STUDIES ON THE PATHOGENESIS OF FELINE T-CELL NEOPLASIA

by

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DECLARATION

This work was done independently by the author apart from the following: all procedures which require a Home Office Licence, which were carried out by Dr. D. Onions. In addition, the analysis of tumour and cell line DNA described in Chapter Six was done in collaboration with Dr. J. Neil of the Beatson Institute for Cancer Research, Glasgow.

SUMMARY

This study was concerned with two principal aspects of T-cell neoplasia in the cat. First, the role that T-cell growth factor (TCGF) had to play in the proliferation of neoplastic T-cells *in vitro* and secondly, the properties of the viruses associated with the feline thymic lymphosarcomas in this series.

The first part of the work (Chapter Two) concerned the production of lymphocyte conditioned medium (Ly-CM) which provided a source of TCGF, or IL-2, as it is better known. This enabled the long-term culture of normal activated feline T-cells which were used in routine blastogenesis assays to determine the level of IL-2 in Ly-CM.

The next chapter (Chapter Three) involved the establishment of neoplastic feline T-cell lines *in vitro*. The cells were derived from thymic lymphosarcomas and were cultured both with and without Ly-CM. Most of these primary cultures required this source of IL-2 for proliferation with two noticeable exceptions (designated T3 and T17) which were extensively studied in the following chapter.

The experiments in Chapter Four were designed to determine the role of IL-2 in T-cell neoplasia. The evidence generated suggests that at least some neoplastic T-cell lines have an altered requirement for, or response to, IL-2 *in vitro*. Recent data from the study of human T-cell neoplasia suggests that enhanced IL-2 receptor expression may be fundamental to the alteration in the requirement for IL-2 observed with some of these cells. The strategy for the further characterisation of the growth requirements of neoplastic T-cells is discussed.

In Chapter Five the nature of the viruses associated with the thymic lymphosarcomas and the resultant cell lines was determined. The frequent presence of a novel FeLV which has recombined with a cellular oncogene (c-*myc*) was reported and the *in vitro*

transmission of these recombinant viruses (FeLV/myc) was demonstrated. The association of additional FeLV subgroups other than A with thymic lymphosarcoma in the series was also observed. The generation of these subgroups and their possible role in the development of neoplasia is discussed.

Finally, in Chapter Six, the in vivo passage of the recombinant FeLV/myc virus from the T3 and F422 cell lines was described. These viruses, which originated from cells from thymic lymphosarcomas, rapidly produced the same tumours in susceptible animals. Furthermore, the cells isolated from these tumours readily established in culture without an exogenous source of IL-2. These cells continued to grow under the same conditions as do the parental T3 and F422 cell lines.

The relationship between the enhanced expression of the myc oncogene and independence from an exogenous source of IL-2 for growth, is discussed in the light of recent evidence, and future experimental approaches are suggested.

ABBREVIATIONS

ALV	Avian leukosis virus.
ATL	Adult T-cell leukaemia.
BSA	Bovine serum albumin.
Con-A	Concanavalin-A.
c-*onc*	Oncogene present in cellular sequences.
CsCL	Caesium chloride.
pCT4 3'	Feline v-*myc* clone.
CTL	Cytotoxic T-lymphocyte.
10% DMEM	Dulbecco's minimum essential medium, containing L-Glutamine, Penicillin-Streptomycin and 10% foetal bovine serum.
DNA	Deoxyribonucleic acid.
EGF	Epidermal growth factor.
FEA	Feline embryo cells of the FEA strain.
F422	Suspension cells derived from a feline thymic lymphosarcoma. They release FeLV-A.
FL74	Suspension cells derived from a feline lymphoid tumour. They release FeLV-ABC/KT.
FeLV	Feline leukaemia virus.

FBS	Foetal bovine serum.
FFU	Titre of virus expressed as focus-forming units/ml.
FOCMA	Feline oncornavirus-associated cell membrane antigen.
HTLV	Human T-cell leukaemia virus.
IFN-γ	Gamma interferon.
IL1	Interleukin-1.
IL2	Interleukin-2.
ppIL2	Partially purified human IL-2.
0.25% Isc.	Iscove's modified Dulbecco's medium containing penicillin-streptomycin, 2-Me and 0.25% BSA.
L/M	Liebovitz L-15 and McCoy's 5A media in equal volumes supplemented with L-glutamine, penicillin-streptomycin and 10% FBS.
LTR	Long terminal repeat.
Ly-CM	Lymphocyte conditioned medium.
2-Me	2-mercaptoethanol.
50:50 medium	Medium composed of equal volumes of RPMI 20% + Me and Ly-CM.
MuLV	Murine leukaemia virus.
PBS	Phosphate buffered saline.
PHA	Phytohaemagglutinin.
PDGF	Platelet-derived growth factor.

RNA	Ribonucleic acid.
RODW	Reverse osmosis distilled water.
RPMI 20% + Me	RPMI 1640 medium containing L-glutamine, penicillin-streptomycin, 2-Me and 20% FBS.
RPMI 0.25% +Me	RPMI 1640 medium containing L-glutamine, penicillin-streptomycin, 2-Me and and 0.25% BSA.
SPF	Specific pathogen free.
Tac-ag	IL-2 receptor designated Tac-antigen.
TCGF	T-cell growth factor.
TGF	Transforming growth factor.
v-onc	Oncogene incorporated into a viral genome.

NOTES ON NOMENCLATURE

Throughout the thesis, the terms TCGF and IL-2 are used interchangeably and refer to the same factor. Ly-CM refers to crude lymphocyte conditioned medium which is a source of IL-2 but has not been purified in any way and therefore retains, in addition, mitogen and other growth factors. The terms Ly-CM and TCGF/IL-2 are not therefore used interchangeably but refer specifically to crude or purified factor respectively. Furthermore, in experimental protocols described in the following chapters the purified factor used is human (provided by Dr. R.C. Gallo, NCI, USA) and is designated ppIL-2 which stands for partially purified IL-2 and always refers to this source of human factor.

GENERAL INTRODUCTION

Pathogenesis of T-cell neoplasia

A number of mechanisms have been proposed to explain the induction of T-cell neoplasia. More than one mechanism may operate in the development of even an individual tumour, as it would appear that multiple steps are required for a cell to become fully malignant (Peto, 1977).

Two groups of viruses have been shown to be the initiating agents of T-cell neoplasia. A herpesvirus of chickens is known to be the aetiological agent of the economically important lymphoproliferative and lymphomatous condition, Marek's Disease (For Review see Powell, 1984). Most attention, however, has been directed at the only known oncogenic RNA virus group, the retroviruses. Viruses of this group have been shown to be the aetiological agents of T-cell neoplasia in mice (Moloney, 1960 and Gross, 1957), cats (Jarrett et al., 1964) and gibbons (Kawakami et al., 1972). The demonstration of these oncogenic viruses in animal populations stimulated a search for a similar virus in human T-cell neoplasia. In 1980 the search proved successful with the isolation of the T-cell leukaemia virus of man designated HTLV-I (Poiesz et al., 1980a). The study of animal retroviruses has had a second important impact on cancer research in that it has revealed a set of cellular genes, known as oncogenes, which have now been implicated in the development of a wide range of viral and non-viral cancers.

Retroviruses have a relatively simple structure and pattern of replication (Varmus and Swanstrom, 1982) (Fig. 1). The genome is in the form of ribonucleic acid (RNA) and is enclosed by a protein core. This is surrounded by an envelope derived from modified plasma membrane from which viral glycoprotein spikes project; these spikes, known as gp70, can bind to receptors on target cells to initiate an infection. Whether or not a cell has a surface receptor for a specific viral gp70 determines if that cell is susceptible to infection by that particular virus. A cell which displays a receptor for the virus is said to be a permissive cell

for infection by that virus (host cell). On infecting a host cell, the virus uncoats to liberate the nucleic acid which is transcribed into deoxyribonucleic acid (DNA) by a virus encoded enzyme called reverse transcriptase (RT). This property is unique to retroviruses and enables the stable integration of a DNA copy of the viral genome (provirus) into the cellular DNA where it behaves, essentially, like a normal cellular gene. The provirus contains three genes: the gag gene codes for the internal proteins of the virus; the pol gene codes for the reverse transcriptase (RT); and the env gene codes for the envelope glycoprotein of the spikes and an envelope-associated protein known as p15E. The provirus is flanked at both ends by long terminal repeats (LTRs) which are non-coding but are essential in that they contain both promoter and enhancer sequences for transcription of the proviral genes. Viral glycoproteins are inserted into the cell membrane and new virus particles assemble beneath this and bud from the cell in a non-cytopathic process.

Oncogenes

Oncogenic retroviruses can generally be classified as strongly or weakly transforming. Weakly transforming viruses have long latent periods before tumour formation and do not usually transform either haemopoietic cells or fibroblasts in vitro. Strongly transforming viruses have short latent periods of a few weeks and often transform fibroblasts and, on occasions, haemopoietic cells in vitro. Strongly transforming viruses usually contain specific oncogenes (v-onc) which are derived from cellular genes (Friend leukaemia virus is one exception). These cellular genes (c-onc) are incorporated into the viral genome by recombination and are responsible for the transforming function (Fig. 2). Two principal differences between the v-onc genes and their cellular homologues have been detected. Firstly, many c-onc genes, like other eukaryotic genes, are divided into exon (coding) and intron (non-coding) regions. The introns are missing in the v-onc gene structure indicating that the recombination process probably involves the reverse transcription of a viral/c-onc hybrid mRNA

similar to the model proposed by Swanstrom et al (1983) for the generation of Rous sarcoma virus. Secondly, in the Harvey and Kirsten strains of rat sarcoma virus, a single point mutation has occurred at position 12 of the v-onc gene compared to its cellular equivalent (Tabin et al., 1982). The amino acid substitution is such that the tertiary structure of the protein at that region would be "stiffened" which could completely change the catalytic function of the protein. This suggests that, at least in some cases, the c-onc gene may require a mutation to convert it to a form which encodes a transforming protein. This example of an altered gene is supportive of the hypothesis that acutely transforming viruses may exert their oncogenic effect by transmitting this altered gene to other cells where it may be inappropriately, continuously, expressed through the enhancing and promoting activity of the viral LTR. Another hypothesis to explain the oncogenic potential of acutely transforming viruses is that they induce excessive amounts of the oncogene coded protein. This gene-dosage model is supported by evidence both from examination of Rous sarcoma virus (RSV) transformed cells which contain a 100-fold increase in the oncogene (src) coded protein kinase over normal cellular levels (Collett et al., 1978), and by experiments in which a normal c-onc gene attached to a retroviral LTR has been transfected into fibroblasts causing transformation (Chang et al., 1982).

Models involving inappropriate or enhanced expression of a cellular transforming gene could also apply to non-viral induced cancers if regulatory sequences controlling c-onc expression were mutated by radiation or other known chemical carcinogens or if c-onc sequences were moved to sites where their expression was placed under altered and active transcriptional control by such events as chromosomal translocation. Support for this model arises from the observations that certain cellular oncogenes cluster in chromosomal regions which are involved in specific rearrangements in leukaemias and lymphomas (Klein, 1981). Similar models have been applied to explain the oncogenic potential of non-acutely (weakly) transforming leukaemia viruses like feline leukaemia virus

(FeLV) and avian leukosis virus (ALV) which do not carry a transforming gene and induce tumours only after a long latent period. Avian leukosis virus in avian B-cell tumours has been shown to integrate next to a cellular oncogene, designated c-myc, and enhance its level of transcription many fold over the normal expression rate by initiation or enhancement of transcription from the viral LTR through the adjacent (c-myc) cellular sequences (Neel et al., 1981). This mechanism of insertional mutagenesis (Fig. 3) is not restricted to the avian system or to B-cell tumours since recently in the cat, FeLV provirus has been found to integrate close to the c-myc locus in a proportion of T-cell tumours (Neil et al., 1984).

A radically different model for the role of retroviruses in T-cell neoplasia has been proposed by McGrath and Weissman (1979). In murine T-cell tumours caused by recombinant murine leukaemia viruses (MuLV), they have suggested that an auto-stimulation model may operate. This model proposes that neoplastic growth can be initiated through a continuous mitogenic signal resulting from repeated presentation of viral antigens to antigen-specific receptors on the surface of cells infected with recombinant MuLV. To date, the receptor responsible for virus binding by leukaemic blast cells has not been clearly identified, neither has its potential for recognition of virus as a mitogenic signal been assessed, which will be essential to establish this theory. Finally, some neoplastic T-cell lines have been shown to have altered production of, and response to, certain growth factors which control the growth and differentiation of normal T-cells (Gootenberg et al., 1981).

Growth factors

Growth factors were first implicated in transformation by DeLarco and Todaro (1978) who, when investigating the properties of murine sarcoma cells, found that they produced a factor, designated transforming growth factor (TGF) which, when plated onto normal cells, caused morphological transformation. T-cell growth factor

(TCGF) or Interleukin-2, (IL-2) as it is also known, plays a fundamental role in the regulation and interaction of normal T-cells, and it has been proposed that a loss of control in this system could lead to the continuous proliferation of T-cells and finally neoplasia (Gillis et al., 1981; Mier and Gallo, 1981). The regulation of T-cell growth has been extensively investigated and an understanding of the TCGF interactions involved in the normal system has facilitated the study of the role that growth factors may play in tumorigenesis.

Regulation of normal T-cell growth

T-cell activation and proliferation is controlled by a complex series of interactions between distinct subsets of T-cells, antigen or mitogen and two interleukins, namely, the macrophage derived factor Interleukin-1 (IL-1) (Oppenheim and Gery, 1982) and the T-cell derived T-cell growth factor, Interleukin-2 (IL-2) (Fig. 4). The most usual mitogens used in T-cell activation studies are plant lectins, normally phytohaemagglutinin (PHA) or Concanavalin A (Con-A) which can substitute for antigen in the accessory cell signal. Accessory cells (macrophages) which are antigen pulsed provide two distinct signals for T-cells inducing them to release IL-2; firstly antigen, presented in the context of proteins of the major histocompatibility complex (MHC) and secondly IL-1 (Smith, 1980). The helper T-cell subset is the major source of IL-2 although all subclasses of T-cells can be induced to produce this factor (Pfizenmaier et al., 1984). IL-2 will bind to any responder T-cell which has been activated (by antigen and possibly one or more interleukins) to express IL-2 receptors, regardless of its subclass or antigenic specificity (Schreier et al., 1980). The binding of IL-2 to a receptor bearing T-cell in this manner induces the proliferation of this cell by causing the cell to progress from the late G1 phase of the cell cycle into the S phase (Klaus and Hawrylowicz, 1984) with a concomitant induction of receptors for transferrin (Neckers and Cossman, 1983). The level and duration of the response to IL-2 is dependent on the concentration of IL-2 present, the number of IL-2 receptors per cell and the continued

expression of the receptors on the cell surface. It now seems clear, from various lines of evidence, that the induction and down-regulation of the high affinity IL-2 receptor mediates the physiological role of IL-2 in T-cell proliferation and is central in controlling the extent of the cellular response (See Robb, 1984 for review).

Other activities of IL-2 in immune responses

The crucial role of IL-2 as a growth factor for activated T-cells is undisputed but recent evidence suggests that this is not its only role and that its activity extends to non-T-cell immune responses (Fig. 5). Farrar et al. (1982) demonstrated the ability of IL-2 to induce secretion of γ-interferon (IFNγ) by T cells. IFN has many effects on immune functions including that on natural killer (NK) cell activity, modulation of the expression of histocompatibility antigens, macrophage activation and the generation of cytotoxic T-cells. Other studies have shown that IL-2 can also play a direct role in stimulating non-T-cell immune responses by causing an increase in NK cell and lymphocyte activated killer cell (LAK) activity beyond that attributable to induction of IFN γ (Henney et al., 1981). In addition, T-cell lines have recently been induced to produce B-cell growth factor (BCGF-1) when stimulated with purified IL-2. This B-cell growth factor induces B-cells to both grow and differentiate to antibody secretion and evidence suggests that the same high affinity IL-2-receptor-ligand interaction is responsible, at least in part, for both the IL-2 induced cell cycle transitions and lymphokine secretion (Howard et al., 1983).

Production and purification of IL-2

Morgan et al. (1976) first demonstrated the presence of a T-cell derived growth factor in crude PHA stimulated lymphocyte conditioned medium (Ly-CM) and its ability to maintain normal human lectin-activated T-cells in continuous proliferative culture. Since then IL-2 has been extensively purified to homogeneity to

enable biochemical and biological characterisation to take place (Gillis et al., 1982). Purification schemes vary in detail but are basically as follows: the crude Ly-CM is first filtered to remove gross debris, then concentrated by ammonium sulphate precipitation. Affinity chromatography can be used to remove contaminating lectin (Fagnani and Braatz, 1980) after which various methods of chromatography and electrophoresis can be applied to remove the majority of remaining contaminating proteins (Mochizuki et al., 1981). These purification procedures are extensive and require large amounts of starting material in order to recover sufficient purified material for analysis. To this end, spontaneous lymphoma lines from murine, human and primate sources, have been established which constitutively produce IL-2, when stimulated with mitogen or antigen (Smith et al., 1980). To maximise the yield of IL-2 from tumour producer lymphoma lines, several investigators have fused high yielding producer lymphoma lines with allo-antigen activated T-cells (Stull and Gillis, 1981). These stable hybridoma lines can be stimulated repeatedly with subtoxic concentrations of lectins to secrete their biologically active IL-2 in relatively large amounts. Furthermore, T-cell hybridomas release a restricted set of lymphokines, mitogen-free, therefore reducing contaminating molecules, an advantage previously displayed by spontaneous producer tumour lines in culture. Recently, Altman et al. (1982) have shown that hybridoma lines can be successfully grown in vivo and retain their ability to produce IL-2 in vitro. Another approach to the production of IL-2 has been the use of Xenopus oöcytes to translate lymphocyte mRNA into biologically active IL-2 (Bleackley et al., 1981). Like the use of producer tumour cell lines this provides IL-2 free from lectin and other major contaminants but generally in very small amounts, about 1% of that from the original line. These problems of quantity and purification have now been overcome by the successful cloning of the human IL-2 gene by Taniguchi et al. (1983) and similar clones are also now available for generating large amounts of pure IL-2 suitable for characterisation at the molecular level.

The IL-2 molecule

Characterisation data reveals that human, gibbon ape and rat IL-2 are single polypeptide chains with molecular weights (mol.wt.) of 19-22000 on gel-filtration chromatography and 15-17000 mol.wt. on SDS-PAGE (Mier and Gallo, 1981; Robb and Smith, 1981). Murine IL-2 appeared at first to differ, being a molecule of 30-40000 mol.wt. on gel filtration but this has now been attributed to non-covalent dimerization of a 15-17000 mol.wt. sub-unit. IL-2 from each of the species is hydrophobic and all have almost equivalent specific activities. Furthermore, evidence from cross-reactivity studies using monoclonal antibodies to the various IL-2s showed that the molecules displayed specific structural features, clearly demonstrating the homology of IL-2 between these species (Paetkau *et al.*, 1984). IL-2 is heterogeneous with respect to size and charge which is due to post-translational glycosylation with a variable sialic acid content. This seems to have little consequence for *in vitro* activity although it may play a role in *in vivo* clearance of the molecule (Robb and Smith, 1981). Human IL-2 has been most extensively characterised and it is now known to be a polypeptide consisting of 133 amino acids with a 20 amino acid signal sequence which is removed prior to secretion (Taniguchi *et al.*, 1983; Robb and Smith, 1981). The polypeptide contains a single intra-molecular disulphide bridge, connecting the cysteines in positions 58 and 105, which is essential for the active configuration of the molecule (Robb, 1984). The human gene for IL-2 is present in single copy per haploid human genome and is located on chromosome 4q. The cat gene for IL-2 is also present in single copy per haploid genome and is isolated on chromosome B1 (Seigal *et al.*, 1984). The IL-2 gene is interrupted by 3 introns and the promoter sequences in the 5' region of the gene contain a prototype "TATA" box. Two points of particular note are: that sequences upstream from this point show remarkable homology to the enhancer region of the IFN-γ gene, (Paetkau *et al.*, 1984) whose expression is under similar control, and also that sequences that closely resemble the core sequences for viral enhancer elements (Olabuenaga *et al.*, 1983) have been found in the second intron

(Fujita et al., 1983). Although the mechanism of gene expression in the suggested "cascade" interactions of the lymphokines remains to be elucidated, (Yamamoto et al., 1982) these observations suggest that the common sequences detected may be associated with tissue specific transcription of lymphokine genes by T-cells on stimulation with antigen or mitogen.

The role of IL-2 in T-cell neoplasia

The possible involvement of IL-2 in T-cell neoplasia of the cat is the subject of Chapter Four. Similar studies on human leukaemias and lymphomas have recently proven rewarding. IL-2 and its interaction with its membrane receptor, is central to the regulation of normal T-cell proliferation (Robb, 1984) therefore the aberrant or inappropriate expression of IL-2 in T-cells could theoretically contribute to their progression into, or maintenance of, the transformed state. Gallo and Wong-Staal (1982) proposed that the HTLV provirus present in adult T-cell leukaemias (ATLs) could activate cellular IL-2 genes resulting in the continuous auto-stimulation of membrane IL-2 receptors and hence uncontrolled proliferation of the cells. This hypothesis initially seemed likely since T-cells from patients with HTLV-positive leukaemias and lymphomas were reported to bind, and in some cases release, IL-2 in culture (Poiesz et al., 1980b; Gootenberg et al., 1981). However, further binding studies revealed that the modulation of the IL-2 receptor (designated Tac) by the anti-Tac monoclonal antibody, observed with normal activated human T-cells (Tsudo et al., 1982) did not occur with ATL cells (Tsudo et al., 1983). This led directly to the proposal that it was the constitutive expression of the Tac receptor on ATL cells and not their hyperproduction of IL-2, that was primarily responsible for the uncontrolled growth of these cells (Yodoi et al., 1983; Gallo et al., 1983). The human IL-2 receptor has recently been molecularly cloned (Nikaido et al., 1984; Leonard et al., 1984) and this should help to elucidate the mechanism of leukaemogenesis, at least of human ATL.

The search for a T-cell leukaemia virus of man was a direct consequence of the study of oncogenic retroviruses in neoplastic conditions of animals. In this respect the feline model has been extremely important in the study of haematological neoplasia because of the association of feline leukaemia virus (FeLV) with this disease. The feline system represents a natural as well as an experimental disease model and enables the assessment of the degree to which the variety of molecular mechanisms identified in experimental systems are involved in spontaneous tumours in a randomly outbred species.

Transmission and epidemiology of FeLV

FeLV was originally isolated from cats with lymphosarcoma (Jarrett et al., 1964) but is, in fact, associated with a whole spectrum of both neoplastic and non-neoplastic diseases of the domestic cat. In nature, the non-neoplastic conditions resulting from virus infection, such as reproductive failure and immunosuppression, are more commonly encountered than are those of the leukaemogenic effects of the virus. Transmission of virus occurs both horizontally, principally through saliva and congenitally, across the placenta (Hardy et al., 1973; Jarrett et al., 1973). The epidemiology of FeLV in the cat population reveals two distinct patterns dependent on whether the cats are maintained in closed multiple cat households (MCH) or are free range. In a closed MCH, into which FeLV is introduced, the incidence of persistent infection is high (about 40%), probably due to the increased incidence of exposure to relatively high doses of virus at an early age. These circumstances are those under which most FeLV related diseases occur (Hardy et al., 1973; Jarrett et al., 1978). The prognosis for persistently infected cats is poor; approximately 90% die within five years of infection compared to 15% of virus-negative cats in the same environment (McClelland et al., 1980). The majority of the cats in these closed households, however, become transiently infected, that is, they appear to recover from the infection and about two-thirds of these produce virus neutralising antibodies which are believed to render the cats

resistant to reinfection (Hardy et al., 1976; Russell and Jarrett, 1978a). In contrast, free-range cats show a much lower incidence of persistent infection (less than 1%) and only a small proportion have neutralising antibodies which appear only to develop following a reasonable degree of virus growth in the cat. Consequently free-range cats manifest fewer FeLV related diseases. The difference observed in the outcome of exposure to virus in these two situations can be explained by the fact that cats become more resistant to virus infection with age, the cut-off being about six months after which time the cat is resistant to infection unless a predisposing immunosuppression occurs concomitantly. Free-range cats are probably exposed to virus when they are older than cats exposed in multicat households, therefore they will be more resistant to infection by FeLV at that time. Furthermore, in multicat households contact between cats is intimate and constant so that far larger doses of virus will be transmitted between cats in closed households than in the free-range situation. It has recently been reported (Rojko et al., 1982; Madewell and Jarrett, 1983) that FeLV has the ability to form a latent infection even in the presence of a virus neutralising antibody response. Treatment with immunosuppressive agents induces the expression of these latent viruses causing viraemia and the release of infectious virus particles in the saliva of cats. Latently infected cats may represent an important reservoir of infectious virus in the population and, up until recently, would have appeared to be virus-negative using standard diagnostic assays.

Subgroups of FeLV

FeLV isolates can be classified on the basis of interference subgroup. The basis of this is that cells previously infected with a virus of one subgroup are resistant to superinfection with a virus of the same subgroup. Sarma and Log (1973) demonstrated three subgroups of FeLV, designated A, B and C, using the interference system. Interference subgroup is determined by the viral envelope (env) gene product and reflects the cell surface receptors utilized by FeLV to infect a host cell. Although each

subgroup can be separated as non-defective components, in nature, subgroups B and C are always found in association with subgroup A (Sarma and Log, 1973; Jarrett, 1980). Experimental studies have shown that the efficient replication of subgroups B and C *in vivo*, and the transmission of subgroup B, are dependent on the concomitant presence of the subgroup A of FeLV (Jarrett and Russell, 1978a). The possible association of FeLV subgroup with disease is discussed further in Chapter Five.

T-cell lymphosarcoma of the cat

The most common neoplastic disease associated with FeLV is T-cell lymphosarcoma. Most tumours of this type are virus-positive unlike the B-cell alimentary lymphosarcoma which are equally common but are virus-negative. Two common forms of T-cell lymphosarcoma are seen; thymic lymphosarcoma, where the tumour mass is predominantly at a single site; that of the thymus, and multicentric lymphosarcoma in which multiple lymph nodes are involved.

Certain strains of FeLV, notably Rickard (Rickard *et al.*, 1969) have been shown to produce thymic T-cell lymphosarcoma *in vivo* after a short period of time, usually 3-4 months (Rojko *et al.*, 1979). The mechanism of transformation by Rickard and related viruses is not understood but several mechanisms of induction of neoplasia, previously discussed in this Introduction, like viral induction of a growth factor or oncogene activation, are possible.

The present study

The experiments described in the following chapters were designed to determine the role of growth factor and oncogenes in the pathogenesis of FeLV induced thymic T-cell lymphosarcoma, both in an experimental system and in tumours occurring spontaneously in the cat population. The growth properties of the cell cultures obtained from the tumours were examined for alterations in the requirement for, or response to, exogenous IL-2 to determine if an

auto-stimulation type of model relating to growth factor could be involved in the generation or maintenance of neoplasia in these cats (Chapters Three and Four). The properties of the viruses associated with the tumours and also circulating in the plasma of the cats were assessed with respect to the subgroup of virus present, in that it has previously been observed than an additional subgroup other than A is more often present in diseased cats than in their normal healthy counterparts (Jarrett, 1980). The association of subgroup with tumours in this series is discussed further in Chapter Five.

Finally, in collaboration with Dr. J. Neil, the involvement of oncogenes in thymic lymphosarcoma has been investigated. Tumour DNA was examined to determine if the c-myc locus was re-arranged by FeLV (indicative of insertional mutagenesis). In order to determine if the infecting FeLV was integrating at a preferred site, the flanking sequences of host DNA adjacent to each provirus must be determined. Since the task of preparing lambda libraries of each integrated provirus is enormous, a tumour was selected (designated 84793) which had a low copy number of integrated proviruses (in this case 3). Two of the three proviruses were successfully cloned by Dr. J. Neil and the flanking sequences of these were analysed initially by screening with known oncogenes. It was hoped that one or more of these probes may have proven positive but a lack of detectable hybridization would not have ruled out the model of insertional mutagenesis as the sequences involved may have been of a novel oncogene. Surprisingly, however, one of the proviruses (pCT4) appeared not to be inserted within or close to a cellular oncogene but in fact to be a defective recombinant virus containing myc sequences within the proviral genome (Neil et al., 1984). This began the search in other tumour DNAs for similar recombinant viruses and also revealed upstream integration of FeLV adjacent to c-myc in some cases. The in vitro properties of these novel feline leukaemia viruses and their oncogeneic potential in vivo are the subjects of Chapters Five and Six respectively.

The elucidation of the pathogenesis of T-cell neoplasia in the cat will be of considerable value in two main respects. Firstly, it will contribute significantly to the understanding of the complex process of neoplasia in general and secondly, as the discovery of retroviruses in animals directly led to the detection of those of man, so may the determination of the role that FeLV may play in the generation of T-cell tumours of the cat, direct the investigation of the role that HTLV may play in the generation of T-cell tumours of man.

FIGURE 1.

Replication of retroviruses

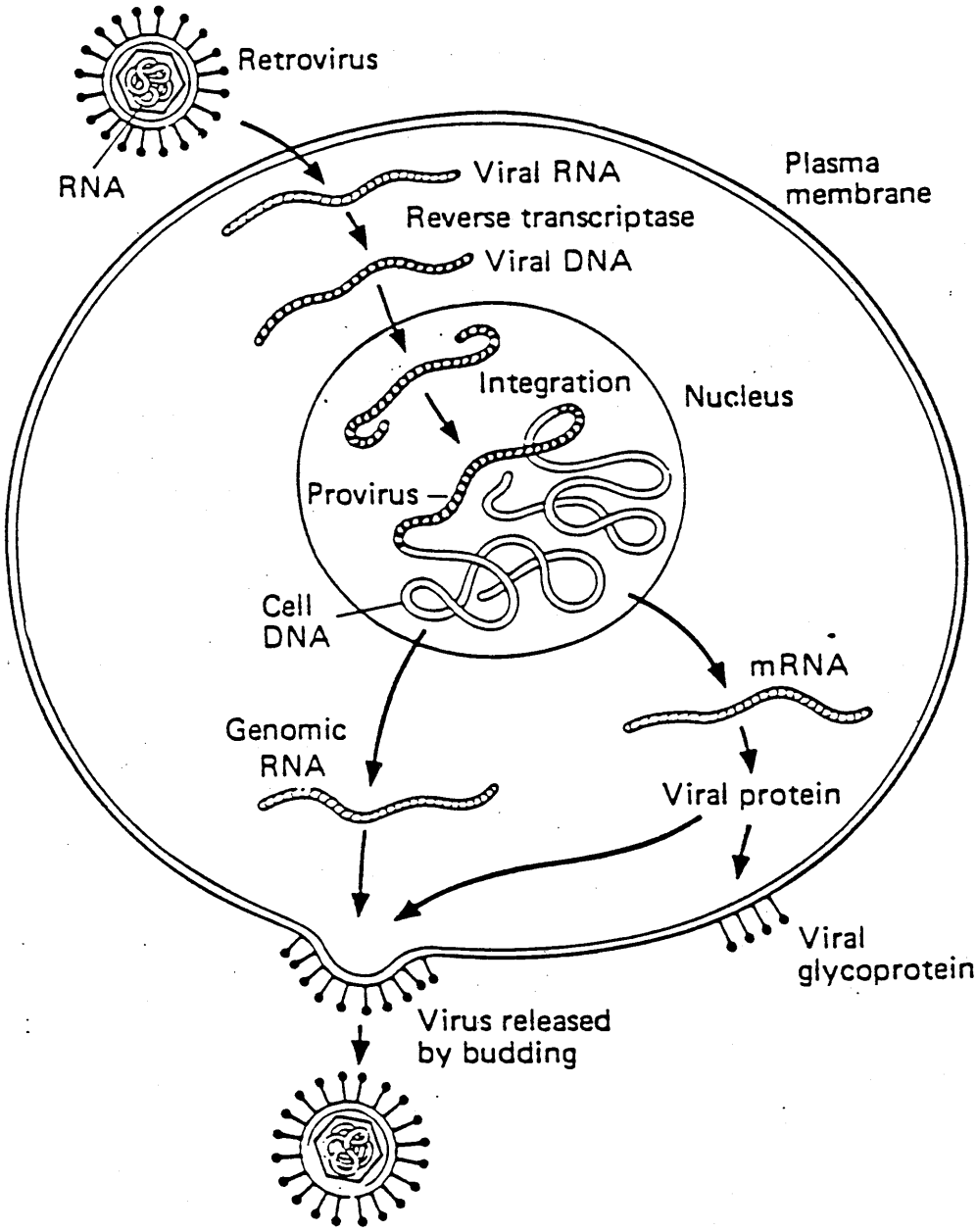


Figure 1 is a schematic representation of the process of retroviral replication. The individual steps are described in the text.

FIGURE 2.

Structure of proviruses

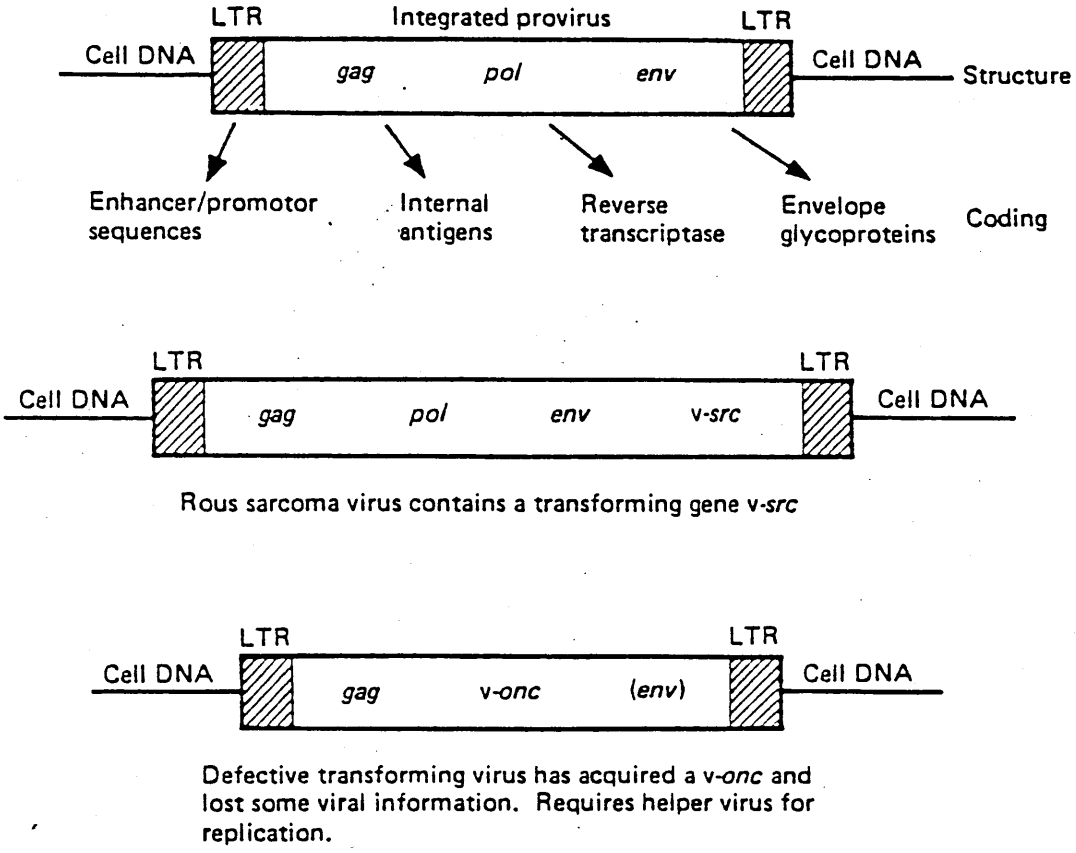


Figure 2 shows the 3 possible structures of the proviral genome. The structure of Rous sarcoma virus is unusual in that it has acquired a v-onc gene without concomitant loss of viral information. This transforming virus is unique in this respect and is said to be 'non-defective' (does not require helper virus for replication.)

FIGURE 3.

Model of insertional mutagenesis

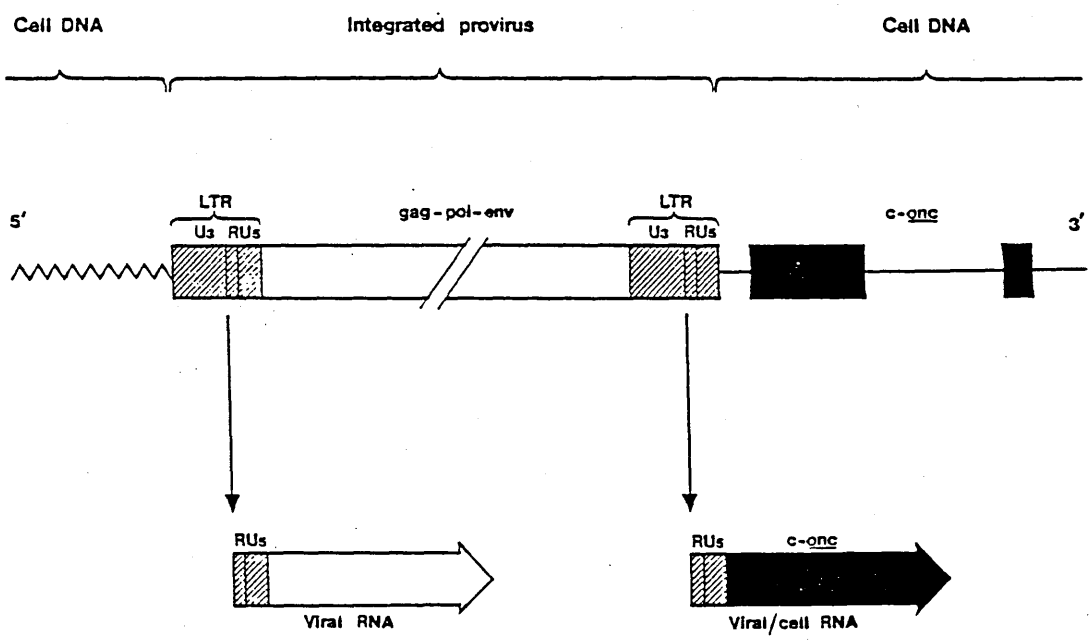


Figure 3 depicts a possible model for insertional mutagenesis. Here the virus has integrated adjacent to the cellular-onc gene and in the same transcriptional orientation. In this orientation it is possible to generate a hybrid transcript, initiating in the 3' viral LTR and carrying on into the c-onc coding sequence. This process will be under the transcriptional control of the viral LTR and may result in the enhanced or inappropriate expression of the c-onc protein.

FIGURE 4

The generation of IL-2 and its principal role as a growth factor inducing cytotoxic T-cell proliferation

MØ, macrophage; T-act, activated T cell; T-resp, responsive T cell; IL-1, interleukin 1; Ag, antigen; MHC, major histocompatibility complex proteins.

FIGURE 4.

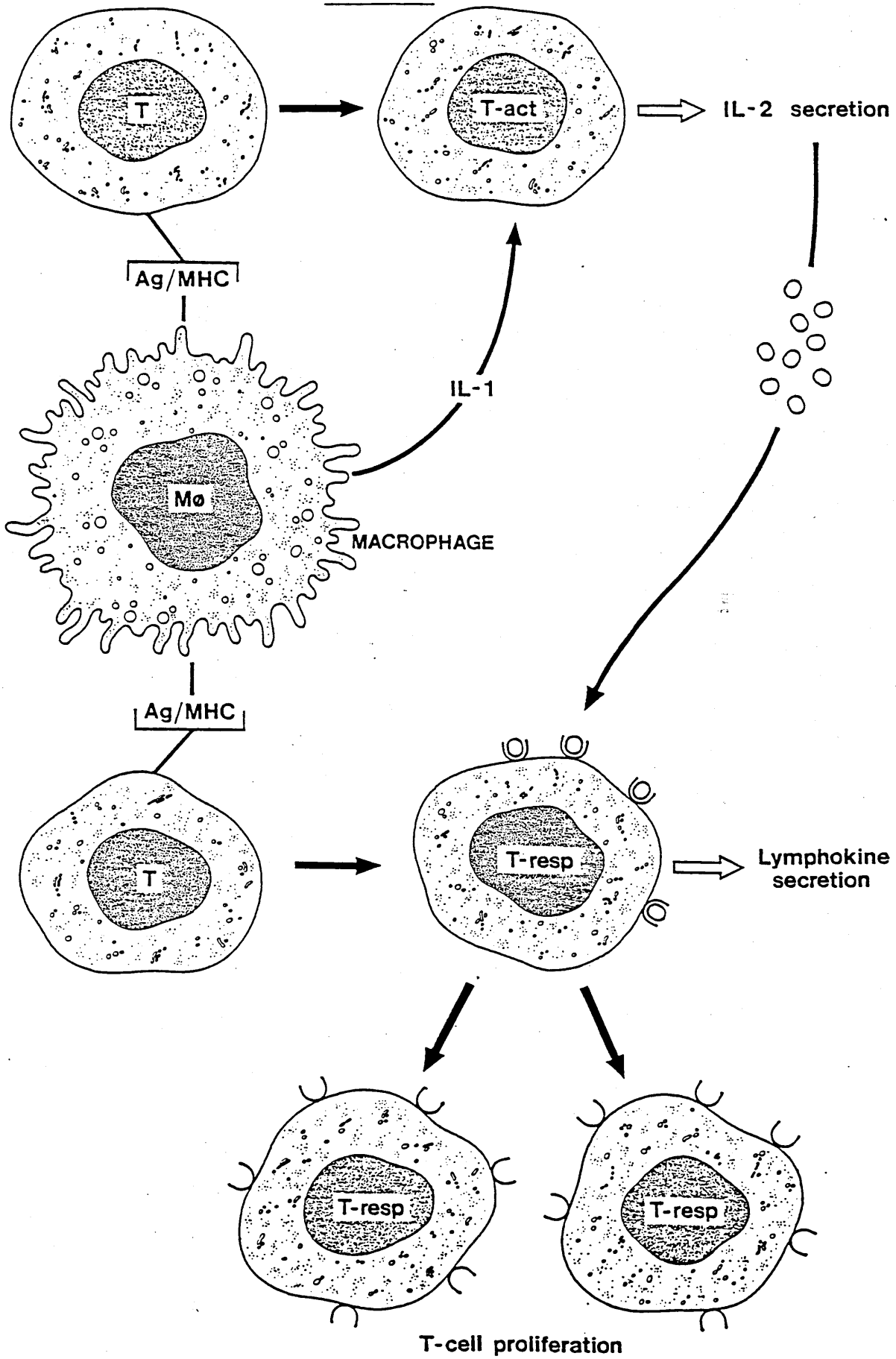
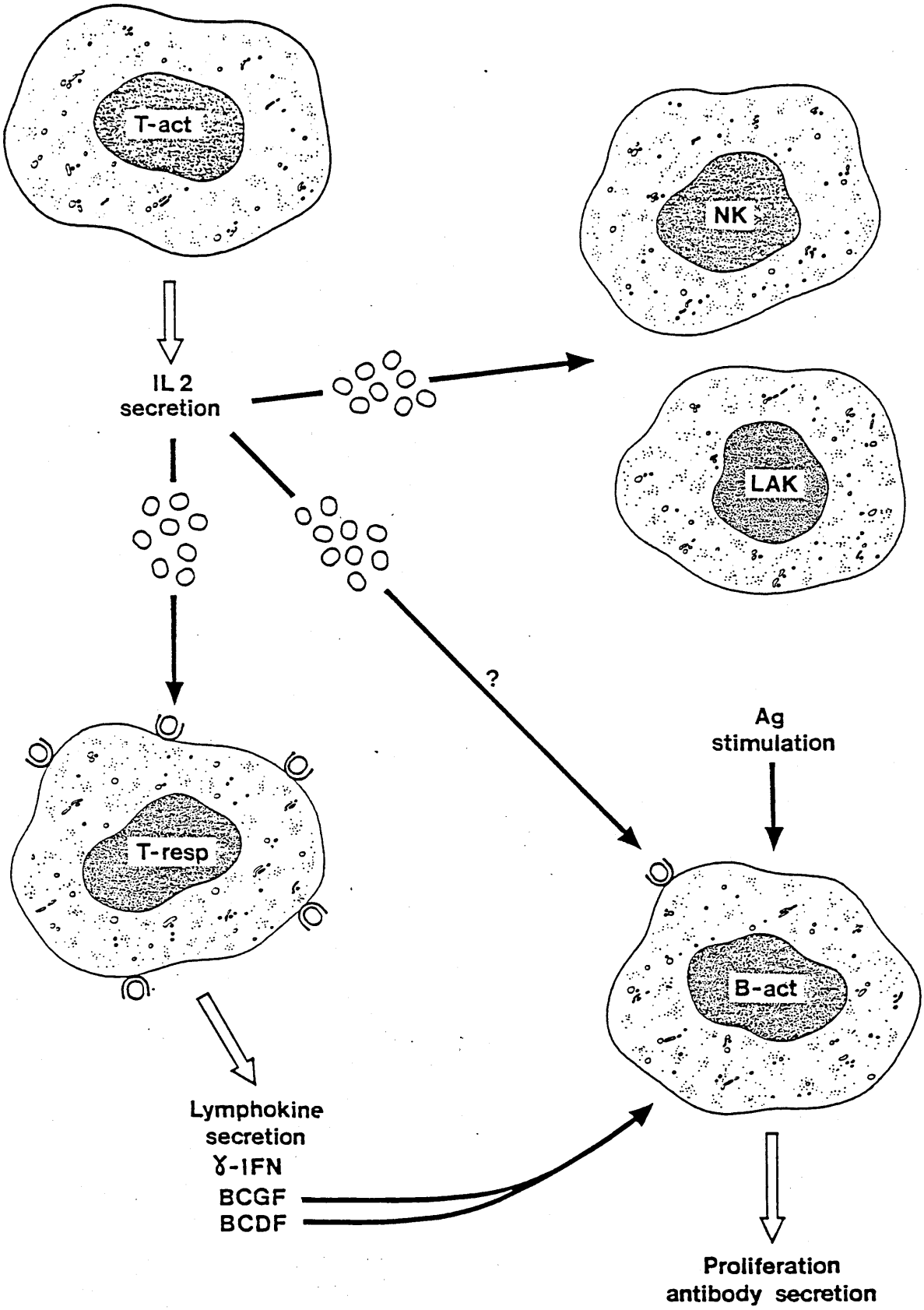

T
T-act
IL-2 secretion
Ag/MHC
IL-1
Mø
MACROPHAGE
Ag/MHC
T
T-resp
Lymphokine secretion
T-resp
T-resp
T-cell proliferation

FIGURE 5.

Involvement of IL-2 in non-T-cell immune responses

T-act, activated T-cell; T-resp, IL-2 responsive T cell; NK, natural killer cell; LAK, lymphocyte activated killer cell; B-act, activated B cell; γ-IFN, immune (gamma) interferon; BCGF, B cell growth factor; BCDF, B cell differentiation factor; Ag, antigen.

FIGURE 5.


T-act
NK
IL 2
secretion
LAK
?
Ag
stimulation
T-resp
B-act
Lymphokine
secretion
γ-IFN
BCGF
BCDF
Proliferation
antibody secretion

CHAPTER ONE

GENERAL MATERIALS AND METHODS

The materials and methods detailed below are used repeatedly throughout the experiments in the following chapters. Any method specifically relating to work in an individual chapter is detailed in the materials and methods section of that chapter.

Culture of monolayer cells

Cells

Feline leukaemia virus is routinely grown in feline embryonic fibroblasts. Feline embryo (FE) cells of the FEA strain (Jarrett et al., 1973), uninfected or infected with FeLV (FEA + FeLV), were used between passages 13 and 40.

Clone 81 (c81) cells derived from the Crandell feline kidney cell line (CCC) contain the genome of Moloney MSV and were used in the FEA/c81 virus detection assay (Fischinger et al., 1974).

Media

The monolayer cells FEA and c81 were maintained in Dulbecco's Minimum Essential Medium (Gibco Biocult Ltd) supplemented with 2mM L-glutamine, 400 units/ml penicillin-streptomycin and 10% foetal bovine serum (FBS) (Gibco Biocult Ltd) and the complete medium is referred to as 10% DMEM.

Cell Culture

Stock monolayer cells were passaged in 250 ml glass bottles with 15 ml of medium or in 2.5 litre rotating bottles with 100 ml of medium. Cells were routinely subcultured 1:4 twice weekly when they were confluent. The cells were removed from the glass by rinsing with versene followed by 0.01% trypsin in 0.02% EDTA obtained from the Institute of Virology, University of Glasgow. Resuspended cells were transferred to fresh bottles and gassed with 5% carbon dioxide in air.

For virus detection by the FEA/c81 assay and for determination of virus subgroup by interference, cells were grown in 5 cm diameter plates (Nunclon) with 4 ml of medium.

Culture of suspension cells

Cells

Two feline tumour T-cell lines were available. They grow as suspension cells, the first of which, the FL74 cell line, originated from a lymphoid tumour in the kidney of a kitten infected with FeLV (Theilen et al., 1969) and continuously release FeLV-ABC/KT.

F422 cells were established by Rickard (1969) from a thymic lymphosarcoma of a kitten inoculated with cell free thymic tumour homogenate. These cells continuously release a subgroup A virus.

Media

The suspension cell lines FL74 and F422 were maintained in medium consisting of equal volumes of Liebovitz L-15 and McCoy's 5A media (Gibco Biocult Ltd.) supplemented with 2mM L-glutamine, 400 units/ml penicillin-streptomycin and 10% FBS. The complete medium is referred to as L/M.

Cell Culture

Suspension cells were grown in stationary cultures at densities of 5×10^5 - 3×10^6 per ml. They were subcultured twice weekly by removing 3/4 of the suspension and replacing with an equal volume of fresh L/M.

The culture of both normal T-cells and other tumour derived T-cells is described in detail in materials and methods in Chapters Two and Three respectively.

Virus purification

Virus was purified and concentrated to high titre for use in some *in vivo* infections discussed later and also for preparation of viral RNA for molecular analysis.

Clarification

Culture supernatants were collected and centrifuged at 10,000 x g for 10 minutes. They were then filtered through a 0.45 μm Nalgene sterile filter and, if necessary, stored at this stage at -70°C.

Concentration

Two methods of virus concentration were used.

(i) Ultrafiltration (Amicon Hollow fibre concentrator Model CH4). The clarified supernatants were concentrated up to 50-fold by passage through a hollow fibre cartridge (Amicon Diaflo hollow fibre cartridge, nominal molecular weight cut-off 1,000,000). In some instance the concentrated virus was then further purified by density gradient centrifugation and pelleted.

(ii) Ammonium Sulphate Precipitation

Equal volumes of clarified supernatant and saturated ammonium sulphate (pH 7.4) were mixed and incubated at 4°C for 30 minutes. The mixture was then centrifuged at 10,000 x g for 10 minutes and the precipitate resuspended in 1/10th original volume of TS buffer (0.1M NaCl, 0.1 M Tris-HCl, 0.001 M EDTA, pH 7.4).

Density gradient centrifugation

The concentrated virus was then further purified by ultracentrifugation in discontinuous sucrose gradients. Gradients were made up in Beckman SW41 rotor tubes by overlaying 0.5 ml of

50% sucrose in TS buffer with 2 ml of 30% sucrose in TS buffer. The tubes were filled with concentrated virus and centrifuged at 40,000 rpm for 45 minutes after which time the virus had banded at the interface of the two solutions. The bands were collected by puncturing a hole in the bottom of the tubes and collecting the fractions by visualizing the light-scattering virus band with a strong light source.

Pelleting

The purified virus collected was diluted in TS buffer and centrifuged at 35,000 rpm for 30 minutes in a Beckman SW41 rotor. The virus pellet was either resuspended for further analysis or stored at -70°C until required.

Assay of FeLV

FeLV was assayed by focus formation in c81 cells (Fischinger et al., 1974). The assay used was a modification of this (Jarrett et al., 1982) and was carried out as follows: 3×10^5 FEA cells and 3×10^4 c81 cells were seeded onto 5 cm plates in 4 ml of 10% DMEM. The next day the virus in 1 ml of medium containing 4 µg/ml polybrene was adsorbed for 2 hours at 37°C. The virus was then removed and replaced by 4 ml of fresh medium. That medium was changed three days later and foci of transformed cells were counted under the microscope 7 days after infection. The titre of the virus obtained by this method was expressed as focus forming units (FFU) per ml.

Preparation of RNA from virus

RNA was prepared from purified virus as follows: to inhibit nuclease damage to the RNA, pelleted virus was disrupted in a modified 4M guanidinium isothiocyanate buffer described by Chirgwin (1979). The buffer used consists of: 4.2M guanidinium isothiocyanate, 17mM Na-N-lauroyl sarcosine, 25mM sodium citrate (pH 7.0), 100 mM β-mercaptoethanol and 0.33% anti-foam A. Carrier

yeast t-RNA (40 µg-100 µg) was added and the RNA layered over 1-2 ml of 56% caesium chloride (CsCl) in TS buffer, in Beckman SW41 rotor tubes. The RNA was pelleted by ultracentrifugation at 35,000 rpm for 20-24 hours, after which time the pellet was resuspended in 200 µ - 500 µ of a solution containing: 10mM Tris-HCl (pH 7.4), 5mM EDTA (pH 8.0), and 1% SDS. The RNA was then extracted once with a 4:1 mixture of chloroform and 1-butanol and the aqueous phase transferred to a fresh tube. The organic phase was re-extracted with an equal volume of the Tris-HCl buffer (pH 7.4). The two aqueous phases were then combined and 0.1 volumes of 3M sodium acetate (pH 5.2) and 2.2 volumes of ethanol added. This was stored at -20°C for a minimum of two hours to precipitate the RNA which was then recovered by centrifugation at 10,000 rpm for 10 minutes. The pellet obtained was dissolved in 1 ml of reverse osmosis distilled water (RODW) and reprecipitated with ethanol. The RNA was stored in 70% ethanol at -70°C until required.

Isolation of RNA from tumour material

Tumour tissue taken for analysis of RNA was flash frozen in liquid nitrogen. To prepare RNA from material stored in this way, the frozen tissue was placed directly into the 4M guanidinium isothiocyanate buffer and minced using scalpels. It was then homogenised briefly and clarified by centrifugation at 10,000 rpm for 10 minutes. The supernatant was layered onto discontinuous CsCl gradients, consisting of 2 ml of 51% CsCl on top of 2 ml of 56% CsCl, and ultracentrifuged at 35,000 rpm for 20-24 hours. The pellet was recovered, extracted and stored according to the protocol for isolation of RNA from virus, previously described (Maniatis et al., 1982).

Isolation of DNA from infected and uninfected FEAs

FEA cells (1×10^9 cells) were removed from 2.5 litre glass bottles by standard procedure and washed once in phosphate buffered saline (PBS). The cells were then resuspended in TNE (10 mM Tris-HCl pH 8.0, 100 mM NaCl, and 10 mM EDTA) which contained 0.5%

SDS and 250 µg/ml proteinase K (Boehringer). This was incubated at 37°C for a minimum of three hours after which time the DNA was extracted by gentle mixing for 10 minutes with an equal volume of phenol pH 8.0. The aqueous phase was then removed to a fresh tube and re-extracted once more with an equal volume of phenol after which the aqueous phase was extracted twice with an equal volume of chloroform then dialysed against TE (10 mM Tris-HCl pH 7.9 and 1 mM EDTA) at 4°C with 2-3 changes of buffer. Ribonuclease A, at a concentration of 100 µg/ml was then added to the DNA and incubated at 37°C for a minimum of three hours. The DNA was then extracted exactly as before and dialysed extensively against TE buffer at 4°C, and stored at 4°C until required.

CHAPTER TWO

THE PRODUCTION AND ASSAY OF T-CELL GROWTH FACTOR (IL-2)

Introduction

A source of feline interleukin-2 (IL-2) was considered essential for three principle reasons: (i) to develop a system for establishing cell cultures from tumours enabling the study of the properties of the viruses associated with them, (ii) to determine if these cultured neoplastic T-cells had altered requirements for, or response to IL-2, *in vitro* and (iii) to maintain normal T-cells in culture as targets for potentially leukaemogenic viruses.

IL-2 is predominantly produced by mature helper T-cells which have been activated by antigen or mitogen and interleukin-1 (IL-1). Since IL-1 is a mitogenic product of macrophages (Rosenstreich and Mizel, 1978) a good source of both mature T-lymphocytes and macrophages was required for optimal IL-2 production. In these experiments mononuclear cells were isolated from the spleens of cats and mesenteric lymph node cells were added to make up the final cell number required.

IL-2 has been produced from several sources. Human peripheral blood lymphocytes (PBL) optimally produce IL-2 when stimulated by 1% PHA with lower activity obtained using Con-A. (Alvarez *et al.*, 1979) which is the reverse of the situation with murine PBLs (Gillis *et al.*, 1978). These differences presumably reflect the distinctive mitogenic response to PHA and Con-A which are higher with PHA on human cells (Arala Charles *et al.*, 1978) and Con-A on rodent cells, (Stobo, 1972). It was, therefore, important to establish which mitogen was optimal for IL-2 production in the cat and this was one of the parameters determined in the first set of experiments. Cell densities used for IL-2 production vary between 1-5 x 10^6 cells/ml. In the human system, the maximal IL-2 production/cell in the culture is obtained using 1x 10^6 cells/ml. However if no purification is performed on the crude lymphocyte conditioned medium (Ly-CM) produced then the use of high cell numbers i.e. 5 x 10^6/ml is most effective for removing contaminating mitogen from the medium. In high density cultures peak IL-2 levels in the medium occur 18-24 hours after mitogen

stimulation, but for low density cultures it is between 48-60 hours. The optimum duration of culture for two different cell densities was studied and a blastogenesis assay, first described by Gillis et al. (1978) to determine the activity of the T-cell growth factor obtained, was optimised for use in the feline system.

The IL-2 produced was used for the culture of normal mitogen-activated T-cells which were derived from the thymus of specific pathogen free cats. The T-cells were characterised by their absolute dependence on T-cell growth factor for their continued proliferation in culture and by E-rosetting with guinea-pig red blood cells.

MATERIALS AND METHODS

Cells

Spleen and mesenteric lymph node cells, for production of Ly-CM, were obtained from specific pathogen free (SPF) cats. Spleen cells provide a good source of macrophages for IL-1 production, therefore the total spleen cells available were passed through Ficoll-Paque (Pharmacia Ltd.) to isolate the mononuclear cells and these were combined with mesenteric lymph node cells to a total number of 2.5×10^6 cells/ml for Ly-CM production.

Normal activated T-cells were derived from the thymuses of SPF cats and were cultured for a minimum of 4 weeks as detailed later in this section. These cells were used to assay the activity of IL-2 in routinely produced Ly-CM. The replication of normal activated T-cells, which have been in culture for a period exceeding 4 weeks, is absolutely dependent on an exogenous source of IL-2.

The cell lines FL74 and F422, previously described in Chapter One, were used to optimise the blastogenesis assay for use in this system.

Media

RPMI 1640 medium (Gibco Biocult Ltd.) supplemented with 2mM L-glutamine, 400 units/ml penicillin-streptomycin, 5×10^{-5}M. 2-mercaptoethanol (2-Me) and 20% FBS was used in equal volume with Ly-CM to maintain normal T-cells. The complete medium is referred to as 50:50 medium.

Iscove's modified Dulbecco's medium (Gibco Biocult Ltd.) supplemented with 400 units/ml penicillin-streptomycin, 5×10^{-5}M 2-Me and 0.25% bovine serum albumin (BSA) was used for production of Ly-CM and the complete medium is referred to as 0.25% Isc. Concanavalin-A (Con-A) (Miles Scientific) was the T-cell mitogen

used both in Ly-CM production and for activation of normal T-cells. It was made up to a stock solution of 1 mg/ml in RPMI 1640 medium supplemented with 400 units/ml penicillin-streptomycin and stored in aliquots at -20°C. The PHA used to determine the most potent mitogen available for IL-2 production was supplied by Gibco Biocult Ltd. and was made up to a stock solution in distilled water and used at a final concentration of 1%.

The production of Ly-CM (Figure 6)

Ly-CM was routinely produced by the following procedure: The spleen and mesenteric lymph nodes of an SPF cat were removed asceptically into 0.25% Isc containing 1% preservative free heparin (Paines and Byrne Ltd.) and rinsed twice in 0.25% Isc. The tissues were transferred independently into 9 ml petri dishes (Nunclon) and diced with scalpels in 0.25% Isc. The slurries were put into sterile plastic stomaching bags (Seward Surgical) and pulped in a Stomacher machine. The single cell spleen suspensions were then layered onto Ficoll-Paque and centrifuged at 2,000 rpm for 10 minutes after which time the mononuclear leucocytes had banded at the interface. These were washed with medium, counted, and made up to a total of 2.5×10^6 cells/ml using lymph node cells in 0.25% Isc. Con-A was added at a concentration of 7.5 µg/ml and the complete culture was incubated at 37°C for 24 hours, after which time the Ly-CM was harvested and clarified by centrifugation at 10,000 rpm for 15 minutes, sterile filtered through 0.22 µm Millipore membranes and stored in aliquots at -70°C.

The culture of normal feline T-cells

Normal T-cells were cultured from the thymus of SPF cats that were less than one year old. They were collected asceptically into RPMI 1640 medium, diced with scalpels then stomached once, briefly. The single cell suspension was washed in fresh medium and resuspended in RPMI 20% +Me at a concentration of 1×10^6 cells/ml. 7.5 µg/ml Con-A was added to activate the T-cells and the culture was incubated for 18 hours. The following day the the cells were

washed free of Con-A and resuspended in medium composed of equal volumes of RPMI 20% +Me and Ly-CM (referred to as 50:50 medium). The cells were maintained by sub-culturing twice weekly into fresh medium at a concentration of 5×10^5/ml.

The determination of the optimum labelling conditions for the blastogenesis assay

This assay was first developed by Gillis et al. (1978) and was described as a specific and quantitative microassay for T-cell growth factor. It is based on the proliferation, as measured by [^{3}H]-thymidine (Tdr) incorporation of a TCGF-dependent T-cell line. The assay was used to determine IL-2 activity in the Ly-CM by titrating the conditioned medium against normal T-cells grown in culture for a minimum of 4 weeks prior to use in the assay. The assay was also used to determine the initial response of tumour cells to TCGF (Chapter Three) and to determine the response of TCGF-independent cell lines to other regulatory factors (Chapter Four). The assay was optimised using FL74 and F422 cell lines which are TCGF independent. Two parameters were studied: (i) concentration of radioactive label used to pulse label the cells and (ii) duration of pulse label applied to the cells. The cells were washed and resuspended in fresh L/M at 5×10^5 cells/ml. 100 µl of cells were plated per microtitre well and the four conditions of label were applied for both four hours and overnight (standardised to 18 hours) on each of three consecutive days after which time the cells were harvested and counted.

Radio-active labelling

[6-^{3}H]-thymidine (Tdr) was obtained from Amersham at a specific activity of 2 Ci/mM and was diluted either in RPMI-1640 medium or in cold thymidine made up to a stock concentration of 1 mg/ml in RPMI-1640 medium and diluted in the same medium. The four conditions of label applied were: (i) 2Ci/mM specific activity; 0.5 µCi per well, (ii) 0.98 Ci/mM specific activity; 2µCi/well, (iii) 0.56 Ci/mM specific activity; 0.8 µCi/well and (iv) 0.32 Ci/mM specific activity; 1.6 µCi/well.

Harvesting the cells

The cells were harvested from the microtitre plates onto glass fibre filter papers using a Titertek cell harvester. The cells were flushed onto the filters using a 20 second pulse of deionised water followed by a 20 second pulse of air to partially dry the filter. The filters were completely dried for 30 minutes in a 37°C hot room then the individual wells on the filters were transferred to scintillation vials.

Scintillation Counting

The scintillation fluid used was Aqua-Luma which is toluene based and the samples were counted on a Packard Tri-Carb 300C liquid scintillation counter.

The determination of the optimum conditions for the production of IL-2 in Ly-CM

IL-2 activity obtained from Ly-CM was optimised with respect to three parameters: (i) Mitogen type and mitogen concentration; which were 1% PHA, 1 µg/ml Con-A, 5 µg/ml Con-A and 7.5 µg/ml Con-A. (ii) Cell density; cells were seeded at either 2.5 x 10^6/ml or 5 x 10^6/ml. (iii) Duration of culture; cultures were incubated at 37°C for either 24 hours, 48 hours or 72 hours. The cultures were set up in 24 well Costar plates and harvested on the appropriate day. IL-2 activity in the supernatants was determined using the standard blastogenesis assay with TCGF-dependent cultured normal T-cells as the target population.

The determination of IL-2 activity in Ly-CM by blastogenesis assay

The level of IL-2 activity present in each batch of lymphocyte-conditioned medium was assayed by incubating normal cultured feline T-cells with serial dilutions of Ly-CM. Normal T-cells were used at confluence and were spun down at 2,000 rpm for 5 minutes then resuspended in RPMI 20% + Me at 5 x 10^5 ml. 100 µl

100 µl of this suspension was plated in each well of a U bottom microtitre plate (Nunclon) and an equal volume of Ly-CM, serially diluted in RPMI 20% + Me, was added and the plate incubated at 37°C overnight (16-18 hours). The next day 0.5 µCi of [^{3}H]-Tdr at a specific activity of 2 Ci/mM was added per microtitre well and the plate was incubated at 37°C for a further 16-18 hours. After this incubation the plate was harvested and the level of [^{3}H]-Tdr incorporation was determined by scintillation counting as described on the previous page. A positive control value was obtained by plating the normal T-cells with a previously assayed, active Ly-CM and a negative control value was determined by plating the cells into conventional tissue culture medium alone (RPMI 20% + ME).

An E-rosette test on normal cultured feline T-cells

The guinea pig erythrocyte (GPE) rosette test is considered to be a specific marker for T-cells (Mackey et al., 1975; Cockerall et al., 1976). This test was carried out on normal cultured T-cells in the following manner. Guinea pig erythrocytes were washed and resuspended to 1% in phosphate buffered saline (PBS). 200 µl of this suspension was added to 200 µl of normal cultured feline T-cells, at a concentration of 2 x 10^6 cells/ml, in a V bottom tube (Sterilin). The mixture of cells was centrifuged at 1,000 rpm for 5 minutes then left at room temperature for 30 minutes. At the end of this time 100 µl of supernatant was removed and replaced with an equal volume (100 µl) of 1% crystal violet. The cells were very gently disturbed from the pellet and an aliquot was removed onto a haemocytometer for rosette counting. A rosette was defined as one T-cell in contact with 3 or more guinea pig erythrocytes and a minimum of 200 T-cells were counted. The number of rosette positive T-cells compared to rosette negative T-cells was expressed as a percentage.

The Geimsa staining of cytocentrifuge preparations of T-cells

Normal cultured feline T-cells were resuspended to a final concentration of 1 x 10^5 cells/ml in RPMI 20% + Me. 500 µl of this

suspension was used per cytocentrifuge slide. The slides were fixed in methanol for 10 minutes at room temperature, stained with 20% May Grunwald Geimsa for 4 minutes then stained twice with 10% Geimsa before washing in phosphate buffered saline (PBS) pH 6.8. (The Geimsa stains were also made up in PBS pH 6.8). The slides were dried at room temperature then visualized and photographed, using oil immersion light microscopy.

RESULTS

The optimum labelling conditions for the blastogenesis assay

Figures 7 and 8 show the response of cell lines FL74 and F422 respectively to the 4 labelling conditions listed in the legends to the figures. The optimum radioactive label for both cell lines was 0.5 μCi/well of [^{3}H]-Tdr at a specific activity of 2 Ci/mM. Maximum incorporation of label occurred overnight at day 2-3 with acceptable incorporation overnight at day 1-2. A 4-hour label applied on any of the 3 consecutive days resulted in suboptimal labelling of the cells.

The optimum conditions for the production of IL-2 in Ly-CM

Figures 9 and 10 graphically represent the results of a blastogenesis assay of normal T-cell growth in response to IL-2 present in Ly-CM produced under the varying conditions of culture shown. Figure 9 represents a cell culture concentration of 2.5 x 10^6 cells/ml which shows optimum IL-2 production 24 hours after stimulation by 7.5 μg/ml Con-A. Similarly 5 x 10^6 cells/ml culture (Figure 10) optimally produces IL-2 under the same conditions but the yield of IL-2 is significantly less.

The demonstration of IL-2 activity in Ly-CM

IL-2 activity was routinely monitored in Ly-CM batches by blastogenesis assay of growth of normal cultured feline T-cells. Serial 2-fold dilutions of the Ly-CM results in a growth response curve for normal T-cells, an example of which is shown in Figure 11.

An E-rosette test on normal cultured feline T-cells

An E-rosette test was carried out in quadruplicate as detailed in Materials and Methods on normal feline T-cells cultured for a minimum of 4 weeks. The experiment gave a mean result of 31.01% E-rosette positive (see Table 1).

The Geimsa staining of cytocentrifuge preparations of T-cells

A typical stained preparation of normal cultured feline T-cells is shown in Figure 12. The mononuclear morphology of the cells is clearly demonstrated.

DISCUSSION

The concentration of radioactive label to be used in the blastogenesis assays was optimised to ensure that maximum counts per minute (cpm) would be detected for statistical analysis without loss of cell viability due to radio-nucleotide damage. From the results presented in Figures 7 and 8 it was determined that 0.5 µCi/well of undiluted label (specific activity 2 Ci/mM) ensured high cpm in a range suitable for analysis without loss of cell viability.

This was, therefore, the concentration of radio-label used for cells in all the blastogenesis assays performed. From the same figures it is obvious that most label is incorporated overnight between days 2 and 3 of the assay. The explanation for this is probably due to the fact that the FL74 and F422 cells used have mean doubling times of 30 hours and 21 hours respectively (Toth, 1980), and are confluent, i.e. in static culture, on day 0 of the assay. They are fed with fresh medium (L/M) prior to plating in the microtitre wells and the observed increase in incorporation of [^{3}H]-Tdr with time is possibly a reflection of a rising number of cells capable of dividing. The 4 hour labels were not successful compared to the overnight labels again probably due to the relatively few cells undergoing division in this period. From the results it was decided to standardise the labelling of the cells to overnight between days 1 and 2 as this gives interpretable cpm and reduces the overall duration of the assay by 24 hours.

Preliminary results from Onions et al. (1980) suggested that Con-A was a more potent mitogen than PHA for inducing the production of IL-2 from feline mononuclear cells. This was confirmed and the optimum Con-A concentration for production of IL-2 was found to be 7.5 µg/ml. Two fairly high cell densities were tested for optimum production (2.5 x 10^6 and 5 x 10^6 cells/ml). No further purification of the IL-2 was planned and high cell densities are more efficient for removing contaminating mitogen from the medium than low cell densities as they adsorb more Con-A onto the surface. For both cell densities maximum IL-2 was

released after 24 hours in culture. A culture of 2.5 x 10^6 cell/ml gave a better yield of IL-2 than did 5 x 10^6 cells/ml. This may be due to the self-adsorption of IL-2 by the activated cells in the culture (Gillis et al., 1978) which is also why, even with the lower cell number, the level of IL-2 produced declines over the three days. A good yield of IL-2, determined by IL-2 dependent normal T-cell blastogenesis, seems to be dependent on a high percentage of spleen cells in the culture. Spleen cells are the best source of macrophages and hence IL-1, the second mitogenic signal required for IL-2 production by mature T-cells. Based on these results, IL-2 was routinely produced by incubating 2.5 x 10^6 cells/ml for 24 hours, using the total mononuclear spleen cell population available, and stimulating with 7.5 µg/ml Con-A.

The blastogenesis assay shown of the determination of IL-2 activity in these cultures of Ly-CM, was typical of the response of normal cultured feline T-cells to conditioned medium produced in this way. However, residual Con-A in the medium can occasionally show slight inhibition of T-cell response at high concentration. Equal volumes of Ly-CM and RPMI 20% + Me (50:50 medium) proved to be most satisfactory for the routine maintenance of T-cells in culture. Even using reasonably high cell density to produce IL-2, the crude Ly-CM used retains in addition, IL-1, interferon and other growth factors (Garland, 1982). Ruscetti and Gallo (1981) have indicated that because of this, the use of crude Ly-CM creates difficulties in assessing cellular response to IL-2. From the data presented it seems certain that the crude feline Ly-CM definitely supports the growth of a mature T-cell population. The source of normal feline T-cells used for culture is the thymus, the overwhelming mononuclear cell population of which is, by definition, T-cell. E-rosette testing with guinea-pig erythrocytes has been used as a specific marker for T-cells and the results of GPE-rosettes with normal cultured feline T-cells, presented in Table 1, show an average value of 30% E-rosette positive. This percentage of T-cells which were E-rosette positive was expected since the T-cell population tested was not synchronised and E-rosette formation is known to be cell-cycle dependent (Cockerell

et al., 1976). Furthermore, the Geimsa stained cytocentrifuge preparation of the same cells, shown in Figure 12, clearly demonstrates the mononuclear appearance of these cells.

In conclusion, a reliable blastogenesis assay has been developed to enable routine screening for IL-2 present in feline lymphocyte conditioned medium. The IL-2 produced has been used both to support the growth of normal IL-2 dependent feline T-cells, and also to establish neoplastic feline T-cells, in vitro, which will be discussed, in detail, in the following chapter.

TABLE 1

E-rosette test on normal cultured feline T-cells.

T-cell sample	Rosette +ve	Rosette -ve	% Rosette +ve
1	68	173	28.21%
2	73	142	33.95%
3	74	162	31.35%
4	69	157	30.53%

Mean = 31.01%

Standard deviation = 2.36

FIGURE 6.

The production and assay of Ly-CM

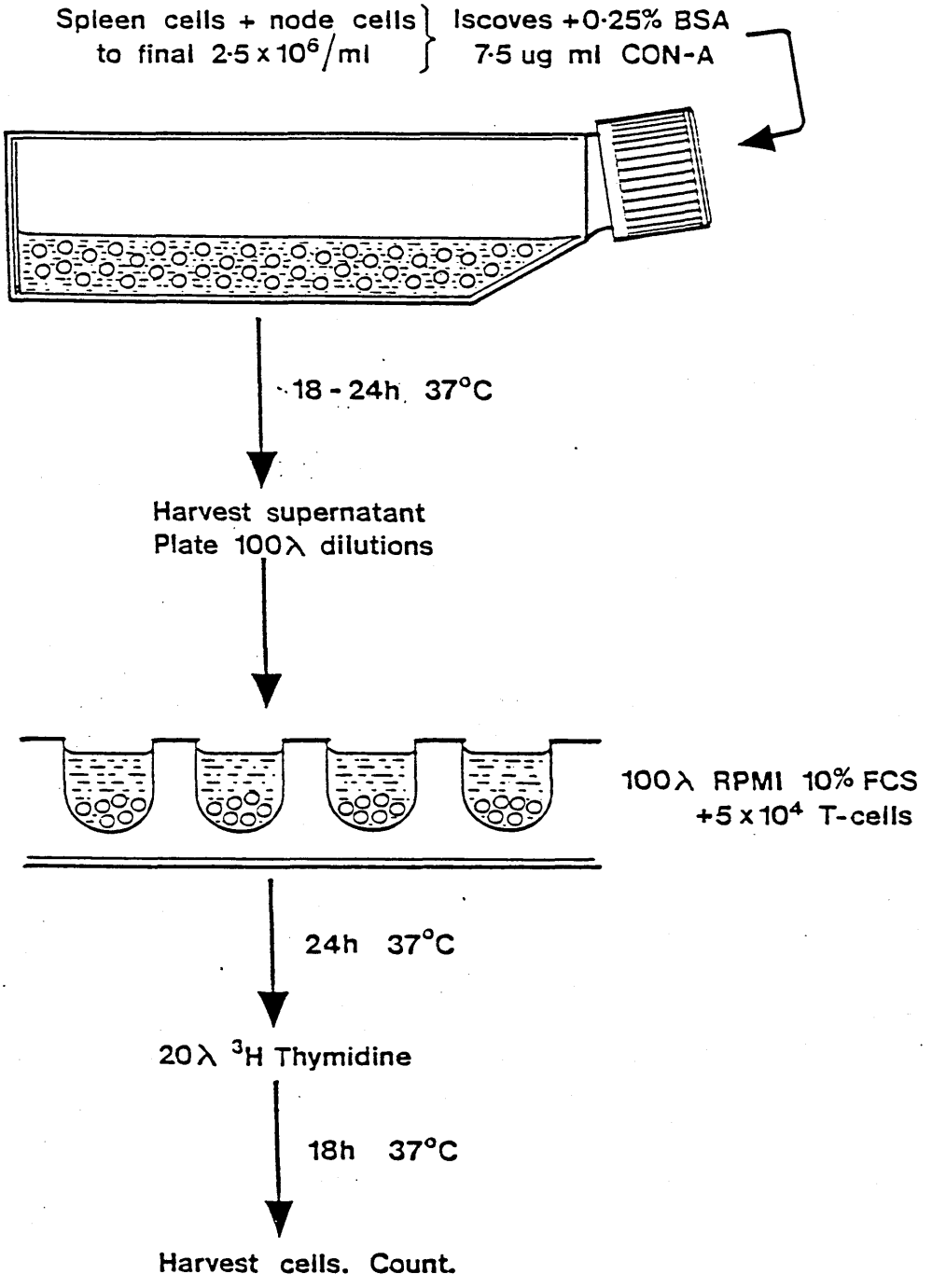


FIGURE 7

Determination of the optimum labelling conditions for cell line FL74

FL74 cells were labelled with 4 concentrations of [^{3}H]-Tdr denoted as follows:

	Symbol	Specific Activity	µCi/well
(i)		2 Ci/mM	0.5 µCi
(ii)		0.98 Ci/mM	2.0 µCi
(iii)		0.56 Ci/mM	0.8 µCi
(iv)		0.32 Ci/mM	1.6 µCi

FIGURE 7.

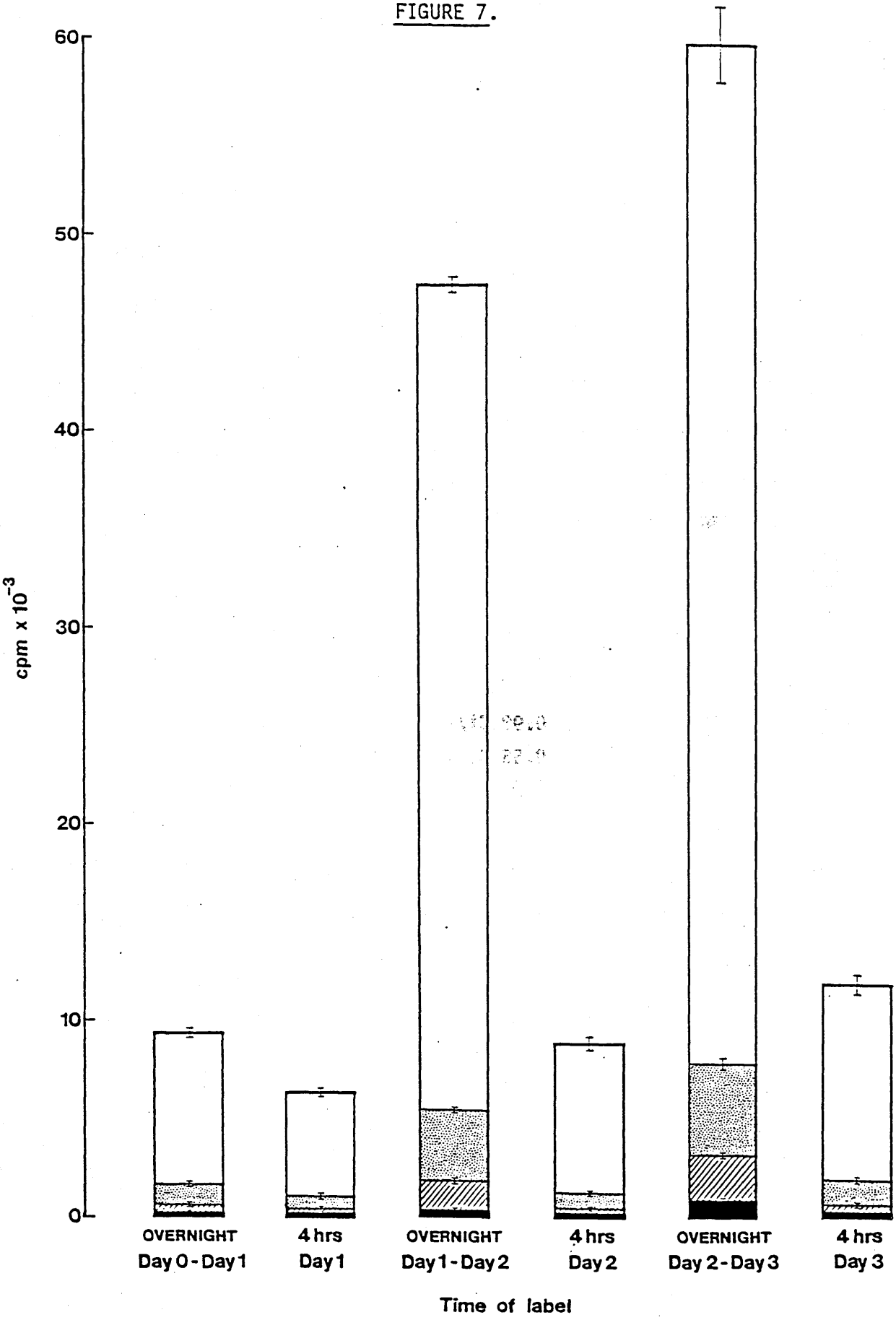

60
50
40
30
20
10
0
cpm x 10^-3
OVERNIGHT
Day 0 - Day 1
4 hrs
Day 1
OVERNIGHT
Day 1 - Day 2
4 hrs
Day 2
OVERNIGHT
Day 2 - Day 3
4 hrs
Day 3
Time of label

FIGURE 8

Determination of the optimum labelling conditions for cell line F422

F422 cells were labelled with 4 concentrations of [^{3}H]-Tdr denoted as follows:

	Symbol	Specific Activity	µCi/well
(i)		2 Ci/mM	0.5 µCi
(ii)		0.98 Ci/mM	2.0 µCi
(iii)		0.56 Ci/mM	0.8 µCi
(iv)		0.32 Ci/mM	1.6 µCi

FIGURE 8.

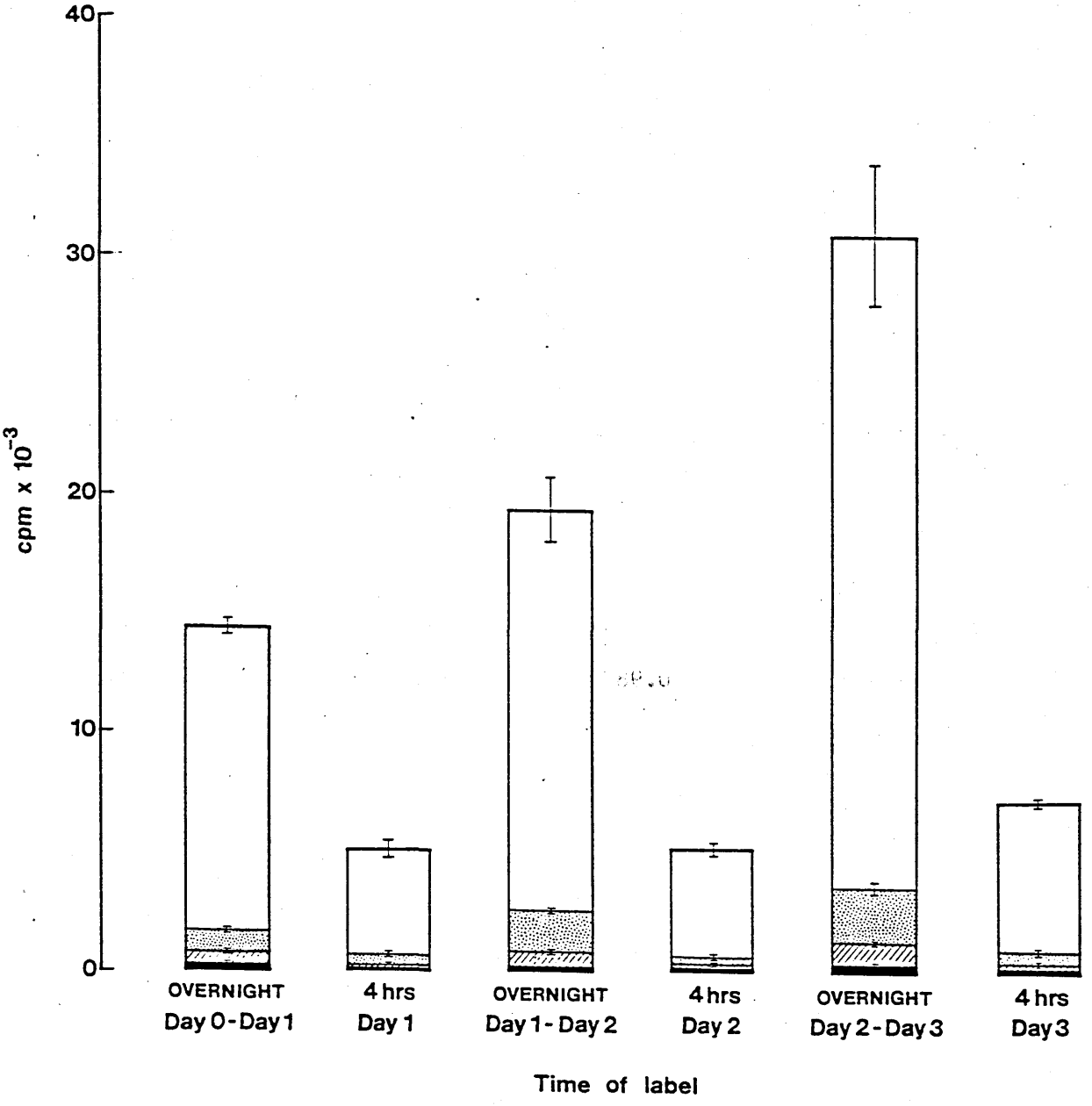

40
30
20
10
0
cpm x 10^{-3}
OVERNIGHT
Day 0 - Day 1
4 hrs
Day 1
OVERNIGHT
Day 1 - Day 2
4 hrs
Day 2
OVERNIGHT
Day 2 - Day 3
4 hrs
Day 3
Time of label

FIGURE 9

Determination of the optimum conditions for IL-2 production using 2.5 x 10^6 cells/ml culture

Mononuclear cells were seeded at a density of 2.5 x 10^6/ml in 0.25% Isc and the supernatants were harvested on the days shown. The mitogen and mitogen concentrations added are denoted as follows:

Symbol	Mitogen concentration
■	7.5 µg/ml Con-A
●	5 µg/ml Con-A
□	1 µg/ml Con-A
○	1% PHA

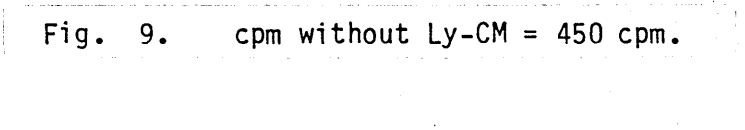


Fig. 9. cpm without Ly-CM = 450 cpm.

FIGURE 10

Determination of the optimum conditions for IL-2 production using 5 x 10^6 cells/ml culture

Mononuclear cells were seeded at a density of 5 x 10^6/ml in 0.25% Isc and the supernatants were harvested on the days shown. The mitogen and mitogen concentrations added are denoted as above.

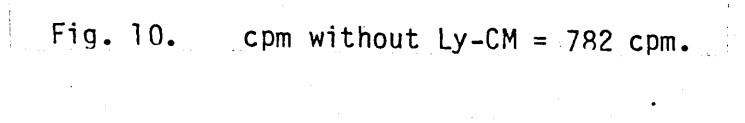


Fig. 10. cpm without Ly-CM = 782 cpm.

FIGURE 9

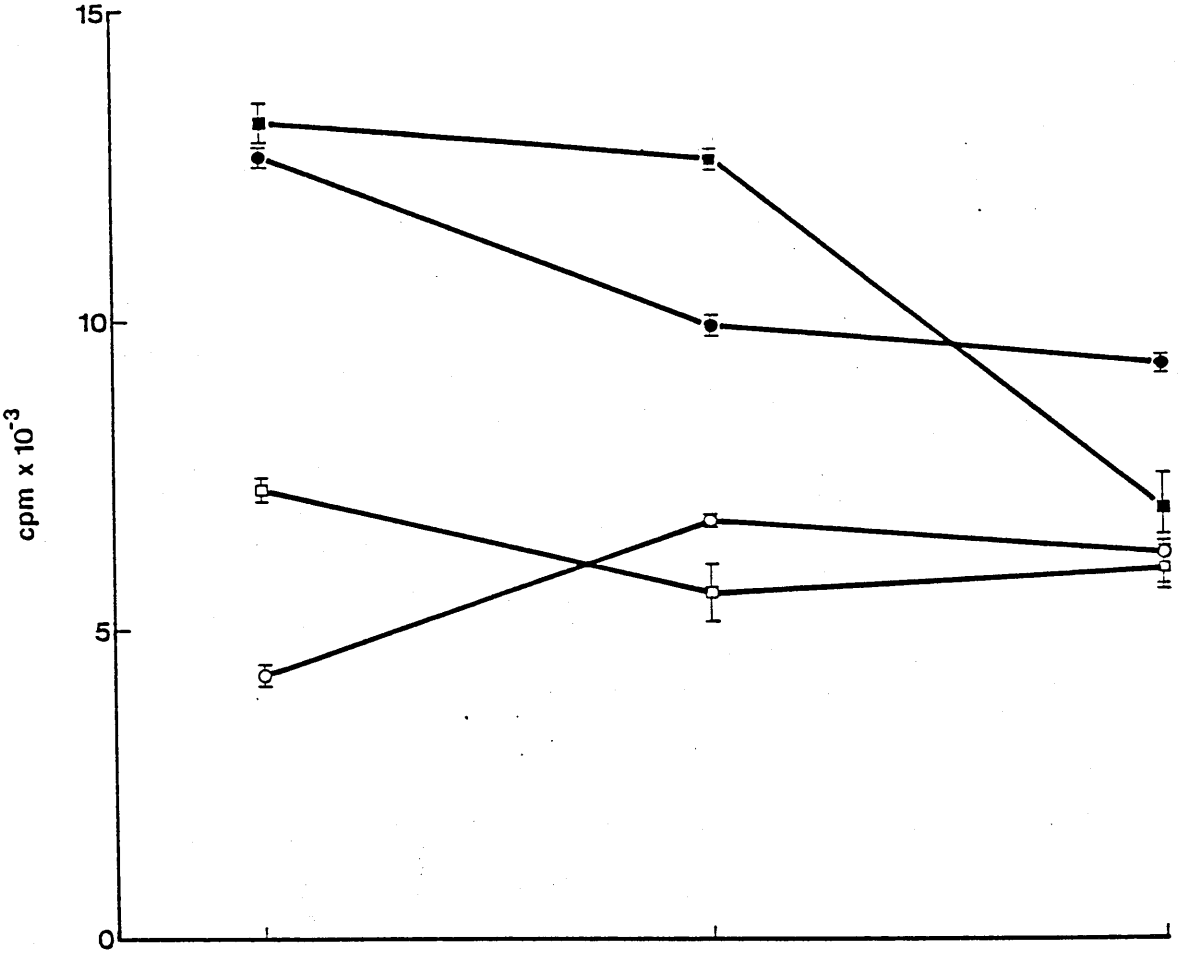
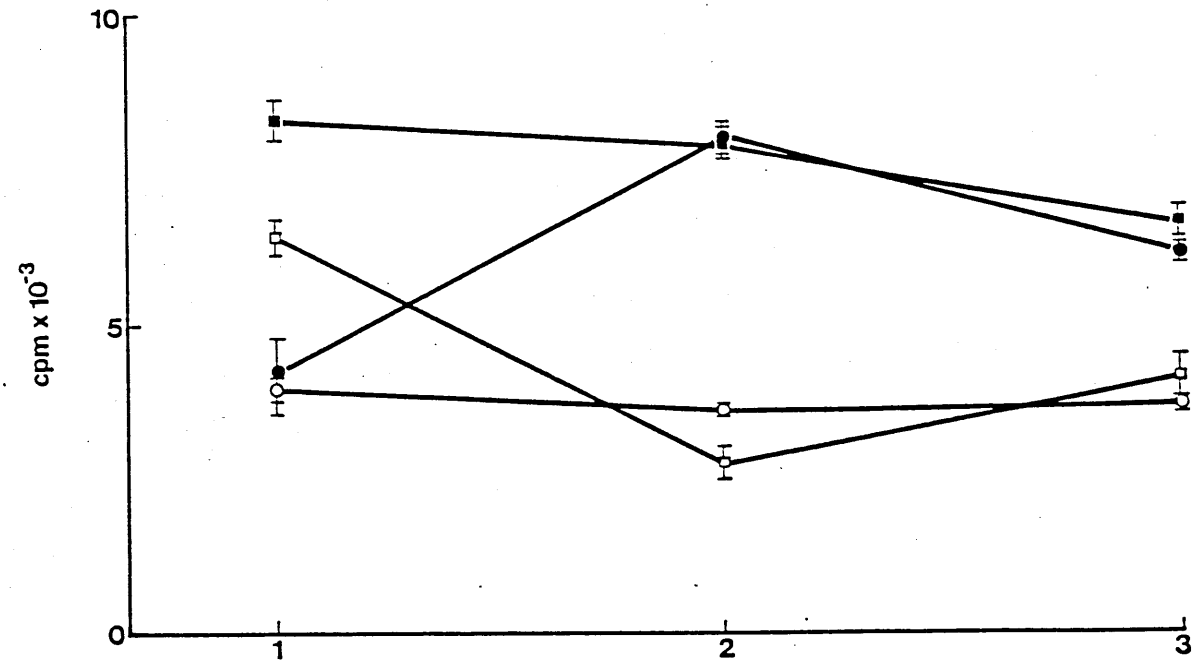


FIGURE 10



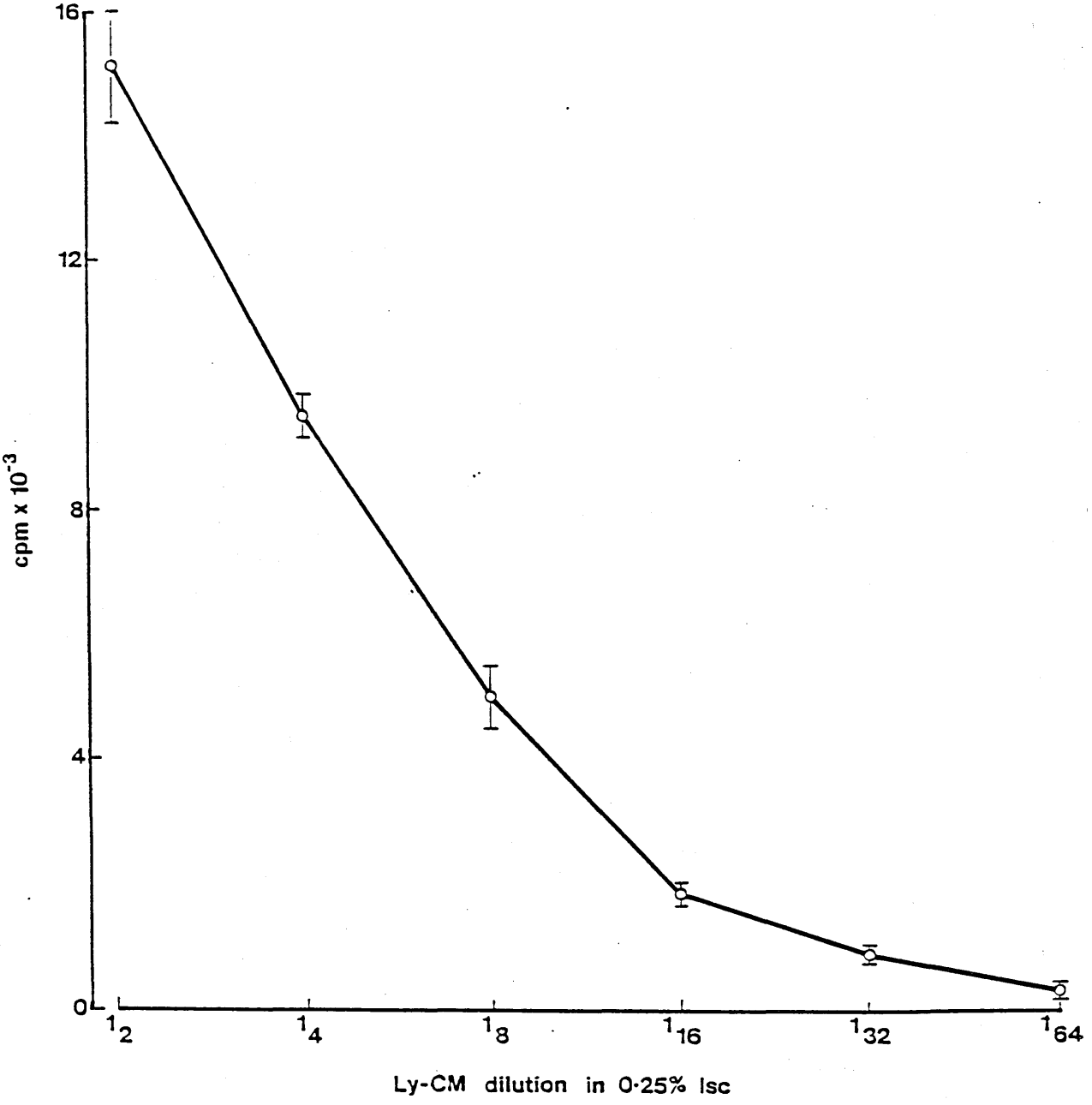


FIGURE 11

Normal cultured T-cell response curve

The graph shows a typical response curve of normal cultured feline T-cells to the IL-2 present in Ly-CM. The Ly-CM was produced from an SPF cat using 2.5 x 10^6 cells/ml culture stimulated with 7.5 µg/ml Con-A for 24 hours.

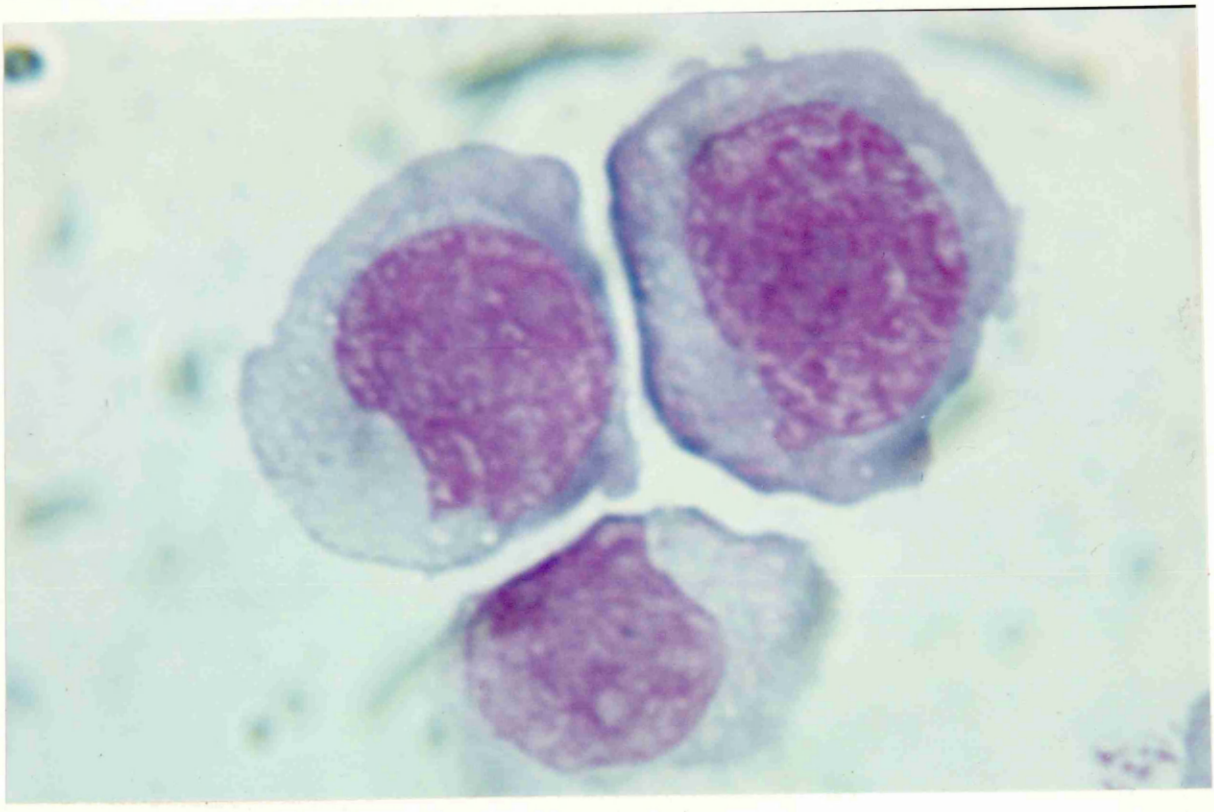


FIGURE 12

Geimsa stained normal cultured T-cells

The photograph shows geimsa stained mononuclear cells prepared by cytocentrifugation of cultured T-cells.

CHAPTER THREE

THE ESTABLISHMENT OF NEOPLASTIC FELINE T-CELLS IN VITRO

Normal activated T-cells can be grown in continuous proliferative culture only if they are first activated by antigen or mitogen and IL-1 to express receptors for IL-2. Their growth can then be sustained for a long period of time by repeatedly supplementing the medium with IL-2 (Ruscetti et al., 1977). In contrast to this, T-cells obtained from T-cell malignancies can frequently be cultured directly with IL-2 without prior activation suggesting that they already display the IL-2 receptor (Poiesz et al., 1980b). Before the discovery of IL-2, relatively few neoplastic T-cell lines were successfully established in culture since the neoplastic tissue was plated directly into conventional tissue culture medium. Human neoplastic T-cell lines derived from patients with acute lymphoblastic leukaemia (T-ALL) were cultured in this manner and only one could be induced to produce free IL-2 in the medium. Both this line (designated JURKAT) and another nine did not display receptors for IL-2 nor did they respond to it (Smith, 1982). Where data was available on these cell lines, Smith reports that most of the cells which were initially placed in culture died and the lines which eventually became established arose from a small number of cells (perhaps even a single cell) in the original inoculum. All of the cell lines which established in culture were initiated after the patients had received chemotherapy which is a potential source of mutagenesis so these lines may not be representative of the original neoplastic T-cells in vivo.

Now that IL-2 is available there has been considerably more success in establishing neoplastic T-cell lines in culture. Poiesz et al. (1980b) have established six out of six human cutaneous T-cell lymphoma cell lines (CTCL) by plating the neoplastic tissue directly into tissue culture medium supplemented with human partially purified, lectin-free, IL-2. Four of these lines have subsequently become independent of exogenous IL-2 for their continued proliferation in culture, a phenomenon never observed with any normal T-cell line. It has subsequently been demonstrated that these cell lines both produce and respond to IL-2, suggesting

that at least some malignant T-cell lines may be capable of of autonomous growth by virtue of this property (Gootenberg et al., 1981). These IL-2 independent T-cell lines produce no detectable IL-2 activity free in the supernatant of the cultures but it can be demonstrated by acid-glycine treatment resulting in the release of IL-2 from the cell membrane. In marked contrast to this a gibbon-ape T-cell line established from a gibbon ape with spontaneous lymphosarcoma (Kawakami et al., 1972) constitutively released detectable IL-2 in the culture supernatant which was capable of supporting the growth of human, primate and murine IL-2 dependent T-cells in vitro (Rabin et al., 1981). This cell line was established directly into conventional tissue culture medium and it has only recently been demonstrated that it can also both produce and respond to IL-2 (Smith, 1982) supporting a hypothetical autonomous growth system similar to that proposed for the IL-2 independent human CTCL cell lines.

Very few neoplastic feline T-cell lines have been successfully established in long-term culture. The FL74 cell line (Theilen et al., 1969) and the F422 cell line (Rickard et al., 1969) were established by plating the neoplastic tissue into conventional tissue culture medium but nothing was known of their response to or production of IL-2. Since a source of feline IL-2 was available (described in the previous chapter), the neoplastic T-cells from the spontaneous lymphosarcomas in the present experiments were plated both into conventional tissue culture medium (RPMI 20% + Me) and the same tissue culture medium + Ly-CM (50:50 medium) in an attempt to establish these T-cells in vitro. Blastogenesis assays of neoplastic T-cell response to Ly-CM on the day that the cells were isolated from the lymphosarcoma were carried out in order to determine if this initial response was indicative of which cells would proliferate in culture. Cellular growth was also compared, by blastogenesis assay, using partially purified, lectin-free, human IL-2 (provided by Dr. R.C. Gallo) and crude feline Ly-CM, to determine if the cells responded in a similar manner to both sources of factor. This would indicate whether or not it was really the IL-2 in the crude feline Ly-CM that was supporting the

growth of these cells or if they were simply responding to residual mitogen (ConA) in the preparation. (Poiesz et al., 1980b).

MATERIALS AND METHODS

Cells

Normal feline T-cells, used for the comparison of the effect of partially purified human IL-2 (pp IL-2) and crude feline Ly-CM on the growth of cells, were isolated and cultured as detailed in Chapter Two.

FL74 and F422 cells used in a similar study were cultured as described in Chapter One.

Media

RPMI-1640 medium, (Gibco Biocult Ltd.) supplemented with 400 units/ml penicillin-streptomycin and 2% FBS was used for isolating tumour material and stomaching these cells. This medium is referred to as RPMI 2%.

Ly-CM was produced as described in Chapter Two and its activity tested on normal cultured feline T-cells prior to use.

The preparation and establishment of cell cultures from tumours

Fourteen thymic lymphosarcomas isolated from field cases were initiated in culture in the following manner. The tumours were removed asceptically into RPMI 2% and washed twice in this medium. They were then diced in petri dishes (Nunclon) with scalpels to a fine suspension and stomached once, as described in Chapter One. The single cell suspension was centrifuged at 2000 rpm for 5 minutes and the cell pellet resuspended to 1 x 10^6 cells/ml either in RPMI 20% + Me or in an equal volume of RPMI 20% + Me and Ly-CM (50:50 medium). If many dead cells were present (determined by trypan blue exclusion test) the cells were passed through Ficoll-paque for 10 minutes at 2000 rpm and the live cells isolated from the interface. These were washed once in RPMI-20% and resuspended as above. Cultures were maintained by sub-culturing

the cells twice weekly, and resuspending them back to 5 x 10^5/ml in fresh medium.

The initial response of primary tumour cells to Ly-CM

Blastogenesis assays were performed on primary tumour cells on the day of isolation from the cat. The cells were prepared as for culturing above but were resuspended to 5 x 10^5 cells/ml in RPMI 20% + Me then 100 µl seeded into microtitre wells giving a final concentration of 5 x 10^4 cells/well. Ly-CM was added in 2-fold serial dilutions in RPMI 20% + Me and the assay was labelled, harvested and counted as described in Chapter One.

The response of normal and cultured tumour cells to Ly-CM and pp IL-2

A comparison was made of the effect of crude feline Ly-CM and human pp IL-2 on the growth of normal feline T-cells and tumour cells (T11) established in culture. Blastogenesis assays were used in the usual way previously described (Chapter One). Both cell lines were grown to confluence before use and were dependent on Ly-CM for growth.

RESULTS

The establishment of tumour cells in culture

Of 14 thymic lymphosarcomas initiated in culture (Figure 13) only one, T17, grew when plated directly into RPMI 20% + Me without added Ly-CM. T17 has now been in culture for more than 6 months. Four of the rest; T10, 11, T14 and T16 formed IL-2 dependent longterm cultures (their continued growth was absolutely dependent on the medium being supplemented with 50% Ly-CM). These cells were routinely sub-cultured into RPMI 20% + Me only but they never grew in the absence of Ly-CM. Another cell line which established in culture, T3, initially required exogenous IL-2, as the cells which were seeded directly into RPMI 20% + Me only, died immediately.

However, within one week the cells which were originally seeded into 50:50 medium, survived and grew when they were subcultured into RPMI 20% + Me alone. This cell line has now been in culture for 2 years and will be discussed extensively in the following chapters.

The initial response of primary tumour cells to Ly-CM

Figure 14 represents the results of blastogenesis assays performed on freshly isolated tumour cells to determine if their initial response to growth factor was a reliable indication of whether or not they would establish in culture. The response to growth factor was expressed as a multiple of the negative control value, which was obtained by seeding the cells into RPMI 20% + Me with no Ly-CM. A typical normal cultured feline T-cell response is shown for comparison. Two virus-positive tumours, T7 and T10 showed a low level response while the others showed virtually no response at all (represented by the area between the dotted lines). The latest two tumours, T16 and T17 are not included in these results as this assay was not done on these cells (see discussion).

The response of normal and cultured tumour cells to Ly-CM and ppIL-2

Figure 15 demonstrates virtually parallel responses of normal and tumour T-cells to crude feline Ly-CM and pp human IL-2. T11, a long-term cultured, IL-2 dependent cell line, represents the response of tumour cells to these factors. Both sources of growth factor are slightly inhibitory at a dilution of 1:2 in 0.25% Isc on both cell types. The response to crude feline Ly-CM is greater, in both cases, than the response to pp human IL-2, which is probably a function of species specificity. The response index is again expressed as a multiple of the negative control value, which was obtained by plating both cell lines into RPMI 20% + Me, without additional growth factor.

DISCUSSION

Cells were isolated from the thymic lymphosarcomas of 14 cats in order to attempt to establish them in culture as neoplastic feline T-cell lines. Feline IL-2, present in Ly-CM produced from SPF cats, was used to initiate these cells in culture as this approach has been more successful in other systems than the use of conventional tissue culture medium alone (Poiesz et al., 1980b). As a control the cells were also plated into conventional tissue culture medium without Ly-CM. Figure 13 summarises the results obtained using these two methods of culture. Of the 14 tumours in the series, 12 were FeLV-positive and two were FeLV-negative, determined by virus isolation from the plasma of these cats. Tumour cells cultured from cats that had infectious virus present in the plasma were also tested for virus release from the cells into the culture supernatant. All of the neoplastic cells that established in culture were FeLV-positive both in the plasma and from the cultured cells with the noticeable exception of T17 which had no infectious FeLV circulating in the plasma but was releasing low levels of FeLV from the cultured tumour cells. The release of virus from the cells and circulating in the plasma of these cats will be discussed further in Chapter Five.

The five cell lines which initially established in Ly-CM supplemented medium remained totally dependent on this source of IL-2 for continued growth with the exception of T3 which rapidly became independent of an exogenous source of IL-2 (within one week of culture). Possibly these cells consisted of both IL-2 dependent and IL-2 independent cells, the latter being a very small percentage of the total population. In this event there may have been too few cells capable of autonomous growth in the culture, when plated directly into tissue culture medium alone, to enable them to establish into a cell line (cloning of single viable cells was not attempted). Alternatively, the cells may have required a mitogenic signal present in the crude lymphocyte conditioned media before they became capable of growth independent of exogenous IL-2. Interestingly, the T3 cell line has always been absolutely

dependent on the presence of mercaptoethanol in the tissue culture medium for continued proliferation. Similarly, T17, which did establish in vitro in the absence of a source of exogenous IL-2, has also always been dependent on the presence of 2-Me in culture. 2-Me provides an IL-1-like mitogenic signal to cells in culture (Chen and Hirsch, 1972) which would support a hypothesis that these two tumour cell lines may be independent of exogenous IL-2 by virtue of their capacity to both produce and respond to this factor, in a self-stimulatory type of mechanism. This possibility was investigated and the evidence for and against this theory is the subject of the next chapter.

Figure 14 demonstrates that the initial response of these cells to Ly-CM on the day of isolation from the tumour (Day 0) gave no indication of whether or not they were going to form cell lines in culture. T10 did respond on Day 0 but very little in comparison to the response of normal cultured T-cells to the same conditioned medium. It was hoped that this initial response assay would provide a fast screen of tumour cells to determine which ones were likely to grow, and whether or not they required an exogenous source of IL-2 in culture. As this assay did not provide any information on subsequent tumour cell growth it was not used after tumour T15. The first day of isolation of these cells from tumour material was possibly not the best time to carry out this type of assay as they had just been traumatised by transferring them from an in vivo to an in vitro environment and usually little cellular growth in tissue culture was observed over the first few days. The IL-2 dependent cell lines, once established in culture, responded to IL-2 in a manner similar to normal T-cells (exemplified by T11, Fig. 15). These cells were unlikely to be a normal population based on the evidence of the nature of the virus associated with them, which will be discussed later (Chapter Six).

Figure 15 also demonstrates the comparison of the effect of crude feline Ly-CM and partially purified human IL-2 on both a normal and a neoplastic (T11) cell line. The parallel response of the cells to the two sources of factor strongly supports the assumption that it is the IL-2 in the crude feline Ly-CM that the

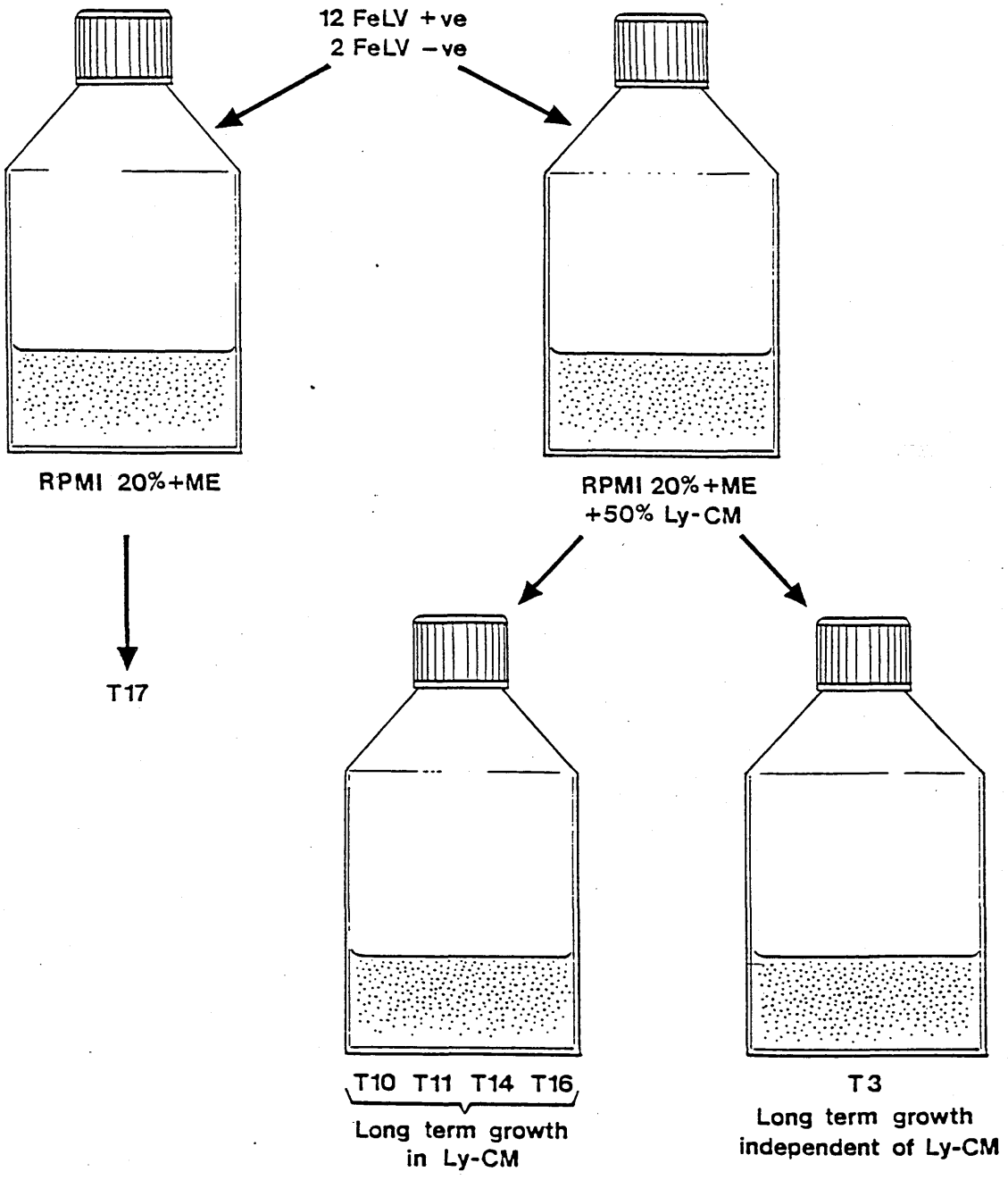
cells are responding to and not some other growth or mitogenic factor. Partially purified human IL-2 was provided by Dr. R. Gallo and has been extensively treated to remove contaminating lectin and other growth factors (Mier and Gallo, 1982). The reduced response observed to the human factor compared to the feline factor is probably due to cross-species restriction but the noticeable difference in activity between the 1:2 and 1:4 dilution is unexpected and more difficult to interpret. An increase in activity occasionally does occur at a 1:4 dilution of crude feline Ly-CM compared to the 1:2 value. Residual lectin in crude conditioned medium often creates this result (Ruscetti and Gallo, 1981) by inhibiting cellular growth at high concentrations. This effect, however, can also be seen here with the pp human IL-2 which is lectin-free and therefore was not expected to produce this effect. It has been stated that continued stimulation with mitogen is equally toxic to cultured transformed T-cells as it is to normal cultured T-cells which is why partially purified preparations can sustain the growth of malignant T-cells in culture longer than can crude preparations (Poiesz et al., 1980b). The IL-2 dependent cell lines presented here were maintained in culture for at least four months and could be grown in numbers sufficient for the preparation of cellular DNA and RNA for analysis.

The successful establishment of these cell lines facilitated further investigation of the properties of these cells and of the viruses associated with them. The role that growth factors may or may not play in neoplasia is controversial and the experiments described in the following chapter were undertaken to try to elucidate the involvement of IL-2 in the T-cell neoplasias described here, and in the classic feline neoplastic T-cell lines FL74 and F422.

Figure 13: Twelve feline leukaemia virus-positive and two feline leukaemia virus-negative tumours were initiated in culture by seeding them both into RPMI 20% + Me alone and this medium supplemented with 50% Ly-CM.

FIGURE 13

The establishment of tumour T-cells in culture



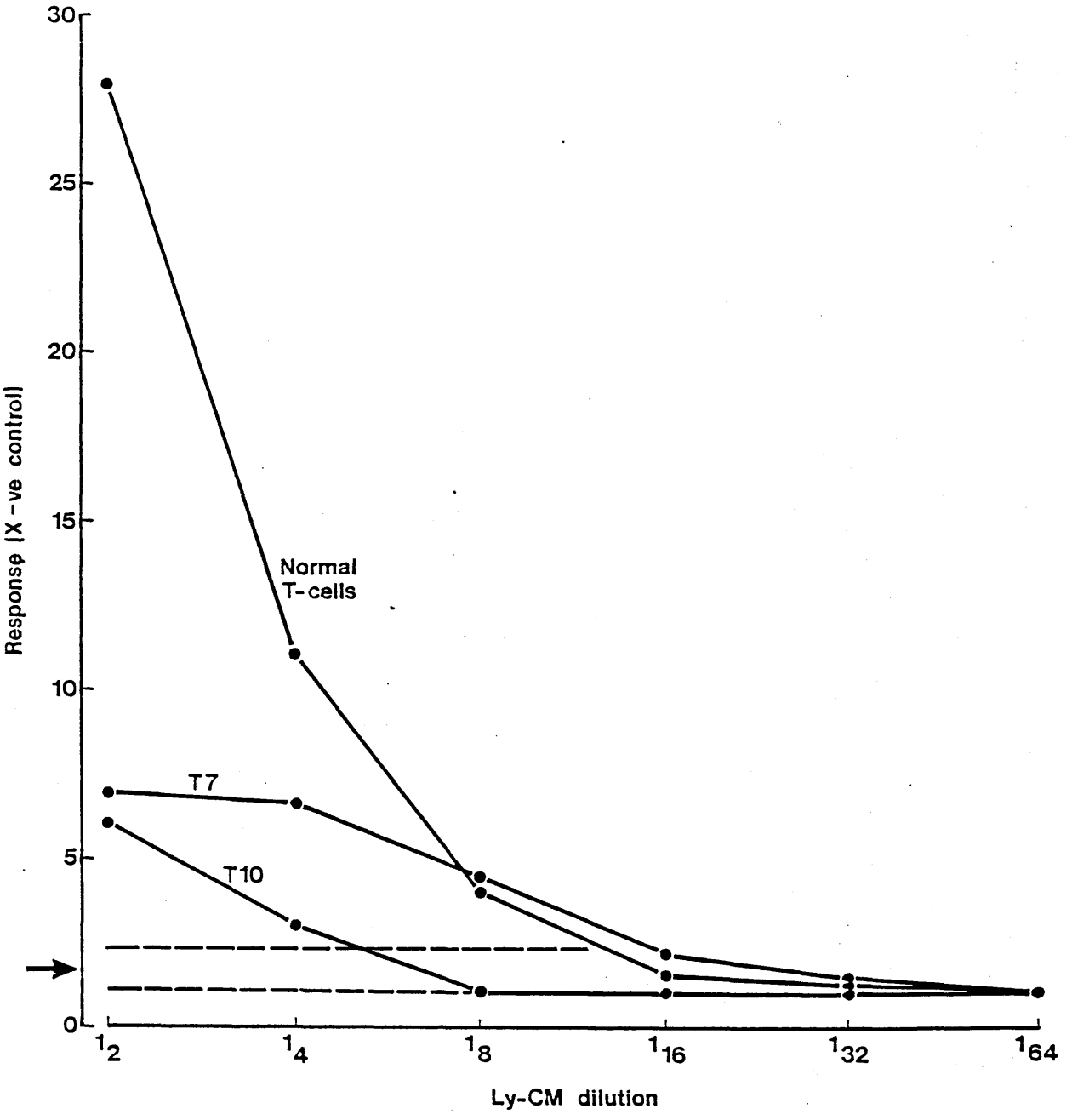


FIGURE 14

The initial response of tumour cells to Ly-CM

The area between the dotted lines on the graph represents the response of T1, T3, T4, T5, T6, T8, T11, T12 and T15 to Ly-CM. Each point on the graph represents the mean of 6 replicates (% S.E. $\leq$ 9.5%).

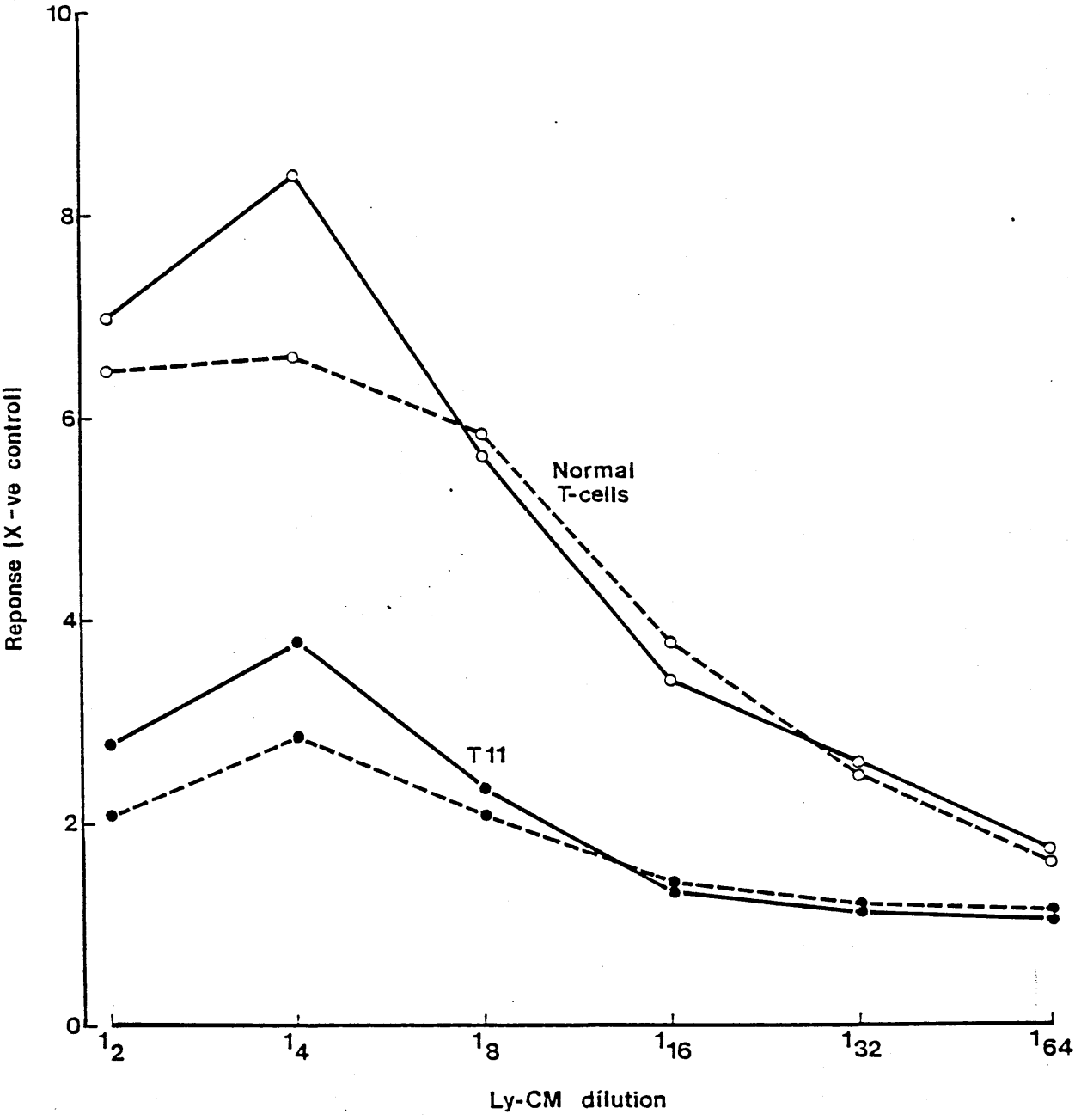


FIGURE 15

The response of normal and IL-2 dependent tumour cells to Ly-CM and ppIL-2

The solid line in the graph represents the response of the cells to crude feline Ly-CM and the dashed line represents the response of the cells to pp human IL-2. Each point on the graph represents the mean of 6 replicates (% S.E. $\leqslant$ 8.7%).

CHAPTER FOUR

INVESTIGATION OF THE ROLE OF IL-2 IN T-CELL NEOPLASIA

The exact nature of the involvement of growth factors in tumourigenesis is still unknown but several mechanisms have been proposed to explain how they may operate in initiating or maintaining the transformed state. Fundamental to the understanding of the role that growth factors may play in neoplasia is the method by which they participate in the regulation of normal cellular proliferation as depicted in Figure 16. A growth factor can be considered to be a mitogenic signal to a cell which induces cellular proliferation by binding to a specific receptor on the cell surface and initiating a sequence of cellular events to transmit this message to the nucleus, culminating in cell division. Therefore it can be envisaged that an alteration at any level of action of the growth factor, either at the level of the molecule itself, the cellular receptor, or within the intracellular messenger system, could result in loss of regulation of cell growth.

Since de Larco and Todaro (1978) first demonstrated the involvement of a growth factor in the neoplastic state (transforming growth factor (TGF) in the murine system) many more examples which should help to elucidate this relationship have come to light. Both from these initial studies by de Larco and Todaro and from subsequent investigations it has been determined that cells infected by Moloney murine sarcoma virus (MuSV) produce an endogenous growth factor called TGF which blocks the receptors for epidermal growth factor (EGF) on the surface of these cells. Furthermore the transforming activity released by MuSV transformed cells is probably due to the combined action of two factors; one EGF-like (TGF-α) and one that, in combination with EGF or TGF-α, induces phenotypic transformation but has no mitogenic activity in itself (TGF-β) (Anzano et al., 1983). Production of TGFs is not restricted to MuSV transformed cells but is a property of several transformed cell lines (Kaplan and Ozane, 1982; Todaro et al., 1980 and Twardzik et al., 1983) and has also been found in normal tissues (Roberts et al., 1981). The use of temperature-sensitive

mutants of Moloney and Kirsten MuSVs has clearly demonstrated the relationship between transformation and TGF production (Todaro et al., 1979; Kaplan et al., 1982). The transformed state was absolutely dependent on TGF production implying that the respective viral oncogene products $p37^{mos}$ and $p21^{k\text{-}ras}$ directly or indirectly regulate TGF synthesis at the transcriptional or translational level. Evidence on the relationship of certain growth factors to known oncogenes is rapidly emerging and is providing data linking different oncogenes to the different levels of action of growth factors. The example of TGF demonstrates how a viral oncogene can induce the production of a cellular growth factor which can bind to the cell's EGF receptor and induce transformation at this level of the signalling system.

Examples also exist of oncogene products which mimic the growth factor molecule. The oncogene v-sis, which is carried by simian sarcoma virus (SSV) has close amino acid sequence homology to platelet-derived growth factor (PDGF) which is one of the mitogenic signals involved in cellular division (Waterfield et al., 1983; Doolittle et al., 1983). Another retrovirus, Parodi-Irgens feline sarcoma virus (FeSV) carries a similar oncogene with homology to c-sis although its exact relationship to the PDGF gene remains to be established (Besmer et al., 1983a). It has been proposed that in these cases the viral gene product functions as a growth factor (PDGF) agonist by interacting with the PDGF receptor and stimulating cell replication.

A structural relationship between a growth factor receptor and a viral oncogene product has also been demonstrated by the strong amino acid homology between the EGF receptor and the transforming protein of avian erythroblastosis virus (AEV), $gp65^{erb\text{-}B}$. AEV appears to have acquired the part of the EGF receptor gene that codes for the internal domain and transmembrane region of the receptor but not the external domain containing the EGF binding region. The lack of a regulatory EGF binding domain may then be accompanied by a constitutive activation of the effector domain

(Downward et al., 1984). A functional homology also exists in that several growth factor receptors and transforming proteins have tyrosine kinase activity, including the PDGF receptor (Heldin et al., 1983), the EGF receptor (Cohen et al., 1982) and many retroviral oncogene products (for review see Heldin and Westermark, 1984).

Of particular interest with respect to the results in the following chapters is the relationship of oncogenes to growth factors at the level of the intracellular messenger system which relays the signal from the stimulated receptor to the nucleus. Evidence has emerged which shows that two hours after mitogenic stimulation by PDGF of stationary Balb/C 3T3 cells, significant increase in the expression of mRNA of a proto-oncogene, c-myc, is detected. This also occurs when mouse lymphocytes are stimulated with mitogen (Kelly et al., 1983). The myc product is a nuclear binding protein (Abrams et al., 1982; Donner et al., 1982) and is transduced by several retroviruses causing transformation of a spectrum of target cells of different histogenetic origins (Graf and Beug, 1978). Other proteins whose level of synthesis is increased after cellular exposure to mitogen have been detected but their relationship to known oncogenes has not yet been determined (Pledger et al., 1981). Similarly, proteins that specifically undergo phosphorylation on stimulation of intact cells have been detected (Cooper et al., 1982) and should eventually enable identification of the target cellular substrates for the receptor-associated kinases.

On the assumption that c-myc has a role to play in the control of normal cellular proliferation, it is easy to envisage that the aberrant expression of c-myc or the transduction of a viral v-myc could result in neoplastic transformation of a wide variety of cells.

Normal cellular proto-oncogenes need not be transduced by viruses to become oncogenic. The aberrant expression of these genes can also be achieved by chromosomal translocation of the gene

to a different location where its expression is under altered control, such as happens to c-myc in Burkitt's lymphoma (Dalla Favera et al., 1982; Taub et al., 1982). Alteration of control of gene expression in any manner, even by conventional mechanisms of mutagenesis, could lead to abnormal production of, or response to, a growth factor with or without viral involvement. In the T-cell tumour series presented here, the involvement of growth factor (IL-2) was investigated to determine if the neoplastic cells had an altered response to, or requirement for IL-2. When these experiments were initially undertaken there was no evidence for the involvement of an oncogene or oncogenic product in feline T-cell neoplasia. Since then the association of the oncogene myc with T-cell tumours has been established (Neil et al., 1984) and the results will be discussed in this light.

The adsorption of IL-2 by neoplastic T-cells

Tumour T-cells, freshly isolated from feline thymic lymphosarcomas, were incubated at a concentration of 10^7 cells/ml in crude feline Ly-CM to determine if the cells were expressing receptors for IL-2 and were actively adsorbing it from conditioned medium. Cells were resuspended at 10^7 in 1 ml of both Ly-CM and 0.25% Isc, the latter serving as a control to detect the possible production of an endogenous growth factor from the tumour cells. The cells were incubated for one hour at 37°C in duplicate. Duplicate tubes were also set up containing each medium alone without cells as negative controls. After 1 hour each tube had 1 ml of reciprocal medium added in order to standardise the Ly-CM concentration in each (final concentration 1/4) (Table 2). All tubes were then centrifuged for 5 minutes at 2,000 rpm at 4°C to pellet the cells. The control tubes of media alone were treated identically. The supernatants were then removed in aliquots and stored at -20°C until assayed. The IL-2 remaining in the media was determined by blastogenesis assay (previously described) using normal cultured feline T-cells.

The spontaneous production of a growth factor by neoplastic T-cell lines

The cultured neoplastic T-cell lines FL74, F422 and T3 were studied to determine if they released detectable quantities of an endogenous growth factor into the culture supernatant. The supernatant of all three cell lines was harvested 21 hrs., 48 hrs. and 72 hrs. after sub-culturing the cells. The cells were spun down at 2,000 rpm for 5 minutes at 4°C and the supernatants removed. Virus was then pelleted at 40,000 rpm for 45 minutes at 4°C in case it interfered with the growth of the normal T-cells to be used in a blastogenesis assay. The supernatants were then sterilised by filtration and growth factor activity was assayed by blastogenesis assay using normal cultured feline T-cells. The

cells were confluent when used and were spun down at 2,000 rpm for 5 minutes then resuspended to 5 x 10^5 cells/ml in RPMI 20% + Me. The supernatants were added in equal volume i.e. they were not titrated out since it seemed unlikely that growth factor would be present in a high enough quantity to be detected at any more than a 1:2 dilution.

The response of neoplastic T-cell lines to crude feline Ly-CM and pp human IL-2

The cultured cell lines used in this experiment were F422, T3, T17 and FL74 which are all independent of an exogenous source of IL-2 for growth in vitro. The classic cell lines FL74 and F422 grow well in culture in 10% FBS supplemented medium (L/M) and do not require 2-Me. In contrast to this, the cell lines T3 and T17, initiated from this tumour series, prefer 20% FBS supplemented medium (RPMI 20% +Me) and are absolutely dependent on 2-Me for growth in vitro. These were the media used to resuspend the cells for assay. Although these cell lines do not require an added source of IL-2, these experiments were designed in order to assess if they were capable of responding to exogenous IL-2, perhaps by an enhanced growth rate. Since crude feline Ly-CM retains mitogen which may be inhibitory to these cells, pp human IL-2 was also used for comparison. The cells were used at confluence and were spun down at 2,000 rpm for 5 minutes then resuspended in fresh medium at a concentration of 5 x 10^5 cells/ml. Blastogenesis assays were performed as usual on each cell line titrating out both crude feline Ly-CM and pp human IL-2 in serial dilutions. A positive control value was obtained by plating the cells in medium supplemented with the optimum concentration of FBS and a negative control value was provided by adding 0.25% Isc to the wells instead of Ly-CM or pp human IL-2 as this is the medium used to generate conditioned medium.

The response of T3 cells in limiting serum conditions to pp human IL-2

T3 cells were plated in 0.25% BSA and a blastogenesis assay was used to determine their response to serial dilutions of pp human IL-2. This serum level was used to try to arrest cell growth and prevent the cells from making an endogenous growth factor which would potentially block receptors for IL-2. T3 cells were spun down and washed in RPMI 0.25% + Me resuspended to 5 x 10^5/ml and 100 µl cells/micro-titre well were plated. Serial dilutions of pp human IL-2, diluted in RPMI 0.25% + Me was added, 6 wells per dilution. RPMI 0.25% + Me was added to the cells to produce a negative control value and RPMI 20% + Me was used to produce a positive control value. The plates were incubated, labelled, harvested and counted as usual.

Dexamethasone inhibition of neoplastic T-cell lines

It has been reported that in the murine system the glucocorticoid dexamethasone specifically inhibits the production but not the utilisation of IL-2 by mature T-cells (Gillis et al., 1979a; 1979b). It has subsequently been shown that glucocorticoids can inhibit the proliferation of some neoplastic T-cell lines (Norman and Thompson, 1977; Smith, 1982) and in the case of IL-2 independent neoplastic T-cell lines, such as those cultured in this series of cats, the inhibition of growth by dexamethasone would indicate that these cells may be producing and responding to an endogenous growth factor. The effect of pharmacological levels (10^{-6}M - 10^{-10}M) dexamethasone on normal cultured feline T-cells was assessed. These cells are dependent on an exogenous source of IL-2 for proliferation in vitro and were therefore cultured in equal volumes of molar concentrations of dexamethasone and Ly-CM. Gillis et al. (1979a) observed a 30% reduction in proliferation of cytotoxic T lymphocyte lines (CTLL) when they were cultured in this manner. This indicates that glucocorticoids exert an inhibitory effect on T-lymphocytes by a dual mechanism; that is, a direct inhibitory effect on T-cell proliferation as well as inhibition of

IL-2 production.

The effect of dexamethasone on the proliferation of Ly-CM supplemented normal T-cells was assayed in the following manner. Normal cultured T-cells at confluence were spun down at 2,000 rpm for 5 minutes and then resuspended to 5 x 10^5 cells/ml in Ly-CM. A volume of 100 µl of this suspension was plated in each well of a Nunc 96 well plate. Dexamethasone was prepared and stored at 4°C in 99% Analar ethanol at a concentration of 2 x 10^{-4}M. A 100 µl aliquot of the solution was evaporated in a well of a 6-well microculture plate (Costar). After evaporation 10 ml of RPMI 20% +Me was added and the microculture plate was incubated at 37°C for 30 minutes. After incubation, this solution (2 x 10^{-6}M) was diluted to give log concentrations ranging from 10^{-6}M to 10^{-10}M dexamethasone. To serve as a positive control value in all experiments, 100 µl of 99% Analar ethanol without dexamethasone was evaporated and subsequently diluted in 10 ml of appropriate tissue culture medium (in this instance RPMI 20% +Me) and 100 µl was added to the cells in the microtitre plate. The effect of dexamathasone was assayed by adding 100 µl/dilution to each of 6 microtitre wells containing 100 µl cells in Ly-CM and measuring ^{3}H-Tdr incorporation following the protocol for a standard blastogenesis assay.

The 4 IL-2 independent T-cell lines FL74, F422, T3 and T17 were also assayed to determine the effect of the same range of concentrations of dexamethasone (10^{-6}M - 10^{-10}M) on cellular proliferation. Each cell line was spun down at 2,000 rpm for 5 minutes then resuspended to 5 x 10^5 cells/ml in appropriate tissue culture medium. A volume of 100 µl of cells were plated per microtitre well and to this 100 µl of either control medium (previously described) or 100 µl of dexamethasone was added to each of 6 wells and the ^{3}H-Tdr incorporation measured by standard blastogenesis assay technique.

The effect of adding an exogenous souce of IL-2 to dexamethasone suppressed cells

It has been shown (Smith, 1982) that the addition of an exogenous source of IL-2 to dexamethasone inhibited MLA-144 gibbon ape T-cells (Rabin et al., 1981) ameliorates the glucocorticoid-mediated suppression of proliferation. If the feline T-cell lines which were suppressed by the addition of dexamethasone were suppressed by the inhibition of production of an endogenous growth factor then the supplementation of the cells with an exogenous source of IL-2 (feline Ly-CM or pp human IL-2) should rescue them. To determine if this was the case with the T3, T17 and F422 neoplastic T-cells 10^{-7}M dexamethasone was added to 5 x 10^5 cells/ml supplemented with a final concentration of 1:2 to 1:32 crude feline Ly-CM or pp human IL-2. The cells were spun down at 2,000 rpm for 5 minutes and resuspended to 5 x 10^5/ml in 10^{-7}M dexamethasone resuspended in appropriate tissue culture medium. A volume of 100 µl of this cell suspension was added to each well of a 96-well microtitre plate. One hundred µl of each Ly-CM or pp IL-2 dilution was added to each of six microtitre wells. ^{3}H-Tdr incorporation was determined using the protocol for a standard blastogenesis assay.

RESULTS

The adsorption of IL-2 by neoplastic T-cells

The adsorption of growth factor by the freshly isolated neoplastic T-cells from conditioned medium is shown in Table 3. Normal activated T-cell adsorption is shown as a control. The adsorption figures are very difficult to interpret since the percentage standard error is so high due to the samples only being run in duplicate. There is some indication that T3 may be adsorbing factor but the rest of the cells do not appear to be adsorbing factor to any great extent. This will be discussed in the last section of this chapter.

The spontaneous production of a growth factor by neoplastic T-cell lines

The results of the assays of the culture supernatants of the T3, F422 and FL74 cell lines are shown in Table 4. Since all three cell lines are capable of growth *in vitro* without an exogenous source of IL-2 the culture supernatants of the lines were assayed to determine if these cells were producing their own growth factor and releasing it into the culture fluid. Clearly none of these cell lines are releasing growth factor in detectable amounts free in the supernatant. However, the following experiments were undertaken to determine if IL-2 was involved in the proliferation of these cells.

The response of neoplastic T-cell lines to crude feline Ly-CM and pp human IL-2

Figures 17 and 18 are graphs representing the response of the neoplastic T-cell lines T3, F422 and T17 to both crude feline Ly-CM and pp human IL-2 respectively. The cell line FL74 was only tested using crude feline Ly-CM since this cell line, although growth factor independent, does not have a *myc* recombinant virus associated with it and has consequently been studied less intensely than T3, T17 and F422.

Figure 17 shows that significant enhancement of growth occurs with all 4 cell lines when they are supplemented with exogenous IL-2 at a 1/8 dilution. Student's t-tests gave p values as follows: $p > 0.001$ for T3, $p > 0.01$ for T17, $p > 0.02$ for FL74 and $p > 0.01$ for F422. The inhibition detected at the 1/2 dilution is most probably due to residual ConA in the crude medium.

Figure 18 shows the response of the cell lines T3, F422 and T17 to pp human IL-2. This preparation (provided by Dr. R. Gallo) has been extensively purified and is reputedly free of contaminating mitogen and other growth factors apart from IL-2. The graph, however, shows both inhibition and enhancement of the T3 and T17 cell lines when supplemented with this source of IL-2. The F422 cell line, however, is markedly inhibited by this preparation and shows no enhancement of growth at all. Student's t-tests performed on the data at a 1/32 dilution of pp IL-2 showed that T3 and T17 responded significantly to this level of factor ($p > 0.001$ for both cell lines) and that F422 was still significantly inhibited ($p > 0.01$). One possible reason for the inhibition of all 3 cell lines could be competition between a putative endogenous factor and the exogenous factor for cell receptors. This was investigated in the following experiment.

The response of T3 cells in limiting serum conditions to pp human IL-2

Figure 19 shows the response of the T3 cell line to pp human IL-2 under limiting conditions for growth (0.25% BSA serum level). Assuming that this renders the cells incapable of producing a putative endogenous factor they now demonstrate significant response to the exogenous IL-2 ($p > 0.001$ at 1/4 and 1/16 dilution points). At high pp IL-2 concentrations (1/2) there was inhibition below the negative control value which is commonly observed when pp IL-2 is plated onto both normal and neoplastic feline T-cells (see also Figures 18, 22 of this chapter and Figure 15 of Chapter Three).

Dexamethasone inhibition of neoplastic T-cell lines

Figure 20 shows that the IL-2 independent T-cell lines T3, T17 and F422 are markedly inhibited by 10^{-6}M dexamethasone, whereas the IL-2 independent FL74 cell line proliferation is only inhibited by approximately 40% of the positive control value (growth in 10% L/M). Smith (1982) showed that for the gibbon ape T-cell line MLA-144 ^{3}H-Tdr incorporation was suppressed by only 20 -25% with 10^{-7} M dexamethasone. This was subsequently determined to be due to the MLA-144 cell population being heterogeneous with respect to glucocorticoid sensitivity and TCGF production. The implication of the results of the dexamethasone inhibition of the feline neoplastic T-cell lines shown in Figure 20 will be discussed in relation to these observations.

The effect of adding an exogenous source of IL-2 to dexamethasone suppressed cells

Figure 21 shows the response of dexamethasone inhibited T3, F422 and T17 cells to IL-2 present in crude feline Ly-CM. A positive control value was obtained by plating cells without dexamethasone into conventional tissue culture medium and a negative control value was obtained by plating cells into conventional tissue culture medium plus 10^{-7} M dexamethasone without an exogenous source of IL-2. Recovery of the cells is expressed as a percentage of the positive control value. T3 and F422 show a maximum recovery of only 6.8% and 8.2% respectively but T17 is rescued to 43% of the positive control value with a 1/4 dilution of Ly-CM. The recovery at this level is greater than that with a concentration of 1/2 Ly-CM probably due to inhibition of the cells by residual mitogen in the crude conditioned medium. Student's t-tests were performed to demonstrate that the observed rescue was statistically significant. For all 3 cell lines response at both 1/2 and 1/4 dilutions of crude factor was shown to be significant ($p > 0.001$) and the same p value was obtained for the rescue of these inhibited cells with pp IL-2, shown in Figure 22 ($p > 0.001$ at 1/2 and 1/4 dilution points). Positive and

negative control values were obtained in an identical manner to that described for the rescue of inhibited cells by crude feline Ly-CM and the recovery was again represented as a percentage of the positive control. T3 and F422 inhibited cells recover to 11.6% and 4.2% of the positive control value respectively and is similar to the levels of recovery detected with crude feline Ly-CM. The T17 rescue by pp IL-2 however, is reminiscent of the response of uninhibited T17 cells to pp IL-2 (see Fig. 18) i.e. there appears to be both inhibitory and enhancing effects operating concomitantly. Maximum rescue occurs with a 1/16 dilution of pp IL-2 and is 27% of the positive control. Differences in recovery obtained with the two sources of IL-2 are most probably due to a combination of factors including different levels of IL-2 activity in the two factors, residual mitogen in the crude Ly-CM and the problem of cross-species reactivity with the human pp IL-2.

The negative control values were depressed to the same extent in Figs. 20, 21 and 22 as follows:

	Fig. 20	Fig. 21	Fig. 22
T3	95%	98%	98%
T17	85%	88%	88%
F422	98%	98%	99%

DISCUSSION

The preceding experiments were designed in order to determine whether or not neoplastic T-cells had an altered requirement for, or response to, IL-2.

Figure 23 depicts two possible relationships that may exist between IL-2 and growth of T-cell tumours. In this series of thymic lymphosarcoma T-cell cultures there are two distinct cell types with respect to requirement for an exogenous source of IL-2 for proliferation. In the case of the IL-2 dependent cultured T-cell lines, exemplified by T11, clearly an exogenous source of IL-2 is essential for continued proliferation of these cells in culture. In those cell lines that are independent of exogenous IL-2 for growth (exemplified by T3), two principal possibilities exist to explain IL-2 involvement in the proliferation of these cells. Either IL-2 plays no role in the growth of these cells in vitro i.e. the cells have escaped the requirement for this factor, or the cells may be producing and responding to IL-2 or an IL-2-like endogenous growth factor. The cell lines T3 and T17 are dependent on the presence of 2-Me (an IL-1 like signal) in the culture medium for growth suggesting that this may serve as a mitogenic signal to these cells to produce IL-2, similar to the induction of IL-2 production by normal helper T-cells. The IL-2 independent T-cell lines FL74 and F422 however do not require 2-Me for growth suggesting that either these cells do not produce their own factor or that they have lost the requirement for this signal.

The first set of experiments performed on this series of thymic lymphosarcomas was carried out to determine if these neoplastic T-cells were displaying receptors for IL-2 and were actively adsorbing it from lymphocyte conditioned medium. There were, however, several problems associated with the design of this experiment since the majority of these tumours did not establish in long-term culture and it was impossible to determine in advance which ones would. It was therefore necessary, in order to standardise the assay, to use cells which were freshly isolated

from tumour material and these cells may not have been actively dividing as will be discussed later. The interpretation of the results was also rendered difficult because of the exceedingly high standard error of the results. The standard errors were unacceptably high due to the necessity to compare only two sets of duplicate points (adsorption medium/control medium). In retrospect, at least four more duplicates for each test set should have been run to bring the standard error down to an acceptable level for analysis of the data. It would, however, appear from the results that there is some indication of adsorption of IL-2 from Ly-CM by the T3 cells. T3 established in culture as an IL-2 independent T-cell line after one week, during which time the cells were dependent on IL-2 for growth. Therefore the low level of adsorption of IL-2 may have been due to only a subset of the T3 cells being responsive to IL-2 or alternatively that IL-2 receptors on the cell surface were already blocked. If the latter were indeed the case then this would imply that these cells are producing their own endogenous factor which can bind to surface IL-2 receptors and block them. This possibility was investigated extensively in subsequent experiments. Of the other cell lines tested T11 established in culture as an IL-2 dependent cell line but these cells showed no IL-2 adsorption in this test and none of the other tumours tested adsorbed IL-2 to the extent that was expected. There are two principal explanations for this: firstly, the necessity to use freshly isolated tumour cells meant that any IL-2 receptors on the cells may have already been blocked *in vivo* rendering them incapable of adsorbing detectable quantities of IL-2 from the conditioned medium in this test. Secondly, since these cells had been isolated from the tumours immediately before use, they may initially have gone through a lag period, during which time IL-2 receptors may not be expressed, before recommencing cell division. Consequently, the lack of detectable adsorption in this assay may not be indicative of either a lack of receptors for IL-2 or a lack of response to it.

The possibility that the IL-2 independent cell lines may be producing an endogenous growth factor was investigated further by

analysing the culture supernatants of the T3, F422 and FL74 cells for IL-2. The results of IL-2 production over a 3-day test period demonstrate that none of these cells are spontaneously producing detectable IL-2 free in the culture medium. Mier and Gallo (1981) have demonstrated acid-glycine buffer elution of IL-2 from the surface of a human IL-2 independent neoplastic T-cell line suggesting that a significant portion of IL-2 synthesised by the cell is immobilized on the plasma membrane. Unfortunately, the acid elution protocol was unsuccessful in my hands. Furthermore, the neoplastic T-cell line described by Mier and Gallo also actively adsorbed IL-2 from conditioned medium in a manner similar to normal activated T-cells, which was not the case with T3.

Mier and Gallo (1981) have also reported that this particular line, although independent of an exogenous source of IL-2 for growth, can respond to added IL-2 by increased proliferation in culture. The FL74, F422, T3 and T17 IL-2 independent feline T-cell lines were similarly tested for IL-2 response by adding two different sources of exogenous IL-2; crude feline Ly-CM and pp human IL-2. Figures 17 and 18 show the response of the cell lines to both factors with nutritionally optimum medium. All four cell lines show a slight increase in proliferation over the positive control value when supplied with crude feline Ly-CM suggesting that they are capable of responding to some extent to exogenous IL-2. The limited magnitude of response could be explained if these cells are indeed producing their own growth factor which is blocking the IL-2 receptor sites either at the external or internal domain. In support of this theory, the T3, F422 and T17 cells show inhibition of positive control levels of growth when supplemented with high concentrations of pp human IL-2. Since this source of IL-2 is reputedly free of mitogen and other contaminating growth factors, it may be that this preparation is competing with the cells own putative growth factor for available receptors. Since, however, Mier and Gallo found that the cells responded maximally to an exogenous source of IL-2 when they were plated under nutritionally sub-optimum conditions, the response of T3 cells to pp human IL-2 was determined under these conditions (Figure 19). Although the

response obtained was 2-3 times that of the negative control value, it was only, at most, 10-12% of the positive control. Ideally various concentrations of serum plus pp IL-2 would require to be assayed but the quantity of pp human IL-2 available to us at that time was limiting. It was concluded that these IL-2 independent T-cell lines were capable of responding to an exogenous source of IL-2 but that further investigation was required to determine if the cells were producing an endogenous factor which could be blocking IL-2 receptors.

Gillis et al. (1979a; 1979b) have shown in the murine system that the glucocorticoid, dexamethasone, specifically inhibits the production but not the utilisation of IL-2 by mature T-cells. Figure 20 demonstrates that dexamethasone similarly inhibited the T3, T17 and F422 cell lines by 90%. The FL74 cell line was inhibited only by 40% maximum and this was possibly due to heterogeneity of the cells with respect to glucocorticoid sensitivity previously reported by Smith (1982). These results strongly support the hypothesis that these IL-2 independent T-cell lines are producing and responding to an endogenous growth factor and that the inhibition of production of this factor terminates cell growth. Since T3, T17 and F422 were inhibited to a greater extent by dexamethasone it is possible that these cell populations are fairly homogeneous with respect to glucocorticoid sensitivity.

These three cell lines were further investigated to determine if the dexamethasone suppressive effect could be ameliorated by the addition of an exogenous source of IL-2 proving the specificity of the inhibition. The results of these experiments were inconclusive but as is shown in Figures 21 and 22 both crude feline Ly-CM and pp human IL-2 are capable of at least partially rescuing these cells from dexamethasone inhibition. Of particular note is the fact that T17 is rescued far better by crude feline Ly-CM than are T3 and F422, furthermore, high concentrations of pp human IL-2 are once again inhibitory to these cells to the extent that maximum rescue occurs at a dilution of 1/16 pp IL-2. The explanation for the incomplete rescue of these cell lines is possibly different for the

two different sources of IL-2. Firstly, the low level of rescue effected using crude feline Ly-CM may be a function of the low level of activity of IL-2 in this medium compared to that normally produced endogenously by the cells. If this were so then only a small proportion of the cellular IL-2 receptors would be stimulated and the response would be minimal like that of T3 and F422. The discordance in the response of T17 could hypothetically be explained by a lesser requirement for IL-2 by these cells for growth and they may consequently be expected to produce less endogenous factor than T3 or F422. Clearly actual cellular levels of IL-2 will have to be determined to test this hypothesis. Secondly, although pp human IL-2 is highly active it may have reduced activity on feline cells and may even be competitive at high concentrations as inhibition is detected at these levels. The comparison of the effect of crude feline Ly-CM and pp human IL-2 on normal feline T-cells and on the IL-2 dependent T11 neoplastic T-cell line (Fig. 12, Chapter Three) suggests that although pp human IL-2 is highly active (800 x concentrated) it has no greater activity on feline cells than does crude feline Ly-CM which is unconcentrated. Furthermore, there was inhibition at high concentration (1:2 pp IL-2) of factor detectable in Fig. 22 which theoretically cannot be due to contaminating mitogen.

In conclusion, preliminary evidence from these results suggest that the IL-2 independent cell lines T3, T17 and F422 (and possibly FL74) may be capable of growth in vitro in the absence of an exogenous source of IL-2 by virtue of their capacity to produce and respond to an endogenous factor. The only definitive way (in the absence of suitable antisera) of determining IL-2 levels in these cell lines will be to examine cellular mRNA for IL-2 gene expression. A human IL-2 probe has now been made available (courtesy of Dr. R. Gallo) which will be tested to determine whether or not it will detect feline IL-2 sequences and to determine if the IL-2 gene is rearranged in the tumours, perhaps as a secondary step in the transformation of these cells.

TABLE 2.

Adsorption test protocol

Tube No.	Cell concentration	1 Hr. in 1 ml medium	After 1 hr + 1 ml medium
1	10^7	Ly-CM	0.25% Isc.
1a	10^7	Ly-CM	0.25% Isc.
2	10^7	0.25% Isc.	Ly-CM
2a	10^7	0.25% Isc.	Ly-CM
3	0	Ly-CM	0.25% Isc.
3a	0	Ly-CM	0.25% Isc.
4	0	0.25% Isc.	Ly-CM
4a	0	0.25% Isc.	Ly-CM

The table shows the protocol used to detect the adsorption of IL-2 from Ly-CM with control tubes to detect production of an endogenous factor and negative controls of media alone. All tubes were incubated at 37°C for 1 hour.

TABLE 3

The adsorption of IL-2 by neoplastic T-cells

Test	Set				$\bar{x}$	n	% S.E.	% Adsorption	% S.E.
T 3A	10^7	cells	Ly-CM	1072	953	5	9.6		
B	"	"	"		1192	6	11.4	40.0	13.9
C	No	"	"	1784	1838	6	6.2		
D	"	"	"		1731	6	14.6		
T 4A	10^7	"	"	568	581	5	5.2		
B	"	"	"		555	6	7.2	15.3	5.5
C	No	"	"	670	693	6	1.2		
D	"	"	"		647	6	6.9		
T 5A	10^7	"	"	2531	2467	6	3.2		
B	"	"	"		2595	6	5.6	24.0	12.8
C	No	"	"	3324	2981	6	4.6		
D	"	"	"		3667	6	7.4		
T 7A	10^7	"	"	6384	6476	6	9.0		
B	"	"	"		6292	6	2.3	18.2	2.3
C	No	"	"	7802	7727	6	5.1		
D	"	"	"		7878	4	6.1		
T 8A	10^7	"	"	7604	7212	6	3.9		
B	"	"	"		7996	6	3.2	22.6	8.5
C	No	"	"	9815	9477	5	5.0		
D	"	"	"		10154	5	7.6		
T10A	10^7	"	"	11181	10033	6	2.7		
B	"	"	"		12397	5	4.1	28.8	18.0
C	No	"	"	15689	14893	6	2.9		
D	"	"	"		16485	6	4.2		
T11A	10^7	"	"	16667	17266	6	0.8		
B	"	"	"		16069	5	1.4	3.1	5.2
C	No	"	"	17193	17492	6	2.3		
D	"	"	"		16895	6	1.3		

TABLE 3 (Cont'd).

Test Set					$\bar{x}$	n	% S.E.	% Adsorption	% S.E.
T15A	10^7	"	"	23064	23685	6	5.0		
B	"	"	"		22443	6	3.5	19.0	6.5
C	No	"	"	28386	27290	5	7.6		
D	"	"	"		29482	6	6.1		
N:TA1	10^7	cells	Ly-CM		3887	4	1.7		
B1	"	"	"	3962	4101	4	3.3		
A2	"	"	"		3808	4	3.6	30.0	3.0
B2	"	"	"		4054	4	4.0		
C1	No	"	"		5650	4	1.9		
D1	"	"	"	5621	5816	4	6.2		
C2	"	"	"		5561	4	2.2		
D2	"	"	"		5459	4	3.3		

T3-T15 are tumour cell lines.

N:T are normal cultured feline T-cells.

TABLE 4

The spontaneous production of a growth factor by neoplastic T-cell lines

Sample set	$\bar{x}$ (cpm)	n	S.E.
FL74 +ve Control	19841	6	350.0
-ve Control	67	6	5.7
24 hr. supernatant	113	6	13.0
48 hr. supernatant	101	6	13.4
72 hr. supernatant	102	6	14.3
F422 +ve Cotrol	19841	6	350.0
-ve Control	67	6	13.0
24 hr. supernatant	84	6	5.7
48 hr. supernatant	63	5	3.1
72 hr. supernatant	111	5	35.3
T3 +ve Control	7413	5	541.6
-ve Control	90	6	4.9
24 hr. supernatant	112	6	15.5
48 hr. supernatant	186	6	42.8
72 hr. supernatant	101	6	14.3

The +ve control value was obtained by plating the normal cultured T-cells in RPMI 20% + Me with an equal volume of Ly-CM and the -ve control value was obtained by plating the cells in RPMI 20% + Me alone.

FIGURE 16.

PROPOSED MECHANISM OF MITOGENESIS IN NORMAL CELLS

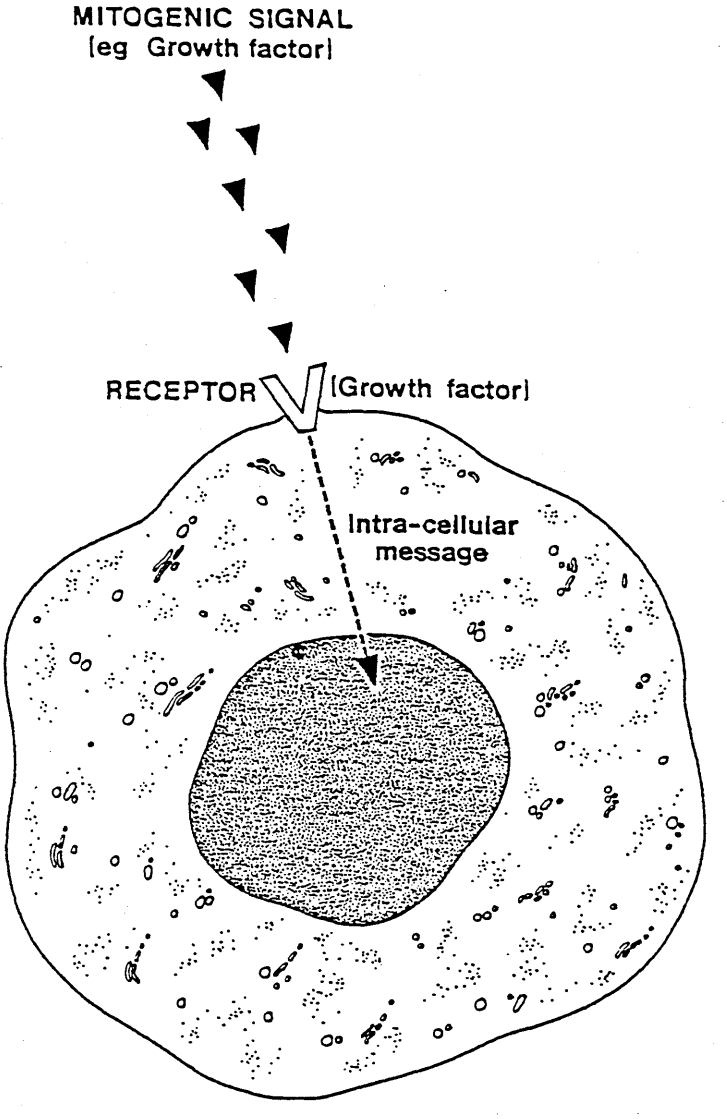


Figure 16 depicts the mechanism of mitogenesis that is thought to induce cell division. One or more mitogenic signals may stimulate a cell membrane receptor to relay a series of intra-cellular messages to the nucleus to instruct the cell to divide.

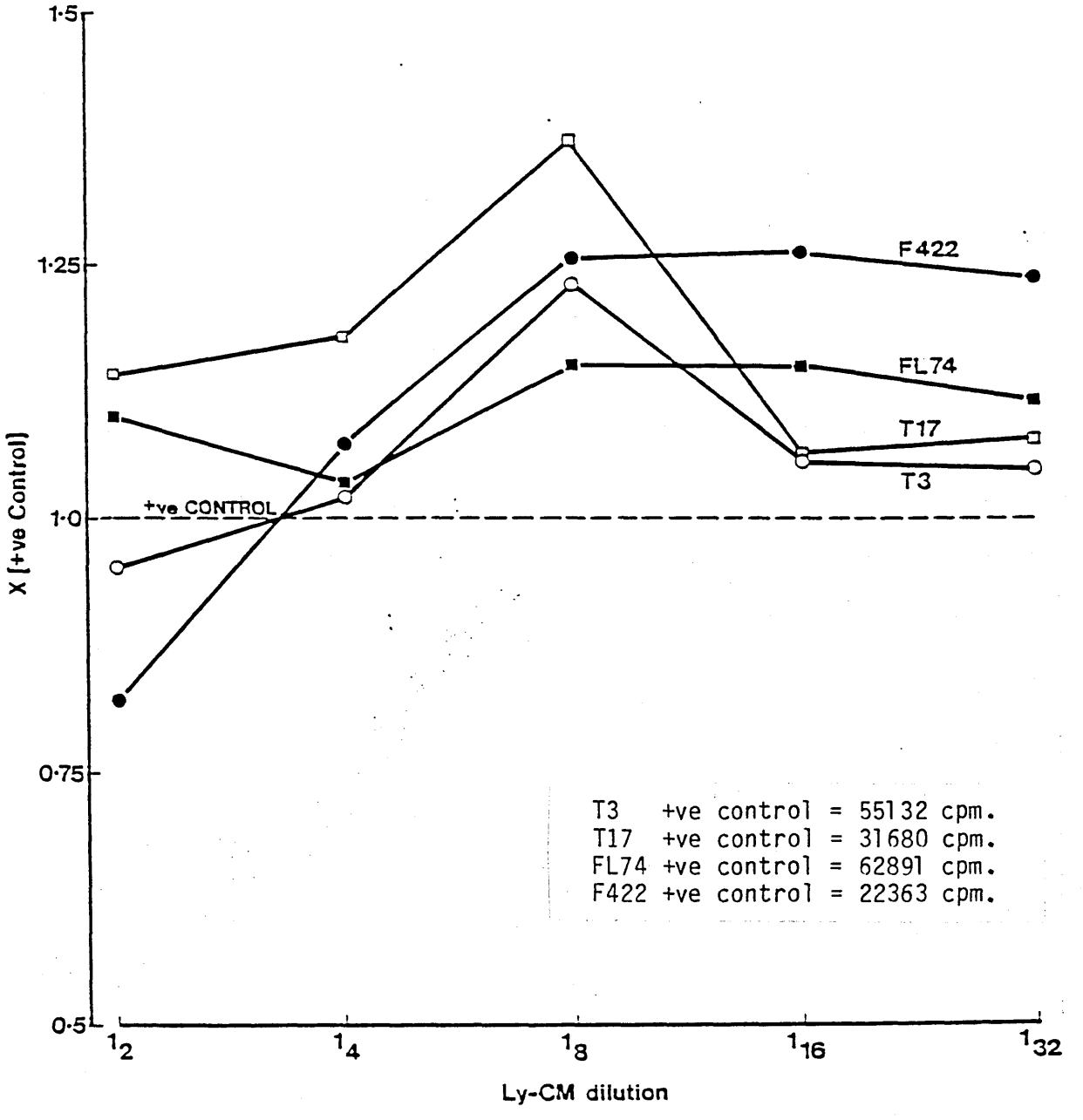


FIGURE 17

The response of neoplastic T-cell lines to crude feline Ly-CM

Students t-tests performed on the data at the 1/8 dilution point revealed significant differences over positive control values for all the cell lines. (F422, $p > 0.01$; T17, $p > 0.01$; T3, $p > 0.001$ and FL74, $p > 0.02$). Each point on the graph represents the mean of 6 replicates (% SE $<$ 5%).

FIGURE 18.

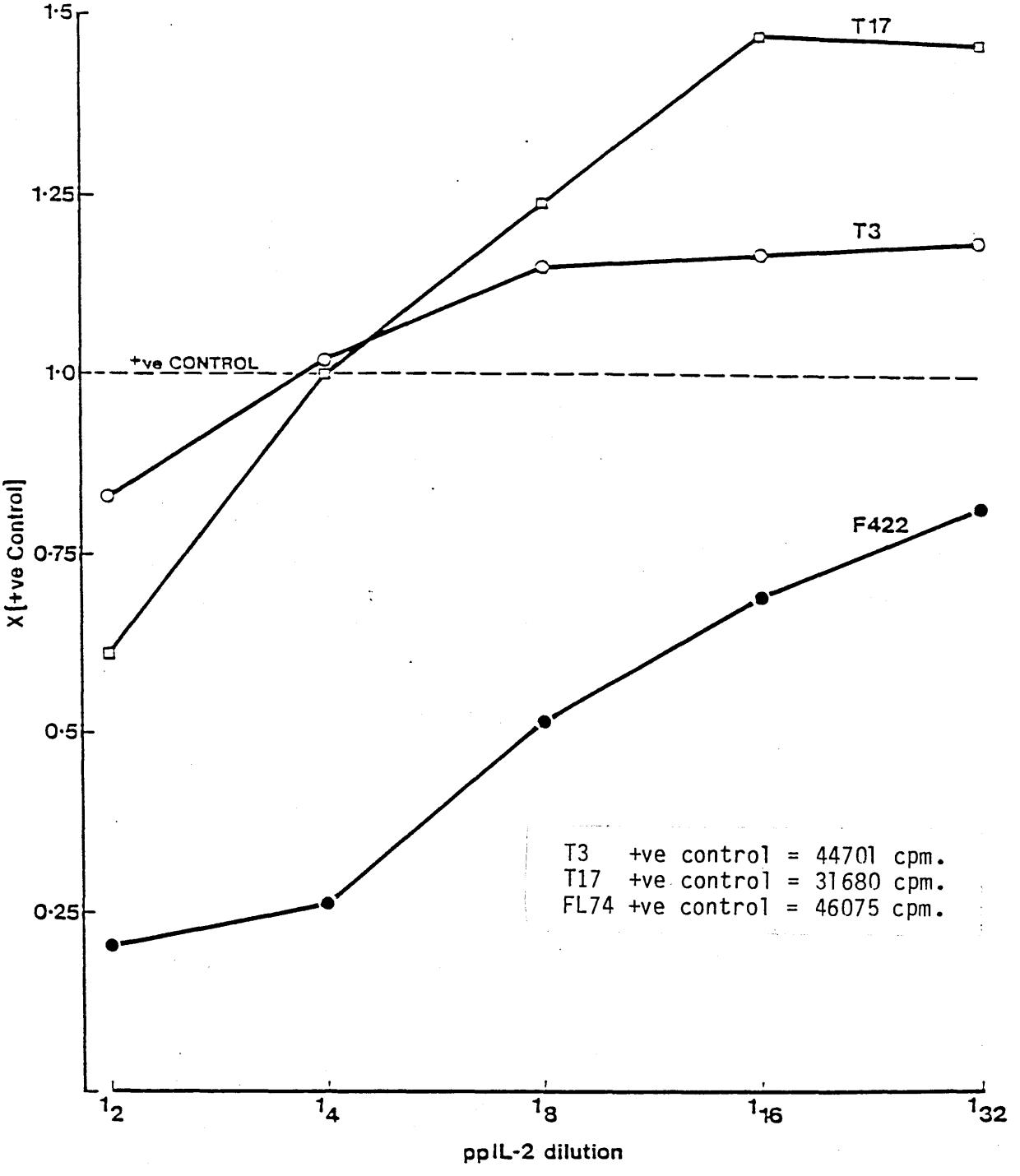


FIGURE 18

The response of neoplastic T-cell lines to pp IL-2

Students t-tests performed on the data at the 1/32 dilution point revealed significant differences over positive control values. The T3 and T17 cell lines show enhancement and the F422 cell line shows inhibition (T3, $p > 0.001$; T17, $p > 0.001$; F422 $p > 0.01$). Each point on the graph represents the mean of 6 replicates (% SE $\leq$ 4%).

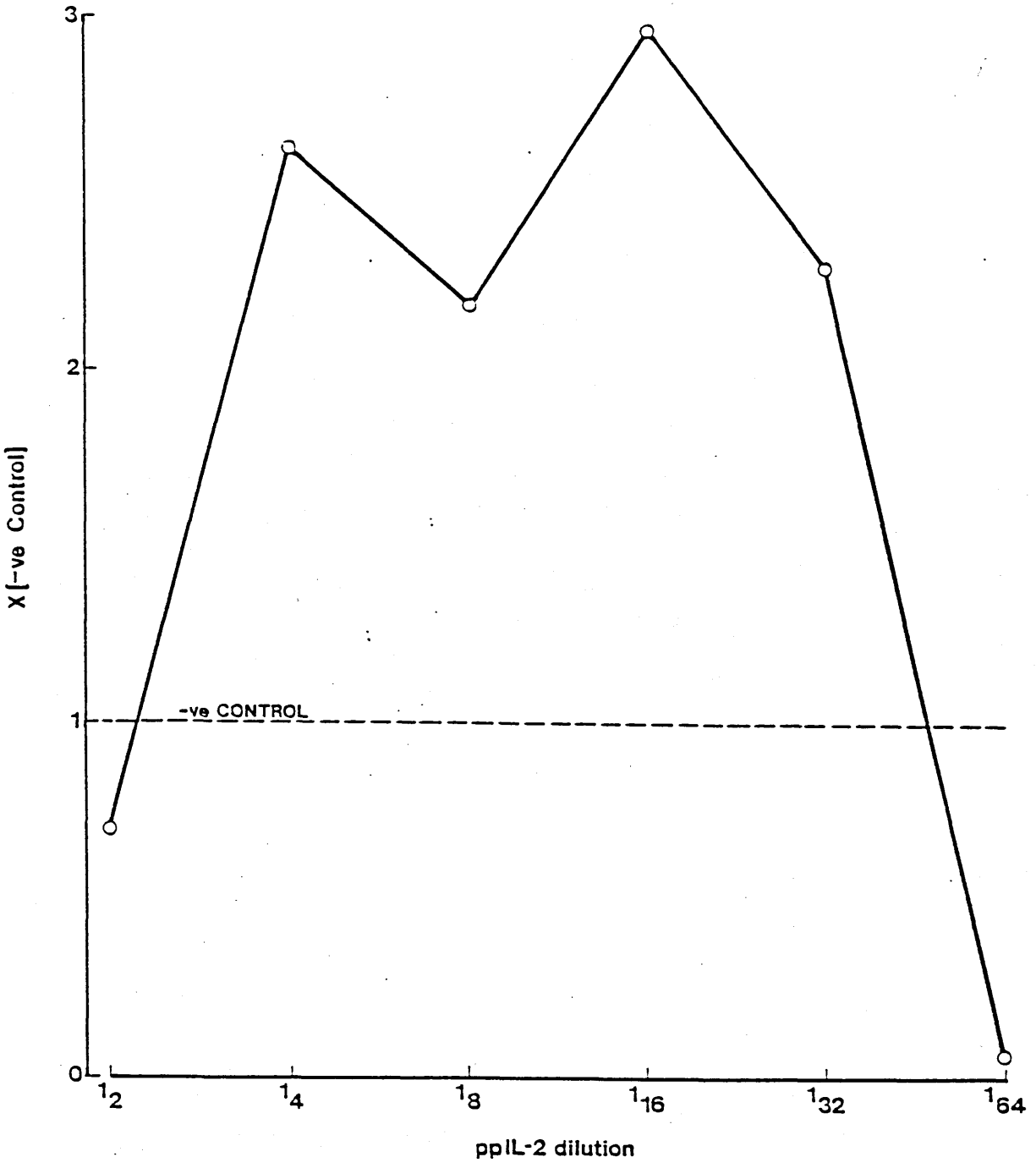


FIGURE 19

The response of T3 cells in limiting serum conditions to pp IL-2

Students t-tests performed on the data at the 1/4 and 1/16 dilution points revealed significant enhancement over negative control values ($p > 0.001$). Each point on the graph represents the mean of 6 replicates (% SE $\leq$ 5.8%).

T3 -ve control value = 3976 cpm.
T3 +ve control value = 79520 cpm.

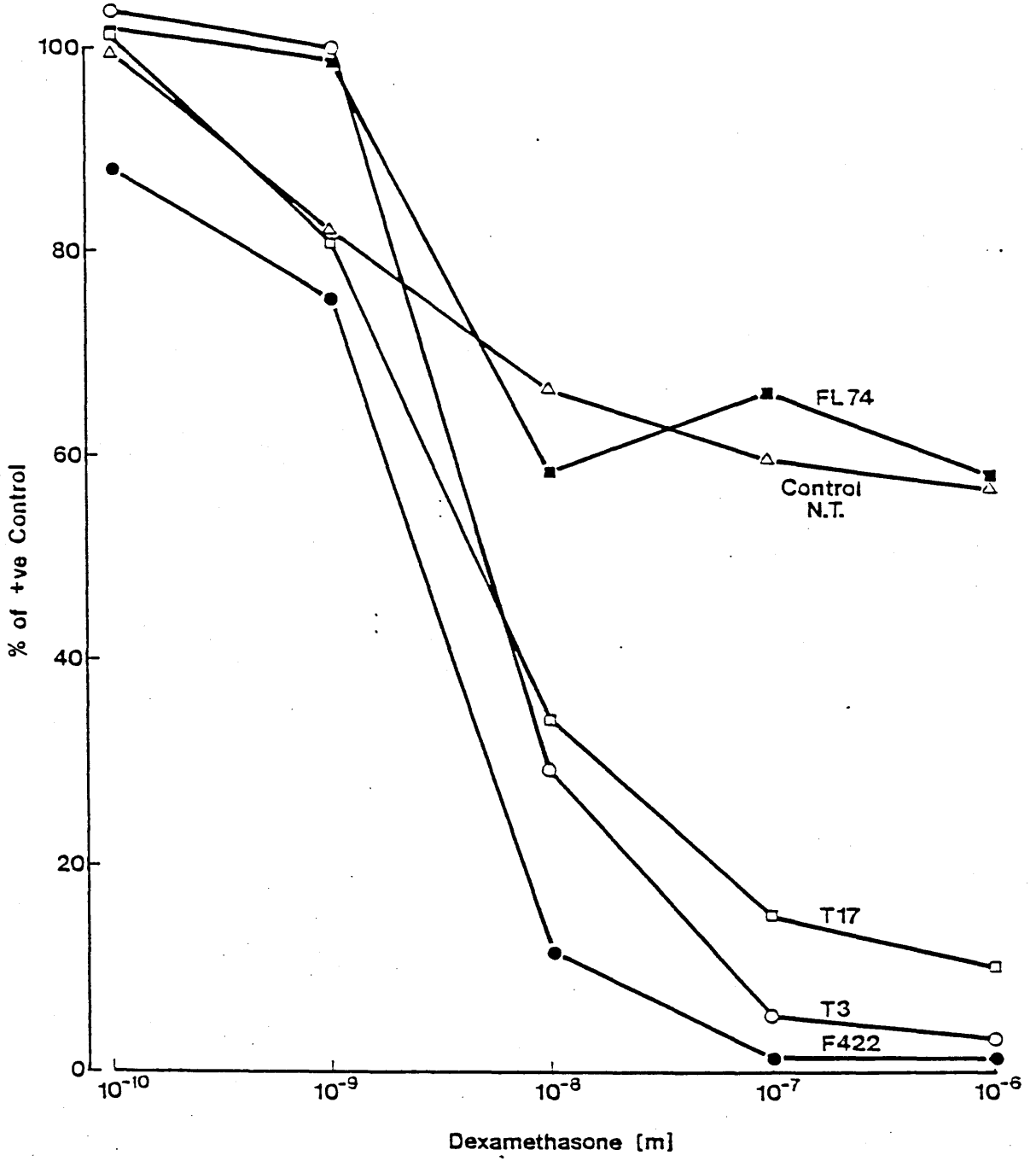


FIGURE 20

Dexamethasone inhibition of neoplastic T-cell lines

This figure shows the inhibitory effect of the glucocorticoid dexamethasone on 4 neoplastic T-cell lines and on normal cultured T-cells. Each point on the graph represents the mean of 6 replicates (% SE $\leqslant$ 10%).

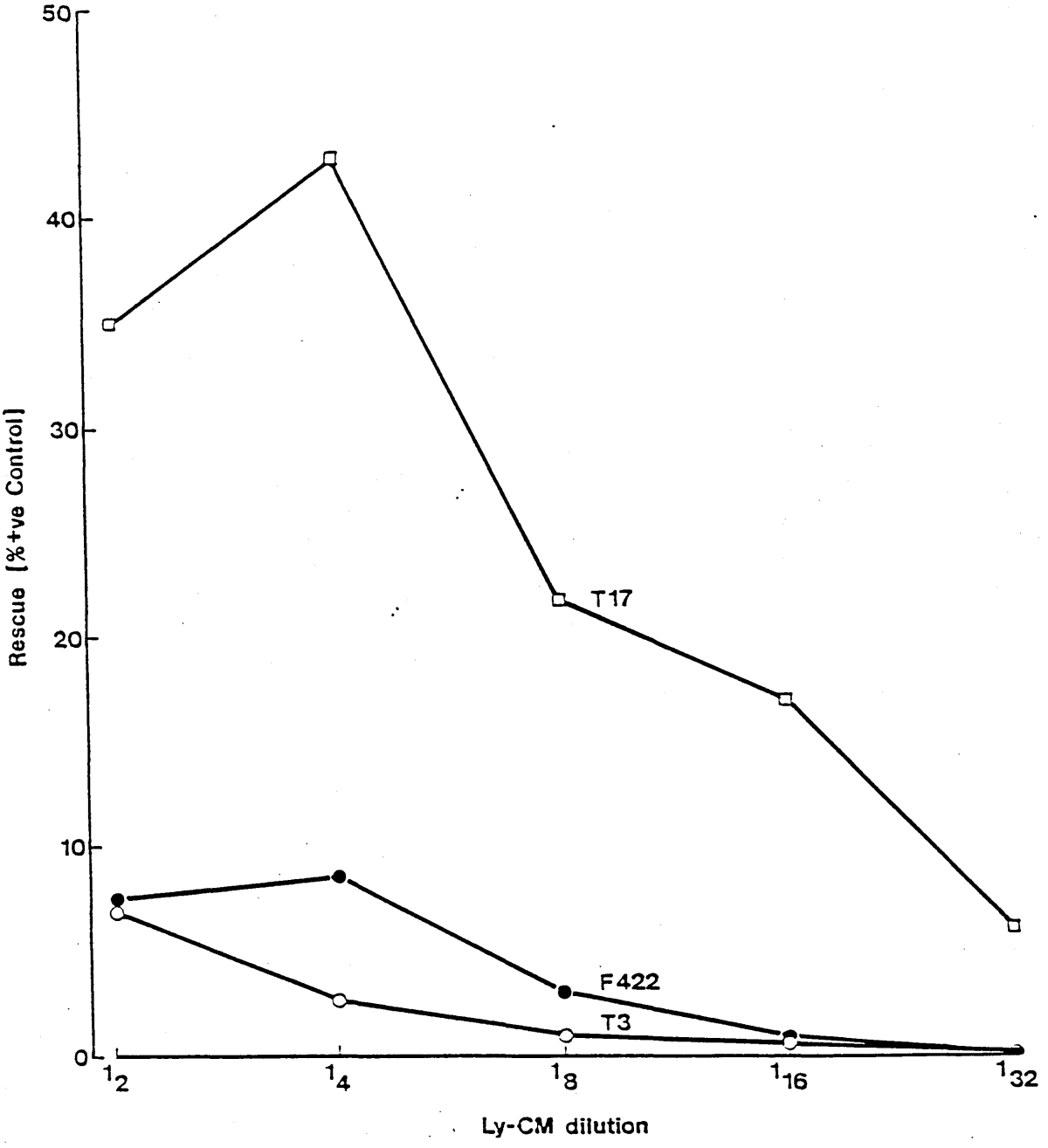


FIGURE 21

The partial rescue of dexamethasone suppressed cells with Ly-CM

Students t-tests performed on the data at the 1/2 and 1/4 dilution points revealed significant differences over negative control values for all 3 cell lines ($p > 0.001$). Each point on the graph represents the mean of 6 replicates. (For T3, % S.E $\leq$ 5.9%, T17, % S.E. $\leq$ 6.6%; F422, % S.E. $\leq$ 12.8%).

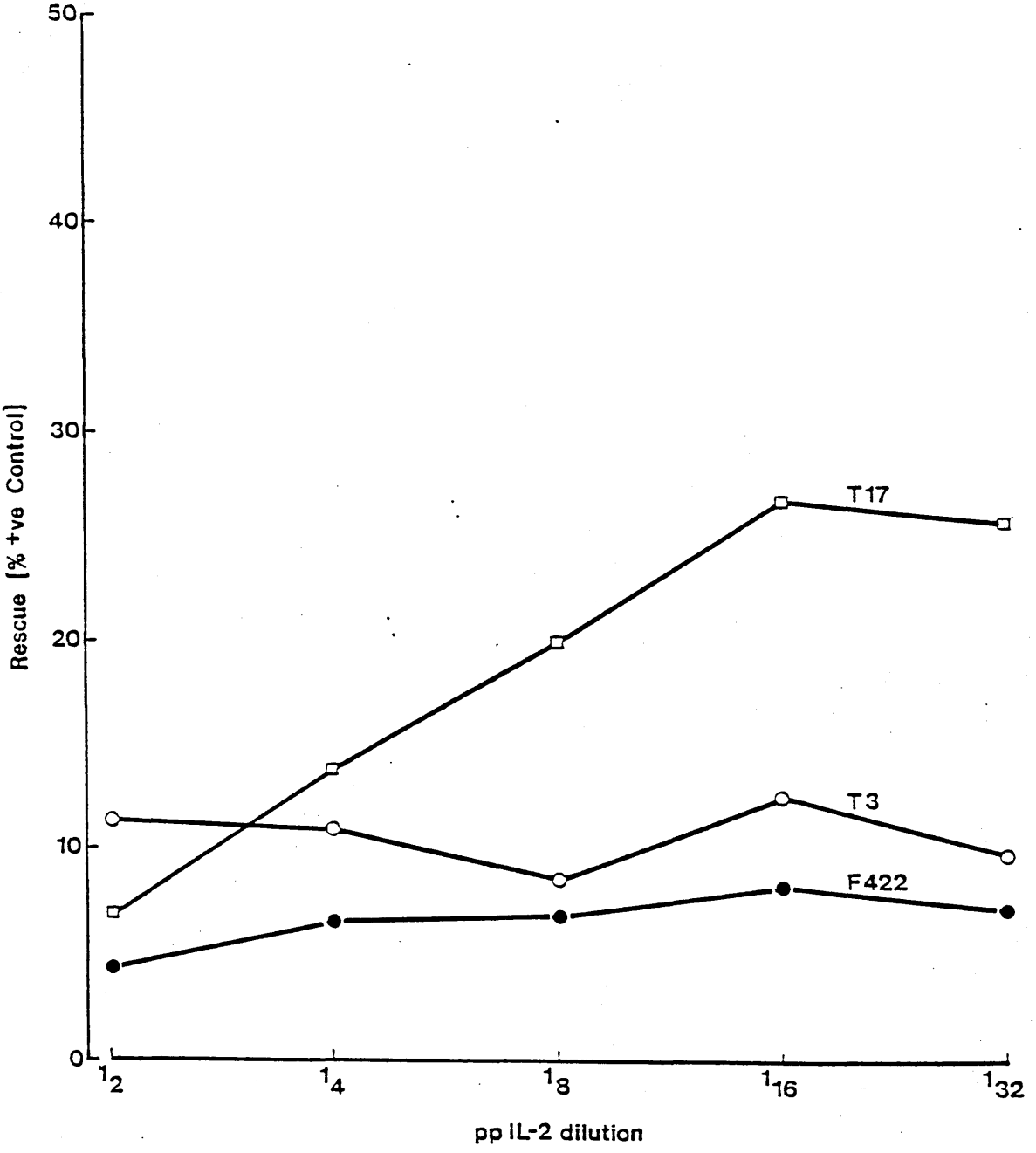


FIGURE 22

The partial rescue of dexamethasone suppressed cells with pp IL-2

Students t-tests performed on the data at the 1/2 and 1/4 dilution points revealed significant differences over negative control values for all 3 cell lines ($p > 0.001$). Each point on the graph represents the mean of 6 replicates. (For T3, % S.E $\leq$ 5.8%; T17, % S.E. $\leq$ 9.7%; F422, % S.E. $\leq$ 9.5%).

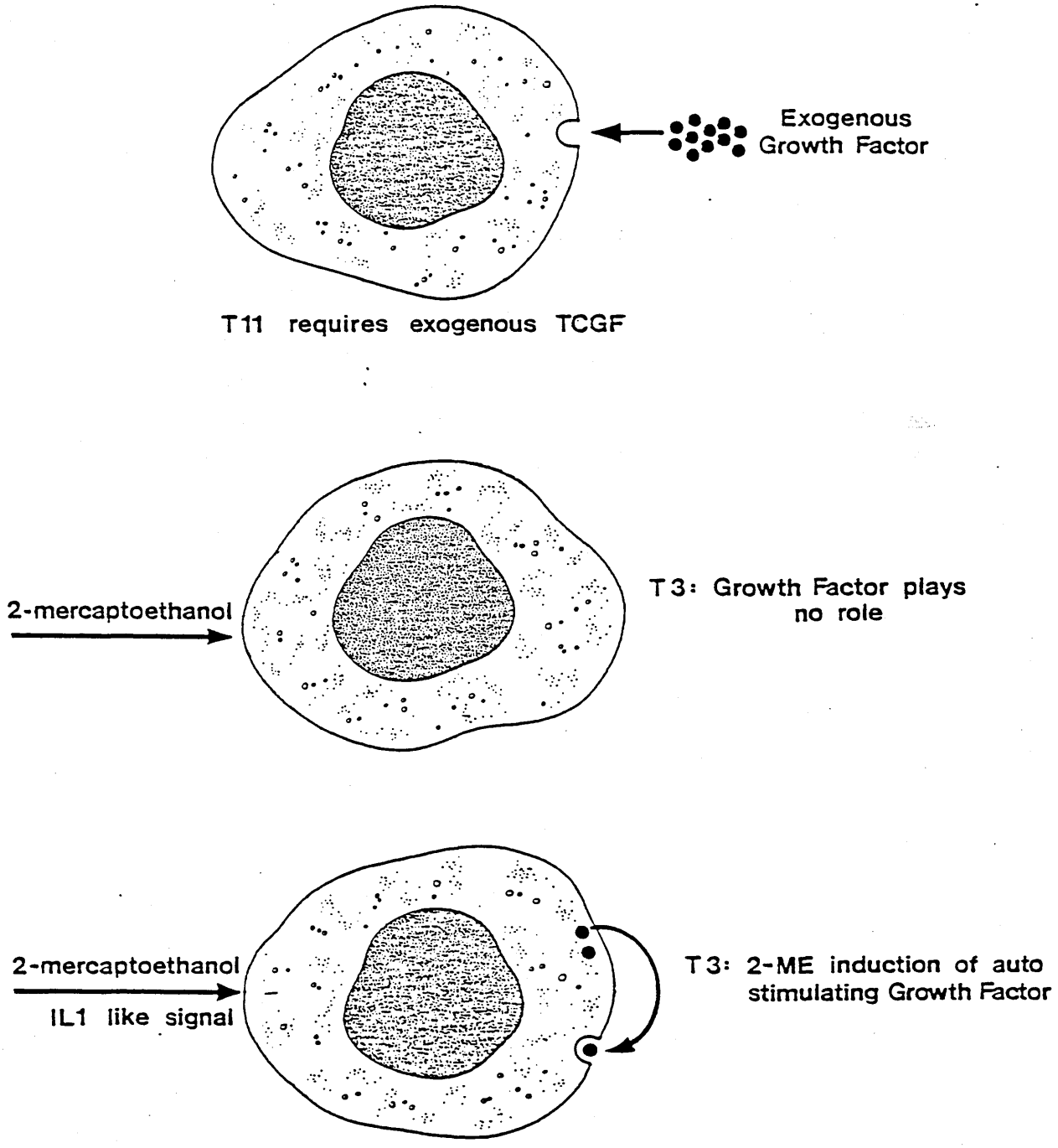
FIGURE 23

The relation of growth factor to tumour cell growth

Figure 23 depicts two possible relationships that may exist between IL-2 and growth of T-cell tumours. In this tumour series, two types of cell were cultured with respect to requirement for an exogenous source of IL-2 for proliferation. The first cell type is absolutely dependent on exogenous IL-2 for growth and is exemplified by Tll. The other cell type which grows independently of an exogenous source of IL-2 and is exemplified by T3, may either have lost the requirement for this signal for cell proliferation or may be producing and responding to an endogenous factor.

FIGURE 23.

The relation of growth factor to tumour cell growth



CHAPTER FIVE

THE NATURE OF THE VIRUSES ASSOCIATED WITH FELINE T-CELL LYMPHOSARCOMAS

Oncogenic retroviruses are generally classified as weakly or strongly transforming and several models (detailed in the General Introduction) have been proposed to explain the pathogenesis of each type. In brief, strongly transforming viruses usually contain a specific oncogene and are consequently defective, requiring a non-defective helper virus for replication. It is thought that the altered or inappropriate expression of the viral oncogene (v-onc) may be how the strongly transforming retroviruses exert their oncogenic effect. Models to explain the oncogenic potential of weakly transforming retroviruses include insertional mutagenesis which is implicated in the pathogenesis of avian B-cell tumours induced by ALV. In this process the virus is found integrated adjacent to the oncogene c-myc and enhances its level of transcription many fold over the normal expression rate (Neel et al., 1981). This model was investigated in the pathogenesis of the thymic lymphosarcomas associated with FeLV presented here. Surprisingly, although integration of FeLV close to the c-myc locus did occur in a proportion of the cats, causing a rearrangement of the c-myc gene, far more frequently a novel feline leukaemia virus was isolated which had acquired a v-myc oncogene (Neil et al., 1984).

In the following chapter these recombinant viruses were studied in three respects; (i) the viral genomic RNA was analysed for myc sequences (ii) in vitro experiments were carried out to determine if these recombinant viruses were infectious for FEA cells and (iii) the subgroup of the viruses both circulating in the plasma of these cats and released from the cultured tumour cells was determined to establish whether or not a specific subgroup of FeLV was associated with thymic lymphosarcoma of the cat.

There are three subgroups of FeLV defined by interference testing which are designated A, B or C (Sarma and Log, 1973). The basis of the interference test is that cells infected with a virus of one subgroup are resistant to superinfection with a virus of the

same subgroup but can be super-infected with a virus of a different subgroup. Although the viruses being tested are leukaemia viruses and are non-cytopathic to the FEA cells, for challenge it is convenient, for detection, to use a sarcoma virus that is a pseudotype with a transforming genome and the envelope of a leukaemia virus of a specific subgroup. These pseudotype viruses produce a morphological alteration of the FEA cells indicating that infection has occurred.

Two principal approaches have been used to establish a correlation between FeLV subgroup and disease. One has been to determine the subgroup of FeLV associated with specific types of disease in individual animals and the other has been to experimentally infect cats with viruses of known subgroup and observe the outcome of infection.

The subgroups FeLV-B and FeLV-C have never been isolated in natural conditions alone but always occur in the cat together with FeLV-A (Sarma and Log, 1971, 1973; Jarrett et al., 1978). FeLV-A grows well in cats, 100% of kittens inoculated at birth becoming persistently viraemic (Hoover et al., 1976; Jarrett et al., 1984). However there is an age-related decline in the permissiveness for FeLV-A infection which is correlated with a decline in the ability of macrophages to support FeLV replication (Hoover et al., 1981). FeLV subgroups B and C are also capable of producing a persistent viraemia to a lesser or greater extent (20% and 100% respectively) when inoculated into newborn kittens but resistance to infection by these viruses develops almost immediately after birth. In experimental in vivo studies by Jarrett and Russell (1978) on the differential growth and transmission of FeLV subgroups A and B, it was determined that virus was quickly recovered from the blood and oropharynx of most animals infected with subgroup A alone, whereas only 20% of cats infected with subgroup B alone developed a viraemia, and that after a long time. In cats infected with a mixture of the two subgroups (FeLV-AB), the results showed that FeLV-A was recovered from the plasma first in a high percentage of cats and FeLV-B appeared later in a much lower percentage of cats.

In this respect, therefore, the viruses appeared to operate independently but evidence of interaction was also apparent in that the percentage of cats which were viraemic with FeLV-B was greater following FeLV-AB infection than afterinfection with FeLV-B alone. In addition, FeLV-B was transmitted by contact from cats infected with, and excreting, FeLV-AB but not from cats infected with and excreting FeLV-B alone.

In similar studies on the interaction of FeLV subgroups A and C, results show that cats over 8 weeks of age, which are normally not susceptible to FeLV-C, can become infected when subgroup C is introduced into the cat in a mixture with FeLV-A (FeLV-AC) (Jarrett et al., 1984). Again FeLV-A is detected in the plasma long before FeLV-C. Assuming that the hypothesis that many more cells in the cat are susceptible to subgroup A infection than are susceptible to subgroups B and C infection is correct, then the observed enhancement in the growth of FeLV-B and FeLV-C by FeLV-A is probably attained by the generation of phenotypic mixtures when both viruses grow in the same cell. This would give rise to viruses with subgroup B and C genomes and mosaic envelopes predominantly subgroup A, enabling the spread of subgroup B and C viruses into cells normally permissive for infection by subgroup A only.

In nature FeLV-C is the only one of the three subgroups of FeLV associated with a specific disease, although epidemiological observations suggest that an additional subgroup other than A, usually B, is more often associated with cats with lymphosarcoma than with their healthy viraemic counterparts (Jarrett, 1980). FeLV-C causes erythroid hypoplasia, a condition in cats which is analagous to pure red cell aplasia in humans (Hoover et al., 1974; Mackey et al., 1975). There are four individual isolates of FeLV-C which were all isolated from naturally occurring cases of severe anaemia and experimentally produce erythroid hypoplasia on inoculation into newborn kittens (Mackey et al., 1975; Onions et al., 1982). In addition, in the experiments of Jarrett et al. (1984) on the interaction of subgroups A and C, it was found that

the appearance of subgroup C in the plasma of the 8 week old kittens inoculated with FeLV-AC strongly correlated with the development of erythroid hypoplasia in all these kittens. The generation of erythroid hypoplasia clearly demonstrates the specificity of this virus (FeLV-C) for the erythroid precursor cell, the BFU-E (Boyce et al., 1981; Onions et al., 1982).

Evidence for the association of an additional subgroup of virus other than A with neoplastic disorders is largely based on epidemiological investigations. Mentioned earlier, was the observation by Jarrett (1980) that in closed MCHs there was a higher proportion of FeLV-AB in cats with lymphosarcoma than in the normal healthy viraemic cats. This may either indicate that FeLV-B has some, as yet undefined, role to play in the generation of lymphosarcoma or it may simply reflect that the household has been endemic with FeLV for a long time, resulting in a greater opportunity for the development of lymphosarcoma which has a long latent period in cats under natural conditions (Jarrett, 1980). Further evidence suggesting that the association of subgroup B with lymphosarcoma is specific comes from the results of the outcome of infection of kittens with FeLV-B alone in the experiments of Jarrett and Russell (1978). In these experiments FeLV-B infected cats were followed longterm and 25% (2/8) developed lymphosarcoma after a long latent period (1-2 years) (Jarrett, 1980) suggesting that the B subgroup may indeed be involved in the development of neoplasia. Strongest support for this hypothesis has emerged recently from experiments by Onions et al. (pers. comm.) demonstrating the rapid induction of myeloid leukaemia by a novel FeLV containing both subgroups A and B, termed FeLV-AB/GM1, which was originally isolated from the spleen and bone marrow of a field case of erythroleukaemia. The development of leukaemia by this virus correlated with the appearance of the subgroup B virus in the plasma. Following infection with the virus, all the cats became viraemic with the subgroup A virus and latently infected with the subgroup B virus. In those cats which remained haematologically normal, this distribution of subgroups was maintained, whereas in those cats which underwent the transition from normal haematology

to leukaemia, there was a concomitant change from subgroup A viraemia with subgroup B latency to subgroup AB viraemia.

In the tumour series presented here the subgroup of the virus associated with the tumour cells and circulating in the plasma was determined to establish whether or not there was any correlation between subgroup and disease in these cats. The rapidity of the onset of leukaemia induced by the FeLV-GM1 virus was unusual as most feline leukaemia viruses have long latent periods *in vivo* before neoplasia develops. This suggests that these viruses may carry an oncogene as FeLV appears to be particularly adept at recombining with cellular proto-oncogenes (Besmer *et al.*, 1983b) and recombinant viruses containing the myc oncogene have been isolated from four of the tumours presented here (Neil *et al.*, 1984). Viral RNA was isolated from tumours known to contain recombinant myc viruses and the infectious nature of these viruses *in vitro* is demonstrated and discussed in this chapter.

Interference test

The basis of interference testing is detailed in the Introduction. Fischinger and O'Conner (1969) demonstrated that feline cells infected with FeLV were resistant to superinfection with a FeLV pseudotype of a murine sarcoma virus (MuSV) of the same subgroup. The interference test used here is a modification of this original test developed by Russell and Jarrett (1976) and was carried out as follows.

FEA cells were seeded at a density of 3×10^5 cells in 4 ml of 10%DMEM in 5 cm plates (Nunclon) and incubated at 37°C for 24 hours. The medium was then removed and either 1 ml of plasma diluted 1:10 with 10% DMEM or 1 ml of a 1:1 ratio of 10% DMEM plus tumour cell culture supernatant (0.45 μm filtered and taken when the tumour cells were actively dividing) was added, with 4μg/ml polybrene and incubated at 37°C for a further 2 hours. At the end of this adsorption period the medium was removed and 10% DMEM added. The infected FEA cells were then routinely subcultured (as described in Chapter One) for a minimum of 3 weeks to ensure spread of the virus throughout the culture.

The subgroup of the infecting FeLV was then determined by interference testing in the following manner. 5×10^3 infected FEA cells were plated in 0.5 ml 10% DMEM with 4 μg/ml polybrene in each of 8 wells of a 24-well Costar plate. The plates were incubated at 37°C overnight then duplicates of one of the following were added, either (i) 10% DMEM, (ii) sarcoma virus (SV) pseudotype A(SV-A), (iii) SV pseudotype B (SV-B) or (iv) SV pseudotype C (SV-C). The plates were further incubated at 37°C overnight then the medium was replaced with 1×10^5 uninfected FEA cells per well in 1 ml 10% DMEM which improves the detection of foci three days later. The presence of foci is indicative of transformation and was detected when the challenging sarcoma virus was of a different subgroup from the infecting leukaemia virus. Hence, if the

subgroup of the infecting FeLV was A, foci would be detected on infected monolayers of FEA cells which had been challenged with SV-B and SV-C but not in the wells challenged with SV-A.

The analysis of viral genomic RNA for myc sequences

(i) Isolation of virus for analysis

Virus released from cell cultures of FL74, F422 and T3 was prepared for Northern blotting (Maniatis, 1982) and molecular analysis with a feline myc probe designated pCT4 3' to determine if the tumour cells F422 and T3, which had previously been shown to contain recombinant FeLV/myc proviruses, (Neil et al., 1984) were releasing these viruses in culture. Growing cells were spun down and resuspended in 500 ml fresh medium at a concentration of 2 x 10^6/ml. The flasks were gassed and incubated at 37°C for 4 hours after which time the cells were spun down again and the supernatant collected for virus isolation.

The supernatant was clarified by centrifugation at 10,000 x g for 10 minutes at 4°C then any remaining cellular debris was removed by filtering the clarified medium through a 0.45 μm Nalgene filter. Throughout the procedure the virus was kept at 4°C as it rapidly degrades at room temperature. (Vogt, 1965). The viral RNA was then prepared for running on an agarose gel by the procedure detailed in Chapter One (virus purification). Virus was concentrated using the second method, that of ammonium sulphate precipitation. After concentration the virus was purified by banding on discontinuous sucrose gradients then pelleted at 35,000 rpm for 30 minutes in a Beckman SW41 rotor.

The RNA was prepared from purified virus according to the protocol in Chapter One then electrophoresed through a 1.5% agarose gel as follows:

(ii) Electrophoresis of RNA through gels containing formaldehyde

For a 150 ml gel, 2.25g of agarose (BRL) was boiled in 109.5 ml of distilled H_2O (dH_2O) by placing in a microwave oven for 4 minutes. The agarose was cooled to 60°C then 15 ml of 10 x MOPS buffer (0.2M morpholinopropanesulfonic acid (MOPS) pH 7.0, 50 mM sodium acetate, and 1 mM EDTA pH 8.0) and 24.3 ml of 37% formaldehyde was added. The gel was the poured onto a flatbed horizontal plate and 1 x MOPS (gel running buffer) was added when the gel had set.

(iii) Viral RNA sample preparation

The samples were prepared for loading onto the gel as follows. The RNA was dissolved in 4.5 µl of dH_2O in a sterile Eppendorf tube and mixed with 2 µl of 10 x MOPS, 3.5µl of 37% formaldehyde and 10 µl formamide and incubated at 55°C for 15 minutes. Restriction fragments of radiolabelled λ DNA were used as molecular weight markers and were treated and run exactly as the RNA samples (10^5 cpm/lane of marker was used). At the end of the incubation period the samples were cooled on ice and 2 µl/tube of sterile loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) was added. The samples were then loaded onto the gel and run at 100 mA for approximately 3-4 hours until the tracking dye had progressed at least 2/3 of the length of the gel.

(iv) Transfer of formaldehyde-denatured RNA onto Gene-screen

Gene-screen membrane (New England Nuclear) was used in the following blotting procedure to transfer the viral RNA from the gel onto a suitable filter for hybridisation and molecular probing. After removing the gel from the electrophoresis tank it was rinsed in 1 x Gene-screen buffer (25 mM$NaPO_4$ pH 6.5).This buffer was used throughout the procedure to presoak all the filter papers used and as the transfer buffer. The gel was cut to size and the Gen-screen filter and two pieces of 3 MM Whatman filter paper were cut

to the same size and a transfer system depicted in Figure 24 was set up and left overnight. After the transfer was complete the Gene-screen filter was removed and rinsed in 1 x buffer then placed between two sheets of Whatman 3 MM filter paper and baked at 80°C for 2 hours.

(v) Nick translation

Nick translation of the pCT4 3' myc probe was carried out using a nick translation kit (Amersham) as follows. 0.5 µg of DNA was added to 10 x buffer solution containing 100 µm dATP, 100 µMdGTP and 100 µMdTTP in Tris/HCl pH 7.8, magnesium chloride and 2-mercaptoethanol in a polypropylene reaction tube in an icebath. ^{32}p-radiolabelled dCTP was added at 100 µCi/reaction (3000 Ci/mMol) and finally 5 units of DNA polymerase 1 plus 100 pg DNase in a buffer containing Tris/HCl pH 7.5, magnesium chloride, glycerol and BSA were added to the reaction and the tube was capped and inverted several times to mix the solutions. The tube was then placed in a constant temperature bath at 14°C for 2.5 hours after which time the reaction was stopped by adding 2 µl of 0.5M EDTA. The specific activity obtained by this method was usually 1 x 10^8 cpm/µg DNA. The nick translated DNA probe was then separated from unincorporated dNTPs by chromatography through a Sephadex G50 column. The first peak was the probe and the second peak the unincorporated nucleotides.

(vi) Hybridisation

The myc probe was then ready to hybridise to the Northern blot of viral RNAs on the Gene-screen filter. The buffer used throughout this procedure consisted of: 5 x SSC (750 mM sodium chloride, 75 mM sodium citrate pH 7.0) 50 mM sodium phosphate, 10% dextran, 5 x Denhardt's solution, 0.1% SDS, 50% formamide (deionised) and 100 µg/ml denatured salmon sperm DNA. The Gene-screen was pre-wetted in 1 x Gene-screen buffer then sealed in a plastic bag in the above buffer and incubated at 42°C in a shaking water-bath overnight. The next day a corner of the bag was cut

open and the probe (boiled, cooled and diluted to 5 ng/ml in hybridisation buffer) was added and the bag re-sealed (using a heat sealer). This was incubated as before at 42°C overnight in a shaking water-bath. The filter was then removed and washed at high stringency by incubating in 0.1 x SSC plus 0.5% SDS at 60°C for 2 x 30 minutes (changing buffer between washes) then in 5 changes of 0.1 x SSC for 1 minute/wash at room temperature. After the filter was dried on a sheet of Whatman 3 MM filter paper at room temperature it was ready for autoradiography.

(vii) Autoradiography

The filter was taped onto a backing of cardboard and pieces of tape marked with radioactive ink were placed at several locations around the filter as markers. Both filter and backing sheet were then wrapped in cling-film to prevent contamination of the intensifying screen which was laid on top (Dupont intensifying screens). This was then placed in an X-ray film holder (in a dark room) and covered with a sheet of Kodak XAR-5 X-ray film. The film was exposed at -70°C for several days and developed in an automatic X-ray film processor.

The analysis of infected FEA cells for acquired myc sequences

(i) The infection of FEA cells with recombinant viruses

Feline embryonic fibroblasts were infected with viruses from tumours known to contain recombinant proviruses. Two sources of virus were used from T3 and T11: that derived from the plasma of the cats and that present in the supernatant of cells cultured from the tumour. FL74 cell supernatant was included as a control since it is known not to release a recombinant virus but does release helper non-defective FeLV. Cell culture supernatant only was used from the F422 cell line as plasma from this tumour is not available and plasma was used as the only source of virus from a case prior to this study designated 84793 since cells were not cultured from this lymphosarcoma. This experiment was designed to determine if

the recombinant viruses associated with these tumours are infectious i.e. can be transmitted *in vitro* via helper FeLV.

FEA cells were infected by the same method used for interference testing previously described in this chapter. After the FEA cells had been routinely subcultured for 3 weeks to ensure spread of the virus throughout the culture, they were grown to 1 x 10^9 cells and the DNA was isolated according to the protocol in Chapter One (The isolation of DNA from infected and uninfected FEA cells). Uninfected FEA cells were used as control DNA in this procedure and both this DNA and that from the infected FEA cells was digested with restriction endonuclease and analysed for the presence of acquired *myc* DNA sequences.

(ii) Restriction of DNAs

The DNAs were digested in sterile eppendorf tubes with two restriction enzymes, KpnI and PstI, both of which cut within the viral LTRs and internally within the virus. 20 µg of each DNA was digested with 0.1 units of each restriction enzyme by incubating the samples plus 0.1 units of enzyme at 37°C overnight then for a further 2-4 hours in the presence of an additional 0.1 units of enzyme. Following the second incubation a test gel of 0.8% agarose was run to check that the DNA had completely digested. Whole λ DNA was used for the marker lanes but digested marker can also be used in this case as the marker lanes are only to indicate that the gel has run properly. When digestion was complete the DNAs were precipitated by adding 3/50volumes 5M NaCl and 2 volumes of ethanol (ETOH) and incubated at -70°C for 1 hour. When the DNAs had precipitated they were spun down for 5 minutes in an Eppindorf centrifuge, then the ETOH was decanted and the DNAs were dried in a lyophiliser for 10 minutes. After drying, 40 µl of 1 x TE buffer (10 mM Tris HCl pH 7.9 and 1 mM EDTA) per tube was added to resuspend the DNAs and this was incubated at 37°C for 10-15 minutes to ensure that the DNAs had resuspended completely before electrophoresis.

(iii) Electrophoresis of restricted DNAs

The total 40 µl of restricted DNAs were loaded onto agarose horizontal gels with 10 µl of tracking dye. For Kpn-1 restricted DNAs a 0.7% agarose gel was used and for Pst-I restricted DNAs a 0.8% agarose gel was used, made up in 1 x TEA buffer (40 mM Tris base, 2 mM EDTA, 20 mM NaCl and 20 mM sodium acetate, pH 8.15) which was also the buffer used for electrophoresis.The marker lanes were radiolabelled Hind III restricted λ DNA at 5,000 cpm/lane in an excess of cold Hind III λ digest. The gels were run overnight at less than 20V then processed in the following manner. The gels were stained for 30 minutes with ethidium bromide which intercalates with the DNA and hence enables visualisation of the restricted DNA under an ultravoilet light source. This provides an indication of whether the gels have run properly and allows accurate trimming of the gels for Southern blotting.

The gels were then prepared for blotting by denaturing for 30 minutes in a denaturing buffer (1.5M NaCl + 0.5MNaOH) then neutralising for 30 minutes in a neutralising buffer (3MNaCl + 0.5M Tris base, pH 7.0). The gels were rinsed in 3 changes of 1 x Gene-screen buffer for 30 minutes/wash and transferred to Gene-screen membrane by the process of Southern blotting which is identical to that of Northern blotting described for the viral RNA. The processing of the blotted membrane, the nick translation of the pCT4 3' myc probe, the probing of the blot and the autoradiography procedures again were identical to those previously described for viral RNA analysis.

RESULTS

FeLV subgroups associated with thymic lymphosarcoma

Table 5 shows the results obtained by interference testing of Fea cells infected by virus derived from the plasma and cell culture supernatants from the feline T-cell tumours. Ten of these 12 cats had an additional subgroup other than A (9 had B, 1 had C) associated with either the tumour cells or circulating in the plasma. T4 and T12 cell culture virus subgroup was not determined as in the case of T4, since the cells died immediately in culture and did not release sufficient virus for FEA infection. In the case of T12 cell culture, the virus infected FEA cells died before the subgroup could be determined by interference testing. The oncogene status of these tumours (which was determined by Dr. J. Neil) is also shown and these results will be discussed in the following section.

The analysis of viral genomic RNA for myc sequences

Figure 25 shows a Northern blot of viral RNA from the cell lines T3, F422 and FL74 probed with FeLV-*myc* and indicates that recombinant *myc*/FeLV viruses are released by the cell lines F422 and T3. FL74, shown in the middle lane, has no recombinant *myc*/FeLV provirus and was therefore not expected to release anything but helper FeLV. The genome size of the two defective recombinant viruses is different. T3 is 5 kilobase pairs, smaller than helper FeLV genome size which is 8.5 kilobase pairs. F422 defective recombinant genome is 9 kilobase pairs long, marginally larger than helper FeLV genome length but not too large to be packaged into virions. Helper FeLV can be visualised in these blots by probing with a U_3 exogenous virus probe (Casey *et al.*, 1981) which hybridised with an 8.5 kilobase band of all three viruses (data not shown).

The analysis of infected FEA cells for acquired myc sequences

Figure 26 shows a test gel of virus infected FEA DNAs and indicates that the DNAs have been completely digested by both Kpn-I and Pst-I. The two flanking lanes in each set of wells was whole DNA marker. The restricted genomes of the FEA cells appear as smears down the lanes in between the marker lanes and are FEA cells infected with virus as denoted in the legends. Control DNA was isolated from uninfected FEA cells. The DNA was visualised by staining with ethidium bromide (EtBr) and photographed using an ultraviolet light source.

The agarose gel electrophoresis of the restricted DNAs to be used in the Southern blotting technique is shown in Fig. 27. The marker lanes here are Hind-III λ restricted DNA and the DNA was visualised in the same way as before (Figure 26). This demonstrated that the gel had run properly and could now be blotted onto Gene-screen for probing with pCT4 3' myc.

Figure 28 demonstrates the in vitro transmission of FeLV/myc recombinant viruses. The recombinant myc is present in FEA cells infected by virus in the supernatant of the F422 and T11 tumour cells and in the plasma of the T3 cat. No additional recombinant myc hybridising band was seen in the control FEA DNA or in the FEA DNA infected by cell supernatant from the FL74 or T3 cell lines or the plasma from the T11 or 84793 cats. These results will be discussed at length in the next section.

DISCUSSION

Epidemiological evidence suggests that an additional subgroup other than A, usually B, is more often associated with cats with lymphosarcoma than with their healthy viraemic counterparts (Jarrett, 1980). The results of the interference tests (Table 5) to determine which subgroups of FeLV were associated with these T-cell tumours support this observation as subgroup B (and in the case of T3, subgroup C) is present in 83% of the lymphosarcomas studied.

The association of the subgroup B virus with disease in these cats is of particular interest with respect to the striking analogy between the highly leukaemogenic mink cell focus forming (MCF) viruses of mice (Famulari, 1983) and FeLV-B. Recent evidence shows that considerable homology exists between certain regions of the *env* gene of FeLV-B (biologically cloned from the Gardner-Arnstein FeLV isolate) and the *env* gene of MCF virus (Elder and Mullins, 1983). MCF viruses arise by recombination of an ecotropic parental virus with endogenous murine leukaemia virus sequences present in the murine genome (Chattopadhyay *et al.*, 1982) and possess an extended host range over the parental virus. FeLV-B has a similar expanded host range, and like MCF viruses forms an interference group distinct from the ecotropic subgroup A viruses (Jarrett,1980; Rein, 1982).

In this respect the observations of Onions *et al.* (pers.comm.) that the onset of myeloid leukaemia in cats infected with the FeLV-AB/GM-1 virus correlates temporally with the appearance of subgroup B virus in the plasma is intriguing and is undoubtedly important in the progression of leukaemia in those cats. Similarly the frequent presence of subgroup B viruses in the present tumour series is suggestive that these viruses may have a role to play in the development of this disease although, since these cats were first encountered when lymphosarcoma had already developed, nothing is known of the temporal appearance of the subgroups prior to the onset of neoplasia. The high frequency with which subgroup B

viruses are associated with these tumours may indicate that the subgroup B envelope gp70 may have a mitogenic effect on the cells causing an early proliferation due to autostimulation similar to that proposed by McGrath and Weissman (1979) in the murine system (see General Introduction). Alternatively the subgroup B viruses may be involved in recombination events with the cellular myc proto-oncogene, although some tumours had no detectable myc alteration and had associated subgroup B virus (Table 5). Subgroup B involvement in recombinational processes is not unlikely since Russell and Jarrett (1978b) have proposed that FeLV-C may arise de novo in infected cats by recombining with endogenous sequences. This emerged from a study on FOCMA (feline oncornavirus-associated cell membrane antigen) which was first described by Essex et al. (1971a, 1971b) as an antigen or antigen complex expressed on the surface of FeLV-producing leukaemic cells of the FL74 cell line. However, experiments by Russell and Jarrett (1978a) first suggested a relationship between FeLV-C and FOCMA. These authors found neutralising antibody to FeLV-C but not to FeLV-A in cats which were known to have antibody to FOCMA. More recently, Vedbrat et al. (1983) have concluded that FOCMA is an FeLV-C envelope glycoprotein. They found that when monoclonal antibodies to FOCMA were used in neutralisation tests they neutralised FeLV-B and FeLV-C but not FeLV-A (Jarrett and Lutz, cited in Vedbrat et al., 1983). These monoclonals therefore bind to an epitope common to FeLV-B/Sarma and FeLV-C/Sarma. Such an antigen was described earlier by adsorption experiments using serum from a cat inoculated with FL74 cells which neutralised FeLV-B/Sarma and FeLV-C/Sarma (Russell and Jarrett, 1978b).

Russell and Jarrett (1978a) proposed that anti-FeLV-C antibody may be evoked by an antigenic determinant which is coded for by the endogenous FeLV-like genes which are present in cat cells (Levin et al., 1976; Okabe et al., 1976) and which are expressed in cats during infection with FeLV-A. Indeed recent evidence from Mullins (pers.comm.) suggests that the endogenous FeLV genes are actively transcribed in both virus-negative and virus-positive leukaemias. Russell and Jarrett (1978a) further

proposed that the origin of the rare, infectious FeLV-C might be a recombination between infecting FeLV-A and these endogenous viral genes implying that each FeLV-C isolate may have arisen de novo in a FeLV infected cat (Russell and Jarrett, 1978b). It may be that both FeLV subgroups B and C arise by this recombination mechanism which would explain why subgroups B and C are never isolated in the field in the absence of FeLV-A. Virus neutralisation data also support this contention since only FeLV-A has been demonstrated to be antigenically monotypic while a great deal of antigenic diversity exists among FeLV-B and FeLV-C isolates (Russell and Jarrett, 1978b) which is understandable if these subgroups have been generated by recombination between infecting FeLV-A viruses and endogenous FeLV *env* sequences.

Recently it has been shown that DNA probes specific for the envelope gene of FeLV-B detect similar sequences in FeLV-C and endogenous viral genomes but not in FeLV-A (Warnock *et al.* pers. comm.). This strongly supports the hypothesis that FeLV-B and FeLV-C arise by recombination. Since subgroup B viruses may therefore evolve by recombinational events they may similarly be involved in the *myc* recombinations detected in these T-cell tumours since FeLV seems able to commonly recombine with cellular proto-oncogenes (Besmer *et al.*, 1983b). Two forms of *myc* alteration are seen in this series of naturally occurring T-cell lymphosarcomas; rearrangement of the cellular proto-oncogene by integration of the FeLV genome into the *myc* locus and recombination of FeLV with part of the *myc* locus generating recombinant *myc*/FeLVs. The thymic lymphosarcomas presented here far more frequently released recombinant *myc*/FeLV than was expected (25%) and a further 25% involved rearrangements of the *myc* gene by adjacent integration of FeLV. Three tumours in this series were derived experimentally: T5, T8 and T10. Cats T8 and T10 were inoculated at birth with the Rickard strain of FeLV which contains subgroups A and B. T5 was a cat experimentally inoculated at birth with FeLV/Glasgow-1 which is a subgroup A virus. Rearrangement of the *myc* gene has occurred in T5 and T8 and also in a field case cat T7.

Rearrangements are possibly seen more frequently than recombinants in tumours induced by known highly pathogenic viruses like Rickard since these viruses have been selected for leukaemogenicity and may contain strong enhancers which can activate or increase expression of myc simply by integrating next to it, whereas non-selected viruses in natural conditions may require recombination with the myc gene to induce neoplasia. Interestingly T7, which was a field case involving a rearrangement of myc, was integrated very close to the gene (within a few hundred base pairs) compared to T8 (several thousand base pairs distal). This may imply that the FeLV did not have a particularly strong enhancer but activated myc by virtue of its close proximity to the gene.

Another tumour of particular note is T17. No FeLV antigen was detected in the plasma of this case and only very small quantities of infectious virus were isolated (1 focus on 1 5cm plate on one of three occasions tested). However FeLV of subgroup A was isolated from cultured tumour cells and was found to contain recombinant myc/FeLV provirus integrated in the cell genome (J. Neil pers. comm.). The extremely low level of virus detectable in the plasma of this cat correlated with the presence of anti-FeLV-A and anti-FeLV-C neutralising antibodies and therefore there may have been neutralised virus circulating in the plasma in immune complexes. Alternatively, as in cattle infected with bovine leukaemia virus, the virus emerging into the plasma from the cells may be incomplete if antibody modulates the viral protein and inhibits viral production (Gupta et al., 1984). Transcription studies of primary tumour material should elucidate which of these mechanisms is correct.

Whichever mechanism operates, the presence of detectable FeLV in the tumour cells of this cat but not free in the plasma, could explain the observations by Hardy et al. (1980) that virus-negative lymphosarcomas are isolated more frequently from cats that have been exposed to FeLV than from cats that have never been exposed. Although it is difficult to determine exactly by what criterion these lymphosarcomas are considered to be virus-negative, and

indeed which category of lymphosarcoma is being referred to for a given set of results (thymic or alimentary), it may have been that the antigen-negative cats which developed thymic lymphosarcoma in Hardy's series did in fact contain FeLV proviruses.

It would then appear that there are two distinct classes of virus-negative thymic lymphosarcomas, probably generated by distinct mechanisms. One class, exemplified by T17, although plasma virus-negative is producing virus from the tumour cells, and the other class is truly virus-negative determined by lack of both exogenous integrated provirus in the cellular DNA, (determined by U_3 probing; Casey et al., 1981) and circulating free plasma virus. Three such true virus-negative thymic lymphosarcomas have been studied, including one in this present series (T6 = 88960) all of which have no detectable circulating plasma virus and no integrated exogenous provirus in the tumour cell DNA (Neil et al., 1984). Furthermore, no myc alterations were detected in any of these three virus-negative thymic lymphosarcomas or in another three virus-negative alimentary lymphosarcomas although rearrangements may have occurred outside the domain analysed in the study. Further experiments are required to investigate the mechanisms of transformation in these cases.

The isolation of recombinant viruses released from tumour cell cultures shown in Figure 25 provided proof that these recombinants were packaged and released from the tumour cells with helper FeLV. Had they not been released from the cells, the possibility of horizontal spread of the recombinant myc/FeLVs in vivo would have to have been ruled out. Since the recombinant viruses were released from the tumour cells it was important to establish if these viruses were infectious in vitro. Figure 28 clearly demonstrates that the recombinant viruses of T3, T11 and F422 can gain entry to permissive cells (FEA) via helper FeLV and become integrated as provirus into the cell genome. The viruses used for these infections were not titrated and were, therefore, plated onto the cells at different concentrations. This was probably why the plasma helper FeLV of T3 (which subsequently turned out to be high

titre: 10^7 ffu/ml) carried sufficient FeLV/myc into the FEA cells to be detected in the genome, whereas the cell culture supernatant did not (it had very low helper titre: 5×10^3 ffu/ml). Similarly the plasma helper virus of T11 and 84793 (a case prior to this series) may have been at too low a titre to infect the FEA cells with sufficient recombinant to be detected. In particular 84793 was the tumour tissue from which the feline myc probe pCT4 3' was cloned and this had a very low copy number(3) of clonally integrated proviruses so relatively little recombinant virus may have been circulating in the plasma of this cat. Both the control uninfected FEA DNA and the DNA from the FEA cells infected with FL74 cell culture virus do not display an additional myc band as expected. No morphological transformation was detected in the FEA cells infected by these recombinant viruses but this may simply reflect that FEA cells are not target cells for transformation by these recombinant viruses. Since these viruses were infectious in vitro further experiments were required to determine their pathogenicity in vivo and these are the subject of the next chapter.

TABLE 5

FeLV subgroups associated with thymic lymphosarcoma

Tumour	Virus subgroup		Oncogene status
	Plasma	Tumour	
T3	AC	A	myc recombinant
T4	AB	N/D	-
T5	AB	A	myc rearrangement
T7	AB	A	myc rearrangement
T8	AB	A	myc rearrangement
T10	AB	AB	-
T11	AB	AB	myc recombinant
T12	A	N/D	-
T14	AB	AB	-
T15	AB	A	-
T16	A	AB	-
T17	-ve	A	myc recombinant

N/D = Not done.

(Data from Neil et al., 1984; Neil, Lees and Onions, unpublished).

FIGURE 24.

TRANSFER OF RNA/DNA ONTO GENE-SCREEN

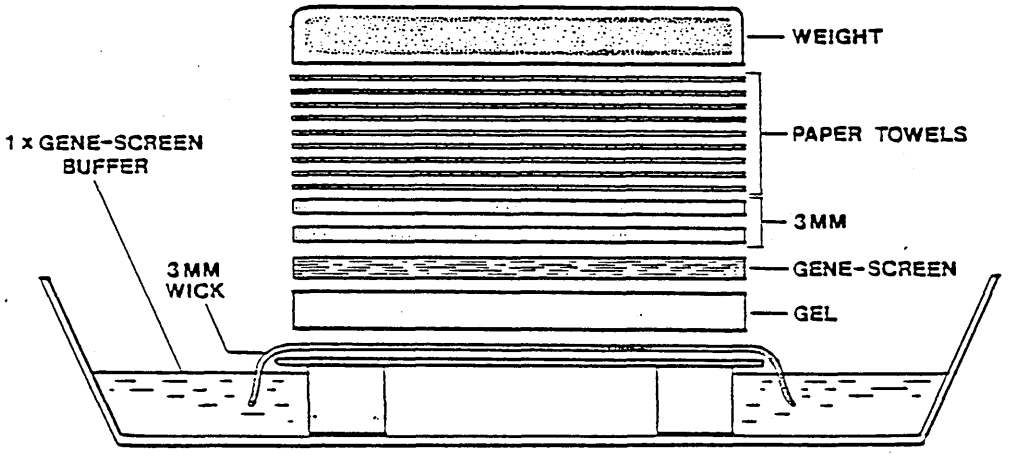


Figure 24 shows the method used to transfer RNA or DNA from agarose gels onto membrane (Gene-screen) suitable for the hybridisation of radioactive probes.

FIGURE 25.

Virion RNA

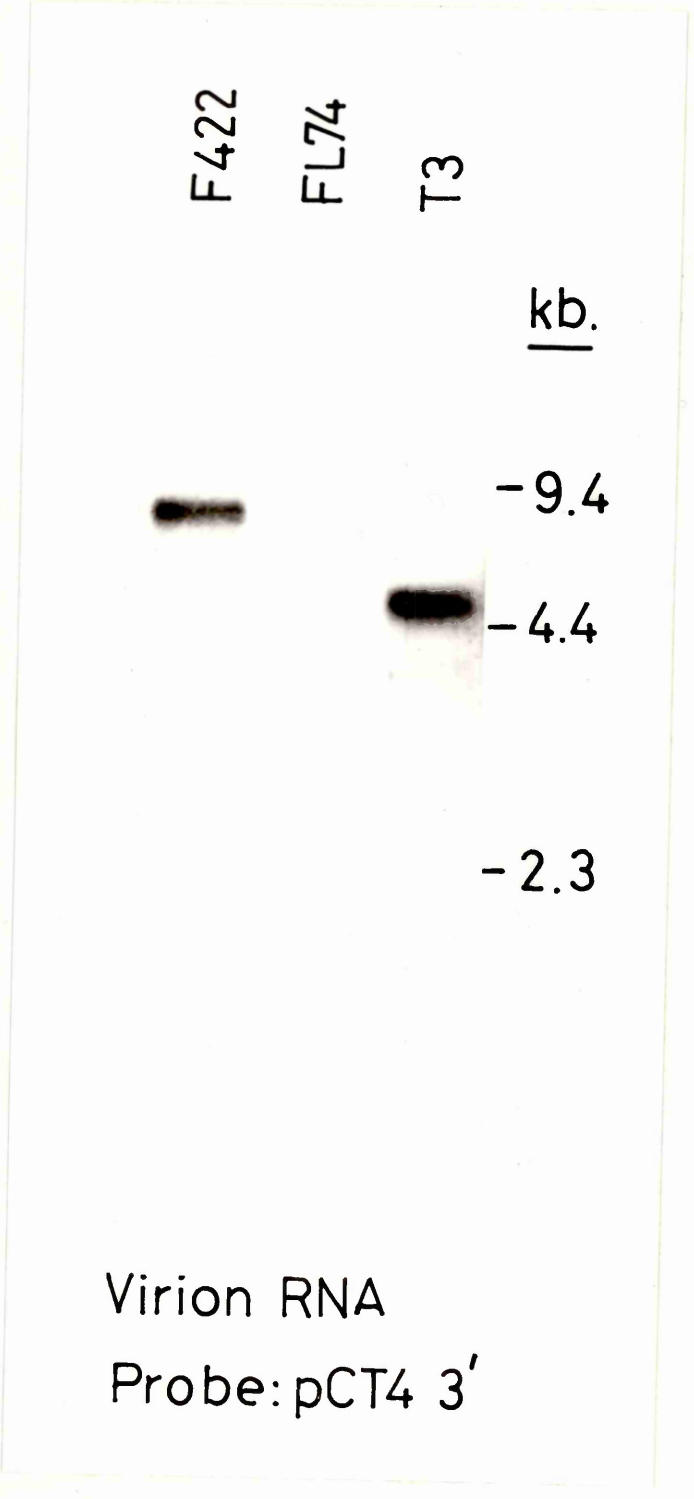
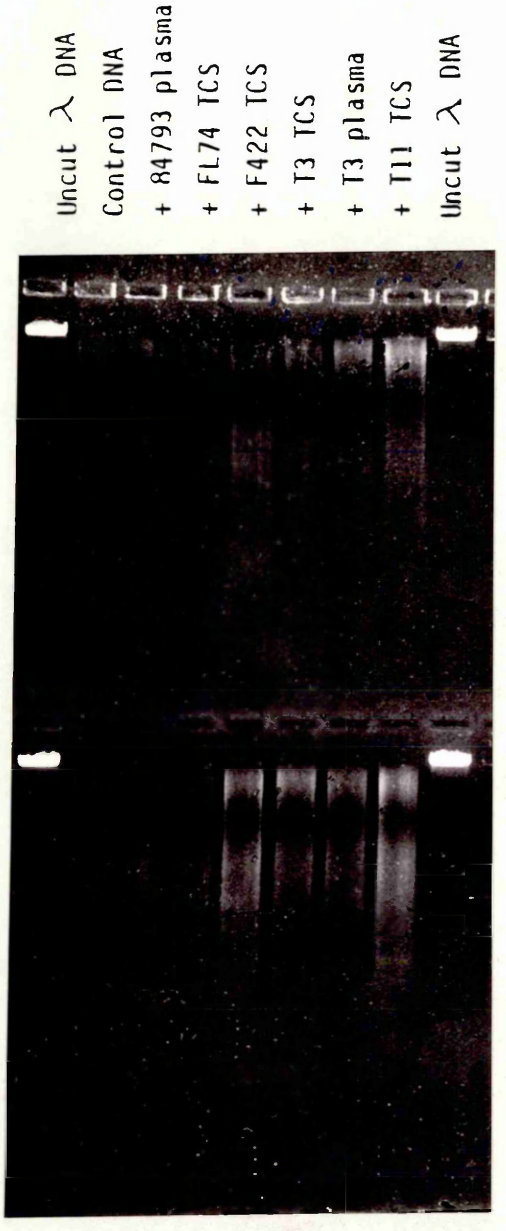


Figure 25 shows a northern blot or viral RNA from the cell lines T3, F422 and FL74 probed with feline v-myc (pCT4 3').

FIGURE 26.

Restriction test gel



Kpn-1 digest

Pst-1 digest

Figure 26 demonstrates that the digestion of the DNAs with restriction enzymes was complete.

The method is described in the text.
TCS = tissue culture supernatant.

FIGURE 27

Gel for Southern blot of DNAs

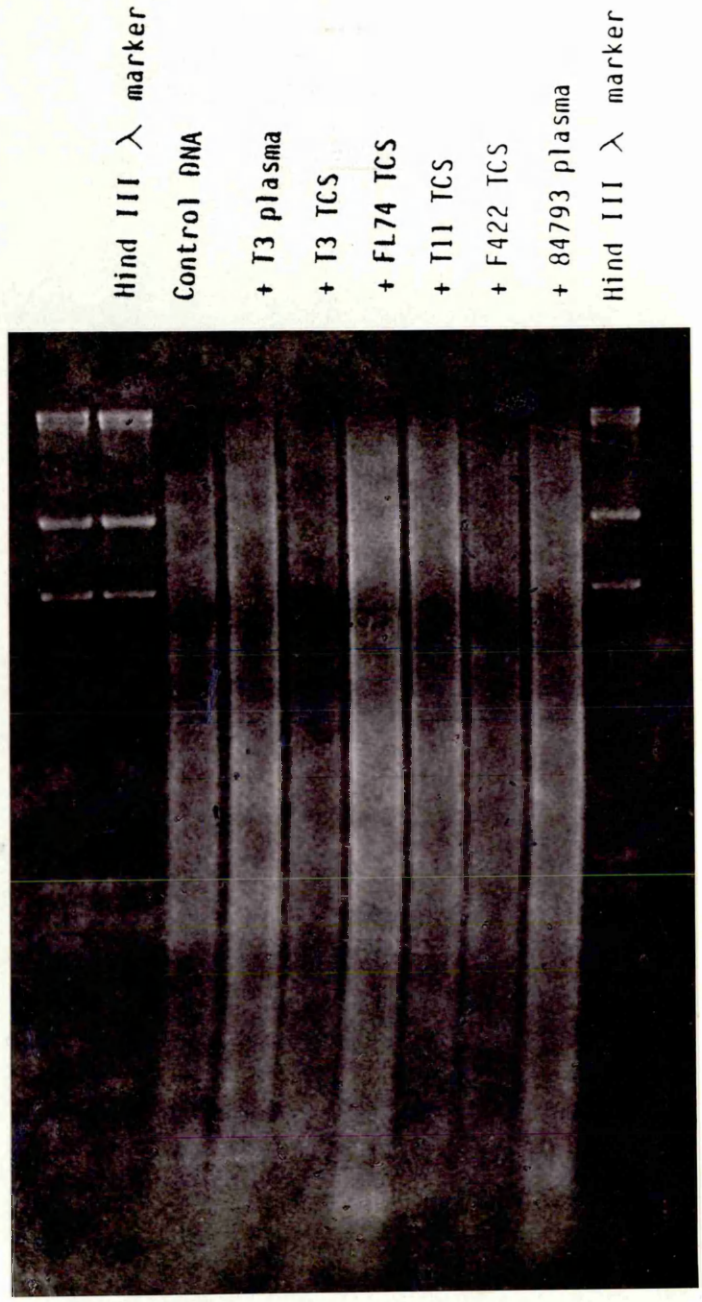


Figure 27 demonstrates that the FEA DNAs digested with Pst-1 had run properly on the agarose gel and that this gel was suitable for blotting onto gene-screen

FIGURE 28

In vitro transmission of FeLV/myc recombinant viruses

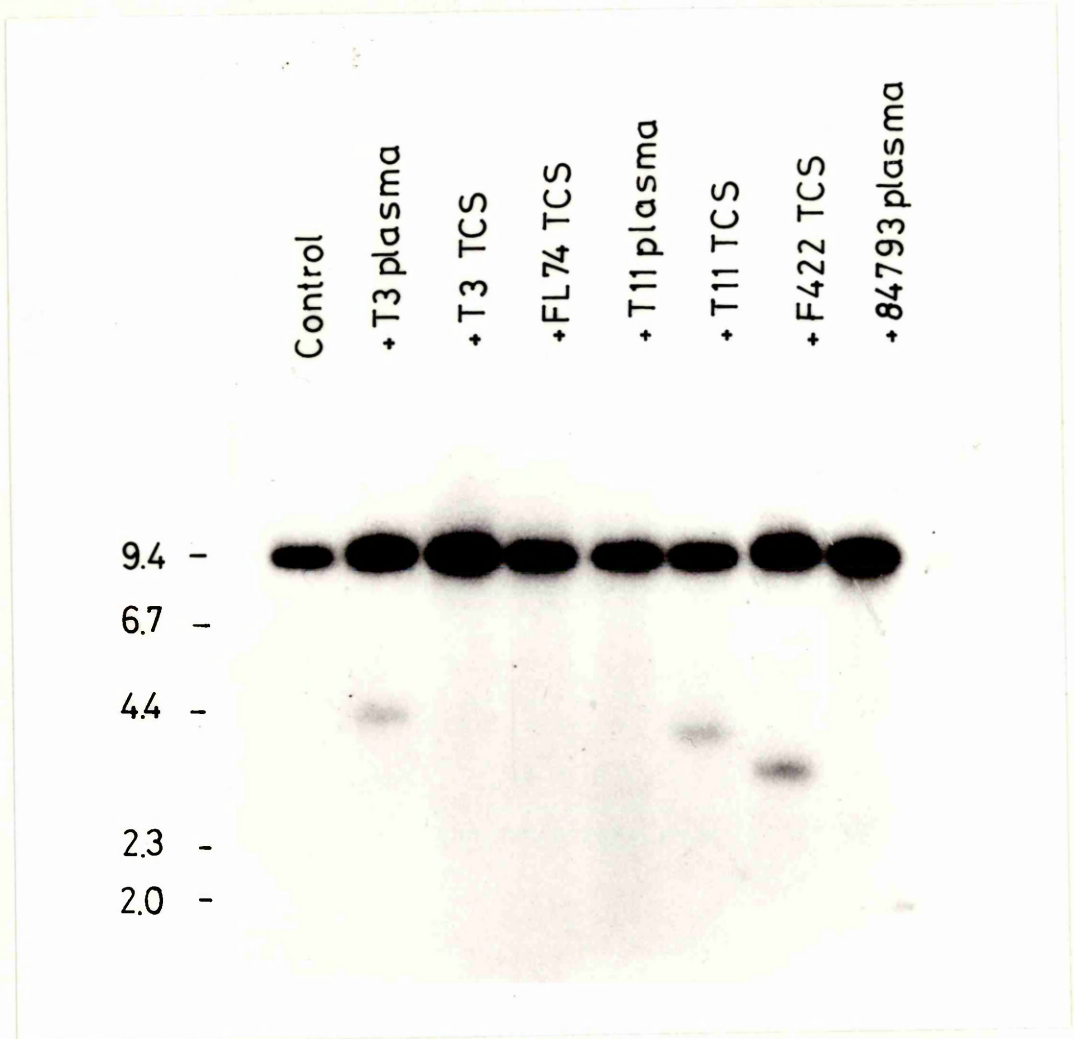


Figure 28 demonstrates the in vitro transmission of FeLV/myc recombinant viruses from F422 and T22 TCS and T3 plasma into FEAs.

TCS = tissue culture supernatant.

(Neil et al., 1984).

CHAPTER SIX

THE IN VIVO PATHOGENESIS OF FeLV/myc RECOMBINANT VIRUSES

INTRODUCTION

Avian and murine acutely transforming leukaemia viruses which carry a viral onc (v-onc) gene are known to rapidly produce a wide spectrum of disease in vivo (Bister 1984; Fischinger, 1984). ALVs do not contain a v-onc gene but integrate near specific host proto-oncogenes and again produce a spectrum of disease in vivo but after a longer latent period than that observed with the acute leukaemia viruses (Crittenden and Kung, 1984). In the cat, some virus-induced thymic lymphosarcomas have recently been shown to involve the oncogene myc either incorporated into the viral genome in the form of an acutely transforming virus or by insertion of an FeLV genome adjacent to the myc locus (Neil et al., 1984; Levy et al., 1984 and Mullins et al., 1984). The transforming properties of the recombinant FeLV/myc viruses F422 and T3, obtained from the tumour cell lines, have been studied both in vivo and in vitro. The in vitro experiments, described in Chapter Five, suggest that these recombinant viruses do not morphologically transform FEA cells in culture unlike avian acute leukaemia viruses containing v-myc sequences. These latter viruses readily transform avian fibroblasts in vitro and one strain, MC29, has been shown in addition to transform fibroblasts of mammalian origin (Ouade, 1979). It may be that FEA cells are not target cells for these recombinant feline viruses or alternatively they may alter a cellular growth property such as anchorage dependence and permit cells to grow in semi-solid agar. Experiments are in hand to try to determine which haemopoietic cells are targets for these viruses in vitro and their effect on the growth properties of the cells they infect.

Since these viruses were originally isolated from thymic lymphosarcomas, attempts have been made to infect cultured mature T-cells from the thymus of SPF cats with these viruses but the infection of mature feline thymocytes with any feline leukaemia virus has proven to be extremely difficult. (Rojko et al., 1980). In conjunction with these in vitro studies, experiments were undertaken to investigate the pathogenicity of the F422 and T3 recombinant viruses in vivo.

The results of this work, with respect to the spectrum of disease induced by these viruses, the speed of development of disease and the clonality of the resultant tumours, is the subject of the present chapter.

MATERIALS AND METHODS

The work in this chapter was done in collaboration with Dr. D. Onions and Dr. J. Neil. I am particularly grateful to Dr. Onions for carrying out procedures requiring a Home Office Licence. I am also grateful to Dr. S. Toth who determined the pathology of the disease produced by these viruses.

The infection of kittens with the FeLV/myc recombinant viruses

Virus was harvested from the cell lines T3 and F422 two days after feeding and was clarified and concentrated by ultrafiltration using an Amicon hollow fibre concentrator (model CH4) as described in Chapter One. Virus was inoculated into newborn kittens at 5 x 10^5 ffu/ml intraperitoneally; seven cats were inoculated with T3 virus and three cats with F422 virus. The two groups of cats were housed separately and four tracer cats which were not inoculated with virus were housed together with the infected cats in each group to attempt to detect horizontal transmission of the virus (Fig. 29). Blood samples were taken from all cats every two weeks and were screened for abnormal haematology and plasma virus subgroup.

The establishment of cell lines from the thymic lymphosarcomas

Cats which developed clinically detectable thymic lymphosarcoma were sacrificed and the tumours removed and established in culture in an identical manner to that described in Chapter Three (Preparation of cell cultures from tumours). In brief, cells were isolated from the tumours in single cell suspensions by dicing with scalpels and stomaching in RPMI 2% + Me. The cells were then washed, counted, and resuspended at 5 x 10^5/ml in either RPMI 20% + Me alone or in 50:50 medium (RPMI 20% + Me + LyCM). Cultures were maintained by subculturing twice weekly and dead cells were removed by centrifugation through Ficoll-paque at 2000 rpm for 10 minutes.

The molecular analysis of tumour and cell line DNA

(i) The extraction of DNA from tumour material and cell lines

Tumour material from cats with thymic lymphosarcoma was stored at -70°C until required. Cells from lines which were established from 5 of 10 of these tumours were grown to a total cell number of 1 x 10^9 by routine feeding with RPMI 20% + Me. The cells were spun down and washed once in PBS then resuspended directly into 4M guanidinium isothiocyanate buffer with a 10 ml agar pipette. The frozen tumour tissue material was thawed in the same buffer and diced with scalpels then stomached gently to break up the tissue. Both this slurry and the cell suspension were clarified by centrifugation at 10,000 rpm for 10 minutes to remove gross debris. The DNAs were then isolated by centrifugation through a discontinuous CsCl gradient (2 ml of 51% CsCl on top of 2 ml 56% CsCl) at 35,000 rpm for 20 hours after which time the DNA had banded at the interface of the two CsCl solutions. The bands were carefully removed using a wide bore pipette and dialysed briefly against 1 x TE buffer at 4°C to remove any residual CsCl. Proteinase K (Boehringer) was added at a concentration of 50 µg/ml DNA in 1 x TE containing 0.5% SDS and the DNAs incubated overnight at 37°C. Phenol, equilibrated to pH 8.0 in 1 x TE buffer was used to extract the DNAs by adding in equal volume to the DNA and mixing gently end over end at room temperature for 10 minutes. The top aqueous phase was then removed to a fresh tube and extracted once more with phenol then twice with chloroform (49 parts chloroform:1 part isoamyl alcohol) and dialysed against 1 x TE at 4°C with two to three changes of buffer. The DNA was next treated with RNase at a concentration of 20 µg/ml overnight at 37°C and extracted with phenol and chloroform exactly as before. Finally the samples were dialysed extensively against buffer at 4°C prior to restriction.

(ii) The restriction analysis of the isolated DNAs

The DNAs were digested in sterile Eppindorf tubes with two restriction enzymes, EcoR1, and Kpn-1. EcoR1 cuts the T3 provirus

both internally and in the flanking sequences which generates unique proviral fragments. However, there is no EcoR1 site within the recombinant F422 provirus. EcoR1 therefore cuts outside the virus in the flanking sequences of F422 generating large unique fragments which makes counting of individual bands on the blots more difficult. This will be discussed further in the results section. 20 µg of each DNA was restricted with 0.1 units of each restriction enzyme following the protocol described in Chapter Five (Restriction of DNAs). The protocol for Southern blotting the DNA, nick translation of the pCT4 3' myc probe, hybridisation of the probe to the blots and autoradiography of the blots were again identical to those described in Chapter Five.

The determination of virus subgroups associated with the infected cats

Blood samples were taken from all the cats every week for four weeks then approximately every two weeks until the final sample and at death. The subgroup of virus present in the plasma was determined by the standard interference test (described in Chapter Five). Supernatant from the tumour cell lines was also tested in the same manner to determine the subgroup of virus being released from the cells in culture.

RESULTS

The spectrum of disease induced by the recombinant viruses

All the cats infected with the T3 and F422 viruses were destroyed *in extremis*. Of the seven cats inoculated with T3 recombinant virus, five ($T3C_4$, $T3C_5$, $T3C_6$, $T3C_7$ and $T3C_8$) developed thymic lymphosarcoma 81-98 days post-infection as shown in Table 6. Pathological examination revealed that in addition to the tumour three of these cats ($T3C_4$, $T3C_6$ and $T3C_8$) had sinus histiocytosis and $T3C_4$, $T3C_5$ and $T3C_6$ had erythroid hypoplasia. T-cell depletion was marked in $T3C_5$ and $T3C_8$. The other two of these seven cats ($T3C_1$ and $T3C_2$) died 103 and 71 days after infection respectively; $T3C_1$ had erythroid hypoplasia and $T3C_2$ had haematopoietic dysplasia, both showed marked T-cell depletion as shown in Table 7. Of the four tracer cats housed with the infected cats, only one ($T3C_{11}$) became persistently viraemic from 147 days post-infection onward but all four became latently infected with FeLV subgroup AB as shown in Table 8. Of the three cats infected with F422 recombinant virus two (F422/1 and F422/3) developed thymic lymphosarcoma 123 and 112 days post-infection respectively. The pathology of the other cat (F422/2) revealed lymphoblastic leukaemia with marked T-cell depletion 71 days post-infection and all the tracer cats are, to date (134 days post infection) plasma virus-negative although whether or not these cats are latently infected in the bone marrow remains to be determined.

The establishment of cell lines from the thymic lymphosarcomas

Of the five cats inoculated with T3 virus that developed tumours, four cell lines were established in tissue culture from the tumours of $T3C_4$, $T3C_5$, $T3C_7$ and $T3C_8$. The tumour from cat $T3C_6$ was very small and too little material was available to establish it in culture. All four cell lines grew immediately in RPMI 20% + Me alone and were dependent on mercaptoethanol for their continued proliferation in culture. Of the two cats inoculated with F422 virus that developed tumours, one established into a cell line *in*

vitro in RPM1 20% + Me alone but not so readily as the cells from the tumours of the T3 infected cats. These cells were cultured in vitro in sufficient quantities for DNA isolation and analysis.

The molecular analysis of tumour and cell line DNA

Figure 30 shows Southern blots of T3 and F422 tumour DNA restricted with Kpn-1 and probed with feline v-myc pCT4 3'. Kpn-1 cuts the provirus internally and therefore generates a unique defective proviral band containing the acquired myc sequences. The results show that the T3 tumours, (A, B, C and D) contain defective proviruses which are representative of those in the original tumour. In addition, however, T3A and T3B are clearly harbouring defective proviruses which have undergone further deletion (small bands under major bands on blot). Similarly, the F422 tumours A and B and resultant cell line, (B), contain defective proviruses which are representative of those of the original tumour.

Figure 31 shows Southern blots of the same DNAs restricted with EcoR1 and probed again with feline v-myc pCT4 3'. EcoR1 cuts within the T3 provirus and also in the flanking cellular sequences enabling the determination of the number of integrated defective proviruses. EcoR1, however, has no restriction site within the F422 defective provirus and is therefore cutting in the flanking cellular sequences only. This generates very large fragments which are difficult to count. Therefore these DNAs will be restricted with another enzyme which cuts within the provirus (Hind III) to clarify the number of integrated defective proviruses in the F422 induced tumours and resultant cell line.

Figure 32 shows Southern blots of T3-induced tumour and homologous derived cell line derived DNA. The DNAs were restricted with EcoR1 as before and probed with v-myc pCT4 3'. From a comparison of the position and number of bands generated by this enzyme digest in the tumour and cell line DNAs it is clear that the cell lines which were established in culture are representative

of the tumours from which they originated. The EcoR1 digest shows that the copy number of integrated defective proviruses and their pattern of integration is similar in the tumours and homologous cell lines.

The virus subgroups associated with the infected cats

The results of the interference tests to determine the subgroup of virus found in the T3 infected cats are shown in Table 9. Blood samples assayed (23-32 days) prior to death revealed that all of the cats had only subgroup A FeLV circulating in the plasma with the exception of $T3C_2$ which had subgroup AC plasma virus detected 10 days before death. Four of the six plasma samples which were assayed at death contained FeLV with an additional subgroup other than A and three of these subgroups were identical to those released by the homologous cells that were cultured. (T3C4, T3C5 and T3C7).

The interference data on the F422 virus infected cats is incomplete but Table 10 shows the virus subgroup circulating in the plasma of the cats 35 days post-infection. Determination both of plasma virus subgroup at death and the subgroup of the virus released from the tumour cells of these cats has proven difficult to assay since the virus appears to have a cytopathic effect on the FEA cells as it spreads through the culture. This will be discussed in the next section.

DISCUSSION

The rapidity with which the T3 and F422 recombinant viruses induce tumours in newborn cats (81-98 days post-infection and 71-123 days post-infection respectively) is typical of acutely transforming leukaemia viruses of other species which rapidly produce disease on inoculation into susceptible animals (Bister, 1984). In particular, in the feline system, the Rickard strain of FeLV was shown to rapidly produce malignant lymphosarcoma of mixed histiocytic-lymphocytic cell type on inoculation into newborn kittens (30-139 days post-infection) (Rickard et al., 1969). In the same experiment a few animals with longer latent periods between inoculation and death (around 160 days post-infection) developed thymic lymphosarcomas. Interestingly $T3C_1$ and $T3C_8$ cats showed lymph node enlargement as early as 40 days post-infection. Examination of an excised popliteal lymph node from cat $T3C_8$ indicated abundant sinus histiocytes and occasional giant cells reminiscent of the mixed histiocytic-lymphocytic lymphoma described by Rickard. However, these lesions regressed in both cats.

The specificity of disease produced by these recombinant viruses is not due to specificity conferred by the oncogene v-myc as myc is associated with a spectrum of neoplasia in several species from avian to human. The myc oncogene is not only incorporated into defective retroviruses (Enrietto et al., 1983) or altered by the integration of retrovirus into the cellular locus (Hayward et al., 1981) but it is also involved in non-viral mechanisms of tumourigenesis such as the chromosomal translocation seen in Burkitt's lymphomas (Croce et al., 1983). Any specificity of disease induced by these recombinant viruses is more probably due to tissue specific enhancer sequences in the viral LTR (suggested by Anderson and Scolnick, 1983) similar to those described for immunoglobulin genes (Boss, 1983). The viral LTRs contain promoter and enhancer sequences for RNA transcription (Temin, 1982) and may therefore increase the level of cellular myc mRNA if myc sequences are incorporated into the viral genome or are adjacent to the viral genome.

Both T3 and F422 cell lines release recombinant and helper FeLV with subgroup A envelopes. The original T3 cat however had both subgroups A and C FeLV circulating in the plasma as well as recombinant virus. The results of the interference tests shown in Tables 9 and 10 demonstrate that although these cats were inoculated with subgroup A virus only, additional subgroups are frequently generated *in vivo*. It is of particular interest that all of the T3 infected cats that had AC subgroup plasma virus developed erythroid hypoplasia with the exception of $T3C_2$ which did, however, display haematological dysplasia. The plasma virus subgroup of $T3C_6$ remains to be determined but this cat also showed erythroid hypoplasia and the association of subgroup C virus with this condition is well documented (Jarrett *et al.*, 1984). Furthermore, in the present series of cats there may be some suggestion of a temporal association between the appearance of these additional subgroups in the plasma and the onset of disease, similar to that observed by Onions *et al.* (pers. comm.) in the GM-1 *in vivo* experiments. It seems probable that the T3 and F422 "A" envelopes are in fact partial recombinants between infecting subgroup A viruses and endogenous FeLV sequences, since additional plasma subgroups have never been generated when Glasgow-1 (subgroup A) FeLV has been inoculated into cats (O. Jarrett pers. comm.). It may be that there is genomic masking of a small subgroup C component present in these viruses but this is unlikely since the virus is grown in FEA cells for a minimum of three weeks before subgroup determination by interference testing. This method of assay should easily permit subgroup C detection and it therefore seems more likely that these subgroup A viruses have some subgroup C envelope sequences present enabling them to readily recombine with other endogenous cellular sequences. Recent evidence from Vedbrat *et al.* (1983) using non-neutralising anti-FeLV-C monoclonal antibody against F422 cells supports this theory. The results suggest that F422 cells display FeLV-C envelope determinants which react with this non-neutralising antibody and it would therefore be intriguing to use this monoclonal against the T3 cell line. Unfortunately, this antibody has not been available and immunofluorescence tests carried out in collaboration with Prof.

O. Jarrett, using neutralising FeLV-C monoclonal antibodies (supplied by H. Lutz and C. Grant) against both the T3 and F422 cell lines have been negative. Further characterisation of these cell lines is currently in progress using a wide range of monoclonal antibodies.

The Southern blot shown in Figure 31 clearly demonstrates that these induced thymic lymphosarcomas are clonal (or at most oligoclonal) in origin, like those induced in the avian system (Hayward, 1981). Even although the bands on the blot of the F422 tumour and cell line DNAs are large, it is obvious that a unique set of fragments has been generated. This indicates that the tumour has arisen from a single cell, or at most a very few cells, otherwise there would have been proviral integrations at a great many sites in the DNA which would have generated a smear on the southern blot. Considering the speed with which these tumours developed and the potent oncogenic nature of the viruses used to induce them, it had seemed likely that several cells may have been transformed at the same time and polyclonal tumours, similar to those induced by FeSV (Besmer, 1984) would not have been surprising. The clonality of these tumours suggests that a second transformation step after the infection of myc is necessary for tumour development or that selection for a rapidly dividing transformed clone occurs.

The possibility that an altered requirement for or sensitivity to a growth factor could play a role at this stage is currently under investigation. The similarity between the restricted tumour and the cell line DNAs (Fig. 32) show that the cultured cells are representative of the tumour cell population although there may be some additional rearrangment in some of the other cell lines which have still to be checked. This often occurs when cells are passaged in vitro. The T3 tumour cells in particular were readily established in vitro in the absence of growth factor. Only one field case, T17, was established in culture in this fashion and this too releases both recombinant and helper FeLV. It would therefore appear that the presence of recombinant virus or the

passage of these viruses *in vivo* is generating transformed cells with little or no growth restriction. Whether or not the presence of the myc oncogene is related to the loss of growth factor control of division of these cells remains to be established.

The successful transmission of these recombinant FeLV/*myc* viruses *in vivo* has clearly demonstrated their acutely transforming potential in nature. Horizontal transmission of these viruses has yet to be demonstrated but one of the T3 uninfected tracer cats ($T3C_{11}$) has now become persistently viraemic and virus has been isolated from the plasma of this cat and will be analysed for the presence of acquired *myc* sequences. Bone marrow cultures have been established from all the latently infected tracer cats and virus induced from these cells has been plated onto FEA cells to produce a sufficient quantity for analysis for acquired *myc* sequences. The DNA of the infected FEAs has been analysed in a similar manner as before but no newly acquired *myc* sequences have been detected (Onions, pers.comm.). The absence of acquired *myc* sequences is, however, inconclusive as this may simply reflect the amount of recombinant virus present; if the titre was too low not enough *myc* would have been present to allow detection by the probe. It will be interesting to determine if horizontal transmission of the FeLV/*myc* virus occurs *in vivo* or if each of these viruses is generated *de novo* in each cat by recombination between infecting FeLV and cellular sequences.

TABLE 6.

TUMOURS PRODUCED BY T3 FeLV-*myc*

CASE	PATHOLOGY	TIME AFTER INFECTION days
T3C4	Thymic lymphosarcoma Sinus histiocytosis Erythroid hypoplasia	81
T3C5	Thymic lymphosarcoma Erythroid hypoplasia T-cell depletion	93
T3C6	Thymic lymphosarcoma Erythroid hypoplasia Sinus histiocytosis	91
T3C7	Thymic lyphosarcoma	91
T3C8	Thymic lymphosarcoma T-cell depletion Sinus histiocytosis	98

TABLE 7.

DISEASE PRODUCED BY T3 FeLV-*myc*

CASE	PATHOLOGY	TIME AFTER INFECTION
T3C1	Erythroid hypoplasia T-cell depletion	103 days
T3C2	Haematopoietic dysplasia T-cell depletion	71 days

TABLE 8.

INFECTION OF TRACER CATS IN FeLV T3 EXPERIMENT

CASE	VIRAEMIA	LATENCY (Days)	
		69-74	115-118
T3C3	At 28 days only	AB	AB
T3C9	Not detected	AB	AB
T3C10	Not detected	AB	AB
T3C11	At 147 days onward	AB	AB

TABLE 9

Subgroups at death

Case	Plasma	Tumour cells
T3C1	AC	-
T3C2	AC	-
T3C4	AC	AC
T3C5	ABC	ABC
T3C6	N/D	A
T3C7	A	A
T3C8	A	ABC

ND = not done.

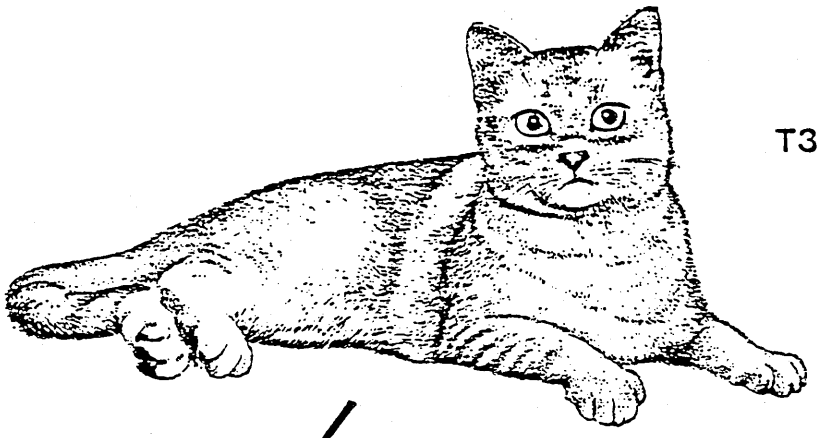
TABLE 10

Subgroups 35 days post infection

Case	Plasma
F422/1	A
F422/2	AB
F422/3	AB

FIGURE 29.

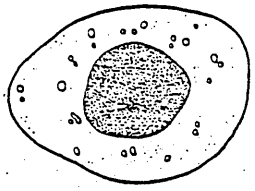
FeLV-myc PATHOGENESIS EXPERIMENT



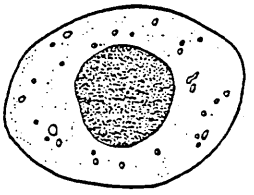
T3 Thymic lymphosarcoma

Viraemia : FeLV-AC

Tumour : FeLV-A



T3 Cell line
FeLV-A/myc



F422 Cell line
FeLV-A/myc

Amicon ultrafiltration

Amicon ultrafiltration

5×10^5 f.f.u. i/p into newborn

5×10^5 f.f.u. i/p into newborn

7 infected

4 littermates
Tracers

3 infected

4 littermates
Tracers

Figure 30 shows DNAs isolated from the T3 and F422 tumours. The DNAs have been restricted with Kpn-1 which cuts the virus internally

M = Hind III λ DNA marker.

A, B, C, D = T3 and F422 induced tumour DNAs.

(B) = cell line DNA derived from the F422 induced tumour B.

CON = control DNA.

FIGURE 30

The demonstration that the proviruses of the T3 and F422 tumours are representative of those in the original cell lines

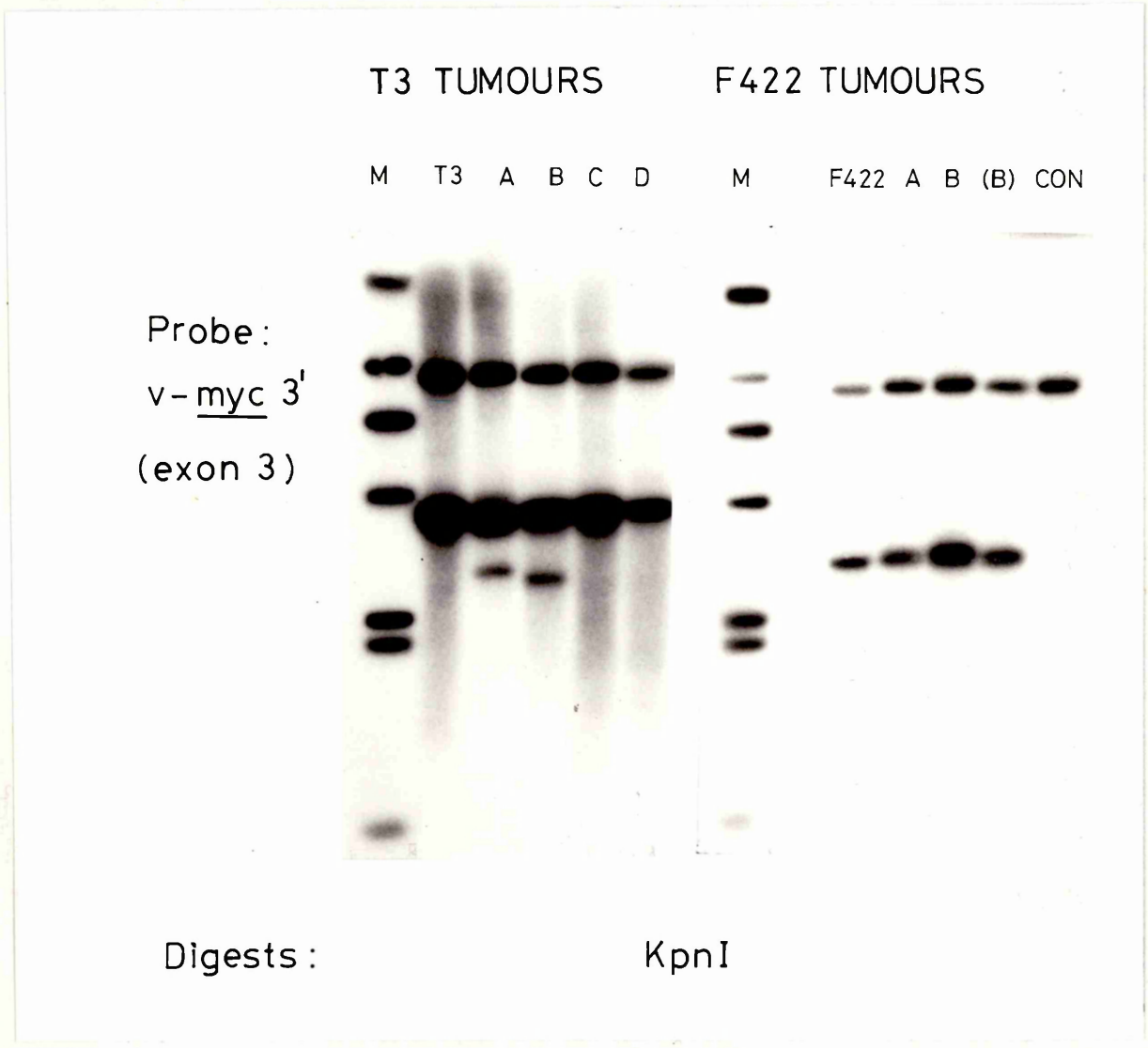


Figure 31 shows DNAs isolated from the T3 and F422 induced tumours. The DNAs have been restricted with EcoR1 which has a restriction site within the T3 provirus and also in cellular flanking sequences. The F422 provirus, however, does not contain a restriction site for EcoR1 therefore this enzyme is cutting these DNAs in cellular flanking sequences only.

M = Hind III λ DNA marker.
A, B, C, D = T3 and F422 induced tumour DNAs.
(B) = cell line DNA derived from the F422 induced tumour B.
CON = control DNA.

FIGURE 31

Clonal origin of tumours demonstrated by
EcoR1 restriction analysis

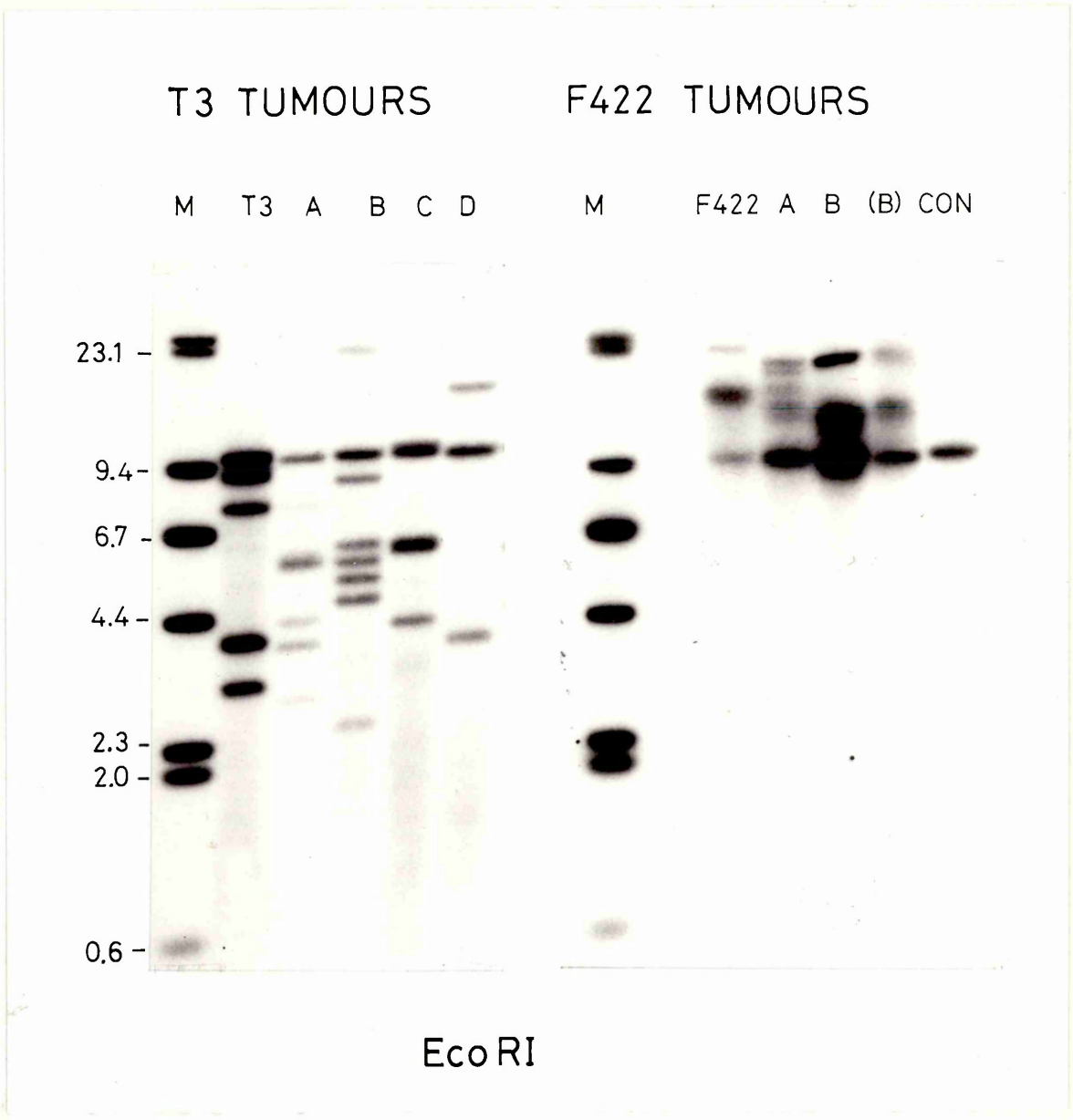
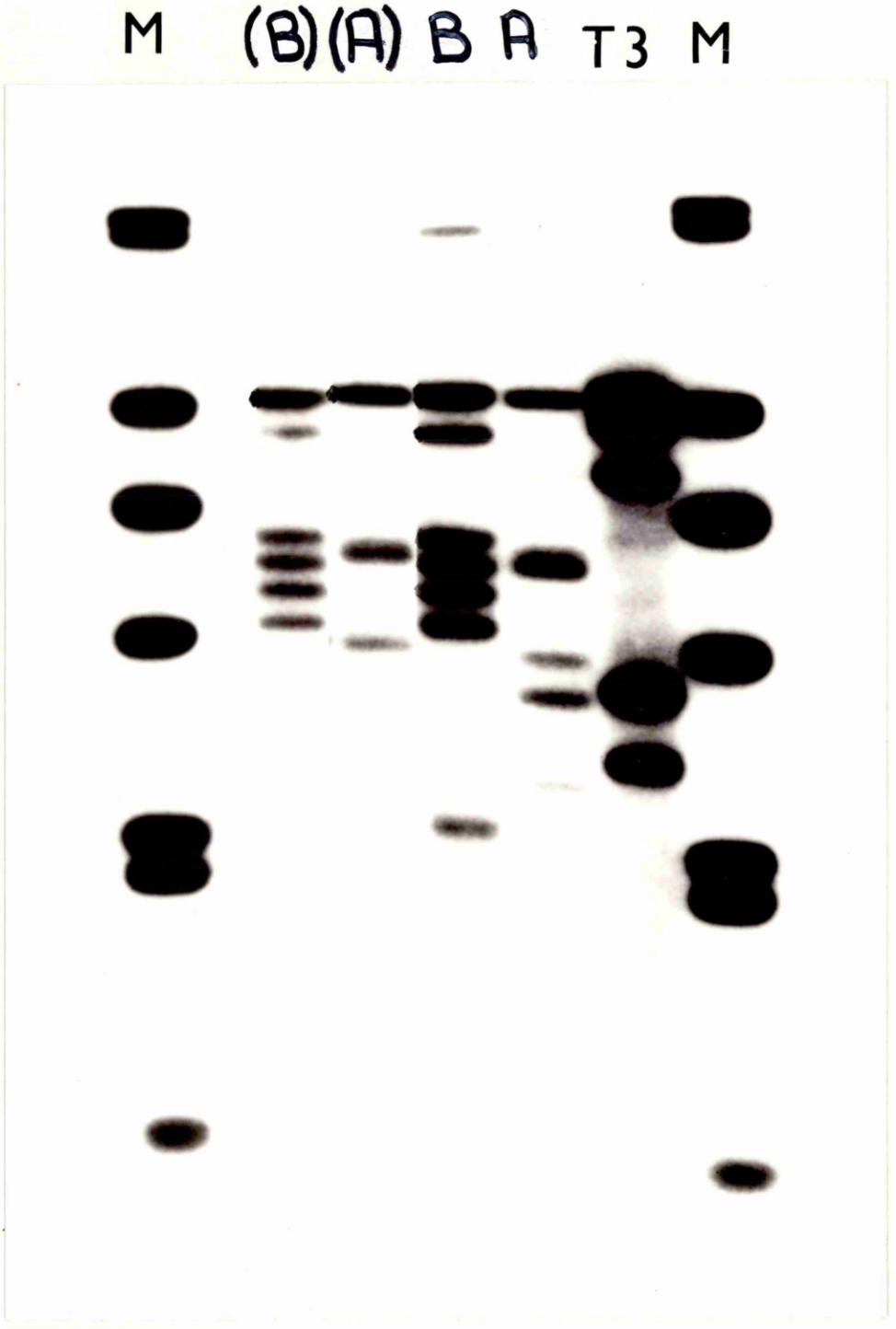


Figure 32 shows a comparison of the integration pattern of the proviruses of tumour and representative cell line DNAs. The T3 DNAs were restricted with EcoR1 which cuts both within the virus and in the flanking sequences.

M = Hind III λ DNA marker.
A, B = two of the T3 induced tumour DNAs.
(A), (B) = the corresponding cell line DNAs.

FIGURE 32

The comparison of the restriction maps of tumour and representative cell line DNAs.



GENERAL DISCUSSION

This study was concerned with two principal aspects of the pathogenesis of T-cell neoplasia in the cat; first, the investigation of the role, if any, of IL-2 in the proliferation of neoplastic T-cells *in vitro* and secondly, the determination of the properties of the FeLVs associated with these tumours.

A prerequisite for this work was a source of feline IL-2 which could support the long-term culture of normal activated T-cells. The production of lymphocyte conditioned medium from SPF cats has answered this requirement and has enabled both the long-term growth of normal T-cells and the establishment of neoplastic T-cell lines.

The investigation into the requirement of T-cells for an exogenous source of IL-2 *in vitro* has been very rewarding. These studies have revealed that although normal T-cells are absolutely dependent on an exogenous source of IL-2 for growth *in vitro*, neoplastic T-cells isolated from FeLV positive thymic lymphosarcomas, fall into one of two categories with respect to this requirement (Figure 33).

Of nine tumours (3 field cases, 4 T3 virus-infected cats and 2 F422 virus-infected cats) from which a recombinant FeLV/*myc* virus has been isolated, seven have established in culture independent of an exogenous source of IL-2. (One of the other two, T11, requires an exogenous source of IL-2 for growth *in vitro*). Strikingly, of nine other tumours which did not harbour an FeLV/*myc* recombinant virus, none established in culture independent of an exogenous source of IL-2 but three grew if IL-2 was added to the medium.

Our recent observations (Stewart *et al.*, submitted) suggest that the level of *myc* RNA in the tumour cells which harbour FeLV/*myc* recombinant virus is increased approximately 100-fold over that in normal activated T-cells.

In the light of recent studies on the relationship of oncogenes to growth factors and growth factor receptors (reviewed by Heldin and Westermark, 1984) and from the results presented here

it is possible to speculate on the possible relationship between myc and the growth factor independence of the neoplastic feline T-cells in this series.

Since myc is a nuclear DNA binding protein it may have a regulatory function in the induction or de-repression of transcription of a regulatory protein involved in cell division. It has been reported that c-myc is an inducible gene and that the expression of c-myc mRNA is increased 10 to 40-fold within 1-3 hours after cell stimulation with a mitogen such as Con-A (Kelly et al., 1983). In addition, this induction not only occurs in the presence of cyclohexamide demonstrating that no new protein species is required, but in the presence of both Con-A and cyclohexamide, superinduction of the gene is observed, suggesting that a labile protein may regulate c-myc levels in these cells.

It has also been well documented that although the increased expression of c-myc "primes" the cell to divide, the binding of IL-2 to the cell membrane is essential for the cell to proceed from the late G1 phase of cell division into S phase (Klaus and Hawrylowicz, 1984). The product of the c-myc gene would therefore appear to provide a link between the mitogenic signal (s) to the cell instructing it to divide and the competent cell, ready to undergo division. The most obvious hypothesis which could emerge from these observations is that the myc proto-oncogene either directly or indirectly (via a secondary intracellular message) controls the expression of the growth factor receptor. In the case of T-cells, this would be the IL-2 receptor and recent evidence from Tsudo et al. (1982; 1983) that the IL-2 receptor on human ATL cells is not down-regulated by the monoclonal antibody (anti-Tac) which modulates the IL-2 receptor (Tac-ag) on normal activated T-cells suggests that the regulation of the IL-2 receptor may be different between normal and malignant T-cells.

One can envisage that the constitutive expression of the IL-2 receptor on IL-2 dependent malignant T-cells could directly lead to

the uncontrolled proliferation of these cells. On the other hand, the constitutive expression of the receptor on so-called IL-2 independent T-cells is not such an obvious advantage..

The crucial fact to be determined in this case is the definition of "IL-2 independent" T-cell growth. Whether or not malignant T-cells are truly independent of an exogenous source of IL-2 for growth or whether they are really hyper-responsive to low levels present in foetal calf serum has not been satisfactorily established, even in the experiments presented in this work. It will be important to study the growth of the T3 cell line in a complete Iscoves medium which is free of FCS. If these cells continue to grow in this medium it can be concluded that they are truly independent of exogenous IL-2 for proliferation in vitro. It has been the assumption in many experiments (my own included) that the cells do not respond to IL-2 in the FCS but one must be wary of this assumption since it was believed that in the murine system BFU-E cells infected with Friend MuLV could differentiate in the absence of erythropoietin when in fact the cells were responding to low levels of this factor in the foetal calf serum in the culture medium (Hankins, 1983).

A constitutive expression of IL-2 receptor could, in theory, confer a hyper-responsive state on a cell. However, adsorption data (presented in Chapter Four) suggests that the neoplastic T-cells studied in this series do not display an excess of available receptors. It may be that the IL-2 receptors on these cells are altered in such a way that they behave as if IL-2 were bound in the absence of the factor or alternatively the receptors may be binding an endogenous factor produced by the cells. If these cells are producing and responding to their own growth factor, it is not produced in detectable levels free in the supernatant of the cells. However, the dexamethasone inhibition and rescue experiments (also presented in Chapter Four) strongly suggest that the IL-2 independent T-cell lines T3 and T17 are producing and responding to IL-2 or a closely related factor. This would not disagree with a receptor-orientated hypothesis which

would postulate that the enhanced expression of myc results in the constitutive expression of IL-2 receptors on these cells. Indeed, although all sub-classes of T-cells have been shown to be capable of producing IL-2, helper T-cells are the major source of this factor (Robb, 1984) and it would be of interest to determine if the T11 neoplastic T-cells were of the same subset as the T3 and T17 "IL-2 independent" cells. The hypothesis, on this basis, would be that enhanced levels of myc results in the constitutive expression of receptors on all T-cell subsets but that when this occurs in a T-cell of the helper subset which is capable of producing IL-2, then these cells would be capable of uncontrolled proliferation.

The results in Chapter Six demonstrate that the tumours produced by the T3 and F422 FeLV/myc recombinant viruses are clonal, or at most oligoclonal in origin. It therefore follows that if these viruses integrate into multiple target cells on infection then a secondary event, such as the activation of a second oncogene, must occur to achieve the fully-transformed cell. Alternatively, the selection for a rapidly growing clone may occur if, for example, a sub-population of the infected cells are capable of continuously dividing by auto-stimulation

Clearly the most immediate questions to be answered now are: (i) are neoplastic T-cell lines such as T3 and T17 truly IL-2 independent in vitro? (ii) what is the level of IL-2 mRNA in these cells? (iii) do these cells constitutively express a receptor for IL-2? and (iv) if so, does this expression correlate with enhanced myc expression in the cells?

Several experiments have been designed to answer these questions. As mentioned previously, the T3 cell line will be grown in a complete Iscoves medium without FCS to examine the possibility that the cells are hyper-responsive to low levels of IL-2 in foetal calf serum. If these cells are truly IL-2 independent they will be probed with a human IL-2 cDNA clone (provided by Dr. R. Gallo) to determine the intracellular level of IL-2 mRNA. If the cross-hybridisation between the human and feline IL-2 is poor, then the

human IL-2 will be used as a detection system for the cloning of the feline IL-2 gene. It is also hoped to have the human IL-2 receptor cDNA clone made available to us in the near future and similar experiments are planned to determine if the IL-2 independent neoplastic T-cells which harbour a recombinant virus constitutively express the IL-2 receptor. Ultimately, it is hoped to introduce combinations of the myc gene and the IL-2 gene into normal cells via retroviral packaging deletion mutants, and observe the outcome of infection. The results of these experiments should either prove or disprove the hypothesis that the enhanced expression of the myc gene leads to the constitutive expression of IL-2 receptors on neoplastic T-cells.

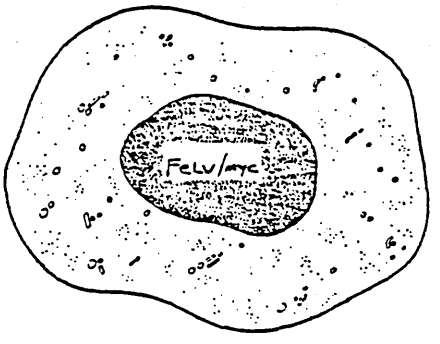
If the normal product of c-myc is essential to the regulation of normal cell division at the level of control of growth factor receptor expression then one can envisage a model whereby the aberrant expression of myc could lead to the transformation of a wide spectrum of target cells of different histogenetic origins (Graf and Beug, 1978).

In this respect the study of the effect of increased myc and Il-2 expression on T-cells should help to elucidate the mechanism of transformation of FeLV/myc positive thymic lymphosarcomas of the cat.

FIGURE 33.

The correlation between the presence of recombinant virus and independence from exogenous IL-2.

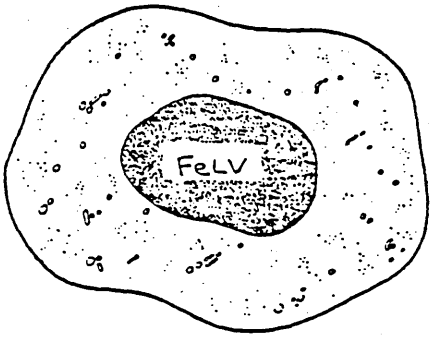
Class 1 - Thymic lymphosarcomas harbouring FeLV/myc recombinant virus.



7/9 grew independently of exogenous IL-2

1/9 grew in the presence of exogenous IL-2

Class 2 - Thymic lymphosarcomas harbouring helper FeLV only.



0/9 grew independently of exogenous IL-2

3/9 grew in the presence of exogenous IL-2

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