MDM. The presence of motile trichomonads was noted using phase contrast light microscope and the number determined using a haemocytometer slide.

The effects of various drugs and inhibitors on the growth of subcutaneous lesions were tested. Inhibitors were administered through several routes (intraperitoneal, subcutaneous, intravenous and oral) and at different times prior to and during infections (as detailed in the individual experiments). The subsequent lesion growth was monitored in the usual way.

2.4.2. The Intravaginal Mouse Assay

2.4.2.1. Oestradiol Treatment

Oestradiol-17β-cypionate (Sigma), a long-lasting oestrogen was routinely administered subcutaneously in the flank, in corn oil, at

40mg (kg body weight)$^{-1}$ two days prior to infection as suggested by Meingassner and Mieth (1976). To determine the stage of the oestrous cycle of a mouse, a vaginal smear was taken. This was carried out using a dental instrument with a curved-shaped head which was dipped in saline, placed inside the mouse vagina and gently scraped along the vaginal wall. Cells on the instrument were then transferred to a drop of saline on a microscope slide. The slide was air-dried, fixed in methanol and stained using Giemsa's stain. The stages of the oestrus cycle could then be recognized by observing, using light microscopy, the colour and form of the epithelial cells present (see Figure 3, Introduction).

2.4.2.2. Spaying Mice

Female Balb/c mice aged approximately 2 months old were anaesthetized using 0.2mg (kg body weight)$^{-1}$ valium followed by 0.18ml sagatal (20% solution in saline), both injected intraperitoneally. Both ovaries were then removed through small incisions in the back of the mouse. The fallopian tubes were tied and the muscle layer and skin sewn up separately and swabbed with an antibiotic spray. The mice were allowed to recover for at least one month before being inoculated with parasites.

2.4.2.3. Inoculation of Parasites

Trichomonad cultures were grown to a density of approximately $5x10^5$ ml^{-1}, and the parasites were harvested and resuspended to a density of $5x10^6$ ml^{-1} in MDM containing 0.32% (w/v) bactoagar (Difco). 0.02ml of this suspension was inoculated into the regions of Balb/c mice using a round-ended needle; the inoculation was normally two days after the administration of oestradiol-17β-cypionate. In early investigations the mice vaginas were immediately plugged with the dental sponge, Sterispon (Allen & Hanbury's), however, this was later

abandoned as studies by Miss Dianne Markham in our laboratory proved the vaginal plugs to be unnecessary.

2.4.2.4. Detecting Infections in Mice

To look for the presence of trichomonads in the vagina of a mouse, the vagina was washed out with 0.02ml MDM using a round-ended needle and syringe. The wash-out was then placed in 1ml MDM in a tissue culture well. Plates were incubated anaerobically (in an anaerobic incubator) at 37°C for at least one week and checked daily for the presence of motile trichomonads using a Leitz inverted microscope. Mice incubated with parasites were monitored for an infection periodically until the infection disappeared.

2.4.2.5. *In vitro* Cultivation of Isolates

A number of isolates which were found to have a high infectivity rate intravaginally were serially subpassaged routinely in mice. Mice infected with the isolate to be passaged were washed out in the usual way. Wells containing wash-out medium were monitored until a well was seen to contain approximately $5x10^5$ trichomonads ml^{-1}. The culture was then removed from the well and placed in a universal containing 20ml MDM. This was then incubated until a density of approximately $5x10^5$ trichomonads ml^{-1} was reached. The culture was then centrifuged (3000 rpm) and resuspended to a density of $5x10^6$ ml^{-1} in MDM containing 0.32% (w/v) bactogar. 0.02ml of the suspension was inoculated into the vaginas of six more mice pretreated with oestradiol two days earlier. Two weeks later these mice were washed out and the process was repeated.

2.5. STUDY ON THE EFFECTS OF INHIBITORS

2.5.1. *In vitro* Tests

2.5.1.1. Trichomonads in Axenic Culture

The sensitivity to inhibitors of trichomonads in axenic culture was determined using two methods. Firstly, for metronidazole, sterile 96-well microtitre plates (Gibco) were used. Trichomonads to be tested were adjusted to a density of $5x10^4$ ml^{-1} in MDM. 0.05ml of metronidazole solution sterilized by filtration was placed in each well of the microtitre plate and 0.2ml trichomonad culture was added. A dilution series of metronidazole concentrations was used, giving working concentrations of 100, 50, 25, 10, 5, 2, and 1μg ml^{-1}. A control well was included, to this was added 0.05ml distilled, deionized water. Each test was carried out in duplicate. Plates were normally prepared in identical pairs, one being incubated aerobically in a dampened box, and the other anaerobically in an anaerobic incubator at 37°C for 24 hours. Wells were then examined for the presence of motile trichomonads using a Leitz inverted microscope and where appropriate double-checked using a standard light microscope. The minimum lethal concentration (MLC) of each drug was taken to be the lowest concentration of metronidazole of which no motile trichomonads were observed after 24 hours.

For other inhibitors, however, I was less interested in determining the minimum lethal concentration than knowing the effects on the growth rate of the trichomonads incubated with the compounds at less than lethal concentrations. To determine this trichomonads were placed in universals containing 10ml MDM supplemented so that an initial density of 10^4 trichomonads ml^{-1} was obtained. A sterile solution, in water, of the relevant inhibitor was made and added to the culture at the appropriate concentration. The cultures were incubated at 37°C and growth of the trichomonads was monitored by

counting the cell density twice daily using a haemocytometer slide.

2.5.1.2. In Mammalian Cell Culture

Mammalian cells were grown in flasks, 0.5ml aliquots were distributed to tissue culture wells and incubated overnight in the usual way. A sterile solution of the inhibitor to be tested was prepared and 0.05ml added to each well containing mammalian cells; 0.05ml distilled, deionized water was added to the control wells. The inhibitor solution was also added to wells containing 0.5ml tissue culture medium but no cells. 0.2ml trichomonad suspension at the density in MDM was then added to all wells except the mammalian cell culture control, and the plates incubated in 5% CO_2, 95% air at 37°C for approximately one week. The effect of trichomonads on the mammalian cell monolayer was monitored twice daily using a Leitz inverted microscope and the time in hours for each of the cell types to disappear in the presence and absence of inhibitors at different trichomonad to mammalian cell ratios was noted.

2.5.2. Studies on the Effects of Inhibitors using the Subcutaneous Mouse Model

Balb/c mice, aged approximately 2-3 months were closely shaved on the right flank and injected with 0.2mls of trichomonad suspension of $5x10^6$ ml^{-1} in MDM with 0.16% agar in the usual way. Inhibitors dissolved in distilled, deionized water to the concentration required were administered either subcutaneously, intraperitoneally, intravenously or orally. Inhibitors were tested by giving various concentrations and also by administering at various times before and after inoculation of parasites; multiple doses were also investigated. Lesion growth was measured daily, that in treated animals being compared to growth of lesions of control mice.

Another approach involved incubating trichomonads in MDM with the inhibitor added for 24 hours prior to inoculating them subcutaneously into mice. In some cases the inhibitor was also included in the medium in which trichomonads were injected.

2.5.3. Studies on the Effects of Inhibitors using the Intravaginal Mouse Model

Balb/c female mice were infected intravaginally in the usual way. Inhibitors, dissolved in distilled, deionized water to the concentration required, were administered, at various times prior to and after parasite inoculation by various routes: subcutaneously, intraperitoneally, intravenously and orally. Again, alternative experiments involved incubating trichomonads in the appropriate concentration of the inhibitor to be tested for 24 hours prior to inoculation. The inhibitor was also included in the medium in which the trichomonads were injected in some cases. Infections were monitored by periodically washing out the vaginas of mice and examining cultures daily in the usual way.

3.0. RESULTS

3.1. DEVELOPMENT OF EXPERIMENTAL MODELS

3.1.1. The Interaction of Trichomonads with Mammalian Cells *in vitro*

The effects of *T. vaginalis* G3 on a number of different mammalian cell lines were investigated. Initially mouse myeloma cell line P3-X63-Ag8-653 was used and the interaction between it and *T. vaginalis*, in Leighton tubes, at a ratio of 20:1 is shown in Figure 4. Trichomonads in HOMEM medium by themselves survived for no more than 2 days, reached a maximum density of only about $2x10^4$ ml^{-1}, and had all died by 44 hours, the time point labelled 'A'. When myeloma cells were incubated in mixed culture with trichomonads, they declined in number relatively rapidly until all had lysed by the time point labelled 'B', in this case approximately 68 hours. Trichomonads in the mixed culture increased greatly in number such that a density of over 10^6 ml^{-1} was reached. They died only after reaching this high maximum density by the time point labelled 'C'. This process is termed 'overgrowing'. In this experiment, the myeloma cells in monoculture were maintained at a similar density throughout, thus the time point 'D', that by which all mammalian cells in monoculture were lysed, was assigned a value of 00. Thus the results can be tabulated as shown in Table 1.

A similar interaction was observed between *T. vaginalis* G3 and Hela cells (Figure 5). This experiment, however, was carried out using the wells of a tissue culture plate rather than Leighton tubes and the ratio of mammalian cells:trichomonads was 1:25. Nevertheless, time points A and C were very similar in this case to those detained for the trichomonad/myeloma interaction (Figure 4). Hela cells in mixed culture, however, declined in number rather more rapidly than the myeloma cells under similar conditions.

It was found that the conditions employed in the interaction experiments largely determined their outcome. If a large number of trichomonads were used they grew to a high maximum density before

Figure 4. The interaction of Trichomonads with Mammalian Cells *in vitro*

The figure shows the mean (± S.D.) of 6 experiments. Myeloma cell line P3-X63-Ag8-653 were used at a ratio of 20:1 with *T. vaginalis* G3 cells. Key to cell types: ●, trichomonads; O, mammalian cells; _ _, cells in mixed culture; ___, cells in monoculture.

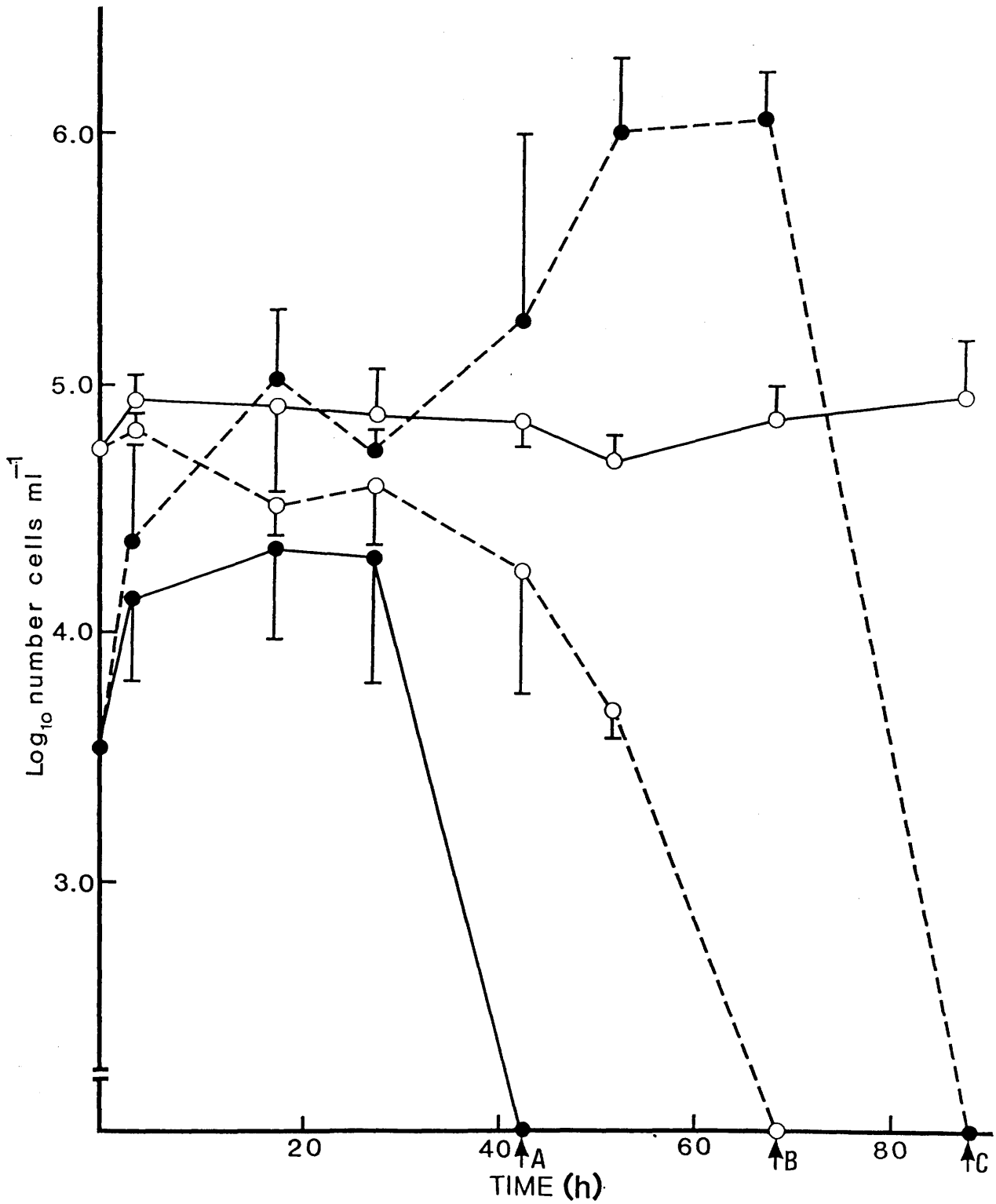
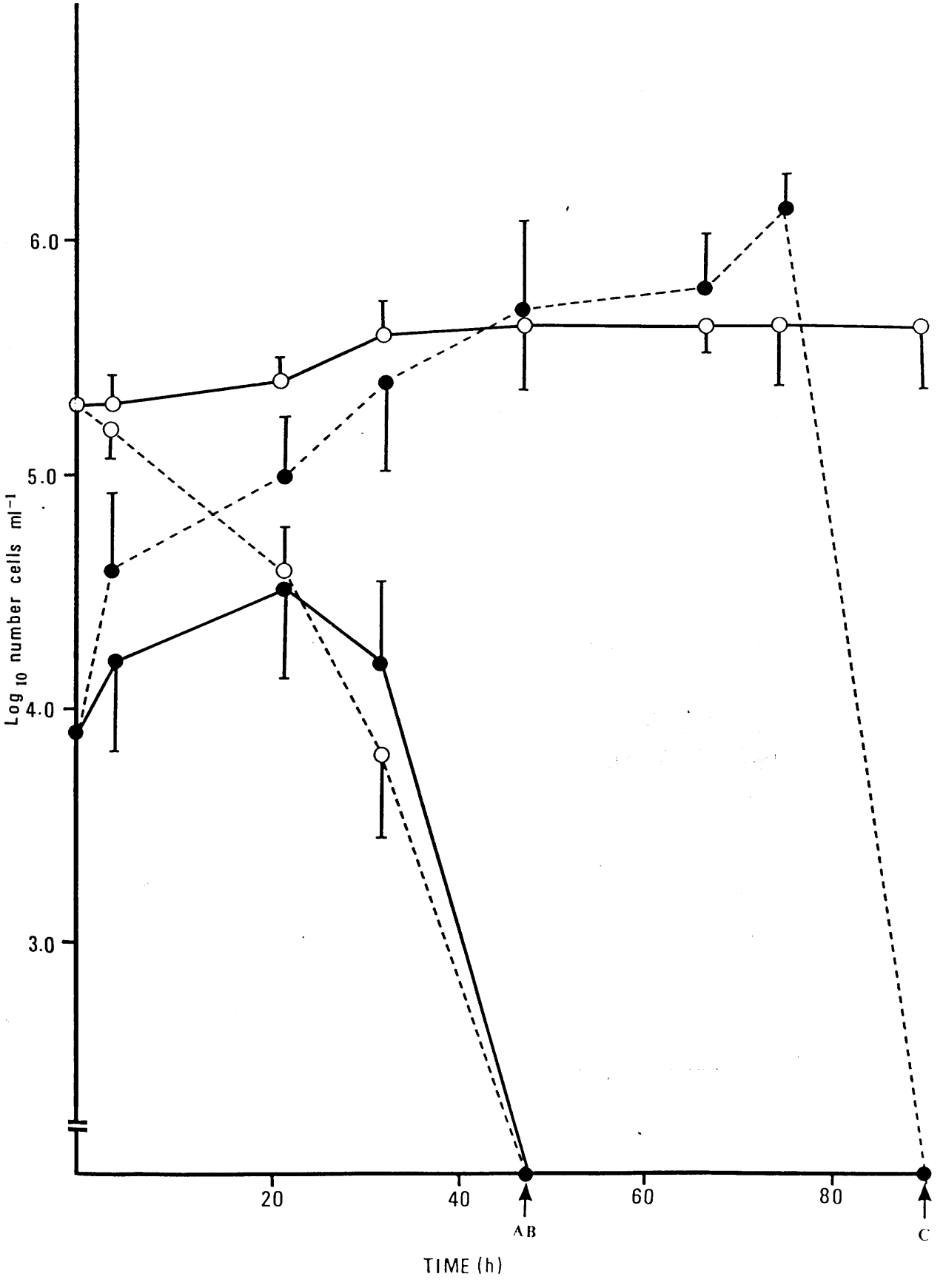

6.0
5.0
4.0
3.0
Log10 number cells ml-1
20
40
60
80
TIME (h)
A
B
C

Table 1. Interaction of Trichomonads with Mammalian Cells *in vitro*

Ratio of myeloma cells: trichomonads	Time (hours) for cell lines to disappear			
	A	B	C	D
20:1	44	68	87	00

The table shows the same experimental data as Figure 4 in tabulated form. *T. vaginalis* G3 was added to a culture of myeloma cells at a ratio of 20:1, mammalian cells:trichomonads. Key: A, time point for trichomonads in monoculture to die; B, time point for mammalian cells in mixed culture to die; C, time point for trichomonads in mixed culture to die; D, time point for mammalian cells in monoculture to die.

Figure 5. The Interaction of *T. vaginalis* and Hela Cells *in vitro*. The experiment was performed three times and the means are given in this figure. *T. vaginalis* G3 was added to a culture of HeLa cells at a ratio of 20:1, mammalian cells:trichomonads. Key to cell types: ●, trichomonads; ○, mammalian cells; _ _, cells in mixed cultures; ——, cells in monoculture.


6.0
5.0
4.0
3.0
Log 10 number cells ml-1
20
40
60
80
AB
C
TIME (h)

dying, that is they overgrew, even in the absence of mammalian cells. In the other extreme, if only a small number of trichomonads were used then no cytotoxic effect on the mammalian cells was observed. Clearly, the number of trichomonads added to the mammalian cell culture must fall between these limits, defined as the maximum and minimum, for there to be a cytotoxic effect upon which the trichomonads are dependent for their survival and growth. The growth and survival of *T. vaginalis* G3 in EMEM in tissue culture with a gaseous phase of 5% CO_2, 95% air, from various initial densities is shown in Figure 6. This demonstrates that an initial density of less than $5x10^4$ trichomonads ml^{-1} must be used in cultures with Hela cells if the interaction is to be dependent upon the cytotoxic potential of the trichomonads towards the Hela cells. The maximum and minimum densities were found to depend upon the mammalian cell type and medium used and whether the experiment was carried out in Leighton tubes or the wells of tissue culture plates. Similarly the kinetics of the interaction depend upon these factors (Table 2).

3.1.1.1. The Effects of Varying the Number of Trichomonads and Mammalian Cells

The effects of varying the ratio of trichomonads to mammalian cells was tested using several starting densities of mammalian cells as shown in Table 2a and 2b. The results given in Table 2a indicate that the cytopathogenic effect is dependent not only on the ratio of trichomonads to mammalian cells but also on the density of mammalian cells. In Table 2b the results for the same experiment are presented in terms of initial densities of trichomonads. These show that with a myeloma cell density of $4x10^5$ ml^{-1} or less, greater than $4x10^4$ trichomonads ml^{-1} must be added for pathogenicity to be observed. If, however, the initial density of *T. vaginalis* is $8x10^5$ ml^{-1} or higher,

Figure 6. Growth of *T. vaginalis* G3 in EMEM under 5% CO_2, 95% air. Twice daily counts of trichomonad densities were taken. ●, Mean (± S.D.) of 3 experiments.

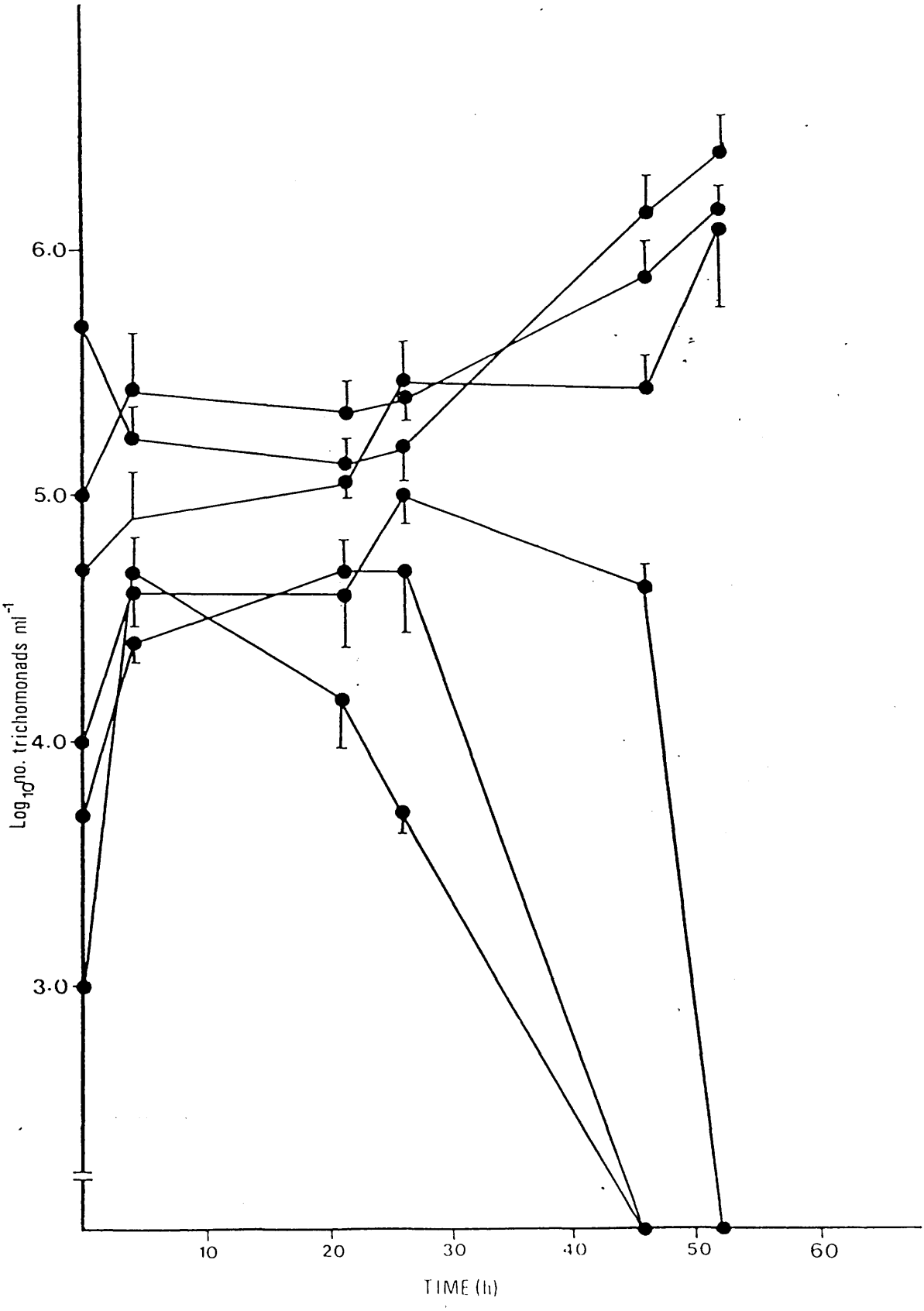

6.0
5.0
4.0
3.0
Log10no. trichomonads ml-1
10
20
30
40
50
60
TIME (h)

Table 2a. *The Effects of Varying the Number of Trichomonads and Mammalian Cells upon their Interaction*

INITIAL MAMMALIAN CELL DENSITIES	TIME (HOURS) FOR CELLS TO DISAPPEAR										SEE 2b FOR NO. TRICHOMONADS ADDED
RATIO	4×10^4		8×10^4		2×10^5		4×10^5		8×10^5		0
MAMMALIAN CELL: TRICHOMONADS	B	C	B	C	B	C	B	C	B	C	A
100:1	00	45	00	45	00	45	00	45	00	45	45
50:1	00	45	00	45	00	45	00	53	53	70*	45
20:1	00	45	00	45	00	45	00	53	53	70*	45
10:1	00	45	00	53	00	53	00	53	53	70*	45
5:1	00	45	00	53	00	53	20	70*	20	45*	45
2:1	00	45	00	53	20	93*	20	70*	20	45*	45
1:1	00	45	20	70*	20	70*	20	70*	20	45*	53
1:2	29	93*	20	70*	20	70*	20	45*	20	45*	53
1:5	20	77*	20	70*	20	53*	20	45*	4	45*	70*
1:10	20	77*	20	45*	20	45*	20	45*	4	20*	70*

Myeloma cells were used; mammalian cells in monoculture survived throughout the experiment at all initial densities. Experiments were carried out in the wells of a tissue culture plate. Key: *, trichomonads overgrew; A, time point for trichomonads in monoculture; B, time point for mammalian cells in mixed culture; C, time point for trichomonads in mixed culture; ▨, cytopathogenic effect observed but trichomonads in monoculture overgrew; ▨, cytopathogenic effect observed; □, no cytopathogenic effect.

Table 2b.

INITIAL MAMMALIAN CELL DENSITIES: / RATIO MAMMALIAN CELLS: TRICHOMONADS	INITIAL DENSITIES OF TRICHOMONADS ADDED: 4×10^4	8×10^4	2×10^5	4×10^5	8×10^5	0
100:1	4×10^2	8×10^2	2×10^3	4×10^3	8×10^3	2×10^2
50:1	8×10^2	1.6×10^3	4×10^3	8×10^3	1.6×10^4	8×10^2
20:1	2×10^3	4×10^3	10^4	2×10^4	4×10^4	2×10^3
10:1	4×10^3	8×10^3	2×10^4	4×10^4	8×10^4	8×10^3
5:1	8×10^3	1.6×10^4	4×10^4	8×10^4	1.6×10^5	2×10^4
2:1	2×10^4	4×10^4	10^5	2×10^5	4×10^5	8×10^4
1:1	4×10^4	8×10^4	2×10^5	4×10^5	8×10^5	2×10^5
1:2	8×10^4	1.6×10^5	4×10^5	8×10^5	1.6×10^6	8×10^5
1:5	2×10^5	4×10^5	10^6	2×10^6	4×10^6	2×10^6
1:10	4×10^5	8×10^5	2×10^6	4×10^6	8×10^6	--

Data from same experiment as Table 2a but presented in terms of initial densities of trichomonads used. Symbols as in 2a.

they grow in monoculture. With a myeloma density of $8x10^5$ ml^{-1}, only $1.5x10^4$ trichomonads ml^{-1} were needed for the cytopathogenic effect to occur.

3.1.1.2. Effects of Varying the Medium

Trichomonads had no observable effect on mammalian cells if the parasites were added as a suspension in HOMEM medium rather than MDM. Hence an experiment was carried out to discover the concentration of MDM requried, the results are shown in Table 3. It was found that the incubation medium must contain at least 25% MDM for a cytotoxic effect to be observed. If the proportion of MDM was increased to 45%, however, the trichomonads grew and overgrew even in monoculture at an initial density of 10^4 ml^{-1}. Consequently a ratio of 30% MDM to 70% HOMEM was used.

An experiment was performed using the murine macrophage-like cell line $P388D_1$ to determine whether resuspending the trichomonads in fresh MDM before addition to the mammalian cell line, as opposed to adding them in the MDM in which they had grown changed the cytotoxicity of the trichomonads. The results are given in Table 4. The observed interaction was quite similar in both cases although at the 2:1, mammalian cell:trichomonad ratio, the macrophages were lysed less rapidly when spent MDM was added with the trichomonads whereas at the same density trichomonads in monoculture died slightly more rapidly when fresh MDM was used.

3.1.1.3. The Effects of Varying the Depth and Volume of the Medium

The effects of using different depths and volume of medium in wells of a tissue culture plate upon the cytopathogenicity of *T. vaginalis* were investigated using a range of trichomonad densities, as shown in Table 5. It was found that if a volume of 1.25ml or more was used with a high density of trichomonads, the trichomonads

Table 3. The Effects of Varying the Media upon the Interaction between Trichomonads and Mammalian Cells

% MDM:HOMEM	TIME (HOURS) FOR CELLS TO DIE: A	B	C	D	$\propto$ MINIMUM RATIO
15:85	43	00	55	00	2:1
25:75	43	00	69	00	2:1
30:70	49	49	144*	00	10:1
40:60	69	43	122*	00	10:1
45:55	122*	43	92*	00	10:1
50:50	122*	25	92*	00	10:1

*. trichomonads overgrew

T. vaginalis G3 cells were added at a ratio of 20:1, mammalian cells:trichomonads, to a culture of myeloma cells at a density of $2x10^6$ ml^{-1}. The experiment was performed in the wells of a tissue culture plate. Key: A, time point for trichomonads in monoculture; B, time point for mammalian cells in mixed culture; C, time point for trichomonads in mixed culture; D, time point for mammalian cells in monoculture; ▨, cytopathogenicity observed but trichomonads overgrew in monoculture; ▨ cytopathogenicity observed and trichomonads did not grow in monoculture; ☐ no effect on mammalian cells; $\propto$, minimum RATIO-lowest ratio of mammalian cells:trichomonads (at a density of $2x10^6$ myeloma cells) at which trichomonads had a cytopathogenic effect on mammalian cells.

Table 4. The Effect of Resuspending Trichomonads in Fresh MDM before Adding to Mammalian Cell Culture

RATIO OF MAMMALIAN CELLS: TRICHOMONADS	Trichomonads resuspended in MDM they had grown in				Trichomonads resuspended in fresh MDM			
	A	B	C	D	A	B	C	D
5:1	46	00	46	00	46	00	46	00
2:1	77	29-53	120*	00	46	53-70	120*	00
1:1	120*	22	70-120*	00	120*	22	70*	00

*, trichomonads overgrew

T. vaginalis G3 cells were added at various ratios to a culture of macrophages at a density of 10^6 ml^{-1}. The experiment was performed in the wells of a tissue culture plate. Data represent a range from 5 experiments normally checked at 6, 22, 29, 46, 53, 70, 77, 94 and 120 hours. Key: A, time point for trichomonads in monoculture; B, time point for mammalian cells in monoculture; C, time point for trichomonads in mixed culture; D, time point for mammalian cells in monoculture; ⊠, cytopathogenicity observed and trichomonads overgrew in monoculture; ▨, cytopathogenicity observed and trichomonads did not grow in monoculture; □, no effect on mammalian cells.

Table 5. Survival of *T. vaginalis* in cultures of different Depths and Volumes.

INITIAL TRICHOMONAD DENSITY (ml^{-1})	TIME FOR CELLS TO DIE (HOURS): VOLUME (ml): 0.25	0.50	0.75	1.00	1.25	1.50
	DEPTH (mm) 1.2	2.4	3.6	4.9	6.1	7.3
8.0×10^4	44	44	44	50	80*	80*
5.5×10^4	20	44	44	44	44	44
3.0×10^4	20	20	20	20	20	20
4.0×10^3	20	20	20	20	20	20

Trichomonads were added in various densities to various depths of HOMEM medium in wells of tissue culture plates and incubated in an atmosphere of 5% CO_2, 95% air.

overgrew, whereas when a low volume was used the trichomonads died very rapidly even at a relatively high density. Consequently, an intermediate volume of 0.75ml was chosen for routine use in experiments.

3.1.1.4 The Effects of Different Sera on the Interaction

The mammalian cell line and trichomonads were grown up under standard conditions but then washed and resuspended in appropriate media containing one of several sera to be tested. The interaction of the cell types in the presence of various sera is shown in Table 6. Minor differences between the interaction occurring in different sera were observed, but major differences were not found. No cytopathogenicity was apparent when serum was omitted altogether. Guina pig serum seemed the most effective at supporting trichomonad/mammalian cell interaction, a cytopathogenic effect being observed even at a ratio of 10:1, mammalian cells:trichomonads. In order to find out how the different sera supported growth of the trichomonads themselves, an experiment was carried out to determine the extent of growth of trichomonads axenically in mixed MDM/HOMEM medium supplemented with different sera and incubating at 37^{o}C under an atmosphere of 5% CO_2, 95% air. It was found that growth was minimal and survival only short term in all sera except guinea pig and mouse serum. In these, however, trichomonads grew rapidly and well even from a low initial density.

3.1.1.5. The Cytopathogenic Effect of *T. vaginalis* on Different Mammalian Cell Lines

The cytotoxic effects of *T. vaginalis* G3 against three other mammalian cell lines, which had been used previously by others to determine the cytopathogenicity of trichomonads, were also investigated using the standard procedure developed for myeloma cells.

Table 6. The Effects of Different Sera on the Interaction

SERA	NO SERUM		FCS				7% FCS		HS				NCS				MS				GPS			
%			5%		10%		3%	HS	5%		10%		5%		10%		5%		10%		5%		10%	
RATIO MAMMALIAN CELL: TRICHOMONADS	AB	CD	AB	CD	AB	CD	AB	CD	AB	CD	AB	CD	AB	CD	AB	CD	AB	CD	AB	CD	AB	CD	AB	CD
20:1	46 00	46 00	46 00	46 00	46 00	46 00	52 00	46 00	52 00	46 00	46 00	46 00	46 00	46 00	46 00	46 00	46 00	46 00	46 00	46 00	70 00	52 00	52 22	52 00
10:1	46 00	46 00	52 00	46 00	70 00	46 00	52 00	46 00	52 00	46 00	52 00	46 00	94 00	52 00	46 00	46 00	52 00	52 00	52 00	52 00	70 00	70 00	70 00	122 00 *
5:1	46 00	46 00	70 00	70 00	122 27 *	144 00 *	52 27 *	122 00	52 27 *	122 00	52 00	52 00	122 46 *	122 00	46 00	70 00	70 22 *	52 00	52 22 *	70 00	70 00	52 00	122 27 *	94 00
2:1	46 00	46 00	70 00	94 00	122 22 *	122 00 *	122 22 *	94 00	122 22 *	94 00	52 00	52 00	122 22 *	94 00	94 22 *	94 00	52 22 *	52 00	46 22 *	46 00	94 22 *	122 00	94 00 *	94 00

The experiment was performed in the wells of a tissue culture plate. Key: A, time point for trichomonads in monoculture; B, time point for mammalian cells in mixed culture; C, time point for trichomonad in mixed culture; D, time point for mammalian cells in monoculture; ⊠, cytopathogenic effect observed but trichomonads in monoculture overgrew; ▨, cytopathogenic effect observed; □, no effect; FCS, foetal calf serum; HS, horse serum; NCS, newborn calf serum; MS, mouse serum; GPS, guinea pig serum.

Their relative susceptibilities to *T. vaginalis* G3 in our model system are given in Table 7. The same basic interaction occurred in all cases. The cytoxic effect was most dramatic when myeloma and Hela cells were used as these cell lines disappeared most rapidly in the presence of trichomonads, and the trichomonads in mixed culture with these cell lines grew and overgrew most quickly. However, far fewer trichomonads were needed to cause an effect on Hela cells than for myeloma cells, the minimum ratios of mammalian cells:trichomonads being 100:1 and 10:1 respectively.

In each case of an isolate interacting with Hela cells and myeloma cells a far greater minimum ratio was required for a cytotoxic effect to occur against the myeloma cell line than for this to occur against Hela cells, and one isolate *T. vaginalis* 45733, did not have any effect on the myeloma cell line although at a ratio of 200:1 mammalian/trichomonads cells, this isolate caused complete destruction of the Hela cell monolayer (Table 4).

Observing the interactions of *T. vaginalis* with mammalian cells with a Leitz inverted microscope, differences were noted between experiments using the two different cell types. Myeloma cells did not adhere to the flask as strongly as the Hela cell line which needed trypsinizing to free the cells. When the trichomonads were added to the mammalian cell culture they did not appear to attach to the myeloma cells, however attachment of many motile trichomonads to the Hela cell monolayer was observed.

3.1.2. The Subcutaneous Mouse Model

3.1.2.1. The Effects of Adding Agar to the Inoculation Medium

In early experiments, published methods were followed and mice were inoculated subcutaneously in a flank with trichomonads suspended

Table 7. Comparison of Cytotoxic Effects of *T. vaginalis* G3 on Different Mammalian Cell Lines

MAMMALIAN CELL LINES	INITIAL CELL DENSITY	n	TIME (HOURS) FOR CELLS TO DIE				MINIMUM* RATIO	MEDIUM
			A	B	C	D		
CHO	$2x10^6$	1	125	37	103*	00	-	HOMEM
MACROPHAGE	10^6	4	46-72	65-72	95-113*	00	20:1	HOMEM
MYELOMAa	10^6	6	41-48	28-53	70-125*	00	20:1	HOMEM
MYELOMAb	10^6	4	44	00	44	00	-	HOMEM
CHICK EMBRYO	$5x10^5$	2	71	71	95*	00	-	HBME
HELA	$2x10^6$	5	90-119*	28-48	71-94*	00	100:1	EMEM

Experiments were carried out in wells of a tissue culture plate except for MYELOMAb. Data given are for 20:1 mammalian cell:trichomonad ratio. Key CHO, Chinese hamster ovary cells; MACROPHAGE, murine macrophage-like cell line $P388D_1$; MYELOMAa, murine myeloma cells (wells); MYELOMAb, murine myeloma cells (tubes); n, number of experiments performed for each cell line; MINIMUM RATIO*, lowest ratio of mammalian cells:trichomonads at which cytopathogenic effect is observed. A B C D - as in previous tables.

in fresh MDM medium. The lesions resulting from this procedure were found to grow slowly and there was great inconsistency within groups of mice (Figure 7). When, however, trichomonads were resuspended in MDM supplemented with 0.16% bactoagar prior to incolution, subsequent lesion growth was found to be more rapid and there was much greater consistency within groups of mice (Figure 7). Inoculation of MDM with 0.16% agar in the absence of trichomonads did not cause lesions. Subsequently, MDM containing agar was used routinely in the subcutaneous mouse model infection.

3.1.2.2. Growth of *T. vaginalis* Subcutaneously in Different Strains of Mice

The rate of lesion growth after subcutaneous inoculation of *T. vaginalis* G3 into the flanks of mice was found to depend upon the strain of mouse used (Figure 8). No lesions appeared in C57 black mice, of the three susceptible strains lesions grew most rapidly in Balb/c mice. Balb/c mice were subsequently used for all experiments with the subcutaneous model.

3.1.2.3. Growth of *T. vaginalis* as Subcutaneous Lesions in Naive and Previously-Infected Mice

Balb/c mice which had been infected subcutaneously with *T. vaginalis* G3 two months previously were re-inoculated with *T. vaginalis* G3 in both flanks. The previously induced lesions had ruptured and disappeared several weeks prior to the second experiment. A second group of Balb/c mice, of the same age as the first group but which had not been previously infected with *T. vaginalis*, were similarly inoculated with *T. vaginalis* G3 at the same time as the first group (Figure 9). The rates of growth of the subcutaneous lesions were found to be very simlar in the two groups of mice.

Figure 7. The Effects of Adding Agar to the Inoculation Medium. Means (± S.D.) of groups of three mice are shown. The last point plotted for each group is the last measurement taken before the first lesion ruptured. Key: ●, MDM with 0.16% bactoagar; □, *T.vaginalis* G3 in MDM; ■, *T. vaginalis* G3 in MDM with 0.16% bactoagar.

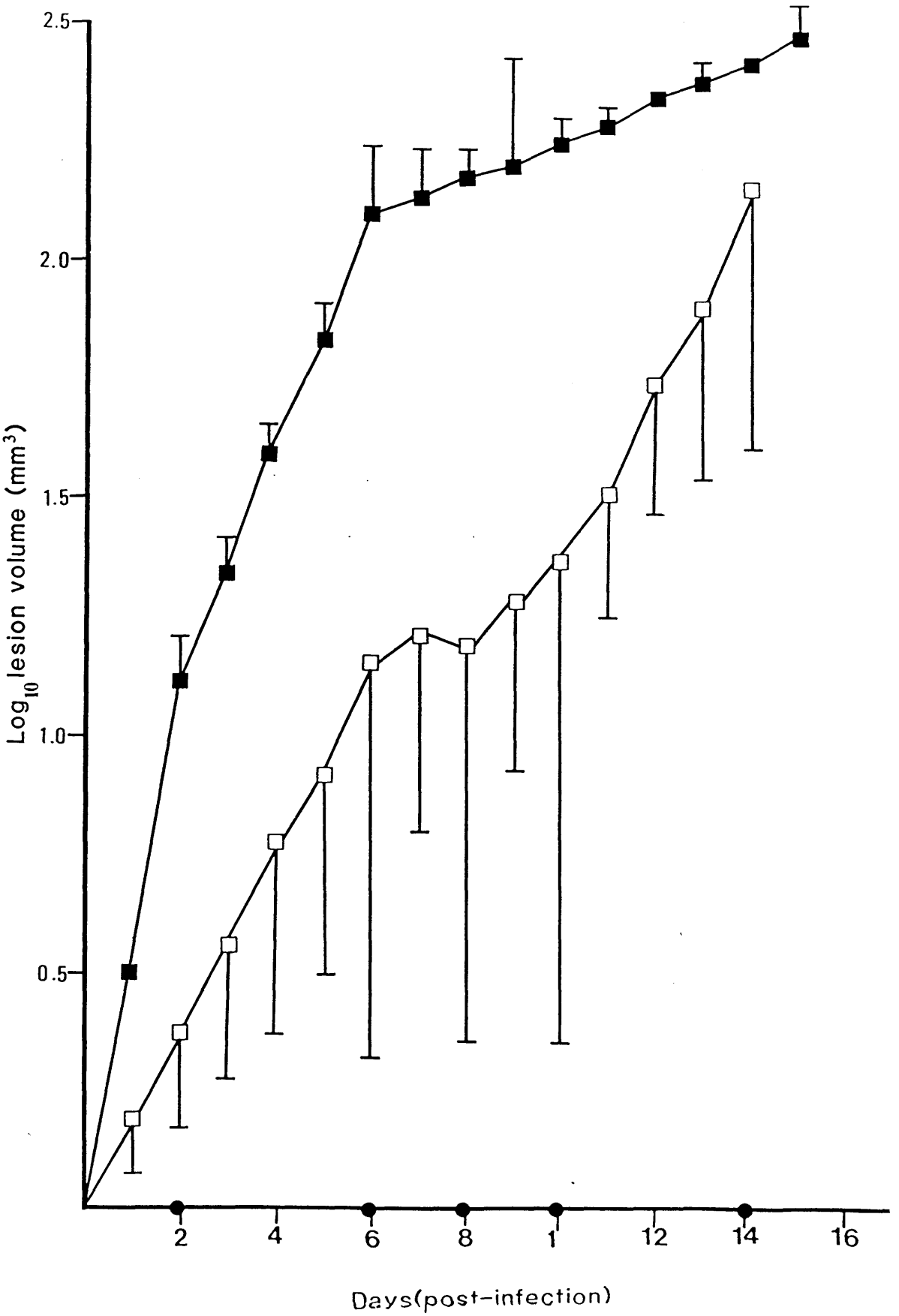

2.5
2.0
1.5
1.0
0.5
Log10 lesion volume (mm3)
2
4
6
8
1
12
14
16
Days(post-infection)

Figure 8. Growth of *T. vaginalis* subcutaneously in Different Strains of Mice

Means (±S.D.) of groups of three mice are shown, female mice aged 2 months were used. Key: ●, Balb/c mice; ■, CBA; ▲, NIH; ◆, C57 Black.

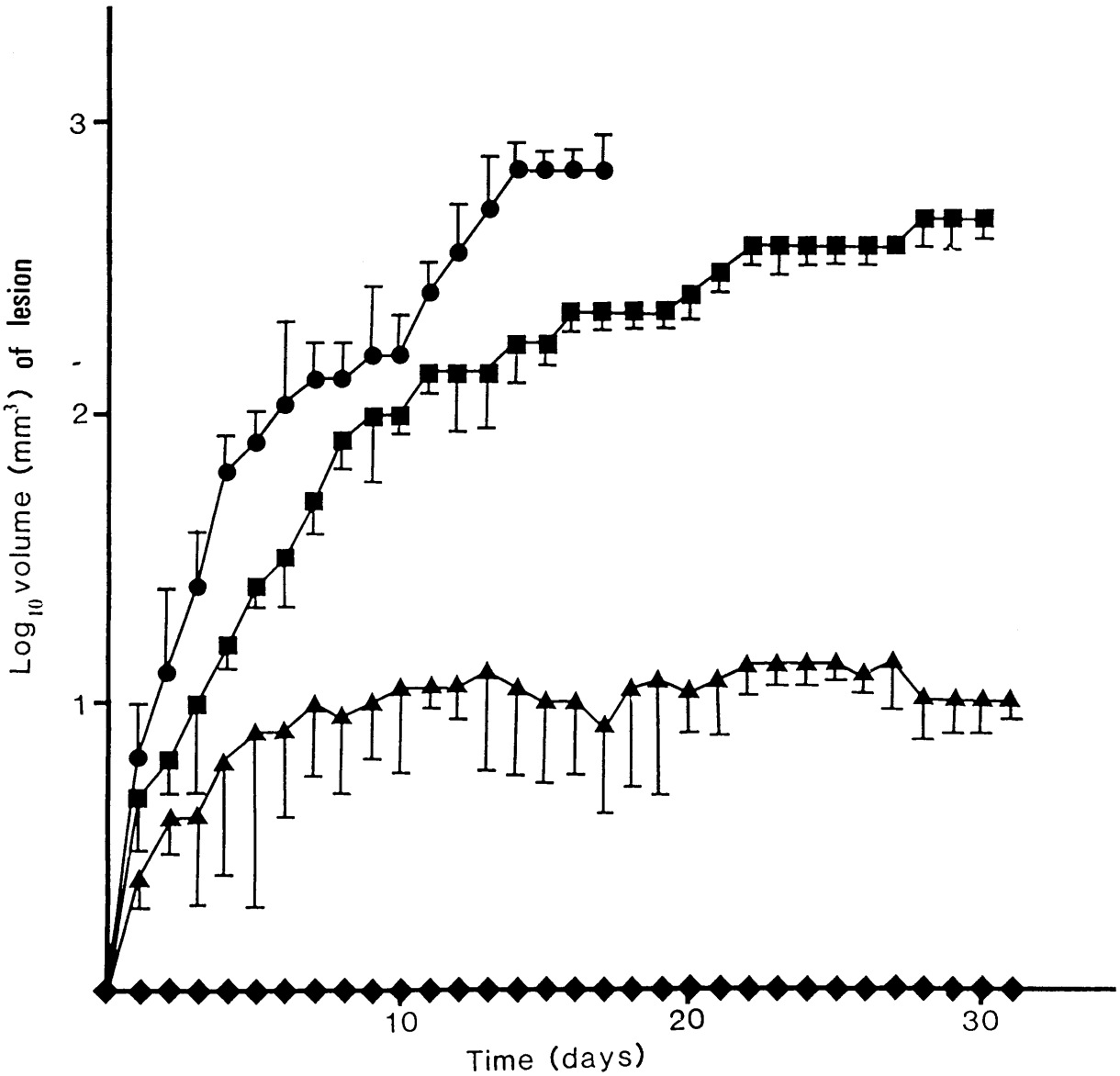
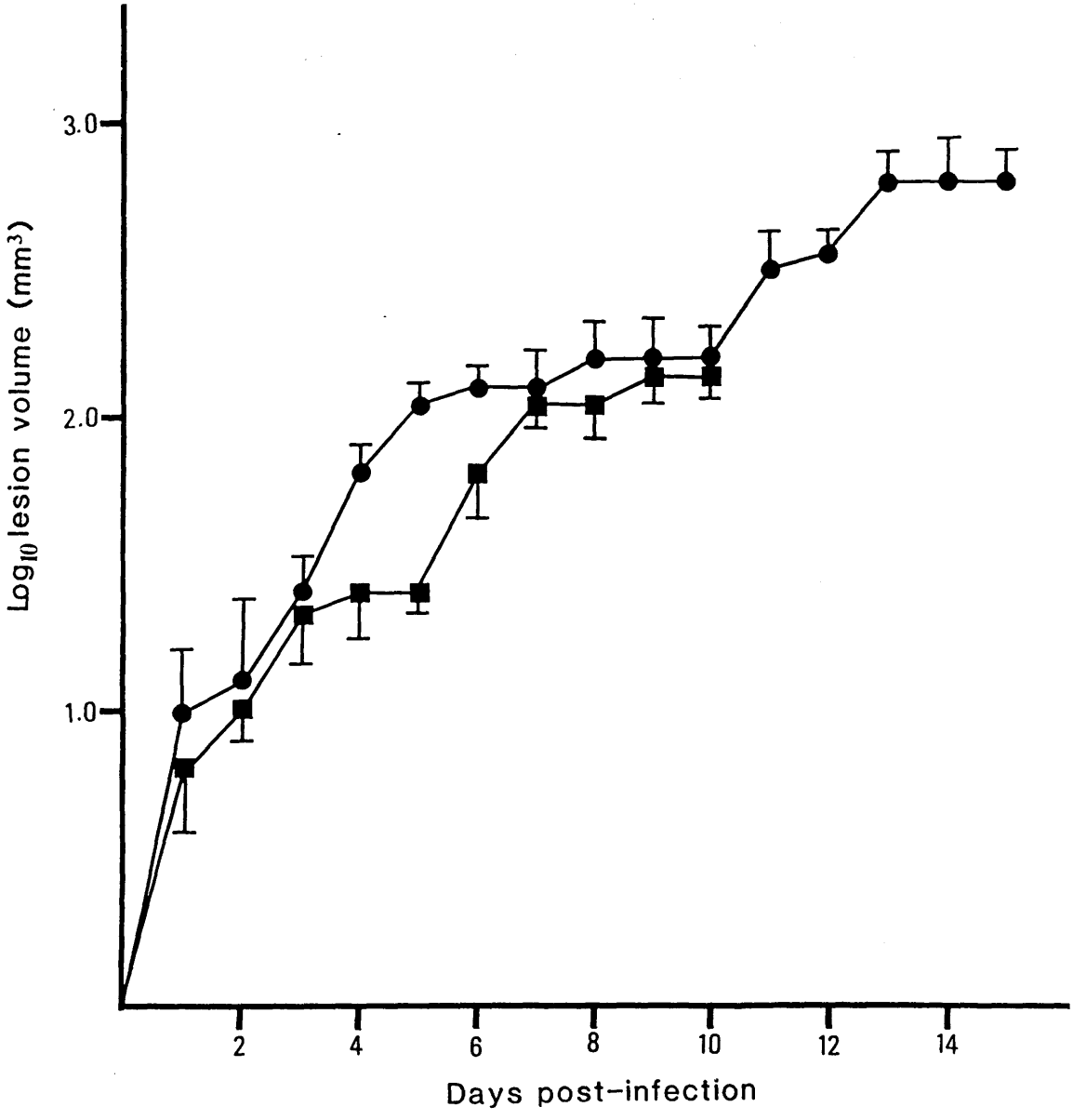

3
2
1
Log10 volume (mm3) of lesion
10
20
30
Time (days)

Figure 9. Growth of *T. vaginalis* as Subcutaneous Lesions in Naive and Previously-Infected Mice

Means (± S.D.) of groups of 6 mice are shown. Female Balb/c mice aged about 2 months were used. Key: ●, naive mice; ■, previously infected mice.


3.0
2.0
1.0
Log10 lesion volume (mm3)
2
4
6
8
10
12
14
Days post-infection

3.1.2.4. Relationship between Lesion Volume and Number of Trichomonads

Lesions produced by subcutaneous inoculation of *T. vaginalis* G3 were dissected out at different times post-infection and the number of motile trichomonads contained within each lesion was counted. The results (Figure 10) showed that there was a direct correlation between lesion volume and number of trichomonads contained within it up to a lesion size of approximately $150mm^3$. Larger lesions generally did not contain greater numbers of trichomonads.

3.1.2.5. Lesion Growth in Mice of Different Ages

Three groups of CBA mice (female, 2 months old; female, 7 months old; male, 5 months old) were compared for susceptibility to *T. vaginalis* G3 inoculated subcutaneously. The rate of lesion growth in each of the three groups was very similar (Figure 11).

3.1.2.6. The Effect of Growth Phase of Trichomonads upon their Infectivity

T. vaginalis 39, harvested from axenic cultures in MDM that were at lag phase (about $5x10^3$ ml^{-1}), log phase (about 10^5 ml^{-1}) and stationary phase (about 10^6 ml^{-1}), were inoculated subcutaneously into the flanks of Balb/c mice using the routine procedure and the volumes of the lesions produced measured for the next 9 days. The results are shown in Figure 12. The lesions caused by each of these populations of *T. vaginalis* grew similarly initially but after approximately three days the growth rate of the lesions caused by the lag phase culture slowed down to a greater extent than the growth rates of the lesions produced by log phase and stationary phase cells. Dead trichomonad cells, killed by metronidazole at log-phase, produced no lesions when injected subcutaneously.

Figure 10. Relationship between Lesion Size and Number of Trichomonads (±S.D.)
Female Balb/c mice, aged 2 months were used. Means of groups of 3 lesions are shown except where otherwise indicated.

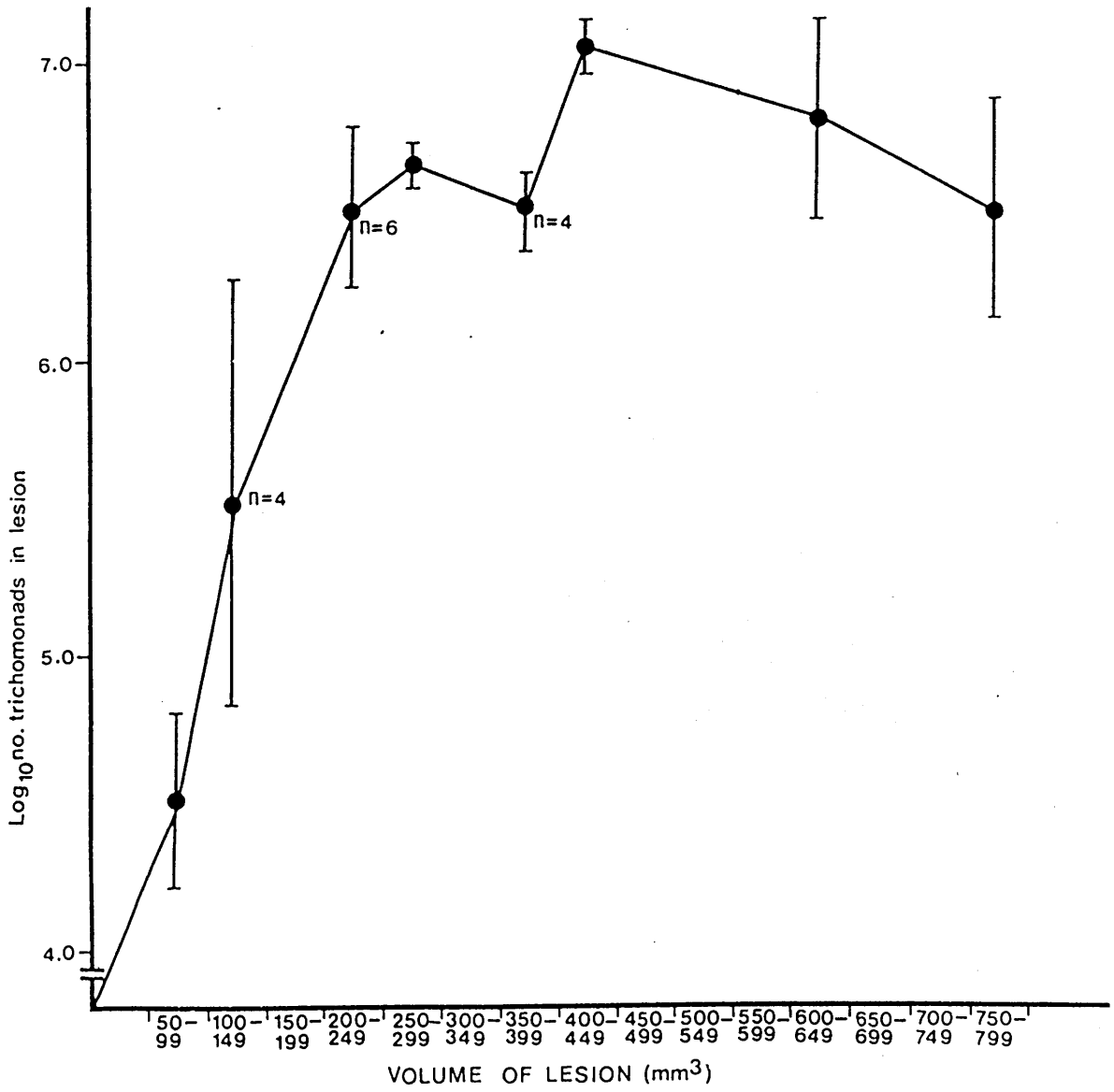

7.0
6.0
5.0
4.0
Log10 no. trichomonads in lesion
n=4
n=6
n=4
50–99
100–149
150–199
200–249
250–299
300–349
350–399
400–449
450–499
500–549
550–599
600–649
650–699
700–749
750–799
VOLUME OF LESION (mm3)

Figure 11. Lesion Growth in Mice of Different Ages (±S.D.)

Means of groups of three mice are shown. Key: ●, CBA mice, aged 5 months, male; ■, CBA mice, aged 2 months, female; ▲, CBA, aged 7 months, female.

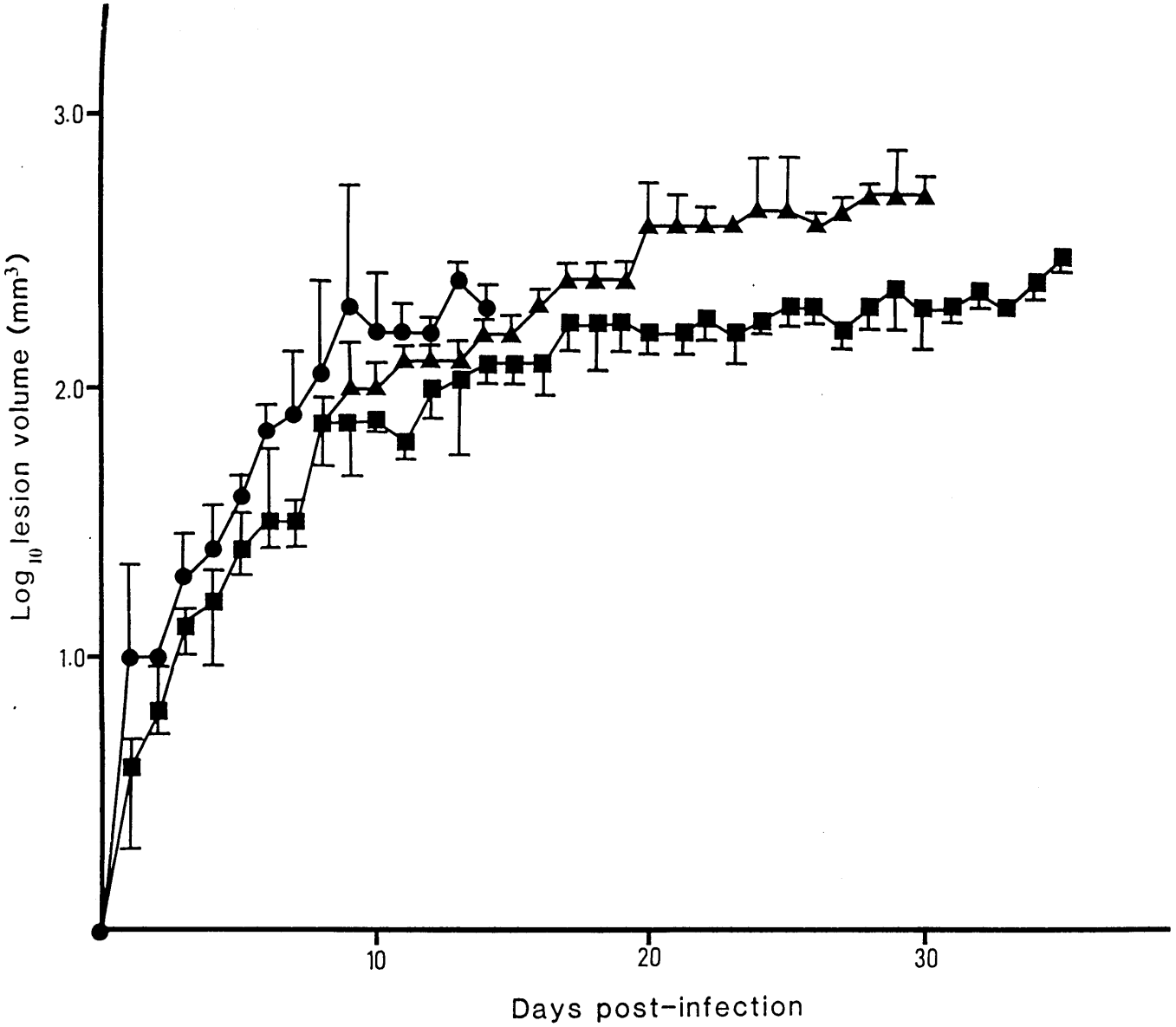
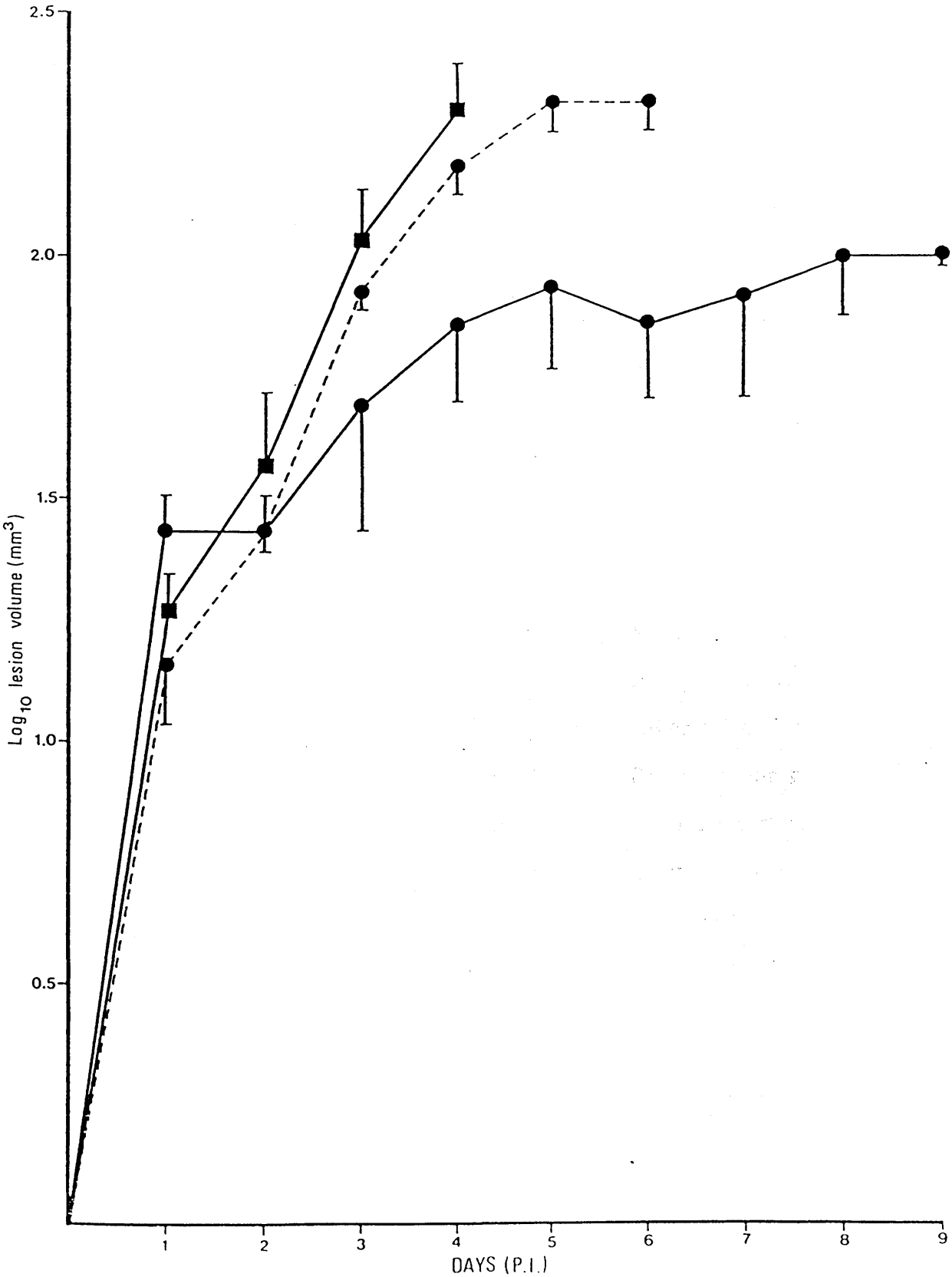

3.0
2.0
1.0
Log10 lesion volume (mm3)
10
20
30
Days post-infection

Figure 12. The Effect of Growth Phase of Trichomonads upon their Infectivity

(±S.D.)

Groups of 3 mice were used and the mean lesion volume plotted. Key: ■, trichomonads in log phase inoculated; ●— trichomonads in lag phase inoculated; ●– –, trichomonads in stationary phase inoculated. A Student's t test was carried out to determine whether lesion volumes were significantly different on day 4 post-infection. This day was chosen as the lesions were at maximum volume before beginning to rupture. It was found that the differences between lesion volumes caused by lag, log and stationary phase cultures were significantly different ($P = 0.1$).


2.5
2.0
1.5
1.0
0.5
Log10 lesion volume (mm3)
1
2
3
4
5
6
7
8
9
DAYS (P.I.)

3.1.2.7. The Effect of Serum Type in the Growth Medium upon the Growth of Subcutaneous Lesions caused by Trichomonads

The rates of growth of lesion produced by the trichomonads cultured in media with different sera or suspended in media with no serum were relatively similar (Figure 13), certainly if the results are compared with those obtained using different mice (Figure 8) or no agar (Figure 9). The lesions produced by trichomonads which had been grown in foetal calf serum or newborn calf serum apparently produced lesions more slowly than trichomonads grown in horse serum, there was also greater diversity within these groups of mice. Interestingly, suspension of trichomonads in inoculation medium without serum had no effect on the subsequent production of lesions.

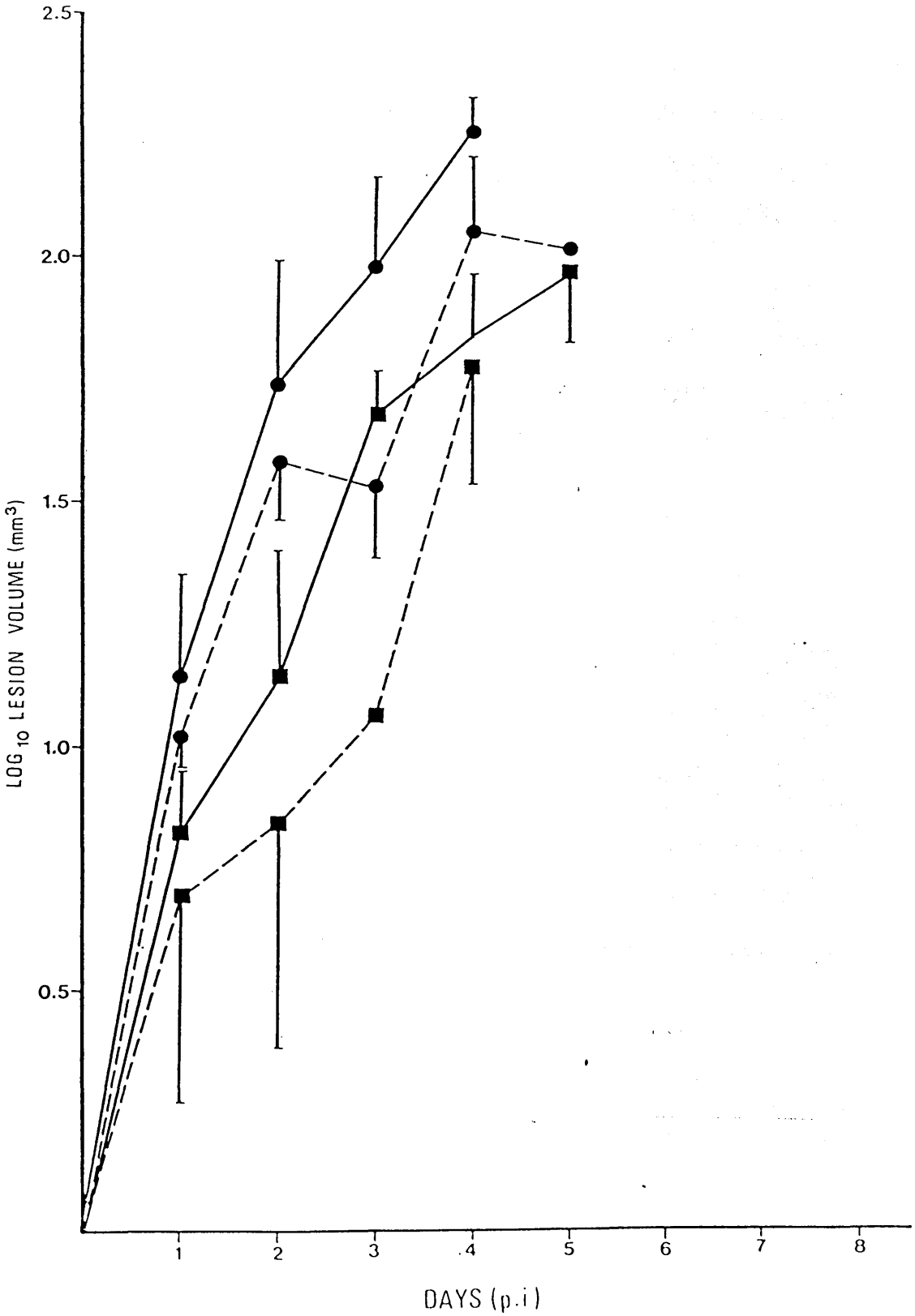
3.1.3. The Intravaginal Mouse Model

3.1.3.1. The Susceptibilities of Normal and Ovariectomized Mice

A group of Balb/c mice were spayed and allowed to recover for 3 months. Subsequently, they and a similar group of mice of the same age, but which had not been spayed, were inoculated with *T. vaginalis* 39 *in vivo* and the infections monitored (Table 8). A higher percentage of the group of mice which had been spayed were infected after 3, 7 and 14 days, than the intact mice. None of the intact mice remained infected past 20 days post-infection whereas at 28 days 30% of spayed mice were still infected and at least one mouse had an infection at 76 days post-infection. Although this particular experiment indicates that spayed mice were more susceptible than intact mice this was not found consistently. In some subsequent experiments, an equally or even higher rate of infectivity was achieved with intact mice than with spayed mice, for example, *T. vaginalis* 39 infected 76% of mice in one case and in one particular mouse the infection lasted 92 days. It appears, however, that the variable results are mainly due to

Figure 13. Effect of Serum Type on Subcutaneous Lesion Growth

Trichomonads were cultured and suspended in media with different sera or suspended in media with no serum. Key: ●— , no serum; ●--, 10% horse serum; ■— , 10% newborn calf serum; ■-- , 10% foetal calf serum. A Student's t test was performed on the results for day 4 post-infection, this day chosen as giving the maximum lesion volumes before lesions began to rupture. It was found that differences between lesion volumes from the different cultures were not significant at 40% confidence limits except for that between the lesions from cultures with no serum and those supplemented with foetal calf serum. (n=3, mean ± S.D.)


2.5
2.0
1.5
1.0
0.5
LOG 10 LESION VOLUME (mm3)
1
2
3
4
5
6
7
8
DAYS (p.i)

differences in susceptibility of the intact mice for reasons not fully understood.

3.1.3.2. The Dependence upon the Number of Trichomonads Inoculated

The infection rates and longevity of infections resulting from inoculation of either 10^5 or 10^6 *T. vaginalis* 39 *in vivo* cells were relatively similar, with the exception that trichomonads were detected in more mice at 3 days post-infection when the higher parasite inoculum was used (Table 9).

3.1.3.3. Investigation of the Relationship between the Condition of the Mouse Vagina on Infection with *T. vaginalis*

Balb/c mice were inoculated intravaginally with trichomonads using the routine procedure. Immediately before infection, however, a vaginal smear was taken and its appearance noted. The various stages of the oestrus cycle of the mouse were defined as indicated in Figure 3. The course of infections was monitored in the usual way, but smears were also taken and the appearance of the vaginal cells noted. The results are given in Table 10.

The results suggest that the susceptibility of the mice depends upon the stage of the oestrus cycle at the time of infection. For instance, 78% of mice which were in early pro-oestrus at the time of inoculation became infected whereas only 31% of mice which were in oestrus at the time of inoculation were found to be infected 7 days later. It is noteworthy, however, that the correlation was not absolute. A 100% infection rate was not achieved and none of the groups of mice were totally refractory.

The data from the wash-outs show that 80% of the mice apparently in meta-oestrus at the time of sampling were infected, whilst only 18% of the mice apparently in early pro-oestrus were. One possible

Table 8. Susceptibility of Intact and Spayed Mice to Intravaginal Infection with *T. vaginalis*

CONDITION OF MICE	NO. OF MICE	% OF MICE INFECTED AFTER				NUMBER OF DAYS AFTER WHICH INFECTION WAS NOT DETECTED
		3 DAYS	7 DAYS	14 DAYS	28 DAYS	
INTACT	14	28	28	28	0	20
SPAYED	10	60	60	60	30	76

Table 9. The Effect of the Numbers of Parasites Inoculated upon the Infection Rates

NO. OF TRICHOMONADS INOCULATED	NO. OF MICE	% OF MICE STILL INFECTED AFTER:				MINIMUM NUMBER OF DAYS AFTER WHICH INFECTION WAS NOT DETECTED
		3 DAYS	7 DAYS	14 DAYS	28 DAYS	
10^5	6	17	17	0	0	7
10^6	6	50	17	0	0	7

Table 10. Likelyhood of infection at various stages of the oestrus cycle.

CELLS PRESENT IN VAGINAL SMEAR	RN P	RN	SC RN	SC	SC P	SC RN P
STATE OF CYCLE :	DI-OESTRUS	EARLY PRO-OESTRUS	LATE PRO-OESTRUS	OESTRUS	META-OESTRUS	LATE META-OESTRUS
% OF MICE IN THIS STATE WHICH BECAME INFECTED.	57	78	59	31	50	40
TOTAL NUMBER OF MICE USED	62	16	73	20	2	7
% OF MICE IN EACH STATE AT TIME OF WASH-OUT WHICH WERE INFECTED	27	18	39	73	80	50
TOTAL NUMBER OF MICE USED	11	22	16	45	10	49

The table shows the number of mice tested that were at each stage of the oestrus cycle, and the percentage of those which were positive for trichomonads present in the vagina. Th first section gives the number of mice found to be at each stage of the cycle on infection and the percentage of those which gained infections which lasted at least 7 days. The second section of the table shows the number of mice tested which were at each stage of the oestrus cycle when washed-out at 7 days post-infection and the percentage of each of these which were infected at that time. The stages of the cycle are as defined in Figure 3. Key SC, square, cornified cells present in smear; RN, round, nucleated cells present in smear; P, polymorphonuclear leucocytes present in smear.

explanation of the results is that the number of trichomonads in the vagina varies with stage of the oestrus cycle and that a low number of trichomonads would not be detected and so scored as negative for infection. Alternatively, the presence of trichomonads may affect the state of the vaginal epithelium and so the apparent stage of the oestrus cycle.

3.1.3.4. The Effects of Oestradiol Treatment of the Mice

In the standard infection procedure, oestriodiol-17B-cypionate was used at a dose of 40mg (kg body weight)$^{-1}$, given as a suspension in 0.2ml corn oil and administered subcutaneously two days prior to infection. The effects of varying this hormone treatment upon the state of the vagina were tested and the results are shown in Table 11. Mice given the standard dose, administered half intraperitoneally and half subcutaneously were brought into oestrus in two days with oestrus lasting 2-3 days. Intraperitoneal dosing only, however, brought the mice into oestrus sooner. A double dose caused the mice to remain in oestrus for longer and mice injected with a half dose took 3 days to come into oestrus, although oestrus lasted for 3 days once reached.

A group of three spayed mice was hormone treated in the standard way and the conditions of the vaginal epithelium monitored. They were not brought into oestrus within 5 days. Non-treated spayed mice did not reach oestrus during this period either. A double dose of oestradiol (i.e. 80mg kg^{-1}) was then given in the same way but again neither the treated nor untreated mice came into oestrus within 5 days.

Another group of 3 spayed mice was smeared daily for 2 weeks and the appearance of vaginal smears noted (Table 12a). Two of the mice were hormone treated in the standard way on Day 0 and all mice were treated on Day 9. None of the mice appeared to reach oestrus at any

Table 11. The Effects of Oestradiol Treatment on the Condition of the Mouse Vagina

MOUSE	HORMONE TREATMENT		OESTROUS DAY					
	mg kg^{-1}	INJECTIONS	0	1	2	3	4	5
A	40	0.1ml ip + 0.1ml SC	x	x	✓	✓	✓	x
B	40	0.1ml ip + 0.1ml SC	x	x	✓	✓	x	x
C	40	0.2ml SC	x	x	✓	✓	x	x
D	40	0.2ml IP	✓	✓	✓	x	x	x
E	80	0.2ml ip + 0.2ml SC	x	x	✓	✓	✓	✓
F	20	0.05ml ip + 0.05ml SC	x	x	x	✓	✓	✓

Oestradiol-17β-cypionate was the hormone used, given as a suspension in 0.2ml corn oil. Key: ✓, oestrous condition; x, non-oestrous condition, SC, subcutaneous injection; IP, intraperitoneal injection.

Table 12a. The Composition of Vaginal Smears from Spayed Mice after Oestradiol Treatment

Day	Cell Types Observed in Vaginal Smears:		
	MOUSE A	MOUSE B	MOUSE C
0	- RN P*	- RN P*	SC RN P
1	SC RN P	SC RN P	SC RN P
2	SC RN P	- RN P	SC RN P
3	SC RN P	- RN P	SC RN P
4	SC RN P	- RN P	SC RN P
5	- RN P	- RN P	SC RN P
6	- RN -	- RN P	SC RN P
7	- RN -	- RN P	SC RN P
8	- RN -	- RN P	- RN P
9	- RN P*	- RN P*	SC RN P*
10	SC RN P	- RN P	- RN P
11	- RN P	- RN P	SC RN P
12	- RN P	- RN P	SC RN P
13	- RN P	- RN P	SC - P
14	- RN -	- RN P	SC RN P

Key; *, mice treated subcutaneously with oestradiol-17β-cypionate at 40mg kg body weight^{-1}; SC, square, cornified cells; RN, round nucleated cells; P, polymorphonuclear leukocytes.

time and it was impossible to detect any change in the composition of vaginal smears that related to the hormone treatment.

A similar experiment was performed on mice inoculated intravaginally with *T. vaginalis* 39 *in vivo*. All mice were hormone-treated in the standard way on Day 0, inoculated with parasites on Day 2 and washed out on Day 6. In addition vaginal smears were made daily. The results (shown in Table 12b) were rather variable and there were no clear cut correlations apparent. Surprisingly, the number of days for a mouse to reach oestrus after hormone-treatment varied; mice D, E and F being in oestrus by Day 2, the day of infection, whereas other mice did not come into oestrus until Day 4 or 5. Nevertheless, the appearance of the vaginal smear on the day of infection did not directly correlate with whether or not a mouse was infected on Day 6 post-infection, neither did the length of time the mouse remained in oestrus after being infected. Also the appearance of the vaginal smear at the time of wash-out appeared to have no bearing on whether the mouse was found to be positive or negative for infection at that time. Although the results apparently do not fit in with those of Table 10, they are not inconsistent for as stated previously, the correlation was far from absolute. It was only possible to use a small number of mice in this experiment and this limited the significance of the results. In addition, the daily manipulations of the vagina may have interfered with the infection. Despite being unable to achieve permanent oestrus in mice by hormone treatment, it was evident that oestradiol treatment was necessary for infection as no trichomonads were ever observed in mice infected without hormone treatment.

3.1.3.5. The Use of Vaginal Plugs

In the early experiments the standard procedure was to plug the vagina of each mouse with a small ball of Sterispon immediately after

Table 12b. Vaginal Smears from Oestradiol-Treated, Intravaginally Infected Mice

	DAY	CELL TYPES OBSERVED IN VAGINAL SMEARS:					
		MOUSE A	MOUSE B	MOUSE C	MOUSE D	MOUSE E	MOUSE F
OESTRADIOL TREATMENT	0						
	1						
INFECTION	2	SC RN - -	RN -	SC - -	SC - -	SC - - -	RN -
	3	SC RN -	SC RN -	SC - -	SC - -	SC - -	SC RN -
	4	SC - -	SC RN -	SC - -	SC - -	SC - P	SC - -
	5	SC - P	SC - -	SC - -	SC - -	SC RN P	SC - -
WASH-OUT	6	SC - P	SC - P	SC - P	SC - P	SC RN P	SC - P
	7	SC RN P	SC RN P	SC RN P	SC - P	SC RN P	SC RN P
	8	SC RN P	SC RN P	SC RN P	SC RN P	SC RN P	SC RN P
	9	SC RN P	SC RN P	SC RN P	SC RN P	SC RN P	SC RN P
	10	- RN P	- RN P	- RN P	SC RN P	SC RN P	- RN P
6 DAY WASH-OUT		⊖	+	+	⊖	+	+
14 DAY WASH-OUT		⊖	⊖	⊖	⊖	⊖	⊖

Key: SC, square, cornified cells; RN, round, nucleated cells; P, polymorphonuclear leucocytes; +, trichomonads present; ⊖, trichomonads not present.

inoculation with the parasites. Subsequently, it was shown by Miss Dianne Markham, working in the same laboratory, that this procedure did not enhance the infection rate, and was hence abandoned.

3.1.3.6. The Course of Intravaginal Infections of *T. vaginalis* in Mice

Vaginal wash-outs were taken from intravaginally-infected mice throughout the course of infection. The growth of trichomonads in the wash-out cultures was then monitored by counting cell numbers using a haemocytometer and the figures used to calculate the approximate number of trichomonads initially present in each culture. The number of parasites seen in a wet smear of the vaginal wash-out was also recorded to determine how good a guide this was to the number of trichomonads present in the vagina. Representative results are shown in Table 13. They suggest that the number of trichomonads present in the vagina may fluctuate during an infection. The figures for wet smears indicate that this is a poor method of estimating numbers of parasites in the vagina.

[illegible] infection in three [illegible] and C. The number [illegible] monads observed in wet [illegible] in 5 fields.

3.1.3.7. Diagnosis of Intravaginal Infection by Detection of Parasite Proteinases in Vaginal Wash-outs

Vaginal wash-outs from mice infected with trichomonads were collected and 0.01ml of each was cultured using standard procedures to detect the presence of viable trichomonads. The other 0.01ml was subjected to electrophoresis on a geletin gel to search for the presence of parasite proteinases. This was carried out by Barbara Lockwood using published methods (Lockwood *et al.*, 1987). Of the 62 isolates tested, however, proteinase activity on gelatin gels was detected in only 2 even though 50 were found to contain trichomonads

Table 13. The Course of Intravaginal Infections

READING		DAYS POST-INFECTION	INITIAL NUMBER ml^{-1} IN CULTURE*	NUMBER PER FIELD (x100 objective) IN WET SMEAR
A	1	2	2.5×10^4	2
	2	5	2.8×10^4	3
	3	9	2.3×10^4	1
	4	14	1.1×10^4	0
	5	41	3.0×10^4	0
	6	56	5.2×10^3	0
	7	79	0	0
B	1	7	1.5×10^4	0
	2	12	1.1×10^4	6
	3	20	8.7×10^4	0
	4	42	-	0
	5	56	1.2×10^4	1
	6	79	0	0
C	1	1	2.0×10^4	1
	2	13	1.2×10^5	6
	3	20	3.7×10^4	9

The table shows the course of intravaginal infection in three different mice: A, B, and C. The number of trichomonads observed in wet smears taken on the days post-infection indicated is given as the mean number in 5 fields.

*Estimation of initial number in culture by extrapolation of cell numbers present during log phase growth.

as revealed by positive cultures. The two wash-outs positive for proteinase activity were from mice with usually high infections, many trichomonads being observed in wet smears of the vaginal wash-out.

3.1.4. Investigation of the Methods used to Determine the Sensitivity of Trichomonads to Metronidazole

Trichomonas lines IR78, Fall River, Boston and Albany, all reported to be metronidazole-resistant isolates, were tested *in vitro* for metronidazole sensitivity. The minimum lethal concentrations (MLC) obtained were somewhat lower than those reported by previous authors (Table 14). It was thought that this may be due to methodological differences, and hence variations on the original method were tested. In particular it was thought that the high levels of ascorbate in MDM could be affecting the sensitivity to the drug, especially as resistance is dependent upon the presence of O_2. Little difference in the sensitivity to metronidazole was found using any of these variations. Nevertheless, the methods used demonstrated differences in sensitivity to this drug of sensitive and 'resistant' lines and was subsequently used to study fresh isolates (Section 3.4.1).

The results (Table 14) indicate that *T. vaginalis* Boston is no longer resistant to metronidazole but has a similar sensitivity as *T. vaginalis* G3.

3.2. COMPARISON OF BEHAVIOUR OF FRESH ISOLATES OF *T. vaginalis* IN THE EXPERIMENTAL MODELS

70 fresh isolates were collected from Glasgow Royal Infirmary Department of Genito-urinary Medicine, over a period of one year (November 1983 - November 1984). Of these, 23 proved viable and usable. Some information on the clinical symptoms relating to each isolate was obtained, unfortunately it proved impossible to use this in any meaningful way as the records were fragmentary and incomplete.

Table 14. Metronidazole Sensitivity of Isolates *in vitro* in Different Media

ISOLATE	MEDIUM	MLC ($\mu g\ ml^{-1}$)		PREVIOUS AUTHORS (AEROBIC)
		AEROBIC	ANAEROBIC	
T. vaginalis G3	MDM	2.0,5.0,1.0	2.0,1.0,1.0	
	GTM	1.0	1.0	
	MDM-ASCORBATE	5.0	1.0	
T. vaginalis Boston	MDM	2.0	2.0	75 (Roe, 1977)
T. vaginalis Albany	MDM	25.0	0.5	
	GTM	25.0	1.0	
T. vaginalis Fall River	MDM	50.0	2.0	250 (Lindmark & Muller, 1976)
	GTM	50.0	-	
T. vaginalis IR 78	MDM	25.0,50.0,50.0	2.0,1.0	150 (Muller, 1982)
	GTM	50.0	-	
	MDM-ASCORBATE	50.0	1.0	
T.foetus F2	MDM	2.0,1.0	1.0,1.0	

Key: MDM, modified Diamond's medium; GTM, Modified trichomonas medium (Clackson and Coombs, 1983)
MDM-ASCORBATE, modified Diamond's medium without addition of ascorbate

Table 15. Summary of Characteristics of Different Trichomonads and *T. vaginalis* Lines

ISOLATE	GENERATION TIME *in vitro* G (hours)	SUBCUTANEOUS GROWTH IN MICE (hours)[a]	INTRAVAGINAL GROWTH IN MICE (% INFECTED AFTER 7 DAYS)	EFFECT ON MAMMALIAN CELL LINES (MINIMUM RATIO)[b]	PROTEINASE[c] ACTIVITY 1*	PROTEINASE[c] ACTIVITY 2*	O.D.C.[d] ACTIVITY *
T.vaginalis G3	13.0 ± 2.1	76 ± 16.5	30	200:1	49.4 ± 4.5	10.2 ± 1.5	1.107 ± 0.446
39	15.9 ± 3.3	71 ± 1.0	10	200:1	43.2 ± 10.5	4.1 ± 1.1	1.465 ± 0.772
45	12.9 ± 4.3	85 ± 0.5	20	-	48.6 ± 5.1	5.0 ± 1.7	1.040 ± 0.330
46	9.7 ± 1.6	70 ± 4.5	60	-	34.2 ± 4.2	5.3 ± 1.6	0.845 ± 0.338
55	9.9 ± 1.8	68 ± 18.0	20	-	36.8 ± 5.3	4.9 ± 1.7	1.677 ± 0.274
45733	10.5 ± 1.7	80 ± 10.0	0	200:1	47.4 ± 7.5	3.2 ± 2.4	1.456 ± 0.663
61	10.6 ± 1.1	18 ± 0.5	17	200:1	49.4 ± 4.0	4.9 ± 2.7	-
6950 Male	9.3 ± 1.4	56 ± 16.0	67	200:1	16.2 ± 5.5	3.7 ± 1.8	0.340 ± 0.142
2755	13.9 ± 2.4	78 ± 0.5	67	200:1	47.6 ± 2.5	5.6 ± 1.1	1.376 ± 0.382
IR78	-	82 ± 1.5	-	10:1	38.6 ± 6.6	3.9 ± 3.7	0.853 ± 0.168
F1297	-	-	-	-	50.6 ± 5.3	5.1 ± 2.3	2.325 ± 0.698
64	-	31 ± 4.0	0	200:1	50.8 ± 12.0	5.6 ± 2.6	2.465 ± 0.300
Fall River	-	-	-	5:1			
51	-	25 ± 4.5	17	-			
63	-	42 ± 5.5	100	-			
65	-	43 ± 15.5	30	-			
52	-	87 ± 10.5	-	-			
66	-	-	67	-			
36130	-	-	30	-			
T.foetus F2	19.7 ± 2.1	00	17	-			
T.batrachorum	25.5 ± 2.2	-	-	-			
P.hominis	-	00	0	-			

Key: a, time (hours) for lesion volumes to reach $50mm^3$; b, minimum ratio at which trichomonads had an effect on HeLa cells; c, results are given in units of g. substrate hydrolysed min^{-1} $(mg.protein)^{-1}$ and are the mean and standard deviation from four independent determinations; d, n mol CO_2 evolved min^{-1} $(mg.protein)^{-1}$, results are the means and standard deviation of four independent determinations. *, courtesy of Drs Lockwood and North. 1, NPAase; 2, A2Case. Generation times: mean (± S.D.) of 3 cultures; subcutaneous growth: means (± S.D.) of 3 mice. ODC, ornithine decarboxylase.

3.2.1. *In vitro* Growth Kinetics

Immediately after collection from the hospital, each sample obtained was subpassaged into fresh MDM and its growth kinetics monitored. In particular the apparent doubling time of the cells in log phase of growth was determined. The results obtained are shown in Table 15. The samples shown represent the range from those studied.

3.2.2. Virulence as Determined Using the *In Vitro* and *In Vivo* Models

3.2.2.1. Effect on Mammalian Cell Lines

The effect of several isolates on myeloma cells and on Hela cells was tested. The results of one experiment using Hela cells are shown in Table 16. This is representative of the many experiments carried out. The results suggest that there are large differences in the cytopathogenicity of different isolates. At one extreme, *T. foetus* F2 had no apparent effect on the mammalian cells except at a ratio of 1:1, at which density *T. foetus* F2 in monoculture in EMEM also overgrew. Isolate *T. vaginalis* 45 also had no effect at any of the ratios tested whereas *T. vaginalis* lines 6950 Male, 45733, 2755 and 39 had a cytotoxic effect even at as low a ratio of 200/1, mammalian cells/trichmonads.

Further experiments were carried out, and the minimum ratio of trichomonads to mammalian cells at which the trichomonads produced a cytotoxic effect on the mammalian cells was determined (Table 17).

3.2.2.2. Growth of Subcutaneous Lesions

Before cultures of new isolates collected from the Royal Infirmary had been sub-passaged for the third time, they were injected subcutaneously into the flanks of Balb/c mice and the rate of growth of the lesions they produced was measured. The growth of the fastest and slowest growing lesions is shown in Figure 14. The fastest growing

Table 16. Effect of Trichomonad Isolates on Mammalian Cells *in vitro*

RATIO OF MAMMALIAN CELLS TO TRICHOMONADS	Time (hours) for cells to disappear:																							
	1:1				25:1				50:1				100:1				200:1				400:1			
ISOLATE	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
G3					48	48	93*	00	48	00	68	00	48	00	22	00	48	00	48	00	48	00	48	0
F2	93*	48	93*	00	48	00	48	00	48	00	48	00	48	00	48	00	48	00	48	00	48	00	22	0
6950 Male					124*	22	76*	00	68	00	68	00	93	48	93*	00	48	68	124*	00	48	00	93	0
45733					124*	48	93*	00	124*	48	100*	00	68	68	124*	00	68	68	124*	00	48	00	76	0
2755					68	48	93*	00	68	48	124*	00	68	68	124*	00	68	68	124*	00	48	00	93	0
39					68	48	93*	00	68	48	124*	00	48	93	124*	00	68	93	124*	00	48	00	93	0
39 *in vitro*					48	68	93*	00	48	00	68	00	48	00	68	00	48	00	68	00	22	00	48	0
45					48	00	68	00	48	00	68	00	48	00	68	00	48	00	68	00	48	00	68	0
64					124*	22	68*	00	48	68	100*	00	48	00	68	00	48	00	68	00	48	00	68	0

Key: A, time point for trichomonads in monoculture to die; B, time point for mammalian cells in mixed culture to die; C, time point for trichomonads in mixed culture to die; D, time point for mammalian cells in monoculture to die; ▨, cytopathogenic effect observed but trichomonads in monoculture overgrew; ▧, cytopathogenic effect observed; □, no cytopathogenic effect; *, trichomonads overgrew.
The experiment was performed using the wells of tissue culture plates.

Table 17. Effect of Trichomonad Lines on Mammalian Cells

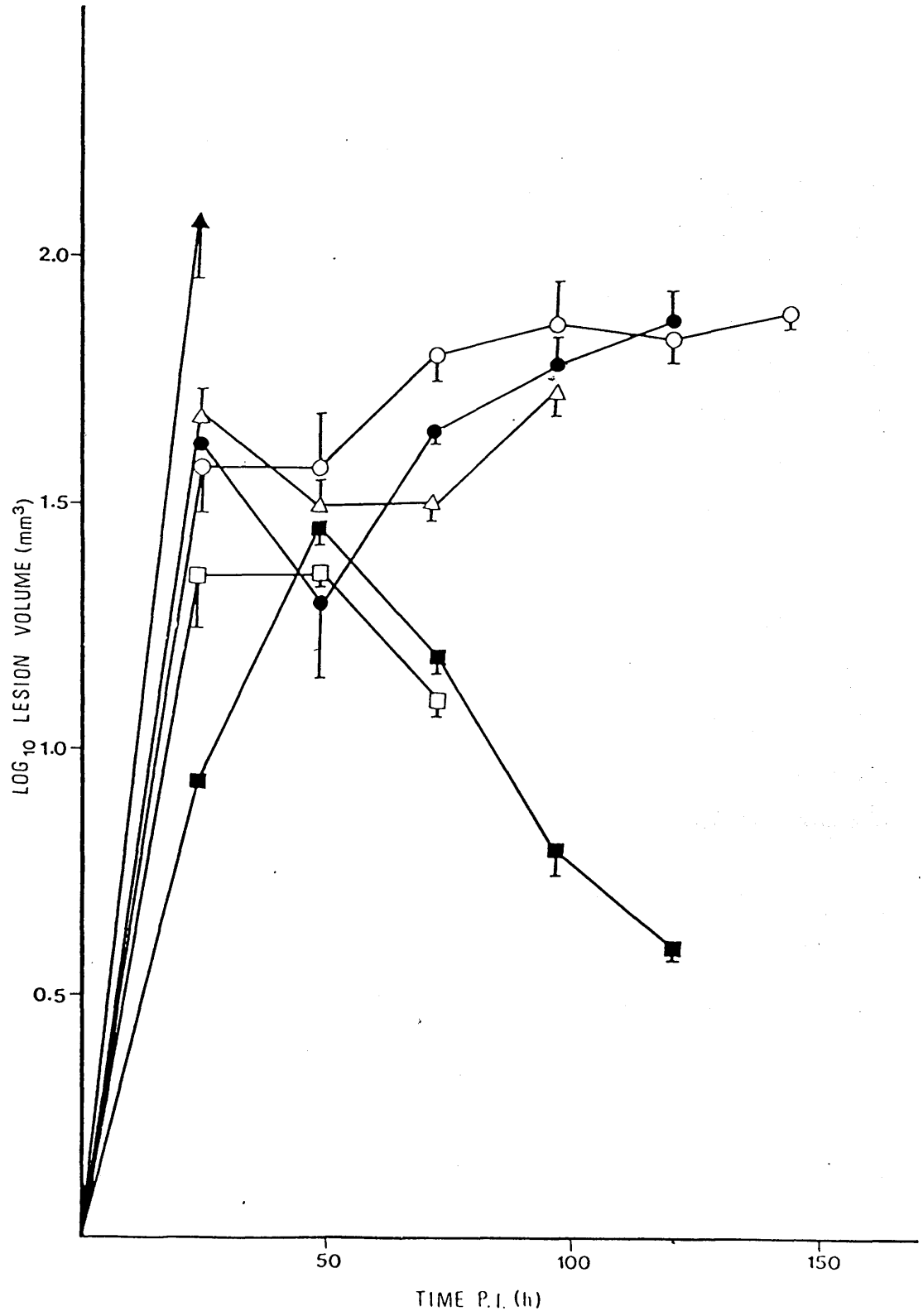
ISOLATE	MAMMALIAN CELL TYPE	MINIMUM DENSITY AT WHICH TRICHOMONADS OVERGREW WHEN IN MONOCULTURE (a)		MINIMUM RATIO OF MAMMALIAN CELLS: TRICHOMONADS WHICH HAD AN EFFECT	n
G3	MYELOMA	2.5×10^5	(2:1)	5:1	2
	HELA	5.0×10^3	(200:1)	<200:1	3
F2	MYELOMA	N.E.		N.E.	2
	HELA	10^6	(1:1)	N.E.	2
39	HELA	2.0×10^4	(50:1)	200:1	2
39 *in vitro*	MYELOMA	2.5×10^5	(2:1)	5:1	2
	HELA	2.0×10^4	(50:1)	200:1	1
39 *in vivo*	MYELOMA	2.5×10^5	(2:1)	10:1	2
	HELA	5.0×10^4	(20:1)	50:1	3
6950 Male	MYELOMA	2.5×10^5	(2:1)	10:1	2
	HELA	4.0×10^4	(25:1)	200:1	2
45733	MYELOMA	N.E		N.E.	2
	HELA	2.0×10^4	(50:1)	200:1	2
P.hominis	HELA	N.E.		N.E.	1
64	HELA	$<4.0 \times 10^4$	(<25:1)	200:1	2
61	HELA	$<2.0 \times 10^4$	(<50:1)	200:1	1
2755	HELA	$<4.0 \times 10^4$	(<25:1)	200:1	1
2755 *in vivo*	HELA	$<2.0 \times 10^4$	(<50:1)	200:1	2
45	HELA	N.E.		N.E.	2
IR78	HELA	$<2.0 \times 10^5$	(<5:1)	10:1	1
Fall River	HELA	$<2.0 \times 10^5$	(<5:1)	5:1	1

The experiments were performed in multiwell plates. Key: (a), the ratio this would give in mixed culture is given in parentheses; <, this is lowest ratio observed at which trichomonads grew although lower densities were not tested; N.E, no effect observed/no growth of trichomonads; n, number of experiments performed.

All isolates were T. vaginalis except for F_2 (Tritrichomonas foetus) and P. hominis.

Figure 14. Growth of Subcutaneous Abscesses caused by Various Trichomonad Isolates

Groups of 3 Balb/c, female mice, aged 2 months were used and the means (± S.D) of lesion volumes plotted. Key: ●, *T.vaginalis* 61; ○, *T. vaginalis* G3; ■, *P. hominis*; □, *T. foetus* F2; ▲, *T. vaginalis* 45733; △, *T. vaginalis* IR78.


2.0
1.5
1.0
0.5
LOG 10 LESION VOLUME (mm3)
50
100
150
TIME P.I. (h)

lesions were produced by *T. vaginalis* 61, other *T. vaginalis* isolates grew at an intermediate rate and the slowest growing lesions were produced by *P. hominis* and *T. foetus* F2. The results for the isolates tested are summarised in Table 15. This shows the time to reach $50mm^3$, a value chosen as most lesions reached this volume and yet none ruptured at a lower volume. The results show that there were fairly large differences in the rates at which subcutaneous abscesses were produced by different *T. vaginalis* isolates.

3.2.2.3. Growth of Isolates Intravaginally in Mice

As well as being inoculated into mice subcutaneously, similarly fresh isolates were also inoculated intravaginally into Balb/c mice using the standard protocol for intravaginal infections. The results are given in Table 18. Again there were large differences between the isolates with respect to the proportion of mice that became infected and how long the mice remained infected. For example, isolate *T. vaginalis* 66 infected 84% of mice initially, 67% were still infected after 28 days and at least one mouse remained infected for at least 63 days. In contrast, isolates 64 and 45733 failed to infect any mice for as long as 3 days.

3.2.3. Resistance to Metronidazole

Isolates of *T. vaginalis* from patients were sent to our laboratory by clinicians from around Britain. these were samples from patients suffering from trichomoniasis, who had proved difficult to cure with the standard metronidazole treatment. The parasites were sub-passaged serially in MDM with antibiotics until an axenic culture was obtained and the sensitivity of this to metronidazole *in vitro* was then measured as soon as possible. *T. vaginalis* lines IR78 and G3 were used as the standard 'resistant' and 'sensitive' lines for comparative

Table 18. Growth of Trichomonad Lines Intravaginally in Mice

LINE	NO. OF MICE USED	% STILL INFECTED AFTER:				MAXIMUM NO. DAYS INFECTED
		3 DAYS	7 DAYS	14 DAYS	28 DAYS	
T vaginalis						
G3	6	30	30	0	0	10
39	10	50	10	0	0	9
45	10	50	20	0	0	7
46	12	58	58	58	25	55
51	6	50	17	17	17	33
55	10	50	20	0	0	7
61	12	34	17	17	8	52
63	6	100	100	0	0	7
64	6	0	0	0	0	0
65	6	30	30	0	0	8
66	6	84	67	50	67	63
6950 Male	6	67	67	67	17	56
45733	5	0	0	0	0	0
2755	6	0	67	67	50	30
36130	6	30	30	30	0	14
T.foetus	6	17	17	0	0	7
P.hominis	6	0	0	0	0	0

Groups of Balb/c female mice aged approximately 2.5 months were used.

purposes. The results are summarized in Table 19. The results show that under aerobic conditions twelve of the isolates were less sensitive to metronidazole than our laboratory strain, *T. vaginalis* G3, and that nine were as resistant as the standard resistant strain, *T. vaginalis* IR78. Drug resistance was not apparent under anaerobic conditions.

3.2.4. Biochemical Differences between Fresh Isolates

Fresh isolates were tested biochemically for ornithine decarboxylase activity by Dr M. North of Stirling University, using published methods (North *et al.*, 1986). The proteinase activity of fresh isolates was also measured at Stirling University by Miss B. Lockwood also using published methods (Lockwood *et al.*, 1987). The results are shown in Table 15.

3.3. THE EFFECTS OF PROLONGED *IN VITRO* AND *IN VIVO* SUBPASSAGE AND CRYOPRESERVATION ON TRICHOMONAD BEHAVIOUR IN THE EXPERIMENTAL MODELS

3.3.1. Growth Rate *in vitro*

One isolate of *T. vaginalis* was continually sub-passaged intravaginally in mice for 6 months and also continually sub-passaged *in vitro* over the same period. Subsequently, the apparent generation time for growth axenically *in vitro* for the two cultures were again determined. It was found that there was no significant difference between the generation time of the original line and those of the lines sub-passaged for 6 months either *in vitro* or *in vivo* (Table 20).

3.3.2. Interaction *in vitro* with Mammalian Cell Lines

T. vaginalis 39 and *T. vaginalis* 2755 were tested for their effects against mammalian cells soon after being obtained. The results are summarized in Table 21. *T. vaginalis* 39 was tested again after

Table 19. Metronidazole-Sensitivity of *Trichomonas vaginalis* Isolates

MLC ($\mu g ml^{-1}$)	AEROBIC			ANAEROBIC		
	ISOLATES	G3	IR78	ISOLATES	G3	IR78
<100	0	0	0	0	0	0
100	0	0	2	0	0	0
50/100	1	0	2	0	0	0
50	2	0	11	0	0	0
25/50	2	0	3	0	0	0
25	4	0	4	0	0	0
10/25	1	0	0	0	0	3
10	0	0	0	0	0	0
5/10	2	0	0	0	0	0
5	5	12	0	4	5	2
2/5	1	1	0	0	0	0
2	2	1	0	3	0	2
1/2	1	0	0	2	0	0
1	1	8	0	13	17	15
Total numbers:	22	22	22	22	22	22
% resistant* :	55	-	100	0	-	14

*, % resistant - resistance defined as isolates having a higher MLC than the standard sensitive strain, *T. vaginalis* G3. Experiments were performed in the wells of multiwell plates.

Each of the isolates was tested along with the T. vaginalis G3 and T. vaginalis IR 78 for comparison.

Table 20. The Effects of Prolonged *in vitro* and *in vivo* Sub-passage on Trichomonad Generation Time

ISOLATE	GENERATION TIME (HOURS)
T. vaginalis 39	15.9 ± 3.3
T. vaginalis 39 *in vitro*	17.1 ± 1.8
T. vaginalis 39 *in vivo*	18.3 ± 2.4

Isolate 39 *in vitro* had been sub-passaged *in vitro* for 7 months, isolate 39 *in vivo* had been sub-passaged *in vivo* intravaginally in mice for 7 months and *T. vaginalis* 39 was freshly isolated from the patient and had not been sub-passaged more than three times. A students t test was performed and it was found that the mean generation times were not significantly different. (n=3, mean ± S.D.)

being sub-passaged *in vitro* for 9 months (line 39 *in vitro*) and also after being sub-passaged intravaginally in mice for 7 months (line 39 *in vivo*). *T. vaginalis* 2755 was similarly tested after being sub-passaged *in vivo* for 6 months. The results (Table 21) suggest that *in vitro* but not *in vivo* sub-passage of this line of *T. vaginalis* reduced its cytopathogenicity towards Hela cells in the *in vitro* test used. *T. vaginalis* 2755, however, appeared to be unaffected in this way.

3.3.3. The Production of Subcutaneous Abscesses in Mice

T. vaginalis 39 which had been sub-passaged intravaginally in mice for 8 months produced lesions which initially grew more rapidly than the lesions produced by the freshly isolated *T. vaginalis* 39 (Figure 15) although lesion growth slowed down more quickly after the first day post-infection. *T. vaginalis* 39 *in vitro* behaved similarly to *T. vaginalis* 39 *in vivo*, indicating that the change was not due to adaptation to mice. Four isolates were injected subcutaneously and lesion growth monitored at various intervals. Between experiments all trichomonad lines were sub-passaged *in vitro* except for *T. vaginalis* 39 *in vivo* which was sub-passaged intravaginally in mice. The results are summarized in Figure 16. There was a reasonable amount of variation in lesion growth rate with time for all four isolates, although in general growth rate appeared to become more rapid with time.

3.3.4. Growth Intravaginally in Mice

The relative infectivity to mice of four lines via the intravaginal route after various periods of *in vitro* and *in vivo* sub-passage were measured. The results are given in Tables 22, 23, 24, 25 and Figure 17.

Table 21. Interaction of Sub-passaged Isolates with Mammalian Cell Lines *in vitro*

ISOLATE	MINIMUM DENSITY (a)	MINIMUM RATIO (b)	n (c)
39	4×10^4 (50:1)	200:1	2
39 *in vitro*	4×10^4 (50:1)	200:1	1
39 *in vivo*	10^5 (20:1)	50:1	3
2755	8×10^4 (25:1)	200:1	1
2755 *in vivo*	4×10^4 (50:1)	200:1	2

Key: 39, *T. vaginalis* 39, fresh isolate; 39 *in vitro*, *Trichomonas vaginalis* 39 after 9 months sub-passaging *in vitro*; 39 *in vivo*, *T. vaginalis* 39 after sub-passaging intravaginally between mice for 7 months; 2755, *T. vaginalis* 2755, fresh isolate; 2755 *in vivo*, *T. vaginalis* 2755 after being sub-passaged intravaginally between mice for 6 months; (a), minimum density at which trichomonads grew in monoculture, ratio of Hela cells/trichomonads this corresponds to is given in parentheses; (b), minimum ratio of Hela cells/trichomonads at which cytopathogenicity was observed; (c), n represents the number of each experiment performed.

Figure 15. The Production of Subcutaneous Abscesses in Mice by *T. vaginalis* 39 after *in vitro* and *in vivo* Subpassage

Key: ●— , *T. vaginalis* 39, fresh isolate; ●- - , *T. vaginalis* 39 *in vivo*, *T. vaginalis* 39 after subpassage intravaginally in mice for 7 months; ○- - , *T. vaginalis* 39 *in vitro*, *T. vaginalis* 39 aftr subpassage *in vitro* for 9 months. The mean (± S.D.) volume of 3 lesions is shown.

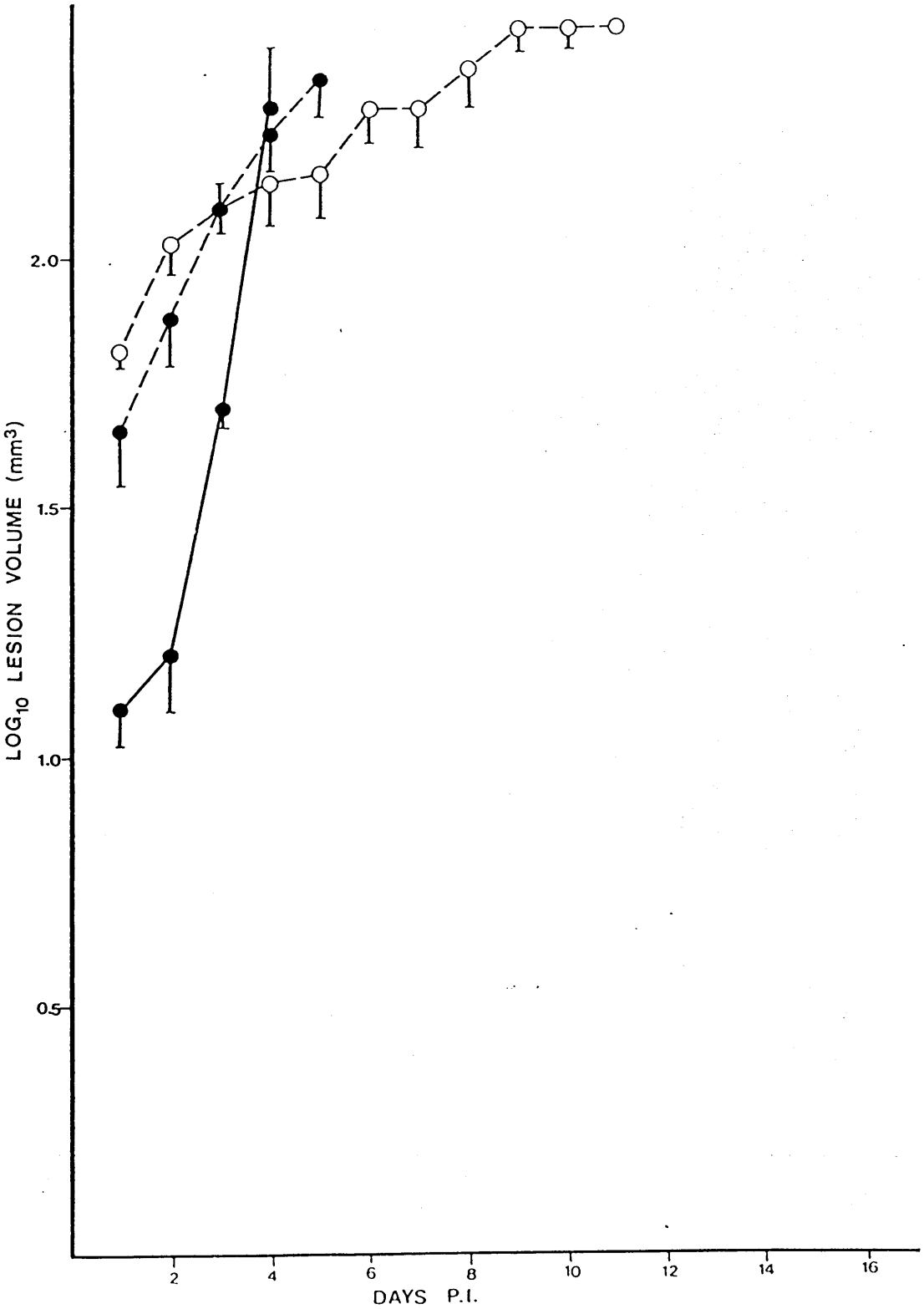
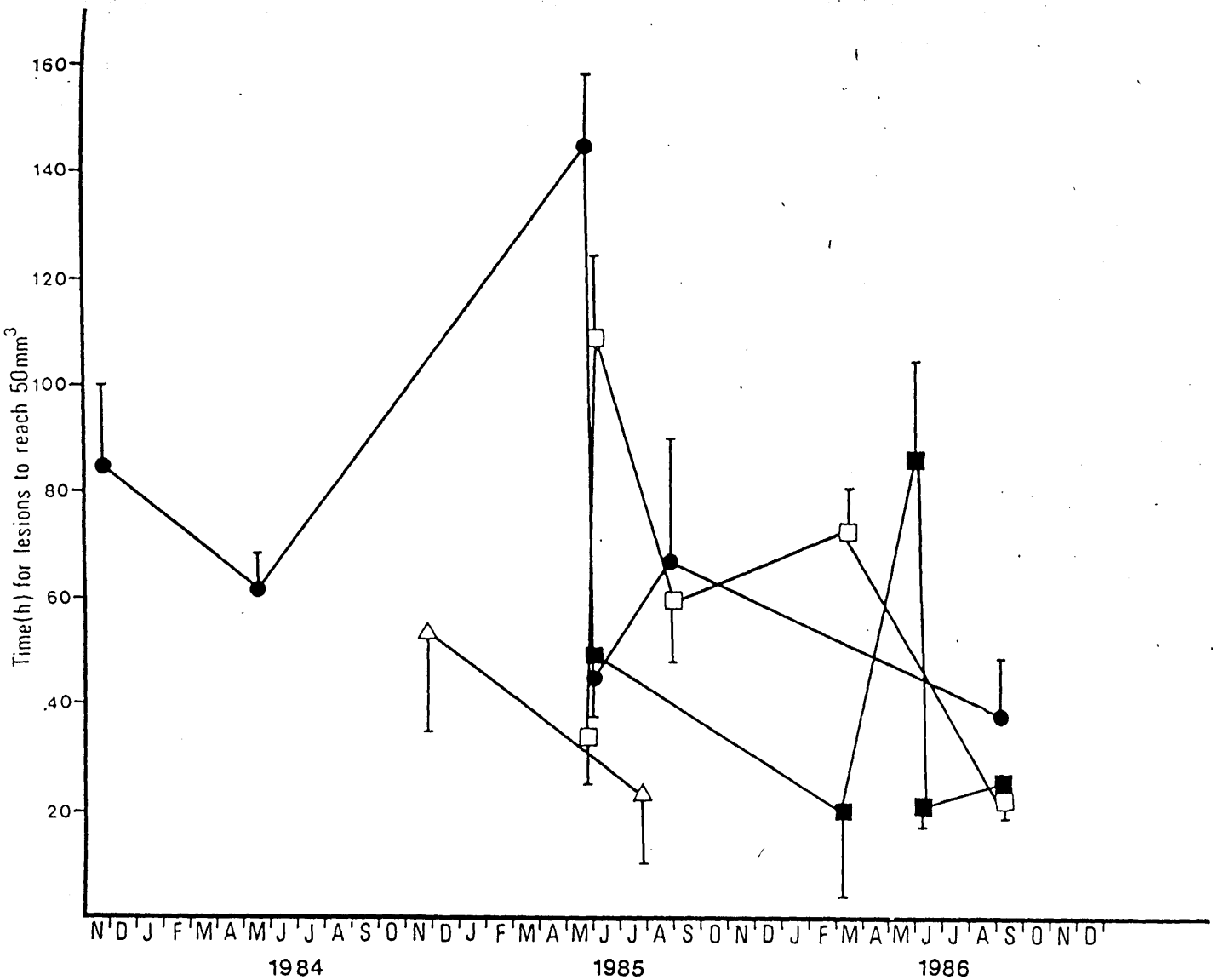

2.0
1.5
1.0
0.5
LOG10 LESION VOLUME (mm3)
2
4
6
8
10
12
14
16
DAYS P.I.

Figure 16. Variation in Growth of Subcutaneous Abscesses with Time

Key: ■, *T. vaginalis* 6950 Male; □, *T. vaginalis* IR78. These trichomonad lines were subpassaged *in vitro* between experiments. △, *T. vaginalis* 39 *in vivo*, this line was subpassaged intravaginally in mice between experiments. The means (± S.D.) from groups of three mice shown

(T.vaginalis G3 : ● .)


Time(h) for lesions to reach 50mm3
160
140
120
100
80
60
40
20
N D J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D
1984
1985
1986

When *T. vaginalis* 39 was sub-passaged *in vitro* it appeared gradually to lose its infectivity to mice (Table 22), whereas the infectivity of *T. vaginalis* 39 *in vivo* remained largely unchanged (Table 23). *T. vaginalis* 2755 *in vitro* and *T. vaginalis* 6950 Male also retained their infectivity toward mice (Tables 24 and 25). In all cases, however, the infectivity fluctuated with time.

Figure 18 shows the change in rate of infectivity with time of the three isolates that were sub-passaged *in vivo*. There appeared to be a correlation between the variation that occured with the three isolates.

3.3.5. Effects of Cryopreservation

Freezing in liquid nitrogen in the presence of the cryoprotectant dimethylsulphoxide (DMSO) at 5% appeared to have no effect on the behaviour of the isolates in any of the models used and was, therefore, used as the standard method of storing fresh isolates.

3.4. METRONIDAZOLE SENSITIVITY OF TRICHOMONADS

3.4.1. The Sensitivity of New Isolates of *T. vaginalis* to Metronidazole *in vitro*

The metronidazole sensitivity of many of the new isolates collected was measured shortly after isolation (Table 19). Many of these were found to be relatively resistant to metronidazole and the isolate *T. vaginalis* 2755 had a minimum lethal concentration (MLC) of $50ugml^{-1}$ under aerobic conditions, which is comparable to that of the standard resistant strain IR78. This isolate infected mice intravaginally and was sub-passaged in this way so that it could be used for further *in vivo* studies on metronidazole sensitivity.

Table 22. Variation with Time of Intravaginal Infectivity of *T. vaginalis* 39 Sub-passaged *in vitro*

WEEKS POST-ISOLATION	NUMBER OF MICE	% MICE INFECTED AFTER:				MAXIMUM[a] NUMBER OF DAYS INFECTED
		3 DAYS	7 DAYS	14 DAY	28 DAYS	
0	14	50	43	28	21	65
8	6	50	50	33	17	37
15	6	33	17	17	0	19
20	6	0	0	0	0	0
28	6	50	50	33	17	46
34	6	17	17	0	0	7
38	6	67	67	0	0	7
43	6	0	0	0	0	0
63	6	17	0	0	0	3

a, at least one mouse remained infected for this number of days

Table 23. Variation with Time of Intravaginal Infectivity of *T. vaginalis* 39 Sub-passaged *in vivo*

WEEKS POST-ISOLATION	NUMBER OF MICE	% MICE INFECTED AFTER:				MAXIMUM[a] NUMBER OF DAYS INFECTED
		3 DAYS	7 DAYS	14 DAY	28 DAYS	
0	14	50	43	28	21	65
1	17	76	59	53	53	92
3	9	22	22	22	0	20
8	6	17	17	17	33	37
11	6	50	50	50	50	33
15	6	50	33	33	33	52
19	6	17	17	17	17	32
22	6	33	33	33	33	40
28	6	67	67	33	17	46
31	6	0	0	0	0	0
47	6	33	33	17	17	38
35	15	53	27	13	7	45
39	5	40	20	20	20	45
44	5	0	0	0	0	0
54	5	40	0	0	0	3
58	5	80	80	80	0	60
62	6	0	0	0	0	0
64	6	83	67	50	0	14
67	6	50	50	50	50	65
75	6	0	0	17	0	14
77	5	100	100	60	60	69
80	6	83	83	17	0	14
88	5	20	0	0	0	7
96	5	0	0	0	0	0
108	5	40	40	40	40	65
112	5	80	60	20	0	14
120	5	20	20	20	0	14

a, at least one mouse remained infected for this number of days

Table 24. Variation with Time of Intravaginal Infectivity of *T.vaginalis* 2755 Sub-passaged *in vivo*

WEEKS POST-ISOLATION	NUMBER OF MICE	% MICE INFECTED AFTER:				MAXIMUM[a] NUMBER OF DAYS INFECTED
		3 DAYS	7 DAYS	14 DAY	28 DAYS	
0	6	0	67	67	50	130
3	5	60	60	60	60	80
7	5	0	20	0	0	7
11	6	67	67	50	67	52
21	5	100	100	60	60	69
24	6	50	50	0	0	7
26	6	67	67	67	33	65
32	6	50	50	33	17	35
40	5	40	20	0	0	7
51	5	40	40	40	20	65
55	5	60	60	60	40	68
62	5	40	20	20	20	36

a, at least one mouse remained infected for this number of days

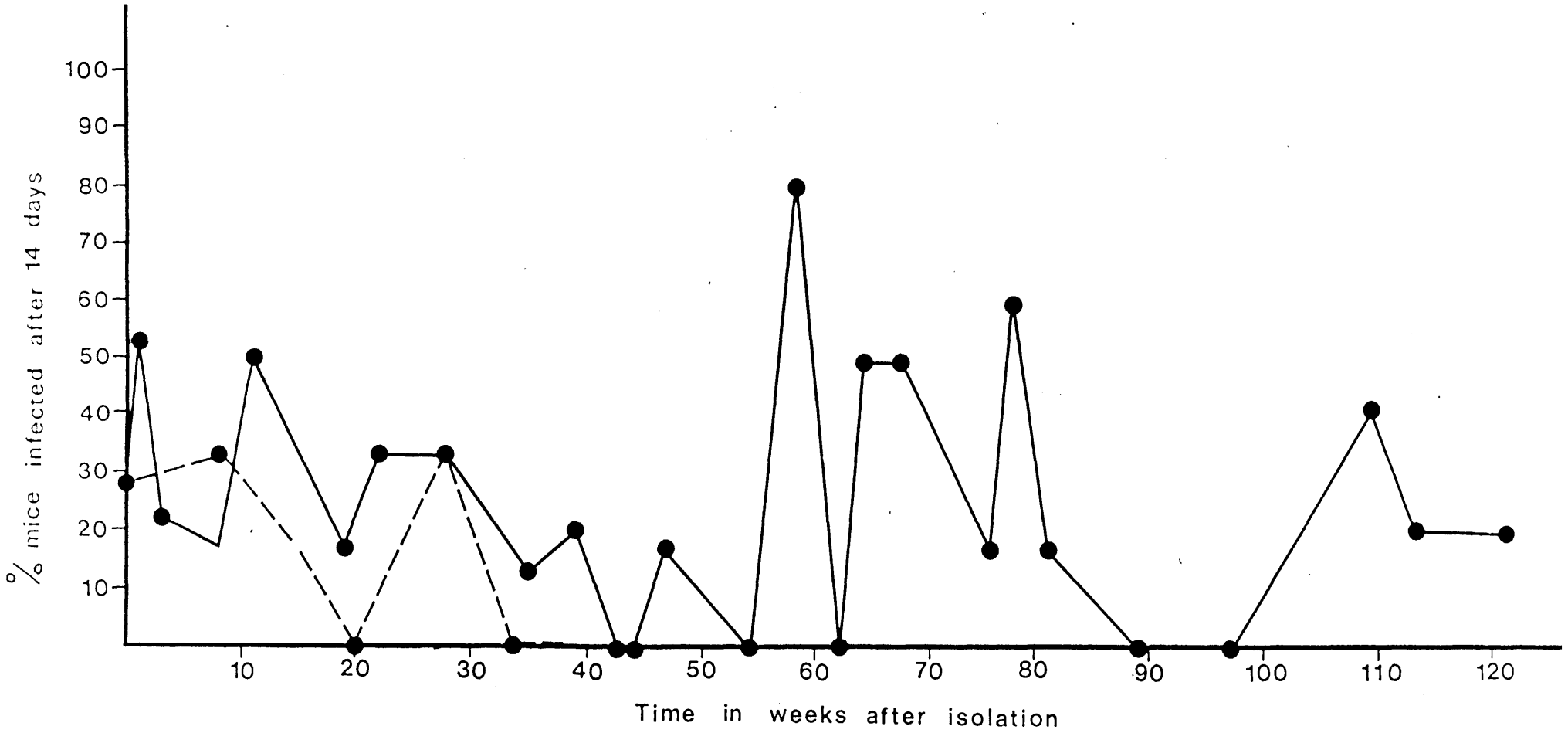
Table 25. Variation with Time of Intravaginal infectivity of *T. vaginalis* 6950 Male Sub-passaged *in vivo*

WEEKS[a] POST-ISOLATION	NUMBER OF MICE	% MICE INFECTED AFTER: 3 DAYS	7 DAYS	14 DAY	28 DAYS	MAXIMUM[b] NUMBER OF DAYS INFECTED
0	6	67	67	67	17	56
32	10	90	90	90	90	125
34	10	0	0	0	0	0
35	5	67	67	67	33	65
40	6	17	17	0	0	7
43	8	13	13	13	0	14
50	5	20	20	20	0	14
53	5	60	60	40	20	35
67	5	100	100	60	40	36

a, *T. vaginalis* 6950 Male was not obtained directly from a patient but had previously been sub-passaged in mice at the Wellcome Laboratories; b, at least one mouse was still infected after this number of days.

Figure 17. Variation with Time of Intravaginal Infectivity of *T. vaginalis* 39 Subpassaged *in vitro* and *in vivo*

Key: ●- - , *T. vaginalis* 39 subpassaged *in vitro* between intravaginal infections; ●— , *T. vaginalis* 39 subpassaged intravaginally in mice.


% mice infected after 14 days
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
100
110
120
Time in weeks after isolation

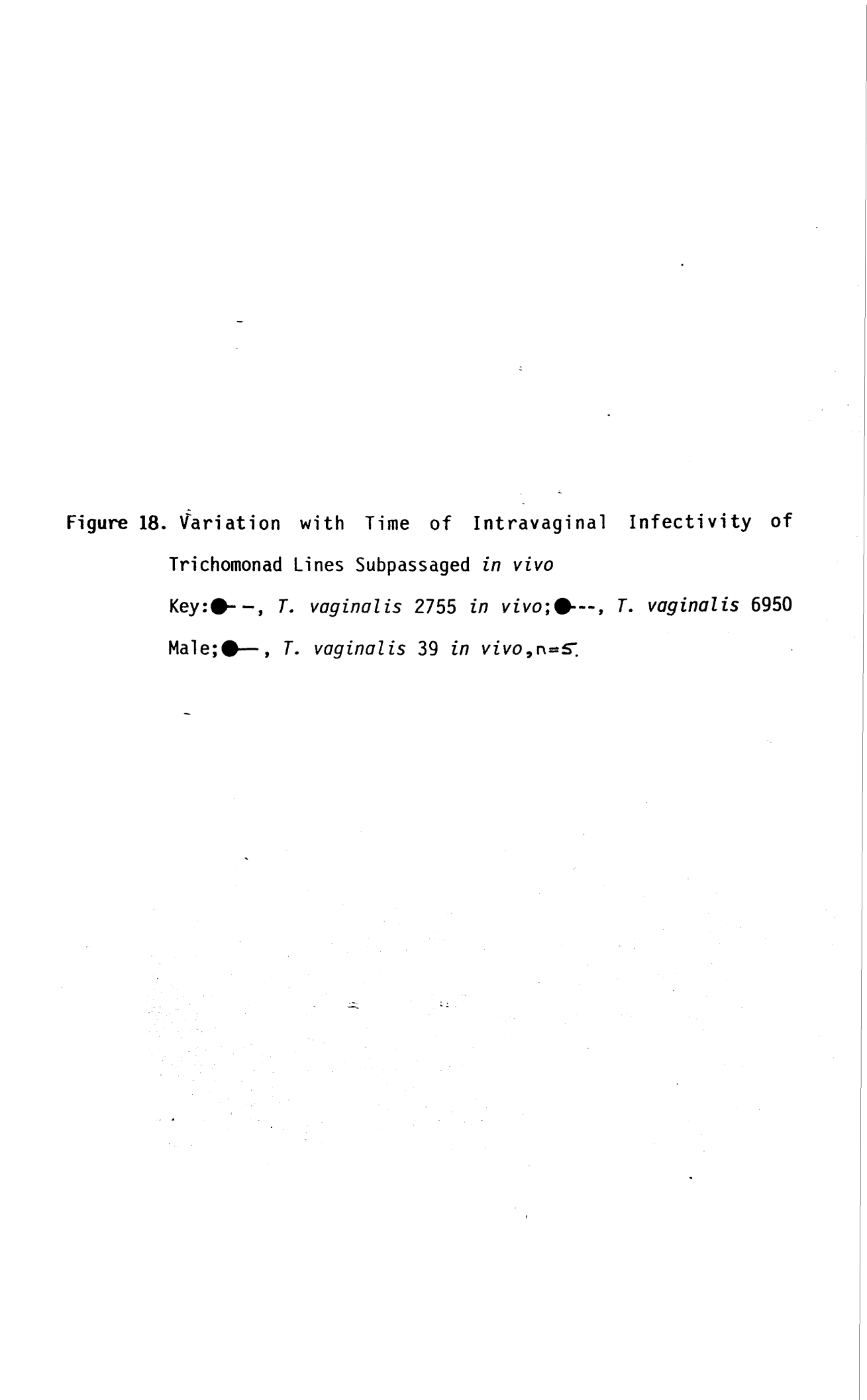
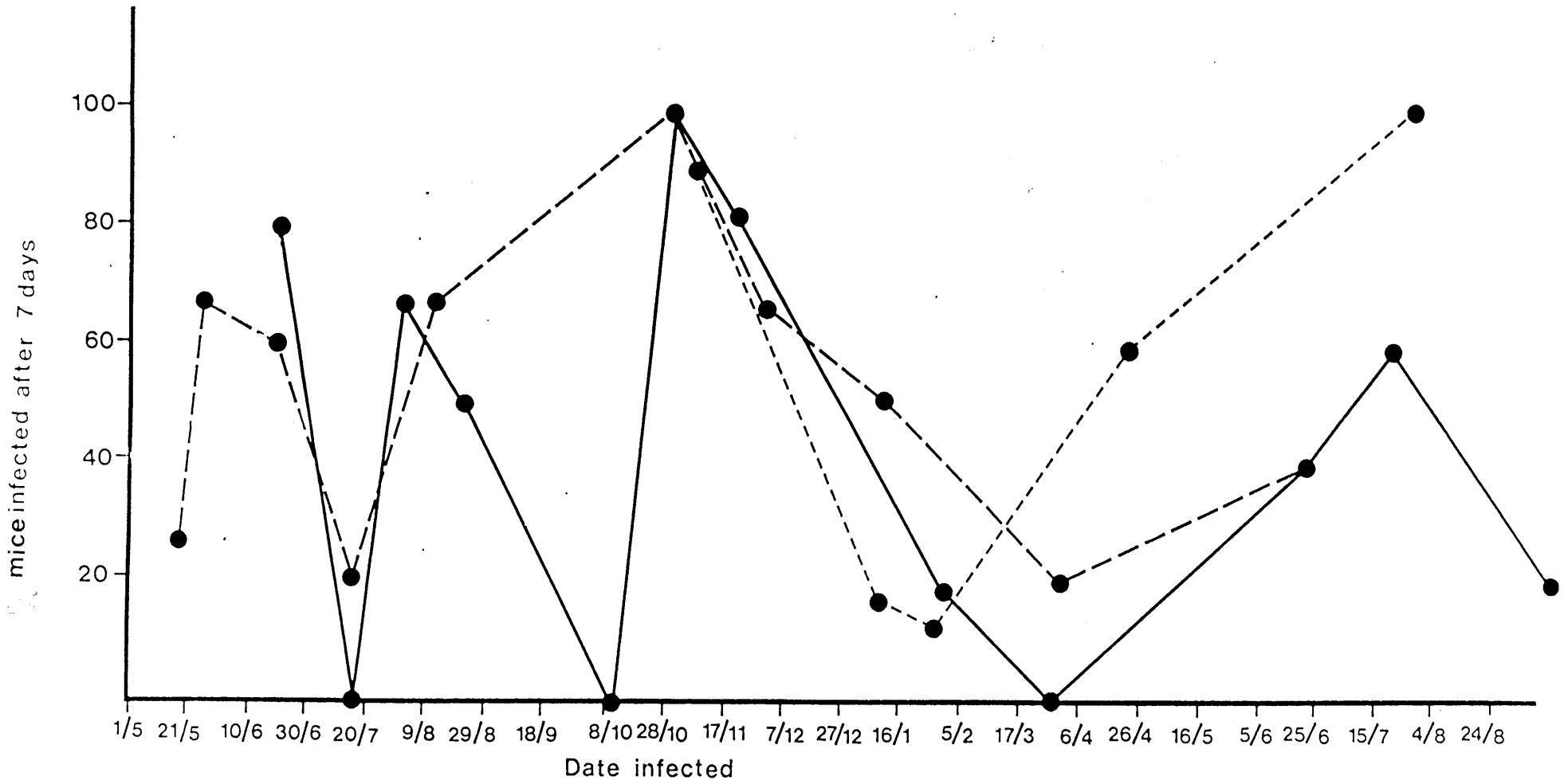


Figure 18. Variation with Time of Intravaginal Infectivity of Trichomonad Lines Subpassaged *in vivo*

Key: ●– –, *T. vaginalis* 2755 *in vivo*; ●---, *T. vaginalis* 6950 Male; ●—, *T. vaginalis* 39 *in vivo*, n=5.


mice infected after 7 days
100
80
60
40
20
1/5
21/5
10/6
30/6
20/7
9/8
29/8
18/9
8/10
28/10
17/11
7/12
27/12
16/1
5/2
17/3
6/4
26/4
16/5
5/6
25/6
15/7
4/8
24/8
Date infected

3.4.2. The Effect of Metronidazole on the Production of Subcutaneous Abscesses by *T. vaginalis*

Groups of Balb/c mice were inoculated subcutaneously in the flank with 10^5 *T. vaginalis* G3 and *T. vaginalis* IR78 cells. Each mouse was then treated with three intraperitoneal doses of 0, 15 or 100mg metronidazole kg body weight^{-1} per dose. Doses were administered at 2, 18 and 24 hours post-infection, as in the schedule used by Meingassner, 1977. Results are shown in Figure 19. No lesions appeared in any of the mice which had been infected with *T. vaginalis* G3 and treated with metronidazole. Lesion growth was slowed in *T. vaginalis* IR78-infected mice which had been treated with metronidazole at 15mgkg^{-1}, whereas when 100mgkg^{-1} per dose was given to *T. vaginalis* IR78-infected mice lesions did not appear until 6 days after infection. Once they appeared, however, they grew at the normal rate.

Further experiments were carried out with various isolates to determine the effects of higher and lower drug doses, and their route and time of administration. The results are summarized in Table 26. As can be seen from the results for *T. vaginalis* G3, the effects of metronidazole were somewhat variable, especially when it was administered orally. Administering the drug in three doses in the first 24 hours appeared to be more effective than giving a single dose and in this case the intraperitoneal route gave the most convincing results. Occasionally no effect on lesion growth was observed even when very high doses of metronidazole were administered. The drug doses, however, caused changes in the conditions in the gut including quite severe diarrhoea. It is probable that this interfered with absorption of the drug. The results for *T. vaginalis* IR78, *T. vaginalis* 2755 *in vivo*, *T. vaginalis* 6950 Male, and *T. vaginalis* 39 *in vivo* showed similar patterns but all were relatively inconsistent.

Figure 19. The Effect of Metronidazole upon Subcutaneous Abscess Production by *T. vaginalis*

Key: ●— , *T. vaginalis* G3 control; ■, *T. vaginalis* G3, 15mgkg^{-1} (3 doses) and 100mgkg^{-1} (3 doses); ○—, *T. vaginalis* IR78 control; ●- - , *T. vaginalis* IR78, 15mgkg^{-1} (3 doses); ○- -, *T. vaginalis* IR78, 100mgkg^{-1} (3 doses).

Means (± S.D.) of groups of three mice

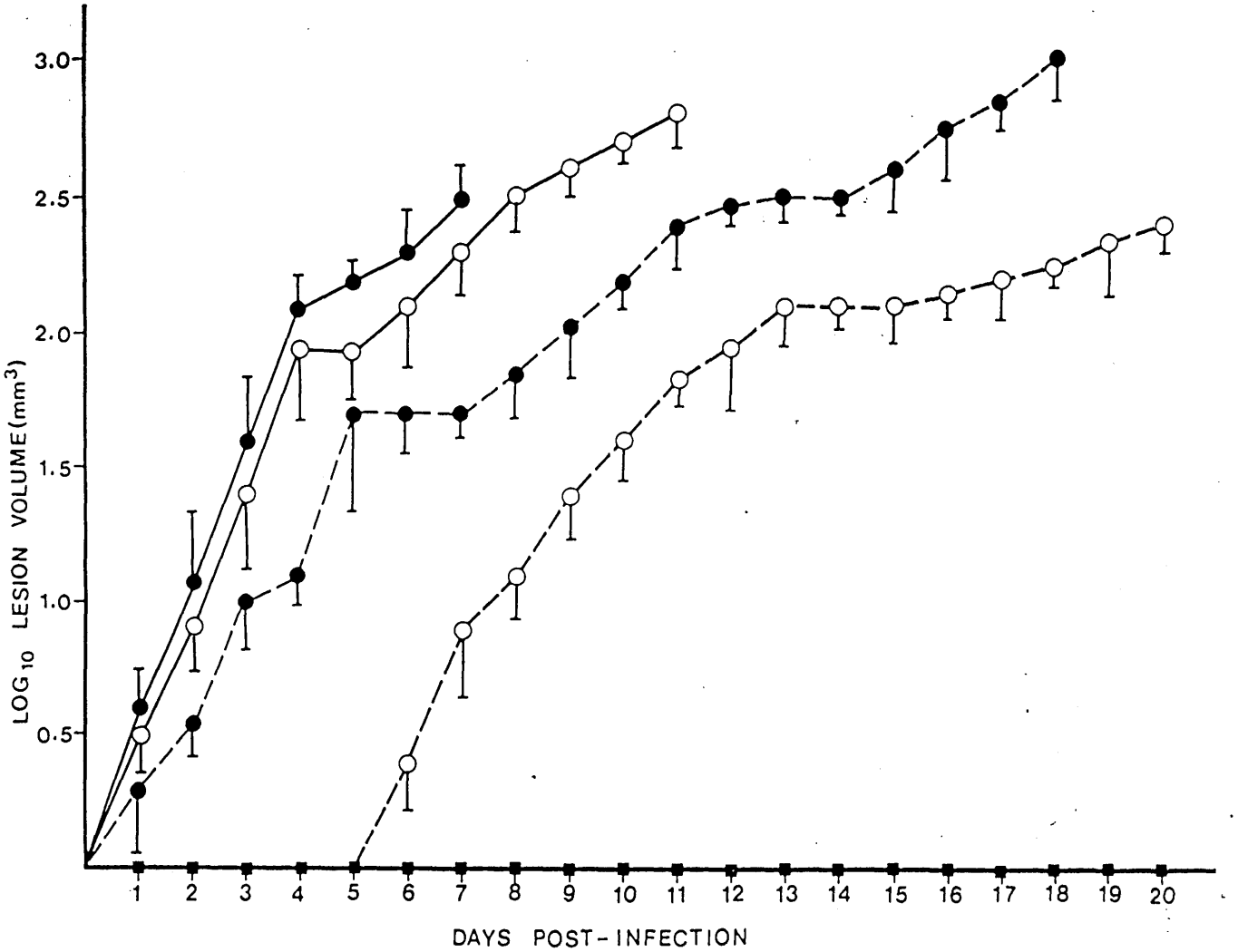

3.0
2.5
2.0
1.5
1.0
0.5
LOG10 LESION VOLUME(mm3)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
DAYS POST-INFECTION

Table 26. Effect of Metronidazole on Subcutaneous Lesion Growth

ROUTE	TOTAL DOSE (mg kg body weight^{-1})	TIMES OF ADMINISTRATION (hrs post-infection)	LINE				
			G3	IR78	2755 *in vivo*	6950 Male	39 *in vivo*
IP	45	2, 18, 24	V	I			
	300	2, 18, 24	V	I			
	45	24	I	0			
	300	24	V	0			
	45	48	0	0			
	300	48	0	0			
IP	25	2, 18, 24	I	0	I	0	0
	50	"	IV_{95}	0	II	0	0
	100	"	0	I	II	0	I
	200	"	0	II	II	0	0
	300	"	IV_{120}	0	II	I	0
ORALLY	45	2, 18, 24	III				
ORALLY	50	2, 18, 24	III				
ORALLY	45	2, 18, 24	I	0			
	150	"	III	0			
	300	"	I	V			
	450	"	III	V			
	600	"	V	V			
ORALLY	45	2, 18, 24	0				
ORALLY	45	2, 18, 24	0	0	0	0	
	100	"	0	I	0	0	
	200	"	0	II	0	0	
	300	"	0	0	0	0	
	400	"	0	II	0	0	

Table 25 (contd.)

ORALLY	50	2, 18, 24	0	IV
	100	"	0	V
	200	"	0	V
	300	"	0	V
ORALLY	50	2	0	0
	100	"	0	IV
	200	"	0	0
	300	"	I	IV
ORALLY	50	24	0	IV
	100	"	0	IV
	200	"	0	0
	300	"	0	IV
ORLLY	50	48	0	0
	100	"	0	0
	200	"	0	0
	300	"	0	0

Key: 0, no effect; I, slight slowing of growth; II, slowing of lesion growth; III, large slowing of lesion growth; IV_x, delay in appearance of lesion until x hours post-infection; V, complete absence of lesion.

3.4.3. Treatment of Intravaginal Infections of *T. vaginalis* in the Mouse with Metronidazole

Balb/c mice were infected intravaginally in the usual way and subsequently treated with metronidazole at a range of doses and by various routes of administration. The results for *T. vaginalis* 6950 Male are shown in Table 27. They show that in some cases infection with *T. vaginalis* was totally cured by 50mgkg^{-1} metronidazole given daily for 5 days after infection. Similar results were found with *T. vaginalis* 39 *in vivo* (Table 28). The results for *T. vaginalis* 2755 *in vivo* are shown in Table 29. They show that infection by this line of the parasite was not cured as early by metronidazole, with parasites being found even after the drug had been adminstered at 300mg (kg body weight)$^{-1}$. Interestingly administering the drug as in 'g' and 'h' (intravaginally at 2, 18 and 24 hours, and intraperitoneally for 5 days plus 2, 18 and 24 hours post-infection) appeared to produce cures. Interpretation of data, however, must be cautious as it was not possible to repeat the experiments and it was previously shown that the infections were variable. Nevertheless, these are interesting pointers to show how drug-resistant infections could be cured.

3.4.4. Comparison of Models for Determining Metronidazole Sensitivity

A summary of the doses of metronidazole needed to cure infections of each of the isolates in the different models is given in Table 30. The table shows that the metronidazole sensitivities of the lines of *T. vaginalis* determined *in vitro* under aerobic conditions are reflected to an extent by the susceptibilities of the lines in the subcutaneous model and particularly by their behaviour in the intravaginal model.

Table 27. Treatment of Intravaginal Infections of *T. vaginalis* 6950 Male with Metronidazole

Total Metronidazole given mg (kg b.w)$^{-1}$	% Mice infected after 7 days								
	0	50	100	150	200	300	400	450	500
a	70	0							
b	70	0	0		0				
c	17	0	0		0	0	0		
d	70		0		0				
e	0		0		0	0	0		0
f	50			0		0		0	
g	0		0		0	0	0		0
h	60		0		0				

Key: a-e, metronidazole was given orally for 5 days starting day 3 post-infection; f, metronidazole given intraperitoneally 3 times in first 24 hours; g, metronidazole given intravaginally 3 times in first 24 hours; h, metronidozole given intraperitoneally daily for 5 days after 2, 18 and 24 hour administration.

Table 28. Treatment of Intravaginal Infections of *T. vaginalis* 39 *in vivo* with metronidazole

Total Metronidazole mg (kg b.w)$^{-1}$	% Mice infected after 7 days								
	0	50	100	150	200	300	400	450	500
a	50			0		0		0	
b	20	0	0		0				

Key: a, metronidazole given orally on days 3, 4, 5, 6 and 7 post-infection; b, metronidazole given intraperitoneally on days 3, 4, 5, 6 and 7 post-infection.

Table 29. Treatment of Intravaginal Infections of *T. vaginalis* 2755 *in vivo* with Metronidazole

Total Metronidazole mg (kg b.w)$^{-1}$	% Mice infected after 7 days								
	0	50	100	150	200	300	400	450	500
a	70	17	35		35				
b	17	17	0		17	0	0		
c	35		0		0	70			
d	50		17		35	35			
e	35			17		0		0	
f	50			0		0		0	
g	35		0		0	0	0	0	
h	40	0	0		0				

Key: a-d, metronidazole given orally for 5 days starting day 3 post-infection; e, metronidazole given intraperitoneally for 5 days starting 3 days post-infection; f, metronidazole given at 2, 18 and 24 hours post-infection; g, metronidazole given intravaginally at 2, 18 and 24 hours post-infection; h, metronidazole given intraperitoneally daily for 5 days after 2, 18 and 24 hour administration.

Table 30. Summary of Susceptibilities of Trichomonad Lines to Metronidazole in Various Experimental Models

T. vaginalis line	*In vitro* MLC ($\mu g\ ml^{-1}$)		*In vivo* Minimum (mg kg b.w.$^{-1}$)*	
	Aerobic	Anaerobic	Subcutaneous	Intravaginal
SENSITIVE				
G3	2	1	45(ip)	-
6950 Male	10	1	100(ip)	50(po) 100(ip)
39 *in vivo*	5	1	-	50(ip)
RESISTANT				
IR78	100/50	5	300(ip)	-
2755 *in vivo*	50	2	> 400(ip)	400(po)

The results are representative of many experiments.

* The *in vivo* results give the minimum doses required to prevent growth of trichomonads subcutaneously or intravaginally.

3.5. STUDIES ON THE ROLE OF PROTEINASES IN THE PATHOGENICITY OF *Trichomonas vaginalis*

3.5.1. Proteinase Activities of Recent Isolates

A range of recent isolates of *T. vaginalis* were assayed for their total proteinase activity. This was performed by Barbara Lockwood at Stirling University using published procedures (Lockwood *et al.*, 1977). The results are shown in Table 15.

3.5.2. The Effect of the Proteinase Inhibitor Leupeptin on the Cytotoxicity of *Trichomonas vaginalis*

The results from four experiments to determine whether leupeptin affected the interaction between *T. vaginalis* G3 and the myeloma cells are summarized in Table 31. In the control experiments in which leupeptin was not used trichomonads were found to have no effect on the myeloma cells when at ratios of 20:1 and 10:1, mammalian cells:trichomonads. At a 5:1 ratio, however, the mammalian cell culture was completely destroyed within 26-65 hours, whereas although the myeloma cells were also completely destroyed at the 2:1 ratio, the trichomonads in monoculture at the same concentration also overgrew. In the presence of 50ugml^{-1} of leupeptin, however, the trichomonads had an effect only when at the 2:1 ratio, and again at this density the trichomonads in monoculture also overgrew.

To determine whether this effect could be explained simply in terms of leupeptin inhibiting the growth of *T. vaginalis*, trichomonads were cultured under the same conditions in mixed HOMEM/MDM medium in the presence of various concentrations of leupeptin and their growth curves monitored. The results are shown in Figure 20. They show that no significant inhibition of trichomonad growth occurred under these conditions even in the presence of 100ugml^{-1} leupeptin. The rather poor growth of *T. vaginalis* in all cases is simply a reflection of the sub-optimal conditions of cultures.

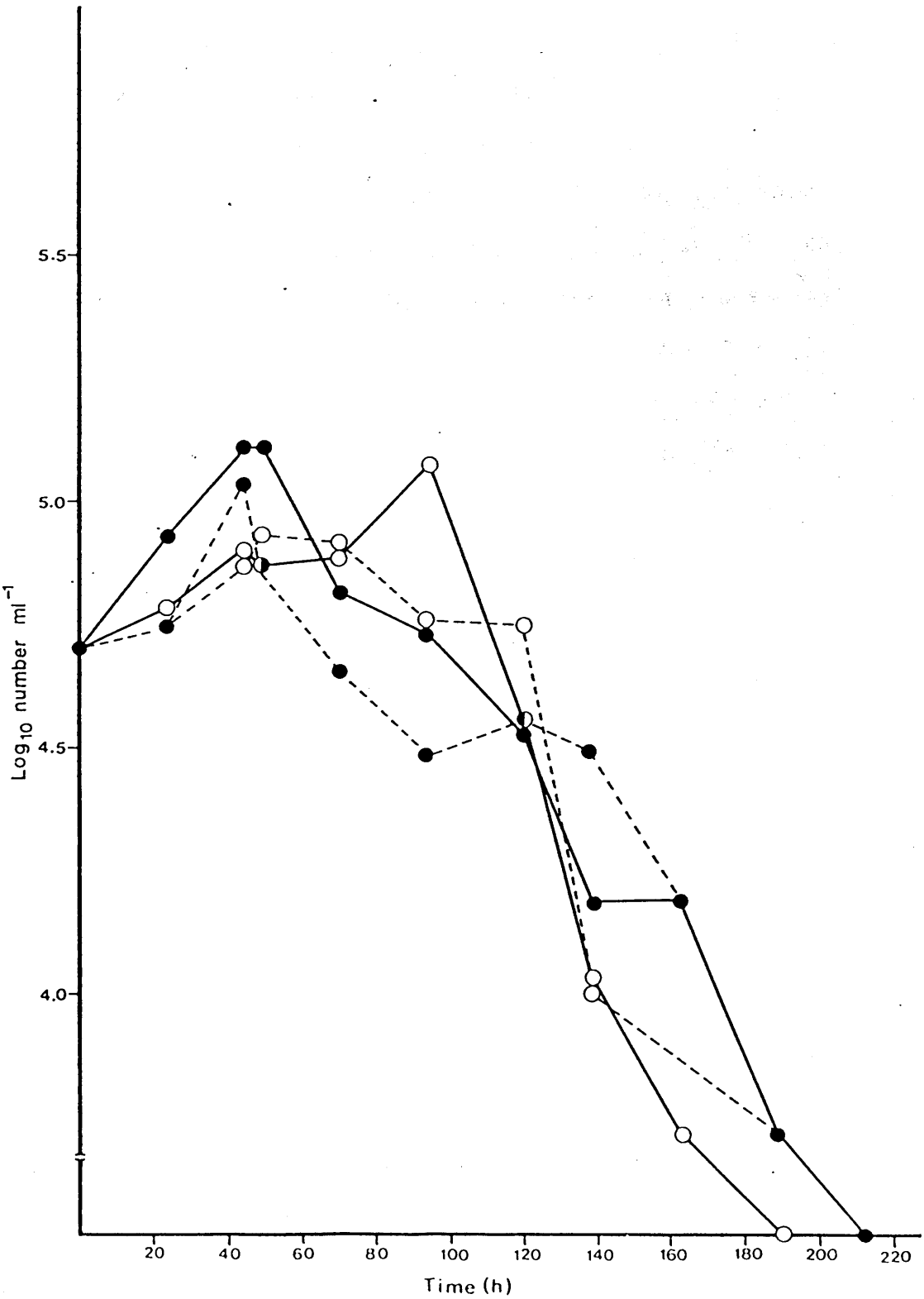
Table 31. Inhibition of the Cytotoxicity of *T. vaginalis* towards Mammalian Cell Lines by Leupeptin

RATIO MAMMALIAN CELLS: TRICHOMONADS	CONTROL				LEUPEPTIN ($50ugml^{-1}$)			
	A	B	C	D	A	B	C	D
20:1	44	00	44	00	44	00	44	00
10:1	44	00	44	00	40-48	00	44	00
5:1	40-70	26-65	95-97	00	40-45	00	40-45	00
2:1	71-95*	20	70-95*	00	89-96*	70-75	113-116*	00

This table shows the range of results found in 4 separate experiments. *T. vaginalis* G3 was the trichomonad line used. Key: A, time point for trichomonads in monoculture to die; B, time point for mammalian cells in mixed culture to die; C, time point for trichomonads in mixed cultures to die; D, time point for mammalian cells in monoculture to die.

Figure 20. Growth of *T. vaginalis* in the Presence of Various Concentrations of Leupeptin

T. vaginalis G3 was incubated in a mixture of HOMEM/MDM medium in 5% CO_2, 95% air. Key:○— , control, no leupeptin added;●— , 25$\mu g ml^{-1}$ leupeptin;○--- , 50$\mu g ml^{-1}$ leupeptin; ●-, 100$\mu g ml^{-1}$ leupeptin.


5.5
5.0
4.5
4.0
Log10 number ml-1
20
40
60
80
100
120
140
160
180
200
220
Time (h)

3.5.3. The Effect of Leupeptin on the Production of Subcutaneous Abscesses in Mice by *T. vaginalis*

The maximum effect which it was found possible to achieve through the administration of leupeptin by various routes to mice infected subcutaneously with trichomonads is shown in Figure 21. There was in this case a delay in appearance of the lesions until 3 days post-infection, after which the lesions grew at the normal rate. Metronidazole, administered as detailed in Figure 21, completely prevented growth of subcutaneous lesions. Other dose levels and administration schedules were tested, as detailed in Table 32, but no combination produced a greater effect than that described in Figure 21. Higher doses of leupeptin than those shown in Table 32 and given intravenously rapidly killed the mice. The subcutaneous administration of the inhibitor caused localised necrosis, even at 50 $mgkg^{-1}$ which severely limited its use by this route.

3.5.4. The Effect of Leupeptin on Growth of Trichomonads Intravaginally in Mice

Mice intravaginally infected with *T. vaginalis* 6950 Male were treated with leupeptin using the same dosage schedule as in the experiment with subcutaneously-infected mice as detailed in Figure 21. The results (Table 33a) show that leupeptin at this level had no effect on the intravaginal infections, whereas mice treated with metronidazole at $50mgkg^{-1}$ orally for 5 days post-infection were completely cured. To investigate whether inhibition of the trichomonad proteinases affected the establishment of infections, a trichomonad culture was incubated overnight in the presence of $50ugml^{-1}$ leupeptin and then inoculated intravaginally into mice. Some mice became infected, however, even though in this experiment, none of the control mice did (Table 33b). This again demonstrates the variability of the

Figure 21. The Effect of Leupeptin on Production of Subcutaneous Abscesses in Mice by *T. vaginalis*

50mg (kg body weight)$^{-1}$ leupeptin were administered to mice at each of the times indicated by the arrows. *T. vaginalis* G3 was used. Key: ■, control; ▲, 50mg (kg body weight)$^{-1}$ leupeptin given three times; ●, metronidazole at 15mgkg^{-1} administered at 2, 18 and 24 hours post-infection (p.o.). Leupeptin was administered intravenously.

Means (± S.D.) of groups of three mice

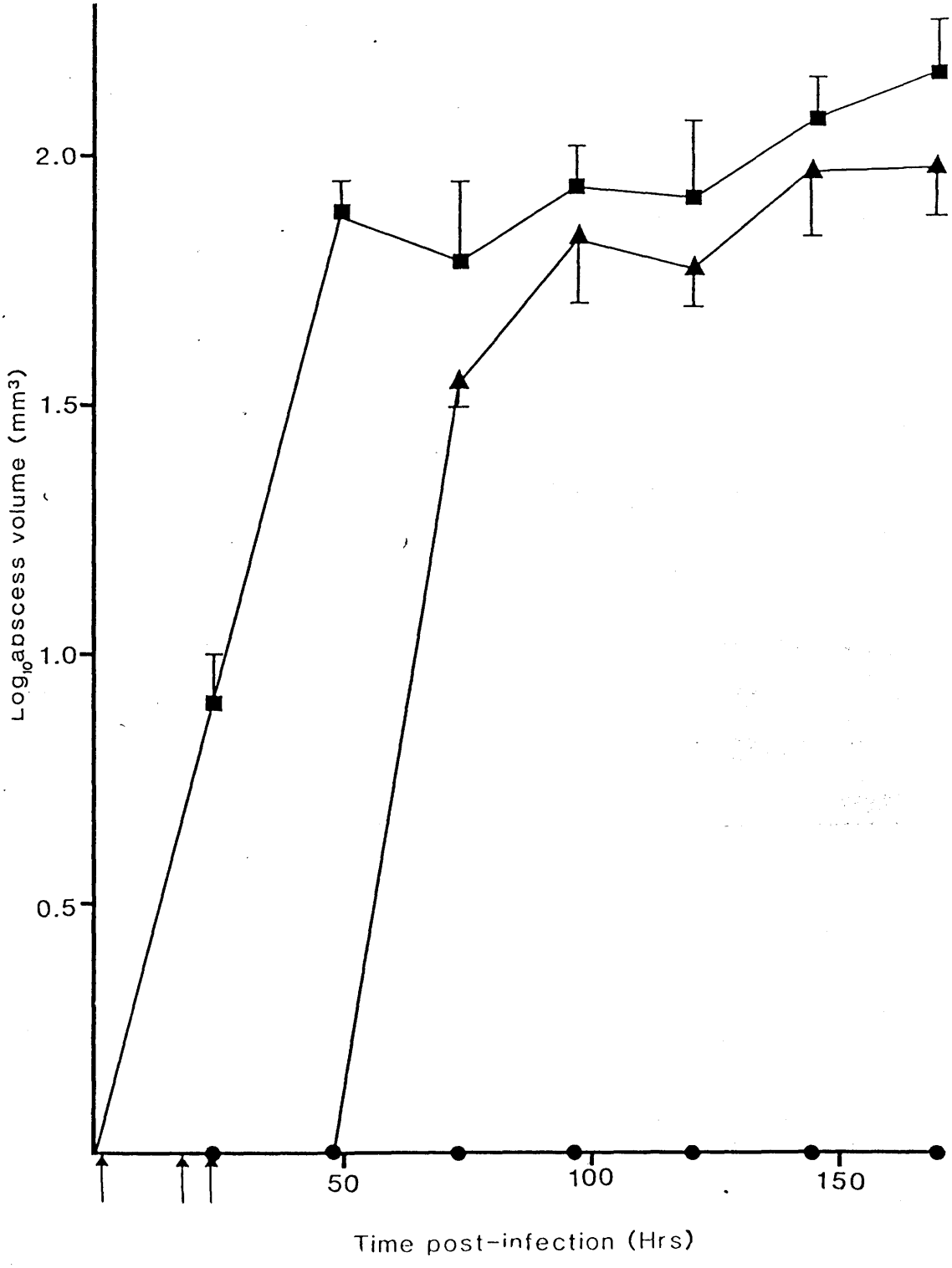

2.0
1.5
1.0
0.5
Log10abscess volume (mm3)
50
100
150
Time post-infection (Hrs)

Table 32. Effect of Leupeptin on the Growth of Subcutaneous Abscess in Mice

Leupeptin per dose ($mgkg^{-1}$)	*T. vaginalis* line	Times of Administration (hrs pi)	Route	Effect
200	G3	2, 18, 24	IP	0
400	G3	2, 18, 24	IP	0
50	G3	2, 18, 24	IV	IV_{48}
50	G3	2, 18, 24	IV	IV_{72}
50	G3	2, 18, 24	IV	0
50	G3	2, 12, 18	IV	0
50	G3	2, 18, 24, 44	IV	IV_{48}
50	G3	2, 18, 24, 44, 68	IV	IV_{48}
50	G3	2, 18, 24	SC	I*
200	IR78	2, 18, 24	SC	I*
350	IR78	-1, 0, 2, 4	IV+IP	I
50	39 *in vivo*	2, 18, 24	IP	0
100	39 *in vivo*	2, 18, 24	IP	0
200	39 *in vivo*	2, 18, 24	IP	IV_{72}

Key: IP, intraperitoneal route; IV, intravenous route; SC, subcutaneous route; 0, no effect; I, slight slowing of abscess growth; II, slowing of abscess growth; III, large slowing of abscess growth; IV_x, delay in appearance of abscess growth until x hours post-infection; V, complete inhibition of abscess growth. *, local toxicity observed.

Table 33. The Effect of Leupeptin on Growth of Trichomonads Intravaginally in Mice

(a)

Experiment	Number of mice	% still infected after		
		7 days	14 days	21 days
CONTROL	7	15	15	15
LEUPEPTIN[a]	7	30	30	15
METRONIDAZOLE[b]	7	0	0	0

a, 50mgkg^{-1} three times in first 24 hours post-infection, intravenous administration; b, metronidazole 50mgkg^{-1} ip for 5 days p.i.

(b)

Experiment	Number of mice	% still infected after		
		7 days	14 days	21 days
CONTROL	10	0	0	0
LEUPEPTIN[a]	10	20	20	20

a, trichomonads incubated overnight in 50μgml^{-1} leupeptin prior to inoculation.

(c)

Experiment	Number of mice	% still infected after		
		10 days	18 days	42 days
CONTROL	6	70	70	70
LEUPEPTIN[a]	6	50	50	50
METRONIDAZOLE[b]	6	0	0	0

a, leupeptin 50mgkg^{-1} p.o. for 5 days post-infection (days 3, 4, 5, 6, 7); b, metronidazole 50mgkg^{-1} for 5 days i.p. *T. vaginalis* 6950 Male was the isolate used for all of these experiments.

model, but the finding of two infected mice in the experiment group provides good evidence that pretreatment of parasites with leupeptin did not abolish infectivity. A further experiment was performed in which mice were treated with 50mgkg^{-1} orally for 5 days post-infection and again leupeptin treated mice remained infected even up to 42 days after infection.

3.6. STUDIES ON THE ROLE OF POLYAMINES IN THE PATHOGENICITY OF *Trichomonas vaginalis*

3.6.1. Ornithine Decarboxylase Activities of Recent Isolates

A range of recent isolates of *T. vaginalis* were assayed for their ornithine decarboxylase activity. This was performed by Dr Mick North of Stirling University, using published procedures (North *et al.*, 1986); the results are shown in Table 15.

3.6.2. The Effect of the Ornithine Decarboxylase Inhibitor, Difluromethylornithine on the Cytotoxicity of *T. vaginalis* towards Mammalian Cell Lines

The results from 5 experiments to determine whether difluoromethylornithine affected the interaction between *T. vaginalis* G3 and mammalian cells are summarized in Table 34. In the control experiments in which difluromethylornithine was not used, trichomonads were found to have no effect on the myeloma cells when at ratios of 20/1 and 10/1. At a 5/1 ratio of mammalian cells/trichomonads, however, the mammalian cell culture was completely destroyed within 26-48 hours, whereas although the myeloma cells were also completely destroyed at the 2/1 ratio, the trichomonads in monoculture at the same concentration also overgrew. In the presence of 1mM α-difluoromethylornithine (DFMO), however, the trichomonads had an effect only when at the 2/1 ratio, and again at this density the trichomonads in monoculture also overgrew.

Table 34. Inhibition of the Cytotoxicity of *T. vaginalis* towards Mammalian Cell Lines by Difluoromethylornithine

RATIO MAMMALIAN CELLS: TRICHOMONADS	CONTROL				DFMO (1mM)			
	A	B	C	D	A	B	C	D
20:1	44	00	44	00	44	00	44	00
10:1	44-48	00	44-48	00	44-48	00	44-48	00
5:1	44-48	26-48	97-116*	00	44-48	00	44-48	00
2:1	97-116*	20-48	70-116*	00	74-97*	70-74	97-116*	00

This table shows the range of results found in 5 separate experiments. *T. vaginalis* G3 was the trichomonad line used. Key: A, time point for trichomonads in monoculture to die; B, time point for mammalian cells in mixed culture to die; C, time point for trichomonads in mixed cultures to die; D, time point for mammalian cells in monoculture to die; *, trichomonads overgrew.

To determine whether the effect could be explained simply in terms of leupeptin inhibiting the growth of *T. vaginalis* trichomonads were cultured under the same conditions in mixed HOMEM/MDM medium in the presence of various concentrations of DFMO and their growth curves monitored. The results are shown in Figure 22. They show that no significant inhibition of trichomonad growth occurred under these conditions even in the presence of 5mM DFMO.

3.6.3. The Effect of Difluoromethylomithine on the Production of Subcutaneous Abscesses in Mice by *T. vaginalis*

The maximum effect which it was found possible to achieve through the administration of DFMO to mice infected subcutaneously with trichomonads is shown in Figure 23. There was in this case a delay in appearance of the lesions until 4 days post-infection, after which the lesions grew at the normal rate. Metronidazole, administered as detailed in Figure 23, completely prevented growth of subcutaneous lesions. Other dose levels and administration schedules were tested as detailed by Table 35, but no combination produced a greater effect than that described in Figure 23.

3.6.4. The Effect of Difluoromethylomithine on Growth of Trichomonas Intravaginally in Mice

Mice intravaginally infected with *T. vaginalis* 6950 Male were treated with DFMO using the same dose schedule as in the experiment with subcutaneously-infected mice as detailed in Figure 23. The results are given in Table 36a. They show that DFMO at this level appeared to have no effect on the intravaginal infections, whereas mice treated with metronidazole at 50mgkg body weight^{-1} orally for 5 days post-infection were completely cured. To investigate if

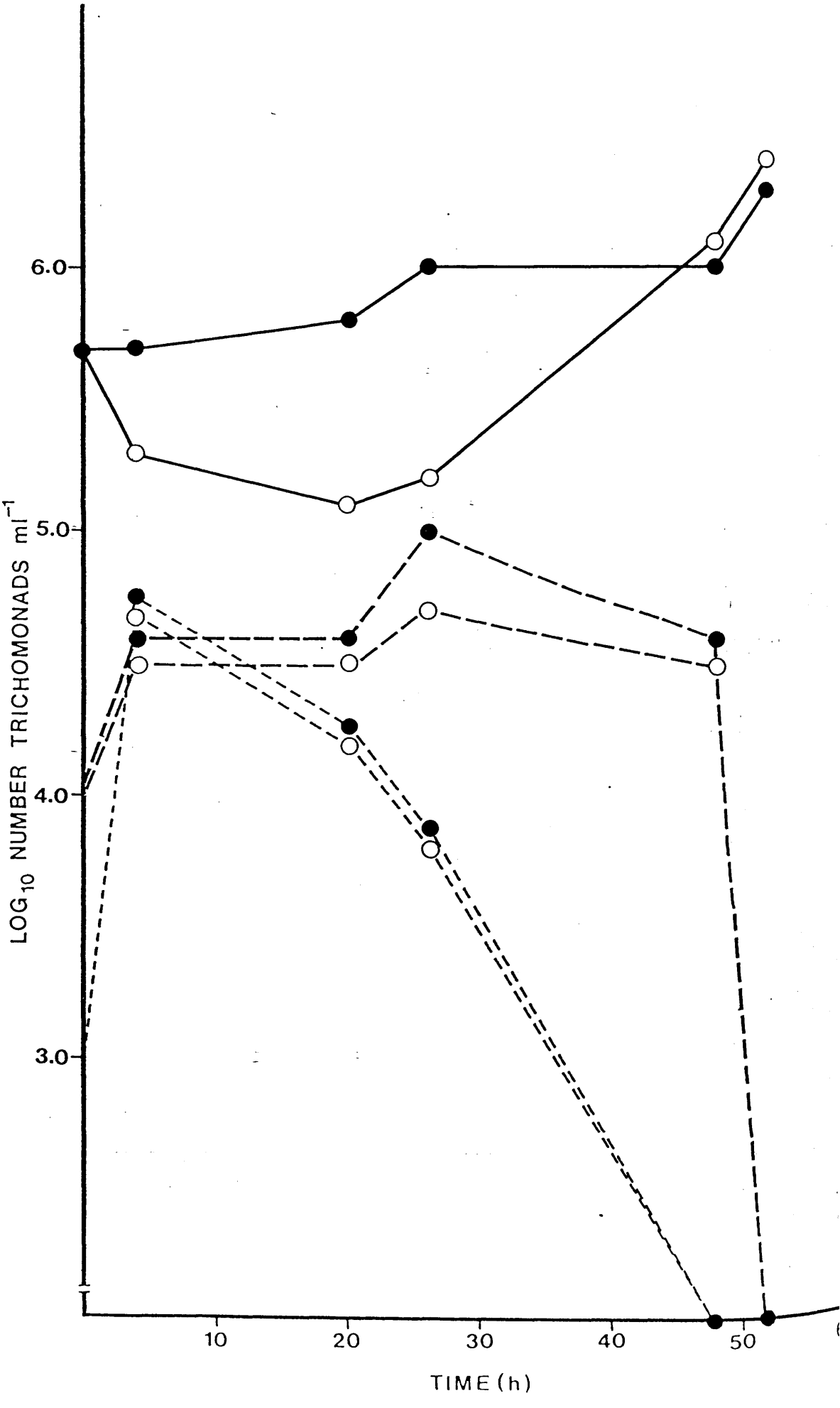

6.0
5.0
4.0
3.0
LOG10 NUMBER TRICHOMONADS ml-1
10
20
30
40
50
TIME (h)

Figure 22. Growth of *T. vaginalis* in HOMEM/MDM in the Presence of Difluoromethylornithine

T. vaginalis G3 was incubated in HOMEM/MDM medium in 5% CO_2, 95% air. Key: ●, control; ○,5mM DFMO.

Figure 23. The Effect of Difluoromethylornithine on Production of Subcutaneous Abscesses in Mice by *T. vaginalis*

T.vaginalis G3 was the trichomonad line was used. Key: ○, control; □, 750mg (kg body weight)$^{-1}$ DFMO at each of times indicated by arrows (p.o.); ●, metronidazole, 15mgkg^{-1} administered at 2, 18 and 24 hours post-infection (p.o.). n=3, means ± S.D.

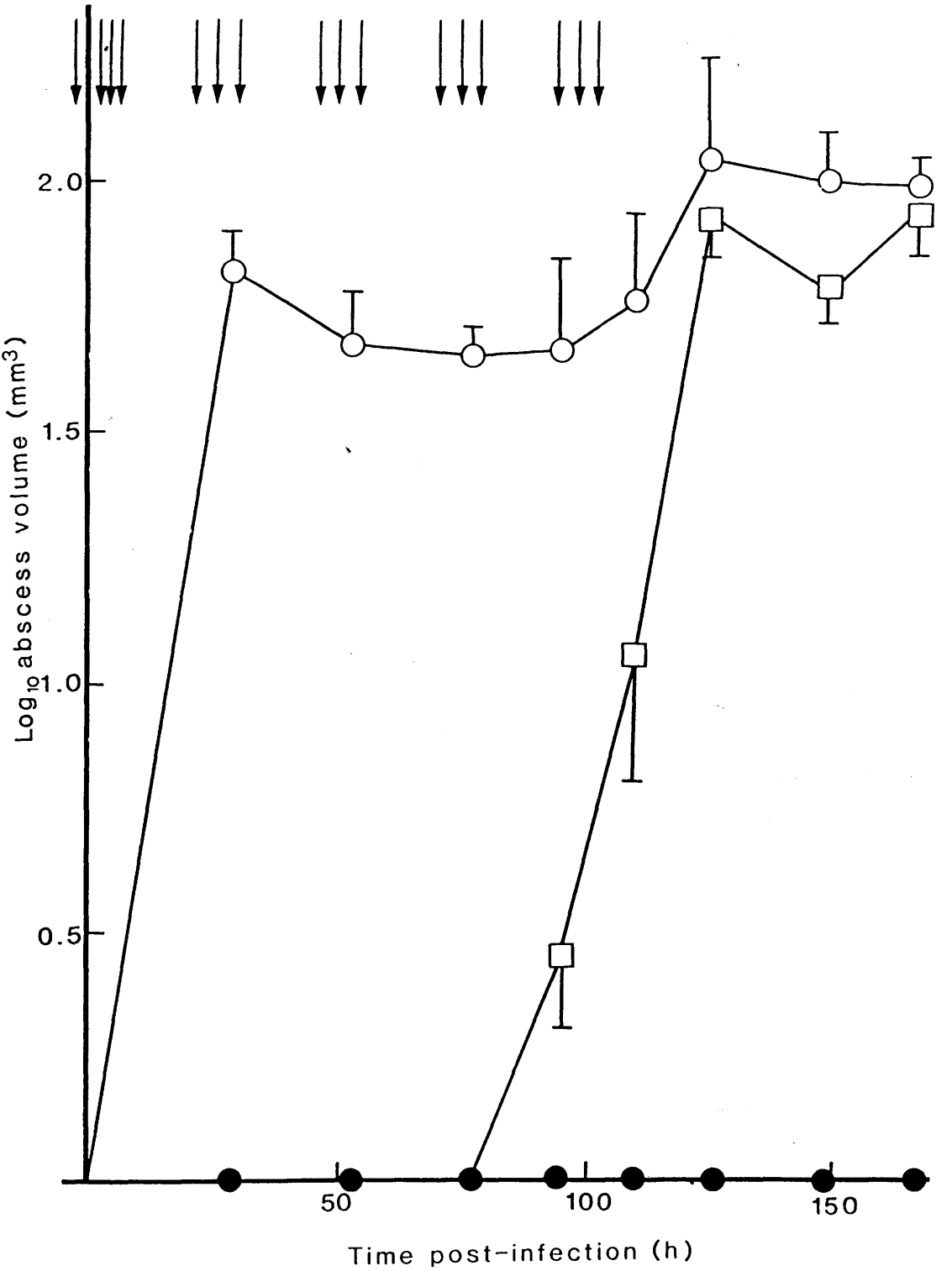

2.0
1.5
1.0
0.5
Log10 abscess volume (mm3)
50
100
150
Time post-infection (h)

Table 35: Effect of DFMO on the Production of Subcutaneous Abscesses in Mice

DFMO/dose mg(kg b.w.)$^{-1}$	Isolate	TIMES OF ADMINISTRATION (hours post-infection)	EFFECT via route: I.P.	P.O	I.V.	S.C.	IV+IP
150	G3	2, 18, 24		0	0,IV_{72}	0	
300					0		
600					0,I		
1000			0				
500	IR78	2, 18, 24		0		0	
75	G3	2, 18, 24, 44, 68		IV_{96}			
150				IV_{96}			
300				V			
600					0		
1000			0		0		
150	IR78	2, 18, 24, 44, 68		0			
150	G3	2, 18, 24 + daily for 6 days		IV_{72}			
750	G3	-2 and 3 x daily for 5 days		IV_{96}			
750	G3	-1, 0, 1, 3, 4		IV			
500	IR78	-1, 0, 2, 4					IV

Key: IP, intraperitoneal route; IV, intravenous route; S.C., subcutaneous route; IV+IP, 50% intravenous and 50% intraperitoneal route; 0, no effect; I, slight slowing of abscess growth; II, slowing of abscess growth; III, large slowing of abscess growth; IV_x, delay in appearance of abscess growth until x hours post-infection; V, complete inhibition of abscess growth.

Table 36. The Effects of Difluoromethylornithine on Growth of Trichomonads Intravaginally in Mice

(a)

Experiment	Number of mice	% still infected after		
		7 days	14 days	21 days
CONTROL	7	15	15	0
DFMO[a]	7	30	30	15
METRONIDAZOLE[b]	7	0	0	0

a, 750mgkg^{-1}, three doses per day for 5 days post-infection and 2 hrs prior to infection (see Figure 23); b, 50mgkg^{-1} p.o. for 5 days post-infection (day 3, 4, 5, 6, 7).

(b)

Experiment	Number of mice	% still infected after		
		7 days	14 days	21 days
CONTROL	10	0	0	0
DFMO[a]	10	30	20	0

a, trichomonads incubated overnight in 1mM DFMO prior to infection

(c)

Experiment	Number of mice	% still infected after		
		10 days	18 days	42 days
CONTROL	12	75	70	70
DFMO[a]	12	92	100	100
METRONIDAZOLE[b]	12	0	0	0

a, 1000mgkg^{-1} p.o, 3 times for 5 days post-infection

b, as for (a)

T. vaginalis 6950 Male was the isolate used for these experiments. Drugs were administered orally.

inhibition of the trichomonad proteinases affected establishment of the infection, a trichomonad culture was incubated overnight in the presence of 1mM DFMO and then inoculated intravaginally into mice. Some mice, again became infected even though none of the control mice did demonstrating the variability of the model but also indicating that pretreatment of parasites with DFMO did not abolish infectivity. A further experiment was carried out increasing the dose of DFMO from 750 $mgkg^{-1}$ three times daily for 5 days to 1000 $mgkg^{-1}$ using the same dose regime, (Table 36c) again, however, no effect was observed on the trichomonad intravaginal infection.

3.7. STUDIES ON THE POSSIBLE ROLE OF METHANETHIOL IN THE HOST-PARASITE RELATIONSHIP

The production of methanethiol within trichomonads has been detected (Thong *et al.*, 1987) although the role of this compound is as yet uncertain. A possible role of methanethiol in the host-parasite relationship was investigated using the homocysteine desulphurase inhibited, D,L-propargylglycine which inhibits methanethiol production. If thiols are important in the interaction, D,L-propargylglycine should antagonise the interaction and methionine should increase it; a series of experiments was undertaken to test this.

The effects of D,L-propargylglycine on *T. vaginalis* in axenic culture in MDM was studied by Dr K.W. Thong who found that the growth rate was not affected by the presence of 10^{-6} M D,L-propargylglycine or 15mM methionine in the medium. The former compound, however, totally inhibited the activities of homocysteine desulphurase and methionine-catabolizing enzymes while the latter resulted in the production of greater amounts of thiol including methanethiol (Thong *et al.*, 1987).

3.7.1. Methane Thiol Production by *Trichomonas vaginalis* Isolates

The rates of thiol production by various trichomonad isolates and their content of related enzyme activities were determined by Dr K.W. Thong in our laboratory, but there were found to be only minor differences between the isolates (Thong *et al.*, 1987). Hence, no correlation levels within trichomonads and pathogenicity was evident.

3.7.2. The Effects of Propargylglyane and Methionine on the Cytopathogenicity of *T. vaginalis*

Experiments were carried out to see if these compounds affected the interaction of *T. vaginalis* with mammalian cells. The results are shown in Tables 37 and 38. It was found that the presence of 15mM methionine or 10^{-5}M propargylglycine in the interaction medium had no effect on the cytotoxicity of the trichomonads towards the Hela cells (Table 37). When similar experiments were carried out using myeloma cells it was found that whereas propargylglycine at 10^{-6}M had no effect on the interaction at 10^{-5}M the compound totally inhibited the cytopathogenicity of the trichomonads when at a 5/1 ratio and even caused a delay in the interaction when at a 2/1 ratio of myeloma/ trichomonads was used. It was found, however, that under the conditions used the compound inhibited the growth of trichomonads in monoculture. Clearly the apparent effect on trichomonad cytopathogenicity could simply be a reflection of this growth inhibition. Propargylglycine at 10^{-4} and 10^{-3}M completely inhibited the cells interaction, even when at a 2/1 ratio.

Table 37. Effect of Propargylglycine and methionine on the Interaction of *T.vaginalis* with Mammalian Cells *in vivo*.

Ratio Mammalian Cels/ trichomonads	CONTROL				METHIONINE (15mM)				PROPARGYLGLYCINE (10^{-5}M)			
	A	B	C	D	A	B	C	D	A	B	C	D
250:1	49	49	95*	00	49	49	119*	00	49	49	95*	00
200:1	49	49	95*	00	49	49	95*	00	49	49	95*	00
150:1	49	49	95*	00	49	49	95*	00	49	49	95*	00
100:1	49	49	95*	00	49	49	95*	00	49	49	95*	00
50:1	119*	28	95*	00	119*	49	95*	00	49	49	95*	00
20:1	119*	28	71*	00	119*	28	119*	00	119*	28	95*	00
10:1	95*	22	71*	00	95*	28	95*	00	95*	22	95*	00
5:1	71*	22	71*	00	95*	22	95*	00	95*	22	95*	00

The trichomonad line used was *T. vaginalis* G3. Key: A, time point for trichomonads in monoculture to die; B, time point for mammalian cells in mixed culture to die; C, time point for trichomonads in mixed cultur to die; D, time point for mammalian cells in monoculture to die; *, trichomonads overgrew; ☐, no cytopathogenic effect; ▨, cytopathogenic effect occurred; ▩, cytopathogenicity but trichomonads also overgrew in monoculture.

Table 38: The Effect of Different Concentrations of Proparagylglycine on the Interaction of *T.vaginalis* and Mammalian Cells

RATIO MAMMALIAN CELLS/ TRICHOMONADS	CONTROL				10^{-6}M				10^{-5}M				10^{-4}M				10^{-3}M			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
20:1	44	00	44	00	44	00	44	00	44	00	44	00	44	00	44	00	44	00	44	00
10:1	44	00	44	00	44	00	44	00	44	00	44	00	44	00	44	00	44	00	44	00
5:1	44	26	97*	00	44	26	113*	00	44	00	44	00	44	00	44	00	44	00	44	00
2:1	70*	20	70*	00	89*	20	89*	00	44	68	168*	00	44	00	52	00	44	00	52	00

The interaction of Hela cells and *T.vaginalis* G3 was used. Key: A, time point for trichomonads in monoculture to die; B, time point to mammalian cells in mixed culture to die; C, time point for trichomonads in mixed culture to die; D, time point for mammalian cells in monoculture to die; *, trichomonads overgrew; ☐, no cytopathogenic effect, ▨, cytopathogenic effect occurred; ▩, cytopathogenicity but trichomonads also overgrew in monoculture.

3.7.3. The Effect of Propargylglycine on *T. vaginalis in vivo*

3.7.3.1. The Subcutaneous Model

Three batches of *T. vaginalis* 6950 Male were grown in MDM. One was incubated for the final 18 hours in the presence of 15mM methionine, whilst a second was incubated for the same period with 10^{-4}M propargylglycine. The third batch was incubated in MDM without additions. The resultant parasites were inoculated into the flanks of Balb/c mice using the standard procedure and the growth of the subcutaneous lesions was monitored.

The results are shown in Figure 24. It was found that all lesions initially grew at a similar rate and all reached approximately the same volume. In one experiment (Figure 24) there was an apparent subsequent decrease in the size of lesions caused by both propargylglycine- and methionine-treated trichomonads. In repeat experiments, however, this was not found and the lesions caused by untreated and propargylglycine-treated parasites grew at a similar rate throughout the experiment.

3.7.3.2. The Intravaginal Model

A number of experiments were performed to test the effect of propargylglycine and methionine on the growth of *T. vaginalis* intravaginally in mice. The result of infecting mice using the standard procedure except that the trichomonads had been incubated for 18 hours prior to use with 10^{-4}M propargylglycine are shown in Table 39. The results show that far fewer of the mice inoculated with propargylglycine-treated trichomonads became and remained infected. In addition, those that became infected had far fewer trichomonads present in the vagina at the time of wash-out than control mice. This was a consistant effect as demonstrated by the results in Table 39a. Treatment of the mice themselves with propargylglycine via the

Figure 24. Effect of Methionine and Propargylglycine on the Growth of Subcutaneous Abscesses

T. vaginalis 6950 Male was the trichomonad line used. Key: ●, control; □, 15mM methionine; ■, 10^{-4}M propargylglycine. The trichomonads were grown for 18 hrs in the presence of the inhibitors prior to inoculation and resuspended in fresh medium. n=3, means (±S.D.).

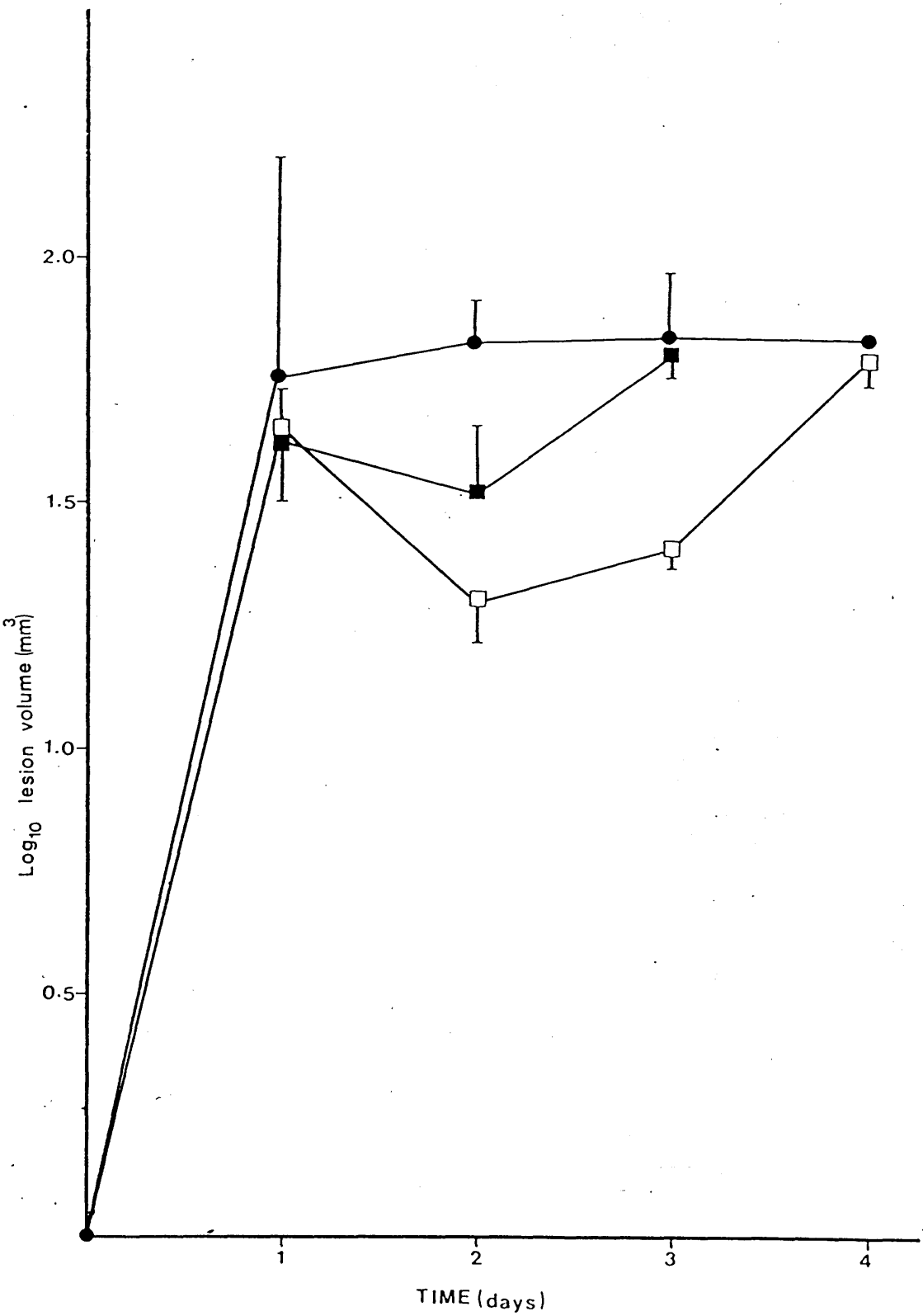

2.0
1.5
1.0
0.5
Log10 lesion volume (mm3)
1
2
3
4
TIME (days)

Table 39. The Effect of Methionine and Propargylglycine on Intravaginal Infectivity of *T. vaginalis* 6950 Male

(a)

	Experiment	Number of mice	% still infected after 7 days	% still infected after 14 days	% still infected after 32 days
A	CONTROL	10	90	90	90
	PROPARGYAGLYCINE	10	50*	30*	40*
B	CONTROL	12	30	17	-
	PROPARGYLGLYCINE	12	17	0	-

Experiments A + B, 18 hour pre-incubation of trichomonads with 10^{-4}M propargylglycine; *, far fewer trichomonads in vaginal wash outs than in those from the control mice.

(b)

Experiment	Number of mice	% still infected after 7 days	% still infected after 14 days	% still infected after 32 days
CONTROL	12	30	17	-
METHIONINE	12	30	30	-

18 hour pre-incubation of trichomonads with 50mM methionine.

(c)

Experiment	Number of mice	% still infected after 7 days	% still infected after 14 days
CONTROL	8	12	12
PROPARGYLGLYCINE[a]	8	38	38

a, 25mg kg^{-1} given 3hr prior to infection and 4hr post-infection and twice daily for the next 4 days

(d)

Trichomonad Line	Number of mice	% still infected after 7 days	% still infected after 28 days
6950 Male CONTROL	10	80	40
METHIONINE[a]	10	60	60
2755 *in vivo* CONTROL	10	40	0
METHIONINE[a]	10	20	0

a, 100mM methionine added to MDM + 0.32% agar prior to infection.

intraperitoneal route had no observable effect. It was found that addition of methionine to the growth medium of the trichomonads did not appear to increase or decrease their infectivity (Table 38b) and similarly infectivity was apparently unaffected by the addition of 100mM methionine to the suspension medium in which the trichomonads were inoculated into the vaginas of the mice.

3.8. STUDIES OF THE MECHANISMS INVOLVED IN THE CYTOPATHOGENICITY OF TRICHOMONADS TOWARDS MAMMALIAN CELLS

As illustrated in a previous section (Section 3.2.1) most of the trichomonad lines tested had a marked effect on mammalian cell lines when placed with them in a mixed culture. The trichomonads caused complete destrucrtion of the mammalian cell monolayers but the mechanisms of this cytopathogenicity were unknown. Experiments were therefore, carried out in an attempt to elucidate the mechanisms involved.

3.8.1. The Effects of Factors Excreted or Secreted from Trichomonads on Mammalian Cells

The effects on a mammalian cell monolayer of the medium from a mixed trichomonad/mammalian cell culture in which the mammalian cells had been lysed (called 'Interaction Medium') was studied and compared with the effects produced by medium in which trichomonads had grown in monoculture, and by lysates of both mammalian cells and trichomonads. The results are shown in Table 40. They show that interaction medium from the three isolates tested all produced a damaging effect on mammalian cell monolayers. This damage was not prevented by leupeptin at 50ugml^{-1} or DFMO at 1mM, although there are indications that they had some inhibitory effect. A clarifying effect was also observed when MDM itself was added to the mammalian cells in large amounts, however

Table 40. The Effects of Factors Excreted or Secreted from Trichomonads on Mammalian Cells

TEST SOLUTION	APPEARANCE OF MONOLAYER AFTER:			
	24 HOURS	48 HOURS	64 HOURS	72 HOURS
INTERACTION $MEDIUM_{G3}$	I	III	III	IV
IM_{45}	0	I	III	III
IM_{2755}	0	III	III	III
IM_{G3} (+ LEUPEPTIN)	0	I	II	III
IM_{45} (+ LEUPEPTIN)	0	0	II	III
IM_{2755} (+ LEUPEPTIN)	0	II	III	III
IM_{G3} (+ DFMO)	I	III	IV	IV
IM_{45} (+ DFMO)	0	0	0	III
IM_{2755} (+ DFMO)	0	0	II	III
MDM	0	II	III	IV
$EMEM_{(HELA)}$	0	0	0	0
MDM + EMEM	0	0	0	0
$EMEM_{G3}$	0	III	IV	IV
$EMEM_{45}$	0	0	II	III
$EMEM_{2755}$	0	III	IV	IV
HELA CONTROL	0	0	0	0
G3 LYSATE	0	0	0	0
45 LYSATE	0	0	0	0
2755 LYSATE	0	0	II	II
HELA LYSATE	0	0	0	0

Hela cell monolayers in the wells of tissue culture plates were used for this experiment. IM_x, interaction medium i.e. medium in which trichomonad line x had destroyed all mammalian cells but trichomonad cells were still in log

Table 40 (contd.)

phase; IM_x + LEUPEPTIN, 50ugml^{-1} leupeptin added to wells prior to the interaction media; IM_x + DFMO, 1mM DFMO added to the wells prior to interaction medium; MDM, 1ml modified Diamond's medium added to Hela cell monolayer; EMEM (HELA), 1ml EMEM in which a Hela cell monolayer had grown was added to the test monolayer; MDM + EMEM, 0.75ml EMEM was mixed with 0.25ml MDM and added to the test monolayer; $EMEM_x$, cultures of trichomonad line x were grown in EMEM by adding a high initial density of trichomonads, also suspended in EMEM until log phase was reached, trichomonads were then removed by centrifugation followed by filtration through 0.45u millipore filter, the used EMEM medium was then added to the test monolayer; x LYSATE, trichomonad lines x were cultured in MDM to a density of $5x10^5$ ml^{-1} then resuspended in EMEM and lysed by sonication. Key to description of Hela cell monolayers: 0, as control; I, looked unhealthy; II, some cells unattached, majority still attached; III, most cells unattached; IV, all cells unattached; V, all cells lysed.

Typical results representative of 4 experiments are shown.

EMEM and the mixture of MDM and EMEM used as the interaction medium had no effect. The media from axenic *T. vaginalis* cultures also had a severely damaging effect on the mammalian cell monolayer, whereas lysates of the trichomonads and Hela cells had little or no effect.

3.8.2. Studies on the 'Cell-Detaching Factor'

Quantities of 'cell-detaching factor' (CDF) as described by Kreigev, Poisson and Rein (1986) were produced and its effect was tested against mammalian cell monolayers. The results are shown in Table 41. The results showed that a damaging effect on the monolayer did occur and this was lessened but not prevented by the presence of leupeptin whereas DFMO appeared to have no effect. In a similar experiment CDF produced no effect (although this CDF was produced without the adjustment of the pH), Table 42, although interaction medium which had not been processed to form CDF did have an effect.

3.8.3. The Effect of Separating the Cell Types by a Membrane

To study whether the cytopathogenicity depended upon contact between the trichomonads and the mammalian cells, experiments were carried out using a 0.45um pore size membrane to separate the cell types but at the same time allowing the same medium to bathe both the trichomonads and the mammalian cells. The results are shown in Table 43. It was found that cytotoxicity was completely blocked by this physical separation.

In a similar experiment using greater numbers of trichomonads it was found again that there was no cytopathogenic effect observed even though the trichomonads in the separated mixed culture reached fairly high densities, for example, of approximately $10^6 ml^{-1}$ (Table 44).

Table 41: Effect of Cell-Detaching Factor on Mammalian Cell Monolayers

TEST SOLUTION	Effect on monolayer after 72 hours:	
	CDF 1	CDF 2
CDF (0.3ml)	III	IV
CDF (0.2ml)	III	IV
CDF (0.1ml)	III	III
CDF (0.05ml)	II	0
CDF (0.3ml) + LEUPEPTIN	I	N.D.
CDF (0.3ml) + DFMO	N.D.	III
EMEM (HELA)	0	N.D.
HELA (CONTROL)	0	N.D.
CONTROL + LEUPEPTIN	0	N.D.
CONTROL + DFMO	N.D.	0
HELA LYSATE	0	N.D.

Hela cell monolayers in the wells of tissue culture plates were used. CDF, cell-detaching factor; CDF + LEUPEPTIN, $50ugml^{-1}$ leupeptin added to well before CDF; CDF + DFMO, 1mM DFMO added to well prior to CDF; EMEM (HELA), medium in which Hela cell monolayer had previously grown, 1ml added to wells; HELA control, Hela cell monolayer with no additions; HELA LYSATE, Hela cell monolayer detached and lysed by sonication, 1ml added to well. Key to description of cell monolayers: 0, as control; I, looked unhealthy; II, some cells unattached, majority still attached; III, most cells unattached; IV, all cells unattached; V, all cells lysed. Representatives of 4 experiments shown.

N.D., not done. CDF_1 and CDF_2 were 2 batches of CDF produced in the standard way.

Table 42. Effect of Cell Detaching Factor and Various Lysates on a Mammalian Cell Monolayer

	APPEARANCE OF MONOLAYER AFTER:				
	22 HOURS	44 HOURS	67 HOURS	88 HOURS	112 HOURS
CDF	0	0	0	0	0
CDF + LEUPEPTIN	0	0	0	0	0
HELA + DFMO	0	0	0	0	0
HELA + LEUPEPTIN	0	0	0	0	0
CDF + DFMO	0	0	0	0	0
EMEM (HELA)	0	0	0	0	0
MDM + EMEM (2755)	0	III	III	IV	V
IM 2755	0	II	III	IV	V
HELA LYSATE	0	0	0	0	0
2755 LYSATE	0	0	II	II	II
MDM	0	0	0	II	II
HELA CONTROL	0	0	0	0	0

Representative of 4 experiments shown. Key: CDF, cell-detaching factor; + LEUPEPTIN, 50ugml^{-1} leupeptin added to well; + DFMO, 1mM DFMO added to well; EMEM (HELA), medium in which Hela monolayer had previously grown, 1ml added to well; MDM + EMEM (2755), *T. vaginalis* 2755 culture grown in EMEM + MDM mixture (3:1 ratio) to log phase, all cells then removed by centrifugation followed by filtration and 1ml added to monolayer; IM 2755, medium from an interaction between *T. vaginalis* 2755 and Hela cells after Helas had been lysed, 1ml added; LYSATE, cells lysed into EMEM by sonication; MDM, 1ml MDM added to monolayer. Key to description of monolayer: 0, as control; I, looked unhealthy; II, some cells unattached; III, mostly unattached; IV, all cells unattached; V, all lysed.

Table 43. The Effects upon Trichomonad Cytopathogenicity of Preventing Contact with the Mammalian Cells

RATIO HELA CELLS: TRICHOMONADS	G3				G3 + MEMBRANE				2755 in vivo				2755 in vivo + MEMBRANE			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
200:1	49	00	69	00	49	00	22	00	49	76	133*	00	49	00	69	00
100:1	49	69	107*	00	49	00	69	00	133*	69	133*	00	69	00	69	00
50:1	83*	69	83*	00	83*	00	107	00	133*	49	133*	00	133	00	133	00
25:1	N.D.				N.D.				133*	49	107*	00	133	00	133	00

Experiments performed using Hela cell monolayers in well of tissue culture plates. ☐, no effect on monolayer; ▨, monolayer destroyed by trichomonads; ▩, monolayer destroyed but trichomonads also grew in monoculture.

Key as in previous tables.

Table 44: Effect of Preventing Contact between Cell Types on Trichomonad Pathogenicity

Experiment	Ratio Hela cells: Trichomonads	Appearance of monolayer + density of trichomonads after: 24 hrs	65 hours		90 hours	
		Monolayer	Monolayer	Trichomonads	Monolayer	Trichomonads
CONTROL	50:1	0	III	0	III	0
	25:1	0	V	5.4×10^5	V	2.3×10^5
	10:1	III	V	5.3×10^5	V	2.1×10^5
	5:1	III	V	4.9×10^5	V	O.G.
	2:1	V	V	5.1×10^5	V	O.G.
	1:1	V	V	4.5×10^5	V	O.G.
MEMBRANE	50:1	0	I	3.0×10^4	I	5.0×10^3
	25:1	0	I	9.3×10^5	I	6.0×10^4
	10:1	0	I	2.1×10^5	I	5.5×10^4
	5:1	0	I	1.1×10^6	I	1.1×10^5
	2:1	0	I	1.6×10^5	I	3.5×10^4
	1:1	0	I	1.3×10^6	I	1.2×10^5
MEMBRANE + LEUPEPTIN ($50ugml^{-1}$)	50:1	0	I	0	I	0
	25:1	0	I	5.5×10^4	I	1.0×10^4
	10:1	0	I	1.5×10^5	I	4.5×10^4
	5:1	0	I	2.2×10^5	I	5.5×10^4
	2:1	0	I	1.0×10^5	I	1.0×10^4
	1:1	0	I	3.0×10^4	I	5.0×10^4
MEMBRANE + DFMO (1mM)	50:1	0	I	0	I	0
	25:1	0	I	1.1×10^5	I	3.5×10^4
	10:1	0	I	1.3×10^5	I	3.0×10^4
	5:1	0	I	2.1×10^5	I	4.5×10^4
	2:1	0	I	4.9×10^5	I	5.5×10^4
	1:1	0	I	1.0×10^4	I	5.0×10^4

Table 44 (contd.)

	50:1	0	0
	25:1	0	0
T. vaginalis in monoculture	10:1	1.0×10^4	1.2×10^5
	5:1	5.0×10^4	1.1×10^6
	2:1	4.5×10^5	2.0×10^5
	1:1	9.5×10^5	O.G.

Hela cell monolayers in the wells of a tissue culture plate were used. Key to description of appearance of monolayer: 0, as control; I, looked unhealthy; II, some cells unattached, most still attached; III, most cells unattached; IV, all cells unattached; V, all cells lysed. *Trichomonas vaginalis* G3 was the trichomonad line used.

4.0. DISCUSSION

4.1. MODELS OF THE HOST-PARASITE INTERACTION

Since the first experiments performed by Hogue in 1943, the interaction of trichomonads with mammalian cells has been used many times to investigate the role of this pathogen within the host. However, the great variety of methods used by different authors had caused difficulties in the interpretation and comparison of results. For example, frequently different mammalian cell lines have been selected, and many of these proved to have differing susceptibilities to trichomonads as reported by Pindak *et al.* (1986). Other methodological differences have included the medium used, the ratio of trichomonad to mammalian cell numbers and the duration of experiments. Previous workers used medium in which both trichomonads and mammalian cells prospered, and used large numbers of trichomonads, the interaction taking place over only a number of hours. My own experiments were designed to introduce a low number of trichomonads to mammalian cell cultures in a medium which would allow trichomonad survival for a short time but not support growth to any great extent (Figure 6). Thus trichomonads only multiplied if cytolysis occurred. Consequently, there were clear differences between the interactions which exhibited cytolysis by the trichomonads and those that did not. If no cytolysis occurred, mammalian cells remained healthy and trichomonads died within a few hours; when cytolysis occurred the mammalian cells totally disappeared and the trichomonads multiplied, surviving for many days before finally overgrowing. In other words, to an extent, this was an 'all or nothing' effect (Figure 4), although there were differences in the time for cell death to occur under different conditions. As shown in Figure 4 there was a minimum or threshold number of trichomonads needed to investigate an effect on the mammalian cells. This parameter was exploited to compare the numbers

of trichomonads of different strains needed to cause cytolysis as a measure of their virulence towards mammalian cells.

The mammalian cell lines used in my experiments were chosen initially for their ability to proliferate rapidly and ease of growth. All the cell lines I tested were lysed by trichomonads and their sensitivity differed very little, although the macrophage-like cell line and primary chick embryo cells appeared to be lysed a little more slowly than some of the other lines tested (Table 7). Myeloma cells were found to proliferate quickly but HeLa cells attached more strongly to the flasks used and were larger and therefore cytolysis was more easily monitored using an inverted microscope. HeLa cells were also thought to be most highly appropriate because of their origin as cervical epithelial cells.

As previously mentioned, a medium was needed which would support growth of the mammalian cells and allow survival but not growth of trichomonads. A medium with these characteristics was obtained by mixing the trichomonad medium, MDM, with the appropriate recommended medium for the mammalian cell line used (Table 3). To prevent trichomonad growth in the resulting mixtures it was necessary to use a low initial trichomonad density but also to inoculate sufficient trichomonads so that they would not die before cytolysis could begin (Table 2a,b). Many mammalian cell densities were also tested to find which would allow a monolayer to form and be complete by the end of the experiment (Table 2a,b). Many of the experiments were performed in tissue culture plates; because I thought it was likely that the oxygen concentration may affect the survival of trichomonads and also the interaction with the mammalian cells the effects of using different volumes of medium in tissue culture wells was investigated. The hypothesis was that the deeper the culture, the lower the oxygen concentration would be at the bottom. Indeed, a correlation was

observed between the number of trichomonads necessary to allow growth and the volumes used (Table 5). Consequently, what appeared to be the most appropriate volume was chosen for experiments.

Trichomonads were routinely grown in medium containing 10% horse serum. Different growth rates were observed when other sera were used and therefore the effects of using se ra of various sources in the interaction medium were investigated. The results indicated, however, that the effects of sera on the interaction mirrored the effects on trichomonad growth in MDM alone and did not appear to have any significant effect on the interaction itself (Table 6).

The model, therefore, was found to be fairly easy to use although a number of days were necessary to prepare the monolayers, hence experiments had to be planned in advance. However, results were obvious, quick and easy to read using an inverted microscope. The model was found to be suitable for use in testing various potential inhibitors of the trichomonad/mammalian cell interaction (Tables 31 and 34), as well as for comparing the interaction between different trichomonad lines and mammalian cells (Table 16). The model was also used for investigating the hypothesis that trichomonads have a pathogenic effect on mammalian cells through mechanical means (Table 44).

Since Honigberg reported 'the subcutaneous mouse assay' in 1961 the growth of trichomonads subcutaneously in mice has been used widely for investigations of the interaction of host and parasite and also for evaluation of antitrichomonal compounds *in vivo*. My own experiments, however, involved certain modifications of this basic method. The addition of agar to the inoculati on medium MDM was found to be necessary for reproducible lesion growth rates to be obtained (Figure 7). Honigberg used BBL thioglycollate medium which already

contained agar. It is unknown why agar is necessary, but possibly it is required in localization of the parasites at the injection site. Several strains of mice were tested for their susceptibility to *T. vaginalis* when inoculated subcutaneously (Figure 8). Interestingly, the strain used by Honigberg, C57/BLACK were least susceptible in my experiments, no lesions were produced by the trichomonads in these mice. The most susceptible strain tested was found to be Balb/c and hence this was used for further experiments. In order to use minimal numbers of mice it was important to ascertain whether a subcutaneous trichomonad infection would produce any lasting effect on the mouse or whether mice could be reused after self-curing and several weeks recovery period. A comparison of infections in naive and previously infected mice revealed that mice could indeed be reused, meaning that fewer mice were used overall (Figure 9).

Honigberg (1961) reported a correlation between the growth of lesions caused by subcutaneous inoculation of *T. vaginalis* into mice with symptoms caused by the same strains in patients and hence believed this model to be a good indicator of virulence levels. However, he made no attempt to correlate the size of lesions to the number of trichomonads they contained. My own experiments did, however, show that there was a correlation between lesion size and number of trichomonads contained within lesions, up to a maximum trichomonad number (Figure 10).

Although results were generally fairly reproducible, certain experiments showed large variations between the rates of lesion growth of different groups of mice; rates within groups were fairly similar. Various experiments were undertaken to attempt to alleviate this problem. Mice used for experiments were normally taken when they were aged about 10 weeks. This was, however, only an approximate age and mice used would have differed in age to an extent. Nevertheless, a

comparison of rates of lesion growth in mice 2 months, 5 months and 7 months old revealed that within this range, age made little difference to the rate of lesion growth (Figure 11). Trichomonads were routinely injected during log phase of growth, although again, there was some variation as to whether mid-log phase or late-log phase trichomonads were used. A comparison of lesion growth rates resulting from inoculation of log-phase and stationary phase trichomonads, however, showed no difference, whereas lesions due to the inoculation of trichomonads from lag phase appeared to grow a little more slowly during the later days of the experiment (Figure 12). It may have been that these lesions were actually growing a little more slowly throughout the experiment, but when lesions are very small it is difficult to measure small differences between them. Indeed, one of the problems associated with this model is the difficulty in accurately qualitating growth especially in the initial stages. Heat-inactivated horse serum from the same source was routinely used in medium for all trichomonads used for subcutaneous inoculation, however different batches were used during different periods of time which may have varied slightly in composition. To see if this may be a problem, an experiment comparing rate of growth of lesions when trichomonads were grown up in, and injected, in media containing different sera was performed (Figure 13).

Lesions grew at slightly different rates in the presence of the various sera. However, as such widely different sera produced such a small variation in lesion growth rate, it is unlikely that the difference in composition of batches of the same sera would have any significant effect on lesion growth.

Hence, although this model did prove of some use in comparing different trichomonad lines (Figure 14) and for testing various

potential inhibitors (Figures 21 and 23), I believe its use to be limited and its relevance to the symptoms of the patient to be doubtful. The method is expensive and time consuming and the results are not easy to read except when large differences in lesion volumes are evident.

Many attempts have been made in the past to develop an intravaginal model of trichomoniasis using various mammals. The most successful appear to have been in monkeys (Johnson *et al.*, 1950) and guinea pigs (Kazanowska, 1962) with some success in rats (Meingassner & Mieth, 1976). Clearly, however, the most economical and convenient mammalian model for such an infection would be the laboratory mouse. Unfortunately, the few attempts made previously to infect vaginas of mice with *Trichomonas vaginalis* were not very successful (Cappuccinelli *et al.*, 1974). Previous workers have suggested that ovariectomy of laboratory rodents followed by hormone treatment were necessary to obtain a trichomonad infection (Newton *et al.*, 1960; Cavier & Mossion, 1956a,b; Combescot *et al.*, 1959; Asami, 1956; Meingassner & Mieth, 1976; Cappuccinelli *et al.*, 1974).

My own investigations revealed that although a greater infectivity rate was observed in spayed mice, intact mice could also be infected and retain the infection for as long as 92 days (Table 23). Spaying mice proved to be time consuming with an unacceptable mortality rate and as I found that it was not essential for infection, intact mice only were used for further experiments. Various numbers of trichomonads were inoculated into mice vaginas but numbers in excess of 10^5 organisms did not appear to improve infectivity rate or length of infection (Table 9). It is probable that a minimum number of trichomonads is necessary to establish an intravaginal infection but that any number above this minimum makes no difference to the infection.

The condition of the mouse vagina at the time of infection was monitored. The pattern of infection rate appeared to follow the oestrous cycle, with the highest infectivity rates being achieved with mice in early pro-oestrus and the lowest when the mice were at oestrus itself (Table 10). Intermediate infectivity rates were achieved with other vaginal conditions. It is unknown at present why this pattern occurs. It is thought that during the oestrus cycle there are many variations within the vaginal environment, e.g. fluctuation of glycogen levels, presence and consistency of vaginal fluid, variation in quantity and quality of the bacterial flora, thickness of the epithelium, proliferation and delamination; and the presence of various amounts of oestrogen circulating in the blood. Glycogen is formed in considerable amounts in the vagina of mice after keratinization. Gregoire and Guiness (1968) studied female hamsters and found that glycogen was maximal on the day preceding ovulation, followed by a rapid decrease on subsequent days; ovariectomy resulted in a decrease in glycogen. From the small amount of evidence available, therefore, there is no correlation between glycogen concentration and infectivity rate as shown by our results.

The consistency of the vaginal fluid alters during the oestrus cycle (Allen, 1922) of the mouse, ranging from very viscuous during meta-oestrus II and di-oestrus then gradually drying up almost completely by oestrus and meta-oestrus I. The lower infectivity rates of the cycle appeared to occur whilst the vagina was rather dry. The quantity of bacteria in the vagina of mice appears to reach a maximum just prior to oestrus and remains high throughout oestrus and meta-oestrus I, decreasing rapidly with the influx of leucocytes which occurs in met-oestrus II (Larsen *et al.*, 1977). Thus it appears that high numbers of bacteria appear to correlate with the lower

susceptibility of mice to *T. vaginalis*. The part played by oestrogen in trichomonad infections is unknown, although Honigberg (1978) reported that high oestrogen doses adminstered to mice restricted the rate of subcutaneous lesion growth at a different site. However, our results did not indicate any direct correlation between infectivity and oestrogen levels except that some oestrogen is necessary to establish the infection. The vaginal epithelium changes rapidly during the oestrus cycle, varying from a time of quiescence at dioestrus when the epithelium is fairly thin, to rapid proliferation during pro-oestrus. The epithelium is thick and cornified during oestrus and begins to delaminate during meta-oestrus when the lumen of the vagina is filled with cell fragments and heavy leucocyte infiltration begins. Maximum infectivity appeared to occur during the period of rapid proliferation and the least infective time was during oestrus itself when the epithelium is thick and cornified. It is likely that these great changes in the microenvironment of the vagina of the mouse have an effect on the numbers of trichomonads present during an intravaginal infection. It is known, also, that the numbers of trichomonads present in the vagina of a patient suffering from trichomoniasis change at different times during the menstrual cycle with greatest infection occurring around the time of the menses (Kurnatowska, 1958) although no explanation has so far been given. Hence, from the information currently available it is not possible to understand why mice are more infective at certain stages of the oestrus cycle, but from a practical point of view it is best to inoculate mice at the proestrus stage.

It was found that some mice gave a negative wash-out whilst still carrying trichomonads in the vagina (Table **24**). Thus mice were continually tested for trichomonads until many consecutive wash-outs were negative. One of the drawbacks of this method is the way in which

infections are detected; it is laborious, not totally accurate and only qualitative. The state of the vagina was also monitored at the time that wash-outs were taken from mice known to be infected with *T. vaginalis* (Table 10). Again there appeared to be variation in infection rate with different stages of the cycle, the highest rate of positive wash-outs was found with mice at oestrus and meta-oestrus, a time when the vaginal epithelium is thick and beginning to delaminate. The lowest number of positive wash-outs was found during dioestrus and proestrus where the thin epithelium begins rapid proliferation. It is possible that trichomonads attach more strongly whilst the epithelium is not changing or growing and are then sloughed off with the cornified cells after oestrus and so are more easily detected. This would mean that many false negative readings would be found. However, the effects of changing glycogen levels, bacterial levels or mucus levels may also be involved. There are no previous reports of investigations into numbers of trichomonads present during infections of any animal. Although a recent study has attempted to estimate quantities of trichomonads present in the infected human vagina (Philip *etal.*, 1988).

The effects of treating mice with oestradiol-17B-cypionate in various ways were tested. Various concentrations of the hormone were injected in combinations of subcutaneous and intraperitoneal inoculations. All mice reached oestrus within 3 days (Table 11). Meingassner & Mieth (1976), who devised the method from which I developed my own, injected half the oestrogen subcutaneously and half intraperitoneally. I found, however, that giving the oestrogen by the single or dual route appeared to make little difference to the susceptibility of the mouse to *T. vaginalis* and, as injecting the oestrogen all by the subcutaneous route was simpler, this method was

adopted. Miss D. Markham, in our laboratory, later confirmed that although oestrogen was indeed necessary for infection, no difference in infectivity occurred when trichomonads were inoculated one or two days subsequent to oestrogen administration. Thus it seems that oestrogen is required but the precise timing is not crucial.

Many authors had stated that permanent oestrus is necessary to achieve trichomonad infections in the vagina of laboratory rodents (Cavier & Mossion, 1956a,b; Asami, 1961; Michaels *et al.*, 1962). However, following the protocol advocated by these authors of injecting spayed mice with the long-lasting oestrogen, oestradiol-17B-cypionate, our results showed that mice did not at any time reach permanent oestrus even after multiple injections of the hormone (Table 12a,b). Nevertheless, it was possible to infect these mice with trichomonads and the infection was maintained for as long as 130 days (Table 24).

Why the oestrogen is necessary is unknown. It is possible that it is necessary to produce certain conditions within the mouse vagina at the time of infection as previously discussed, however, it may be that the oestrogen has some unknown effect, e.g. on the mouse immune system.

The course of infection was monitored by determining the change in parasite numbers with time and the density was found to be surprisingly consistent around $1\text{-}2\text{x}10^4$ ml^{-1} (Table 13). In one case the number appeared to drop off dramatically approximately 20 days prior to the infection d ying out, indicating the decline of the infection although reasons for the sudden decline are unknown. Counts used for monitoring infections were estimates of the numbers present and therefore inaccurate, therefore, although fluctuations were observed in the numbers of parasites present during an infection it is unknown if these counts represent the situation actually in the mouse

vagina. The lack of efficiency of using wet smears for detection of trichomonads was demonstrated as many cultures indicated that a large number of trichomonads were present in the vagina whilst wet smears were negative. The number of trichomonads seen in wet smears did not appear to correlate with the density of the infection in the vagina (Table 13).

The development of a sensitive method of detecting trichomonad proteinases by my collaborators Dr Barbara Lockwood and Dr Michael North of Stirling University (Lockwood *et al.*, 1987, 1988) led to attempts to use this method to detect the presence of trichomonad proteinases in vaginal wash-outs of infected mice. Although wash-outs of particularly highly infected mice showed that this is possible, the method appeared not to be sensitive enough to detect trichomonads in the majority of infected mice.

Hence the intravaginal model, despite the problems with reading the results, proved to be efficient and useful and was subsequently used to compare trichomonad lines and to test potential inhibitors of the host-parasite relationship.

The three models developed, the subcutaneous mouse model, the intravaginal mouse model, and the tissue culture model were then used to study the host-parasite interaction in trichomoniasis, and in so doing compared the usefulness of each of the models. The model systems I developed were used to compare and assign virulence levels to a number of fresh isolates of *T. vaginalis*. Using the mammalian cell model virulence appeared to differ widely, and results proved to be fairly consistent (Tables 15 and 16). The subcutaneous mouse model also showed variation between isolates, although there was much overlapping of lesion growth rates and it did not prove to be easy to use this method to distinguish between isolates with similar

virulence, that is, the method proved rather insensitive (Figure 14, Table 15). The intravaginal model showed differences among isolates in two ways, the number of mice which were infected at all by the trichomonads, and the duration of the infection (Table 18'). Correlation between virulence levels assigned in each of the three models was very low, with a greater comparison between the intravaginal and subcutaneous models than either of these with the mammalian cell model (Table 15).

There was also little correlation between the axenic growth rates of isolates *in vitro* and their growth in the models used. Although details of clinical symptoms in patients from whom the trichomonad lines were requested from Glasgow Royal Infirmary these were unfortunately unavailable.

The lack of correlation between trichomonad growth in the *in vitro* and *in vivo* models is perhaps to be expected as many factors which probably have an effect on trichomonad growth *in vivo* are not present in the tissue culture model. The lack of correlation between growth of trichomonads in the subcutaneous mouse model and the intravaginal mouse model is interesting as, in the past, the subcutaneous model has been used to measure virulence of trichomonad lines and to test potential inhibitors of the host-parasite interaction under the assumption that this model mirrors the effect of trichomonads in the human vagina. Although, it is unknown whether the growth of trichomonads either in the subcutaneous model or intravaginal model, correlates with growth and virulence in the patient my results suggest that the subcutaneous model should be investigated more thoroughly before its use in this way.

Since the development of the subcutaneous mouse assay in 1961 (Honigberg, 1961), it has been used by some workers to show that there is an attenuation of virulence of trichomonads with prolonged axenic

in vitro cultivation (Kulda & Honigberg, 1969), although there has been some dispute over this (Dohnalova & Kulda, 1975; Alderete & Gavza, 1985). My own experiments on isolates which had been cultivated for many months *in vitro* and the same isolates which had been cultured *in vivo* intravaginally in mice, varied with the model used to test them. The interaction of these isolates with mammalian cells did not reveal any change of effect (Table 21). There was rather large variation of subcutaneous growth rate with time, although a general trend of reduction in rate was observed (Figs 15 and 16). There was, however, a definite reduction in infection rate in the mouse vagina and reduced length of intravaginal survival was seen when an isolate was cultured *in vitro* for over 14 months (Fig 17, Table 22). No such change occurred when the same isolate was passaged *in vivo* intravaginaly in mice for a similar length of time (Tables 23, 24 , 25; Figures 17, 18). Thus my results indicate that trichomonads do lose infectivity when cultured for prolonged periods *in vitro* and that this loss of infectivity could be prevented by growing the parasites *in vivo* intravaginally in mice. The key changes that occurred are unknown, but my work has provided the basic model required for this to be studied in detail. Changes in antigenic structure, the structure of the outer membrane and biochemical changes could all be studied to give some ideas as to what, in fact, gives the trichomonads their pathogenic ability.

Advantages and disadvantages were apparent with each of the model systems developed and used. The mammalian cell model was an improvement on previous similar models as the growth of trichomonads was totally dependent on their cytolysis of the mammalian cells and hence must be a direct effect rather than an effect of a large accumulation of metabolic by-products caused by 'overgrowing' of

trichomonads. Other models reported suffer from this problem, although it has been claimed by many that they are useful. This 'all or nothing' effect of my own model taking place over a number of days rather than several hours, as used by previous authors, allowed a very simple and direct method of analysis. Both trichomonads and mammalian cells were clearly observed through an inverted microscope and the interaction could therefore be monitored continuously throughout.

The *in vitro* system was very easy to manipulate to investigate aspects of pathogenicity e.g. in the addition of inhibitors to the interaction and blocking of attachment. However, the disadvantages of this model included problems with contamination by yeast cells, and the need for tissue culture facilities. Also inherent differences between experiments may account for some variation, e.g. differences in serum batches, there may well have been unequal diffusion of gases, and difficulty in ensuring an equal mixture of cells by shaking the wells of the tissue culture plate.

Controversy has existed over the reliability of the subcutaneous mouse model in assigning virulence values which relate to the patient (Alderete, 1983). I found small differences between growth rates of lesions caused by different isolates but there was relatively wide variation between experiments which could not be accounted for by serum batch or growth phase of the flagellates. This clearly makes interpretation of the results difficult. The environment of the *T. vaginalis* when growing subcutaneously is clearly very different from that when in vagina, so must be of limited value. Nevertheless, the model has some clear uses, for example, when testing drugs the model does give some information on pharmokinetics of drugs. Indeed, this model has been used with some success in the testing of antitrichomonal compounds (Meingassner & Mieth, 1976). However, I found it only of limited value in testing for metronidazole

sensitivity (see later).

The attempt to develop a mouse intravaginal model was an ambitious project as the few previous reports suggested that it would not be easy. The great advantages of our model include the use of mice which are economical both to buy, keep and to take up little space and are also easy to handle. An advantage of this model is that clear cut results are observed in that the infection is either positive or negative and comparisons are, therefore, very simple, although negative wash-outs can occur when a mouse is in fact still infected so care must be taken. The low infectivity rate we achieved was disappointing but not unsurprising when compared to the rate of infectivity in human experimental models (Asami & Nakamura, 1955). Another disadvantage is the lack of establishment of a true pathogenesis, most mice remain apparently symptomless, although the infection can last for several months. However, this model is a more appropriate one than the subcutaneous infection for the study of pathogenicity and other aspects of the host-parasite interaction (e.g. immune response, about which little is known) and also for anti-trichomonal drug testing.

Unfortunately, although virulence levels could be assigned to fresh isolates using each of the models developed, the results from the three did not correlate well. This suggests that a different aspect of the trichomonads and trichomoniasis is revealed by each of the models. It would appear that future studies of trichomoniasis should advance this point and clearly the results produced from use of just a single method must be interpreted with caution and should not be extrapolated to the human infection indiscriminately.

4.2. METRONIDAZOLE RESISTANCE

Reports of metronidazole resistant trichomonad strains have increased since 1966 when De Carneri found that a *T. vaginalis* strain, from a patient refractory to treatment with metronidazole, had a minimum lethal concentration of $12ugml^{-1}$ assessed *in vitro*. Much greater resistance has been found since e.g. Lossick *et al.* (1986) found eleven isolates that could survive up to $200ugml^{-1}$ of metronidazole.

Our own testing of isolates from patients refractory to metronidazole treatment sent to us by clinicians supported the view that lines of *T. vaginalis* resistant metronidazole to occur widely in Britain. There are not previous reports on this. This poses some problems for the clinician because of the risk of carcinogenesis and mutagenesis with increased doses of metronidazole. Although metronidazole has proved to be safe at the doses used routinely for treatment of trichomonads, the effects of ever increasing doses to combat resistant isolates is unknown. It has recently been reported that higher intravenous doses of metronidazole are effective in curing resistant cases (Dubrowski *et al.*, 1987). Nevertheless, there is no generally accepted treatment for resistant cases and there is a need for new drugs. However, at the moment no alternative to metronidazole is available so the models I developed were used to devise drug regimes for treating metronidazole-resistant lines.

The subcutaneous mouse assay was used to test the effects of metronidazole *in vivo* and clearly demonstrated that there are large differences between the concentrations needed to cure our laboratory strains, *T. vaginalis* G3, and the known resistant strain, IR78 (Figure 19). However, great variation was observed between experiments and various dose regimes and routes of administration were tested in an attempt to minimize this. It was found that the greatest effects of

metronidazole were achieved when the drug was administered in three doses at 2, 18 and 24 hours post-infection (Table 26). This may be because when three doses are given the level of metronidazole in the mouse's body remains high throughout the period when the trichomonad infection is becoming established. This indicates that the dose given may inhibit the establishment of infection rather than be directly lethal to the trichomonads themselves. More consistent results were obtained when metronidazole was administered intraperitoneally rather than orally probably due to metronidazole having an effect on gut flora as sometimes, with high doses, diarrhoea was observed which may flush out any metronidazole which has come out of solution in the gut. Also uptake may be more efficient or quicker when the intraperitoneal route was used. Although the subcutaneous model proved very variable, a difference between a metronidazole sensitive strain and a metronidazole-resistant strain was detectable (Figure 19). Differences in metronidazole sensitivity could also be detected using the intravaginal mouse model (Tables 27,28,29), in fact, similar metronidazole concentrations were needed to cure infections of the same isolate in subcutaneous and intravaginal infections. These levels were also reflected in the minimum lethal concentrations of metronidazole *in vitro* (Table 30). Thus, in this case, the various models gave similar results.

Hence, the *in vivo* models developed may be used to determine drug concentrations and regimes necessary to cure metronidazole resistant trichomonad infections and to test new drugs and compare these with the effects of metronidazole.

4.3. THE ROLE OF PROTEINASES IN PATHOGENICITY OF *Trichomonas vaginalis*

Proteinases have been found in many parasitic protozoa including *T. vaginalis* (Aissi *et al.*, 1983; Goldberg *et al.*, 1982; Coombs, 1982;

North, 1982; Coombs & North, 1983). The function of these enzymes in *T. vaginalis* has not been elucidated, however, a role in the host-parasite relationship is suggested by a similar role of proteinases in the parasitic protozoan, *Entamoeba histolytica* (Lushbaugh & Pittman, 1982).

Lockwood *et al.* (1987) found that trichomonad proteinases were of the cysteine type and that the different isolates collected had different enzyme patterns on haemoglobin and gelatin gels. Using these gels it was also possible to detect proteinase activity released into the medium by *T. vaginalis* isolates; extra-cellular enzymes present during log phase were qualitatively different from intracellular proteinases. As I have reported *T. vaginalis* proteinases could also be detected in the vaginal wash-outs of mice intravaginally infected with *T. vaginalis*. Thus, there was circumstantial evidence to suggest that proteinases may play a crucial role in the host-parasite interaction in trichomoniasis.

Our mammalian cell culture model was used to detect inhibition of cytotoxicity by the cysteine proteinase inhibitor, leupeptin. The addition of leupeptin to the model prevented the interaction of trichomonads and mammalian cells indicating that cysteine proteinases are involved in this interaction (Table 31).

The effect of leupeptin on the growth of subcutaneous abscesses in the mouse was examined. Although inhibition of lesion growth was not total a delay in lesion development occurred (Figure 21), thus indicating that whilst the concentration of leupeptin within the mouse was sufficiently high, trichomonad and lesion growth did not occur. However, once the concentration of leupeptin had dropped the trichomonads and hence, the lesion, were able to grow. This indicates that although cysteine proteinases may be involved in the host-

parasite interaction they are not essential for survival of trichomonads within this site of the host for some days at least.

The effects of leupeptin on the intravaginal infection of mice was also studied. Leupeptin administered intraperitoneally decreased the number of mice infected by the trichomonads; this was compared to the effect of metronidazole in this model which completely cured the infection. Again this outlines the fact that metronidazole is lethal to the trichomonads themselves through its toxicity whereas leupeptin is not toxic to the trichomonads at the concentrations used but deprives the parasites of cysteine proteinases which may be necessary for the host-parasite interaction.

Leupeptin, therefore, totally blocks cytotoxicity *in vitro* although its effects on *in vivo* models of the trichomonad infection are less dramatic suggesting that although cysteine proteinases are involved in the host-parasite relationship other factors may play an important role and the picture is more complex in the host.

The findings from the use of leupeptin show the value of the models developed as, axenically *in vitro* leupeptin has no effect on trichomonads whatsoever, probably because the complex medium, MDM, supplies in useable form, many of the factors needed for trichomonad growth which are not present in the environment of the host. Hence, testing compounds for potential use as drugs for treatment of trichomoniasis should not be limited to the use of trichomonads in axenic culture.

4.4. THE ROLE OF POLYAMINES IN PATHOGENICITY OF *Trichomonas vaginalis*

Linstead and Cranshaw (1983) revealed that the pathway of arginine catabolism in trichomonads involved the production of ornithine for biosynthesis of polyamines, the presence of the primary enzyme of this pathway, ornithine decarboxylase, confirmed this. The

exact metabolic function of polyamines is unknown although a role in the host-parasite relationship is suggested by reports of these compounds in vaginal washings from patients with trichomonads (Chen *et al.*, 1982). North *et al.* (1986, 1987) found that the major polyamine present in *T. vaginalis* and *T. foetus* was putrescine, the product of ornithine decarboxylase reaction. Treatment of *T. vaginalis* with α-difluoromethylomithine (DFMO), a specific irreversible inhibitor of ornithine decarboxylase, reduced the level of intracellular ornithine decarboxylase activity, yet growth axenically *in vitro* was only slightly affected.

The ornithine decarboxylase activity of fresh isolates was assayed by Dr Michael North of Stirling University (Table 15). A wide variation in activities was observed, although unfortunately these did not appear to correlate with virulence as determined by our *in vivo* or our *in vitro* models.

Experiments with the ornithine decarboxylase inhibitor, DFMO, in the mammalian cell model revealed, as did leupeptin, that cytotoxicity was totally inhibited in the presence of this compound (Table 34), this also was shown to be an effect on the interaction rather than on the cell types alone (Figure 22).

An effect of DFMO was observed on the growth of subcutaneous lesions in mice, although, once again inhibition of lesion growth was not total. A delay in lesion growth of approximately 3 days was observed as the maximum effect (Figure 23), once the lesions did appear, however, they grew at a similar rate to control lesions. The most consistent effects with DFMO were observed when the inhibitor was administered orally to the mice in multiple doses (Table 35).

The effect of DFMO on the intravaginal infection was studied. Results were similar to those found for leupeptin in that although metronidazole was shown to completely halt infections, no inhibition

was observed when mice were treated similarly with DFMO (Table 36a,b,c).

It is, therefore, likely that polyamines have some role in the trichomonad host-parasite relationship as cytotoxicity *in vitro* is prevented by the ornithine decarboxylase inhibitor, DFMO, however, the role is more complicated than may first appear as suggested by the results in the *in vivo* models.

Again, there appears to be some potential for DFMO as an anti-trichomonad agent particularly due to its low toxicity and tests in humans have already shown some success against trypanosomiasis.

4.5. THE ROLE OF METHANETHIOL IN THE TRICHOMONAD HOST-PARASITE RELATIONSHIP

Methanethiol is produced by *Trichomonas vaginalis* as a result of methionine catabolism (Thong *et al.*, 1987). This compound is thought to be of importance in some plants for its antifungal and antibacterial qualities as a deterrant against invading pathogenic microorganisms. As the role of methanethiol in trichomonads was unknown, we attempted to discover if it played a role in the interaction of trichomonads with other microorganisms in the vagina as well as host cells and so was important in aiding establishment of the parasite.

It was hypothesized that methanethiol might aid infection of the host by trichomonads by repelling competing bacteria and fungi in the vaginal environment. Studies were undertaken using both an inhibitor of methanethiol production and methionine, the presence of which stimulates the production of methanothiol.

The effects of both of these compounds on the interaction of *T. vaginalis* with mammalian cells showed that neither one inhibited or stimulated cytolysis (Table 37). Inhibition of cytolysis by

propargylglycine was achieved when high levels of the inhibitor were added but at these levels the inhibitor also has an effect on trichomonad growth alone and hence the inhibition of cytolysis in this case was probably an indirect effect of this inhibition of trichomonad growth. Using the subcutaneous mouse model, neither methionine nor propargylglycine appeared to have any appreciable effect on lesion growth (Figure 24).

Overnight incubation of trichomonads in the presence of propargylglycine and methionine prior to infection intravaginally in mice did, however, show some interesting results. Although methionine appeared to have no effect on the infection rate, propargylglycine greatly inhibited the percentage of mice that became infected by *T. vaginalis* and those mice which did become infected lost the infection more rapidly than control mice (Table 39a,b,c,d). However, the inhibition of infectivity by propargylglycine did not occur when mice were treated with the compound instead of preincubation of trichomonads. Only a small dose was used in the experiment, however, which may account for this negative result. Clearly more experiments should be carried out to follow up these findings.

Hence, results suggest that methanethiol doses have a role in the host-parasite relationship, but it is one that can be detected only using the intravaginal mouse model and therefore supports the hypothesis of an involvement in repelling competing micro-organisms rather than a role in cytotoxicity.

4.6. MECHANISMS INVOLVED IN CYTOPATHOGENICITY OF TRICHOMONADS TOWARD MAMMALIAN CELLS

Controversy has existed for many years as to the nature of pathogenicity of *T. vaginalis* towards its human host. There have been those who advocate the theory that attachment or contact is necessary

for *T. vaginalis* to lyse mammalian cells and those who believe that cytotoxicity is mediated by an unknown diffusible, toxic substance. There are also a few authors who have stated that the most probable answer is that a combination of both these methods is involved as discussed in Section 1. Our mammalian cell model was used to investigate the mechanisms involved in cytotoxicity.

The effect of factors secreted from trichomonads in the presence of mammalian cells was tested in the mammalian cell model. Table 40 shows that filtered medium in which trichomonads had lysed mammalian cells was capable of cytolysis and that leupeptin and DFMO delayed this effect. Although the trichomonad medium MDM alone was detrimental to mammalian cells the combination used for interaction experiments supported mammalian cell growth. Medium in which trichomonads had grown axenically also had an effect on the mammalian cells. Lysates of all but the most highly virulent trichomonad strain tesed had no effect on mammalian cells and lysates of HeLa cells also had no effect on whole cells of the same type (Table 40).

The data, therefore, indicate that trichomonads do secrete some toxic substances while growing both in the presence and absence of mammalian cells. Pindak *et al.* (1986) asserted that although a toxic substance was secreted by trichomonads this was too weak to be detected unless used in a concentrated form which they called 'cell-detaching factor (CDF). We tested our own CDF made in the same way as described by Pindak *et al.* (1986) (Tables 42,41). CDF did in fact have an effect on mammalian cells although this appeared no stronger than that of non-concentrated media in which trichomonads had grown. Again leupeptin and DFMO had no effect on cytolysis caused by CDF.

The effect of physical separation of the trichomonads and mammalian cells in the mammalian cell model, by a membrane allowing

free diffusion of molecules was studied. Table 43 shows that the separation of *T. vaginalis* G3 from mammalian cells by a membrane totally inhibited cytolysis. Again with *T. vaginalis* 2755 cytolysis was totally inhibited although trichomonads still overgrew at higher initial densities. Table 44 shows that although trichomonads do grow to a relatively high density within millipore cells their effect on mammalian cells is minimal.

The data suggest that although trichomonads do secrete some toxic substances which aid cytolysis of mammalian cells, contact is of great importance also. The fact that trichomonads grow to a higher density when physically separated from mammalian cells than when in totally axenic culture suggests that the flagellates do again something from the presence of mammalian cells and this view is supported by the observation that in this situation mammalian cell monolayers suffer some damage although are not totally disrupted.

5.0. REFERENCES

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