

Studies on the Growth
of *Theileria* infected Bovine Cells
in Immunodeficient Mice

by

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DECLARATION

I declare that this thesis was composed by myself, and the work it contains is my own.

ABSTRACT

The experiments described in this thesis investigated the growth of bovine cells infected with *Theileria* sp. macroschizonts in various strains of immunodeficient mice. It was hoped that it would be possible to create a system for the growth of *Theileria* infected cells *in vivo* without using bovine hosts. Previous work had shown that *T.parva* infected cells would establish as subcutaneous tumours in irradiated Swiss and nude mice (Irvin et al, 1977: Veterinary Parasitology 3, p.141-160). Most of the work in this thesis concerned the related parasite, *T.annulata*.

T.annulata infected cells were found to grow as subcutaneous tumours in irradiated Balb/c, irradiated NIMR and Balb/c nude, and unirradiated C.B-17 scid (severe combined immunodeficiency) mice. Infected cells failed to establish in C57 beige mice, with or without irradiation. The growth and survival of the tumours was dependent on the degree of immunosuppression of the host, and the size of the cell dose administered. In Balb/c mice, *T.annulata* tumours regressed as mice recovered from irradiation. Analysis of lymphocyte subsets using a fluorescence-activated cell sorter (FACS) showed differential susceptibility of B-cells, T-helper and cytotoxic T-cells to a sublethal dose of ionising radiation (4Gy). The numbers of these lymphocytes increased more rapidly after irradiation in tumour bearing mice than in those without tumours.

In irradiated (4Gy) Balb/c nude and scid mice, subcutaneous *T.annulata* tumours failed to regress, despite the development of general haemorrhage and central necrosis. In scid mice, high doses (2×10^7) of *T.annulata* infected cells injected intraperitoneally gave rise to ascites. However, low doses (2×10^6 cells) did not. The presence of macroschizont infected cells in the peritoneal cavity was accompanied by a proliferation of macrophages. Several lines of evidence indicated that natural killer (NK) cells were not effective against *T.annulata*-infected cells in scid mice, but that macrophages were capable of controlling and eliminating the cells, particularly in the peritoneal cavity.

In all the strains of mice examined, subcutaneous *T.annulata* tumours appeared to be damaged by natural immune mechanisms (macrophages and neutrophils) leading to haemorrhage and necrosis. However, the ability of the mice to completely reject the tumours depended on the presence of T and B- cells. Tumour Necrosis Factor was not detected in serum, or in tumour extracts. Attempts to induce tumour rejection

using a preparation of rabbit TNF were not successful. Attempts to affect the growth of *T.annulata*- infected cells, injected i/p into scid mice, with human alpha and gamma interferons (HuIFN- α , HuIFN- γ), were also unsuccessful. The neutralisation of endogenous murine IFN-g with a monoclonal antibody allowed increased growth of *T.annulata* and *T.parva*-infected cells in the intraperitoneal site in scid mice. Of the mouse strains examined, the scid mouse was the most favourable host for *Theileria*-infected cells. Attempts to establish uninfected bovine cells in scid mice were not successful, and these results cast doubt on the use of scid mice as hosts for uninfected cells.

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CHAPTER 1

INTRODUCTION

1.1 Aims of the project

The aim of this project was to investigate the growth of *Theileria*- infected bovine cells in murine hosts. It was hoped that this would lead to the development of a "mouse model" for theileriosis, so that some future *in vivo* studies on the parasitised cells could be carried out without using cattle, which are large, expensive and inconvenient to work with in large groups. Most of the work in this thesis concerned *Theileria annulata* infected bovine cells, but some experiments were conducted with *Theileria parva* infected cells to compare the behaviour of the two parasite species in the mouse host.

1.2 Literature Review

1.2.1 Taxonomy, distribution and life cycle

Theileria spp. are protozoan parasites belonging to the phylum Apicomplexa. All the species so far described infect ruminants, and are transmitted by ticks. The two species of major importance are *Theileria annulata* (Dschunkowsky and Luhs, 1904), which infects cattle in North Africa, the Middle East and India, and *Theileria parva*, occurring in Eastern and Southern Africa. The diseases caused by these parasites are commonly referred to as tropical theileriosis and East Coast fever respectively (Uilenberg, 1981). Indigenous cattle breeds and buffalo tend to be resistant to infection, so theileriosis is a particular problem for cattle which are imported into endemic countries to improve milk and beef yields.

Although difficult to distinguish morphologically, the two species can be separated on several grounds. Clinically, *T.annulata* infection is associated with a less acute disease and lower mortality rate than *T.parva*. Immunologically, sera against the different parasite stocks do not cross react; there are differences in the immune response to the two species, which will be discussed later; *T.annulata* infections can

be transmitted by the passage of infected blood (Sergent et al, 1924) or allogeneic infected cells, while *T.parva* cannot. *T.parva* schizont infected cells carry bovine T-cell markers, while *T.annulata* infected cells do not, implying that the two species infect different mononuclear cell subsets (Spooner et al, 1988). The two species are transmitted by different genera of ticks (*Rhipicephalus appendiculatus* for *T.parva*, *Hyalomma* sp. for *T.annulata*) and their geographical ranges do not overlap.

Infection of cattle with *Theileria* spp. is initiated when an infected nymphal or adult tick bites the animal. Sporozoites enter the bloodstream and rapidly infect mononuclear cells. The sporozoite first develops into a trophozoite and then into a multinucleate macroschizont. This causes transformation of the infected cell, which undergoes repeated mitosis. Each division of the host cell is accompanied by simultaneous division of the parasite (Hulliger et al, 1964). A population of infected cells accumulates, first in the lymph node draining the infection site and subsequently as disseminated tumour-like foci both in peripheral lymph nodes and in other organs. The erythrocytic phase of the life cycle begins when macroschizonts develop into microschizonts, which produce merozoites. The infected cell finally bursts releasing the merozoites to infect erythrocytes. Piroplasms develop in the infected red cells, and when infected blood is ingested by a suitable tick gametogony occurs. The cycle is completed when the infected tick, after moulting, takes its next blood meal (Irvin and Cunningham, 1981).

1.2.2 Pathology and immunology

The pathology of *Theileria* infections is closely related to the unusual life cycle of the parasite. The typical lesion of both tropical theileriosis and East Coast fever is a cluster of multiplying schizont infected cells which becomes haemorrhagic and then necrotic. These "tumour-like foci", usually presenting as petechial haemorrhages, have been recorded from a range of tissues by various authors. These include: the lymph nodes, thymus and spleen; the epicardium and endocardium of the heart; the mucous membrane of the abomasum; the kidneys, liver and adrenal glands; the lungs and subcutaneous tissue (Dschunkowsky and Luhs, 1904; Sergent et al, 1924; Cowdry and Danks, 1933; De Kock, 1957; Neitz, 1957; Srivastava and Sharma, 1981). There does not appear to be any distinct difference between *T.annulata* and *T.parva* infections in the distribution of these foci. These lesions are found initially in the local lymph node draining the infection site, which becomes oedematous and

increasingly haemorrhagic. The other lymph nodes become involved as the disease progresses (DeMartini and Moulton, 1973; Eisler, 1988). After initial proliferation, the affected lymph nodes become depleted of lymphocytes (Cowdry and Danks, 1933; De Kock, 1957).

Theileria infections are usually accompanied by pyrexia, cachexia, loss of appetite and diarrhoea (Dschunkowsky and Luhs, 1904; Neitz, 1957). Anaemia is more marked in *T.annulata* than *T.parva* infections, and is a major cause of death from tropical theileriosis. In *T.parva* infection the multiplication of infected lymphoid cells appears to be more important as a cause of mortality. Anaemia is probably caused by the destruction of infected erythrocytes, and is accompanied by icterus.

Cattle which recover from tropical theileriosis and East Coast fever show long-term immunity to reinfection (Sergeant et al, 1924; BurrIDGE et al, 1972). Cattle can be vaccinated against *T.annulata* infection with live infected cells attenuated by prolonged passage *in vitro* (Pipano, 1981). Vaccines of this type are in use or under development in Israel, Iran, Turkey, Morocco and India (Pipano, 1981; Hashemi-Fesharki, 1988; Sayin, 1986; Ouhelli, 1986; Singh, 1986). This vaccination scheme is not effective against *T.parva* unless the immunising cell line is of the same BoLA type as the recipient (Dolan et al, 1984). Cattle can be immunised experimentally against *T.parva* by infection with virulent sporozoites combined with drug treatment (Radley, 1981). Although effective in field trials, this scheme has not been used on a large scale (Irvin and Morrison, 1987; Dolan, 1987).

In the case of *T.parva*, cytotoxic cells specific for autologous infected cells have been identified in infected or immune cattle (Emery and Kar, 1983) and in *in vitro* cultures of peripheral blood mononuclear cells (PBMC) from immune cattle (Pearson et al, 1982; Emery and Kar, 1983; Goddeeris et al, 1986). This specific cytotoxic activity is directed against infected cells bearing the autologous bovine lymphocyte antigen (BoLA)-A type, which is the bovine MHC class I antigen (Amorena and Stone, 1978). Cytotoxic cells generated *in vitro* carry the BoT8 antigen, corresponding to the human CD8+ cytotoxic T- cell marker (Goddeeris et al, 1986; Morrison et al, 1986). Cells with the BoT4+ phenotype have also been generated *in vitro* from cultures of immune PBL. These cells probably represent a T-helper subset.

Emery et al (1981) reported non-specific cytotoxic activity in leucocyte cultures from cattle with lethal *T.parva* infections. Lysis of both allogeneic infected cells and xenogeneic cells (the murine lymphoma cell line YAC-1) was observed in *in vitro*

assays. This activity was greatest in cultures from moribund calves, and in animals which recovered, non-specific cytotoxicity disappeared after elimination of the parasite. Calves immunised against *T.parva* by simultaneous sporozoite infection and drug treatment developed specific cytotoxic cells from day 14 after immunisation.

Cytotoxic cells were generated *in vitro* by co-culture of PBMC from immune cattle and autologous *T.parva* infected cell lines (Pearson et al, 1982; Emery and Kar, 1983). The cytotoxicity generated in these mixed leukocyte cultures (MLC) was mainly specific for the autologous infected cell line, but there was a distinct non-specific component which seemed to be due to second type of killer cell, rather than to "bystander" killing.

Non-specific effectors seem to be more important in *T.annulata* infections. Preston, Brown and Spooner (1983) found two peaks of cytotoxicity in calves infected with *T.annulata*, occurring in the first and third weeks after infection. The first peak consisted of BoLA-restricted activity, while the second was not genetically restricted. This unrestricted activity could be due to natural killer (NK) cells, macrophages or both. Macrophages from *T.annulata* immunised calves inhibited the proliferation of autologous and allogeneic infected cell lines *in vitro* (Preston and Brown, 1988). The cytostatic effect could be exerted when the macrophages and infected cell lines were separated by a membrane, so a cytokine may have been involved.

There is circumstantial evidence to implicate cytokines such as Tumour Necrosis Factor (TNF), interferon gamma (IFN- γ) and interleukin-1 (IL-1) in the pathology and immunology of theileriosis. TNF and IL-1 can both act at the systemic level to induce pyrexia (fever) (Dinarello and Wolff, 1982; Dinarello et al, 1986), and cachexia (wasting) in the case of TNF (Beutler and Cerami, 1986 and 1987). Pyrexia and cachexia are observed in tropical theileriosis; sick animals become progressively emaciated (Dschunkowsky and Luhs, 1904; Neitz, 1957). TNF may be important in other protozoan diseases: raised levels of TNF have been detected in the serum of humans suffering from malaria (*Plasmodium* spp. infection) and kala-azar (visceral *Leishmania donovani* infection) (Scuderei et al, 1986). Kwiatowski et al (1989) found that in addition to generally raised levels of TNF in the serum of patients infected with *P.falciparum*, mononuclear cells from these patients produced higher levels of TNF when stimulated with endotoxin *in vitro* than cells from healthy volunteers. Anti-TNF antibodies can alleviate the development of cerebral malaria in mice (Grau et al, 1987). *In vitro*, *Plasmodium* sp. infected cells can induce TNF production by

macrophages (Bate, Taverne and Playfair, 1988).

TNF also acts at a local level to induce inflammation and haemorrhage, particularly in the relatively delicate capillaries of tumours. This is the activity for which TNF was originally named (Old, 1988). Haranaka, Satomi and Sakurai (1984) described haemorrhagic necrosis of several different transplanted subcutaneous tumours in mice treated with murine TNF. Meth A sarcoma tumours in mice regressed in response to treatment with recombinant human TNF (rHuTNF), and this regression correlated with haemorrhage and necrosis of the tumours (Palladino et al, 1987). In contrast to these encouraging results, Havell, Fiers and North (1988) reported that the action of TNF against another murine sarcoma (SA1), growing as a subcutaneous tumour, was limited by the toxicity of the TNF preparation, and depended on the immunocompetence of the host.

As well as being pyrogenic, IL-1 also enhances the proliferation of T-lymphocytes, and like TNF, promotes local inflammatory responses. It does not appear to have any direct effects on parasites, although cytotoxic activity against some tumour cell lines has been described (Onozaki et al, 1985; Lachman et al, 1986).

Gamma interferon (IFN- γ) has been shown to have antiparasite activity. Clark et al (1987) showed that recombinant gamma interferon (rIFN- γ) could inhibit *Plasmodium chabaudi* infections in mice when given on a daily basis. The onset of parasitaemia was delayed, peak parasitaemia was lowered and parasite "crisis forms" were observed. In the same experiments, recombinant TNF had a similar effect. Clark et al suggested that the cytokines were causing macrophages to undergo "oxidative bursts", producing toxic oxygen radicals. Other authors have claimed that IFN- γ acts on the liver stage of malaria infection. Administration of IFN- γ simultaneously with *P.berghei* sporozoites reduced the subsequent erythrocytic parasitaemia, but there was no such effect if the IFN was given after completion of the exoerythrocytic cycle (Ferreira et al, 1986). IFN- γ also prevents the development of exoerythrocytic schizonts in hepatoma cell lines *in vitro* (Schofield et al, 1987a).

The experiments described above were carried out with exogenously administered cytokines. Neutralisation of endogenous IFN- γ with a monoclonal antibody lead to a breakdown in immunity to *P.berghei* infection in mice and rats. The endogenous IFN- γ again appeared to be acting against the exoerythrocytic schizonts in the liver, and in conjunction with CD8+ T-cells (Schofield et al, 1987b).

Taken together, these experimental results suggest that if cytokines are important in *Theileria* infections it will be as mediators of local and systemic immune responses (which may not necessarily be beneficial) rather than as direct anti-parasite agents.

1.2.3 Growth of *T.parva* infected bovine cells in mice

All the *Theileria* species identified so far are parasites of ruminants (Uilenberg, 1981). There is no species which naturally infects an animal smaller than a sheep. Attempts to infect laboratory animals such as mice and rabbits with *T.parva* infected cell lines or sporozoites failed (Irvin, Brown and Crawford, 1972; Irvin et al, 1975a).

Immunodeficient mice have been widely used as hosts for xenogeneic tumours. Berenbaum et al (1974) used CBA strain mice which had been thymectomised, sublethally irradiated (3Gy), and finally treated with anti- lymphocyte serum as recipients for explants from malignant human tumours. There was considerable variation in the growth of the transplanted tumours: most regressed rapidly. The authors were able to carry out a chemotherapy study using this system.

Nude mice are congenitally athymic and are therefore deficient in T-cell responses (Wortis, 1971). Human tumour cells and tumour transplants can be grown in nude mice without further treatment, and this system has been used for studies on chemotherapy (Poulsen and Jacobsen, 1975; Giovanella et al, 1978), endocrine therapy, and radiotherapy (reviewed by Giovanella and Fogh, 1978).

With the development of reliable methods for the establishment and maintenance of *Theileria* infected bovine cell lines *in vitro* (Malmquist, Nyindo and Brown, 1970; Brown, 1983) a similar "mouse model" for the growth of *Theileria* infected cells became feasible. The growth of *T.parva* infected cells as tumours in various sites in a variety of immunodeficient or immunosuppressed mice was investigated as described below.

Initial experiments showed that Swiss mice irradiated at 9Gy and inoculated subcutaneously (s/c) with 6×10^7 *T.parva* (Muguga stock) infected cells developed subcutaneous tumours at the inoculation site (Irvin et al, 1972). The tumours were found to contain large numbers of parasitised lymphoid cells. Parasitised cells were also found in a blood smear and impression smears of the spleen of one mouse. Cell lines were successfully isolated from the tumours and reestablished *in vitro*:

karyotyping of these cell lines showed them to be bovine.

Inoculation of irradiated (8-9 Gy) Swiss mice with similar ($2-5 \times 10^7$) doses of *T.parva* infected cells by the intravenous route did not result in the establishment of any detectable infected cells (Irvin et al, 1977). In the same experiment, two out of seven irradiated mice given the same dose of cells intraperitoneally developed ascites, but infected cells in the ascites appeared to be dying by day 14 post-infection. Six out of eight mice which were irradiated and injected with infected cells subcutaneously developed "tumour-like masses" at the inoculation site. These regressed after two to three weeks. The subcutaneous site was therefore shown to be more permissive to growth of *T.parva* infected cells than the intraperitoneal site in the irradiated mouse. Further experiments with lower radiation doses (4 and 6Gy) showed that the size and duration of the subcutaneous tumours increased with increasing doses of radiation. Treatment of the mice with anti-lymphocyte serum (ALS) did not significantly enhance tumour growth (Irvin et al, 1977).

When neonatal Swiss mice were used as recipients for *T.parva* infected cells, irradiation was again required for the infected cells to establish. Subcutaneous tumours were short-lived, disappearing by day 12. Intraperitoneal inoculation of infected cells resulted in the development of ascites, with parasitised cells being seen in biopsy smears. Thymectomy of neonatal mice combined with irradiation allowed longer survival of the *T.parva* infected cells in the peritoneal cavity (Irvin, 1975 and Irvin et al, 1977).

In summary, there were disadvantages to the use of both irradiated adult and irradiated, thymectomised neonatal Swiss mice: the radiation doses required are lethal to a large proportion of the mice; tumour growth is limited by regression; tumours appeared to regress faster in neonatal mice than in adults; a number of neonatal mice were lost to cannibalistic mothers, particularly after thymectomy. Nude (congenitally athymic) mice were then used as hosts, as it was expected that because they lack full T-cell immune mechanisms, they would be more permissive hosts for *T.parva* infected cells.

Inoculation of 5×10^7 infected cells s/c into irradiated (8Gy) adult homozygous nude mice caused the development of large tumour-like masses at the inoculation site. These tumours persisted throughout the experiment (up to 50 days) until the surviving animals were destroyed. No regression of the tumours was seen, although many became ulcerated at the surface. The tumours were found to be infiltrating the

musculature of the body wall, but no macroscopic metastases were seen in tissues away from the inoculation site. However, parasitised cells were detected in impression smears from the liver, kidney, spleen and particularly the lungs, where infiltration of infected cells lead to terminal respiratory distress (Irvin, 1975 and Irvin et al, 1977). Only transient subcutaneous tumours were seen in unirradiated nude mice given the same cell dose at the same time. No survival of infected cells was seen when irradiated nude mice were injected i/p: this was attributed to the action of peritoneal macrophages. Attempts to grow infected cells s/c in irradiated neonatal nude mice were not successful and this approach was abandoned in favour of the irradiated adult nude (Irvin, 1975). Experiments were carried out using this "model" to investigate the following: development of piroplasms in murine hosts, and transmission to ticks (Irvin, 1975; Irvin et al, 1975b); comparison with bovine lymphosarcoma cells (Irvin et al, 1975c); passage of cells between mice and the possible attenuation of cell lines by repeated mouse passage (Irvin, 1975 and Irvin et al, 1976).

These experiments therefore showed that out of those tested, the subcutaneous site in the irradiated adult nude mouse was the most useful for propagation of *T.parva* infected bovine cells *in vivo*. High (near lethal) doses of irradiation were required for tumour establishment, suggesting that a radioresistant natural immunity was present in the nude mice. Irvin et al (1977) suggested that this might be due to Natural Killer (NK) cells: nude mice have been shown to have higher levels of NK cell activity than normal mice (Kiessling et al, 1975).

1.2.4 Lymphocyte subsets in irradiated mice

The use of irradiation as an immunosuppressive agent raises the question, what immune mechanisms are operating in the irradiated mouse to cause tumour rejection? The general effects of irradiation on the immune system are well known. Lymphocytes in general are relatively radiosensitive: moderate doses (above 1Gy/100Rads) cause a rapid drop in numbers in the first two to three days after irradiation (Kataoka and Sado, 1975; Anderson and Warner, 1976; Nias, 1988). The radiosensitivity of lymphocyte subsets was initially studied by the impairment of lymphocyte functions: T-cell "help" was shown to be radioresistant compared to antibody production (Kettmann and Dutton, 1971; Hamaoka, Katz and Benacerraf, 1972).

Surface labelling of lymphocytes with antibodies to differentiation markers allows the direct identification of subsets. B-lymphocytes form a homogeneous radiosensitive population compared to T-lymphocytes, which fall into two populations: one slightly more resistant than B-cells, and a smaller group of extremely radioresistant cells (Kataoka and Sado, 1975). The cutoff point between the two groups occurred at 7Gy. Immunofluorescent analysis, three days after irradiation, of mouse spleen cells using antibodies to the Ly- 1 (predominantly T- helper/inducer cells), Ly-2 (cytotoxic and suppressor cells), and L3T4 (specifically T-helper cells) surface antigens showed that all these T-cell subsets had a radioresistant subpopulation, between 2.5% for Ly-2+ cells and 10% for L3T4+ cells (Sado et al, 1988). These radioresistant populations probably consist of memory cells and mature effector cells (Anderson and Warner, 1976; Sado et al, 1988; Nias, 1988).

Suppressor T-cells appear to constitute a particularly radiosensitive subset. Low doses (0.15Gy) caused augmentation of the immune response to sarcoma I cells in primed mice (Anderson, Williams and Tokuda, 1988). This inhibition of suppression was associated with damage to Ly-1-,2+ cells. However, in the same experiments tumour regression was associated with the presence of cytotoxic Ly-1+ cells, which were also sensitive to the low doses of radiation employed.

Compared to lymphocytes, NK cells and macrophages are extremely radioresistant. Macrophages can undergo doses of 100Gy *in vitro* without any affect on their phagocytic activity. *In vivo*, doses of 10Gy do not impair antigen presentation function (Anderson and Warner, 1976). NK cell activity was observed to rise in the spleens of C3H mice given increasing doses of irradiation (0-6Gy): when corrected for the fall in the total number of spleen cells, this became a slight decline (Sado et al, 1988). Death of the more sensitive T and B lymphocytes therefore enriched the spleens for NK cells.

Antibody formation and cellular reactions mediated by T-cells are therefore relatively sensitive to sublethal doses of ionising radiation. Natural immune mechanisms are far more resistant. No study so far seems to have directly addressed the question of the recovery of lymphocyte subsets in sublethally irradiated mice.

1.2.5 The beige and scid mutants

Since the original experiments with *T.parva* infected cell lines were carried out, new mutant strains of immunodeficient mice have become available. The beige mutant, originally discovered in C57BI/6 mice, is expressed as a defect in the lytic mechanism of the NK cell (Roder and Duwe, 1979). Spleen cells from beige mice were unable to lyse NK- sensitive target cells in an *in vitro* assay, although other cytotoxic functions, eg cytotoxic T-cell function, antibody dependent cell-mediated cytotoxicity and killing by macrophages, remained normal. Talmadge et al (1980) found that an NK-sensitive tumour cell line grew faster and had increased metastatic ability in beige mice, compared to syngeneic heterozygotes which have normal NK functions. Another study published at the same time showed that beige mice had a lowered resistance to the growth of syngeneic leukaemia cells, and this deficiency was particularly marked in the first two weeks after tumour implantation (Kaerre et al, 1980). Depletion of NK cells from nude mice resulted in enhanced growth of NK sensitive tumours (Habu et al, 1981). Together, these results suggest that in the normal mouse NK cells act as an early response to control neoplasms, and that they have a special role in controlling metastasis.

The scid mutation was discovered in C.B-17 mice during routine screening of serum immunoglobulin allotypes (Bosma, Custer and Bosma, 1983), and was subsequently shown to be an autosomal recessive mutant on chromosome 16. The mutation causes errors in the rearrangement of immunoglobulin and T- cell receptor (TCR) genes, so that functional B and T-cells cannot develop (Schuler and Bosma, 1989). C.B-17 scid/scid (hereafter referred to as scid) mice do have normal macrophage functions (Bosma, Schuler and Bosma, 1988) and NK cells (Kumar et al, 1989).

Some scid mice do develop detectable levels of serum immunoglobulin (Bosma et al, 1988). This "leakiness" of the scid phenotype cannot be selected for by breeding, becomes increasingly apparent with age, and is more common in mice kept under specific pathogen-free (spf) conditions than in mice kept in isolators. Leaky scid mice (or scid(Ig+)) also show some T- cell activity (Bosma et al 1988), and low numbers of T-cells can be detected by flow cytometry (Carroll et al, 1989). B and T-cell populations in leaky scid mice consist of as few as 3-4 clones, and the proportions of lymphocyte subsets are skewed relative to normal mice (Carroll et al, 1989; Kearney et al, 1989; Gibson, Bosma and Bosma, 1989). Leakiness therefore appears to arise as

a result of the rare reversion of progenitor lymphocytes to the wild type, together with the occasional production of functional lymphocyte receptors by the defective recombinase (Gibson et al, 1989).

Transplanted human tumours which can be grown in nude mice will also grow readily in scid mice. Most tumours seem to grow equally well in either host, but certain tumours, notably osteosarcomas and retinoblastomas, grow significantly better in scid mice (Phillips, Jewett and Gallie, 1989). These authors list further advantages of scid over nude mice as tumour hosts: they are easier to breed, and it may be possible to transplant human lymphocytes along with the human tumour to study human anti-tumour immune mechanisms *in vivo*.

Several authors have reported attempts to transplant human leukocytes into scid mice. Mosier et al (1988) claimed to have transferred a functional human immune system to scid mice by intraperitoneal injection with human peripheral blood lymphocytes (PBL). The transplanted cells survived for up to six months and colonised lymphoid tissues. The authors were able to elicit specific antibody production in response to tetanus toxoid immunisation. Mice which received high doses of cells from donors who were seropositive for Epstein-Barr virus (EBV+) developed B-cell lymphomas. Graft versus host disease (GVHD), which had been anticipated, did not occur.

Repopulation of scid mice with human cells was also achieved by implanting pieces of human fetal thymus under the kidney capsule, and injecting the mice with human fetal liver cells as a source of haematopoietic stem cells. Precursor cells were found to move to the thymus implant, and mature human T-cells were detected in the peripheral circulation. Functional human B-cells (secreting antibody) were only produced when the mice were additionally engrafted with human fetal lymph node. The authors describe these mice as scid-hu haematopoietic chimaeras (McCune et al, 1988).

Human lymphocytes from lung cancer patients and healthy volunteers were successfully established in scid mice, assayed by the production of human immunoglobulin (Bankert et al, 1989). In these experiments, the authors could not elicit specific antibody from the scid-hu mice, and GVHD occurred in several cases, particularly in mice which had received large doses of cells. Pfeffer et al (1989) did not find GVHD when they injected mice with human PBL. However, the injected cells remained localised to the peritoneal cavity. Colonisation of lymphoid organs

was only observed if the mice had first been implanted with human fetal thymus.

Experiments with human tissues therefore show that the scid mouse is a promising model for investigating the behaviour of tumours and anti-tumour effector mechanisms *in vivo*. However, there are conflicting reports on the occurrence of GVHD, the induction of specific human immune responses in the reconstituted or "chimaeric" mouse, and the colonisation of lymphoid organs by transplanted cells.

Scid-hu chimaeric mice have been infected with Human immunodeficiency virus 1 (HIV-1). Namikawa et al (1988) were able to show infection of human fetal lymphoid tissues, transplanted to scid mice, by HIV-1. Mice which had been reconstituted with mature human PBL supported HIV-1 replication and, apparently developed an AIDS like syndrome characterised by weight loss and depletion of human T-lymphocytes (Mosier et al, 1989).

A further experimental use for the scid mouse is to investigate natural immunity without the complication of B and T-cell mechanisms. *Scid* mice have normal macrophages and NK cells. In scid mice infected with *Listeria monocytogenes*, macrophage activation occurs through a T-cell independent mechanism: the mice are not able to clear the infection but develop chronic rather than acute disease (Bancroft, et al 1986). Treatment of the mice with monoclonal antibody to IFN- γ resulted in acute disease (Bancroft et al, 1987): depletion of NK cells also exacerbated the disease (Bancroft, Schreiber and Uanue, 1989). *Scid* mice therefore show a system of macrophage activation with NK cells acting in a "helper" role. The NK cells appear to be stimulated to secrete IFN- γ by adherent cells, presumably themselves stimulated by the pathogen. These results, like those already described for beige mice (Talmadge et al, 1980; Kaerre et al, 1980) suggest that NK cells may have a role in the early stages of infection or neoplasia. Activated macrophages may also have an antitumour role which would be relevant to studies on tumour growth in scid mice (Benomar, Gerlier and Dore', 1987).

1.3 Approach to the project

(i). Experiments were carried out to investigate the growth of *T.annulata* schizont infected cells in irradiated Balb/c mice. The use of nude and beige mice as hosts for *T.annulata* infected cells was also investigated. Experiments to investigate the growth of *T.parva* infected cells in irradiated Balb/c and nude mice were conducted.

(ii). The effect of rabbit serum containing Tumour Necrosis Factor (TNF) activity on the growth of *T.annulata* tumours was investigated.

(iii). The effect of a sublethal radiation dose on lymphocyte subsets in the spleens of Balb/c mice, and their recovery over three to four weeks, was studied using flow cytometry. The effect of the presence of a *T.annulata* tumour on the recovery from radiation damage was also investigated. NK cell activity in the irradiated and irradiated, tumour-bearing mice was measured.

(iv). The growth of *T.annulata* and *T.parva* infected cells in various sites in scid mice was examined. The dissemination of cells from the inoculation site was studied histologically and by radioactive labelling of inoculated cells.

(v). The immune responses of the scid mouse to *Theileria*-infected cells were studied: assays for NK cell activity and for cytokines (IL-1, TNF, IFN- γ) were performed. The effect of treatment with recombinant cytokines and with anti-IFN- γ antibodies was investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Theileria*-infected cell lines

2.1.1 Designation of cell lines

Theileria-infected cell lines are named according to the *Theileria* species, stock or strain and the identification number of the bovine donor. Thus "TaHis110" is a *Theileria annulata* infected cell line of the Hissar stock, in cells from calf 110. This animal has provided cells for infection with several *Theileria* stocks.

All the cell lines used in this project were derived by *in vitro* infection. The *T.annulata* cell lines were of the "Hissar" stock (Gill, Bhattacharyulu and Kaur, 1976) and the *T.parva* cell line was of the "Muguga" stock (Brocklesby, Barnett and Scott, 1961).

TaHis110 (*T.annulata*, Hissar stock, calf 110)

TpMug110 (*T.parva*, Muguga stock, calf 110)

TaHis249,250 (derived from germ-free calves 249 and 250).

TaHis110 and TpMug110 were originally established by Mr. C.G.D.Brown of the Centre for Tropical Veterinary Medicine, Roslin, Edinburgh. TaHis249 and 250 were established by myself using a ground-up tick supernatant (GUTS) prepared from infected ticks, which was provided by Mr. Brown. The method for the preparation of GUTS is described in Appendix A.

2.1.2 In vitro infection of bovine cells

Calves 249 and 250 were being kept under gnotobiotic conditions at the Moredun Institute, Edinburgh. Blood was obtained from these animals and peripheral blood leukocytes (PBL) were prepared by centrifugation on a ficoll-isopaque gradient (Appendix A).

The PBL suspensions were adjusted to 2×10^6 cells/ml in RPMI with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml kanamycin and 2mM glutamine. 1ml of the cell suspension was plated into each well of a 24 well plate. The GUTS were diluted to 1.0, 0.5, and 0.25 tick equivalents/ml and each dilution was plated on to four wells of PBL at 1ml/well. Plates were incubated at 37°C in 5% CO₂ in a flow incubator and inspected regularly for transformation of infected cells. When large, blastoid cells became numerous, and infected cells were seen in cytopins, the cultures were transferred to culture flasks and maintained as usual.

2.1.3 Maintenance of *Theileria*-infected cell lines *in vitro*

TaHis110 was maintained on RPMI-1640 supplemented with 10% newborn calf serum (NCS), penicillin (100U/ml), streptomycin (100µg/ml), kanamycin (100µg/ml) and glutamine (2mM) (Brown, 1983). TaHis249 and 250 were also maintained on 10% NCS/RPMI with 2mM glutamine, but without antibiotic supplementations. TpMug110 was maintained on RPMI supplemented with 10% FCS, with antibiotics and glutamine as for TaHis110. For experiments in scid mice, TpMug110 was maintained without antibiotics. Cells were passaged once a week and fed every two to three days in between. All cell cultures had a passage number less than twenty when used for experiments.

2.2 Mice

2.2.1 Strains, origin and maintenance

Balb/c mice were bred under clean conditions at this Department. NIMR nu/nu and Balb/c nu/nu mice were purchased from the National Institute for Medical Research, Mill Hill, London. C57 beige mice were purchased from Harlan Olac Ltd., Oxfordshire. Balb/c and C57 beige mice were female; NIMR and Balb/c nu/nu mice were male. All mice were approximately three to four months old at the start of experiments. These mice were housed under clean conditions and provided with food (SDS No.3 diet, SDS, Witham, Essex) and water *ad libitum*.

CB-17 scid mice were obtained from the National Institute for Medical Research,

and housed in a positive pressure isolator (Moredun Animal Health, Edinburgh). They were fed on irradiated food (SDS) and autoclaved water.

2.2.2 Inoculation of Theileria infected cells into mice

Cells were counted by trypan blue exclusion using a haemocytometer. The required volume was then centrifuged, and the cells were washed twice with Dulbecco's phospho-buffered saline "A" (PBS) before being resuspended in cold PBS at 0.2 ml/dose.

Mice were irradiated using a caesium-137 source, and injected with the cell suspension subcutaneously (s/c) in the right flank or intraperitoneally (i/p). The mice were inspected daily for tumour or ascites development. The size of the tumours was measured using calipers. Initially, two measurements were made, of the longitudinal and vertical axes: these were averaged to give a mean measure of tumour size for each mouse. It was found that tumours were generally circular or nearly so, so in later experiments only a single measurement was taken. Any haemorrhage or necrosis was noted.

2.2.3 Preparation of cell lines from tumours and ascites

Tumour pieces were chopped and teased apart in cold, sterile PBS. The suspension was sieved to remove large pieces of tissue and centrifuged at 1000 rpm. The cell pellet was resuspended in RPMI supplemented with 10% foetal calf serum, antibiotics and glutamine as usual. Peritoneal exudates from mice which had developed ascites were centrifuged and resuspended in medium in the same way. Cell lines were initiated in four well (1ml/well) plates and subsequently transferred to 10ml flasks.

2.2.4 Preparation of chromosome spreads

Chromosome spreads were prepared by a modification of the method described by Wurster and Benirschke, 1968. Actively growing cells were incubated with 0.2 µg/ml colcemid for 90-120 minutes to arrest mitosis. The cells were then lysed by incubation in 0.75% sodium citrate at 37 C for 35 minutes. The nuclei were washed

twice by centrifugation in cold acetic alcohol. The pellet was resuspended in an equal volume of acetic alcohol and the suspension was dropped onto ice-cold, pre-wetted slides from a height of 1m. The resulting spreads were stained with Giemsa's stain. A cytocentrifuge preparation was made at the same time to find the percentage of macroschizont-infected cells in the sample.

The results were evaluated by counting the number of chromosomes/spread in 100 spreads. This was compared to results obtained from the original infecting cell line, maintained *in vitro*.

2.3 ^{51}Cr tracking experiments

This schedule was modified from the technique described by Waterfall (1985).

The cells to be injected were labelled with ^{51} chromium by the method described in Appendix A for YAC-1 cells in the NK cell cytotoxicity assay. Mice were injected intraperitoneally (i/p) with 2×10^6 labelled cells in 0.1 ml of PBS. After a time interval (4 hours up to 9 days) mice were bled as described in Appendix A, and a 200 μ l sample of blood was added to 70% ethanol in a Luckham tube. Peritoneal exudates were prepared as described in Appendix A, and 2mls of peritoneal exudate from each mouse was counted. Other organs were removed into 70% ethanol in Luckham tubes. All samples were counted with an automated gamma counter.

2.4 Methods for flow cytometry

2.4.1 Fluorescent labelling of spleen cells

(i). Suspensions of spleen cells were prepared by the method described for YAC-1 assays. Viable cells were counted by Trypan blue exclusion and resuspended in PBS supplemented with 0.1% bovine serum albumin (BSA), 0.1% sodium azide and 0.05% EDTA. This PBS formulation was used throughout the flow cytometry procedures wherever PBS is indicated.

(ii). The cell suspensions were adjusted to a concentration of 1×10^7 cells/ml and dispensed into round-bottomed, 96 well plates at 50 μ l per well.

(iii). 20 μ l of the diluted antibody (table 1) was added to the cells. The cells were incubated with the primary mcab for 45 minutes at 4°C. There were two control wells: one to which no mcabs were added (unstained), and one which recieved only the goat anti-rat IgG conjugate (second step control).

(iv). The plates were centrifuged at 1000rpm for 30 seconds, the supernatants were discarded and the cells were resuspended in approximately 100 μ l of PBS.

(v). This washing procedure was repeated twice. After the third wash, 20 μ l of the fluorescent conjugate or of the directly conjugated mcabs was added as appropriate. The cells were again incubated for 45 minutes at 4°C.

(vi). The cells were washed three times in PBS as before, resuspended in 100 μ l of 1% paraformaldehyde/RPMI and transferred to 12x75mm, round- bottomed tubes (Falcon) for FACS analysis. Tubes were stored at 4°C if analysis was not to be carried out immediately.

2.4.2 Analysis of labelled cells

Cells were analysed using a Beckman FACS4 flow cytometer. Briefly, cells were passed through a laser beam and the size, complexity and fluorescence intensity of the individual cells were measured automatically. A computerised system was used to select cell populations of interest (ie lymphocytes), and the percentage of this population staining positively with the relevant antibody could then be measured separately. From this percentage, the proportion of all the cells analysed falling into the lymphocyte population, and the total number of cells extracted from the spleen, it was possible to calculate the number of cells positive for a particular marker in each spleen.

2.5 Radial Immunodiffusion (RID) assay for mouse immunoglobulins

Serum obtained from scid mice was tested for the presence of immunoglobulins to detect any "leakiness" of the scid phenotype in the experimental mice. Assays were carried out using a mouse monoclonal typing kit supplied by The Binding Site, Birmingham, according to the schedule supplied with the kit. Serum was tested at

Table 1. Monoclonal antibodies used in FACS experiments.

Antibody	Dilution	Cell subset labelled	Source
Sheep anti-mouse IgG	1/40	B-cells	Sigma
Rat anti-mouse Ly-2	1/100	Cytotoxic T-cells (Cantor and Boyse, 1975)	Seralab
Rat anti-mouse L3T4	1/100	Helper T-cells (Dialynas et al, 1989)	Seralab
Goat anti-rat IgG FITC	1/100	(Second step to develop primary antibody)	TAGO

dilutions from neat to 1:1000 PBS, and compared to Balb/c serum as well as to the positive controls supplied with the kit.

2.6 Cytokines and anti-interferon gamma antibody

Recombinant murine TNF and recombinant human IL-1 were obtained from Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., Bell Lane, Lewes, Sussex. Human IFN- α and IFN- γ (not recombinant) were obtained from SIGMA Ltd., Poole, Dorset. All cytokines were aliquoted on arrival and stored at -70 C until required. Cytokines were diluted in PBS for injection into mice.

Rabbit tumour necrosis serum (TNS) and control rabbit serum were prepared as described in Appendix A. TNS was aliquoted, stored and diluted as for recombinant cytokines. TNF activity in rabbit TNS was assayed using the L-929 assay, as described in Appendix A.

The hybridoma cell line R46A2 was donated by Dr. G. Entrican of the Moredun Institute, Edinburgh, with the kind permission of Dr. A. Morris, Interferon and Cellular Immunity Research group, University of Warwick. This hybridoma was originally established, and the antibody product characterised by Dr. E.A. Havell, Trudeau Institute, Saranac Lake, New York (Havell, 1986). R46A2 is a rat-mouse hybridoma secreting a rat IgG monoclonal antibody capable of neutralising the activity of murine IFN- γ *in vitro* and *in vivo* (Havell, 1986; Mowat, 1989).

R46A2 was purified from culture supernatants by precipitation with 50% ammonium sulphate. The precipitate was redissolved in distilled water and dialysed for 48 hours against two changes of PBS at 4°C. The purified protein was sterile filtered and stored at 4°C. The presence of rat antibody was confirmed by radial immunodiffusion against goat anti-rat IgG: the IFN- γ neutralising activity was assayed by Dr. Entrican as described in Appendix A.

CHAPTER 3

GROWTH OF *THEILERIA*-INFECTED CELLS IN IRRADIATED BALB/C MICE

3.1 Introduction

The general aim of these experiments was to develop a system for the growth of *Theileria*-infected bovine cells which could be used to test the effects of TNF on the cells *in vivo*. The experiments concentrated on *T.annulata*, but some results with *T.parva* infected cells are also included.

Starting from the work described in the General Introduction, it was expected that subcutaneous tumours would develop in irradiated mice inoculated with *T.annulata* cell lines. The doses of cells and radiation required, the rate of growth and the time of onset of regression for various doses were not known, and these experiments were intended to answer these questions. They also yielded histological information.

3.2 Inoculation of TaHis110 s/c

3.2.1 Methods

The mice were irradiated and injected subcutaneously with TaHis110 cells as described in "Materials and Methods". Three doses of radiation were combined with three doses of cells to give a total of nine groups of mice. There were at least four mice per group. The data presented were pooled from several experiments: the raw data from which graphs were drawn is therefore presented in Appendix B.

The radiation doses used were 0, 4 and 6 Grays (Gy). The doses of TaHis110 cells were 8×10^5 , 4×10^6 and 2×10^7 cells per mouse.

3.2.2 Results

The results are presented in graphs 1 and 2. No tumours developed in the 0Gy group, except at the highest cell dose, where small (<3mm) swellings were seen at the inoculation site in 3 out of 4 mice up to 14 days post-infection.

In the 4Gy group, subcutaneous tumours first became palpable in the mice given 2×10^7 cells. Tumours reached a size of 5-7mm diameter before regressing after two to three weeks. In the lower dose groups tumours took longer to become detectable and did not grow as big. In the 8×10^5 cells group, tumours were transient, regressing completely about one week after becoming palpable.

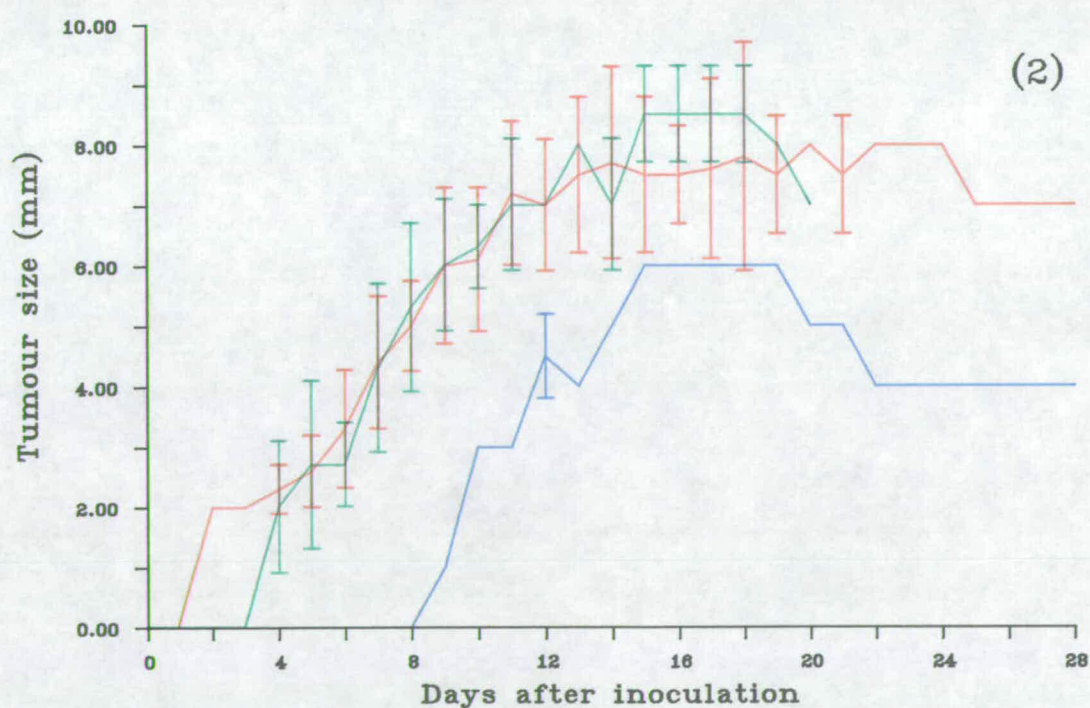
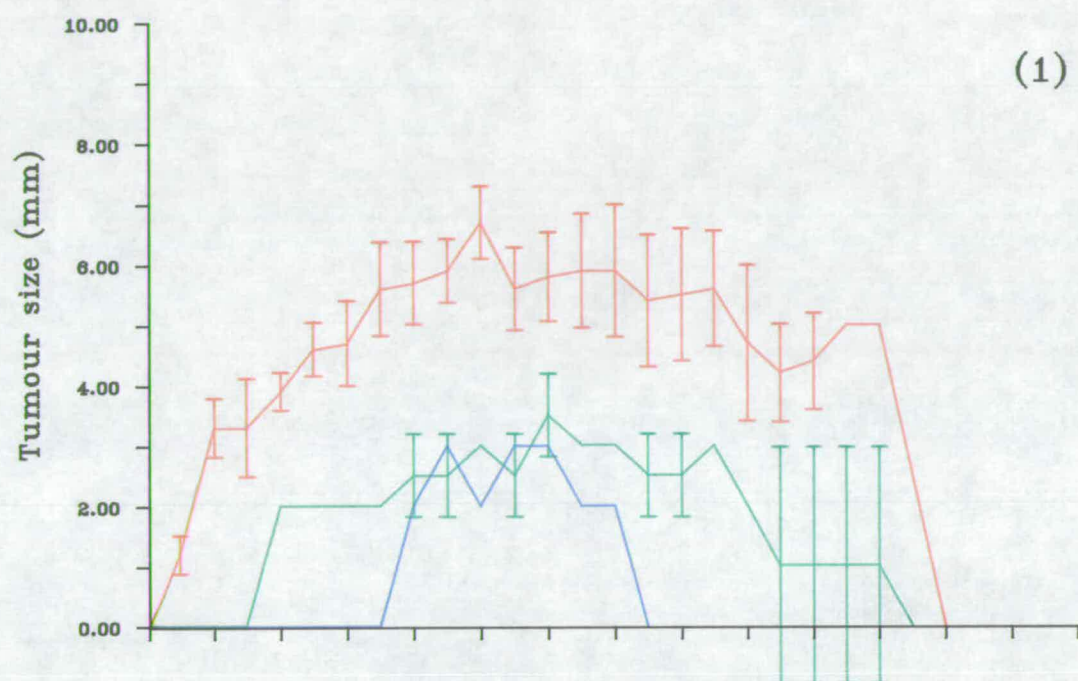
This dose dependence did not seem to hold up so well in the 6Gy group (graph 2). Tumours in the medium and high dose groups reached approximately the same size, and grew at the same rate. Tumours in the low dose group grew at a similar rate once established, but took longer to appear.

Mice irradiated at 6Gy appeared more unhealthy than 4Gy mice. In an early experiment (data not shown) mice were also irradiated at 8Gy, and became severely ill due to irradiation.

Externally, tumours became reddened three to four days after appearance, indicating some degree of internal haemorrhage. Black, necrotic scabs developed about 6-8 days post-infection, and these necrotic areas spread to cover the entire tumour surface within a few days. Sloughing of this necrotic crust resulted in healing of the lesion by twenty to thirty days post- infection. In mice with small ($\leq 2-3$ mm diameter) tumours, regression occurred without outward necrosis.

On dissection, the tumours were found to be discrete subcutaneous masses, solid in texture and usually attached to the dermis. Larger tumours were also attached to the abdominal wall. The tumours were primarily white in colour, but tended to become progressively reddened as haemorrhage set in. Haemorrhage into the surrounding subcutaneous tissue was also seen in some cases, usually in mice given radiation doses of 6Gy or more.

A dose of 2×10^7 TaHis110 cells combined with 4Gy of irradiation was adopted as a standard dose for subsequent experiments, as this gave a good compromise between the health of the mice and satisfactory growth of the tumour.



Red=2x10⁷ cells
Green=4x10⁶ cells
Blue=8x10⁵ cells

3.3 Histology

3.3.1 Methods

A group of 10 mice were given 4Gy and 2×10^7 cells s/c. Pairs of mice were killed 4, 8, 12, 16 and 20 days later. Dab smears were prepared from the tumours, inguinal lymph nodes, liver, spleen and lungs. Pieces of the tumours and of the other tissues were prepared for histology as previously described.

Lymph node samples from calves experimentally infected with *T.annulata* were provided by Mr. C.G.D.Brown, Centre for Tropical Veterinary Medicine, Edinburgh. Bovine tissues were processed and stained as for mouse tissues.

3.3.2 Presence of Macroschizonts in dab smears

Macroschizont infected cells and free schizonts were seen in tumour dab smears on all days. The total schizonts and infected cells in 10 fields at a magnification of $\times 1000$ were counted and averaged for each pair of mice. These results are presented in graph 3. The numbers of macroschizonts peaked on day 8, when tumours were reaching their maximum size, then fell off as tumours resolved.

No schizonts or parasitised cells were seen in dab smears from other organs on any day.

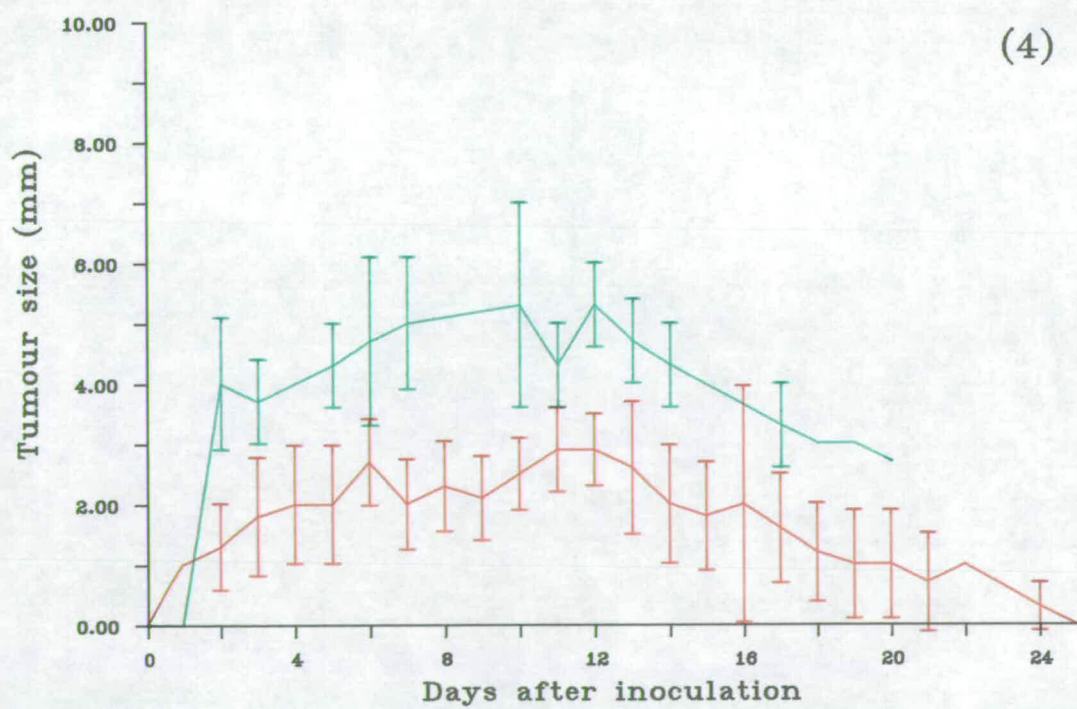
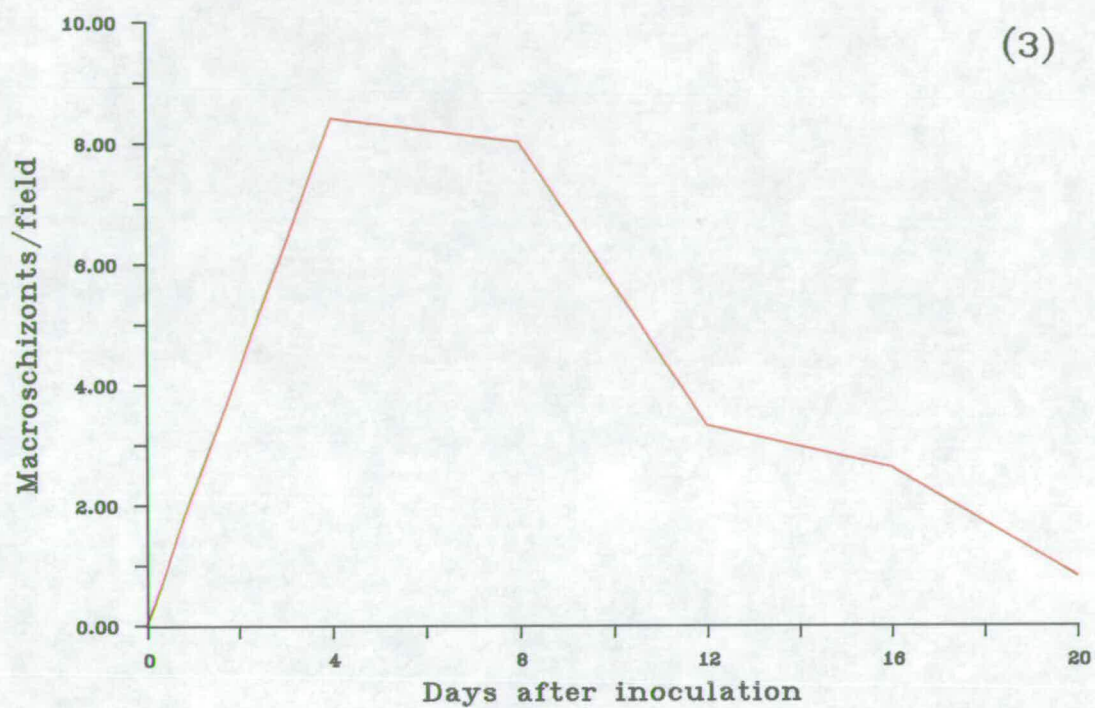
3.3.3 Sections of tumours

Tumours were found to consist of masses of lymphoid cells infiltrating subcutaneous muscle and adipose tissue. Schizonts could occasionally be seen in sections, but were generally difficult to identify with certainty. The presence of infected cells was confirmed by dab smears.

Sections of TaHis110 tumours 4 days after inoculation show large numbers of fat cells, surrounded and infiltrated by tumour cells. Large blood vessels are present, surrounded by masses of tumour cells (plate 1). At higher magnification, erythrocytes could be seen lying among the tumour cells. Some appeared to be in capillaries. Erythrocytes can be distinguished in plate 1 as orange-pink areas between tumour

(3). Counts of *T.annulata* macroschizonts from dab smears of tumours of Balb/c 4Gy mice injected with 2×10^7 TaHis110 cells s/c. Each point represents a mean of at least two mice. A minimum of 10 fields were counted from each slide.

(4). Growth of s/c tumours in Balb/c 4Gy mice injected with TpMug110 cells. Green= 4×10^7 cells; red= 2×10^7 cells.



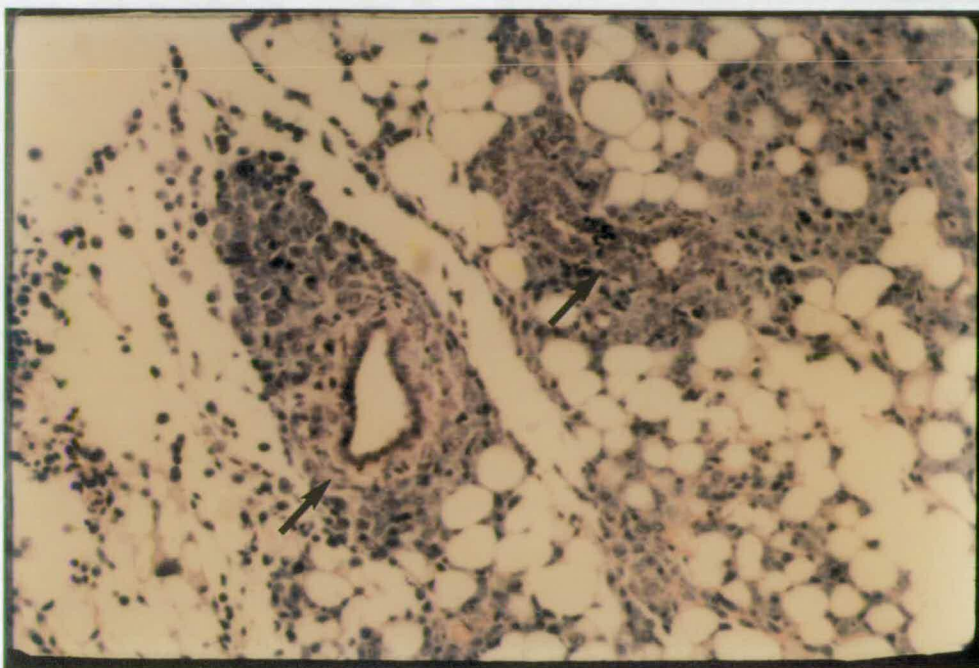
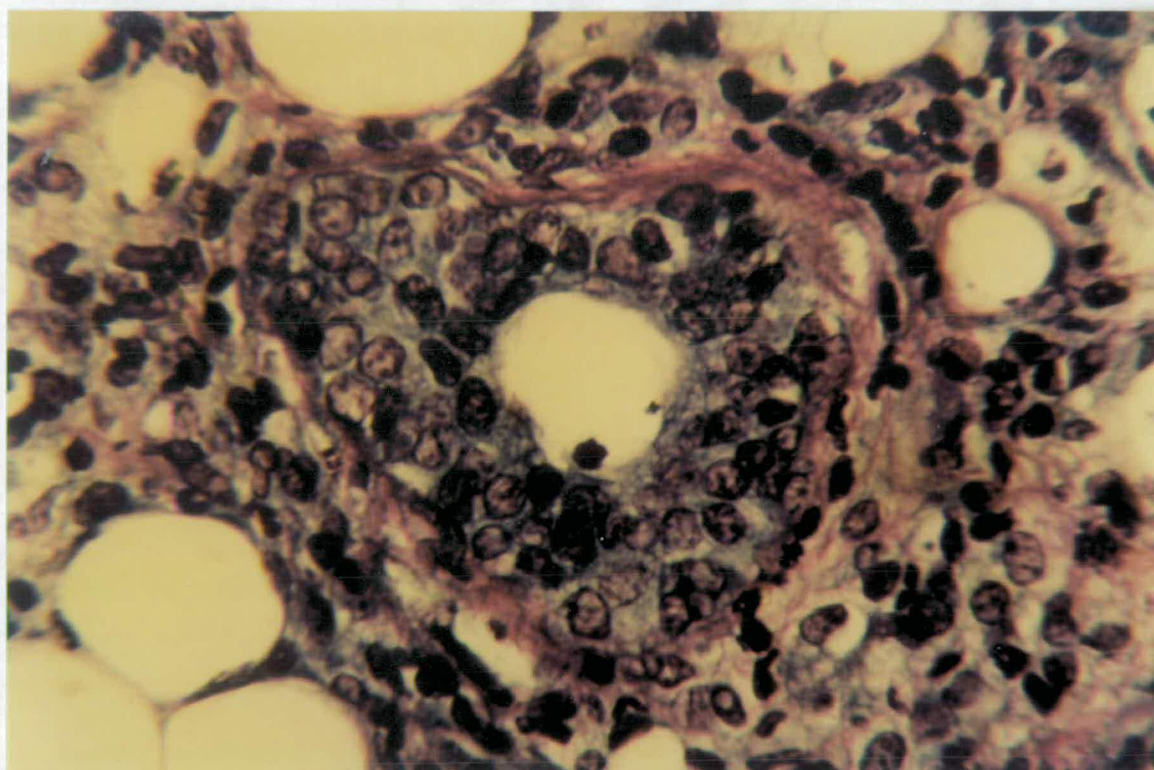


Plate 1. *T.annulata* tumour in Balb/c 4Gy mouse, 4 days after inoculation of 2×10^7 cells (x100). Proliferating tumour cells can be seen infiltrating subcutaneous fat. Two blood vessels can be seen (arrows).

Plate 2. *T.annulata* tumour in Balb/c 4Gy mouse, 4 days after inoculation. A blood vessel is lined and almost occluded by large, mononuclear cells.



cells. Large, mononuclear cells with pale nuclei were seen lining and sometimes obscuring blood vessels (plate 2). The tumours were moderately vascularised, with haemorrhage developing later. Necrotic areas were usually seen at the outer surface of tumours, corresponding to the black scabs which developed at the surface.

12 days after inoculation, necrosis of the tumours was advanced. Sections showed large numbers of infiltrating cells with irregular nuclei (plate 3).

Sections of lymph nodes from cattle with acute theileriosis showed areas of proliferating lymphoblastoid cells, together with areas of lymphodegeneration, haemorrhage and the deposition of extracellular protein (plate 4). Infected cells and schizonts were seen in smears of the cut surfaces of these lymph nodes. There were clear similarities in the histological appearance of the lymph nodes of *T.annulata* infected cattle, as described here and in a previous study (Eisler 1988), and that of subcutaneous *T.annulata* tumours in irradiated Balb/c mice.

3.4 Inoculation of TpMug110 s/c

3.4.1 Methods

Mice were irradiated and injected as previously described.

The radiation doses used were: 4Gy (all cell doses), 6Gy (2×10^7 cells only). The doses of TpMug110 cells were: 2×10^5 , 2×10^6 , 2×10^7 and 4×10^7 cells per mouse.

3.4.2 Results

No tumour development was observed in mice which recieved 2×10^5 or 2×10^6 cells.

In the 2×10^7 cells/6Gy group, tumours reached a size of approximately 5mm diameter and regressed within 25 days without apparently undergoing haemorrhage or necrosis.

The results for the 4Gy group are shown in graph 4. At the highest cell dose (4×10^7 cells), tumours reached a diameter of 5mm and regression began after 12 days.

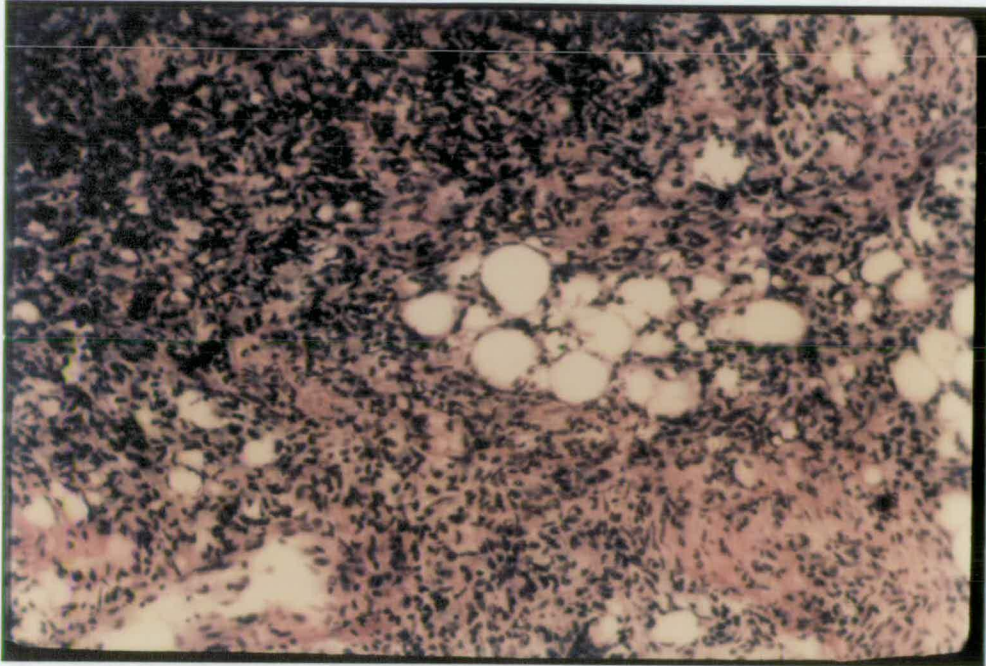
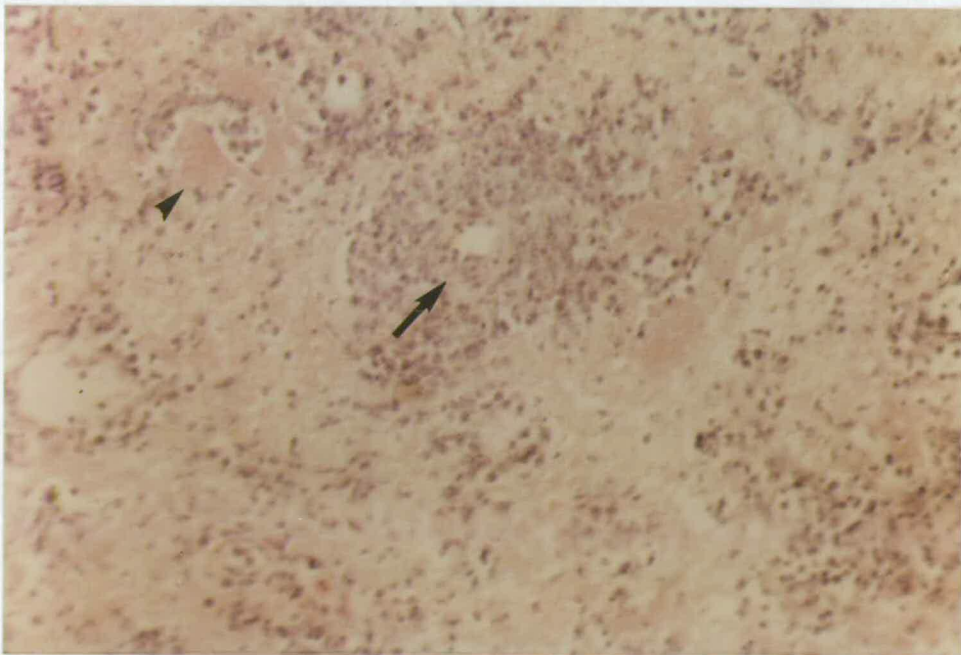


Plate 3. *T.annulata* tumour in Balb/c 4Gy mouse, 12 days after inoculation of cells (x100). Infiltrated fatty tissue can be seen: there are large numbers of polymorphic cells infiltrating the tissue, especially at the top left.

Plate 4. Section of right prescapular lymph node from a calf with an acute *T.annulata* infection (x100). There is a blood vessel surrounded by proliferating cells (arrow): elsewhere there is a general oedema and depletion of cells, with deposition of intercellular protein (arrowhead).



A similar pattern was observed in the 2×10^7 cell/4Gy group, but with tumours reaching a diameter of 3-4mm. Regression again occurred without conspicuous external necrosis.

T.parva induced tumours tended to grow flatter than *T.annulata* tumours, which generally presented as raised lumps even at small doses. This feature was more readily observed in mice which had been shaved at the injection site prior to infection.

3.5 Inoculation of *T.annulata* infected cells i/p

3.5.1 Methods

Eleven mice were irradiated at 4Gy and injected intraperitoneally with 2×10^7 TaHis110 cells. A further nine mice were injected with the same dose of cells without preirradiation. Mice were killed from both groups on days 5, 12 and 30, a cytopsin was made of the peritoneal exudate and dab smears were prepared from the inguinal lymph nodes, liver, spleen, kidney, mesentery and lung. Some additional mice from the 4Gy group were found dead or had to be killed on day 7.

3.5.2 Results

None of the mice developed ascites during the experiment. No schizonts or infected cells were seen in dab smears or cytopsin from any unirradiated mouse on any day. Schizonts and parasitised cells were seen in smears from irradiated mice on days 5 and 7: these results are presented in table 2. It can be seen that there was some survival of *T.annulata* infected cells in the peritoneal cavity, with dissemination into the tissues. The numbers of infected cells present fell rapidly; none were detected on day 12.

Table 2. Schizont counts from Balb/c 4Gy mice injected with 2×10^7 TaHis110 cells i/p

Days post-infection	5	7 (n=2)	12
Peritoneal exudate	33	8	0
Lymph node	0	0	0
Spleen	8	0	0
Mesentery	200	6	0
Kidney	1	4.5	0
Lung	0	2.5	0

All counts are total schizonts plus infected cells per field. At least 10 fields at a magnification of approximately $\times 1000$ were counted on each slide. Cytospins and dab smears of tissues from unirradiated mice given the same dose of cells did not reveal any schizonts.

3.6 Inoculation of uninfected bovine cells into Balb/c 4Gy mice

3.6.1 Introduction

This experiment was done to see if the tumour formed on injection of TaHis110 cells into irradiated mice was composed to some extent of mouse cells infiltrating the injection site. Dab smears showed that infected cells were present in tumours, but it was thought that a local inflammatory reaction might be responsible for part of the observed lump. It was expected that if infiltrating mouse cells were a major component of the tumour, then a similar reaction would develop at the site of injection with uninfected bovine cells.

3.6.2 Methods

Bovine PBL were prepared by the buffy coat method from a blood sample obtained from calf 110. Four mice were given 4Gy and injected s/c with 2×10^7 cells in the usual way.

3.6.3 Results

No palpable tumours developed at the inoculation site. All the mice became sick within three days of treatment; they were emaciated, had a hunched appearance and showed piloerection. One was found dead on day 5, and the rest were killed on the same day. On dissection, one mouse had a small (1mm) nodule at the inoculation site. The mice appeared to have suffered a severe reaction to the injected cells, possibly associated with some form of graft versus host disease. There was no formation of haemorrhagic or necrotic tumour-like lesions.

3.7 Discussion

T.annulata infected cells could be grown as subcutaneous tumours in irradiated Balb/c mice. The size of the tumour depended on the number of cells inoculated and the degree of immunosuppression by irradiation. Tumours were rejected by a process

of haemorrhage and necrosis: all mice which survived went on to reject the tumours. *T.annulata* infected cells injected i/p survived for up to a week, but did not establish ascitic tumours.

There was a marked difference between the growth of *T.annulata* tumours and *T.parva* tumours. *T.parva* infected cells showed a poorer rate of establishment, grew more slowly and were rejected more rapidly than *T.annulata* infected cells injected subcutaneously. The results obtained for *T.parva* are consistent with the previous observations on the growth of *T.parva* schizont infected cells in mice, in which very high (8-10Gy) radiation doses were used (Irvin 1975, Irvin et al 1977). TpMug110 did not grow as vigorously *in vitro* as the *T.annulata* infected cell lines: the cells were sensitive to reductions in cell density and were more stringent in their medium requirements than TaHis110 cells.

Sections of tumours showed vigorous infiltration of subcutaneous tissue at the inoculation site, but no macroscopic metastasis was seen, and no schizonts were detected in dab smears from other organs. The infected cells were therefore confined to the subcutaneous site. No local inflammatory response was elicited in response to subcutaneous inoculation with uninfected bovine PBMC: it was concluded that "tumour" development depended on the presence of *Theileria* infected cells rather than on a host reaction to them.

The finding that *T.annulata* infected cells could be grown in Balb/c mice treated with a moderate radiation dose was an encouraging result, considering the physiological as well as immunological differences between the original bovine host and the mouse. The next experiments used nude (athymic) mice as hosts, to see if their greater immunodeficiency would permit more prolonged growth.

CHAPTER 4

GROWTH OF *THEILERIA*-INFECTED CELLS IN ATHYMIC (NUDE) MICE

4.1 Introduction

Congenitally athymic nude mice have been widely used as hosts for allogeneic and xenogeneic tumours, including subcutaneous growth of *T.parva* infected cells (Irvin et al, 1975d and 1977). This work has already been discussed in the "General Introduction".

After the successful growth of *T.annulata*-infected cells in irradiated Balb/c mice, it was decided to attempt similar experiments using nude mice. It was expected that growth would be more vigorous in these mice, and that larger, "healthier" tumours would be a better system in which to look at the effect of TNF on the *T.annulata* tumours.

Two strains of nude mice were used: the NIMR nude mouse is based on an outbred background and is relatively robust; the Balb/c nude is from an inbred background stock.

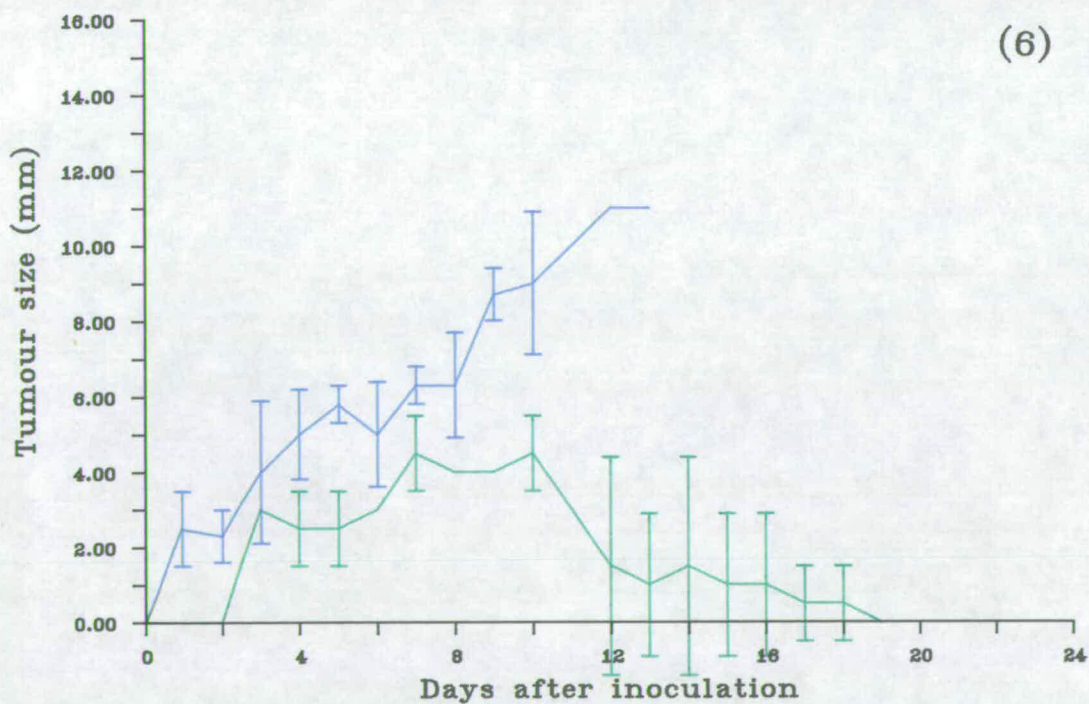
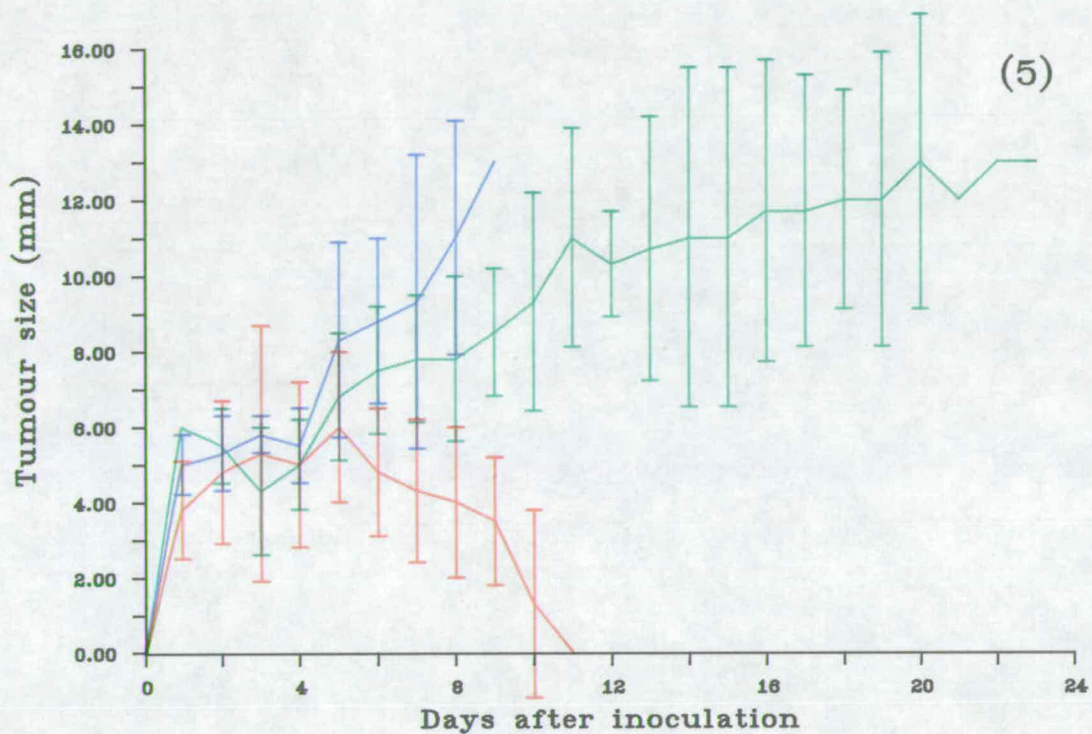
4.2 Inoculation of TaHis110 and TpMug110 into NIMR nude mice

4.2.1 Methods

The mice were given 0, 4 or 8Gy and injected s/c with 2×10^7 TaHis110 or TpMug110 cells. The high dose of 8Gy was used in order to make a comparison with the experiments of Irvin et al (1977).

(5). Growth of s/c tumours in NIMR/nude mice irradiated at various doses and injected with 2×10^7 TaHis110 cells s/c. There were four mice in each group at the start of the experiment. Errorbars are 95% confidence limits. Blue=8Gy; green =4Gy; red=unirradiated.

(6). Growth of tumours in NIMR/nude mice irradiated at various doses and injected with 2×10^7 TpMug110 cells s/c. Blue=8Gy; green=4Gy.



4.2.2 Results

(i).TaHis110 mice. All the animals developed subcutaneous tumours at the inoculation site: these results are displayed in graph 5. Up to day 4, tumours grew at approximately the same rate in the three groups. In the unirradiated mice, tumours began to resolve from day 5 onwards, without obvious haemorrhage or necrosis. In the 8Gy group, tumours continued to grow rapidly, with marked reddening from days 5-6 onwards. The 8Gy mice were very sick and had all died or had to be destroyed by day 10 post-infection.

In the 4Gy group, tumours became progressively reddened from day 6. Necrosis of the centre of the tumour became apparent between days 15 and 20, but there was some individual variation in the time of onset of necrosis. Once the necrotic area appeared at the surface of the tumour, it spread over the whole surface in three to four days, with the necrotic areas becoming increasingly eroded until the entire crust was sloughed off. Two mice which survived until day 40 post-infection had small (<5mm) scars where the scabs were sloughed.

(ii).TpMug110 mice. All the mice in the 8Gy group developed subcutaneous tumours: 2/4 mice in the 4Gy group developed tumours. These results are presented in graph 6. One mouse in the unirradiated group developed a transient swelling up to day 3 (not shown).

In the 8Gy group, tumours grew quite rapidly but did not show any signs of haemorrhage or necrosis. Three of the mice in this group died or were killed by day 11: one survived until day 14.

In the 4Gy group, tumours grew slowly, resolving from day 10 onwards. No reddening or surface necrosis was observed as tumours resolved.

TpMug110 tumours presented as flattened, white subcutaneous masses, rather than as the prominent tumours seen in the TaHis110 mice.

4.2.3 Histology

Schizonts and infected cells were seen in dab smears from tumours of irradiated mice (table 3). No schizonts were seen in a smear of a subcutaneous lump from a

Table 3. Schizont counts in dab smears from NIMR nude mice.

	Tissue	Tumour	Lymph node	Liver	Spleen	Lung
Mouse:						
TaHis110	4Gy d.8	7.0	0	0	0	0
	d.11	14.0	0.8	0	0	0
	8Gy d.8	12.0	0	0	0	0.7
	" "	2.2	0	0	0	0
	d.10	1.2	0	0	0	0
	d.20	1.9	0	0	0	0
TpMug110	8Gy d.8	2.5	0	0	0	0
	d.10	3.2	0	0	0	0

All counts are total free schizonts plus infected cells per field. A minimum of 10 fields were counted, at amagnification of approximately x1000. Each row represents the results from one mouse.

TaHis110/0Gy mouse killed on day 11. Schizonts were rarely seen in smears from other organs: some were found in the inguinal lymph node of a TaHis110/4Gy mouse killed on day 11, and in the lungs of a TaHis110/8Gy mouse killed on day 8.

4.3 Inoculation of TaHis110 and TpMug110 into Balb/c nude mice

4.3.1 Methods

From the results of the first experiment, it was decided to inject the mice with a range of cell doses while using only the 4Gy radiation dose. The unirradiated nude did not appear to be very promising as a host, and the side effects of higher radiation doses were too severe. The doses used were 2×10^5 , 2×10^6 and 2×10^7 cells for both cell lines.

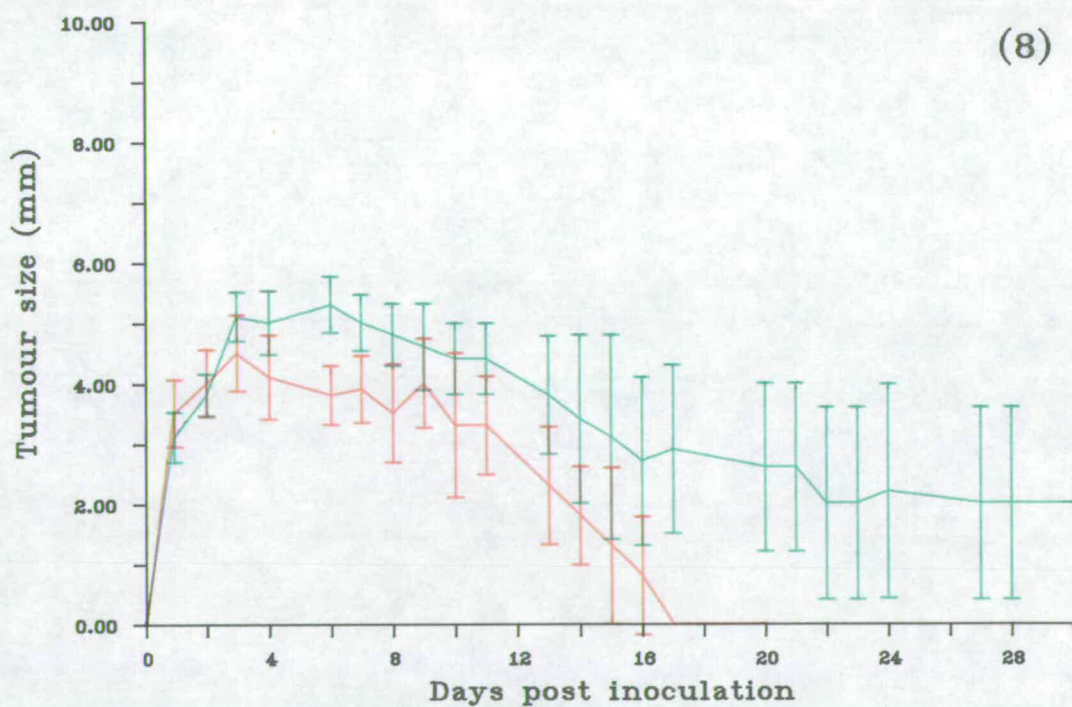
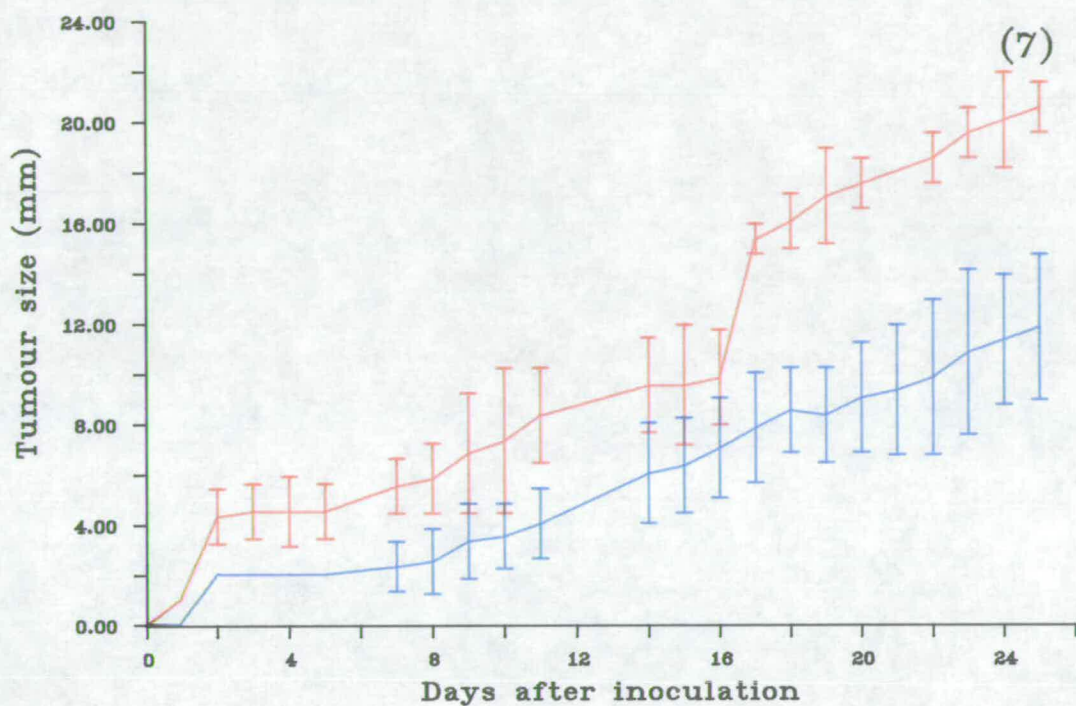
4.3.2 Results

4.3.2.1 TaHis110 mice

The results are presented in graph 7. No visible tumours developed in the low dose (2×10^5 cells) group. In the other two groups, prominent subcutaneous tumours developed at the injection site. In the high dose group, tumours became reddened from days 5-6, with surface necrosis developing by day 14. Medium dose tumours became reddened around day 10, with necrosis from days 18- 19. The tumours were typically rounded, prominent lumps with a red shiny appearance over most of the body of the tumour, and a central necrosed pit where dead material had been eroded. Non-haemorrhaged, healthy tumour tissue could be seen around the edge of the tumours, and tumours continued to grow in size until the mice were destroyed on days 19-25 in the high dose group, and days 25, 29, 36 and 39 in the medium dose group. The mice killed on days 36 and 39 had tumours 18 and 15mm in diameter, respectively.

(7). Growth of tumours in Balb/c nude 4Gy mice injected with TaHis110 cells s/c. There were four mice in each group. Red= 2×10^7 cells; blue= 2×10^6 cells. No tumours developed in mice injected with 2×10^5 cells.

(8). Growth of tumours in C57 beige mice injected with 2×10^7 TaHis110 cells s/c. Green=4Gy (11 mice); red=unirradiated (8 mice). 8 Balb/c 4Gy mice injected at the same time with the same dose of cells developed tumours as shown in graph (1).



4.3.2.2 TpMug110 mice

Only mice in the high dose group developed tumours. These presented as flattened, white subcutaneous masses. The tumours grew slowly, reaching a size of approximately 5mm diameter by day 20, and 10mm diameter by day 52. There was no reddening or obvious necrosis of the tumour, and tumours did not regress.

4.3.3 Histology

4.3.3.1 Dab smears

Large numbers of schizonts and infected cells were seen in dab smears prepared from tumours. In two cases (one TaHis110 and one TpMug110) some schizonts were seen in dab smears from the kidney. These results are shown in table 4.

4.3.3.2 Sections

Sections of *T.annulata* tumours from Balb/c nude 4Gy mice showed similar features to those seen in Balb/c 4Gy mice: infiltration of subcutaneous fat and muscular tissue by tumour cells, and development of general haemorrhage and central necrosis (plates 5 and 6). However, the cellular infiltration seen in Balb/c 4Gy mice was not noted to the same extent in Balb/c nude mice.

In general, TpMug110 tumours did not appear to be as well vascularised as TaHis110 tumours, and did not develop the same degree of internal haemorrhage (plate 7).

4.3.4 Establishment of cell lines and Karyotyping

Cell suspensions were prepared from TaHis110 tumours from mice killed on days 19, 25 and 29. These cell lines were maintained *in vitro* for 10-14 days before karyotypes were prepared. The results are presented in table 5. Mice have a diploid chromosome number of twenty, compared to sixty for cattle. Karyotypes of TaHis110 cells prepared at the same time as those of the tumour cell lines had a mean chromosome number of 53 (see table 5). These results do not provide any evidence

Table 4. Schizonts and infected cells in dab smears from Balb/c nude mice.

Mouse	Schizont count
TaHis110 d19 (tumour)	6.7
TaHis110 d19 (kidney)	0.6
TaHis110 d25 (tumour)	16.7
TaHis110 d29 (tumour)	10
TaHis110 d36 (tumour)	10.1
TpMug110 d44 (tumour)	16.8
TpMug110 d44 (kidney)	0.2
TpMug110 d56 (tumour)	23.7

Schizont count= (free schizonts + infected cells) per field for at least 10 x1000 fields.

Mice are identified by the cell line with which they were injected and the experimental day after injection on which they were killed.

Table 5. Chromosome counts from cell lines derived from tumours in Balb/c nude 4Gy mice.

Mouse	Mean count	Range	% Cells infected
TaHis110 d19	54 (9.8)	24-91	88%
TaHis110 d25	51 (7.4)	32-71	(nd)
TaHis110 d29	52 (8.4)	25-76	91%
TaHis110 control	53 (8.7)	17-73	90%

The TaHis110 control was a cell line grown in vitro of the same stock as that which had originally been used to infect the mice. The table shows: the mean number of chromosomes per spread for 100 spreads, with the sample standard deviation (s.d.); the highest and lowest counts recorded for each sample; and the percentage of cells which were seen to be infected.

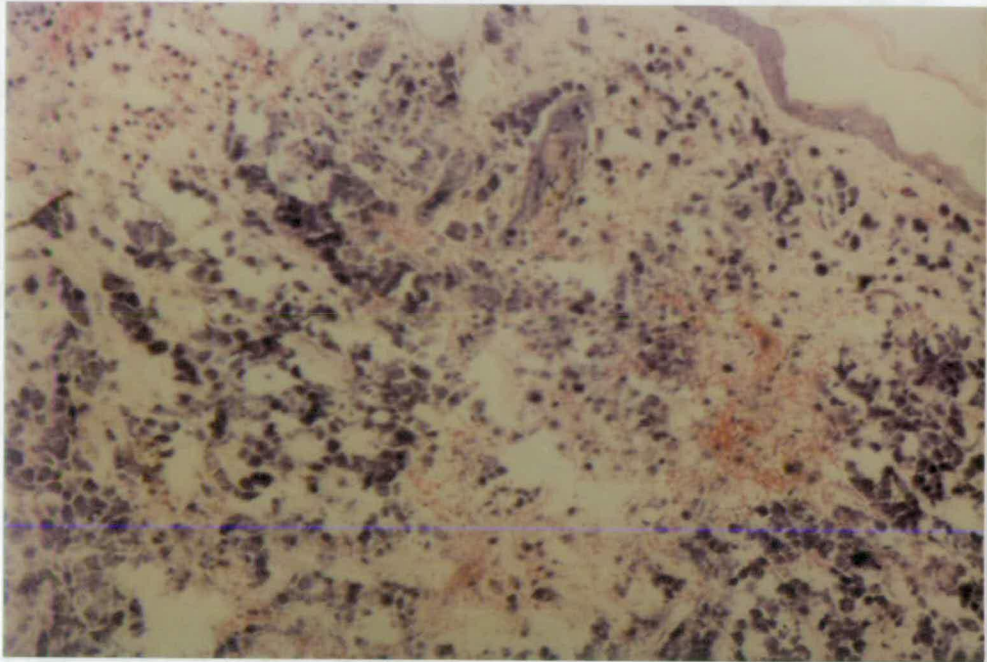
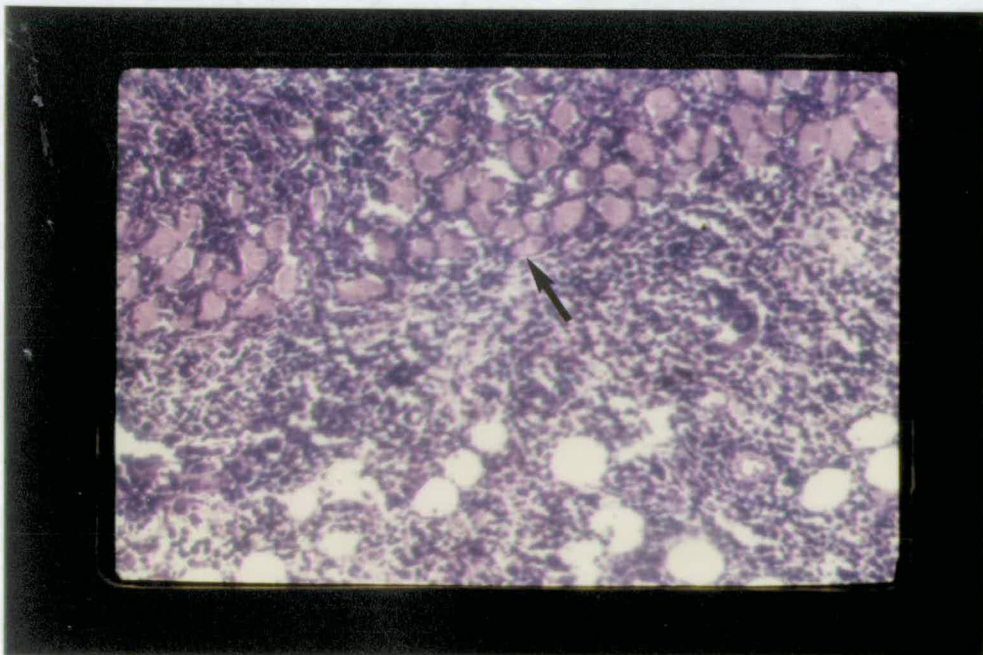


Plate 5. *T.annulata* tumour in a Balb/c nude 4Gy mouse, three weeks after inoculation of cells. The epidermis is at the top right hand corner. There are areas of proliferating cells in the centre of the field and to the lower right; the tumour is extensively haemorrhaged (red areas). The tissue is increasingly necrotic towards the upper left hand side.

Plate 6. *T.annulata* tumour in Balb/c nude 4Gy (x100). Isolated adipocytes can be seen, and a layer of muscle has been infiltrated by tumour cells (arrow). A small blood vessel can also be seen.



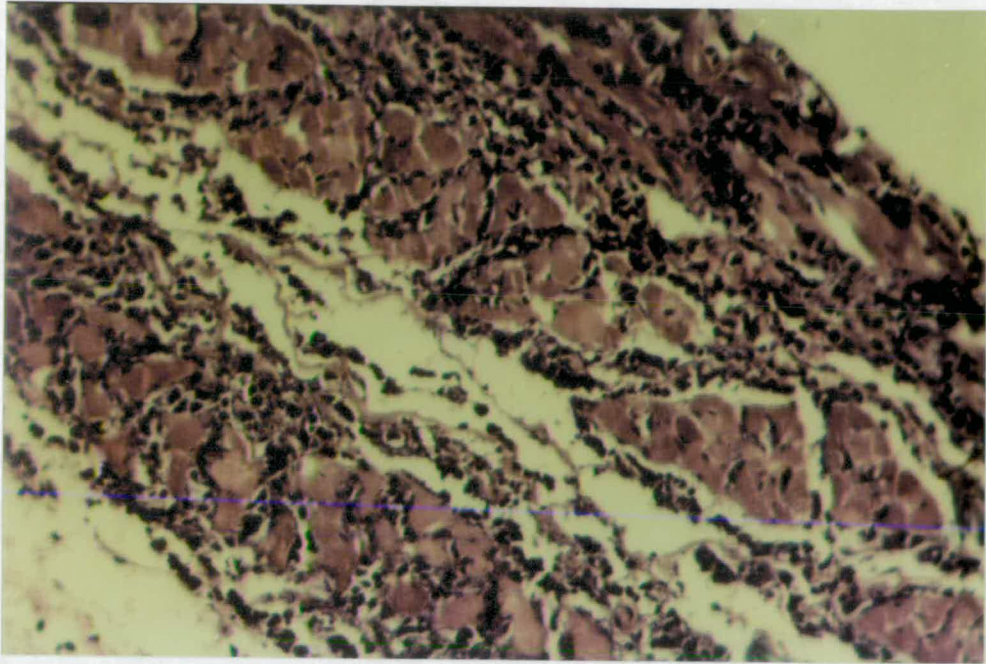


Plate 7. *T.parva* tumour in Balb/c nude 4Gy mouse, showing infiltration of subcutaneous muscle by tumour cells (x100).

for the infection of murine cells by *T.annulata*.

4.4 Discussion

Both TaHis110 and TpMug110 cells grew more rapidly in nude mice than in Balb/c mice at the same cell and radiation doses. Tumours grew to a larger size and persisted for longer. The two cell lines showed the same relative differences as in the Balb/c 4Gy mouse: TaHis110 tumours grew quite rapidly, developed haemorrhage and finally became necrotic; TpMug110 tumours grew much more slowly and did not develop haemorrhage or necrosis. In the irradiated NIMR nude mouse, tumours eventually resolved: tumours in the Balb/c nude mouse showed no sign of resolving despite the development in TaHis110 tumours of central necrosis.

In the relationship between tumour and host, there is a balance between: the rate of growth of tumour cells; natural immune mechanisms; physical factors such as the supply of blood to the tumour. In the Balb/c 4Gy mouse, the equilibrium lies well towards regression, particularly as specific immune mechanisms recover from radiation damage. In the NIMR nude the situation is more finely balanced but eventually leads to regression. In the Balb/c nude (4Gy), the growing tumour is able to "outrun" the immune responses to it: the development of central necrosis does not lead to the destruction of the whole tumour.

The temporary immunodeficiency induced in the Balb/c mouse by irradiation allows *T.annulata* infected cells to establish and grow. In the irradiated Balb/c nude mouse, tumours grow faster and to a larger size. There should be no difference in the physiological environment of the inoculated cells in the two mice. Tumours in both mouse strains develop the same symptoms of haemorrhage and necrosis. The difference in tumour growth between the two strains must depend on the deficiency of specific immune mechanisms, in relation to the size of the tumour burden. In the Balb/c 4Gy mouse, the size of the tumour and its growth may be limited by residual B and T-cell dependent mechanisms. The tumours are damaged by natural mechanisms, which initiate necrosis. As the tumours are relatively small, they are easily destroyed as specific immune responses recover from radiation damage. In the nude 4Gy mouse, the tumours are able to grow to a larger size initially, presenting the recovering immune system with a much larger burden. The limited specific immune responses possessed by nude mice are apparently permanently abolished by

irradiation. The uncontrolled growth of the tumours in irradiated Balb/c nude mice does not mean that natural immunity has been impaired: the congenital lack of a thymus may prevent the recovery of specific immune effector cells after irradiation (Globerson, Fiore-Donati and Feldman, 1962).

CHAPTER 5

ATTEMPTS TO GROW TAHis110 CELLS IN C-57 BEIGE MICE

5.1 Introduction

The previous experiments showed that nude mice could reject *T.annulata* tumours, and that doses of irradiation were required to allow satisfactory growth. This experiment was done to see if mice with the beige mutation were more permissive hosts for *T.annulata*-infected cells than Balb/c or nude mice. The beige mutant is more fully discussed in the General Introduction: to briefly recapitulate, mice with this phenotype lack cytotoxic Natural Killer (NK) cells.

5.2 Methods

A total of 37 C-57 beige mice were used. They were divided into four groups and treated as follows:

Group 1: 2×10^7 TaHis110 s/c, 4Gy (11 mice)

Group 2: 2×10^7 TaHis110 i/p, 4Gy (10 mice)

Group 3: 2×10^7 TaHis110 s/c, 0Gy (8 mice)

Group 4: 2×10^7 TaHis110 i/p, 0Gy (8 mice).

A control group of 8 Balb/c mice were given 4Gy and 2×10^7 TaHis110 cells s/c at the same time.

Two mice from each group, including the controls, were killed on days 7, 14, 21 and 28: cytopins of peritoneal exudates and dab smears of tumours and tissues were prepared and examined for schizonts.

5.3 Results

Swellings developed at the inoculation site in all the mice injected s/c (see graph 8). In the unirradiated mice, these swellings did not appear to grow and had all disappeared by day 17. The tumours grew slightly in the 4Gy group, but never exceeded 6mm in diameter, and steadily regressed from day 6 onwards. The tumours did not become haemorrhagic.

In the Balb/c (4Gy) mice, tumours appeared as previously described, grew to 6-8mm diameter, and regressed through haemorrhage and subsequent necrosis.

None of the mice injected i/p developed ascites.

A single free schizont was seen in a cytospin of a cell suspension prepared from the "tumour" of a 4Gy beige mouse, killed on day 7. This was the only parasite seen in material taken from beige mice during the experiment. Schizonts were seen in dab smears from tumours of both Balb/c mice killed on day 7 (0.8 and 36 schizonts/field) and in one of the Balb/c mice killed on day 14 (0.2 schizonts/field). No schizonts or infected cells were seen in peritoneal exudates, or dab smears from other organs, of any mouse on any day.

5.4 Discussion

The finding that beige mice were more resistant to the growth of *T.annulata* infected cells than Balb/c 4Gy mice was surprising. This may have been due to some other aspect of the beige mutation, such as greater radioresistance, or a difference in the background of the mice. As the beige mice did not appear to be potential hosts for *Theileria*-infected cells, these possibilities were not investigated further. The actual defect in beige mice may be in the cytolytic mechanism of the NK cell, rather than an absence of NK cells (Roder and Duwe, 1979): it is possible that beige NK cells are still capable of acting as "helper" cells.

CHAPTER 6

TREATMENT OF TAHIS110 TUMOURS WITH TUMOUR NECROSIS SERUM

6.1 Introduction

Several authors have described the treatment of various subcutaneous tumours in mice with preparations containing TNF activity. These results have already been discussed in the General Introduction. The experiments described in this chapter were done to investigate the effect of rabbit serum, containing TNF activity, on subcutaneous TaHis110 tumours established in irradiated mice.

The selection of a dose of rabbit tumour necrosis serum (TNS) to give to the mice was based on several considerations: the doses which proved efficacious in published results; the possibility of TNF-related toxicity; and the amount of TNS available, bearing in mind the need to use a reasonably sized group of animals. A dose of 10^4 U/mouse was decided upon. Serum taken from the same rabbit before injection with lipopolysaccharide was diluted by the same amount to act as a control.

It was hoped to detect endogenous TNF activity in mice in which tumours were resolving. TNF activity had previously been shown in mice bearing the SA- 1 murine sarcoma, when haemorrhagic necrosis was induced by administration of endotoxin (North and Havell, 1988). *T.annulata* tumours were already undergoing haemorrhagic necrosis.

Three experiments involving the treatment of tumours with TNS are presented here. The first used two consecutive doses of TNS intravenously in Balb/c 4Gy mice; the second used repeated doses intratumourally in Balb/c nude 4Gy mice; and the final experiment used intratumoural injection of TNS into Balb/c 4Gy mice. A control experiment to look at TNS toxicity was also carried out.

6.2 Control Experiment

Four Balb/c mice were given 4Gy and injected subcutaneously with 10^4 U TNS or control serum (CS) diluted in PBS. No signs of toxicity other than the loss of condition associated with irradiation were observed.

6.3 Experiment 1: Balb/c 4Gy mice i/v

6.3.1 Methods

Ten Balb/c mice were irradiated and injected s/c with 2×10^7 TaHis110 cells. The injection site was shaved before the start of the experiment. The mice were divided into two groups of five, one group to receive TNS and the other CS. 10^4 U TNS or CS, diluted in PBS, were injected into the tail vein on days 5 and 9.

6.3.2 Results

The results are shown in graph 9. There was no apparent difference between the two groups in tumour size, time of onset of tumour regression, or rate of tumour regression.

6.4 Experiment 2: Balb/c nude 4Gy mice

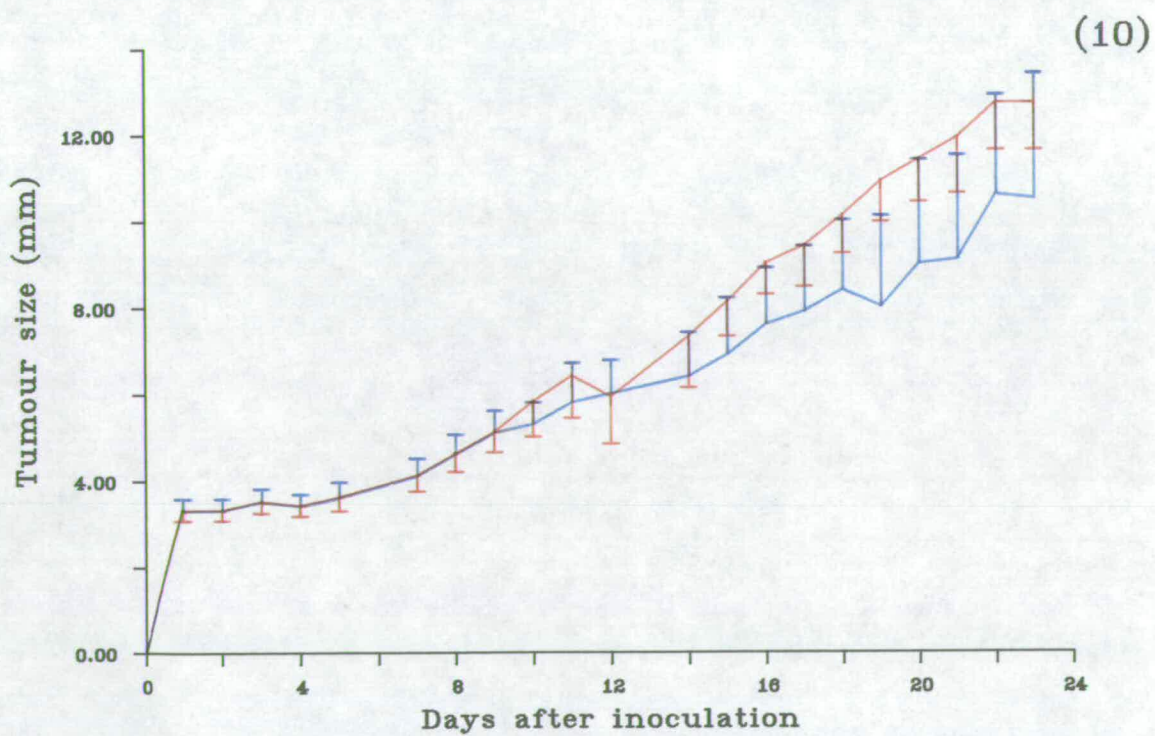
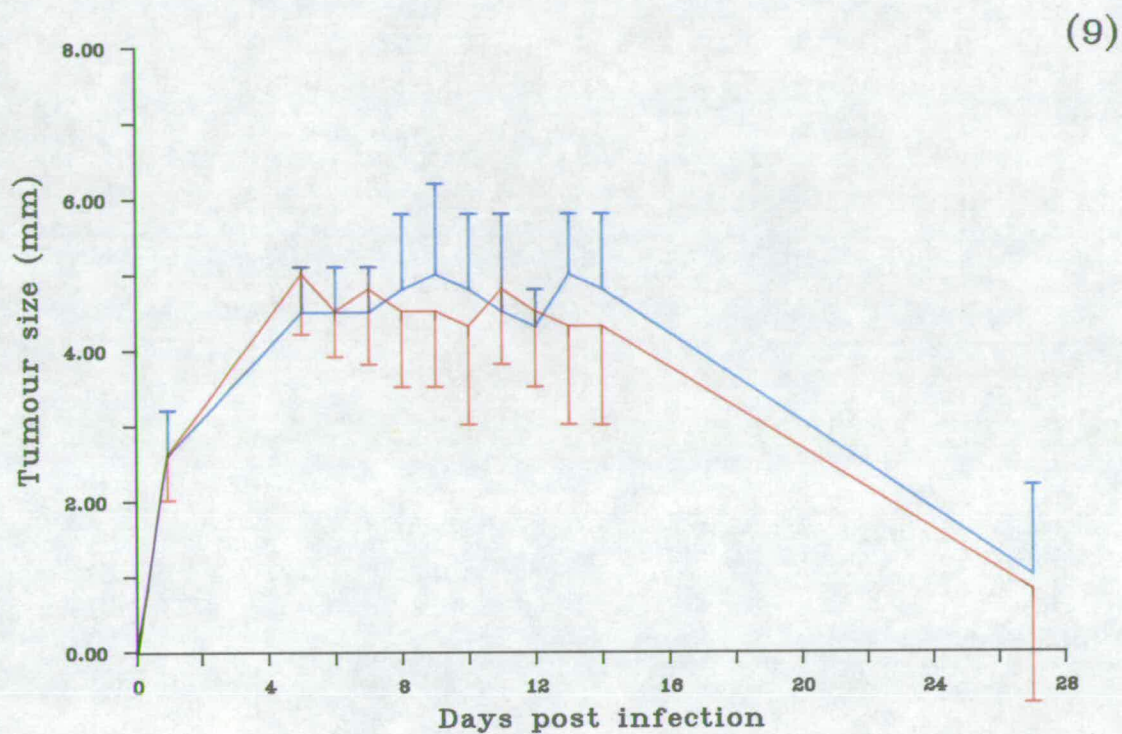
6.4.1 Methods

Sixteen Balb/c nude mice were irradiated and injected with 1.5×10^7 TaHis110 cells s/c. The animals were divided into two groups of eight, one group to receive TNS and the other CS. 10^4 U/ mouse of TNS or CS were given i/t on days 9, 14 and 18.



(9). Effect of rabbit tumour necrosis serum (TNS) given intravenously (i/v) on the growth of s/c TaHis110 tumours in Balb/c 4Gy mice. Red= 10^4 U TNS on days 5 and 9; blue=equal dilution of control rabbit serum on days 5 and 9. Errorbars are 95% confidence limits.

(10). Effect of rabbit TNS given intratumorally (i/t) on s/c TaHis110 tumours in Balb/c nude 4Gy mice. Red= 10^4 U TNS/mouse on days 9,14 and 18; blue=equal dilution of control serum on the same days.



6.4.2 Results

Tumours developed at the inoculation site as described in chapter 2. Graph 10 shows the mean tumour size, with 95% confidence limits, in each group. The tumour sizes are plotted as a single group up to day 9, when the first dose of TNS was given. As in the previous experiments in Balb/c nude 4Gy mice, the tumours continued to grow until the animals were destroyed, despite the development of visible haemorrhage and central necrosis. It can be seen from the graph that the mean tumour size was slightly higher in TNS treated mice: there is however no significant difference between the groups, based on comparison of 95% confidence limits. Tumours treated with TNS did not show an earlier onset or a greater degree of necrosis than tumours treated with CS.

6.5 Experiment 3: Balb/c 4Gy mice

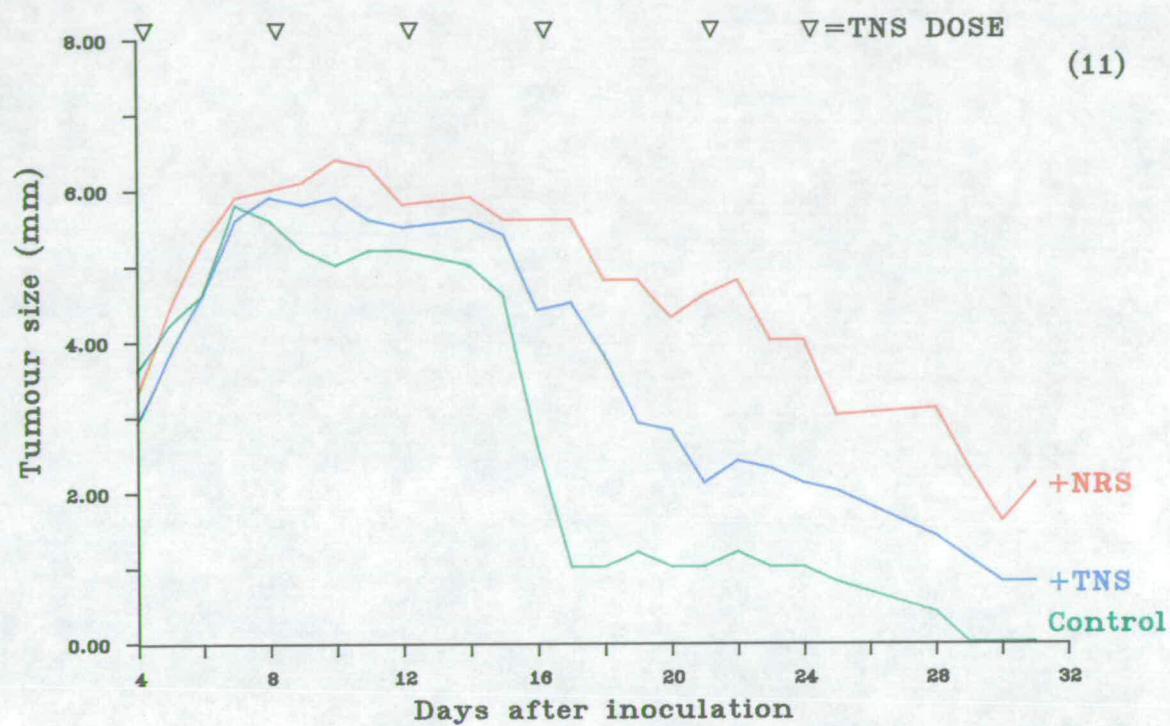
6.5.1 Methods

Twenty-two mice were irradiated and injected s/c with TaHis110 cells as previously described. Prior to irradiation, the inoculation site was shaved and the weights of the mice were recorded. The animals were divided into three groups: nine were to receive TNS i/t, eight were to be given CS i/t, and the remaining five were left as untreated controls. TNS or CS was administered at the usual dose on days 4, 8, 12, 16 and 21 post-infection. The tumours were measured daily using calipers, and any haemorrhage or necrosis was noted.

6.5.2 Results

The results are presented in graph 11. Considerable variation in tumour size was seen within groups, but there was no difference between the groups. The onset of haemorrhage and necrosis also occurred at the same time in the different groups. Comparison of data using the Mann-Whitney test did not show significant differences between the groups.

The weights of the mice did not differ significantly between groups: all mice



showed a drop of 3-4g between day 0 and day 3 due to irradiation, but weights had recovered to normal (approximately 20g) by day 23.

6.6 Tests for endogenous TNF activity

6.6.1 Methods

Balb/c 4Gy mice with TaHis110 tumours were used as sources of material for L-929 assays. Serum was obtained as described in "Materials and Methods". Tumours were excised, chopped with sterile razor blades in cold PBS and homogenised in PBS. Spleens were removed and homogenised in cold PBS. The tumour and spleen homogenates were sieved to remove debris, centrifuged and sterile-filtered to produce a cell-free extract. Sera, tumour and spleen extracts were applied to L-929 monolayers at a dilution of 1/4.

6.6.2 Results

No cytotoxic effect was seen on L-929 monolayers treated with serum, tumour extract or spleen extract obtained 4 and 8 days after initiation of the tumours. Spleen extracts taken on days 12 and 16 caused proliferation of L-929 cells. No tumour extracts were made on these days due to advanced necrosis of the tumours.

6.7 Discussion

A rabbit TNF preparation with anti-L-929 activity did not show activity against *T.annulata* tumours in mice, although administered by two different routes. This preparation had show anti-*Theileria* activity *in vitro* (Preston, unpublished data). The lack of activity *in vivo* could be because: (i) the preparation was not effective against *T.annulata* infected cells *in vivo*, or at recruiting inflammatory responses in the mouse; (ii) the doses were insufficient to have an effect; (iii) the effect was masked by the action of endogenous TNF. The failure to detect endogenous TNF in L-929 assays does not rule out its presence: TNF is known to have a very short half life in circulation, and may have been released only at a local level, and rapidly cleared.

CHAPTER 7

RADIATION-INDUCED CHANGES IN LYMPHOCYTE SUBPOPULATIONS

7.1 Introduction

The previous experiments had shown that a radiation dose of 4Gy would permit the growth of *T.annulata* infected cells in Balb/c mice. Regression of the tumours occurred after two to three weeks. It was presumed that this was because of the recovery of the mouse's immune system from radiation damage. This experiment was designed to investigate: the effects of the 4Gy dose on the main lymphocyte subsets (B, cytotoxic T and helper T-cells); the influence the presence of a subcutaneous *Theileria* tumour would have on the recovery of lymphocyte numbers; natural immunity in irradiated and tumour-bearing mice.

At an earlier stage, attempts were made to label macrophages and NK cells for flow cytometry using the anti-Mac-3 antibody (Boehringer) as a macrophage marker, and anti-asialo GM1 (WAKO) as an NK cell marker. Trial experiments did not show the activity or specificity required for FACS analysis. Alternative methods of identifying macrophages and NK cells were therefore used: macrophages were counted directly in Giemsa-stained smears, and cytotoxic NK cells were detected by YAC-1 assays.

7.2 Methods

47 female Balb/c mice were split into four groups and treated as follows:

Group A: untreated controls (13 mice)

Group B: irradiated 4Gy (13 mice)

Group C: 4Gy and 2×10^7 TaHis249 cells s/c (13 mice)

Group D: unirradiated, 2×10^7 TaHis249 cells s/c (8 mice)

2 mice from groups A,B and C were killed on days 2,6,9,13 and 16. Group D mice were killed on days 2,5,9 and 16. The surviving mice in groups A and C were killed on day 27: one mouse in group B died on day 6.

Peritoneal exudates were prepared, and total and differential cell counts were made. Spleens were removed and spleen cell suspensions were made from each mouse. Cells were labelled with antibodies as described in Materials and Methods. Spleen cells were also prepared for YAC-1 assays as described in Appendix A. In addition, a differential count was made from a cytopsin of the spleen cell suspension.

Antibodies used

<i>Antibody</i>	<i>Cells labelled</i>
Anti-mouse IgG FITC	Surface IgG (sIgG) on B-lymphocytes
Anti-Ly-2	Ly-2+ cells are predominantly cytotoxic T-cells.
Anti-L3T4	Helper T-cells

(See also "Materials and Methods".)

7.3 Results

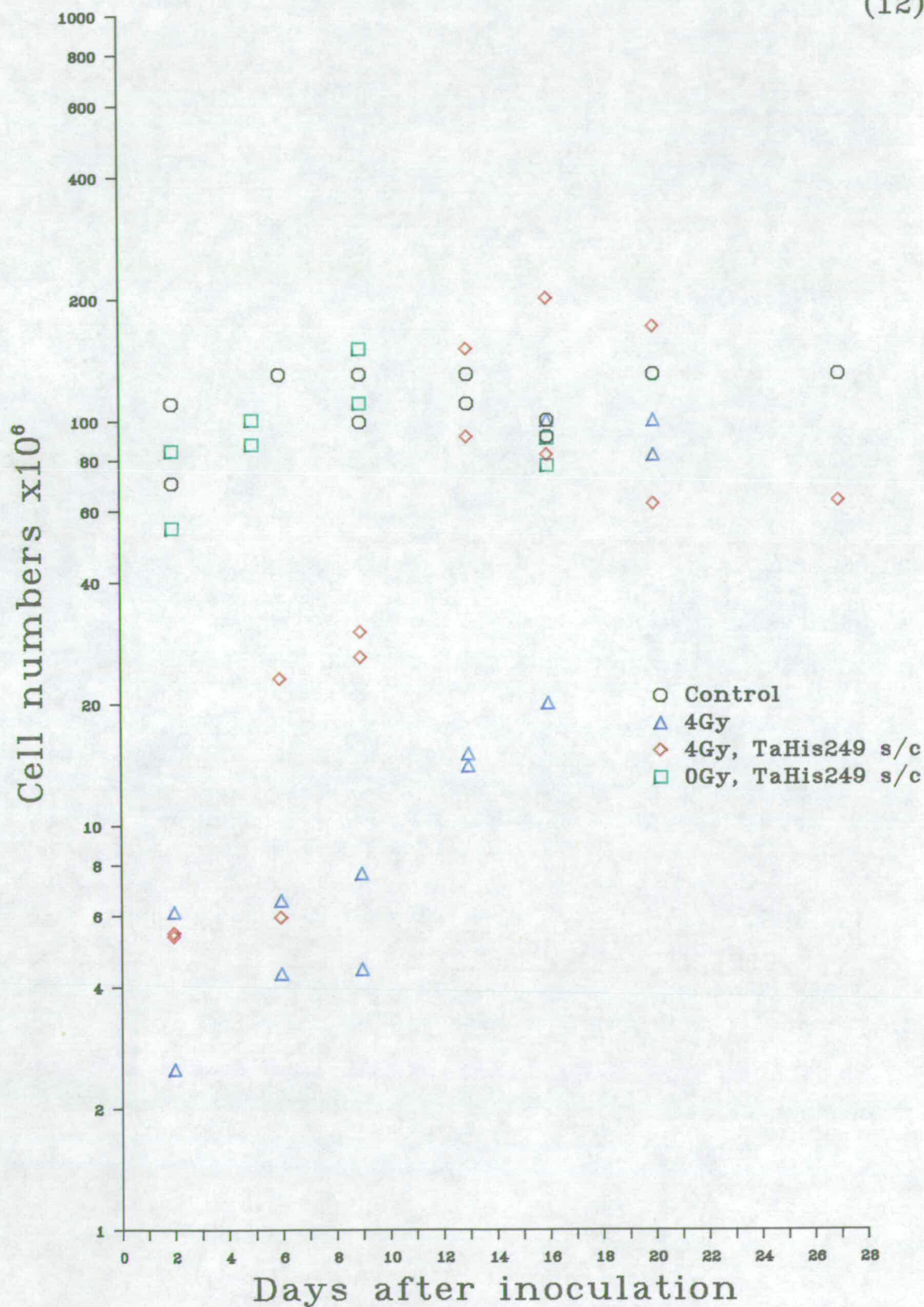
In both irradiated groups, the total spleen cell count on day 2 was 4-5% of control values, falling from over 1×10^8 cells/spleen to approximately 4×10^6 cells/spleen (graph 12). The cell counts returned to normal levels by day 13 in the tumour-bearing group (C) but not until day 20 in the mice which received radiation only (group B).

The drop in cell numbers up to day 2 was reflected in the lymphocyte subsets, but was not evenly distributed among them. Figure 1 shows the proportion of sIgG+ cells in the spleens of one mouse from each group on day 2. The left hand peak represents unstained cells. The numbers next to each right-hand peak are the percentages of cells analysed which were measured as sIgG+. The percentage of sIgG+ cells was lower in the irradiated groups than in the control. Similarly, figures 2 and 3 show the proportion of Ly-2+ and L3T4+ cells, respectively. The Ly-2+ population did not show a steep decline with respect to the total cells present. The percentage of L3T4 cells increased in the irradiated mice relative to the controls.

(12). Total spleen cell counts from control, irradiated and tumour bearing Balb/c mice. Each point represents a single spleen. Circles, untreated mice; blue triangles, irradiated (4Gy) mice; red diamonds, 4Gy mice injected with 2×10^7 TaHis249 cells s/c; green squares, mice given TaHis249 cells only. All cell counts are $\times 10^6$.

Total spleen cells

(12)



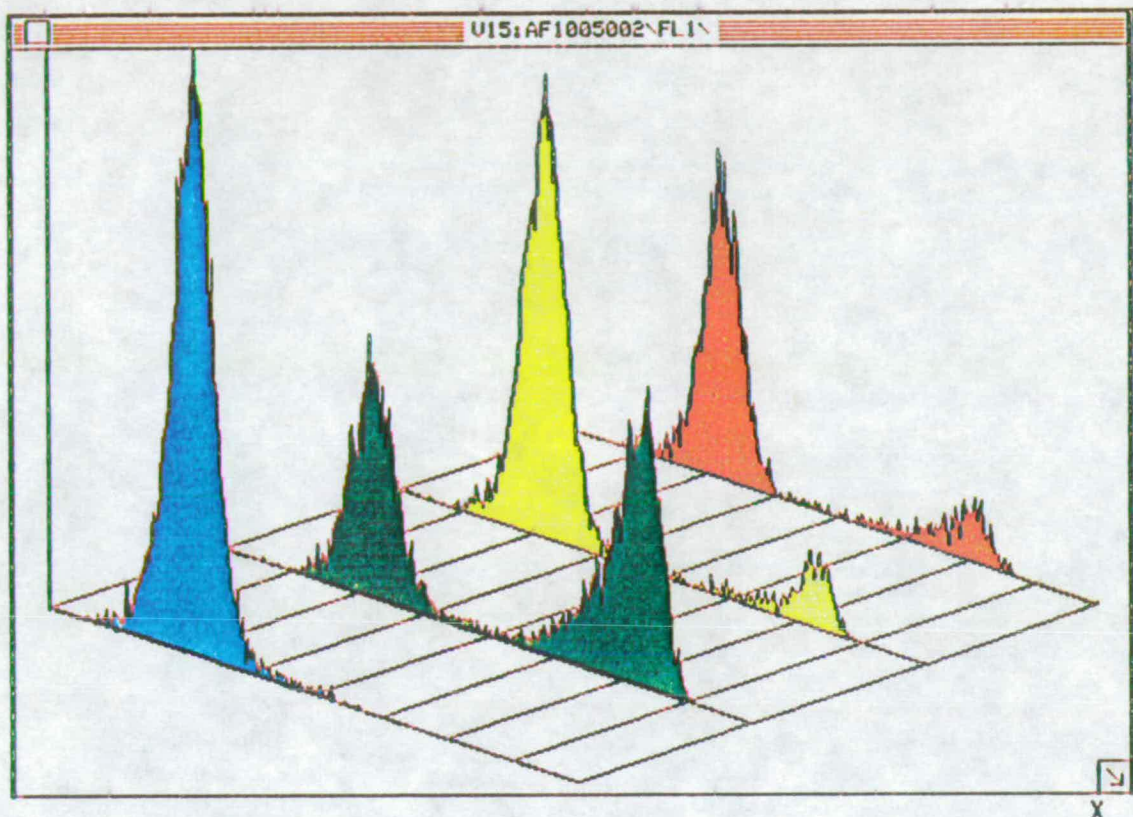


Figure 1. Percentages of sIgG+ cells in the spleens of control, irradiated and irradiated, tumour-bearing Balb/c mice two days after irradiation. The vertical axis is a percentage scale: the horizontal axis is a logarithmic fluorescence scale. The blue peak represents unlabelled lymphocytes; green represents cells from a control mouse; yellow, a 4Gy irradiated mouse; and red, an irradiated, tumour-bearing mouse. The right-hand peaks therefore correspond to sIgG+ cells, while the left-hand peaks represent sIgG- cells. The percentage of sIgG+ cells is reduced in both the irradiated and irradiated, tumour-bearing mice.

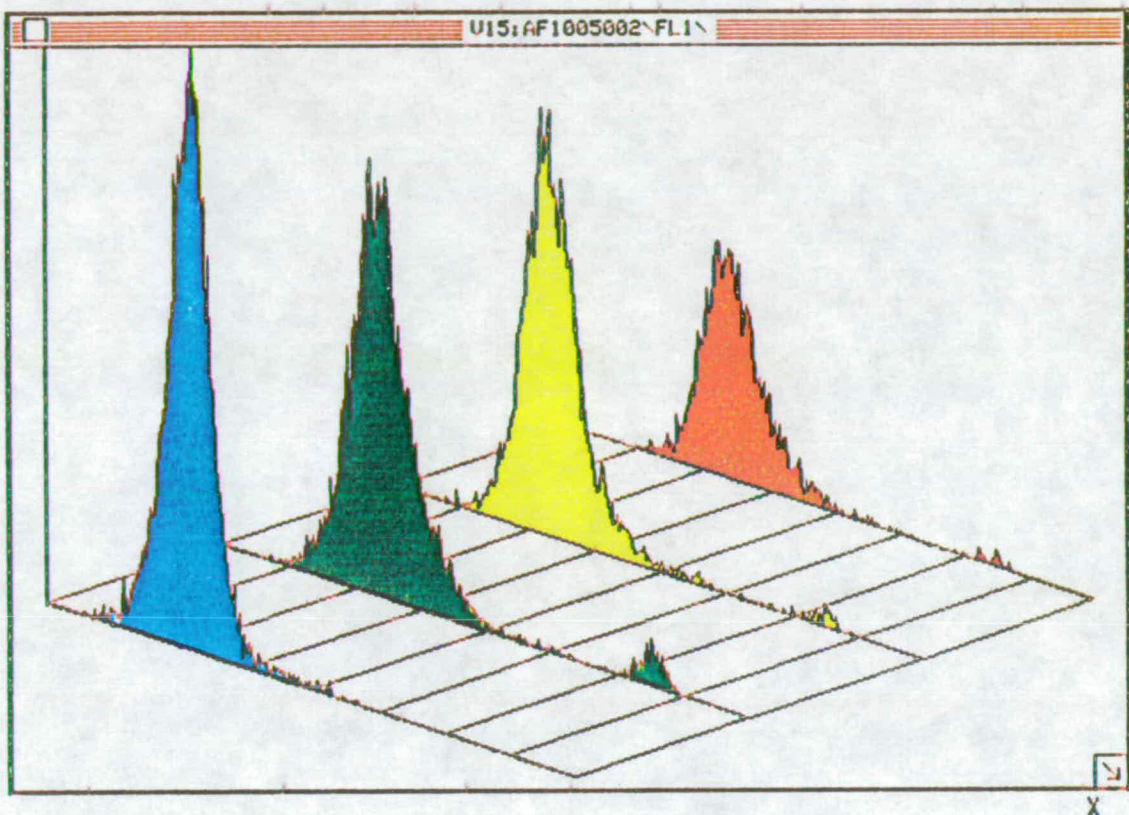


Figure 2. Percentages of Ly-2+ cells in the spleens of Balb/c mice two days after irradiation. The axes and colour-coding are the same as in the previous figure. Ly-2+ cells are less numerous in control mice (green) than sIgG+ cells, but are still markedly reduced in irradiated and irradiated, tumour-bearing mice.

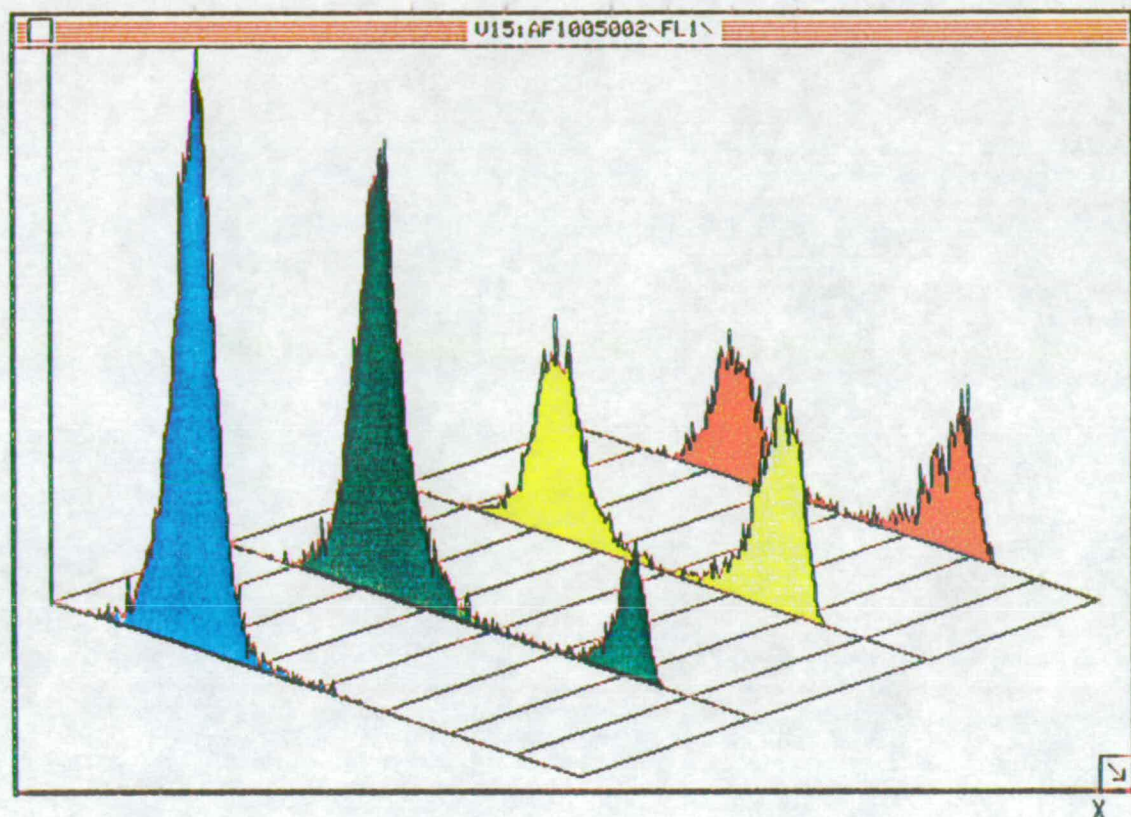


Figure 3. Percentages of L3T4+ cells in the spleens of Balb/c mice two days after irradiation. The axes and colour-coding are the same as in the previous two figures. Irradiation led to a relative increase in the proportion of L3T4+ cells in the spleen.

Table 6 shows the number of sIgG+, Ly-2+ and L3T4+ cells as a percentage of the number of cells positive for these markers in the group A mice on day 2. It can be seen that the drop in cell numbers is relatively greatest in the sIgG+ (B-cell) population, and least in the L3T4+ (T-helper) population, while the Ly-2+ (putative cytotoxic) cells occupy an intermediate position.

Graph 13 shows the number of sIgG+ lymphocytes/spleen for each mouse. The number of sIgG+ cells fell sharply in both irradiated groups, and then recovered to approach control values by day 20. Recovery appeared to occur more quickly in the C group mice between days 6 and 16, but then seemed to be overtaken by the B group although there was some individual variation. Group D mice, which received TaHis249 cells but no irradiation, did not show any changes in cell numbers relative to the controls. Similar results were obtained for the Ly-2+ and L3T4+ lymphocyte subsets (graphs 14 and 15): recovery of cell numbers was more rapid in the C group than in the B group, while group D exhibited little variation from control. There was no apparent difference between the lymphocyte subsets in rate of recovery of numbers.

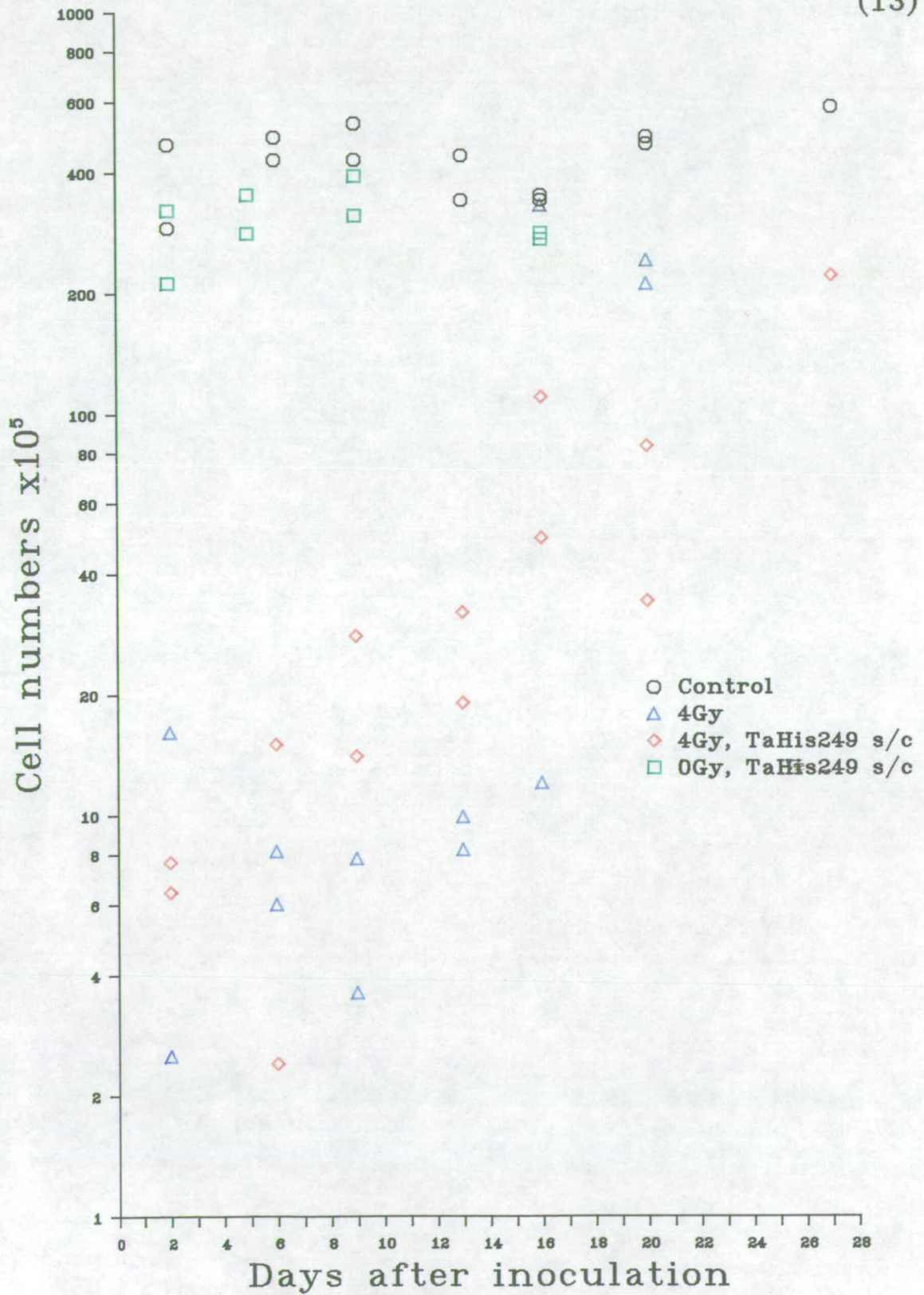
It was noted that the spleens of group C mice became enlarged by day 13 to more than double their normal size, and this enlargement was reflected in an increase in total cell numbers above control levels. This spleen enlargement was associated with the onset of necrosis of the tumours. Figures 4 a,b and 5a,b are three-dimensional plots of complexity (x), size (y) and % of cells (z) for one mouse from each of groups B and C on days 2 and 13. In the B group, the single spike represents the lymphocytes. There was a "tail" of larger cells, and a small cluster of granular cells. In the day 2/C group spleens, the lymphocyte peak could still be identified, but there was a considerable body of larger, granular cells. These large, granular cells were excluded during the analysis of FACS data because of their non-lymphocytic nature. In the day 13/C spleen, a high proportion of large, relatively non-granular cells was present. Differential counts made from spleen cytopspins on days 13 and 16 showed a marked increase in the number of macrophage-type cells.

In the C group, the proportions of sIgG+, Ly-2+ and L3T4+ cells in the lymphocytic population (as identified and analysed with the FACS) decreased sharply on day 13 (table 7), although there was no corresponding fall in the absolute numbers/spleen of these cells. There was therefore a proliferation of cells negative for these surface markers.

(13). Total numbers/spleen of sIgG+ cells, calculated from FACS data. Each point represents a single spleen. Symbols as for graph 12: all counts are $\times 10^5$.

sIgG+ cells

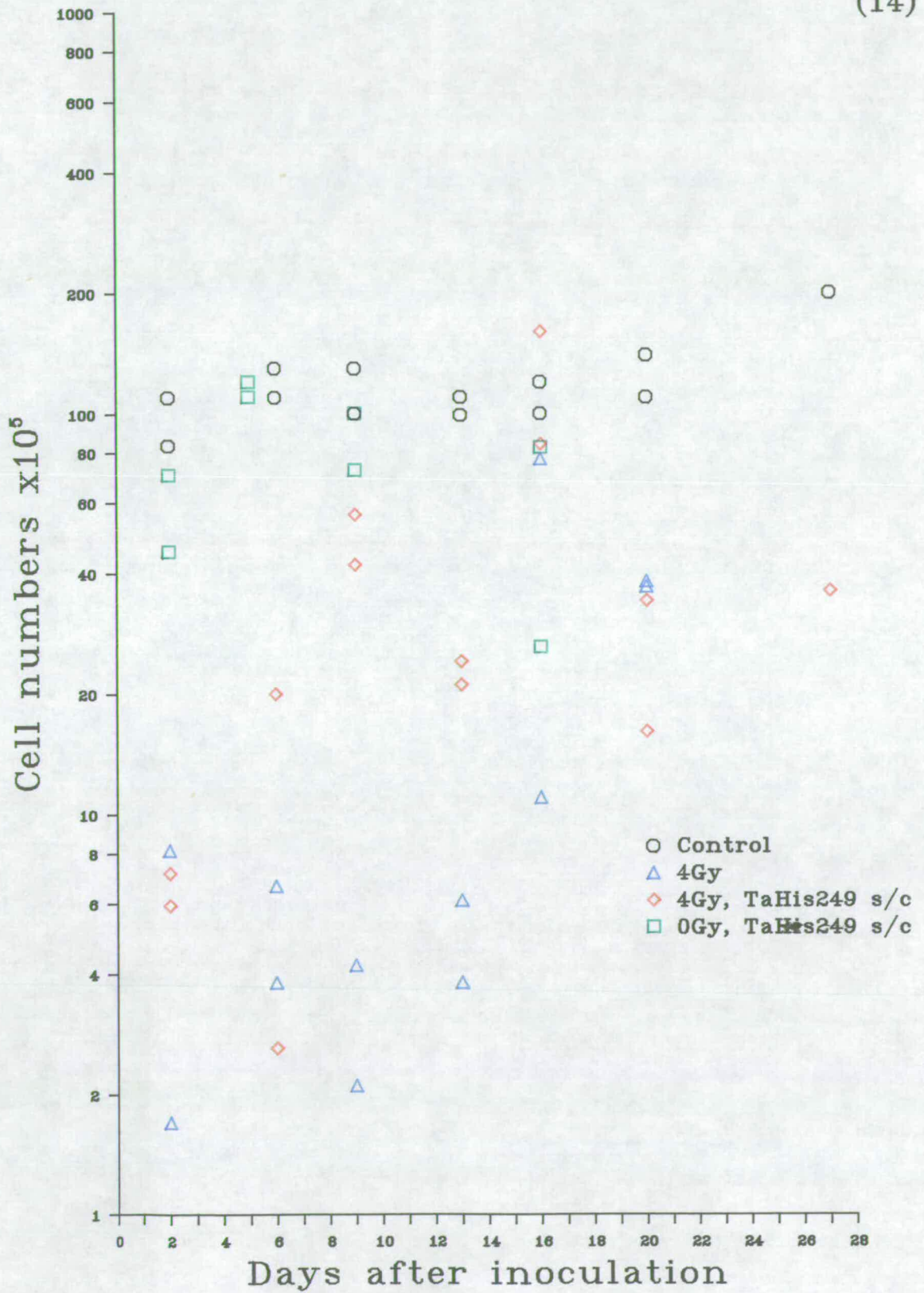
(13)



(14). Total numbers/spleen of Ly-2+ cells, calculated from FACS data. Each point represents a single spleen. Symbols as for graphs 12 and 13.

Ly-2+ cells

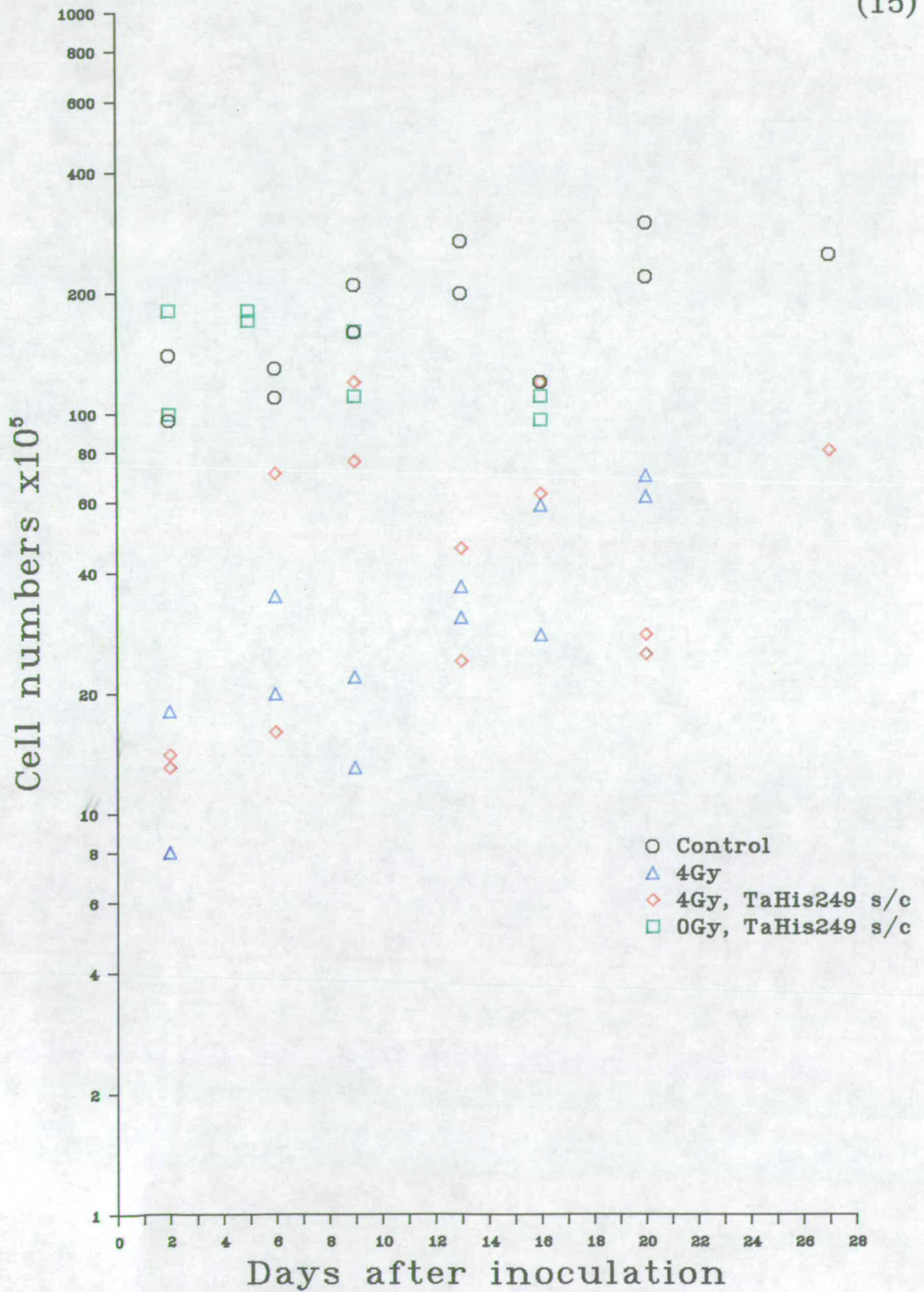
(14)



(15). Total numbers/spleen of L3T4+ cells, calculated from FACS data. Each point represents a single spleen. Symbols as for graphs 12, 13 and 14.

L3T4+ cells

(15)



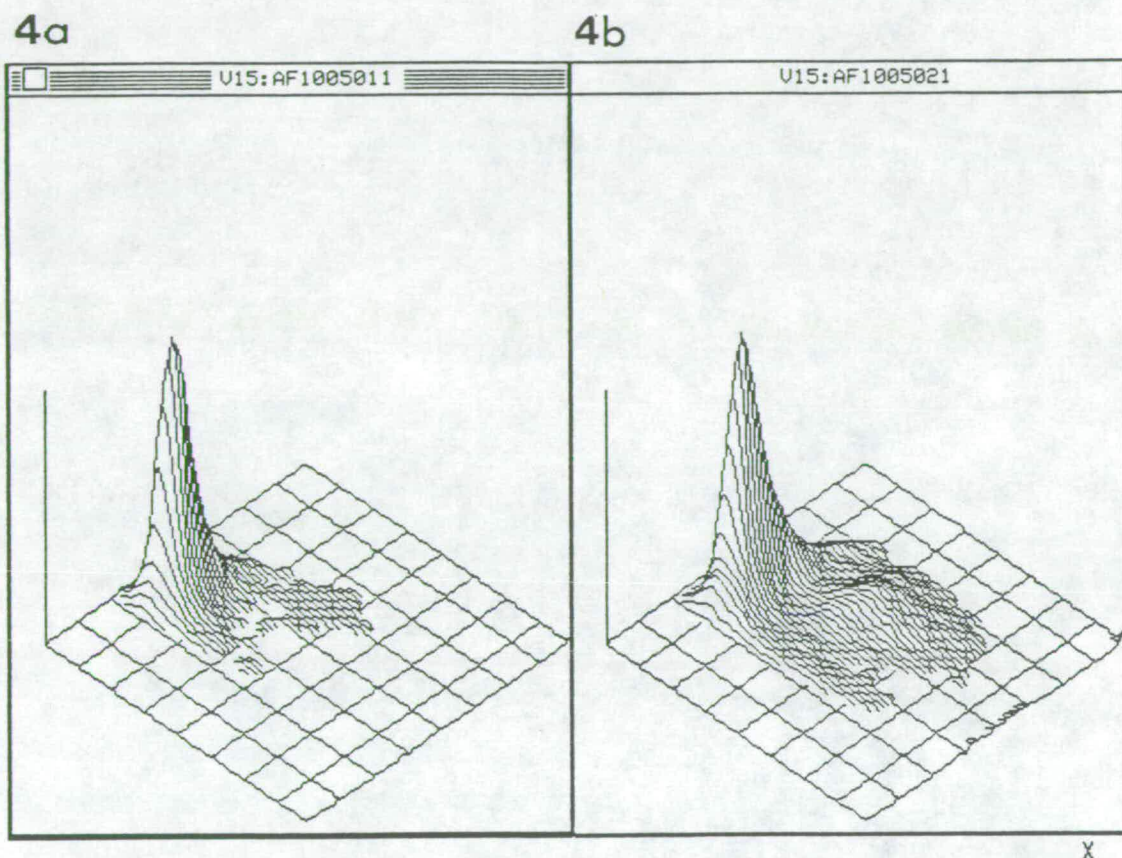


Figure 4a. Three dimensional plot of complexity (x), size (y) and percentage of cells (z) for total spleen cells from a Balb/c 4Gy mouse, two days after irradiation. The main "spike" represents lymphocytes.

Figure 4b. Three dimensional plot of spleen cells from a Balb/c 4Gy, tumour-bearing mouse two days after irradiation and injection of cells. The axes are the same as in figure 4a. There is a body of large, granular cells present in addition to the lymphocytes.

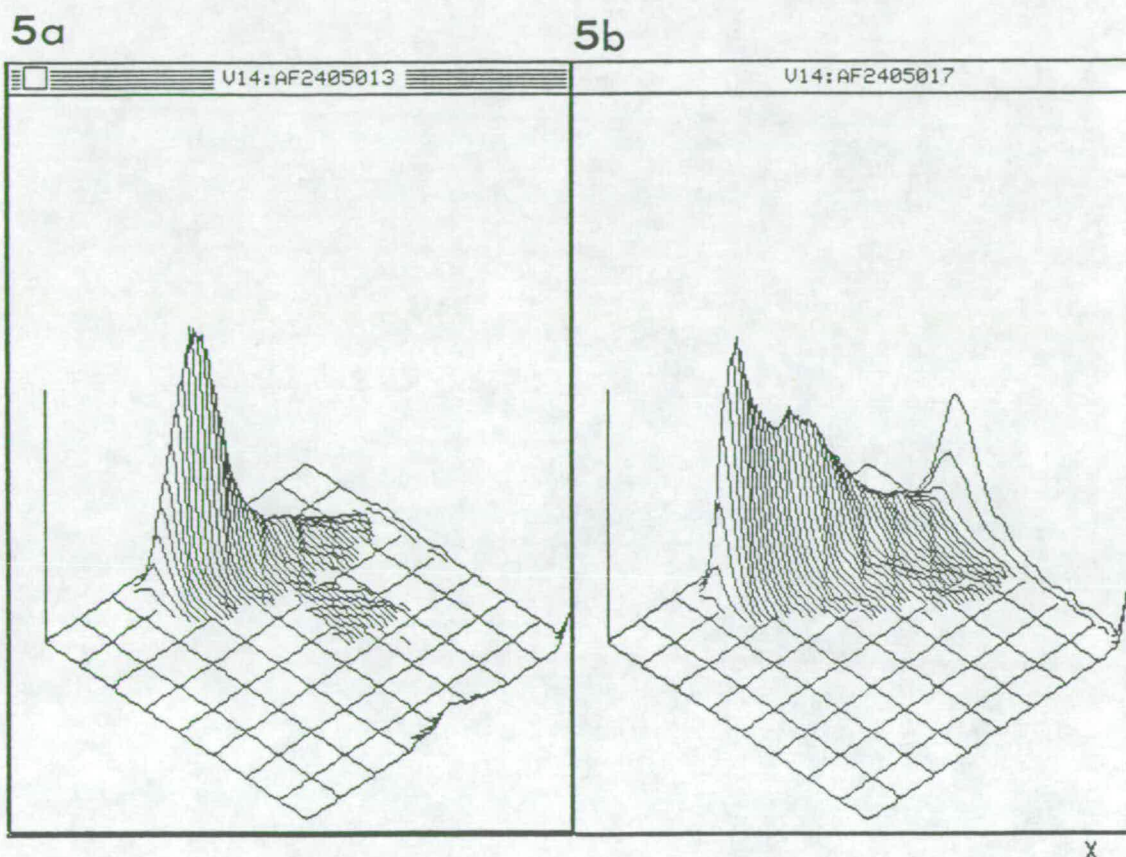


Figure 5a. Three dimensional plot of spleen cells from a 4Gy mouse, thirteen days after irradiation. There is a "tail" of large, macrophage-type cells as well as a small cluster of granular cells.

Figure 5b. Three dimensional plot of spleen cells from a 4Gy, tumour- bearing mouse, thirteen days after irradiation. There is a marked proliferation of large, macrophage-like cells. The total cell count in this spleen was approximately 10-fold higher than that for the spleen represented in figure 5a.

7.3.1 Total and differential counts from peritoneal exudates

The total count of peritoneal exudate cells (Pec) fell by approximately 60% after irradiation. Graph 16 shows the numbers of lymphocytes in peritoneal exudates from group A, B and C mice. Each point represents one mouse. The numbers of peritoneal lymphocytes were consistently lower in the irradiated groups. There was no clear difference between the B and C groups. Graph 17 shows the total numbers of peritoneal macrophages in groups A, B and C. The values for the three groups overlapped and did not show any consistent differences between the irradiated and control mice.

7.3.2 Anti-YAC-1 activity

Graphs 18, 19 and 20 show the percentage killing of labelled YAC-1 cells by spleen cells at an effector to target (E/T) ratio of 100:1. There were significant levels of anti-YAC-1 activity in C group spleens on days 2 and 9 (graphs 18, 19), and in one B group spleen on day 9 (graph 20). The difference between the B and C group spleen was not significant ($p > 0.05$) in a Mann-Whitney test. Spleen cells from D group mice did not show cytotoxicity significantly above control levels.

7.4 Discussion

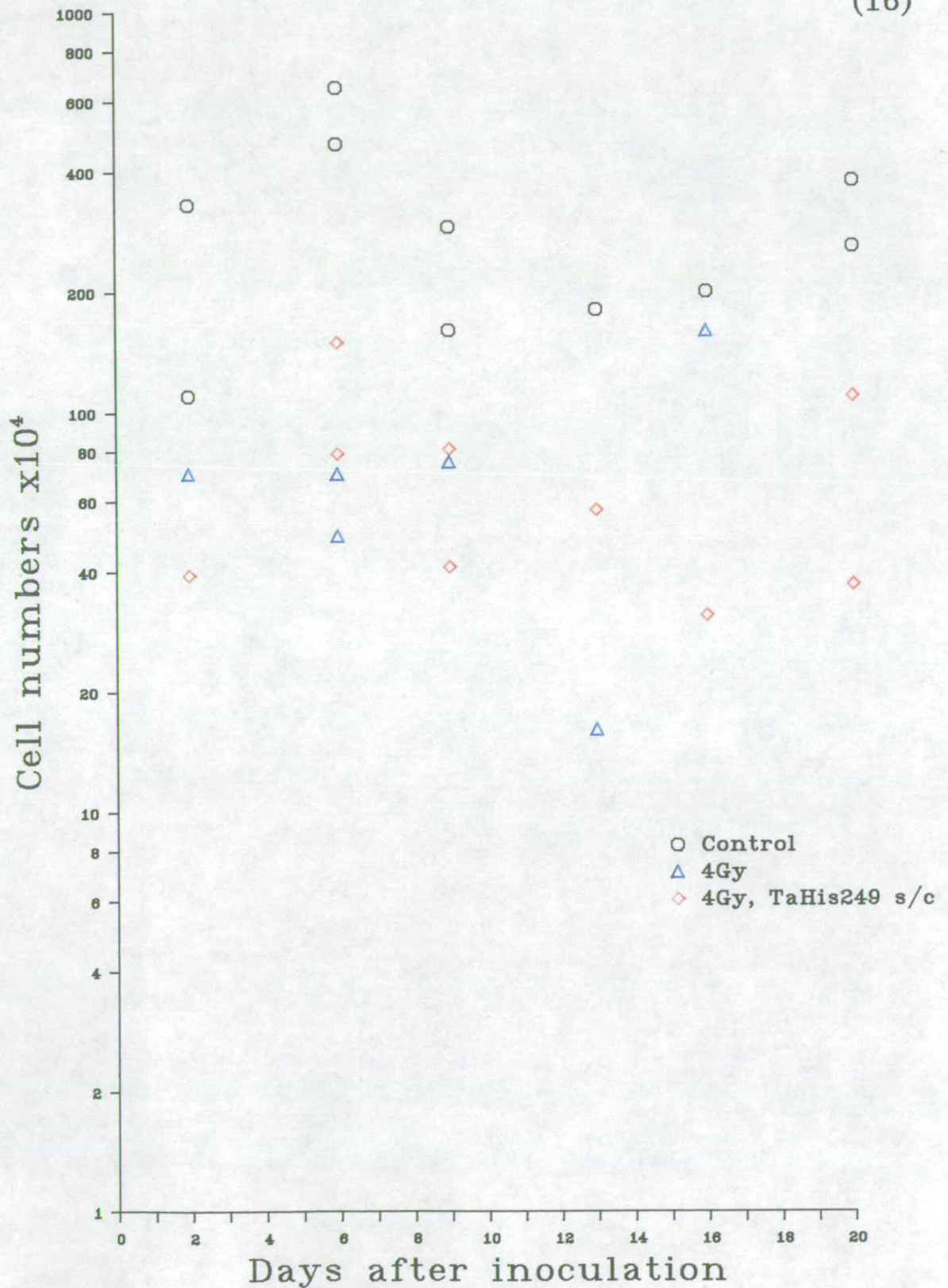
A radiation dose of 4Gy caused a rapid drop in the numbers of spleen cells. Of the lymphocyte subsets examined, sIgG+ B-cells were the most susceptible, Ly-2+ cells were slightly more resistant and L3T4+ T-helper cells were the least sensitive. These results are consistent with published data (Kataoka and Sado, 1975; Sado et al, 1988). Lymphocytes were also depleted from the peritoneal cavity.

The drop in cell numbers up to day 2 was the same in groups B and C, so there was no "protective" effect due to the presence of the tumour. Absolute cell numbers recovered more quickly in tumour-bearing mice than in mice which were only irradiated. This enhanced proliferation affected all three subsets; there was no differential increase in the numbers of one subset. As the tumours became haemorrhagic and started to resolve, a massive increase in spleen cell numbers was seen. FACS analysis and cytopspins showed an increase in macrophage type cells. The

Lymphocytes in Pex.

10-01120170

(16)

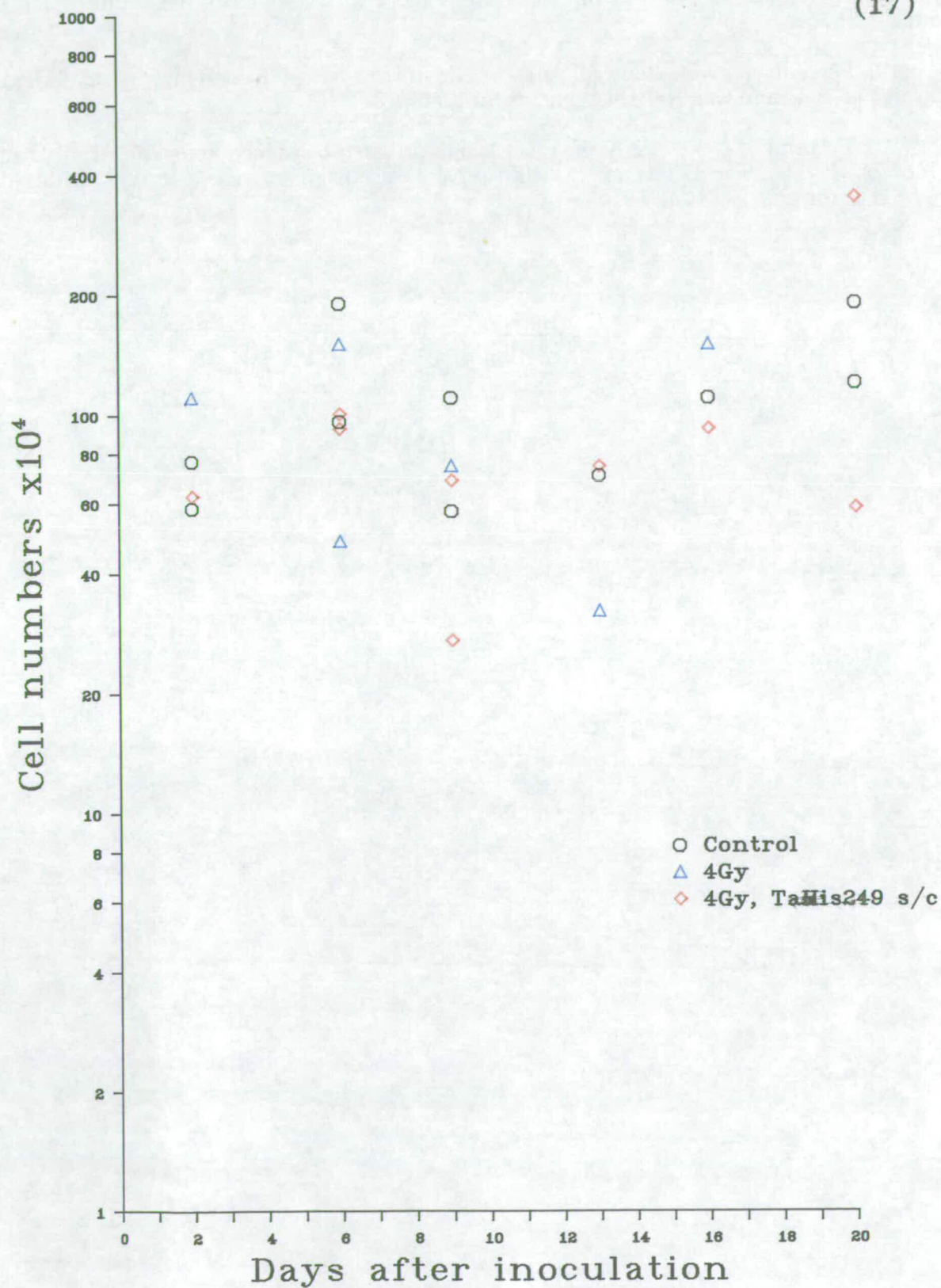


(16). Total counts of lymphocytes in _____ peritoneal exudate cell suspensions. Each point represents one mouse. Circles, untreated mice; blue triangles, irradiated mice (4Gy); red diamonds, irradiated mice injected with TaHis249 cells s/c. All counts $\times 10^4$.

(17). Total counts of macrophages in _____ peritoneal exudate cell suspensions.
Each point represents one mouse. Symbols as for graph 16.

Macrophages in Pex.

(17)

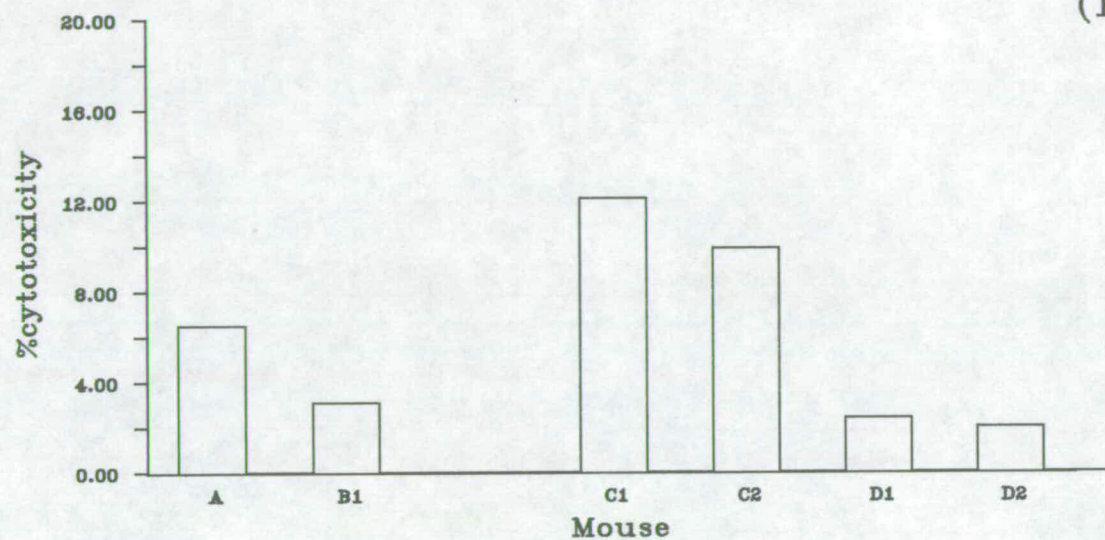


(18). Percentage cytotoxicity of YAC-1 cells *in vitro* by Balb/c spleen cells from mice killed on day 2. The effector:target ratio (E:T) was 100:1. A, untreated mice; B, irradiated (4Gy) mice; C, irradiated, tumour-bearing mice; D, unirradiated mice given TaHis249 cells s/c. Each bar represents an individual mouse, except for A which is the mean of the two untreated mice.

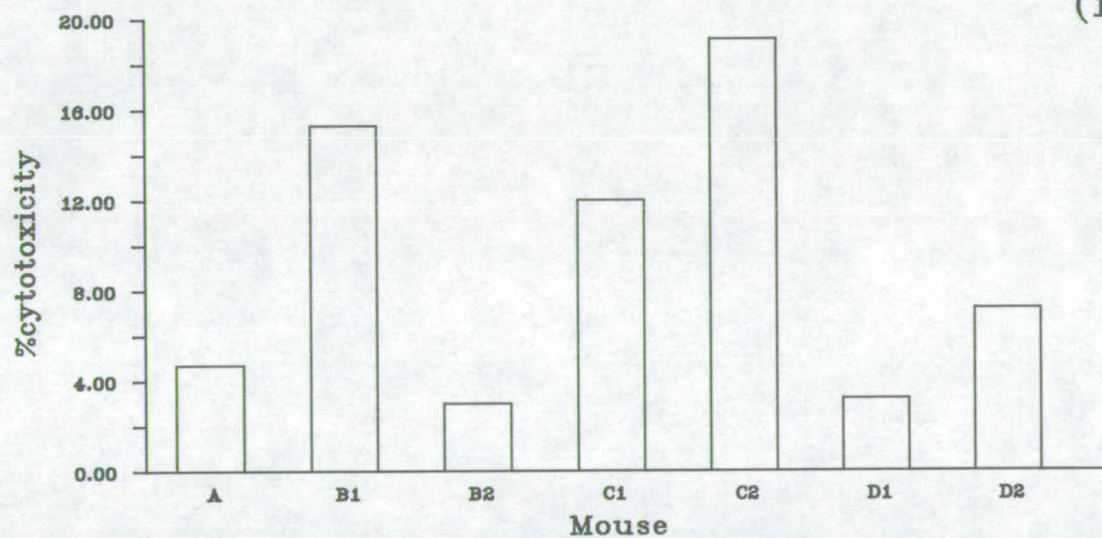
(19). Percentage cytotoxicity of YAC-1 cells *in vitro* by spleen cells from mice killed on day 9. The E:T ratio was 100:1. Legend as for graph 18.

(20). Percentage cytotoxicity of YAC-1 cells *in vitro* by spleen cells from Balb/c mice killed on day 16. The E:T ratio was 100:1. No D group mice were sampled on this day. Legend as for graphs 18 and 19.

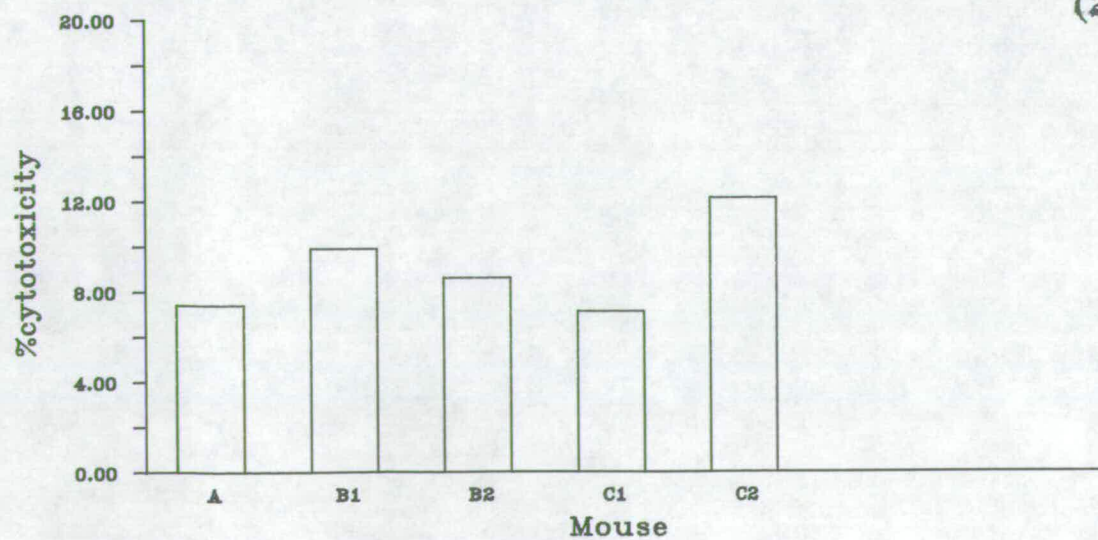
(18)



(19)



(20)



proportion of lymphocytes staining with the B and T cell markers fell drastically on day 13, without an accompanying drop in absolute numbers: this implied a proliferation of null cells. Increases in the numbers of splenic null cells have been recorded in mice bearing other subcutaneous and ascitic tumours (Garnis and Lala, 1978; Lala and McKenzie, 1982). The numbers of granulocytes and macrophages were observed to rise in the spleens of mice bearing subcutaneous tumours (Lala and McKenzie, 1982). Some of the increase in numbers may also have been due to erythropoiesis, as haemorrhage was occurring into the tumour.

Irradiation did not lead to an increase in NK cell activity compared to controls. If, as has been suggested, NK cells are radioresistant, then the death of other cell types should enrich for NK cell activity (Sado et al, 1988). This was not observed. Raised levels of NK activity were seen in C group mice on days 2 and 9, and in one B group mouse on day 9. The activity compared to the group A controls was higher on day 9 than on day 2. It would therefore seem to be the case that NK activity is increased in response to the tumour, rather than being enriched for by irradiation.

Tumours composed of *T.annulata* schizont-infected cells were therefore able to establish and grow in Balb/c mice in which B-cells, T-helper and T- cytotoxic cells were temporarily depleted by irradiation, and the numbers of these lymphocytes recovered more rapidly in tumour bearing mice. However, necrosis of the tumours began well before the numbers of B and T-cells returned to control levels. NK cells were activated at an early stage, and large numbers of macrophage type cells were seen at the same time as regression began.

These results are consistent with those discussed in chapter 4. Although seriously depleted, B and T-cells, particularly T-helper cells, are present in the spleen 2 days after irradiation, and these surviving cells may be sufficient to limit early growth of the tumour. Necrosis of the tumour initiated by natural immune mechanisms (macrophages and NK cells) would then be completed by the recovery of specific immunity.

CHAPTER 8

INTRODUCTION TO SCID MOUSE EXPERIMENTS

The following chapters describe experiments conducted with scid mice. The origin of the scid mutation, experiments by other workers involving the transfer of xenogeneic cells and tumours to scid mice, and the possible immune mechanisms in the scid mouse, have already been discussed in the "General Introduction", section 2.5. The comments below concern the background to the scid mouse project in this laboratory. There were two types of background experiments: the first, carried out by Dr. J.D. Ansell's group, attempted to establish bovine PBL in scid mice; the second, carried out by the author, attempted to establish *T.annulata*-infected bovine cells in scid mice.

The success of experiments with human cells (Mosier et al, 1988 and others, described in the General Introduction), encouraged the hope that scid-bovine chimaeras could be created by the injection of scid mice with bovine cells. Three separate experiments using different bovine donors were carried out by Dr Ansell's group to transplant bovine PBL to scid mice.

The successful establishment of bovine cells was found in only one experiment. Mice injected i/p with 5×10^7 BoPBL from a single bovine donor were sampled 17, 38, 42 and 44 days after injection. Bovine cells in lymphoid tissues and other organs were identified: (i) by electrophoresis of glucose phosphate isomerase (GPI) isoenzymes (procedures performed by Helen Taylor); (ii) by immunofluorescent labelling in the FACS (procedures performed by Kay Samuel). The following tissues were analysed: whole blood; PBL; bone marrow; spleen; peritoneal exudate; thymus; kidney, liver and lung; mesenteric, peripheral and parathymic lymph nodes. GPI analysis showed that the day 17 mouse had bovine cells only in the peritoneal exudate. However, bovine cells were detected in all the tissues examined on the later days (except thymus). FACS analysis showed that all the invading cells stained with an antibody to bovine MHC class I antigens, except those in the peritoneal exudate. (Unpublished data provided by Kay Samuel).

These results suggested that: (i) there might be a "donor effect", with PBL from some cattle establishing more readily than those of others; (ii) scid mice were able to

eliminate xenogeneic PBL in some cases.

An initial experiment with *T.annulata* infected cells was carried out by the author using scid mice provided by Dr J.D.Ansell. The results of this experiment have been published elsewhere (Fell, Preston and Ansell, 1990). A copy of this paper is bound into this thesis. To briefly summarise, 6 female scid mice, housed in a laminar flow filter rack under clean conditions, were injected i/p or s/c with 2×10^7 TaHis110 cells. Mice injected s/c developed subcutaneous tumours at the inoculation site, while mice injected i/p developed ascites. Extensive dissemination of infected cells to other tissues was observed, particularly in mice injected i/p.

For subsequent experiments, it was decided to house scid mice under germ-free conditions in a positive pressure isolator: (i) to remove the possibility of infection and contamination of experiments; (ii) to avoid accidental activation of the natural immune mechanisms of the scid mouse by exposure to antigens. As a result of this "germ-free" policy, steps were taken to ensure that the cells to be administered to the scid mice were also free of contamination: bovine PBL were obtained from calves maintained under germ-free conditions in isolators at the Moredun Institute, Edinburgh; *T.annulata* infected bovine cell lines were established from the PBL of these calves; both the *T.annulata* (germ-free) cell lines and the *T.parva* infected cell line used were maintained without antibiotics prior to injection into scid mice, so that any contamination of the cultures would be readily detected.

The use of the isolator to house the experimental animals meant that batches of 80 mice could be accommodated at a time. These mice were committed to particular experiments at an early stage. Obviously, new batches of mice (and new sets of experiments) could not be begun until the previous batch was finished, and the isolator cleaned and sterilised. This restricted the numbers of mice which could be used for some experiments.

Initial experiments repeated the attempt to establish bovine PBL in scid mice, and further explored the possibility of growing *Theileria*- infected cell lines in scid mice. Investigations into natural (ie non-T, non- B cell mediated) immunity in the scid mice were then carried out. Seven sets of experiments are described in the following chapters:

(i) The first experiment was another attempt to transfer bovine PBL to the scid mice, under the new germ-free conditions. This experiment was carried out in collaboration with Dr. Ansell's group.

(ii). The second part looked at the establishment or failure of *T.annulata* and *T.parva* infected bovine cells in the intraperitoneal and subcutaneous sites, at two different cell doses. The dissemination of infected cells from these sites was studied histologically.

(iii). The dissemination of cells from the intraperitoneal site was further studied using radiolabelled cells to determine the fate of low doses of cells injected i/p.

(iv). An experiment was carried out to look at natural immunity in scid mice *in vivo*, through the phenomenon of allogeneic lymphocyte cytotoxicity (ALC). This again involved the use of radiolabelled cells.

(v). Some "background" experiments were carried out to determine the numbers and composition of peritoneal and spleen cell populations in scid mice; assays for NK cell activity *in vitro* were also done. Mice were screened for "leakiness" of the scid phenotype by assaying for serum immunoglobulins.

(vi). Changes in the numbers of peritoneal and spleen cells, *in vitro* NK cell activity, and in the levels of TNF, IL-1 and IFN- γ in response to the inoculation with *Theileria* infected cells were assayed.

(vi). Attempts were made to affect the growth of *Theileria*-infected cells in scid mice by treatment with exogenous interferons, or by the suppression of endogenous IFN- γ with an anti-murine IFN- γ antibody.

CHAPTER 9

INOCULATION OF BOVINE PBL INTO SCID MICE

9.1 Introduction

The object of this experiment was to inoculate bovine peripheral blood leukocytes from the germ-free calves 249 and 250 into scid mice, to see if the bovine cells would survive, establish and possibly grow in scid mice. The reasoning behind this approach has already been outlined in the "Introduction to scid mouse experiments": briefly, it was thought that by housing scid mice under germ free conditions and injecting them with cells from germ-free bovine donors, the activation of natural immune mechanisms would be avoided and more reliable establishment of bovine cells would be observed. The experiment was conducted in collaboration with Dr. J.D.Ansell of this Department. Various procedures were carried out by Dr. Ansell, Mrs. H. Taylor, and Ms. K. Samuel.

9.2 Methods

At the time of the experiment, calves 249 and 250 were being maintained in isolators at the Moredun Research Institute, Edinburgh.

Bovine PBL were prepared from blood samples from the calves by centrifugation on a Ficoll-isopaque gradient, as described in Appendix A, section 2. The cells were resuspended in PBS at two doses: 1×10^7 cells/dose and 2×10^6 cells/dose. The mice were divided into two groups of 20 to receive the different calf cells: for each calf donor, 10 mice received a high dose of cells, while 10 received a low dose. All injections were done i/p.

Two mice from each group were sampled 8 weeks after injection. Two methods were used to detect bovine cells: staining of cell suspensions with monoclonal antibodies to bovine lymphocyte antigens and flow cytometry (procedures performed by K.Samuel); electrophoresis of glucose-phosphate isomerase isoenzymes (procedures performed by H.Taylor).

9.3 Results

No bovine cells were detected by FACS analysis or by isoenzyme electrophoresis in any of the sampled mice.

One mouse died during the experiment, 6 weeks after injection. The cause of death was presumed to be graft-versus-host disease (GVHD): autolysis of the corpse was too advanced for useful analysis.

9.4 Discussion

In their initial report, Mosier et al (1988) described the survival and proliferation of transplanted human cells in scid mice up to 6 months after injection. No evidence was found in this experiment for the establishment of bovine cells in scid mice. Previous experiments with bovine cells have shown similar results, although one was successful (Samuel, personal communication: see previous chapter).

Following this disappointing result, it was decided to concentrate on the growth of *Theileria*-infected cell lines in scid mice.

CHAPTER 10

GROWTH OF *THEILERIA* INFECTED CELLS IN SCID MICE

10.1 Introduction

The results of experiments with irradiated Balb/c and irradiated nude mice suggested that *Theileria* infected bovine cells would establish and grow in scid mice. A preliminary experiment showed that this was the case (Fell et al, 1990). As already described, attempts to establish uninfected bovine cells in scid mice met with only limited and occasional success. It was therefore decided to investigate the growth of *Theileria*- infected bovine cells in scid mice in more depth. The two main objectives were: to investigate the effect of dose and route of administration on the establishment, growth and dissemination of the infected cells; and to elucidate the immune mechanisms by which scid mice could eliminate the administered cells. It was hoped that the information provided would be ^{of} use (i) in work involving scid mice as hosts for *Theileria* -infected cells; (ii) in suggesting ways in which scid-bovine chimaeras could be constructed with the minimal rejection of transplanted cells.

The experiments presented in this chapter deal with the questions of the dose and route of administration of infected cells, the behaviour of cells infected with the two parasite species, and the dissemination of infected cells. Investigations of the immune responses of scid mice and experiments involving manipulation of the scid immune system are presented in subsequent chapters.

Tumour growth in both the subcutaneous and intraperitoneal sites was investigated: the subcutaneous site was used in previous experiments in irradiated mice, while relatively little investigation of the intraperitoneal site had been carried out. However, in scid mice this site appeared to be much more promising than in irradiated mice. The cell doses chosen were based on those previously used in irradiated Balb/c nude mice, and in the preliminary "scid+cell lines" experiment.

10.2 Methods

A total of 55 mice were used. The cell lines to be injected into the mice were maintained without antibiotics for at least two weeks beforehand. The doses and numbers of mice in each group are given in table 8.

The mice are referred to in the text by an abbreviated description of the treatment they received: Ta or Tp indicates the parasite species, Hi or Lo indicates the cell dose and the route of administration is given in brackets. Thus TaHi(i/p) denotes a mouse injected with 2×10^7 TaHis250 cells intraperitoneally.

Mice were monitored for tumour and ascites development. Mice injected with TaHis250 cells were killed at 7 day intervals, and smears of tumours, peritoneal exudates and various organs were prepared. Mice which received TpMug110 cells were killed at longer intervals, as tumours developed.

10.3 Results

General results are summarised in table 8, together with the numbers of mice in each group. Results are described in more detail below.

10.3.1 Development of subcutaneous tumours

All mice injected subcutaneously with TaHis250 cells developed tumours at the inoculation site (graph 21). In the high dose group, the tumours became reddened 7-8 days post-infection, with central necrosis developing within 2-3 days. By day 12, all these tumours had a central area of necrosis about half the diameter of the tumour in size. This did not affect the growth of the tumours. The last surviving mice in this group were killed on day 28. A macroscopic metastasis was seen in a mouse killed on day 21: a small (2mm) white nodule was found on the left side of the ribcage. An impression smear showed 5.2 schizonts/field, compared to 7.1 from the subcutaneous tumour of the same mouse.

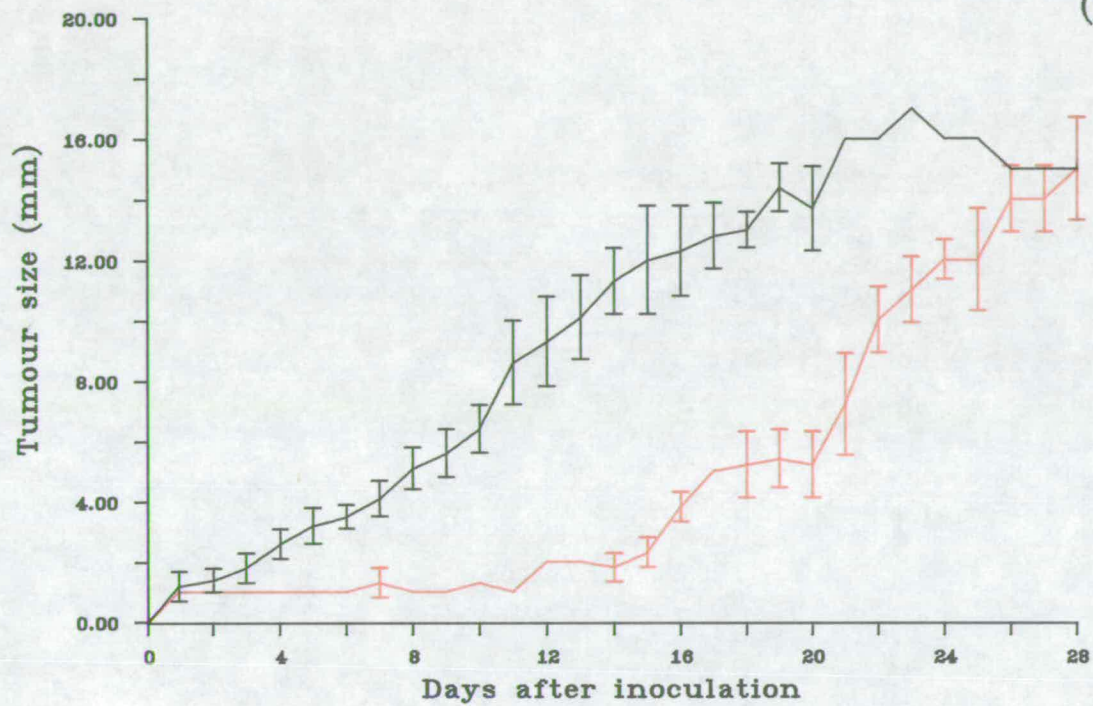
In mice injected with a low dose of TaHis250 cells s/c (TaLo(s/c)), the tumours grew more slowly, but steadily increased in size (graph 21). Tumours became

Table 8. Summary of cell doses and routes of injection in scid mice.

Cell line, route, dose	n	Number of mice with:		Dissemination
		Tumours	Ascites	
TaHis250 s/c 2×10^7	10	10	-	++
" " 2×10^6	10	10	-	++
" i/p 2×10^7	10	-	10	+++
" " 2×10^6	10	-	-	+/-
TpMug110 s/c 2×10^7	3	3	-	+/-
" " 2×10^6	4	1	-	+/-
" i/p 2×10^7	4	2 [#]	-	++
" " 2×10^6	4	-	-	-

"Tumours" refers to solid tumours. Mice were scored as having ascites if they were visibly swollen. Dissemination, as assessed by microscopic examination of Giemsa-stained smears, was scored on an arbitrary scale from -, no infected cells found other than at the tumour site, to +++, extensive and heavy infiltration of multiple organs by schizont- infected cells. #: mice injected i/p, developed solid tumours attached to mesentery.

(21)



reddened from day 26 onwards, but did not become necrosed in most cases. Nine of the mice in this group had been killed by day 29: the survivor, which had a smaller tumour than the rest, developed central necrosis from day 45 onwards.

Mice injected subcutaneously with the high dose of TpMug110 cells also developed tumours at the inoculation site. In contrast to the mice injected with *T.annulata* infected cells, the tumours were flattened rather than prominent, and did not become haemorrhagic or necrotic, reaching a size of 15- 20 mm diameter by day 50. One mouse in the TpLo(s/c) group developed a palpable subcutaneous tumour by day 52. No tumours were found in the other mice in this group, which were killed on days 60 and 63.

10.3.2 Development of ascites

Mice injected with the high dose of TaHis249 cells i/p (TaHi(i/p)) developed ascites by day 8. All the surviving mice in this group died or were killed on days 10 and 11. The abdomens were very swollen, and the ascitic fluid was very turbid. On dissection, the gut had an inflamed appearance, with enlarged blood vessels visible through the serosa. The mesenteries were also inflamed, with multiple small haemorrhages. There were white tumour-like swellings on the mesenteric cords. In some cases the pancreas was reddened and enlarged. The liver was lighter in colour than usual, often with a speckled or blotchy appearance. The spleens were not enlarged.

In contrast, mice which received a high dose of TpMug110 cells i/p (TpHi(i/p)) did not develop ascites. One mouse killed on day 52 had a solid tumour on the mesentery, but did not show the inflammation of gut and mesenteric tissue seen in the TaHi(i/p) group. A mouse killed on day 60 had a small tumour on the abdominal wall, probably due to spillage of the inoculum. The other mice in this group (killed days 17 and 60) did not show macroscopic tumours.

Scid mice which received a low dose of cells of either cell line i/p did not develop ascites, macroscopic tumours or any of the clinical signs described above.

10.3.3 Dissemination of infected cells in mice injected s/c

In TaHi(s/c) scid mice, the largest numbers of schizonts and schizont- infected cells were seen in the tumour (graph 22). As tumours increased in size more parasitised cells were found in other sites, particularly the lungs and kidneys. Schizont infected cells were also detected in the liver and gut.

In TaLo(s/c) mice, the pattern was generally the same as in the high dose mice, with the highest counts again being seen in the tumours (graph 23). Some disseminated cells were found in other organs as tumours grew in size. In a mouse killed on day 59, high counts were recorded from the gut, mesentery and particularly from a smear of the gut mucosa.

In contrast, there was little dissemination of infected cells from the tumour site in mice given either dose of TpMug110 cells s/c (graph 24).

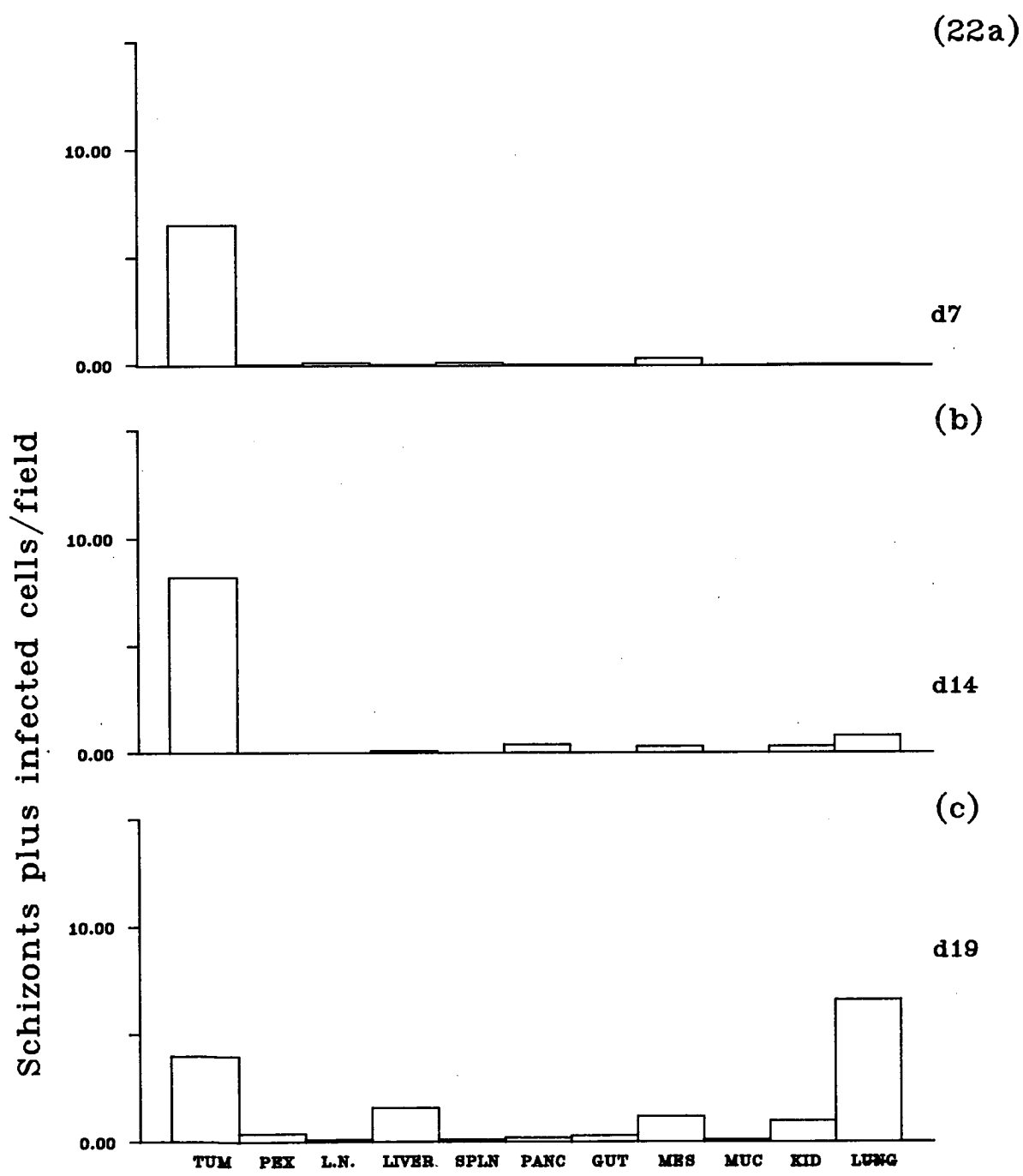
10.3.4 Dissemination of infected cells in mice injected i/p

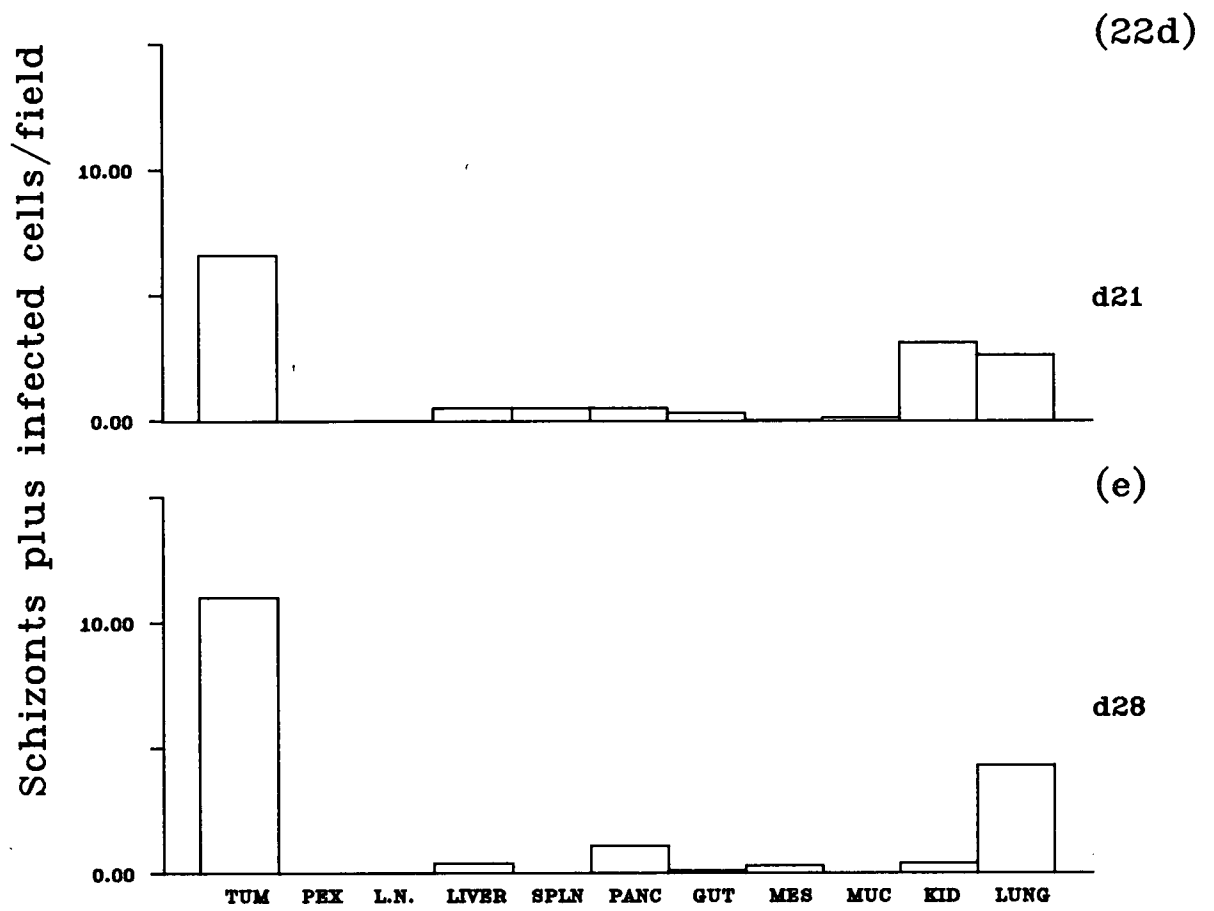
In mice with ascites, ie those which received a high dose of TaHis250 cells i/p, relatively high counts of schizonts were found in smears of the gut and mesenteries. Plate 8 shows a typical schizont infected cell in a cytospin of peritoneal exudate. Mitotic figures were seen in peritoneal exudates and in smears of the gut and mesentery. More infected cells were found in the abdominal organs (liver, spleen, gut, mesentery) than in the lungs and kidney (graph 25). Plate 9 shows free schizonts and a schizont-infected cell in a dab smear from the liver of a TaHi(i/p) mouse.

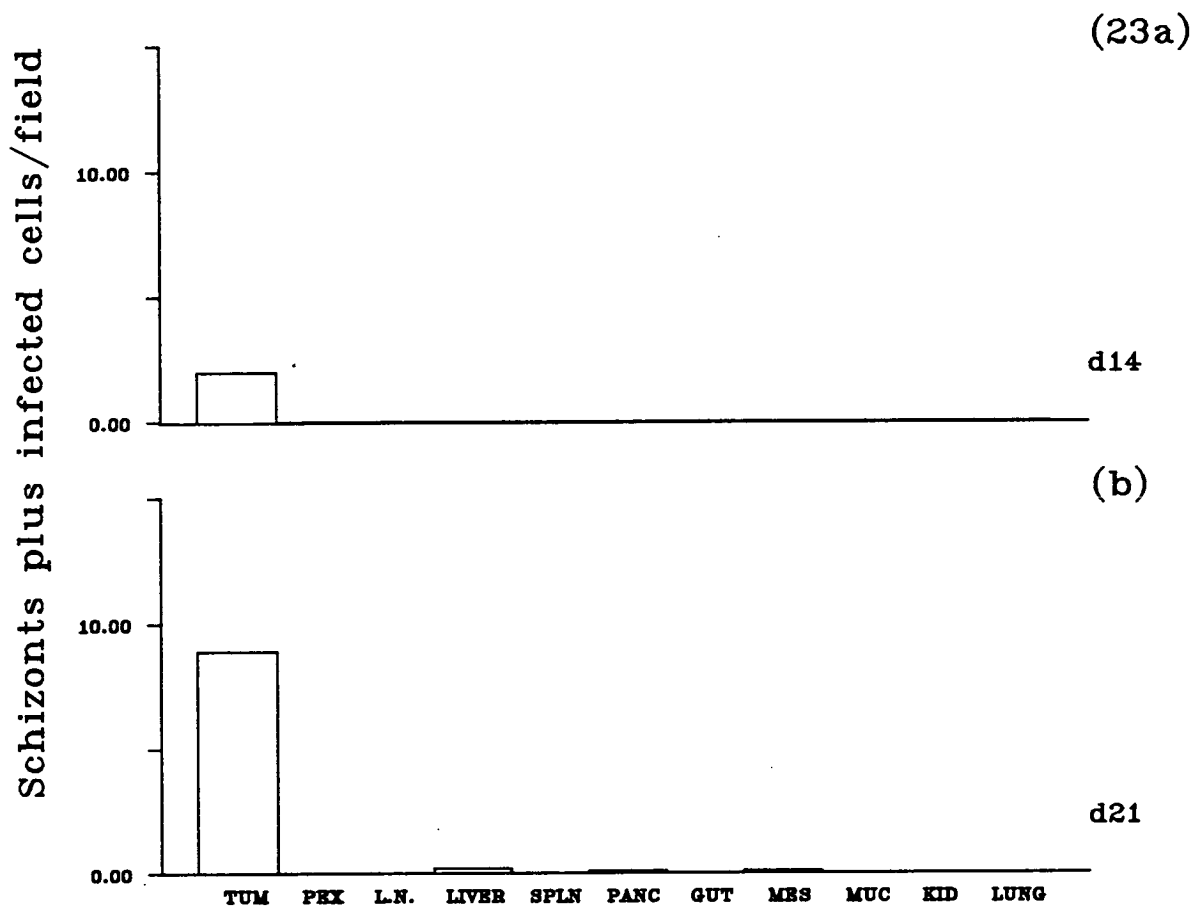
The distribution of infected cells in TpHi(i/p) mice is shown in graph 26. The mouse killed on day 52 showed a similar pattern to TaHi(i/p) mice with high counts in the peritoneal exudate, pancreas and mesenteries, but with a higher count from the inguinal lymph node. The mouse killed on day 60 had a subcutaneous tumour (as described above), and the infected cell distribution was more like that seen in mice injected with TpMug110 cells s/c (graph 24), with little involvement of other organs. The lymph node local to the tumour was greatly enlarged, and smears showed that infected cells had spread to it.

No infected cells were detected in smears from mice injected with the low dose of either cell line i/p, with the exception of one of two TaLo(i/p) mice killed on day 14,

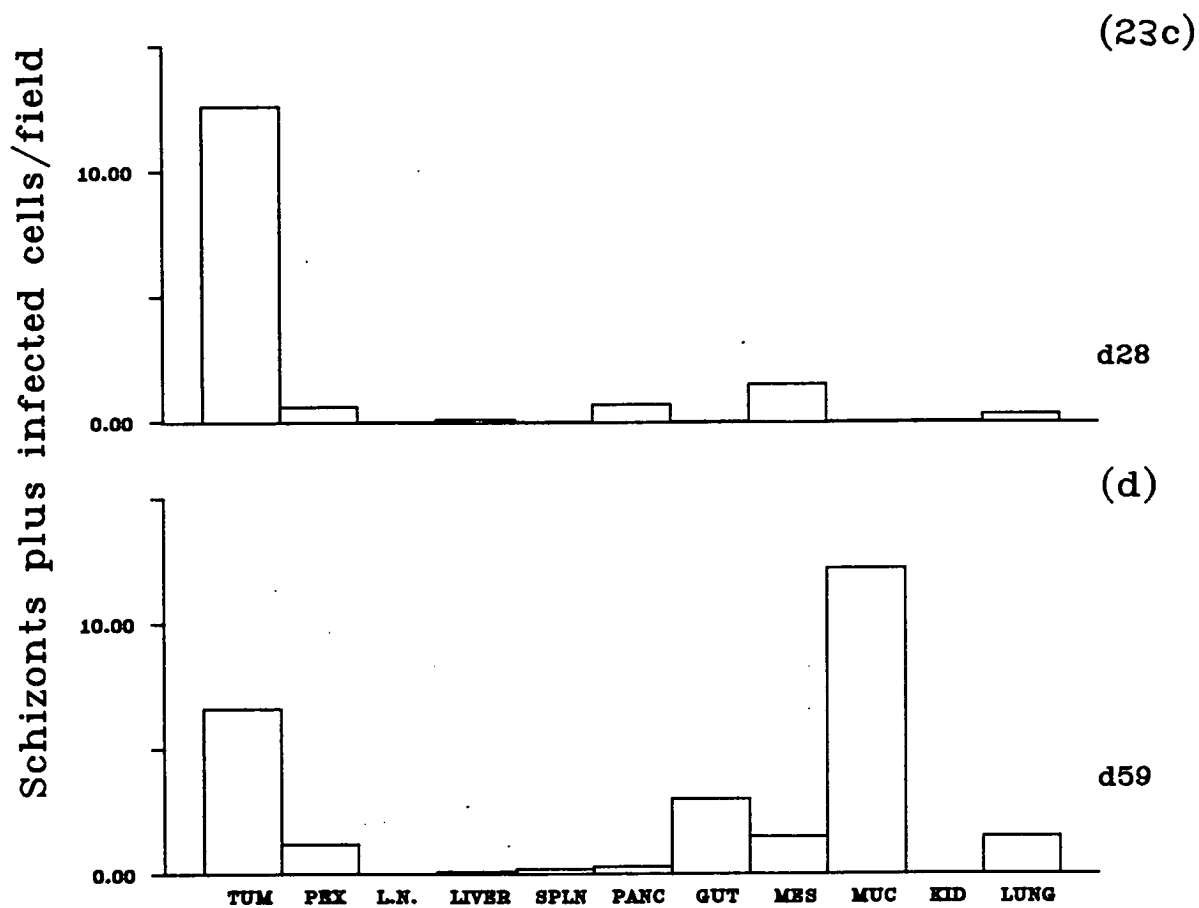
(22). Presence of schizonts and schizont-infected cells in smears made from scid mice injected with 2×10^7 TaHis250 cells s/c. Mice were killed (a),7; (b),14; (c),19; (d),21 and (e),28 days after inoculation, and cytopins and dab smears were prepared as described in the text. For each smear, at least 10 fields, or a minimum of 100 host cells, were counted at a magnification of approximately $\times 1000$. The number of schizonts present was then divided by the number of fields counted. Each histogram presents mean counts from a pair of mice, except (e) which shows the counts from a single mouse. Abbreviations: Pex., peritoneal exudate; L.N., inguinal lymph node; Panc., pancreas; Mes., mesentery; mucosa, scraping of mucosa of small intestine.

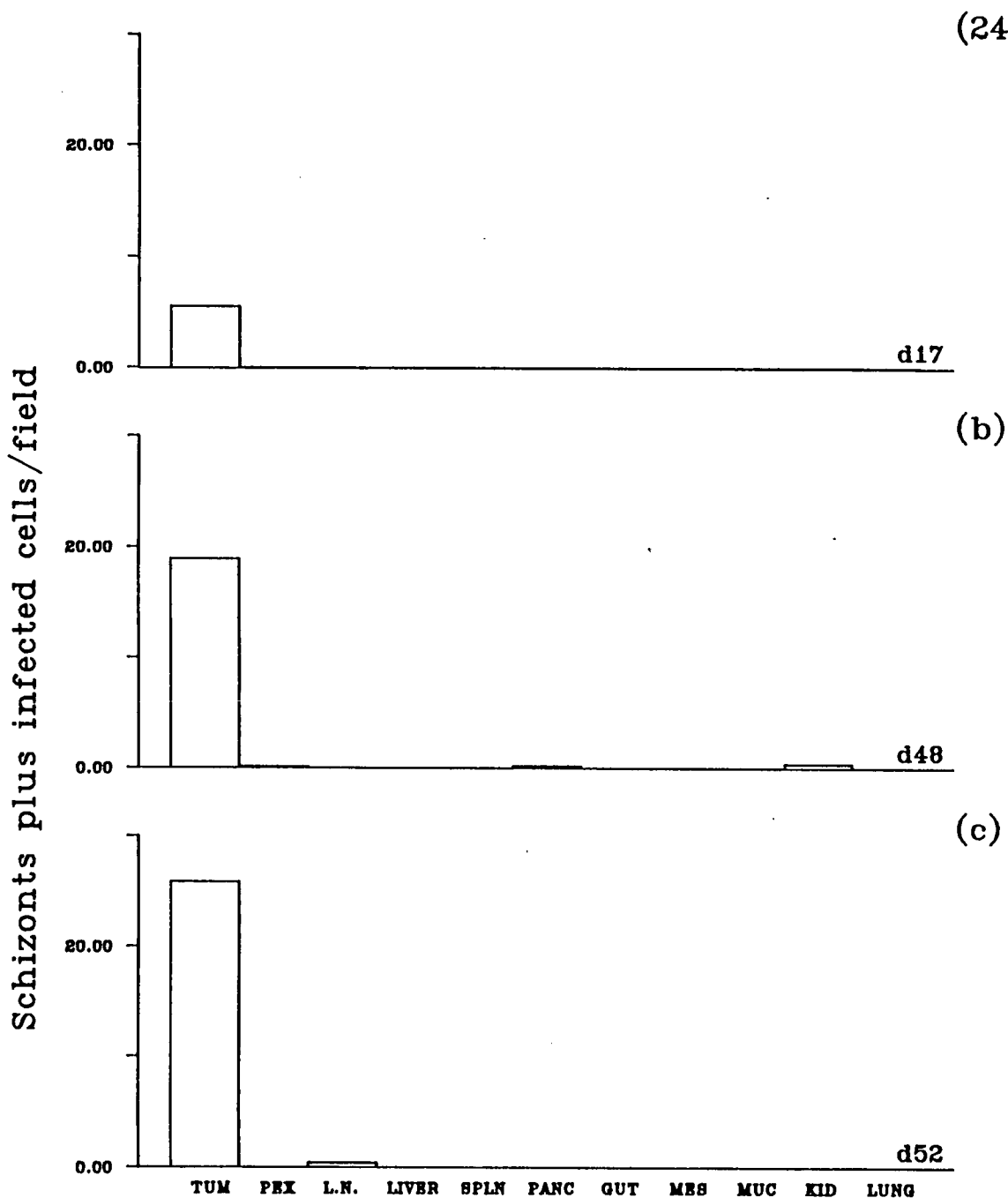






(23). Presence of schizonts and schizont-infected cells in dab smears from scid mice injected with 2×10^6 TaHis250 cells s/c. Mice were killed (a),14; (b),21; (c),28 and (d),59 days after inoculation. Each histogram presents the mean results from a pair of mice, except (d) which is from a single mouse. Abbreviations as (22) above.





(24). Presence of schizonts and infected cells in dab smears from scid mice injected with TpMug110 cells s/c. Graph (a), 2×10^7 cells, day 17; (b), 2×10^7 cells, day 48; (c), 2×10^6 cells, day 52. Each histogram presents the data from a single mouse. Abbreviations as for graphs (22) and (23).

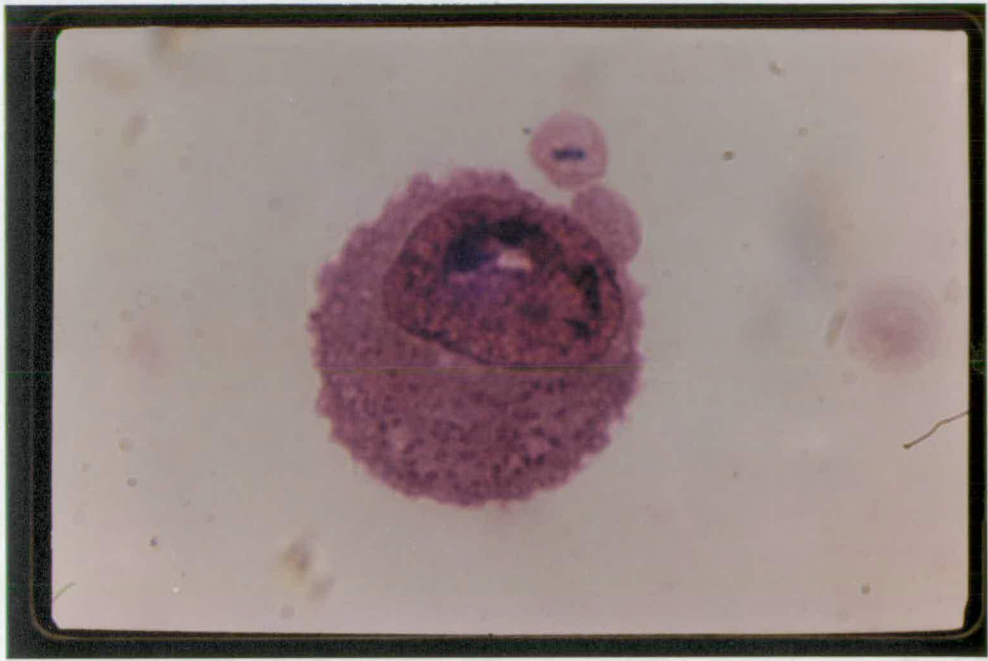
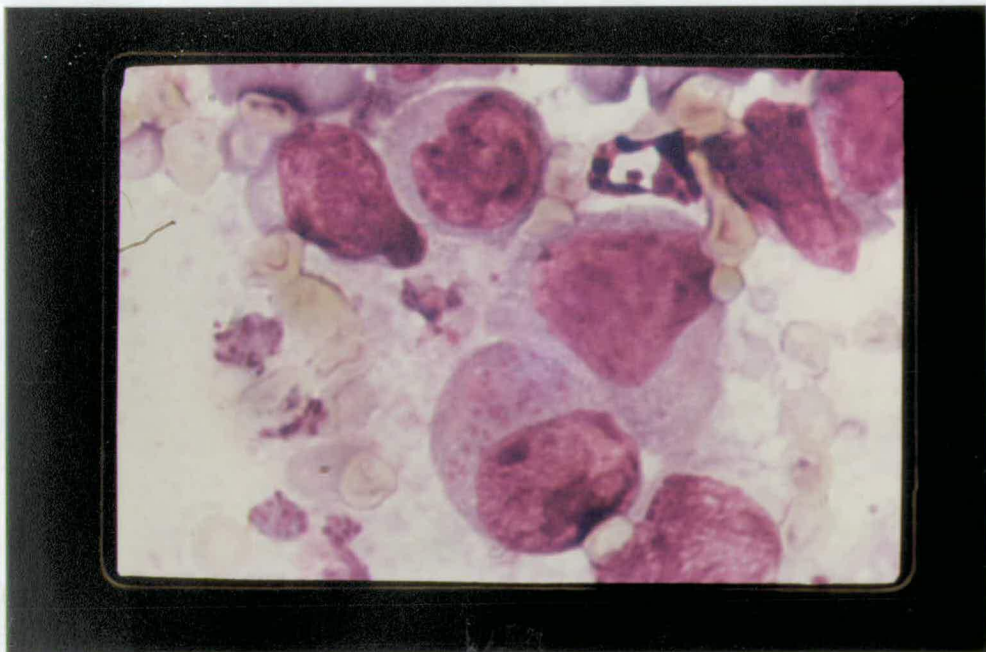


Plate 8. *T.annulata* macroschizont-infected cell in a cytospin of peritoneal exudate from a scid mouse inoculated i/p (x1000).

Plate 9. Free schizonts and a schizont-infected cell in a dab smear from the liver of a scid mouse inoculated with 2×10^7 *T.annulata* infected cells i/p (x1000). A neutrophilic polymorphonuclear leukocyte can also be seen.



which had a schizont count of 0.1 in the liver and 0.6 in the gut.

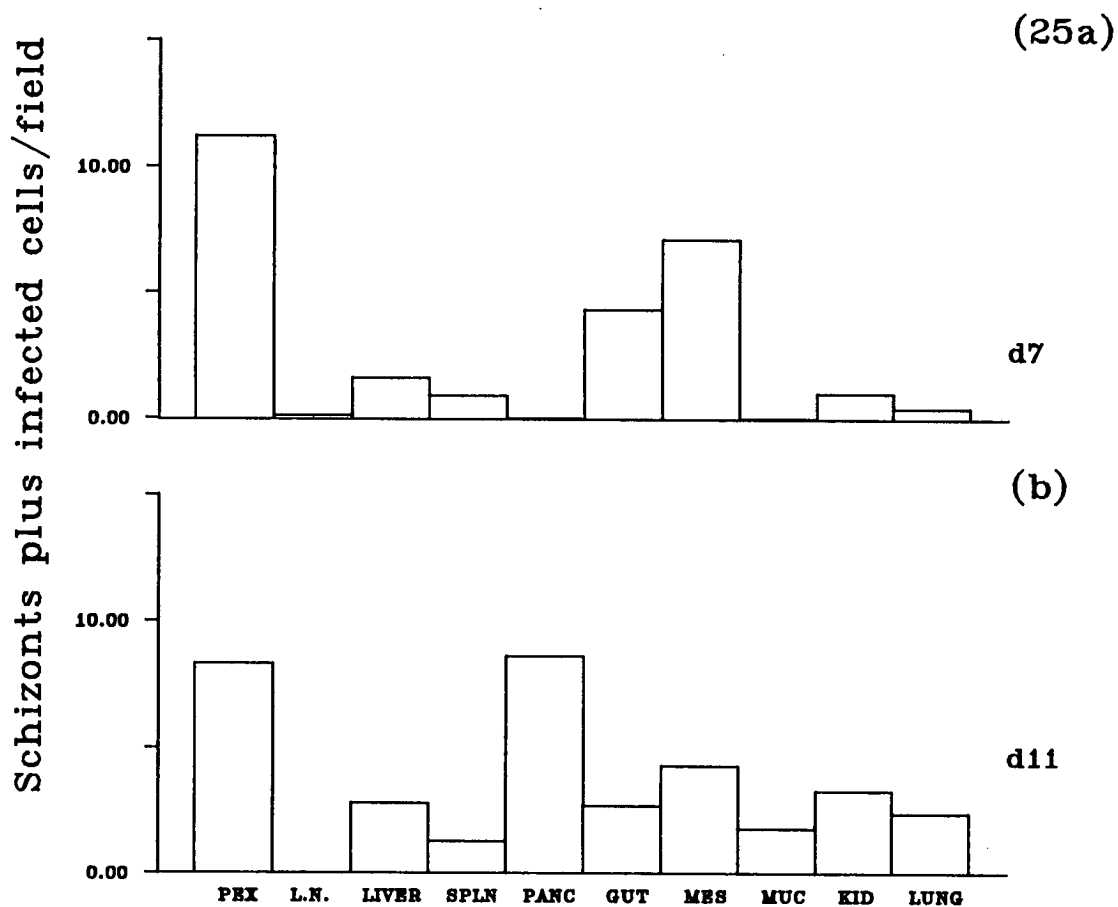
10.3.5 Histology

Sections of subcutaneous tumours showed the same features as in irradiated Balb/c and nude mice injected with *Theileria*-infected cell lines: large, lymphoid cells infiltrating subcutaneous tissue. TaHis250 lumps were vascularised and showed internal haemorrhage with surface necrosis. Large areas of healthy tissue remained, particularly at the base of tumours. Schizonts and mitotic figures were seen in sections. TpMug110 tumours were structurally similar, but with less haemorrhage.

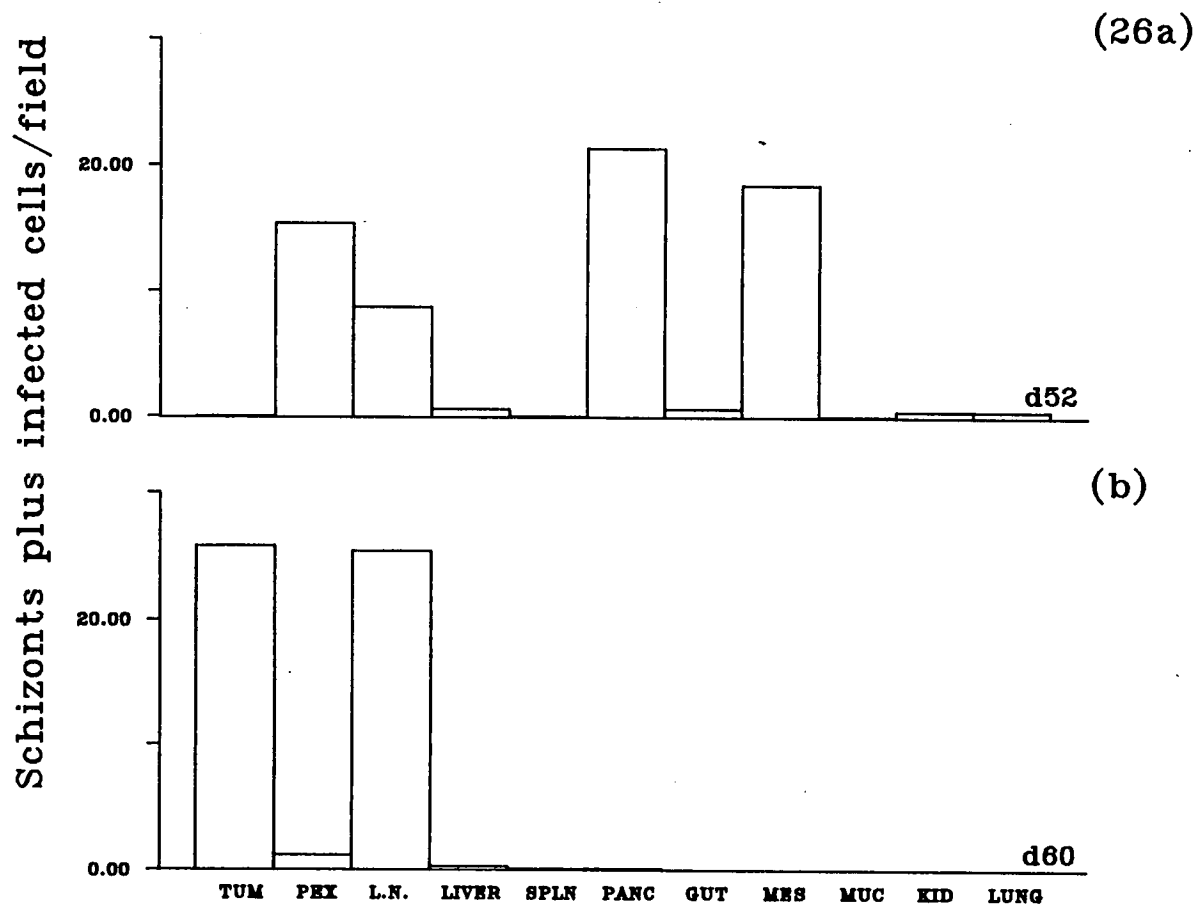
Plate 10 shows a section of the mesenteries of a TaHi(i/p) mouse. Tumour- like accumulations of lymphoid cells were seen in sections of the mesenteries of these mice. Schizont infected cells could be seen in these mesenteric tumours at high magnification. Some of these tumours may have been mesenteric lymph nodes: any distinctive structure was obliterated by tumour cells. Other sections of mesentery from TaHi(i/p) mice showed tumour cells infiltrating the fatty tissue of the mesenteric cords. Schizont infected cells could be seen in these areas. The small intestine was infiltrated by lymphoid cells, but not so severely as to cause major disruption to the submucosa. There was no apparent atrophy of villi, or crypt hyperplasia.

In some TaHi(i/p) mice, the pancreas was noticeably enlarged and haemorrhagic. Sections showed that the structure of the pancreas had been damaged by the infiltration of schizont infected cells, resulting in severe haemorrhage (plate 11). The tissue shown could be identified histologically as pancreas by the presence of Islets of Langerhans, and some remaining glandular structure.

The mesenteric tumour taken from a TpHi(i/p) mouse killed on day 52 was found to consist of masses of tumour cells and some fatty tissue, with few obvious blood vessels. The tumour was divided into lobes approximately 1- 2mm in diameter by bands of necrosis. An adjacent piece of small intestine showed almost total atrophy of the villi (plate 12).



(25). Presence of schizonts and infected cells in dab smears from scid mice injected with 2×10^7 TaHis250 cells i/p. Graph (a), mean of two mice killed on day 7; (b), mean of four mice killed on day 11. Abbreviations as above.



(26). Presence of schizonts and infected cells in scid mice injected with 2×10^7 TpMug110 cells i/p. Mice were killed (a), 52 and (b), 60 days after inoculation. Graph (a) presents data from one mouse; graph (b) presents mean data from a pair of mice. Abbreviations as above.

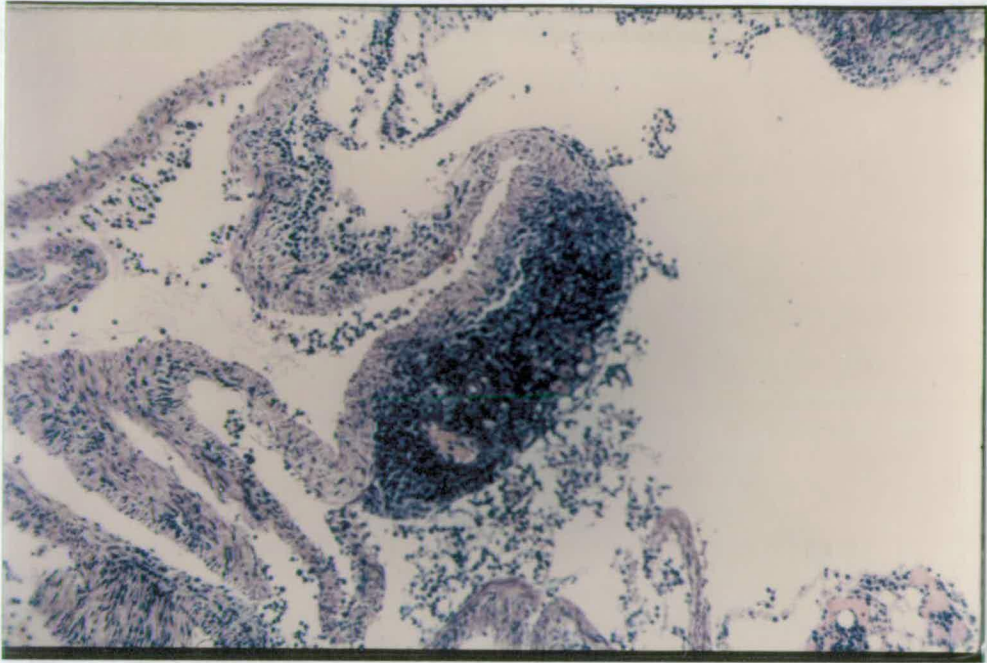


Plate 10. Section of mesenteric tissue from a scid mouse injected with 2×10^7 TaHis249 cells (x100). There is a large accumulation of cells forming a mesenteric tumour in the centre.

Plate 11. Section of pancreas from scid mouse with ascites due to i/p injection of *T.annulata* infected cells. An Islet of Langerhans can be seen to the centre-left. The structure of the tissue has been damaged by the infiltration of cells.

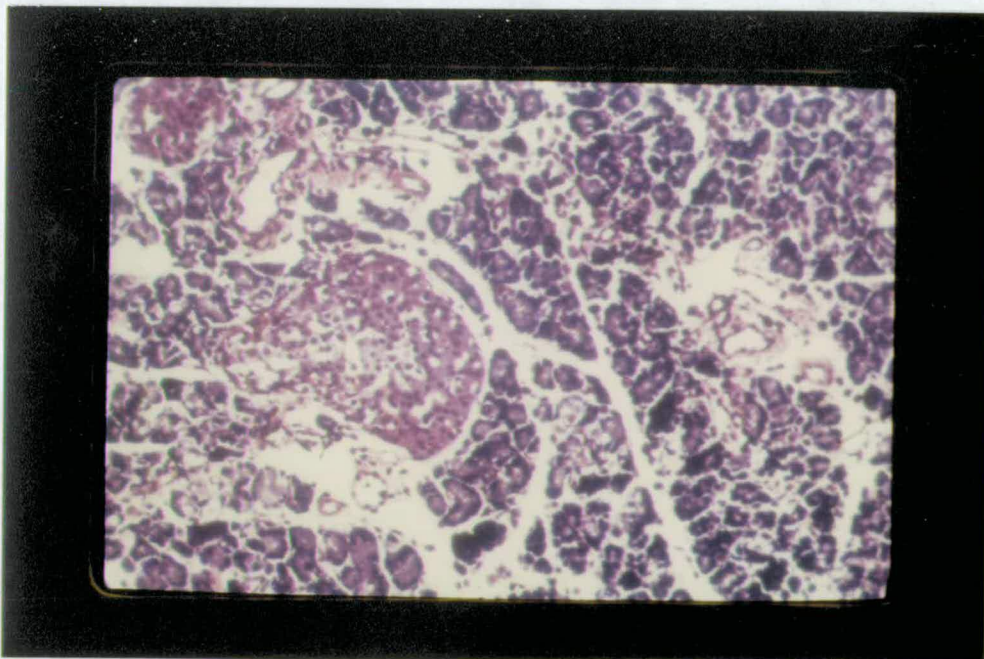




Plate 12. Section of small intestine from a scid mouse injected with 2×10^7 *T.parva* infected cells (x100). This section was taken from a piece of gut adjacent to a large, mesenteric tumour (bottom). The gut villi are in an atrophied state.

10.4 Discussion

T.annulata infected cells grew as subcutaneous tumours in scid mice in a similar manner to that observed in Balb/c nude mice irradiated with 4Gy. The tumours became haemorrhagic and developed central necrosis, but did not resolve. *T.parva* infected cells also grew as subcutaneous tumours in scid mice; the pattern of growth observed was again similar to that seen in Balb/c nude 4Gy mice given the same dose of cells. The major difference between subcutaneous tumour growth in the scid mouse and in the irradiated nude mouse was that dissemination of infected cells from the subcutaneous site occurred more readily in the scid mouse.

In the scid mouse, *T.annulata* infected cells injected intraperitoneally grew rapidly, forming an ascites. This contrasted with the failure of *T.annulata* infected cells to establish in the intraperitoneal site in Balb/c 4Gy mice (chapter 1): previous experiments had shown that *T.parva* infected cells did not survive in the peritoneal cavity of heavily irradiated nude mice (Irvin et al, 1977). The growth of infected cells injected i/p into scid mice was dose dependent: 2×10^7 cells caused severe ascites in 8-10 days, but a dose of 2×10^6 cells i/p could not be detected by the methods described above. Infected cells were able to spread to other organs from the i/p site more readily than from subcutaneous tumours. *T.parva* infected cells were also able to grow intraperitoneally, although more slowly than *T.annulata* infected cells. Both cell lines appeared to form solid tumours on or in the mesentery. The tissue of the mesenteric cords is fatty and appears to be fairly loosely organised. The mesenteric tumours had a similar histological appearance to subcutaneous tumours.

Infected cells probably disseminate from subcutaneous tumours into capillaries in the tumour and thus into the bloodstream. The finding of relatively large numbers of schizonts and infected cells in the lungs of tumour-bearing mice supports this hypothesis, as after entering the venous system and passing through the heart, the cells would next encounter the capillary bed of the lungs. Cells which were not trapped in the lungs would return to the heart and pass on to the peripheral circulation, to become trapped in the capillaries of other organs.

The route by which infected cells leave the peritoneal cavity is less clear. Infected cells are found in the peritoneal cavity both in suspension in the peritoneal exudate, and as solid colonies or tumours on the mesenteries. Three possible routes of dissemination can therefore be considered: (i) into the capillary network and hence

the general circulation; (ii) through the lymphatics draining the peritoneal cavity, and then into the bloodstream via the thoracic duct; (iii) some process of direct infiltration and colonisation of tissues, leading directly into the abdominal organs. (i) and (ii) both lead to the circulation, so a similar distribution of infected cells to that in mice bearing subcutaneous tumours would be expected. In fact, far more infected cells were found in abdominal organs such as the liver and gut in mice with ascites, than in the same sites in mice with substantial subcutaneous tumours. This tends to favour option (iii). However, three other factors need to be taken into account. Firstly, the peritoneal cavity may present such a large area for the "absorbtion" of cells by routes (i) and (ii), that a direct comparison with the same dose of cells injected s/c is not possible, because a far higher proportion of the injected cells may have spread into the circulation. Secondly, as *Theileria*-infected cells are lymphoid in origin, they may "home in" on lymphoid organs such as the spleen. Thirdly, the multiplication of infected cells in various tissues may vary, markedly altering the observed tissue distribution. The methods described in this chapter were not sufficiently sensitive or quantitative to resolve these questions. As described in the next chapter, radiolabelling of injected cells was used to provide more accurate data.

The fact that high doses of cells tend to establish whereas low doses do not, and that a *T.annulata* infected cell line, which grows more rapidly in vitro, establishes more readily and disseminates more vigorously in scid mice than the *T.parva* infected cell line, suggest that some "natural" immunity is operating, but that it can be overcome by large doses of infected cells particularly if those cells are multiplying rapidly. The intraperitoneal site seems to be a more hazardous environment for injected cells than the subcutaneous site, where low doses of cells establish relatively easily. The most numerous cell type in the scid peritoneal exudate is the macrophage (chapter 13, table 1), which could potentially arrest the growth, kill and phagocytose infected cells. Natural Killer (NK) cells may also be involved. As discussed in the "General Introduction", the control of metastasis in nude mice has been attributed to NK cells. The data discussed above show that scid mice apparently have less capacity to control metastasis from the inoculation site than Balb/c nude 4Gy mice, although they can control cells injected i/p. Experiments described later attempted to discover whether macrophages and NK cells played a role in the control of *Theileria* tumours.

The observation of villus atrophy in the small intestine adjacent to *T.parva* mesenteric tumours was of interest because villus atrophy is mediated by IFN- γ in graft versus host reactions in mice (Mowat, 1989). The atrophy seen in the presence of a *T.parva* tumour could therefore indicate a host reaction to the tumour involving

the release of murine IFN- γ .

CHAPTER 11

TRACKING OF RADIOLABELLED CELLS IN SCID MICE

11.1 Introduction

The experiments described in the previous chapter showed that *Theileria*-infected cells, injected s/c or i/p into scid mice, could spread from the tumour site to other sites. This information was derived from differential counts of dab smears and cytopins, and was therefore primarily qualitative: it showed which tissues contained the highest proportion of schizont-infected cells, but could not show the overall distribution of infected cells. The experiments described in this chapter were done to determine the major sites colonised by *T.annulata* infected cells injected i/p into scid mice, and to clarify the routes of dissemination of infected cells from the intraperitoneal site. It was also thought that an accumulation of radioactivity in certain sites, particularly the liver and kidneys, would indicate the elimination of labelled cells.

Two experiments are described in this chapter. The first was designed to compare the fate of *T.annulata* infected cells injected i/p into scid and Balb/c mice: in which organs would the cells be found? The second experiment was intended to track a low dose of TaHis250 cells, injected i/p into scid mice, over a period of several days. It was hoped that this would show any trend to move out of the peritoneal cavity to secondary lymphoid organs such as the spleen. Alternatively, an accumulation of activity in the liver and kidneys would indicate gradual rejection of the cells. A more thorough sampling of tissues was carried out in this experiment than in the first, as a result of data from counts of dab smears.

11.2 Methods

All cells were labelled with $^{51}\text{chromium}$ as described in Appendix A (section 4.3), adjusted to 2×10^6 cells/dose in PBS, and injected intraperitoneally. Mouse tissues were removed and counted as described in "Materials and Methods", (section 3).

11.2.1 Experiment 1.

3 scid and 2 Balb/c mice were injected i/p with radiolabelled TaHis110 cells. Mice were killed after 18 hours and the following tissues were removed for counting: 200ul peripheral blood; 2mls peritoneal exudate; axial and brachial lymph nodes (4); both femurs (for bone marrow); liver; spleen; both kidneys; both lungs.

11.2.2 Experiment 2.

6 scid mice were injected with radiolabelled TaHis250 cells. Pairs of mice were killed 3, 6 and 9 days after injection and organs were removed for counting. In addition to the organs sampled in experiment 1, the small intestine (including the stomach), large intestine (including the caecum) and as much mesenteric tissue as possible were removed for counting.

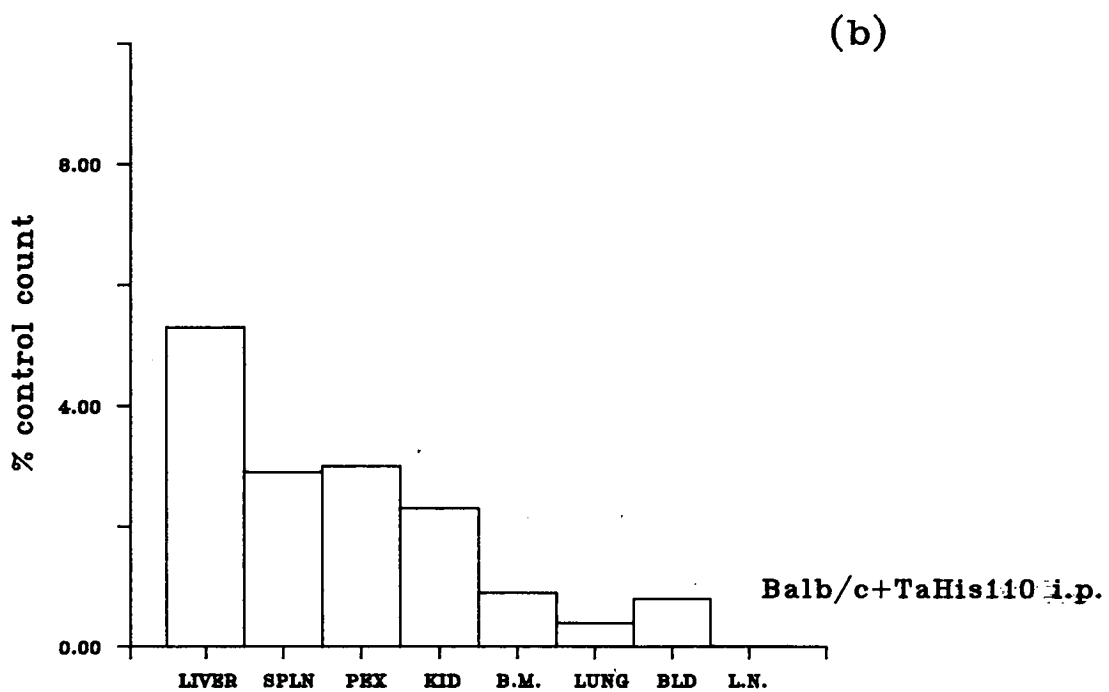
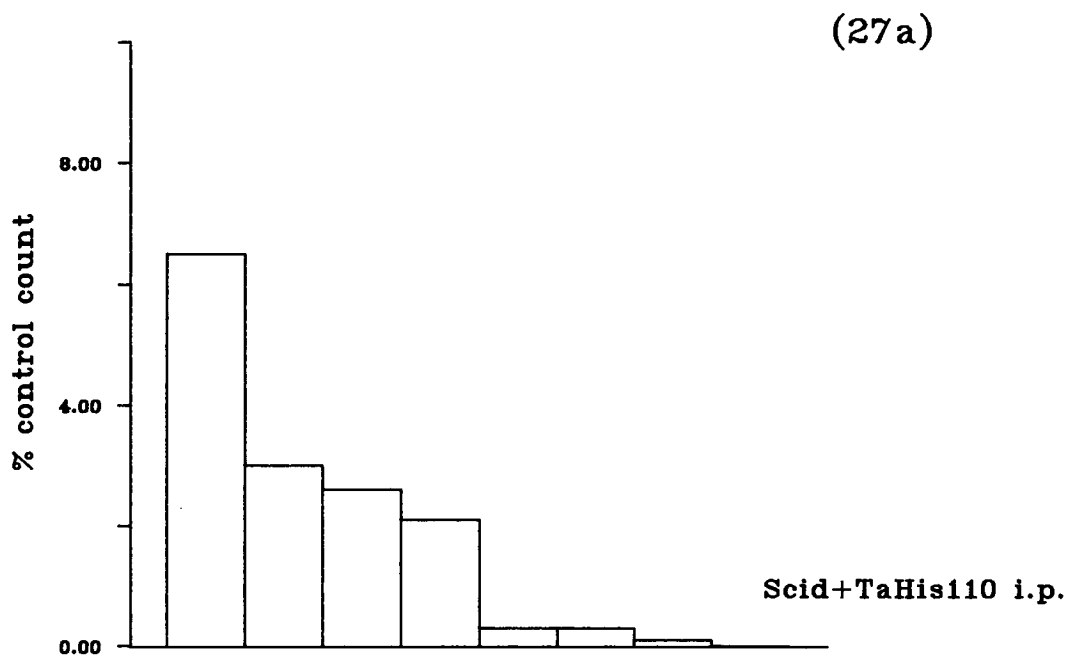
11.3 Results

11.3.1 Injection of TaHis110 cells into scid and Balb/c mice

The highest counts were recovered from the liver, followed by the spleen, peritoneal exudate and kidney (graph 27a and b). No major differences were observed between the distribution of cells in the Balb/c and scid mice. The overall recovered count was low, approximately 12- 15% of control.

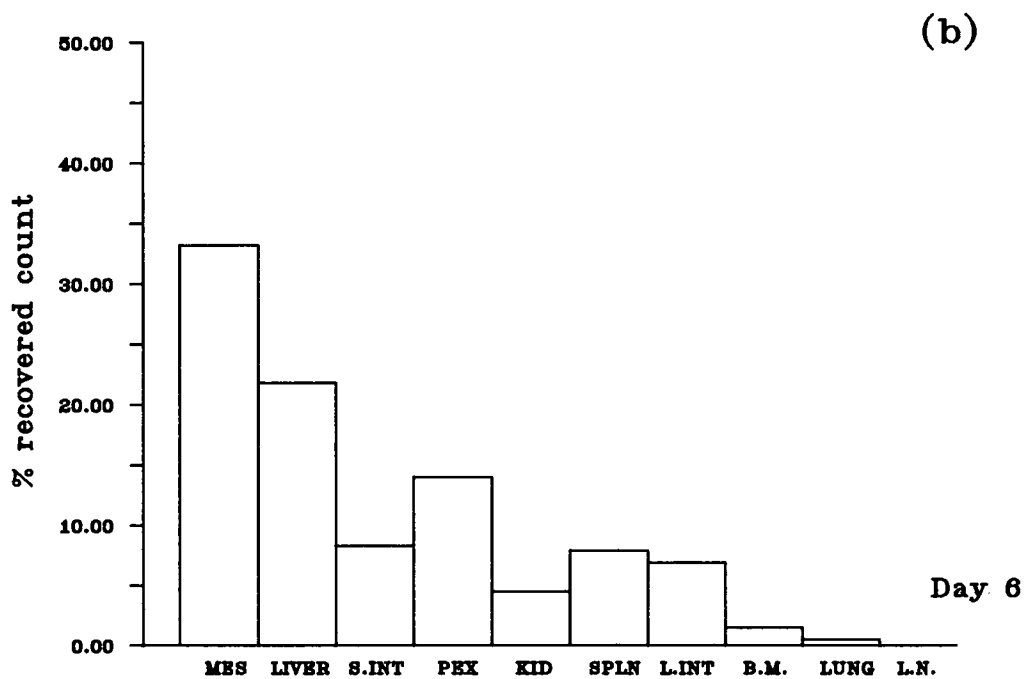
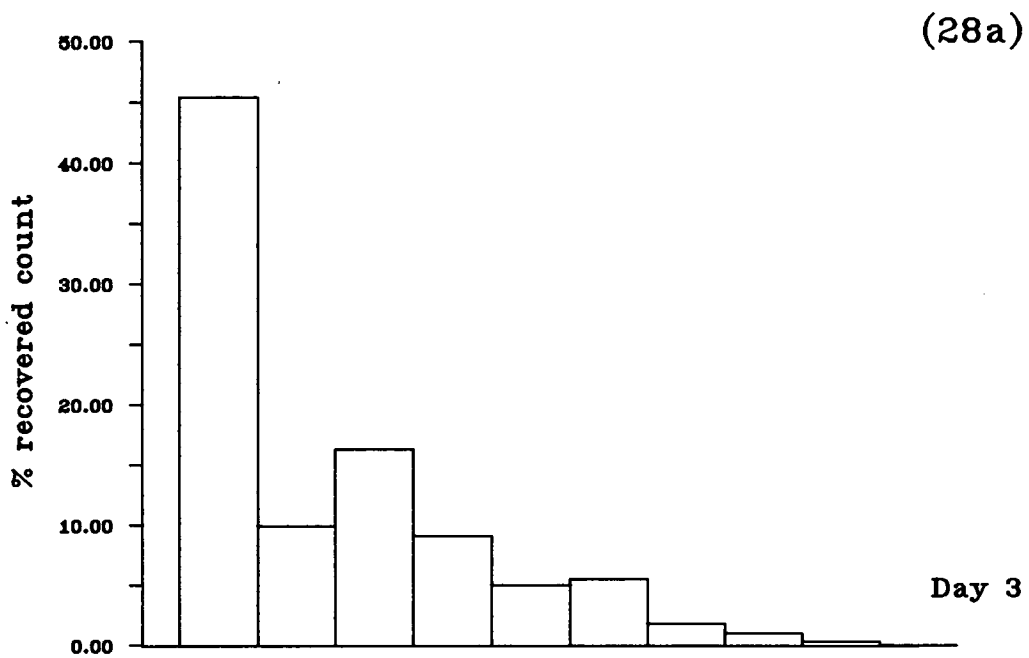
11.3.2 Tracking of labelled cells: timecourse

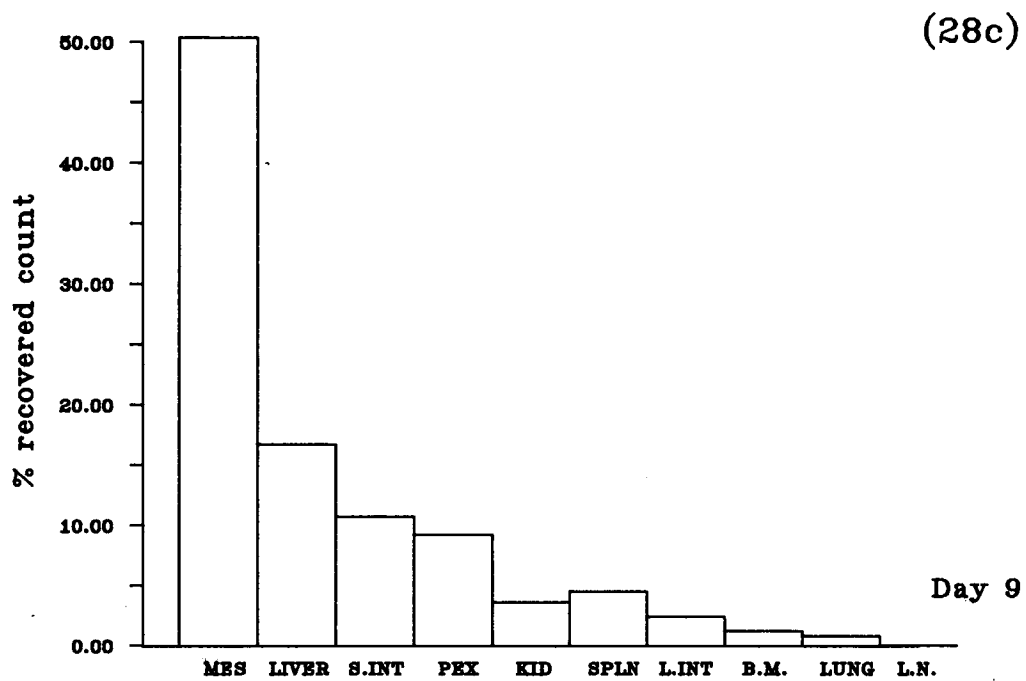
In this experiment the mesenteries were removed as a whole and counted separately. This was because: (i) dab smears had shown the presence of infected cells in the gut and mesenteries; (ii) the counts recovered in experiment 1 were relatively low, so it was assumed that cells must be present in other (unsampled) sites. Up to 50% of the total recovered count was found in the mesenteries. High counts were also recovered from the liver, small intestine and peritoneal exudate on all days (graph 28a, b and c).



(27). Radioactivity of organs removed from mice injected with ^{51}Cr labelled TaHis110 cells i/p, as a percentage of the activity of the same number of cells retained as a standard. Tissues were harvested 18 hours after injection of labelled cells. Graph (a), cells injected into scid mice (mean of three mice); graph (b), cells injected into Balb/c mice (mean of two mice). Abbreviations as for graphs 22-26, plus B.M.=bone marrow.

(28). Tracking of ^{51}Cr labelled TaHis250 cells injected i/p into scid mice. Tissues were harvested (a),3; (b),6 and (c),9 days after inoculation with radiolabelled cells. The activity of the tissues is expressed as a percentage of the total activity recovered. Each histogram presents the mean results from a pair of mice. Abbreviations as above.





No major movement of labelled cells from the mesenteries to other tissues was seen during the experiment. The liver was the second most important site of activity on days 6 and 9, but there was no consistent rise in the activity recovered from the liver. Counts from lymphoid tissues such as the lymph nodes and spleen remained low throughout the experiment.

11.4 Discussion

Experiments by other workers with heat-killed and disrupted $^{51}\text{chromium}$ labelled cells have shown that radioactivity recovered from the lymph nodes and spleen represents live labelled cells, while dead and damaged labelled cells are sequestered in the liver and kidneys (Bainbridge, 1966; Heslop and McNeilage, 1983).

Radiolabelled *T.annulata* infected cells, injected i/p into scid mice, were found to localise in the mesentery. Counts recovered from the mesentery were much higher than those for the peritoneal exudate alone. Other important sites were the small intestine and liver. Small but definite counts were recovered from the kidneys, spleen and lungs. Comparison of the data from experiments 1 and 2 shows that although the mesenteries and gut were not fully sampled in all experiments, there was no inconsistency between the results in the distribution of recovered activity.

No gross movement of radiolabelled TaHis250 cells injected i/p into scid mice was seen over 3-9 days. There was no obvious accumulation over time of activity in the liver or kidneys, which would indicate rejection of the cells. Most of the radiolabelled cells were recovered from the mesentery. The liver was the second most important site on days 6 and 9, indicating some sequestration and killing of TaHis250 cells. Establishment in other organs away from the peritoneal cavity, such as the bone marrow and lungs, was low.

$^{51}\text{chromium}$ labelling of *T.annulata* infected cells made possible the tracking of a low dose of cells (2×10^6 cells). The fate of a dose of this size injected into scid mice i/p could not be determined by the histological methods described in the previous chapter. High doses (2×10^7) i/p lead to extensive infiltration of tissues: the experiments with radiolabelled cells confirm that the same tissues (mesentery, gut, liver and kidney) are colonised by low doses of cells as by high doses.

In summary, these experiments showed that when *T.annulata* infected cells were

injected i/p into scid mice, up to 50% of the cells recovered were found in the mesenteries. Large numbers were also found in the small intestine and peritoneal exudate. Cells found in the liver are conventionally supposed to be damaged or dying: however, dab smears had previously shown apparently healthy schizont-infected cells in the livers of scid mice. The low counts recovered from the bone marrow and lymph nodes may partly reflect the size of these sites, but there did not appear to be a widespread colonisation of lymphoid tissues by labelled cells. Low counts were recovered from the lungs: this would suggest that infected cells do not disseminate from the intraperitoneal site through the venous drainage.

CHAPTER 12

ASSAY FOR ALLOGENEIC LYMPHOCYTE CYTOTOXICITY

12.1 Introduction

The experiments described in chapter 7 demonstrated that while high doses of *T.annulata* infected cells grew rapidly in scid mice, the mice were apparently able to limit the growth of smaller numbers of cells. The growth of *T.parva* infected cells could also be limited by the mice. These results suggested the operation of some form of immune defence against the infected cells. It was thought that NK cells might be responsible for the control of tumour growth: the experiment described in this chapter was carried out as an *in vivo* assay for NK cell activity in the scid mice. Experiments designed to look at NK cell activity *in vitro*, and at the role of macrophages in natural immunity in scid mice, are described in the following chapters.

When allogeneic lymphocytes are injected into mice or rats, rapid elimination of the injected cells has been reported to occur by a mechanism which is antibody and T-cell independent, radioresistant and apparently independent of differences in MHC types. This phenomenon is known as allogeneic lymphocyte cytotoxicity (ALC) (Heslop and McNeilage, 1983; Ford, Rolstad and Fossum, 1984). ALC represents a form of non-acquired, natural immunity to allogeneic cells, and is believed to be mediated by NK cells (Ford et al, 1984). ALC is measured experimentally by the injection of allogeneic or syngeneic radiolabelled cells and the subsequent recovery of radioactivity from lymphoid and other organs. For any particular organ or tissue, the ratio of the count recovered in mice given allogeneic or xenogeneic cells to the count recovered from mice given congenic cells provides a measure of the localisation of labelled cells in that tissue, due either to establishment of the cells or their sequestration by immune mechanisms. This ratio is called the tissue localisation ratio (TLR). An accumulation of radioactivity in the lymph nodes and spleen indicates establishment, whilst a build-up in the liver and kidneys is taken to indicate the presence of damaged or dead cells. The actual counts recovered from the liver and spleen can also be compared as a measure of rejection versus establishment (Martin, 1969; Heslop and McNeilage, 1983).

The experiment described here was intended to investigate ALC in scid mice in comparison to Balb/c mice. Bovine and Balb/c spleen cells, rather than *T.annulata* infected cells, were used for labelling and injection into the mice, for several reasons: (i) scid mice are congenic with Balb/c mice; (ii) murine spleen forms an easily harvested source of large numbers of lymphoid cells, and it was therefore desirable to have bovine cells of similar origin; (iii) it was assumed that the presence or absence of the parasite would make little difference to the overall "foreignness" of the bovine cells in a murine host; (iv) as the tissues were to be harvested after 18-20 hours, and the experiment was to look at rejection mechanisms rather than establishment, the fact that the cells were not transformed would not be important.

12.2 Methods

Balb/c spleen cells (Balb/c SC) were prepared as described in the appendix (section 4.2). Bovine spleen was obtained from the Gorgie Abattoir, Edinburgh, and bovine spleen cells (BoSC) were prepared as for mouse spleen. All cells were labelled with ⁵¹chromium as described in the Appendix (section 4.3) and adjusted to 2x10⁶ cells/dose in PBS. 12 scid mice and 12 Balb/c mice were injected intraperitoneally with radiolabelled bovine or murine (Balb/c) spleen cells (6 mice/group). Organs were removed for counting after 18 hours. Unfortunately, the mesenteries were not removed separately in this experiment: mesenteric tissue was counted with the small intestine. For each mouse strain, the mean percentage of cells localising in each organ from the BoSC group was compared to the mean % for the same organ in the Balb/c SC group using Student's t- test. The tissue localisation ratios (TLRs) for each mouse strain were calculated as follows:

$$\text{TLR (organ)} = \frac{(\text{mean \% "organ" BoSC})}{(\text{mean \% "organ" Balb/c SC})}$$

(Heslop and McNeilage, 1983).

The ratio of mean liver cpm:mean spleen cpm was also calculated for each group.

12.3 Results

Table 9 shows the mean total recovered count as a percentage of the control count, and the mean liver:spleen ratio in each group. No significant differences between the liver: spleen ratios were found.

The % counts recovered from the various tissues and organs are shown in graphs 29a-d. In all four groups, the highest counts were recovered from the small intestine. High counts were also recovered from the peritoneal exudate in all groups except scid+BoSC. The counts recovered from the liver, spleen, large intestine and kidneys were lower, but in approximately the same range as each other; the lungs, bone marrow, peripheral blood and lymph nodes accounted for only a small percentage of the total count.

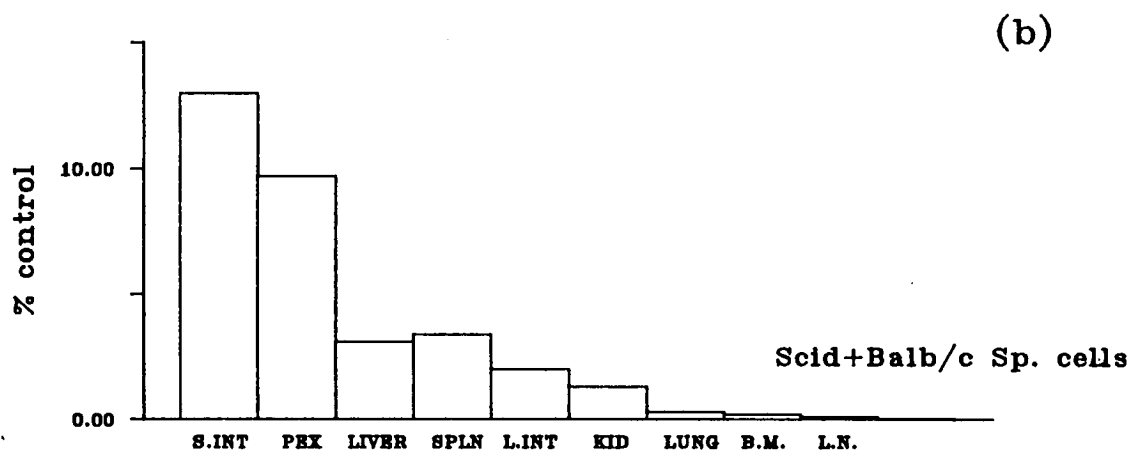
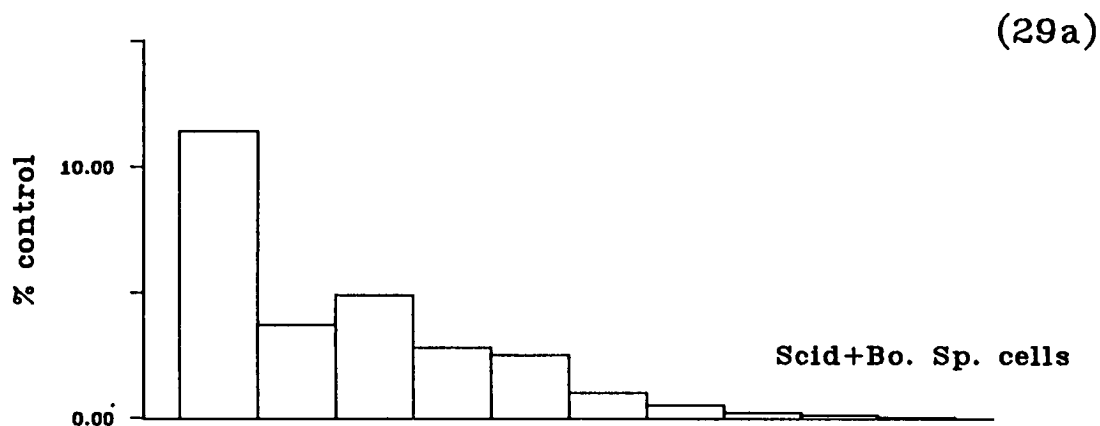
Tissue localisation ratios (TLR) for both mouse strains are given in table 10. These ratios should indicate whether mice given bovine SC have more label in a particular site than mice given congenic (Balb/c) SC. The p values correspond to the probability of the actual TLR being 1.0, i.e. there being no difference between the BoSc and Balb/c SC groups. Three results are significant at $p < 0.01$: Balb/c blood, Balb/c liver and scid peritoneal exudate. The Balb/c blood result would indicate that there is more radiolabel in the peripheral blood of mice which received bovine cells than those which received Balb/c cells; however, the actual counts recovered from the blood were very low (all $\leq 0.1\%$ of control). In comparison, high levels of activity were recovered from the livers of Balb/c mice given BoSC. There was a trend in this direction in the scid mice, but the result was not significant. High TLRs in the lymph nodes and lungs of Balb/c mice (4.27 and 3.57, respectively) were not significant in t-tests. Reanalysis of the data using the non- parametric Mann- Whitney test, which is less sensitive to extreme "outlier" values than the t- test, did not alter the level of significance of any result except that for the Balb/c lung, where $p = 0.0082$ instead of 0.18. This result could therefore be regarded as significant, and indicative of ALC in the lungs: however, the actual counts recovered from the lungs were low (0.5, 0.3; 0.8, 0.2).

The highly significant result for the scid peritoneal exudate indicates that BoSC disappeared from the peritoneal site more rapidly than Balb/c SC. The percentage counts from the other tissues were summed and this "Non-Pex. % count" was compared for the two groups of scid mice. No significant difference between the two

Table 9. Percentage of activity recovered in scid and Balb/c mice injected i/p with ^{51} chromium labelled bovine or Balb/c spleen cells.

	% total recovered	%liver count/%spleen count
Scid/bovine spleen cells	26.8 (6.84)	2.12
Scid+Balb/c spleen cells	36.1 (10.53)	2.39
Balb/c+bovine spleen cells	34.3 (4.26)	2.97
Balb/c+Balb/c spleen cells	27.4 (6.35)	2.64

The activity recovered is shown as a percentage of the activity of a control aliquot of labelled cells, counted at the same time as the mouse tissues. All percentages are means; figures in brackets are standard deviations. Comparison of liver:spleen ratios using the Mann-Whitney test showed no significant differences between the groups.



(29). Location of ^{51}Cr labelled bovine spleen cells (BoSC) and Balb/c spleen cells (Balb/c SC) injected i/p into scid and Balb/c mice. Tissues were harvested after 18 hours. Activity is expressed as a percentage of the activity of a retained dose of labelled cells. Graph (a), scid mice plus BoSC (mean of five); (b), scid plus Balb/c SC (mean of six); (c), Balb/c plus BoSC (mean of six); (d), Balb/c plus Balb/c SC (mean of six).

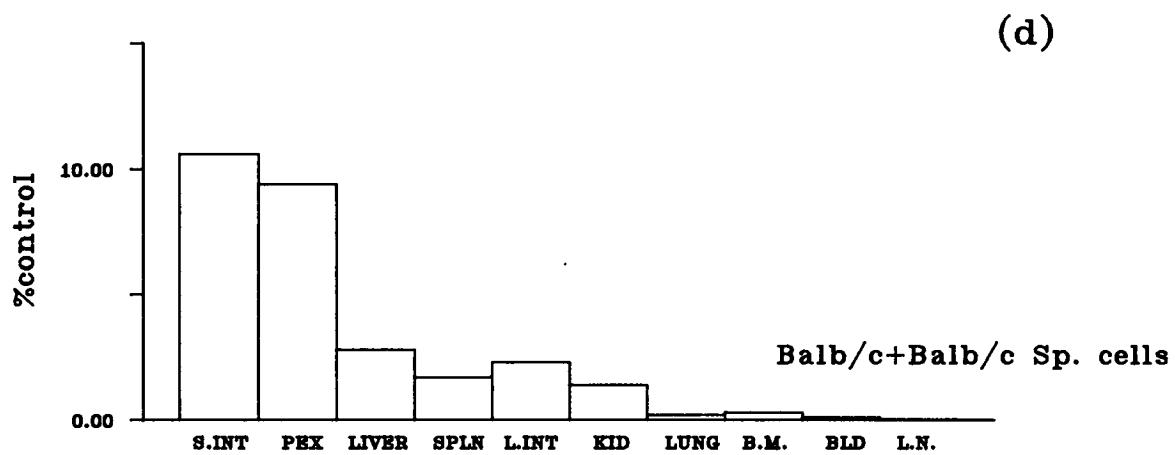
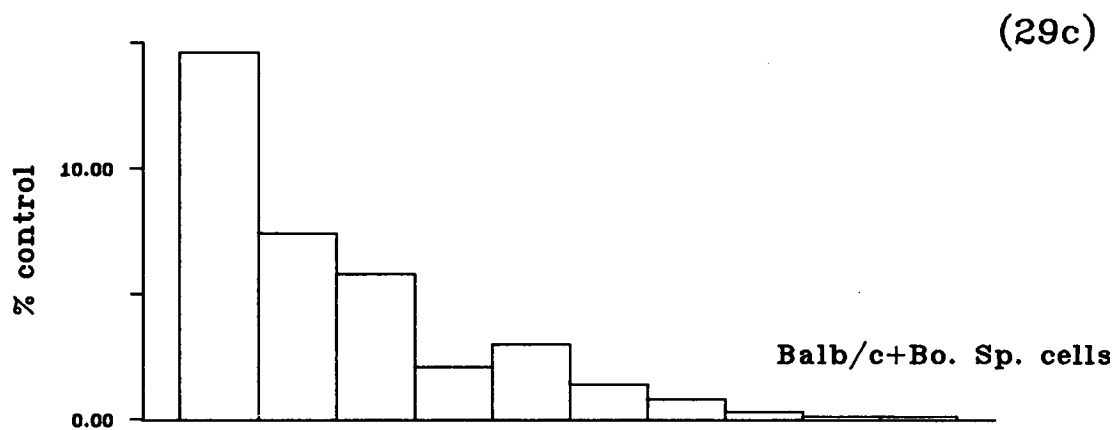


Table 10. Tissue localisation ratios (TLR) for (i) scid mice given bovine or Balb/c spleen cells; (ii) Balb/c mice given bovine or Balb/c spleen cells.

Tissue	Scid TLR	p	Balb/c TLR	p
Blood	1.4	0.054	1.7	0.012
Pex.	0.38	0.000	0.79	0.79
L.N.	0.26	0.055	4.27	0.43
B.M.	0.81	0.26	0.94	0.8
Liver	1.57	0.51	2.04	0.0048
Spleen	0.84	0.73	1.24	0.5
Sm.Int.	0.88	0.56	1.38	0.093
L.Int.	1.21	0.62	1.30	0.52
Kidney	0.75	0.14	1.06	0.6
Lung	1.87	0.26	3.57	0.18

$$\text{TLR} = \frac{(\text{mean tissue cpm in mouse} + \text{Bo. SC})}{(\text{mean tissue cpm in mouse} + \text{Balb/c SC})}$$

Values of p calculated from t-tests comparing mean cpm from mice with Bo. SC and mice with Balb/c SC for each organ.

groups was found.

12.4 Discussion

This experiment did not show significant differences in total activity recovered between the four groups. There was no significant difference between the liver:spleen ratios of the four groups. The liver:spleen ratio has been used as a measure of rejection versus establishment (Martin, 1969). By this measure, there was therefore no difference between the scid and Balb/c mice in the rejection and establishment of xenogeneic and congenic cells.

TLRs were calculated to see if the xenogeneic bovine cells in scid mice were eliminated: (i) more rapidly than congenic Balb/c spleen cells; (ii) as rapidly as in Balb/c mice. The tissue localisation ratios for the Balb/c mice clearly show rejection of BoSC compared to Balb/c SC, as a significant accumulation of radioactivity in the liver and lungs of mice injected with BoSC. A high mean TLR in the lymph nodes was not significant. The only significant TLR in the scid mice was in the peritoneal exudate, where less bovine cells were recovered than Balb/c cells. There are three possible explanations for this result, detailed below.

The first is that the bovine spleen cells were able to migrate out of the peritoneal cavity and into other tissues more rapidly than the Balb/c cells. This does not seem likely, as it would be expected that Balb/c spleen cells would migrate more readily in mice than bovine cells. It was reasoned that if the bovine cells were leaving the peritoneal cavity and establishing elsewhere, then relatively higher counts should be recovered from the rest of the body. No significant difference between the groups for the "whole body- Pex." site was found, but because the total recovered counts were only approximately 30% of the control count, there could be an accumulation of label in the unsampled tissues. However, if the drop in the peritoneal exudate count were due to a migration of the bovine cells to other tissues, then a similar phenomenon should be observed in Balb/c mice: although the "Pex." count for the Balb/c+BoSC group was slightly lower than in the Balb/c+Balb/c SC group, the difference was not significant.

The second explanation is that the difference in the TLR between the scid and Balb/c mice is due to a change in the numbers of murine SC in the peritoneal exudate, not to a change in the numbers of bovine SC. An examination of the data in graph 29

shows that this is not the case.

The third explanation is that bovine cells in the peritoneal cavity were rapidly killed and the label was removed. As over 90% of the cells in the scid peritoneal exudate are macrophages (chapter 13, table 11) these are the obvious candidates as effector cells. It might be expected that the peritoneal macrophages of Balb/c mice would also be effective against xenogeneic cells in the peritoneal cavity, but there could be other mechanisms preventing full macrophage activity in the Balb/c mice. If the bovine cells were rapidly killed by peritoneal macrophages, then their label would have been excreted before the mice were sampled. ⁵¹chromium released from dead cells does not spontaneously attach to other cells *in vivo* (Bainbridge^y 1966).
et al

The calculation of TLRs clearly showed significant rejection of bovine cells in Balb/c mice, as high TLRs in the liver and lungs. This probably indicates NK cell cytotoxicity (Ford et al, 1984). In scid mice, TLRs did not indicate conventional ALC, but did appear to show significant clearance of bovine cells from the peritoneal cavity. Therefore peritoneal macrophages might be playing a significant role in the destruction of intraperitoneally injected cells in scid mice.

CHAPTER 13

NATURAL IMMUNITY IN UNINFECTED SCID MICE

13.1 Introduction

The experiments presented here looked at the immune system of uninfected scid mice. As described previously (chapter 7), attempts to label macrophages and NK cells for flow cytometry were not successful, so macrophages were identified by direct counts of Giemsa-stained cytopins, while NK cell activity was assessed by *in vitro* YAC-1 cytotoxicity assays. The YAC-1 cell line is very sensitive to NK cell cytotoxicity (Kiessling, Klein^{Prus} and Wigzell, 1975) and is widely used as a standard target cell in in vitro assays for murine NK cells.

There were three objectives: (i) to screen the mice for "leakiness" of the scid phenotype; (ii) to obtain control data on numbers of peritoneal and spleen cells, and on NK cell activity *in vitro* (anti-YAC-1 activity); (iii) to see if spleen cells from uninfected scid mice could lyse *Theileria* infected cells *in vitro*, and if so, whether there was a difference in susceptibility between *T.annulata* and *T.parva* infected cells. Some experiments were also conducted to develop an assay for macrophage activity, by the cytostasis of EL-4 cells (Dazor et al, 1978). The results obtained in control experiments were not sufficiently consistent to be useful, and this approach was abandoned.

13.2 Methods

Mice were screened for immunoglobulin production by the radial immunodiffusion technique (RID) as described in "Materials and Methods" section 5.

Viable cell counts were made from peritoneal exudates and spleen cell suspensions. In some cases cytopins were prepared for differential counts of cell suspensions.

Spleen cells from two mice were used as effector cells in an 18 hour YAC- 1

cytotoxicity assay, as described in the Appendix. In addition, ^{51}Cr labelled TaHis249 and TpMug110 cells were used as targets in 18 hour cytotoxicity assays, with scid spleen cells as effector cells.

13.3 Results

13.3.1 RID for serum immunoglobulin

Sera from one-tenth of the scid mice, including some bearing *Theileria* tumours, were tested for the presence of IgG1, IgG2a, IgG2b, IgG3, IgM and IgA. All the sera were immunoglobulin negative at dilutions of 1/10 to 1/1000. As the detection range of the kit used was 1-20 μg protein/ml, there were $<1 \times 10^{-3} \mu\text{g}$ Ig/ml in the samples tested. Serum from a Balb/c 4Gy mouse with a 7 day TaHis110 tumour was strongly positive in the same assays.

13.3.2 Peritoneal exudate and spleen cell counts

The mean numbers of peritoneal exudate and spleen cells, and the relative proportions of the different cell types in scid mice, are presented in table 11. The equivalent data for Balb/c mice are also shown. Total counts for both the spleen and peritoneal exudate were much lower in scid mice than in immunologically intact Balb/c mice of the same age and sex. Lymphocytes were particularly depleted, forming approximately half of the spleen cells and being virtually absent from the peritoneal cavity. Macrophages made up the remainder of the peritoneal cells in scid mice. This depletion of lymphocytes did not cause a relative increase in the numbers of neutrophilic polymorphonuclear leukocytes (neutrophils) and basophilic granulocytes (basophils) in the peritoneal site.

13.3.3 YAC-1 cytotoxicity assay

The results for two mice are shown in graph 30. In both cases, cytotoxicity rose sharply at an effector:target (E:T) ratio of 100:1, although the levels of killing observed were different. Mann-Whitney tests showed that the percentage cytotoxicity at an E:T ratio of 100:1 was significantly higher than spontaneous release in both

Table 11. Peritoneal exudate/spleen cell counts from scid mice.

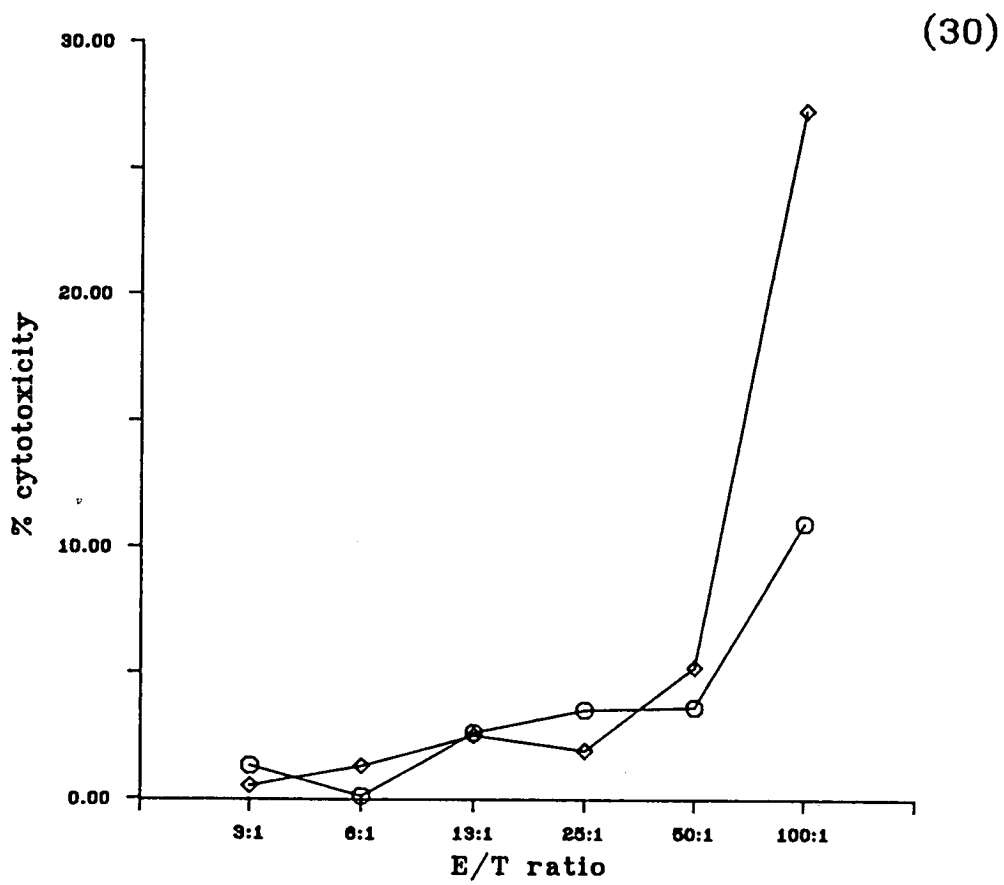
	Total viable count	%Lymphos	%MØ	%PMNs	%Basos
<u>Scid</u> Pex.	7x10 ⁵	4	92	4	-
Balb/c Pex.	40x10 ⁵	66	30	1	3
<u>Scid</u> Spln.	100x10 ⁵	51	42	5	-
Balb/c Spln.	1100x10 ⁵	85	11	4	-

The total viable count was assessed by trypan blue exclusion. The percentages given were calculated from differential counts of cytopspins stained with Giemsa. At least 10 fields/slide were counted at a magnification of x1000. Lymphos=lymphocytes, MØ=macrophages, PMNs=polymorphonuclear leukocytes, Basos=basophilic granulocytes. Pex.=peritoneal exudate, spln.=spleen.

Table 12. Attempts to label TaHis249 or TpMug110 cells for cytotoxicity assays.

Experiment	(Spontaneous release)x100 Maximum release	% viability
1. TaHis249	49	90%
TpMug110	56	75%
2. TaHis249	51	90%
TpMug110	39	67%
3. TaHis249	46	75%
TpMug110	40	(nd)

The maximum release of ⁵¹chromium was measured by incubation of labelled cells with Triton-X100. Spontaneous release was measured by incubating the cells in medium alone. Percentage viability of the labelled cells was measured by trypan blue exclusion immediately before the cells were plated out for experiments.



(30). Percentage cytotoxicity of YAC-1 cells *in vitro* by spleen cells from two untreated scid mice.

cases.

13.3.4 Attempts to use TaHis249 and TpMug110 cells as targets

Radiolabelled TaHis249 and TpMug110 cells were used as targets in cytotoxicity assays in three separate experiments. In each case, the amount of radioactivity released spontaneously from the target cells was high compared to that released from target cells lysed with Triton X-100. The equivalent values for labelled YAC-1 cells were typically 20-25%. No significant cytotoxicity of *Theileria*-infected cells was detected in these experiments (table 12).

13.4 Discussion

No "leakiness" of the scid phenotype was detected by the RID assay for immunoglobulins.

The total and differential cell counts from the peritoneal exudate and spleen provided useful background information on the scid mutant. The paucity of lymphocytes led to an increase in the proportion of macrophages, particularly in the peritoneal cavity. It is interesting that the total numbers of peritoneal cells were depressed, as macrophages are not directly affected by the scid mutation: this may be due to a secondary effect of lymphocyte depletion, such as a failure to recruit monocytes into the peritoneal cavity; or it may be due to the germ-free status of the scid mice. Depletion of T and B lymphocytes may have enriched the spleens for NK cells: it would not be possible to clarify this point without conducting more extensive experiments specifically on this subject.

Anti-YAC-1 cytotoxic activity was detected in scid mice, at higher levels than those seen in Balb/c mice at the same E:T ratios (see chapter 7: typical values 4-6% at an E:T ratio of 100:1). This activity was variable: the two mice examined had widely different levels of cytotoxicity. In subsequent experiments, it would therefore be difficult to make an "external" comparison with uninfected scid mice.

No positive cytotoxicity was found in experiments using labelled TaHis249 or TpMug110 cells as targets. These cell lines showed high levels of spontaneous release compared to the maximum release from lysed cells. This appeared to be due

both to a poor uptake of ^{51} chromium, and a relatively high rate of non-specific release of ^{51} chromium. These factors may have been related to the condition of the cells when they were labelled; but all experiments were done with cells which were healthy by visual inspection, and there was no apparent relationship between the viability of the cells after labelling and maximum/minimum release ratio. In contrast, YAC-1 cells showed consistently good labelling, with spontaneous release only 20-25% of maximum release, suggesting that the YAC-1 cell line is intrinsically better suited to this sort of assay than the *Theileria* infected cell lines.

Cytotoxicity against TaHis249 and TpMug110 cells could perhaps have been measured by a cold-target inhibition assay, in which unlabelled TaHis249 or TpMug110 cells would be added to a YAC-1 assay. Under appropriate conditions, lysis of *Theileria* infected cells by a limiting number of NK cells would lead to a decrease in the cytotoxicity of YAC-1 cells.

CHAPTER 14

FURTHER STUDIES ON NATURAL IMMUNITY IN SCID MICE INJECTED WITH *THEILERIA* CELL LINES

14.1 Introduction

The experiments described in the previous chapters showed that "high" doses of *Theileria*-infected cell lines could establish and grow as both subcutaneous and ascitic tumours in scid mice. However, at low doses cell lines failed to establish intraperitoneally, and *T.parva* infected cells did not establish as readily as *T.annulata* infected cells in either site. The scid mice were therefore able to eliminate, or limit the growth of, a certain number of cells. As no leakiness of the scid phenotype was detected by RID assays for immunoglobulin, the mechanism involved must have been antibody, B and T-cell independent. The experiments in the previous chapters did not show conventional ALC in scid mice injected with bovine spleen cells, but did seem to indicate that resident macrophages were able to clear bovine cells from the peritoneal cavity. *In vitro* assays showed variable levels of NK cell activity in the spleens of uninfected scid mice.

The experiments presented in this chapter were done to investigate which immune responses of the scid mouse were activated when the mice were injected with *Theileria*-infected cell lines. Macrophage activation was assessed by differential counts of peritoneal exudate and spleen cell suspensions. These counts also gave information on the proliferation of other cell subsets such neutrophilic polymorphic leukocytes (neutrophils) and basophilic granulocytes (basophils), as well as on the multiplication of *Theileria*-infected cells in these sites.

Scid spleen cell suspensions were tested for NK cell activity (as anti- YAC-1 cytotoxicity) *in vitro*. This was to complement the *in vivo* ALC assay described in chapter 12.

Assays for two macrophage-derived cytokines (IL-1 and TNF) were carried out. As discussed in the "General Introduction", IL-1 is an important mediator of

inflammation; TNF can also promote inflammation, as well as necrosis of tumours, and has cytotoxic effects on some tumour cell lines. Assays for IFN- γ were also performed: IFN- γ is released by lymphocytes and NK cells and has a range of effects, including macrophage activation. The presence or absence of these mediators might indicate the type of immune mechanisms acting to prevent establishment of *Theileria*-infected cells in the scid mice.

14.2 Methods

A total of 40 scid mice were injected i/p or s/c with TaHis249 or TpMug110 cells at the "high" and "low" doses described previously. The intention was to sample at an "early" and "late" stage: therefore mice were killed after 8-12 days or 15-21 days, depending on tumour development or the severity of ascites. Serum samples, peritoneal exudates, spleen cells and tumour extracts were prepared as described in the "Materials and Methods" (section 2.3) and Appendix A (section 5). Serum samples were pooled for cytokine assays. Peritoneal exudate cells were counted, resuspended in 10% FCS/RPMI at 1×10^6 cells/ml, and incubated for 24 hours. Peritoneal cells from mice from identically treated mice killed on the same day were pooled for the purpose of making peritoneal cell supernatants. The supernatant was removed, centrifuged and sterile-filtered to remove cells, and stored at -70 C. Assays for anti-YAC-1 activity, IL-1, TNF and IFN- γ were carried out as described in Appendix A (sections 3 and 4). The significance of the results was assessed by the Mann-Whitney test.

14.3 Results

14.3.1 Total and differential counts

14.3.1.1 Peritoneal exudates

In mice injected intraperitoneally with the high dose of TaHis249, there was a vast increase in the total number of cells up to day 9, when the mice were killed (table 13). More than half of these cells were schizont-infected, but there was also a substantial increase in the number of macrophages present. Lymphocytes were the

Table 13. Differential counts of peritoneal exudates from scid mice injected with TaHis249 or TpMug110 i/p

All counts $\times 10^5$, to 2 significant figures and one decimal place.

Dose/day	Total	Inf.cells	Macros.	Lymphos.	Basos.	Neutros.	(n)
Controls	7.0	-	6.4	0.3	0	0.3	5
<u>TaHis249</u>							
Hi/6	780	440	190	150	0	0	2
Hi/9	1600	940	400	0	0	260	2
Lo/6	14	0.1	10	2.7	0.6	0.4	1
Lo/15	10	0	9.4	0.1	0.4	0.3	3
<u>TpMug110</u>							
Hi/11	5.6	0.1	4.0	0.9	0.2	0.4	1
Hi/29	44	3.1	26	9.2	1.3	4.4	1
Lo/11	8.0	0	5.9	1.0	0.5	0.6	1
Lo/29	6.1	0	4.5	0.8	0.4	0.4	2

Total cell counts were calculated by trypan blue exclusion, and the numbers of the cell subsets were calculated from percentages obtained by differential counts of cytopins (at least 10 fields counted, $\times 1000$ magnification).

Inf. cells, schizont-infected cells; macros, macrophages; lymphos, lymphocytes; basos, basophilic granulocytes; neutros, neutrophilic polymorphic leukocytes.

second most numerous group on day 6, but had been replaced by neutrophils on day 9. In contrast, mice which received a low dose of TaHis249 cells did not show a major increase in cell numbers, and few infected cells were found in the peritoneal exudate. In mice injected with TpMug110 i/p, total peritoneal exudate counts remained around control levels, except in the high dose mice. A total and differential count from a high dose mouse, made on day 29, showed six times as many cells as normal, mostly macrophages. Some infected cells were also present.

Total and differential counts of peritoneal exudates from scid mice injected with TaHis249 or TpMug110 subcutaneously (table 14) showed that major (>5- fold) increases in the total cell count were only seen when infected cells were also detected in cytospins (TaHis249 day 16, high dose; TaHis249 day 21, low dose). Most of this increase in cell numbers was due to an increase in the number of macrophages.

Basophils were seen in most peritoneal exudate samples from infected mice. Their frequency in the peritoneal exudates of control mice was in comparison very low.

14.3.1.2 Spleen cells suspensions

Total and differential counts of spleen cells from all four groups of scid mice showed an increase in total spleen cells over uninfected controls (table 15). This was most marked in mice bearing large subcutaneous tumours, such as the TaHi(s/c) mouse killed on day 12. For each cell line and route of inoculation, the increase in spleen cellularity was dose- and time- dependent: high-dose mice had higher counts than low-dose mice, and mice killed later in the experiment showed higher counts than those killed earlier. The numbers of lymphocytes were increased 2-5 fold in most cases, but the biggest relative increase in the spleen was in the neutrophil count, which increased more than 20-fold in some cases: these mice (TaHi(i/p) day 9, TaHi(s/c) day 12 and 16) had ascites or large subcutaneous tumours. A high neutrophil count was seen in the ascites of the TaHi(i/p) mouse killed on day 9, but no such infiltration by neutrophils was seen in dab smears of subcutaneous tumours.

Infected cells were only seen in the spleens of mice with severe ascites, ie those injected with the high dose of TaHis249 i/p.

Table 14. Differential counts of peritoneal exudates from scid mice injected with TaHis249 or TpMug110 s/c

All counts are $\times 10^5$, to 2 significant figures and one decimal place.

Dose/day	Totals	Inf.cells	Macros.	Lymphos.	Basos.	Neutros.	(n)
Controls	7.0	-	6.4	0.3	0	0.3	5
<u>TaHis249</u>							
Hi/12	11	0	10	0	0.6	0.2	1
Hi/16	43	5.6	35	0	2.6	0	1
Lo/12	6.5	0	6.0	0	0.5	0	1
Lo/16	8.0	0	7.0	0.6	0.4	0	1
Lo/21	34	0.3	30	0	0.7	2.7	2
<u>TpMug110</u>							
Hi/11	4.1	0	3.4	0.2	0.3	0.2	2
Hi/29	6.8	0	5.9	0.6	0.3	0	2
Lo/11	5.5	0	3.5	1.3	0.4	0.3	1
Lo/29	2.9	0	2.6	0.1	0.2	0	2

Counts were calculated as described for table 13. The abbreviations are the same as for table 13.

Table 15. Differential counts from spleens of scid mice injected i/p or s/c with TaHis249 or TpMug110 cells

All figures are counts $\times 10^5$ to 2 significant figures.

Dose/day	Totals	Inf.cells	Lymphos.	Macros.	Neutros.	(n)
Controls	100	-	51	42	5	9
<u>TaHis249 i/p</u>						
Hi/9	220	8.8	60	49	100	2
Lo/15	120	1.2	18	72	30	1
<u>TpMug110 i/p</u>						
Hi/11	220	0	150	31	36	2
Hi/29	300	0	210	54	30	3
Lo/11	210	0	140	32	42	1
Lo/29	150	0	130	11	4.2	1
<u>TaHis249 s/c</u>						
Hi/8	220	0	100	55	62	2
Hi/12	570	0	270	160	140	1
Hi/16	510	0	260	77	170	1
Lo/12	150	0	80	29	42	1
Lo/16	110	0	40	31	40	1
Lo/21	230	0	110	32	90	1
<u>TpMug110 s/c</u>						
Hi/11	150	0	100	27	20	2
Hi/29	330	0	260	29	40	2
Lo/11	130	0	100	21	9.1	1
Lo/29	230	0	160	25	51	2

Total and differential counts were made from spleen cell suspension using the same methods described for peritoneal exudate cells (tables 13 and 14). The abbreviations used are the same as in table 13.

14.3.2 YAC-1 assays

The percentage cytotoxicity by spleen cells from all mice bearing subcutaneous TaHis249 tumours was significantly higher than spontaneous release, with values nearing 40% in four animals (graph 31a). The cytotoxicity in the day 16 high dose mouse was significantly higher than that in the low dose mouse killed on the same day, which had a smaller tumour. Both the day 21 low dose mice displayed higher levels of cytotoxicity than the day 16 low dose mouse.

Mice injected with a high dose of TaHis249 cells i/p, which had ascites and were killed on days 8 and 9, showed consistently low levels of cytotoxic activity (graph 31b). Only the mice killed on day 9 showed cytotoxicity significantly above spontaneous release. In contrast, mice with a low dose of cells i/p showed high levels of NK activity (approximately 20%) on days 15 and 19, although lower than the levels seen in mice with tumours growing s/c.

Levels of cytotoxicity were generally low (less than 5%) in mice given TpMug110 cells i/p or s/c (graph 31c and d). The only significant results were from two mice given a high dose of cells s/c, whose spleen cells showed 10% cytotoxicity. No significant results were seen in mice injected with TpMug110 cells i/p.

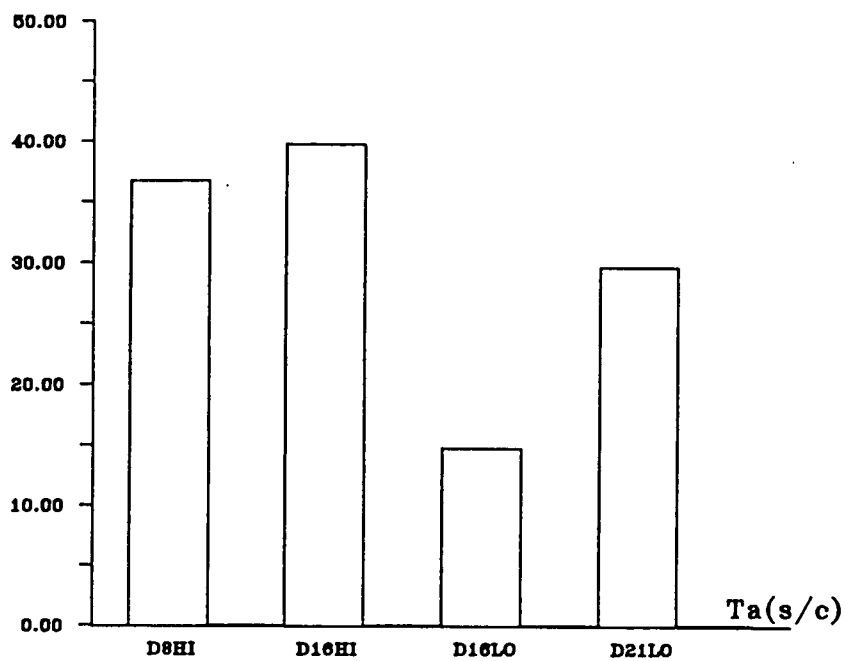
14.3.3 TNF assay results

Samples of serum, peritoneal exudate cell supernatants and tumour homogenates were tested for TNF activity in L-929 assays as described in the Appendix. No definite positive results were seen in repeated trials. The recombinant human TNF, which was used as a standard in the experiments, gave positive results in these assays.

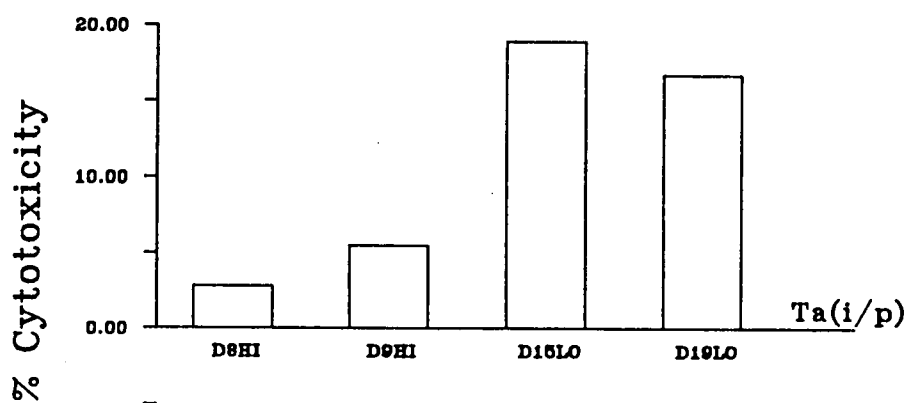
14.3.4 IL-1 assay results

IL-1 was assayed by the ability of samples to induce proliferation of murine thymocytes *in vitro*. Serum samples from infected mice, and tumour homogenates, did not induce thymocyte proliferation, whilst some samples of peritoneal cell culture supernatant induced high levels of proliferation (graphs 32a and b). High levels of IL-1 activity were observed in samples from mice injected with TaHis249 cells s/c.

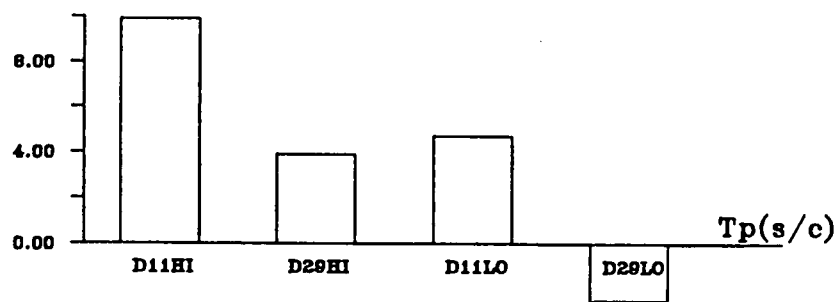
(31a)



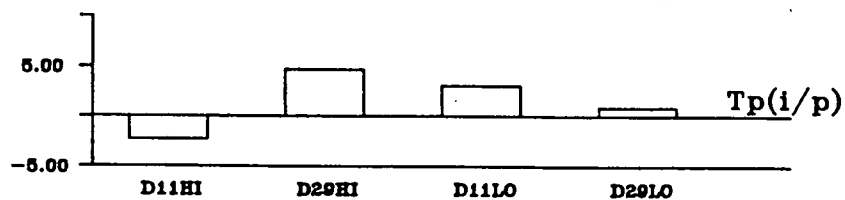
(b)



(c)



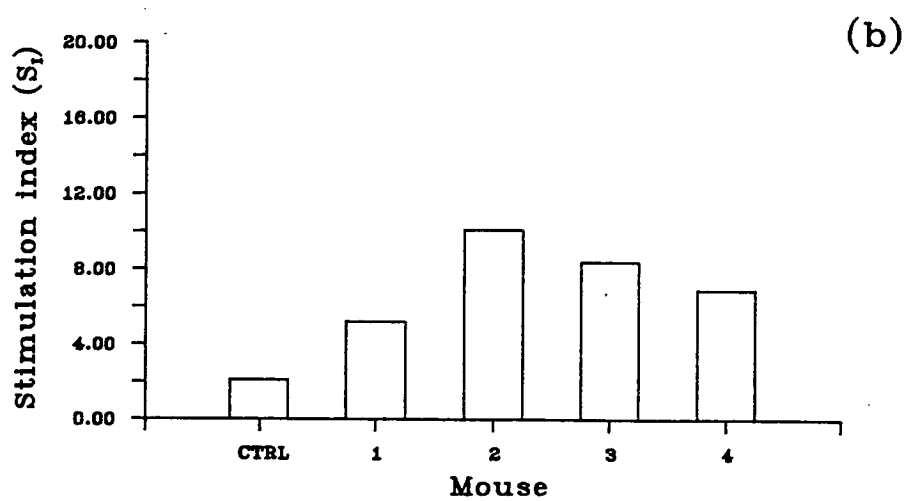
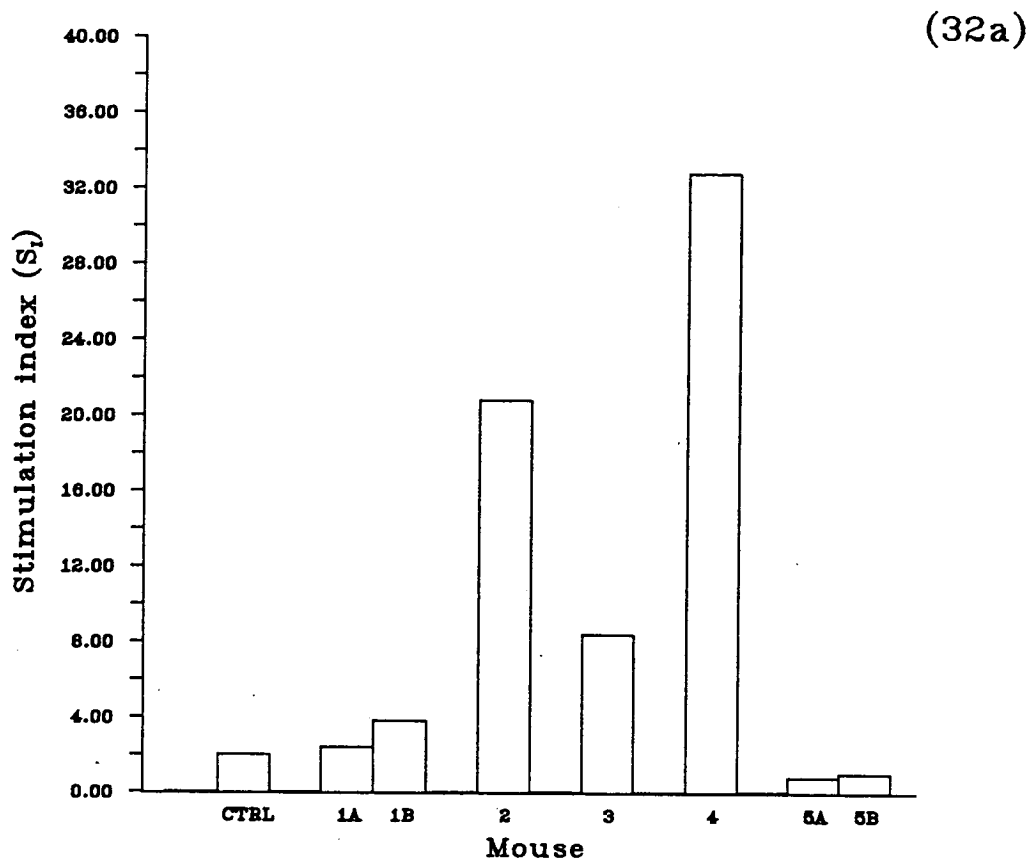
(d)



(31). Percentage cytotoxicity of YAC-1 cells *in vitro* by spleen cells from scid mice injected with *Theileria*-infected cells. Labels on x- axes indicate the day on which the mice were killed and the dose of cells which they had received (Hi= 2×10^7 cells, Lo= 2×10^6 cells). Graph (a), scid mice injected with TaHis249 cells s/c: d8Hi, one mouse; d16Hi, two mice; d16Lo, one mouse; d21Lo, two mice. Graph (b), mice injected with TaHis249 cells i/p: d8Hi, three mice; d9Hi, two mice; d15Lo, two mice; d19Lo, two mice. Graph (c), mice injected with TpMug110 cells s/c: d11Hi, two mice; d29Hi, three mice; d11Lo and d29Lo, two mice. Graph (d), mice injected with TpMug110 cells i/p: d11Hi, d29Hi, d29Lo all two mice; d11Lo, one mouse.

(32a). Proliferation induced in *in vitro* cultures of murine thymocytes by supernatants of peritoneal cells from scid mice injected with TaHis249 cells. Stimulation index (S_I) was calculated as the percentage of ^3H -thymidine incorporation in thymocyte cultures exposed to samples, compared to the incorporation of ^3H - thymidine in control cultures (see Appendix A). Supernatants of peritoneal cells from: ctrl, uninfected controls; 1A/B, mice given 2×10^7 cells s/c, killed on day 8; 2, high dose s/c mice, day 16; 3, low dose s/c mice, day 16; 4, low s/c dose mice, day 21; 5A/B, high dose i/p, day 9. Results were compared using the Mann-Whitney test. Peritoneal cell suspensions were pooled to produce supernatants, except for 1A, 1B, 5A and 5B which represent supernatants of cells from individual mice.

(32b). Thymocyte proliferation caused by peritoneal cell supernatants from mice injected with TpMug110 cells. Ctrl, uninfected controls; 1, high dose s/c; 2, low dose s/c; 3, high dose i/p; 4, low dose i/p. All mice were killed on day 29. S_I was calculated and compared between groups as previously described.



Visual inspection of cultures before supernatants were harvested showed that IL-1 positive cultures contained a high proportion of adherent cells. The level of IL-1 activity increased with time after inoculation of the mice, and was higher in high-dose mice than in low-dose mice. No significant activity was detected in supernatants from i/p mice. In TpMug110 mice, only day 29 samples were tested. There appeared to be little significant variation in activity between experimental groups, although in all groups the levels of thymocyte stimulation were higher than those recorded for uninfected mice.

In all cases, recombinant human IL-1 caused high levels of thymocyte stimulation.

14.3.5 IFN- γ assay results

Murine IFN- γ was assayed using a commercial ELISA kit. Samples of serum from scid mice in all experimental groups at various times during the experiment were tested, as were peritoneal cell supernatants and tumour homogenates. Only two samples gave positive results: peritoneal cell supernatants from a TaHi(s/c) mouse killed on day 8, and from TaLo(i/p) mice killed on day 15. These samples contained 13.5 and 3.8 units of IFN- γ /ml by comparison with the standards provided with the kit.

14.4 Discussion

These experiments were intended to look for evidence of macrophage and NK cell activity in scid mice injected with *Theileria*-infected cells. An increase in the total macrophage count was seen in all mice in which schizont- infected cells were found in the peritoneal site. In mice which developed ascites, the number of infected cells was greater than the number of macrophages; but in mice which received the low dose i/p, or had s/c tumours, or were injected with the slower growing TpMug110 cell line, quite large increases in macrophage number were associated with relatively small numbers of infected cells in the peritoneal exudate.

In TaHis249 mice, which had vigorously growing subcutaneous or ascitic tumours, an increase in the number of spleen cells was noted. In most of these cases, much of this increase was due to the proliferation of lymphocyte type cells. However,

the cell type which increased most radically in number was the neutrophil. The highest total neutrophil counts were seen in those mice which had the highest spleen counts, and the largest tumours: TaHi(s/c) day 12 and 16. A high spleen neutrophil count was seen in the TaHi(i/p) mice killed on day 9: 1×10^7 cells out of a total of 2.2×10^7 . Mice injected with TpMug110 also showed increases in total spleen cell counts, with associated neutrophilia in the spleen: this was most apparent in mice which were injected s/c, or which received a high dose of cells i/p.

Do these increases in macrophage and neutrophil counts indicate an effective immune response? The macrophages in the peritoneal exudate were observed to increase in number in the presence of infected cells. Spleen macrophages did not show such increases in number, and there was some tendency to a decrease in number. This might indicate some recruitment to the peritoneal site: the proliferation of macrophages in the peritoneal cavity must also involve recruitment from the circulation, and possibly the generation of new macrophages. An increase in the neutrophil count in the peritoneal exudate was observed in TaHi(i/p) mice killed on day 9, which also showed neutrophil proliferation in the spleen. However, the highest levels of neutrophil proliferation were seen in the spleens of mice bearing large s/c TaHis249 tumours although few neutrophils were found to be infiltrating the tumours of these mice.

High levels of NK cell activity (as *in vitro* cytotoxicity of YAC-1 cells) were found in the spleens of TaHis249 mice. This activity was greatest in mice bearing large, subcutaneous tumours, more than two weeks after injection. Mice injected i/p also showed cytotoxicity against YAC-1 cells, which was highest in TaLo(i/p) mice killed on days 15 and 19. Mice with ascites did not show particularly high levels of NK cell activity. Low levels of cytotoxicity were recorded for spleen cells of TpMug110 mice.

The high levels of *in vitro* NK cell activity did not therefore correlate with the suppression of tumour growth or the actual regression of tumours, but rather with the presence of a large, subcutaneous tumour burden (8-12mm diameter in the TaHis249 mice). However, in mice injected with TaHis249 i/p, more cytotoxicity was seen in spleen cells from low dose mice, which were controlling the growth of cells. The ALC experiment described in the previous chapter did not indicate NK cell activity *in vivo* against injected bovine cells, and scid spleen cells did not show lysis of TaHis249 and TpMug110 cells *in vitro*. Taken together, these results make it unlikely that NK cell cytotoxicity is a restraint on the growth of *Theileria* infected cells *in vivo*

in scid mice.

Tumour Necrosis Factor (TNF) was not detected in samples from any of the infected scid mice using the L-929 assay. This is consistent with the earlier finding that TNF was not detectable in samples from Balb/c 4Gy and nude 4Gy mice bearing *Theileria* tumours. TNF might still however be acting, but at an extremely local level: membrane bound forms of TNF which are cytotoxic for tumour cells have been described by other workers (Decker, Lohmann-Mathes and Gifford, 1987).

Supernatants of peritoneal cell cultures from scid mice injected with TaHis249 or TpMug110 showed significant thymocyte stimulatory activity. This activity could be due to IL-2 as well as IL-1, but the presence of large numbers of macrophages in the peritoneal cell cultures makes it much more likely to be IL-1 activity which is being measured. The development of IL-1 activity in TaHis249 mice seemed to be time and dose-dependent: very high levels of activity were detected in some cases (>50U/ml by comparison with rHuIL-1 standard). Significant levels of activity were also detected in samples from TpMug110 mice killed on day 29. IL-1 activity was not detected in samples from mice with ascites: this is probably because peritoneal macrophages were overgrown by TaHis249 cells. It was not possible to separate TaHis249 cells from the macrophages as they are semi-adherent: over the 70 hours of the assay, even a small number of contaminating TaHis249 cells would multiply rapidly. IL-1 activity was not found in any of the serum samples. IL-1 was not, therefore, being produced systemically. Its production by the peritoneal macrophages may reflect an increase in macrophage activity mediated by some other factor.

Murine IFN- γ was not detected in serum samples from any mouse. IFN- γ was detected in two samples of peritoneal cell supernatants, but was not consistently present. These results could be accidental, with IFN- γ being produced in the cultures for some reason unconnected with the experiment, or it could be the case that IFN- γ release is transient and localised, making it difficult to detect. IFN- γ may be removed by filtration, which was done to sterilise peritoneal cell supernatants; however, none of the serum samples were filtered. As IFN- γ is produced by NK cells but not by macrophages (Munakata et al, 1985), some NK cells may have been present in these peritoneal cell cultures. Comparison of differential counts with the IFN- γ results did not show any consistent change in the cell population which might reflect the presence of NK cells. No infected cells were detected in these differential counts.

Taken together, the data from differential counts, *in vitro* NK cell assays and

cytokine assays do not provide evidence for a role for NK cells in the control of the growth of *Theileria* infected cells. However, mice with large subcutaneous tumours showed less general dissemination of infected cells to other sites than mice with ascites: this might be connected with the high levels of NK cell activity observed in scid mice with subcutaneous tumours. The data suggest that macrophages were more important than NK cells in the control of the tumours, particularly when cells were given i/p: the appearance of small numbers of infected cells in the peritoneal exudate was accompanied by an increase in the macrophage count. Macrophages have been implicated in the control of both subcutaneous and intraperitoneal tumours (Russell, Doe and Cochrane, 1976; Russell and McIntosh, 1977; Suzuki et al, 1987). Neutrophils are also candidates as effector cells: the numbers of neutrophils were drastically increased in the spleens of mice with tumours and ascites; infiltration of the ascites by neutrophils was noted, but no similar infiltration of subcutaneous tumours by neutrophils was observed. Neutrophils are normally regarded as being associated with inflammation and bacterial infections: their occurrence in scid mice injected with *Theileria*-infected cells may therefore reflect an inflammatory response to the growth of the infected cells.

CHAPTER 15

EXPERIMENTS TO INVESTIGATE THE ROLE OF INTERFERON IN THE PROGRESSION AND CONTROL OF ASCITIC *THEILERIA* TUMOURS IN SCID MICE

15.1 Introduction

The experiments in the previous chapter indicated a role for macrophages in the control of the growth of *Theileria* infected cells in scid mice, particularly in the peritoneal site. Interferon gamma (IFN- γ) is known to be a potent mediator of macrophage activation (Stegg, Johnson and Oppenheim, 1982; Wong et al, 1983), and has been shown to be involved in the control of peritoneal tumours in tumour-dormant mice (Suzuki et al, 1987). As discussed in the "General Introduction", IFN- γ has also been implicated in the activation of macrophages in scid mice by a T-cell independent mechanism (Bancroft et al, 1987).

IFN- α and IFN- γ have been shown to be produced by some *T.annulata* and *T.parva* infected bovine cell lines *in vitro* (Entrican et al, manuscript in preparation). Human IFN- α and HuIFN- γ are both capable of inhibiting the development of *T.annulata* trophozoites into macroschizonts in bovine cells infected *in vitro* (Preston, unpublished data). These cytokines have no consistent effect on mature *T.annulata* and *T.parva* infected cell lines (in vitro) (Preston, unpublished data). There is therefore considerable scope for interactions between the murine host and the infected bovine cells mediated by interferons, assuming that there is some degree of cross-reaction between the species.

The finding of villus atrophy in the gut of a scid mouse with a large mesenteric TpMug110 tumour (chapter 10, plate 12) also suggested the action of IFN- γ , either of mouse origin as a result of some immune response to the tumour, or of bovine origin from the tumour cells themselves. Endogenous IFN- γ released during graft versus host reactions is known to mediate this type of intestinal pathology (Mowat, 1989).

The experiments described below were carried out to examine: (i) the effect of

HuIFN- α and HuIFN- γ on *T.annulata* infected cells in an *in vivo* situation; (ii) the role of IFN- γ in the response of the scid mice to the *Theileria*-infected cells.

In the first experiment, mice were given a high dose of TaHis250 cells i/p and then dosed with HuIFN- γ or HuIFN- α at regular intervals, to see if there was any effect on the development of ascites. The human interferons show some cross-reactivity with bovine cells *in vitro* by inhibiting the development of macroschizont-infected cells.

In the second experiment, scid mice injected i/p with *T.annulata* or *T.parva*-infected cell lines were treated with the IFN- γ neutralising McAb R46A2. It was thought that if endogenous IFN- γ was a factor in causing the inflammatory, haemorrhagic reaction to the presence of ascites, then administration of the neutralising antibody to mice which were expected to develop ascites might modify the pathological effects. Secondly, it appeared from the results of previous experiments that peritoneal macrophages were responsible for controlling the growth of low doses of *T.annulata* infected cells, and of high doses of *T.parva* infected cells in the peritoneal cavity. It was therefore postulated that the IFN- γ neutralising antibody might prevent macrophage activation and allow a "breakthrough" in the growth of the infected cells.

15.2 Methods

15.2.1 IFN- γ , IFN- α versus *T.annulata* infected cells i/p

Eight mice were injected i/p with 2×10^7 TaHis250 cells. Four of these mice were then treated with 1×10^4 U/dose of HuIFN- γ on days 1,3,5 and 7; four were treated with the same dose of HuIFN- α on the same days. The mice were killed on days 8 and 9: peritoneal exudates were prepared, and dab smears were made from the inguinal lymph node, liver, spleen, pancreas, small intestine, mesentery, kidney and lung. Smears were also made of mucosal scrapings from the small intestine. A further two uninfected mice were injected with the same dose of each cytokine, and killed on day 8.

15.2.2 Treatment of mice with R46A2

Four scid mice were injected with 2×10^7 TaHis249 cells i/p; four with 2×10^7 TpMug110 cells i/p; and eleven with 2×10^6 TaHis249 cells i/p.

The monoclonal antibody R46A2, which neutralises murine IFN- γ activity (Havell, 1986) was prepared from culture supernatants as described in "Materials and Methods", section 2.6. The purified antibodies were diluted in PBS to 20000 NU/ml. Mice received a dose of 0.2ml of diluted antibody (approximately 4000 NU) or 0.2ml PBS i/p. In the TaHi and TpHi groups, two mice were treated with R46A2 and two with PBS; in the TaLo group, six mice received R46A2 and five PBS. The TaHi mice received antibodies or PBS daily; the other groups were injected with antibodies or PBS on alternate days (1,3,5,7 etc). When they were killed, peritoneal exudates were made and the total and differential count was determined. Pieces of the gut and mesenteries were formalin-fixed for histology. Any general changes in the condition of the mice were noted. Serum samples from some mice were tested for the presence of murine IFN- γ using the ELISA test previously described (Chapter 14).

15.3 Results

15.3.1 1. Treatment of mice with IFN- γ and IFN- α

The mice all developed ascites, as described in chapter 14. They were killed on days 8 and 9 post-infection. The ascitic fluid was turbid and bloody, there was inflammation of the gut, and development of mesenteric tumours and haemorrhages. Dab smears showed extensive infiltration of other organs and tissues by infected cells. There was no apparent difference in the development of ascites, or the degree of infiltration of infected cells into the tissues, between the IFN treated and untreated scid mice: the distribution of infected cells was the same as that seen in the experiments described in chapter 10.

No ill-effects were seen as a result of treatment with HuIFN- γ or - α alone. Total and differential counts from the peritoneal exudate and spleen cell suspensions did not show any significant changes as a result of cytokine treatment.

15.3.2 Treatment of mice with R46A2 antibody

All 5 mice given the high dose (2×10^7) of TaHis249 cells developed ascites, whether or not they were treated with R46A2. The total peritoneal cell counts were all $>1 \times 10^8$ cells; differential counts showed that most of these cells were schizont-infected (table 16).

In contrast, in mice given the same dose of TpMug110 cells, there was a clear difference between the control mice and those treated with the R46A2 antibody. Control mice were not ascitic and had low counts of peritoneal exudate cells (table 16). No schizont-infected cells were detected in the peritoneal exudate from one of these mice; this result is consistent with earlier findings (chapter 10). However, in both mice treated with the R46A2 antibody, a mild ascites developed by day 35: the mice were not noticeably swollen, but the peritoneal exudate was turbid; there was no evidence of haemorrhage into the peritoneal cavity. Peritoneal cell counts were high; in both cases 90% of these cells were schizont-infected. Infected cells were also seen in dab smears of the mesentery.

As the high dose of TaHis249 was not affected by treatment with R46A2, an experiment was conducted to investigate the effect of anti-IFN- γ treatment on a low dose of the TaHis249 cell line. Two out of the six mice given a low dose (2×10^6) of TaHis249 cells and treated with R46A2 developed ascites by day 15: the ascitic fluid was turbid and bloody, and inflammation and haemorrhages of the gut and mesenteries were observed. Due to accidental puncture of the peritoneum, a peritoneal cell suspension could only be prepared from one of these mice. A small number of infected cells were detected in the peritoneal cavity of one other mouse in the experimental group, but this mouse had not developed ascites (table 17).

None of the control mice, which received a low dose of TaHis249 cells and injections of PBS, developed ascites. A small number of infected cells were found in the peritoneal cell suspension of one mouse (table 17).

No circulating IFN- γ was detected in the serum of control or antibody- treated mice.

Table 16. Total and differential counts from scid mice given high doses of TaHis249 or TpMug110 cells i/p and treated with R46A2.

Mouse	Total	Inf. cells	Macros.	Lymphos.	Neutros.	Basos.
TaHis249						
Ctrl1	3700	3000	590	0	110	0
Ctrl2	4200	4000	130	0	42	0
R4 1	1400	780	620	0	0	0
R4 2	3000	2400	480	0	60	0
R4 3	3900	3700	160	0	78	0
TpMug110						
Ctrl1	14	0	4.5	0	9.2	0.3
Ctrl2	21*					
R4 1	1000	920	60	20	10	0
R4 2	1200	1100	24	36	12	0

All counts are $\times 10^5$ to 2 significant figures. *: no differential count obtained. Mice received 2×10^7 TaHis249 or TpMug110 cells i/p. R46A2 or PBS was given i/p every day to TaHis249 mice, and every other day to TpMug110 mice. TaHis249 mice were killed 9 days after inoculation of cells; TpMug110 mice 35 days after inoculation of cells. Abbreviations: Inf. cells, schizont infected cells; macros, peritoneal macrophages; lymphos, lymphocytes; neutros, neutrophilic polymorphonuclear cells; basos, basophilic granulocytes; ctrl, mice treated with injections of PBS; R4, mice treated with injections of R46A2.

Table 17. Total and differential counts from the peritoneal exudates of scid mice injected with a low dose of TaHis249 cells and treated with R46A2

Mouse	Total	Inf. cells	Macros.	Lymphos.	Neutros.	Basos.
<u>Controls</u>						
1	13	0	7.7	3.8	0.9	0.6
2	18	0	8.6	8.6	0.4	0.4
3	17	0	10	3.2	0.9	2.7
4	11	0.4	7	2.5	1.0	0.1
5 (No cell suspension made: no ascites or signs of inflammation)						
<u>R46A2 treated</u>						
1	580	350	220	0	5.7	0
2 (Ascites, containing inf. cells, but no total count)						
3	14	0.1	11	0.8	1.8	0.4
4	8.1	0	7	0.2	0.5	0.5
5	10	0	8.7	0	0.9	0.4
6	11	0	8.3	0.3	2.1	0.3

All mice received 2×10^6 cells i/p and PBS or R46A2 on alternate days. All mice were killed on day 15.

15.3.3 Histology

Sections of gut from scid mice injected with TaHis249 or TpMug110 cells and treated with PBS or R46A2, did not show any differences between antibody treated and control mice. No evidence of villus atrophy was seen in either group.

15.4 Discussion

Human IFN- α and IFN- γ had no apparent effect on the growth of *T.annulata* infected bovine cells *in vivo*. This might have been predicted from the results of *in vitro* studies, although these were not complete at the time the experiment was performed. In a preliminary experiment, culture supernatants containing recombinant MuIFN- γ secreted by a Chinese Hamster Ovary cell line transfected with the murine IFN- γ gene had no effect on the proliferation of TaHis249 or TpMug110 cells *in vitro*.

Treatment with IFN- γ neutralising antibody had no effect on the rapid progression of ascitic tumours in scid mice injected with 2×10^7 *T.annulata* infected cells i/p. There was therefore no evidence for a major role for endogenous IFN- γ in the development of the inflammatory ascites.

Neutralisation of endogenous IFN- γ allowed a "breakthrough" multiplication of *T.annulata* infected cells in 2/6 mice, and of *T.parva* infected cells in 2/2 mice. None of the control mice showed a similar proliferation of infected cells, and no such proliferation had been observed in any previous experiment with these cell doses, involving a further 25 mice injected with a low dose of *T.annulata*-infected cells and 9 injected with a high dose of *T.parva*-infected cells.

The proliferation of infected cells appeared to be very much an "all or nothing" phenomenon: mice either had very high counts of infected cells, or they had virtually none. This may indicate a very fine balance between control of the *Theileria* infected cells by peritoneal macrophages, and uncontrolled multiplication of the infected cells.

In mice injected with TpMug110 which developed mild ascites as a result of R46A2 treatment, there appeared to be fewer macrophages relative to the number of infected cells than in TaHis249 mice which developed ascites. Since the significance

of this result could not be tested with such a small group of mice, the hypothesis that the proliferation of infected cells was due to the suppression of macrophage activation could not be directly tested.

An alternative possibility is that the antibody stimulated the growth of *Theileria*-infected bovine cells directly. This seems unlikely, for three reasons: there is no obvious mechanism whereby a rat immunoglobulin specific for a mouse cytokine would cross-react with bovine cells to stimulate increased proliferation; *Theileria*-infected cells are already transformed and multiply at a high rate, so it is difficult to see how the antibody could have an additional effect; if the antibody was stimulating the *Theileria*-infected cells to multiply more rapidly, then an earlier onset of ascites might be expected in scid mice given the high dose of *T.annulata*-infected cells i/p, and this was not observed.

Histological studies did not reveal any signs of villus atrophy, crypt hyperplasia or other pathological changes associated with the local release of IFN- γ . The previous observation of villus atrophy was made in sections of small intestine immediately adjacent to a large, solid mesenteric tumour: this mouse was killed 52 days after inoculation. These results suggest that villus atrophy only occurs in the presence of a chronic tumour.

The specific neutralisation of endogenous murine IFN- γ clearly affected the ability of the scid mice to control the multiplication of *T.annulata* and *T.parva* infected cells in the intraperitoneal site, although not all antibody treated mice developed ascites. Direct evidence that this effect was due to an inhibition of macrophage activation was not obtained. However, as it is known that macrophages in scid mice are activated to control *Listeria monocytogenes* infections by IFN- γ released by NK cells (Bancroft et al, 1987), and the results described in previous chapters implicated macrophages in the control of *Theileria* infected cells in the peritoneal cavity (chapters 12 and 13), it seems likely that the neutralising antibody releases the brake on infected cell proliferation exerted by macrophage activation.

CHAPTER 16

GENERAL DISCUSSION

As stated in the "General Introduction", the main aim of the work described in this thesis was to investigate the growth of *Theileria*-infected bovine cells in mice, with the ultimate objective of developing a system for studying *Theileria*-infected cells *in vivo*, without using cattle. The growth of *Theileria*-infected cells in immunodeficient mice cannot strictly speaking be described as a "murine model" for bovine theileriosis. A true murine model would be a *Theileria* sp. infection of rodents of sufficient similarity to bovine theileriosis to make possible predictions about the behaviour of the bovine parasite from that of the murine parasite. However, the growth of *Theileria*-infected cells as tumours in immunodeficient mice might reflect some aspects of their growth in the bovine host more accurately than *in vitro* culture.

Experiments using mice with increasing degrees of immunodeficiency led to investigations of the recovery of specific immune mechanisms in irradiated mice, and into mechanisms of natural immunity in scid mice. Some attempts were also made to carry out further investigations, by treating tumour-bearing mice with serum containing TNF activity, and with other cytokines.

The results of these experiments led to conclusions concerning: (i) the interaction of tumour and host in terms of immunodeficiency; (ii) the immune responses to xenogeneic tumours in a variety of immunodeficient mice; (iii) the comparative behaviour of *T.annulata* and *T.parva*-infected bovine cells *in vivo*; (iv) the routes by which intraperitoneally administered cells disseminate to other sites; (v) the relevance of mouse models for theileriosis, and the prospects for the development of scid/bovine chimaeras; (vi) the possible use of mouse models to carry out *in vivo* experiments to investigate the role of cytokines in theileriosis.

The "discussion" sections at the end of each chapter summarise and discuss the results presented in that chapter. This section of the thesis will be confined therefore to a general recapitulation of the results, and an attempt to relate the results from various mouse strains to each other.

16.1 Relationship between immunodeficiency and the growth of subcutaneous tumours

The mouse strains used in these experiments represent a spectrum of immunodeficiency. Balb/c mice are immunologically intact, but for most of the experiments described in this thesis received a temporary, radiation-induced lesion of the immune system. Nude mice lack T-cell responses and therefore also have impaired B-cell responses (Wortis, 1971): they do have normal NK cell and macrophage activity and it has been suggested that NK cell activity in nude mice is actually enhanced compared to normal mice (Kiessling et al, 1975). Scid mice lack B and T-cells, but have normal macrophages and NK cells (Bosma, Schuler and Bosma, 1988). "Leakiness" of the scid mouse phenotype, leading to the development of some B and T-cell clones and resulting in the rejection of allografts, has been reported (Bosma et al, 1988) but did not appear to be a problem in the scid mice used here (chapter 13).

It was expected that the more immunodeficient the murine host, the more permissive it would be for the growth of *Theileria*-infected cells. In general, this was confirmed by the experiments described in earlier chapters. In Balb/c mice, *T.annulata* tumours grew to a larger size and persisted for longer in mice irradiated at 6Gy than in those irradiated at 4Gy, and in unirradiated Balb/c mice tumours did not establish at all. In Balb/c nude mice irradiated at 4Gy, tumours grew faster and to a greater size, and showed no signs of regression despite the development of haemorrhage and necrosis. *T.parva* infected cells also grew much more readily in Balb/c nude 4Gy mice than in Balb/c 4Gy mice. In scid mice injected subcutaneously, *T.annulata*- infected cells again grew more vigorously than *T.parva*-infected cells. There was more dissemination of infected cells from the tumour site in scid mice than in irradiated Balb/c nude mice.

T.annulata-infected cells did not establish as subcutaneous tumours, or in the peritoneal cavity, in C-57 beige mice. It was expected that the beige NK cell defect, combined with a 4Gy radiation dose, would allow more rapid growth of infected cells than that seen in Balb/c 4Gy mice. In fact, the beige mice appeared to be quite resistant to the growth of *Theileria*- infected cells as subcutaneous tumours or ascites. Unfortunately, no studies on immune function in beige mice were carried out to determine if there was some compensatory factor which made them more resistant than the Balb/c mice.

The relationship between immunodeficiency and the establishment and growth of *Theileria*-infected cells was also seen in the intraperitoneal site. *T.annulata* infected cells survived for about a week when injected i/p into Balb/c 4Gy mice, and no ascites developed. In scid mice, the same dose of *T.annulata* infected cells multiplied rapidly, forming an ascites in 8-10 days. The mice were clearly unable to control the growth of this number of cells, although a 10-fold smaller dose could be suppressed. No experiments were carried out with *Theileria*-infected cells injected i/p into nude mice, as at the time nude mice were being used it was felt desirable to concentrate on the growth of cells in the subcutaneous site: experiments by other workers suggested that the intraperitoneal site in nude mice would not be suitable for the growth of *Theileria*-infected cells (Irvin et al, 1977).

No evidence was found for the transfer of *T.annulata* macroschizonts to mouse cells in any of the mouse strains in which *Theileria*-infected cells established successfully. Horizontal transfer of parasites is believed to occur in cattle infected with *T.annulata*, as allogeneic cell lines can be used to establish infections in cattle and to immunise cattle against subsequent challenge, but has not been demonstrated directly.

Overall, the results obtained were consistent with previous work by other authors using *T.parva*-infected cell lines (Irvin, 1975b; Irvin et al, 1972, 1975c, 1975d, 1977). These experiments showed increased survival and growth of tumours in increasingly immunodeficient mouse strains (irradiated Swiss and nude mice), and that tumour growth was dependent on the size of the cell dose and the degree of immunosuppression. No transfer of macroschizonts from bovine to mouse cells was recorded in these experiments, although transformation to microschizonts did occur, and some infection of mouse erythrocytes by piroplasms was reported (Irvin, 1975b). Development to microschizonts and erythrocyte invasion were not observed in any of the experiments included in this thesis.

16.2 Immune responses to *Theileria* tumours

Subcutaneous tumours generated by the injection of *T.annulata*-infected cells developed general haemorrhage and central necrosis in all the immunodeficient mouse strains tested, although the survival of the tumours differed markedly between irradiated Balb/c mice, irradiated nude mice and scid mice. This suggested that this

necrosis of the tumours was caused by the same underlying mechanism in all these mouse strains, but that it did not lead to tumour regression in the absence of other immune mechanisms. The experiments described in chapters 7,12 and 14 allowed some dissection of the immune response of the immunodeficient mice to subcutaneous *T.annulata* tumours.

T.annulata- infected cells were only able to establish in mice which were immunodeficient at the time of inoculation. A radiation dose of 4Gy was sufficient to allow growth of cells in Balb/c mice, although rejection eventually occurred: the same dose in Balb/c nude mice led to unlimited growth of the tumours. The experiments described in chapter 7 showed that 4Gy caused severe depletion of B and T-lymphocytes in Balb/c mice, although there was some difference in the sensitivity of the major lymphocyte subsets (sIgG+, Ly-2+, L3T4+). The numbers of these lymphocytes recovered more rapidly in irradiated mice bearing subcutaneous tumours than in mice which received radiation only. There was no obvious proliferation of one lymphocyte subset in contrast to the others: therefore this enhanced recovery of lymphocytes probably represents the generation of a mixture of humoral, cytotoxic and helper responses to the tumour. The recovery of T and B cell numbers was accompanied by the destruction of tumours.

Balb/c nude mice irradiated at 4Gy, and scid mice, were assumed to be deficient in T and B-cells: scid mice were agammaglobulinaemic. These mice were therefore unable to mount a specific, T and B-cell mediated antitumour response, and so were unable to reject the tumours despite the development of haemorrhage and central necrosis.

Irradiated Balb/c mice bearing *T.annulata* tumours showed increased NK cell activity *in vitro*. There was no evidence to suggest that NK cell cytotoxicity played a major role in the destruction of tumours: beige mice were resistant to tumour growth, and scid mice bearing subcutaneous *T.annulata* tumours, although displaying high levels of NK cell cytotoxicity *in vitro*, were clearly unable to control tumour growth. These results suggest that the NK cell activity detected *in vitro* was a response to the presence of a chronic tumour load in scid mice.

While NK cells may not have been responsible for the control of the growth of *Theileria* tumours, they may have restrained the dissemination of infected cells from the tumour site. Scid mice with a large subcutaneous tumour burden and high levels of NK cell cytotoxicity *in vitro* showed less dissemination of schizont-infected cells

to other tissues than mice with ascites, which had comparatively low levels of NK activity *in vitro*. This may also have been because the peritoneal cavity was a more favourable site for access to other tissues.

Sections of resolving subcutaneous tumours in Balb/c 4Gy mice clearly showed infiltrating mononuclear cells. This infiltrate was composed of macrophages and neutrophils, with the latter being seen particularly in the later stages of rejection. Large, macrophage-type cells were seen lining and obstructing tumour blood vessels in Balb/c 4Gy mice as early as 4 days after inoculation. FACS data also showed large numbers of large, macrophage-type and granular cells in the spleens of Balb/c 4Gy mice when tumours began to regress. Macrophages have been shown to play a major role in the regression of Moloney sarcomas in mice (Russell, Doe and Cochrane, 1976; Russell and McIntosh, 1977). Sections and smears of tumours in Balb/c nude 4Gy and scid mice did not show extensive infiltration of macrophages, although there was a considerable increase in the number of these cells in the spleen.

The presence of neutrophils was probably a consequence of tumour destruction rather than a cause of it: as death of tumour tissue occurred, neutrophils would be recruited into the site to phagocytose dead material. However, neutrophils are also recognised as mediators of inflammation and endothelial cell injury, and might therefore be involved in the development of haemorrhage in the tumours. The infiltration of neutrophils into tumours prior to regression has been noted previously (Russell and Cochrane, 1974).

The main requirement for the establishment and growth of *T.annulata* infected bovine cells as subcutaneous tumours in Balb/c mice was therefore the temporary or permanent ablation of B and T-cell responses. The recovery of these responses accompanied the destruction of the tumour. "Natural" immune mechanisms (ie macrophages and NK cells) were apparently able to restrain the dissemination of infected cells from the tumour site, and were probably involved in initiating the haemorrhage and necrosis of the centre of the tumours. Activation of these natural effectors must have been occurring through T-cell independent pathways in irradiated nude and scid mice (Bancroft et al, 1986: see "General Introduction", section 2.5). In irradiated Balb/c mice, the L3T4+ lymphocyte subset was the least affected by irradiation, so macrophage and NK cell activation may have occurred through the conventional T-cell dependent pathway.

The haemorrhagic reaction did not lead to regression of tumours unless some B

and T-cells were present. There was an apparent equilibrium between growth and rejection of the tumour, influenced by the size and rate of growth of the tumour on one hand, and the immune response to it on the other. In irradiated Balb/c mice, the balance was tipped towards regression as the B and T-cell populations recovered from irradiation. In nude mice, the balance was inclined towards survival of the tumour, although the outbred NIMR nude 4Gy mice were able to reject tumours even after they had grown to considerable size. These mice were apparently able to damage the tumour sufficiently through natural mechanisms to allow rejection. Scid mice, lacking all B and T-cell responses, were unable to limit the growth of subcutaneous tumours.

Theileria-infected cells were only successfully established in the peritoneal cavity in scid mice. In Balb/c 4Gy mice injected with *T.annulata*-infected cells i/p, only short term survival of cells was seen. Scid mice injected with *T.annulata*-infected cells i/p showed relatively low levels of NK cell activity *in vitro*, and no significant allogeneic lymphocyte cytotoxicity (ALC), of the type indicative of NK cell activity, was detected against bovine spleen cells. In contrast, there was good evidence for the importance of peritoneal macrophages in controlling the proliferation of cells in this site. In scid mice, the presence of quite small numbers of infected cells in the peritoneal cavity, either by inoculation i/p or by dissemination from a subcutaneous tumour, was accompanied by a proliferation of peritoneal macrophages. Although cytotoxic or cytostatic activity was not detected directly, these macrophages were assumed to be in an activated state because they produced high levels of IL-1 activity when cultured *in vitro*. The ALC experiment described in chapter 12 also provided evidence that scid peritoneal macrophages were capable of clearing *T.annulata* infected cells from the peritoneal cavity.

As discussed in the "General Introduction", both specific (T-cell mediated) and non-specific (NK cell and macrophage mediated) cytotoxic responses are generated in cattle infected with *T.annulata* or *T.parva*, and in *in vitro* cultures of bovine cells and infected cells (Emery et al, 1981; Pearson et al, 1982; Emery and Kar, 1983; Preston, Brown and Spooner, 1983; Preston and Brown, 1988). Experiments in scid mice show that *Theileria*- infected cells are also controlled by murine non-specific cellular mechanisms. Peritoneal macrophages appeared to be particularly effective against cells in the peritoneal cavity, but high levels of NK cell activity *in vitro* did not correlate with tumour regression in scid mice. Spleen cells from uninfected scid mice did not show cytotoxic activity against *Theileria*-infected cell lines *in vitro*, although they did show anti-YAC-1 activity. *Theileria*- infected cells therefore seem

to be more susceptible to macrophage than NK cell activity in the scid mouse. It is possible that this differential susceptibility may also occur in infected cattle: while macrophages from immune cattle do show anti-theilerial activity *in vitro* (Preston and Brown, 1988), NK-enriched bovine cells have not been tested for anti-theilerial activity.

16.3 Differences in growth *in vivo* between *T.annulata* and *T.parva* infected bovine cells

Experiments in all strains of mice showed that *T.annulata* infected cells established more readily, grew more rapidly and disseminated more aggressively from the tumour site than *T.parva* infected cells. As subcutaneous tumours, *T.annulata* infected cells formed prominent lumps, with marked reddening and the development of central necrosis: *T.parva* infected cells formed white tumours which appeared to grow laterally through the subcutaneous tissue rather than piling up into a solid mass. Histologically, *T.annulata* tumours seemed to be more vascularised than *T.parva* tumours. In Balb/c nude 4Gy and scid mice, *T.annulata* tumours reached thicknesses of almost 10mm, which could not have been possible without an adequate blood supply to the tumour centre. These tumours did of course develop central necrosis on the outer surface, but this was not generally observed to extend very deep into the tumour.

T.parva tumours did not become haemorrhagic, possibly because of inadequate vascularisation. This lack of blood supply would also have limited the growth of the tumours by reducing the availability of oxygen and nutrients.

Establishment and growth of *Theileria*-infected cells in the intraperitoneal site was only achieved in scid mice. 2×10^7 *T.annulata* infected cells caused the development of ascites, with very large numbers of infected cells in suspension in the peritoneal exudate, and forming tumours on the mesentery. The same number of *T.parva* infected cells resulted in only slow growth of infected cells, and the occasional formation of mesenteric tumours.

The results obtained with irradiated Balb/c and nude mice were consistent with those obtained by Irvin et al using *T.parva*-infected cells in irradiated Swiss and nude mice (Irvin, 1975b; Irvin et al, 1972, 1975c and 1977). In these experiments mice received higher doses of irradiation (8-9Gy) and also received higher cell doses (up to

5x10⁷ cells), than in the experiments described in this thesis, resulting in a more rapid growth of subcutaneous tumours in nude mice. Tumours regressed in irradiated Swiss mice, but growth was progressive in irradiated nude mice. Subcutaneous *T.parva* tumours were described as being moderately vascularised. No widespread haemorrhage followed by central necrosis was reported, although some surface ulceration of the tumours was noted.

The poor growth of *T.parva* infected cells, relative to *T.annulata* infected cells, in immunodeficient mice could have been due to several factors: (i) *T.parva* infected cells grow more slowly *in vitro* than *T.annulata* infected cells; (ii) they are more sensitive to their environment, eg nutrients, cell density etcetera; (iii) they are more sensitive to natural immune mechanisms due to their slow growth rate. This latter point is supported by the result of the experiment in chapter 15 (section 3), where neutralisation of endogenous IFN- γ allowed proliferation of *T.parva* infected cells in the peritoneal cavity of scid mice. Therefore immunological responses were of more importance in limiting the growth of *T.parva*-infected cells than physiological incompatibilities.

In the bovine host, *T.parva* infections generally have a higher mortality than *T.annulata* infections, and tend to follow a more acute course: cattle which succumb to infection generally do so in the early stages of infection, when macroschizont-infected cells are multiplying throughout the body. In *T.annulata* infections, animals are more likely to die during the erythrocytic phase, with acute anaemia and cachexia (Neitz, 1957). There is therefore no obvious parallel between the growth of *T.parva* infected cells in the bovine and in the immunodeficient mouse. *T.annulata* and *T.parva* are thought to infect different subsets of bovine leukocytes (Spooner et al, 1988). It is unclear whether this accounts for the differences in pathology between tropical theileriosis and East Coast fever in cattle. In immunodeficient mice, the differences in establishment, rejection and general behaviour between the two parasite species can be explained on the basis of growth rate alone.

16.4 Dissemination of *Theileria* infected cells from the inoculation site

The degree of dissemination of infected cells to other tissues was greater in more

immunodeficient mice: none was observed in Balb/c 4Gy mice; occasional infected cells were found in sites such as the lung and kidney in NIMR and Balb/c nude 4Gy mice, while quite large numbers of infected cells were found in secondary sites in scid mice. If it is assumed that infected cells are constantly shed from the tumour site, then the more immunodeficient the mouse, the less capable it would be of destroying individual "metastatic" cells. In addition, the larger size and longer duration of tumours in more immunodeficient mice would provide a greater pool of cells available to spread to other sites. *T.annulata* tumours showed similar patterns of growth in Balb/c nude 4Gy and scid mice: the potential for the spread of infected cells would therefore be about the same in both mouse strains, but far more dissemination from the subcutaneous site was seen in scid mice than in the irradiated nude mouse. This may be because of enhanced NK cell activity in the nude mouse, as mentioned above, although scid mice with subcutaneous tumours also showed high levels of *in vitro* NK cytotoxicity.

The question of how *Theileria* infected cells disseminate from the tumour site in scid mice has already been addressed in the discussion section of chapter 10. Some further points will be raised here concerning the route of migration of infected cells from the peritoneal cavity, and the apparent tropism of cells to the gut, even from the subcutaneous site.

The most obvious route for the dissemination of *T.annulata* infected cells from the peritoneal cavity would be through the diffuse lymphatic drainage of the cavity, into the thoracic duct and thus into the circulation. It might then be expected that infected cells would be found in the lungs, as this would be the next major capillary bed to be encountered. Large numbers of infected cells were seen in the lungs of scid mice with subcutaneous tumours, but in mice with ascites, large numbers were found in the mesenteries, liver, kidney, small intestine, pancreas and spleen with relatively small proportions being seen in the lungs. Other mechanisms must therefore be invoked to explain the dispersal of infected cells from the intraperitoneal site: these are described below.

(i) The mesenteries present the largest surface area to the peritoneal fluid, and therefore constitute a natural attachment site for the semi- adherent *T.annulata* infected cells. Settlement of infected cells on the mesenteries lead to the formation of tumours infiltrating the fatty tissue of the mesentery and perhaps gaining access to blood vessels and lymphatics in the mesentery.

(ii) Infected cells could reach the liver through the general circulation: the liver receives approximately 28% of the cardiac output in a resting adult human (Ganong, 1977), and it can be assumed that the a similar proportion would reach the liver in a mouse. Cells could also enter the portal circulation from mesenteric tumours and so reach the liver more directly. In radiolabelling experiments, some of the activity recovered from the liver represented infected cells killed by natural mechanisms. Direct penetration of the liver capsule by infected cells from the peritoneal exudate seems unlikely, but cannot be ruled out if cells formed a tumour on the liver surface.

(iii). Direct penetration of the kidney is unlikely due to the capsule, but like the liver the kidneys receive a large percentage of cardiac output (23% in a human): in addition, the kidney is a relatively smaller organ than the liver, so a smaller number of invading cells would lead to a relatively higher schizont count.

(iv). High counts were routinely seen in impression smears of both the small intestine and of mucosal scrapings. Again, direct penetration of the serosa seems unlikely. Access from mesenteric tumours via the arterial supply also seems unlikely due to the tough, muscular nature of arterial walls. The only potential route would seem to be via the general circulation, but the gut receives a far lower proportion of cardiac output than the liver or kidneys (<5%). The accumulation of infected cells in this site therefore remains mysterious.

(v). Some scid mice with ascites showed marked enlargement of the pancreas, with accompanying destruction of glandular structure and haemorrhage. As with the small intestine, the only potential route for entry to the pancreas would seem to be through the general circulation. However, the damage done to pancreatic tissue seems to indicate that *T.annulata* infected cells were actively multiplying in this organ. Coupled with the fact that pancreatic invasion was not always seen, this suggests that relatively few cells reached this site, but that they then found it a favourable site for rapid multiplication.

(vi). The spleen is an obvious site for the settlement of cells of lymphoid origin. It would be difficult however to prove any "homing" of infected cells to the spleen, but infected cells might be likely to become lodged in the spleen as blood and lymph are filtered through it. Like the other abdominal organs, the spleen is encased in a tough capsule, so direct penetration is unlikely.

Some infected cells were also found in the gut, mesenteries and peritoneal exudates of scid mice with large subcutaneous tumours. Large, subcutaneous

T.annulata tumours were frequently found to be attached to the peritoneum, and infected cells were able to infiltrate muscular tissue. Infected cells may therefore have been able to enter the peritoneal cavity directly from the subcutaneous tumour, without entering the circulation. The finding of schizont-infected cells in the gut might represent a tropism of *T.annulata* infected cells for this tissue: foci of multiplying infected cells in the mucosa of the abomasum and gut are a feature of *T.annulata* infections in cattle (Srivastava and Sharma, 1981). On the other hand, the presence of infected cells in these sites may simply reflect the relative volume of blood output to these organs. The data presented here were insufficient to resolve this point.

No evidence was found to suggest that *T.parva* infected cells would colonise different sites to those infiltrated by *T.annulata* infected cells: there was no difference in distribution between the two parasite species which could not be accounted for on the basis of rate of multiplication.

A note should be added on the difficulties of interpreting data concerning the migration of cells or parasites in hosts. The presence of infected cells in a tissue may mean several things: that the cells are settling and multiplying in this tissue; that the cells are entering this tissue by some positive mechanism, en route for a different final site; that the cells are in fact trapped in this tissue, representing a small but immobile, and therefore readily detected, proportion of the total cells present. For example, a high count of infected cells would not be expected in the peripheral blood, as although cells must be moving through the bloodstream to other sites, they probably do not remain in circulation for long: the impedance of this tissue to cells is low. ⁵¹chromium experiments showed very low levels of activity in blood samples, and some of that was probably released from dead cells. Accurate studies on the migration of cells from the inoculation site to other tissues are not feasible unless the cells present can be accurately and reliably quantified, and a consistently high proportion recovered on sampling.

16.5 Relevance of "mouse models" to bovine theileriosis

16.5.1 Subcutaneous tumours

Striking similarities were observed between the histological appearance of subcutaneous tumours composed of *Theileria*-infected cells, and lymphoproliferative foci in the tissues of cattle infected experimentally with *T.annulata*, particularly in the lymph nodes. In both cases, areas of proliferation of schizont-infected cells were seen, with subsequent local haemorrhage and necrosis. Proliferative foci in cattle could be seen to be infiltrating and replacing host tissue, and were vascularised. There is therefore some potential for comparative studies, for example in cytokine treatments and chemotherapy, using subcutaneous tumours in immunodeficient mice as a model for the proliferative phase of *T.annulata* infections. The experiments with TNS described in this thesis did not yield positive results, but further studies could be conducted after *in vitro* trials.

16.5.2 Ascitic tumours

As multiplying cells in the peritoneal exudate do not form a vascularised tumour, the effect on them of administered cytokines may be closer to that observed *in vitro*. Other workers have found that intraperitoneal Meth A sarcoma cells were insensitive to treatment with recombinant human TNF, whereas the same cells were sensitive to rHuTNF when grown as subcutaneous tumours (Palladino et al, 1987). The peritoneal site is therefore of limited use for studies on *Theileria* infected cells as tumours, but may be more useful for studies involving the regulation of parasite multiplication by cytokines. Under appropriate conditions, *Theileria*- infected cells could be cultured in scid mice without passaging for considerably longer than would be possible *in vitro*.

The results obtained by the injection of *Theileria*-infected cells *i/p* into mice were also of interest because they showed the ability of scid mice to control the growth of cells in this site, and what might happen if uninfected bovine PBL were injected into scid mice and then infected with sporozoites.

16.5.3 Prospects for the establishment of scid/bovine chimaeras

The results obtained so far have not been encouraging. There appears to be a donor effect, with PBL from some cattle establishing more readily than those from others. This phenomenon may be due to the presence of latent viruses in the bovine cells: human PBL from donors seropositive for Epstein- Barr virus (EBV) have been reported to establish in scid mice more readily than PBL from EBV- donors (Mosier et al, 1988).

While in most of the experiments which have been published cells were only successfully established by intraperitoneal injection, the experiments described in this thesis strongly suggest that scid peritoneal macrophages are capable of clearing *T.annulata*-infected cells from the peritoneal cavity. Three approaches could be adopted to avoid this problem: increased dose, further immunosuppression and tissue transplants.

(i) Scid mice can clear 2×10^6 *T.annulata*-infected cells but a dose of 2×10^7 cells invariably leads to a runaway ascites. Therefore the peritoneal macrophages can be overcome by using a very high dose of cells. Unfortunately, the dose would probably need to be still higher if relatively inactive, non-dividing cells such as calf PBL were used.

(ii) The experiments in chapter 15 indicate that treatment with anti-IFN- γ antibodies allows a breakthrough in cell growth, probably by preventing macrophage activation. At present this technique involves injections of antibodies on alternate days, which could itself be seen as affecting the course of the experiment. Further experiments could be done to determine minimum doses and injection schedules to achieve a satisfactory blockade of macrophage activation.

(iii) The successful construction of scid/human chimaeras has been reported by the transplantation of fragments of fetal human tissue into scid mice (McCune et al, 1988). Using this technique, the peritoneal cavity can be avoided altogether. However, surgical procedures are more time consuming and technically difficult than simple injections.

The subcutaneous tumour model could therefore be of some value in cytokine and chemotherapy studies, but a scid/bovine immunological chimaera, which can be infected with *Theileria* sporozoites and develop pathology comparable to that seen in

bovine theileriosis, has not yet been achieved. The scid mouse now seems less promising as a host for uninfected cells from other species such as man and cattle, and the parasites of these cells such as HIV and *Theileria* sp., than was originally hoped. There are several reasons for this: leakiness and natural immunity; inconsistency of establishment; failure to reproducibly reconstitute immune function, and dissimilarity between HIV multiplication in chimaeric mice and in human acquired immunodeficiency syndrome (AIDS).

As described in the "General Introduction" (section 2.5), some scid mice do develop a limited number of T and B-cell clones. This phenomenon was not observed in the experiments described in this thesis: this may be because the mice were housed in an isolator and used at a relatively early age (3-4 months). Nevertheless, phenotypic leakiness is clearly going to be a problem if one is attempting to transfer functional immune cells from another species to the scid mouse. In addition, as noted above, scid mice are capable of considerable natural immune responses to cells transplanted i/p.

As described in the "General Introduction", various authors have reported varying degrees of success in the transfer of human PBL to scid mice. Colonisation of lymphoid organs was observed in some cases (Mosier et al, 1988) but not in others (Pfeffer et al, 1989). Graft versus host disease was noted by some authors (Bankert et al, 1989), and specific human immune responses could not be elicited in all cases (Mosier et al, 1988; Bankert et al, 1989).

HIV has been shown to multiply in human cells and lymphoid tissues transplanted to scid mice. However, this infection of the human cells progresses in a time and dose-dependent fashion (Namikawa et al, 1988) leading to ablation of the transplanted cells (Mosier et al, 1989). This syndrome does not therefore appear to closely resemble the more chronic development of human AIDS. Establishment of bovine cells in the scid mouse, followed by infection with *Theileria* sporozoites, might not therefore closely resemble bovine theileriosis, but rather the intraperitoneal tumour growth described in this thesis (chapter 10).

16.6 Investigations of the role of cytokines in theileriosis using "mouse models"

Some experiments were carried out to investigate the effect of cytokines on *Theileria*-infected cells *in vivo* in immunodeficient mice. Initial experiments looking at the effect of administered rabbit TNS on subcutaneous tumours in irradiated mice did not yield positive results. Later experiments on the role of interferons in the control of cell growth in scid mice produced more interesting results.

Subcutaneous *T.annulata* tumours in all strains of mice developed general haemorrhage and central necrosis, typical of that induced by tumour necrosis factor (TNF) in experimental murine tumours (Haranaka et al, 1984; Palladino et al, 1987). Despite this similarity, no endogenous TNF production was detected in the serum or tumour homogenates of mice with resolving tumours. This might be a purely technical difficulty with the assay system, or might reflect the rapid clearance of circulating TNF: the half life of circulating TNF could be as short as 6 minutes (Beutler, Milsark and Cerami, 1985). If endogenous TNF was acting to cause tumour regression, the activity might not have been recovered in tumour homogenates because: (i) the active TNF was bound to the surface of antitumour macrophages; (ii) the TNF was acting specifically against endothelial cells, which make up a relatively small proportion of the tumour. TNF activity may therefore be extremely localised and short-lived.

Rabbit TNS administered intravenously (i/v) or intratumorally (i/t) did not cause accelerated necrosis of *T.annulata* tumours. There are four possible reasons for this: failure to act on tumour cells; dose; lack of cross- reactivity and irrelevance compared to haemorrhagic response already in progress.

(i) Recent data indicates that recombinant bovine TNF actually enhances the growth of *Theileria*-infected cell lines *in vitro* rather than inhibiting it (Preston, unpublished data).

(ii) The dose used (10^4 U/injection) was relatively low compared to those reported in other studies, but significant regression has been reported with doses of this magnitude (Haranaka, 1984); the dose size was limited by availability of TNS, and it was hoped that multiple treatments would enhance any effect.

(iii) Rabbit TNS, although effective against murine L-929 cells *in vitro*, may not

have been effective against *T.annulata*-infected bovine cells in the form of a subcutaneous tumour, or the murine cells forming the capillary bed of the tumour. The inhibitory effect of this preparation against *Theileria*-infected cell lines *in vitro* could have been due to another factor separate from the TNF activity. This factor appeared to be lipid associated (Preston, unpublished data) and may have been labile *in vivo*.

(iv) If endogenous murine TNF was already causing haemorrhage of the tumours, then the activity of administered TNF might be irrelevant: however, doses of rabbit TNS given as early as 4 days after inoculation with *T.annulata* cells did not cause an early or more rapid onset of necrosis.

HuIFN-a and IFN-g did not prevent the development of ascites in scid mice, but neutralisation of endogenous IFN-g did allow ascites to develop in mice which were otherwise capable of controlling the growth of cells. Recombinant MuIFN-g can prolong the survival of mice bearing ascitic MethA sarcoma tumours (Palladino et al, 1987). The negative results reported in chapter 15 may therefore reflect a lack of cross-reactivity between human interferons and the mouse/bovine model: however, HuIFN-a and HuIFN-g could inhibit the development of *T.annulata* trophozoites into macroschizonts in bovine cells exposed to sporozoites *in vitro* (Preston, unpublished data) so there was some evidence of human/bovine cross-reactivity.

16.7 Concluding remarks

This study has shown that *T.annulata* and *T.parva*-infected bovine cells will grow in immunodeficient mice. The scid mouse in particular has considerable potential as a host for *Theileria*-infected cells. The prospects for the establishment of scid/bovine chimaeras are however rather worse than was originally hoped, for the reasons set out above.

In addition to this main point, original, useful and interesting information was gained on several other topics: the sensitivity of murine lymphocyte subsets to irradiation; the recovery of the murine immune system from a sublethal irradiation dose, and the effect of a tumour load on such recovery; the means by which intraperitoneally injected cells might leave the peritoneal cavity, and immune mechanisms in scid mice which might prevent the establishment of xenogeneic cells.

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APPENDIX A

GENERAL METHODS

This appendix includes techniques which while relevant to the work described were either performed by other people (ie preparation of ground-up tick supernatant) or are standard laboratory methods.

A.1 Preparation of ground-up tick supernatant (GUTS)

T.annulata, Hissar stock sporozoites were prepared from adult *Hyalomma anatolicum* as described by Brown (1983).

(i). The ticks were fed on rabbits to stimulate sporozoite maturation, removed and washed in running water before being surface-sterilised in benzalkonium chloride (1%) followed by three changes of 70% ethanol. They were then washed four times in warm Minimal Eagle's Medium (MEM) with Hank's salts and penicillin (200 u/ml), streptomycin (200 ug/ml) and nystatin 100 u/ml.

(ii). The wash mixture was discarded and the ticks transferred to a sterile mortar in 5ml ice-cold MEM with 3.5% bovine plasma albumin (BPA). As the ticks were ground up, the supernatant suspension was pipetted off and fresh MEM/BPA was added until a concentration of 4 tick equivalents per ml was reached.

(iii). The suspension was centrifuged at 100g for 5 minutes to remove debris, and the supernatant was filtered through an 80µm filter.

A.2 Isolation of uninfected bovine peripheral blood lymphocytes

Uninfected bovine PBL were prepared by two methods. For establishment of cell lines, PBL were isolated from fresh blood by centrifugation on a ficoll- isopaque gradient (Ford, 1978).

(i). Heparinised blood was diluted by 50% with PBS. 20ml aliquots were layered on to 8ml of ficoll-paque (Pharmacia). The layered blood was then centrifuged at

1800 rpm for 45 minutes.

(ii). The serum supernatant was discarded, and the lymphocytes, which form a layer at the interface between the serum fraction and the isopaque, were collected with a pasteur pipette.

(iii). The collected cells were washed twice in PBS. If necessary, contaminating red blood cells were lysed by resuspending the cell pellet in sterile distilled water for 10 seconds, then adding an equal volume of double- concentrated saline.

(iv). Finally, cells were resuspended in complete medium (FCS/RPMI) and counted by trypan blue exclusion or using lymphocyte counting fluid.

Bovine PBL were also prepared by the "buffy coat" method.

(i). Undiluted, heparinised blood was centrifuged at 1800 rpm for 45 minutes.

(ii). The serum supernatant was discarded, and the lymphocytes, which form a layer on top of the erythrocytes (the "buffy coat") were collected with a pasteur pipette.

(iv). The cells were washed, erythrocytes were lysed, and the cells were counted as for the ficoll-paque method.

A.3 Cytokine assays

The mouse thymocyte assay for interleukin-1.

This assay was adapted from the method described by Hamblin and O'Garra (1987), based on an assay developed by Satsangi et al (1987). The published methods for this assay suggest the use of phytohaemagglutinin (PHA) as a co- mitogen to increase the proliferative response. A preliminary experiment showed that high levels of proliferation were obtained without the addition of PHA, so it was not included.

(i). Female Balb/c mice, about three months old, were used as a source of thymocytes. The mice were killed and their thymuses were removed to cold 2% NCS/PBS under sterile conditions. A cell suspension was prepared by teasing the thymuses apart and gently crushing them between the frosted ends of microscope slides. The suspension was pipetted off into a test tube, and any large pieces of tissue

were allowed to settle out.

(ii). Viable cells were counted, and the suspension was then spun down and resuspended in 10% FCS/RPMI with 5×10^{-5} M mercaptoethanol, at a concentration of $3-4 \times 10^6$ cells/ml.

(iii). Doubling dilutions of the samples to be tested were prepared in round bottomed 96-well plates. The starting dilution for most samples was 1/4. Most samples were prepared in triplicate: duplicates were prepared if only small volumes were available. Doubling dilutions of recombinant human interleukin 1 (IL-1) (Boehringer) were made on each plate, to act as standards.

(iv). The thymocytes were dispensed into the wells at 100 μ l/well. The plates were then incubated at 37°C in 5% CO₂ for 48 hours before proliferation was measured.

(v). Tritiated thymidine (Amersham) was added to each well at 0.5 μ Ci/well, diluted in 20 μ l medium (as above) per well. After 18 hours, the cells were harvested onto nitrocellulose filters using a Titertek automatic harvester (Flow), processed by conventional techniques for liquid scintillation, and counts per minute were measured using an automatic beta- counter (Packard).

(vi). The proliferation of thymocytes in the presence of samples was expressed as the stimulation index (S_I), where

$$S_I = \frac{\text{cpm (thymocytes+sample)}}{\text{cpm (thymocytes only)}}$$

ELISA for murine interferon gamma.

Murine interferon gamma (IFN- γ) was assayed using an enzyme-linked immunosorbent assay (ELISA) kit purchased from Holland Biotechnology bv., Leiden, the Netherlands. Assays were performed according to the schedules supplied with the kit.

Assay for MuIFN- γ neutralising activity of R46A2 antibody

The ability of preparations of the R46A2 antibody (see "Materials and Methods" to neutralise murine IFN- γ was measured using an assay for the inhibition of the invasion of L-929 cells by Semliki Forest Virus (SFV). This assay was performed by Dr. G.E. Entrican of the Moredun Research Institute, Edinburgh.

(a). Virus inhibition assay method

(i). L-929 cells were plated out in 96-well plates (Costar) at a density of 5×10^3 cells/well in 100 μ l of IMDM, supplemented with 5% FBS (Northumbria Biologicals). The cells were incubated for 24 hours at 37°C in 5% CO₂.

(ii). Samples to be tested for IFN activity were added to triplicate wells in 100 μ l volumes, and the cells were incubated with the samples for a further 24 hours.

(iii). The medium in the wells was removed and replaced with 100TCID₅₀ of Semliki Forest Virus in 200 μ l of IMDM, supplemented with 2% FBS. The plates were incubated for a further 48 hours before the cytopathic effect (CPE) was read. Titres of IFN were expressed as the inverse of the highest sample dilution which inhibited the CPE by greater than 50%.

(b). IFN neutralisation assay for R46A2 antibody

The levels of IFN- γ neutralising activity in supernatants of R46A2 cultures were determined by measuring the ability of the antibody to neutralise a standard preparation of recombinant murine IFN- γ . The rMuIFN- γ was derived from supernatants of cultures of Chinese Hamster Ovary cells transfected with the gene for MuIFN- γ . This cell line was kindly supplied by Dr.A.Morris, University of Warwick (Morris and Ward, 1987). Dilutions of antibody were reacted with an equal volume of rMuIFN- γ for 3 hours at 37°C /5% CO₂. 100 μ l of the mixture were then added to triplicate wells and tested for residual biologically active IFN- γ as described above.

The highest dilution of R46A2 which inhibited the protective effect of 1000U/ml of rMuIFN- γ was 1/64. The IFN- γ neutralising activity of the undiluted antibody preparation was therefore 64000 neutralising units (NU)/ml.

The L-929 assay for Tumour Necrosis Factor

This assay was modified from the technique of Flick and Gifford (1984).

(a). Maintenance of L-929 cells

The mouse fibroblast-derived cell line L-929 was grown as a monolayer in 10% FCS/RPMI with 2mM glutamine but without antibiotics. Cells were detached from the flask for passaging by incubation with trypsin-EDTA in Puck's saline (Gibco). The cell line was passaged three times a week. The LR cell line, a subclone of L-929

which is highly resistant to TNF, was maintained in the same way.

(b). Assay method

(i). Near-confluent L-929 cells were detached from the culture flask with Trypsin-EDTA, resuspended in assay medium (as for cell culture, but with antibiotics) and viable cells were counted by trypan blue exclusion. The suspension was then adjusted to 2×10^5 cells/ml, and dispensed into 96 well, flat-bottomed microtiter plates (Flow or Nunc.) at 100 μ m/well. The plates were incubated at 37°C in 5% CO₂ overnight.

(ii). The medium was aspirated and replaced with 100 μ m of assay medium supplemented with actinomycin D at 1 μ g/ml. A further 50 μ l of assay medium with 2 μ g/ml actinomycin D was added to the end wells, and 50 μ l of the sample to be tested was added also. Doubling dilutions were then carried out from these wells, and the last 100 μ l was discarded. Dilutions therefore started at 1/4. Human recombinant Tumour Necrosis Factor (200 U/ml) (Boehringer) was included as a standard. The plates were incubated for a further 18-20 hours.

Replicate plates using LR cells were set up at the same time to control for cytotoxic factors other than TNF.

(iii). The medium was discarded and replaced with 40 μ l of 0.5% crystal violet in 20% methanol. The plates were stained for 2 minutes, then washed vigorously with tap water and allowed to dry.

(iv). Plates were inspected visually for disruption of monolayers. Positive results were quantified by measuring the absorbance of each well at 540nm using an automatic ELISA reader.

A.4 YAC-1 assay for Natural Killer Cell activity

Maintenance of YAC-1 cells

A cryopreserved stabilate of these cell line was obtained from the European Collection of Animal Cell Cultures (ECACC), Porton Down, Salisbury. It was grown as a suspension culture in 10% FCS/RPMI with antibiotics and glutamine and fed and passaged as for *Theileria*-infected cell lines.

Preparation of effector cells

(i). Mice were killed by cervical dislocation and their spleens removed under aseptic conditions to cold 2% NCS/PBS. Cell suspensions were prepared by teasing out the cells with bent needles, and breaking up cell clumps by gentle pipetting with a pasteur pipette.

(ii). The cell suspension was transferred to a test tube, and any large pieces of tissue were allowed to settle. The suspension was then decanted into a second test tube. Cells were counted by trypan blue exclusion, centrifuged at 1000 rpm for 10 minutes, and resuspended at 1×10^7 cells/ml in 10% FCS/Leibovitz L-15 with antibiotics and glutamine. L-15 was used instead of RPMI for this assay, because the assay plates were incubated in a humidified polythene sandwich box gassed with CO_2 , rather than in a flow incubator. Under these conditions L-15 was found to maintain a better pH than RPMI, due to its higher buffering capacity.

(iii). 200 μ l of the effector cell suspension was pipetted into the row "B" wells of a conical-bottomed 96 well microtiter plate. Doubling dilutions were then made down the plate using a multichannel pipette (100 μ l/well). All dilutions were made in quadruplicate. The plates were incubated at 37°C /5% CO_2 while the target cells were prepared.

Preparation of ^{51}Cr labelled target cells

(i). Approximately 5×10^6 cells were taken from a growing culture and centrifuged at 1000rpm for 10 minutes. The cells were then resuspended in 0.2 ml PBS plus 0.1ml NCS, with 100 μ Ci (3.7×10^6 Bq) $^{51}\text{chromium}$ (as sodium chromate solution)(Amersham). This suspension was incubated at 37°C in a shaking water bath for 1 hour. (ii). 5mls of 2% NCS/PBS were added. The diluted cell suspension was layered on to 1ml of NCS and centrifuged at 1000 rpm for 7 minutes.

(iii). The supernatant was discarded, the cells were resuspended in 10ml of 2% NCS/PBS, and spun down again.

(iv). The pellet was resuspended in 1ml of 10% FCS/L-15, and a viable cell count was made by trypan blue exclusion. The target cells were then diluted to 1×10^5 cells/ml in medium and plated onto the effector cells at 100 μ l per well. At least four wells were also plated as positive and negative controls: in negative (spontaneous release) controls, target cells were added to 100 μ l of medium; in positive (maximum

release) controls they were lysed with 100µl of 2.5% Triton X-100.

(v). Effector to target ratios therefore ranged between 100:1 and 3:1. After 18 hours incubation, the plates were centrifuged at 1000rpm for 5 minutes, and 100µl of the supernatant in each well was transferred to Luckham tubes for counting. Activity was measured as counts per minute (cpm) using a automatic gamma counter.

Percentage cytotoxicity was calculated according to the formula:

$$\% \text{ Cytotoxicity} = \frac{(\text{cpm in test wells} - \text{cpm in negative control})}{(\text{cpm in positive control} - \text{cpm in negative control})} \times 100$$

Analysis of results

Counts per minute or %cytotoxicity in each quadruplicate group were compared using the Mann-Whitney test.

A.5 Mouse techniques

Preparation of peritoneal exudate cells from mice

Mice were killed by ether inhalation, swabbed with 70% alcohol and the skin was cut and peeled back to expose the abdomen. 5mls of cold 2% NCS/PBS was then injected intraperitoneally to wash out peritoneal cells. The volume recovered was usually 3.5 to 4 mls. Peritoneal cell suspensions were kept on ice to prevent adherence.

Serum preparation

Mice under terminal anaesthesia were bled from the brachial artery. Approximately 1ml of blood could be obtained in this way. The blood was allowed to clot at room temperature, and the clot was then separated from the sides of the test tube using a sterile wooden splint. The blood was incubated at 37°C for 1 hour, then transferred to 4°C overnight. It was then centrifuged at 1800 rpm for 10 minutes and the supernatant serum was removed. Any remaining contaminating erythrocytes were removed by further centrifugation. Serum samples were stored at -70 C.

A.6 Preparation of Rabbit Tumour Necrosis Serum

This procedure was carried out by Dr.P.M.Preston according to the method described by Matthews (1978).

(i) A female, New Zealand strain rabbit was injected intravenously in the ear with $50\text{-}250 \times 10^6$ viable BCG organisms (percutaneous BCG vaccine BP, Glaxo).

(ii) Two weeks later, a dose of 100 μ g of lipopolysaccharide (~E.coli~ E026.B66, Sigma) was administered i.v. 10mls of blood were removed by cardiac puncture immediately prior to injection: this was used to produce control serum. 1.5 hours after injection with LPS, the rabbit was bled by cardiac puncture. Serum was separated as previously described.

(iii) TNF activity in the serum was titrated in an L-929 assay. The dilution of serum which causes 50% cytotoxicity is defined containing 1U/ml: the activity of the undiluted serum is therefore the reciprocal of this dilution. The activity of the serum from rabbit 68 was 5×10^5 U/ml.

A.7 Histology

Impression (dab) smears prepared from various tissues were allowed to dry, fixed in methanol for 30 seconds, and stained with 10% Giemsa's stain (Gurr's R66 formulation, BDH) for 20-30 minutes. Cytocentrifuge preparations (cytospins) were stained in the same way.

Tissues were fixed in 5% formol-saline and processed by conventional techniques using an automatic tissue processor. The tissues were embedded in paraffin wax and sections were cut at 5 μ m thickness. The sections were stained by Bayley's method (Drury and Wallington, 1980):

(i). Sections were dewaxed and rehydrated as usual. They were then stained in 10% Giemsa overnight.

(ii). The slides were rinsed with tap water to remove excess stain, and differentiated with 0.5% ethanoic (acetic) acid.

(iii). Differentiation was stopped by rinsing with tap water. Excess water was blotted off, and the sections were dehydrated with propan-2-ol (isopropyl alcohol).

(iv). The sections were cleared through two changes of xylene and mounted in canada balsam.

APPENDIX B

ADDITIONAL DATA

This appendix contains the "raw" data from two experiments: firstly, the data which was used to plot graphs 1,2 and 3 in chapter 3, and secondly the data which was used to calculate tissue localisation ratios (TLRs) in chapter 12. The criteria for including this information were that in the first case, data was pooled from several experiments to draw some graphs, and in the second case that it might be helpful to have the raw data accessible to understand how results were obtained.

Growth of TaHis110 cells injected s/c into irradiated Balb/c mice.

The data shown here were used to plot graphs (1) and (2) in chapter 1.

(1). 8×10^5 cells, 4 or 6 Gy.

Day	<u>4Gy</u>			<u>6Gy</u>		
	Tumour size	n	95%	Tumour size	n	95%
6	0	-	-	0	-	-
7	0	-	-	0	-	-
8	2	1	-	0	-	-
9	3	1	-	1	1	-
10	2	1	-	3	1	-
11	3	1	-	3	1	-
12	3	1	-	4.5	2	0.7
13	2	1	-	4	2	0
14	2	1	-	5	2	0
15	0	1	-	6	2	0
16	-	-	-	6	2	0
17	-	-	-	6	1	0
18	-	-	-	6	1	0
19	-	-	-	6	1	0
20	-	-	-	5	1	0
21	-	-	-	5	1	0
22	-	-	-	4	1	0
23	-	-	-	4	1	0
24	-	-	-	4	1	0
25	-	-	-	4	1	0
26	-	-	-	4	1	0
27	-	-	-	4	1	0
28	-	-	-	4	1	0

There were four mice in each group at the start of the experiment.

No subcutaneous tumours were observed in unirradiated mice injected with this dose of cells.

All tumour sizes are in mm: mean results are given where n"1. "95%" values are 95% confidence limits calculated from the sample standard deviation.

(2). 4×10^6 cells, 4 or 6 Gy.

Day	<u>4Gy</u>			<u>6Gy</u>		
	Tumour size	n	95%	Tumour size	n	95%
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	2	2	0	2	3	1.1
5	2	2	0	2.7	3	1.4
6	2	2	0	2.7	3	0.7
7	2	2	0	4.3	3	1.4
8	2.5	2	0.7	5.3	3	1.4
9	2.5	2	0.7	6	3	1.1
10	3	2	0	6.3	6	0.7
11	2.5	2	0.7	7	3	1.1
12	3.5	2	0.7	7	3	0
13	3	2	0	8	3	0
14	3	2	0	7	3	1.1
15	2.5	2	0.7	8.5	2	0.8
16	2.5	2	0.7	8.5	2	0.8
17	3	2	0	8.5	2	0.8
18	2	2	0	8.5	2	0.8
19	1	2	2.0	8	1	0
20	1	2	2.0	7	1	0
21	1	2	2.0	0	1	0
22	1	2	2.0	-	-	-
23	0	2	0	-	-	-

There were four mice in each group at the start of the experiment.

No subcutaneous tumours were detected in unirradiated Balb/c mice given the same dose of cells.

Abbreviations as for previous table.

(3). 2×10^7 cells, 0,4 or 6Gy.

Day	<u>0Gy</u>			<u>4Gy</u>			<u>6Gy</u>		
	Size	n	95%	Size	n	95%	Size	n	95%
1	-	-	-	1.2	13	0.3	-	-	-
2	-	-	-	3.3	4	0.5	2	1	0
3	2.8	2	1.6	3.3	6	0.8	2	2	0
4	2.4	4	1.1	3.9	13	0.3	2.3	6	0.5
5	2.6	4	1.5	4.6	15	0.5	2.6	7	0.6
6	2.6	4	1.5	4.7	11	0.7	3.3	4	1.0
7	1.9	4	1.4	5.6	16	0.8	4.4	7	1.1
8	1.9	4	1.3	5.7	16	0.7	5.0	7	0.8
9	2.0	4	1.4	5.9	14	0.5	6.0	7	1.3
10	2.1	4	1.7	6.7	7	0.6	6.1	7	1.2
11	2.0	4	1.6	5.6	14	0.7	7.2	6	1.2
12	2.0	4	1.6	5.8	14	0.7	7.0	6	1.1
13	1.8	4	1.7	5.9	12	1.0	7.5	6	1.3
14	1.3	4	1.9	5.9	12	1.1	7.7	6	1.6
15	0	4	0	5.4	9	1.1	7.5	6	1.3
16	-	-	-	5.5	11	1.1	7.5	6	0.8
17	-	-	-	5.6	10	1.0	7.6	5	1.5
18	-	-	-	4.7	6	1.3	7.8	4	1.9
19	-	-	-	4.2	6	0.8	7.5	2	1.0
20	-	-	-	4.4	5	0.8	8.0	2	0
21	-	-	-	5.0	3	0	7.5	2	1.0
22	-	-	-	5.0	3	0	8.0	1	0
23	-	-	-		(nd)			(nd)	
24	-	-	-	0	3	0	8.0	1	0
25	-	-	-	-	-	-	7.0	1	0

Data from allogeneic lymphocyte cytotoxicity experiment (chapter 10)

The data shown here were used to calculate tissue localisatiuon ratios (TLRs) and the values of "p" quoted in chapter 10.

(1). Scid mice plus bovine spleen cells.

Tissue	Mouse:1	2	3	4	5
Blood	(nd)	0.079	0.103	0.091	0.12
Pex.	4.6	3.65	3.72	3.8	2.72
L.N.	0.015	0.003	0.002	0.004	0.002
B.M.	0.165	0.128	0.16	0.168	0.183
Liver	4.46	7.07	4.37	2.41	6.3
Spleen	5.45	2.04	2.13	0.973	3.56
S.Int.	9.29	17.1	9.0	5.12	16.3
L.Int.	1.38	1.72	5.59	2.19	1.58
Kidney	0.895	0.965	0.876	0.84	1.47
Lung	0.393	0.332	0.352	0.256	1.19

(2). Scid mice plus Balb/c spleen cells.

Tissue	Mouse:1	2	3	4	5	6
Blood	0.078	0.08	0.046	0.101	0.067	0.048
Pex.	6.96	13.3	7.95	10.8	8.74	10.3
L.N.	0.041	0.018	0.023	0.016	0.018	0
B.M.	0.133	0.154	0.186	0.336	0.175	0.214
Liver	4.07	3.82	4.33	11.5	9.53	3.16
Spleen	3.45	1.30	1.15	9.60	2.45	2.30
S.Int.	10.9	12.7	14.0	19.5	10.8	10.3
l.Int.	1.89	2.66	2.04	2.12	2.55	1.05
Kidney	1.10	1.30	1.10	2.15	1.29	1.10
Lung	0.306	0.274	0.161	0.504	0.253	0.117

(3). Balb/c mice plus bovine spleen cells.

Tissue	Mouse:1	2	3	4	5	6
Blood	0.095	0.071	0.076	0.098	0.135	0.102
Pex.	12.4	6.76	7.29	(nd)	3.36	7.35
L.N.	0.007	0.007	0.003	0.007	0.012	0.345
B.M.	0.398	0.357	0.359	0.421	0.368	0.002
Liver	5.53	3.51	4.57	6.46	6.96	7.72
Spleen	1.31	1.57	1.72	1.83	3.48	2.42
S.Int.	12.6	8.68	16.7	19.3	16.1	14.2
L.Int.	2.43	1.45	1.33	7.52	3.34	1.97
Kidney	1.39	1.09	1.40	1.72	1.58	1.41
Lung	0.483	2.46	0.413	0.459	0.311	0.345

(4). Balb/c mice plus Balb/c spleen cells.

Tissue	Mouse:1	2	3	4	5	6
Blood	0.053	(nd)	0.032	0.046	0.067	0.08
Pex.	13.5	9.77	5.36	6.28	7.05	10.3
L.N.	0	0	0.048	0.011	0.011	0.018
B.M.	0.304	0.310	0.347	0.317	0.42	0.315
Liver	3.58	2.89	2.05	3.31	2.47	2.72
Spleen	0.94	3.13	0.40	1.65	2.95	0.865
S.Int.	8.78	7.39	9.14	13.6	8.16	16.6
L.Int.	3.89	2.37	2.03	1.58	2.29	1.73
Kidney	1.17	1.29	0.894	1.75	1.44	1.55
Lung	0.182	0.175	0.103	0.248	0.313	0.212

Abbreviations: Pex., peritoneal exudate; L.N., lymph nodes; B.M., bone marrow; S.Int., small intestine and adjacent mesentery; L.Int., large intestine and adjacent mesentery.

APPENDIX C

PAPER

Brief communication

Establishment of *Theileria*-infected bovine cell lines in *scid* mice

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Summary Bovine cells transformed by infection with the protozoan parasite *Theileria annulata* were inoculated subcutaneously or intraperitoneally into C.B.-17 *scid* mice. Mice injected subcutaneously developed solid tumours at the injection site, whilst those injected intraperitoneally developed ascites. Schizont-infected cells were found in other tissues: infected cells spread much more easily from the intraperitoneal site. Karyotyping of cells isolated from tumours showed no evidence of transfer of parasites to murine cells. These results show that the *scid* mouse can be used as a host for *Theileria*-infected bovine cells.

Keywords: *Theileria*-infected bovine cells, *scid* mice

Tropical theileriosis is a major lymphoproliferative disease of cattle, initiated by transformation and multiplication of macroschizont-infected cells in lymph nodes draining the site of inoculation with *Theileria annulata* sporozoites. As the disease progresses, discrete tumour-like foci of schizont-infected transformed cells and uninfected lymphoblastoid cells develop throughout the body. Macroschizont infected cell lines derived either by isolation of parasitized cells from *Theileria annulata*-infected cattle or by *in vitro* infection of peripheral blood leucocytes from uninfected cattle with sporozoites can be maintained *in vitro*. Cell lines attenuated by long term culture will protect cattle against tick-borne challenge with sporozoites (Pipano 1981).

Members of the genus *Theileria* are specifically parasites of ruminants and past *in vivo* studies on theileriosis have therefore had to be conducted in cattle with the attendant scientific and practical problems of working with large, expensive and outbred animals. The development of a small animal model would greatly facilitate research into the biology of these parasites and the diseases they cause. To date attempts to develop *in vivo* models have had to rely on the establishment of macroschizont-infected bovine cells in mice immunosuppressed by irradiation. Irvin *et al.* (1977) showed that *Theileria parva*-infected cells could be grown as subcutaneous tumours in heavily irradiated mice, and recent experiments in this laboratory have shown that *T. annulata*-infected cells can be grown in the same way at lower radiation doses (Fell 1988). However, these models are complicated by radiation sickness, and the precise effects of sublethal radiation on the mouse immune system remain poorly defined. The recent development of human/mouse haematopoietic chimaeras in the C.B-17 *scid* mouse (Mosier *et al.* 1988; McCune *et al.*

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1988) and their use as models for HIV infection (Namikawa *et al.* 1988) therefore encouraged us to investigate the *scid* mouse as a host for *T. annulata*-infected cells. The *scid* mutation effectively abrogates T and B cell differentiation such that the mice are agammaglobulinaemic and lack functional B and T cells. Macrophage and NK cell functions are unimpaired (Bosma, Schuler & Bosma 1988). The experiment described here was carried out to see if *T. annulata*-infected cells would establish in *scid* mice, as a preliminary guide to the feasibility of using a *scid*-bovine chimaera as a small animal host for *Theileria*.

Female C.B.-17 *scid* mice (3 months old) were housed in a laminar flow filter rack. Serum from mice in the same batch was assayed for immunoglobulins using a mouse monoclonal typing kit (Binding Site Ltd), and found to be negative for all the antisera used (IgG1, 2a, 2b, 3; IgM; IgA).

An *in vitro* derived *T. annulata*-infected cell line of the Hissar stock (Gill, Bhattacharyulu and Kaur 1976), maintained on RPMI-1640 supplemented with 10% newborn calf serum, penicillin (100 U/ml), streptomycin (100 mcg/ml), kanamycin (100 mcg/ml) and glutamine (2 mM) (Brown 1983) was used for infection. Cells were washed twice in phosphate-buffered saline (PBS), and *scid* mice were injected subcutaneously or intraperitoneally with 2×10^7 cells in 0.2 ml PBS. Seven control BALB/c mice, 3 of which had been given 4 Gy from a caesium-137 source immediately beforehand, were injected intraperitoneally with the same dose of cells. 4 Gy is a dose which permits the growth of subcutaneous tumours in BALB/c mice. Peritoneal exudates were prepared from all mice by post mortem lavage with 5 ml of RPMI. Cytospin preparations of ascitic fluid, tapped 11 days after inoculation, and of peritoneal exudates were stained with Giemsa's stain. Impression smears were prepared from subcutaneous tumours, liver, kidney, pancreas, lung, inguinal lymph nodes and spleen, stained with Giemsa's stain and examined for the presence of free macroschizonts and macroschizont-infected cells. Free parasites probably resulted from rupture of infected cells during smear preparation. Fixed tissues were processed conventionally and embedded in paraffin wax. Sections were cut at 5 μ m and stained with Giemsa's stain by a modification of Bayley's method (Drury and Wallington 1980).

Results are summarized in Table 1. *Scid* mice injected subcutaneously developed subcutaneous tumours, whilst mice injected intraperitoneally developed ascitic tumours. Subcutaneous tumours did not show signs of haemorrhage until 8 days post infection. None of the control (BALB/c) mice developed ascites, and peritoneal exudates and impression smears prepared 12 days post infection did not contain any macroschizonts.

Subcutaneous tumours consisted of lymphoid cells infiltrating subcutaneous tissue, with some haemorrhage and necrosis, but few macroschizonts or infected cells were found in other organs. Similar observations have been made in irradiated BALB/c and BALB/c nu/nu mice inoculated subcutaneously with *T. annulata* infected cells: haemorrhage sets in on days 4 to 5 in these mice (Fell 1988; unpublished data).

The ascitic fluid tapped from *scid* mice inoculated intraperitoneally was turbid and bloody. It contained large numbers of cells, some of which were infected. Peritoneal exudates also contained macroschizont-infected cells and macrophages. In contrast to *scid* mice injected subcutaneously, mice injected intraperitoneally had large numbers of macroschizont-infected cells in their abdominal organs. Sections of a kidney from mouse 4 showed very heavy infiltration and tissue destruction by lymphoid cells (Figure 1). There was also extensive haemorrhage under the capsule. Impression smears from this kidney

Table 1. Presence of *T. annulata* schizonts and infected cells in impression smears from various organs, expressed as schizont index

Mouse	Subcutaneous route			Intraperitoneal route		
	1	2	3	4	5	6
Tumour	(+)	84	440	—	—	—
Peritoneal exudate	0	12	7	49	(+)	82
Liver	0	0	0	58	61	17
Kidney	0	0	0	84	25	3
Pancreas	0	0	0	nd	nd	57
Lung	24	2	<1	69	41	6
Lymph node	0	0	nd	40	2	0
Spleen	<1	0	0	nd	<1	6

(+) = Infected cells present but not quantifiable.

nd = not done.

$$\text{Schizont index} = \frac{(\text{total free schizonts} + \text{total infected cells})}{(\text{total host nuclei})} \times 100$$

in at least ten $\times 1000$ fields.

All mice were killed on day 12.

showed that 32% of cells were infected. Sections of tissues from other mice injected intraperitoneally showed extensive infiltration, inflammation, haemorrhage and necrosis of the pancreas and mesentery. The liver was found to be haemorrhagic, and the small intestine was slightly inflamed.

A single cell suspension was prepared from the subcutaneous tumours of 2 *scid* mice by fine chopping and sieving in cold PBS, and maintained in RPMI supplemented with 10% foetal calf serum and antibiotics. After 2 weeks in culture a cytospin preparation showed at least 95% of cells to be infected with macroschizonts. Karyotypic analysis performed at the same time showed the cell line to be bovine.

Previous experiments showed that *T. annulata*-infected cells would only grow for 2 to 3 weeks in irradiated BALB/c mice before tumours regressed due to haemorrhagic necrosis: a schizont index of 60 (mean of 2 mice) in tumour impression smears was typical on day 12 (Fell 1988; unpublished data). In irradiated BALB/c nu/nu mice, subcutaneous tumours grew without regression although they did develop haemorrhagic necrosis. Disseminated cells were only occasionally found in irradiated mice, e.g. in the lung and kidney, and the spread of infected bovine cells was directly related to the radiation dose. In contrast, as described here, *T. annulata*-infected, transformed bovine cells grew readily both as subcutaneous tumours and in ascitic fluid in unirradiated *scid* mice. The pathological effects of the parasitized cells were determined by the site of infection: cells growing intraperitoneally spread much more aggressively into the viscera than those growing subcutaneously.

The *scid* mouse therefore appears to be a more favourable environment for the cultivation of *T. annulata* infected bovine cells than the irradiated BALB/c or BALB/c nu/

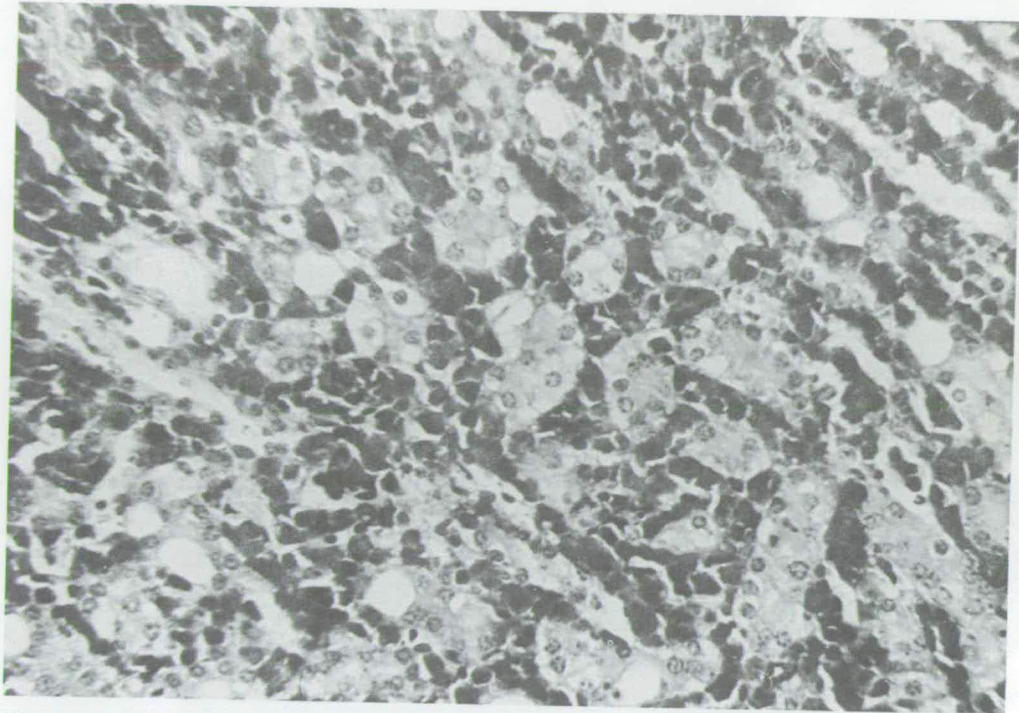


Figure 1. Section of kidney from *scid* mouse injected intraperitoneally showing infiltration of kidney cortex (light nuclei) by lymphoid cells (dark nuclei).

nu mouse. Stimulated by these preliminary results, work is in hand: (1) to study the long-term survival and proliferation of *Theileria*-infected cell lines as ascites and solid tumours in *scid* mice; (2) to construct *scid*-bovine haematopoietic chimaeras for inoculation with sporozoites of *T. annulata* and *T. parva*, the two major *Theileria* pathogens.

The successful establishment of *Theileria*-infected cell lines in *scid* mice and sporozoite induced infections in *scid*-bovine chimaeras respectively should provide suitable *in vivo* systems for studying many immunological aspects of the host-parasite response in *Theileria* infections which cannot be studied *in vitro*. These include: (1) sustained interactions between selected cellular components of the bovine immune system and recombinant bovine cytokines on the one hand and *Theileria* parasites and the tumours in which they grow on the other hand; (2) the response of the bovine immune system to putative candidate antigens.

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