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IMMUNOLOGICAL AND VIROLOGICAL INVESTIGATIONS INTO SPORADIC OVINE LYMPHOMA

A THESIS PRESENTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY
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[illegible]

ABSTRACT

In New Zealand malignant ovine lymphoma is a low--prevalence, sporadic disease affecting sheep 4 years and older. The aetiology of the disease is unknown but a previous study showed that when cell-free extracts of ovine lymphomas were inoculated into *in utero* and new-born lambs they developed a persistent lymphocytosis and cell-mediated immunity to the lymphoma extracts. Furthermore, particles interpreted as virus-like were observed by electron microscopy in phytohaemagglutinin-stimulated lymphocytes from these sheep. This thesis reports the continued investigation of sporadic ovine lymphoma including its immunological characterisation and a search for evidence of conventional retroviruses.

Techniques for the detection of T cell-specific antigens, surface immunoglobulin, complement receptors and Fc receptors on lymphocytes from the blood and mesenteric lymph nodes from normal sheep were established and ranges of values for T and B cells determined. The use of α naphthyl acetate esterase (ANAE) as a specific marker for sheep T cells was investigated. No correlation was found between T lymphocytes and ANAE-positive cells.

Cells from 17 cases of lymphoma were characterised for the presence of T cell antigens and surface immunoglobulin, markers for T and B cells respectively. Seven cases were T cell and 6 were B cell in origin; in one case cells displayed both markers and in another there were similar numbers of both T and B cells; the cells from the remaining 2 cases had neither marker.

An apparent correlation was found between the patho-anatomical classification of the disease and the immunological origin of the lymphoma. All the T cell lymphomas were of multicentric distribution, whereas 4 of the 6 B cell neoplasms were confined to the alimentary tract and its associated lymphoid tissue. There was no correlation between the cellular morphology of the lymphomas and whether they were T or B cell in origin.

Electron microscopy of several ovine lymphomas and their suspension cultures revealed only a few nonbudding

particles with the dimensions of retroviruses. Smaller vesicular structures were seen associated with cells from a single suspension culture and in control cultures but these were considered unlikely to be of viral origin.

Attempts were made to establish continuous cell cultures derived from ovine lymphomas. These included the culturing of lymphoma cells either over normal fibroblast "feeder" monolayers in various combinations of media and sera, or in simple suspension cultures. Alternatively, lymphoma tissue was used in plasma clot explant cultures. Limited success occurred only with the plasma clot explant cultures where cells from one lymphoma survived 2 passages. Several fibroblast cell lines derived from the lymphoma explant cultures have been developed and passaged over 20 times.

To detect the presence of retroviruses in a variety of materials derived from ovine lymphomas, 2 biochemical techniques were used. The first involved the assay of culture supernatants for ^{3}H uridine-labelled virus in density gradients, and the second the search for RNA-dependent DNA polymerase (RDDP) activity in various preparations. Four ovine lymphoma cell suspension cultures were assayed for the production of RNA-containing virus particles. Tritiated uridine was added at the time the cultures were established and the media harvested after 96 hr. The pellets obtained following differential ultracentrifugation were centrifuged through 15 to 60 percent sucrose gradients and fractions of the gradient were assayed for acid-precipitable radioactivity. Although radiolabelled material was detected at densities of 1.15 to 1.18 gm per ml in preparations of 2 lymphoma cultures, normal lymph node cultures yielded similar results. Radiolabelled material treated with sodium dodecyl sulphate and sedimented through a sucrose gradient had a sedimentation value of approximately 7S. No high molecular weight RNA consistent with that of retroviruses was found. Further experiments using normal ovine fibroblasts led to the conclusion that the radiolabelled material detected in the 15 to 60 percent sucrose gradient was probably slowly sedimenting cellular RNA.

The RDDP assay was performed on ultracentrifuged

preparations from media of lymphoma and normal lymph node cultures, and Rous sarcoma and bovine leukaemia viruses were used as positive controls. Incorporation of ^{3}H thymidine triphosphate into acid-insoluble material was detected in 4 of 16 ovine lymphoma cultures but was also found in material from control lymph node cultures. Little variation in incorporation kinetics could be evinced by altering the assay conditions, and the observed activity was not associated with a particle of density 1.15 gm per ml. Furthermore, RDDP could not be detected in preparations from the homogenates of 6 lymphomas. It was concluded that the activity observed in both the lymphoma and control lymphocyte preparations was not due to RDDP.

Depressed responsiveness by lymphocytes to nonspecific mitogens has been associated with infections by retroviruses in other species. However, there were no differences in responses to phytohaemagglutinin by lymphocytes from lymphoma-inoculated and control sheep.

Although conventional retroviruses have not been clearly demonstrated in association with sporadic ovine lymphoma in these experiments, the failure to detect virus does not rule out the possibility of retroviral involvement at some stage of lymphomagenesis. The development of more sensitive techniques might allow the detection of low levels of virus or viral nucleic acid sequences within cells.

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ERRATA

*p.44, line 34: r^2=0.0484 not r^2=0.484

*p.71, line 33: defective not detective

*p.110, line 32: exogenous not endogenous

*p.111, lines 7 to 9: read deoxyribonucleoside triphosphates not ribonucleoside triphosphates

*p.130, line 6: 1.15 not 1.5

*p.130, line 10: gradients not gradinets

*p.132, line 14: were not was

*p.190, line 17: aseptically not asceptically

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ABBREVIATIONS USED IN TEXT

AEV	avian erythroblastosis virus
ALL	acute lymphoblastic leukaemia
ALV	avian leukaemia virus
AMV	avian myeloblastosis virus
ANAE	alpha naphthyl acetate esterase
ATV	antibiotics-trypsin-versene
BLV	bovine leukaemia virus
BLV-FOS	bovine leukaemia virus-infected foetal ovine spleen cells
C	complement
CLL	chronic lymphocytic leukaemia
Con A	Concanavalin A
c src	cellular equivalent of the *src* gene
DMBA	7,12-dimethylbenz(a)anthracene
E	erythrocyte
EA	erythrocyte-antibody complex
EAC	erythrocyte-antibody-complement complex
env	envelope gene of retroviruses
erb	oncogene of avian erythroblastosis virus
ES	equine serum
FBS	foetal bovine serum
FeLV	feline leukaemia virus
FITC	fluorescein isothiocyanate
FOCMA	feline oncornavirus-associated cell-membrane antigen
FOSK	foetal ovine skin cells
^{3}H TTP	tritiated thymidine triphosphate
IgG-latex	IgG-coated latex particles
LTR	long terminal repeat
mac or *myc*	oncogene of MC29 virus
MC29	strain of avian myelocytomatosis virus
MEM	minimum essential medium (Eagle's)
MuLV	murine leukaemia virus
myb	oncogene of avian myeloblastosis virus
OGG	ovine gamma globulin
OLF	ovine lymphoma fibroblasts
OS	ovine serum
PBS	phosphate buffered saline
PHA	phytohaemagglutinin

PRC-CEF	Prague strain Rous sarcoma virus-infected chick embryo fibroblasts
PWM	pokeweed mitogen
RDDP	RNA-directed DNA polymerase
RNAase	ribonuclease
ROK	a diploid foetal ovine kidney cell line
RSV	Rous sarcoma virus
SDS	sodium dodecyl sulphate
SIg	surface immunoglobulin
src	oncogene of Rous sarcoma virus
TCA/NaPP	trichloroacetic acid and sodium pyrophosphate mixture
TdT	terminal deoxynucleotidyl transferase
ZC	complement-coated zymosan particles

PREFACE

The animal lymphomas that are used as research models for human lymphomas and leukemias have, in most cases, a retroviral aetiology (Kaplan, 1978). Although these models provide a framework for human studies, their relevance could be questioned because there is little epidemiological evidence for the horizontal transmission and hence for the involvement of an infectious agent in the human diseases (Kaplan, 1978). Other animal lymphomas such as sporadic bovine lymphoma (Burny *et al.*, 1978; Kettmann *et al.*, 1978), canine lymphoma (Onions, 1980) and a certain proportion of feline lymphomas (Francis *et al.*, 1979) are reported in which retroviruses are not detected as readily as those of the conventional laboratory species. These may be of importance in establishing whether or not retroviruses have a role in the human lymphoproliferative disorders.

In New Zealand, sporadic ovine lymphoma is a low-prevalence lymphoid neoplasm which has not been observed to occur in specific areas or flocks (Johnstone, 1974). As it was of unknown aetiology, Johnstone *et al.* (1979a) attempted to transmit the disease by inoculating *in utero* or new-born lambs with cell-free extracts of the neoplasm. The initial results were encouraging, in that a persistent lymphocytosis developed in 19 of the 28 sheep inoculated. Electron microscopy of phytohaemagglutinin-stimulated lymphocytes from these sheep showed unenveloped particles 80 to 100 nm in diameter which were interpreted as being virus-like by the authors. These particles were seen in large cytoplasmic vacuoles but budding forms were not found. Cell-mediated immunity to cell-free extracts of ovine lymphomas was demonstrated in the sheep showing persistent lymphocytosis and this was considered further evidence in support of a viral aetiology (Johnstone *et al.*, 1979b). However, detectable lymphomas did not develop in any of these animals (Johnstone, unpublished data).

The aim of the experimental work reported in this thesis on sporadic ovine lymphoma was to investigate further this disease both as an entity in itself and as a possible model for lymphomas in man. The research involved immunological

and virological investigations and is reported here in 2 sections. The first of these, Section A, describes the application of immunological and cytochemical methods to the identification of ovine T and B cells. After ascertaining the reliability of these methods with lymphocytes from normal sheep, they were then applied to a number of ovine lymphomas.

The second section, Section B, reports on experiments to clarify whether or not a retrovirus is associated with the disease. In addition to the electron microscopic examination of neoplasms for virus and attempts at establishing long-term suspension cultures, other experimental procedures were applied to enhance the sensitivity of retroviral detection in lymphomas and their culture materials and to provide information about the nature of any particles isolated. These experiments included attempts to detect RNA-containing particles and to identify RNA-directed DNA polymerase in preparations from sporadic ovine lymphomas. Finally, the mitogenic responsiveness of blood lymphocytes from the lymphoma-inoculated sheep of Johnstone *et al.* (1979a) were compared with those of normal sheep as altered responses could be considered indirect evidence for retroviral infection (Dent, 1972).

SECTION A:

THE IMMUNOLOGICAL CHARACTERISATION OF SPORADIC OVINE LYMPHOMA

CHAPTER 1

LITERATURE REVIEW: LYMPHOCYTE CELL SURFACE PHENOTYPING AND ITS APPLICATION TO LYMPHOMAS AND LEUKAEMIAS

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(iii) Sporadic lymphoma

5. Sheep

C. Summary and conclusion

CHAPTER 1

LITERATURE REVIEW: LYMPHOCYTE CELL SURFACE PHENOTYPING AND ITS APPLICATION TO LYMPHOMAS AND LEUKAEMIAS

INTRODUCTION

The recognition of T and B lymphocytes by their phenotypic characteristics has aided the investigation of the normal immune system and has provided a better understanding of the malignant lymphoid diseases. This review therefore has 2 aspects. Firstly, it considers the immunological and cytochemical identification of lymphocytes, and secondly, it reviews the application of cell surface markers to the human and animal lymphomas and leukaemias. This approach aims to place the experimental work described in this thesis into the overall context of mammalian lymphoma research.

PART 1: THE IMMUNOLOGICAL AND CYTOCHEMICAL IDENTIFICATION OF LYMPHOCYTES

Lymphocytes show differences in their surface structures (Table 1) and in their physical (Table 2) and cytochemical properties (Table 3). Whilst the physical and cytochemical properties tend not to be associated exclusively with a single lymphocyte subpopulation, the cell surface features are of value in the identification of T and B lymphocytes. Recently, attention has focused on the cytochemical identification of these cells as an alternative to immunological techniques.

A. IMMUNOLOGICAL IDENTIFICATION OF T AND B LYMPHOCYTES

The cell surface markers which are used to define lymphocyte subpopulations are detected by reagents which identify receptors and other cell surface features that are membrane specialisations. Some of these are acquired or lost during the process of cell differentiation and reflect changes in lymphocyte function. These changes account, in part for the considerable heterogeneity of markers found within the T and B cell subpopulations (Winchester and Kunkel, 1979).

TABLE 1

HETEROGENEITY OF MARKERS ON HUMAN LYMPHOCYTES (FROM DWYER, 1976)

Presumed cell type	SIg	IgGFc	EA-ox	EA-hu	EAC	EBV	E	E-Rh	E-hu	Anti-T Sera
B	+	+	+	–	+	+	–	–	–	–
B	+	–		–	+					
B	+	+			–					
?					+					
?K cell				+			–			
?K cell	–	±		+	±		–			
T	–	–	–	–	–	–	+	–	–	+
T	–	–	–	–	–	–	+	+	–	
T	–	–	–	–	–	–	+		+	+
T	–	+					+			+
T	+						+			
T					+		+			
T			+				+			
Monocyte	+	+	+	+	+	–	–	–	–	–

SIg: surface membrane immunoglobulin
IgGFc: receptor for aggregated IgG
EA-ox: receptor for antibody-coated bovine erythrocytes
EA-hu: receptor for antibody-coated human erythrocytes
EAC: receptor for erythrocyte-antierythrocyte antibody-complement complexes
EBV: receptor for Epstein-Barr virus
E: receptor for sheep erythrocytes
E-Rh: receptor for rhesus monkey erythrocytes
E-hu: receptor for human erythrocytes

TABLE 2

PHYSICAL PROPERTIES DISTINGUISHING HUMAN T AND B LYMPHOCYTES (FROM HAYWARD AND GREAVES, 1977)

	T		B	References
Adherence of acrylic acid polymer beads to lymphocyte surfaces	-		+	Jondal (1974)
Adherence to nylon	-/±		+/++	Brown and Greaves (1974)
Charge	(---)	>	(-)	Durandy *et al.* (1975)
Cell surface projections (scanning electron microscopy)	+	<	++	Polliack (1978)
Density		>		Yu *et al.* (1973)

TABLE 3

CYTOCHEMICAL IDENTIFICATION OF HUMAN T LYMPHOCYTES, B LYMPHOCYTES AND MONOCYTES (FROM TAYLOR, 1976a)

	CAE	ANAE	ACP	MGP	GLUC	PAS
T cell	-	±	- ->++[a]	- ->++[c]	++	- ->++[d]
B cell	-	-	- ->++[b]	- ->++++[c]	-	- ->++[e]
Monocyte	±	+++	+++	- ->+	-	- ->++

CAE: chloroacetate esterase

ANAE: acid alpha naphthyl acetate esterase

ACP: acid phosphatase

MGP: methyl green pyronine

GLUC: beta glucuronidase

PAS: periodic acid Schiff

(a) convoluted T cell lymphoma, T ALL

(b) B lymphocyte usually negative, plasma cell positive

(c) generally positive in dividing cells, or in immunoglobulin secreting cells such as plasma cells

(d) Sézary cell characteristically positive

(e) lymphocytes secreting carbohydrate IgA

1. THE IDENTIFICATION OF T LYMPHOCYTES

T lymphocytes from a number of species can be identified either by their ability to form nonimmune rosettes with xenogeneic erythrocytes or by the use of T cell-specific antisera.

(i) Erythrocyte (E) Rosette Formation

Human T lymphocytes bind sheep erythrocytes to form rosettes (Brian *et al.*, 1970; Lay *et al.*, 1971; Jondal *et al.*, 1972). The T lymphocytes of species other than man also bind erythrocytes, usually of xenogeneic origin. Table 4 summarises these combinations.

The factors influencing E rosette formation have been reviewed by Dwyer (1976), Taylor (1976a), Winchester and Ross (1976), Lukes *et al.*, (1978) and Stein (1978). The range of normal values recorded for human E rosetting lymphocytes may be a reflection of differences in technique and hence there have been recommendations for their standardisation (Aiuti *et al.*, 1975).

A number of procedures have been developed to stabilise the E rosette. For example, neuraminidase treatment of erythrocytes enhanced rosette formation by human (Weiner *et al.*, 1973) and cattle lymphocytes (Grewal *et al.*, 1976; Higgins and Stack, 1977). The binding between erythrocyte and lymphocyte may be strengthened by the exposure of more reactive sites or, following the treatment of lymphocytes, more cells may be recruited with the removal of immunoglobulin bound via Fc receptors (Warner, 1974). Grewal and Babiuk (1978) reported still higher values of E rosettes in cattle when erythrocytes were treated with the sulphydryl reagent 2-amino-ethylisothiouronium bromide hydrobromide (AET) and the rosetting was carried out in the presence of dextran and Ficoll. AET-treated sheep erythrocytes also stabilised human T cell rosettes (Kaplan and Clark, 1974) as did the presence of dextran (Brown *et al.*, 1975). Dextran alone is reported to enhance rosette formation with cattle (Wardley, 1977; Binns, 1978), sheep, pig and goat lymphocytes (Binns, 1978). Finally, the use of papain-treated erythrocytes with guinea pig (Wilson

TABLE 4

SPONTANEOUS ERYTHROCYTE ROSETTES FORMED WITH T LYMPHOCYTES OF DIFFERENT SPECIES

Lymphocyte	Erythrocyte	References
Guinea pig	Rabbit	Stadecker *et al.* (1973)
Guinea pig	Rabbit	Wilson and Gurner (1975)
Rabbit	Rabbit	Wilson *et al.* (1975a)
Cat	Guinea pig	Cockerell *et al.* (1976a)
Cat	Guinea pig	Mackey (1977)
Dog	Guinea pig	Bowles *et al.* (1975)
Dog	Human	Bowles *et al.* (1975)
Pig	Sheep	Escajadillo and Binns (1975)
Pig	Sheep	Salmon (1979)
Goat	Sheep	Binns (1978)
Cattle	Sheep	Grewal *et al.* (1976)
Cattle	Sheep	Paul *et al.* (1979)
Buffalo	Sheep	Kaura *et al.* (1979)
Horse	Guinea pig	Tarr *et al.* (1977)
Owl monkey	Sheep	Wallen *et al.* (1973)
Rhesus macaque	Sheep	Terrell *et al.* (1977)

and Gurner, 1975), rabbit (Wilson *et al.*, 1975a) and human lymphocytes (Wilson *et al.*, 1975b) enhanced rosette formation in contrast to similar manipulations with the cat (Mackey, 1977).

The mechanism and biological significance of rosette formation are unknown. Owen and Fanger (1974) have suggested that the receptor on the lymphocyte is a glycoprotein. Rosette formation is inhibited by anti-T cell antisera which indicates that these receptors are part of, or are localised near T cell-specific structures (Wortis *et al.*, 1973).

Subsets of E rosetting cells can be identified by the presence of Fc receptors for IgM and IgG (Moretta *et al.*, 1975, 1977), by the presence of complement (C) receptors (Chiao *et al.*, 1974) or by the degree of affinity for erythrocytes that T cells have under suboptimal rosetting conditions (Wybran and Fudenberg, 1973; Borella and Sen, 1975; Taniguchi *et al.*, 1976). The identification of these specific E rosetting subsets may further define a particular T cell lymphoma or leukaemia.

(ii) T Lymphocyte-Specific Antigens

Alloantisera to T cell antigens provide the most specific way of identifying these cells. In the mouse over 40 different genetic loci have been found to code for membrane alloantigens (McKenzie and Potter, 1979).

As alloantisera are generally not available for outbred animals, heterologous anti-T cell antisera have been prepared for a number of species including the mouse (Shingeno *et al.*, 1968), rat (Waksman, 1971), rabbit (Sell, 1979), chicken (Schlesinger, 1972), cow (Waksman, 1971), sheep (Bohs *et al.*, 1976) and humans (Aisenberg *et al.*,1973: Aiuti and Wigzell, 1973; Greaves and Janossy, 1976; Chess and Schlossman, 1977). As these antisera are generally raised against lymphocytes expressing both functional and differentiation heterogeneity, they are unable to discriminate T cell subsets or between thymocytes and mature T lymphocytes (Chess and Schlossman, 1977). In the human, this lack of specificity has been

partially overcome with improved subpopulation separations and absorptions of antisera with autologous lymphoblastoid B cell lines, to the extent that helper and suppressor T cells can be identified (Evans *et al.*, 1977) and that thymocytes can be discriminated from mature T lymphocytes (Chess and Schlossman, 1977).

The problems of specificity are overcome by the use of monoclonal antibodies which can be used to determine both the level of differentiation and the functional role of the lymphocyte (Gupta *et al.*, 1980; Reinherz and Schlossman, 1980). The dissection of the T lymphocyte subpopulation into subsets by this technology is of value in the evaluation of lymphoid disease (Borowitz *et al.*, 1981; Crosier and Beard, 1981).

2. THE IDENTIFICATION OF B LYMPHOCYTES

B lymphocytes are identified by their surface immunoglobulin, by their receptors for the Fc portion of the IgG molecule and for fragments of activated complement, and by the presence of B lymphocyte-specific antigens.

(i) Surface Immunoglobulin (SIg)

Readily detectable SIg, acting as a receptor for specific antigenic determinants (Naor and Sulizeanu, 1967; Byrt and Ada, 1969; Vitetta and Uhr, 1975) is the hallmark of the B lymphocyte. A subpopulation of lymphocytes bearing membrane-bound SIg was first detected by Moller (1961) and since then SIg has been detected on lymphocytes from a number of species including the mouse (Raff, 1970), chicken (Rabellino and Grey, 1971), rat (Avrameas and Guilbert, 1971), guinea pig (Davie *et al.*, 1974), rabbit (Pernis *et al.*, 1970), cat (Taylor *et al.*, 1975; Mackey, 1977), dog (Zander *et al.*, 1975; Kelly, 1980), sheep (Ey, 1973; Beh and Lascelles, 1974; Symons and Binns, 1975; Cahill *et al.*, 1978), pig (Salmon, 1979), cattle (Muscoplat *et al.*, 1974a), buffalo (Kaura *et al.*, 1979), rhesus macaques (Terrell *et al.*, 1977) and man (Klein *et al.*, 1968; Coombs *et al.*, 1969; Froland *et al.*, 1971; Wilson and Nossal, 1971).

SIg can be detected by anti-immunoglobulin antisera labelled

with fluorescein (Johnson *et al.*, 1978), radioactive isotopes (Bankhurst and Warner, 1971) or enzymes (Aiuti *et al.*, 1975), or by the mixed antiglobulin rosette test (Hallberg *et al.*, 1973).

The range of values recorded for SIg-bearing lymphocytes reflects, in part, the difficulties associated with reagent specificity and whether or not the immunoglobulin detected is intrinsic to the cell or simply cytophilic. A number of authors including Aiuti *et al.* (1975), Preud'homme and Labaume (1975), Siegal (1978) and Lukes *et al.* (1978) have discussed the problems associated with detecting SIg by immunofluorescent methods, with the latter 2 papers concentrating on the relevance of these to the study of lymphomas and leukaemias.

Winchester *et al.* (1975a), Horwitz and Lobo (1975) and Kumagai *et al.* (1975) showed that the SIg on many IgG-positive non-T lymphocytes was not intrinsic but was adherent through Fc receptors. These cells bind IgG avidly (Kurnick and Grey, 1975) and appear to be a subset that mediates antibody-dependent cellular cytotoxity (Horwitz and Garrett, 1977). The immunoglobulin adherent through Fc receptors or otherwise not intrinsic to the cell may be removed by short-term culture in serum-free medium or by enzymatic stripping of the cells (Horwitz and Lobo, 1975; Lukes *et al.*, 1978).

Another source of artifacts derive from the fluorescent reagents where aggregation may occur, or immune complexes form with either contaminant homologous serum or with soluble antigens deriving from the absorption procedures. These aggregates or complexes will adhere to the lymphocyte via the Fc receptor and should be prevented either by using $F(ab')_2$ fragments for fluorescein labelling, or by filtering or centrifuging the antisera before use (Winchester *et al.*, 1975a).

Even if these technical artifacts are avoided, the B cell origin of a lymphoma or leukaemia can be shown only when the idiotypic monoclonality of the SIg has been

demonstrated (Siegal, 1978).

(ii) Receptors for Complement Components (C receptor)

Receptors for certain fragments of activated complement components are found on lymphocytes (Bianco *et al.*, 1970), polymorphs, monocytes, eosinophils, basophils (Brown and Greaves, 1974) and mast cells (Sher and McIntyre, 1977). Most lymphocytes bearing C receptors also have SIg (Ross *et al.*, 1973). However, some E rosetting cells also have C receptors (Shevach *et al.*,1974; Chiao *et al.*, 1974) and may be immature forms of T lymphocytes (Gatein *et al.*, 1975; Stein and Mueller-Hermelink, 1977). C receptors have been found on cytotoxic T cells (Perlmann, 1976, cited by Stein, 1978) and on some non-T, non-B lymphocytes (Ross, 1977; Siegal, 1978). Although C receptors are present on different types of lymphocytes, the receptors for specific complement components may be useful in identifying subsets within subpopulations (Ross and Polley, 1975; Stein, 1978).

C receptors are detected by using IgM-coated erythrocytes that form erythrocyte-antibody complement (EAC) rosettes with C receptor-bearing cells (Winchester and Ross, 1976), indirect immunofluorescence (Ross and Polley, 1975) and the use of zymosan particles that activate complement by the alternative pathway to form zymosan-complement (ZC) complexes (Mendes *et al.*, 1974; Kajdacsy -Balla and Mendes, 1976). These complexes have proven useful in double marker studies (Bankhurst *et al.*, 1978).

The nature and function of C receptors are unknown despite considerable research (Katz, 1977). Because C receptors are found on a range of lymphocytes, their detection on cells from lymphomas and leukaemias should be only one of a battery of markers for lymphocyte identification.

(iii) Receptor for the Fc Portion of IgG (Fc Receptor)

The receptor for the Fc protion of IgG is found on most non-T lymphocytes, some normal (Basten *et al.*,1975; Arbeit *et al.*, 1976; Bankhurst *et al.*, 1978) and activated T cells (Yoshida and Andersson, 1972), monocytes (Huber *et al.*, 1968) eosinophils, granulocytes (Lay and Nussenzweig, 1968; Gupta

et al., 1976c) and some neoplastic cells (Tonder *et al.*, 1974). Little species specificity by the receptor has been observed in animals or man (Dickler, 1976a).

Fc receptors are detected by aggregated antibody (Dickler, 1976b), erythrocyte-antibody (EA) complexes (Hallberg *et al.*, 1973), IgG-coated latex particles (Sjoberg and Inganas, 1979), antigen-antibody complexes (Arbeit *et al.*, 1976; Winchester and Ross, 1976), or an indirect aggregate binding assay (Siegal, 1978). In these techniques the crucial factor for bond stability between the immunoglobulin and its receptor is that the antibody must be either complexed with an antigen (Basten *et al.*, 1972) or heat-aggregated (Dickler, 1976b).

The nature of the Fc receptor is unknown. Rask *et al.* (1975) isolated a 65,000 dalton glycoprotein with a high affinity for aggregated IgG. The receptor is distinct from other membrane components including SIg, complement receptors and histocompatibility antigens, but may be closely associated with the Ia antigens (Dickler, 1976a; Dwyer, 1976).

Apart from a role in antibody-dependent cellular cytotoxicity by "killer" (K) cells (Horwitz and Garrett, 1977) the function of the Fc receptor is unknown (Moller and Cortinho, 1975; Katz, 1977).

As SIg is the most reliable marker for B lymphocytes the detection of Fc receptors is useful only in identifying cells with little or no SIg. This is the case with some chronic lymphocytic leukaemias (Augener *et al.*, 1974) and diffuse "histiocytic" lymphomas (Koziner *et al.*, 1977).

(iv) B Lymphocyte-Specific Antigens

B lymphocyte antigens have been studied extensively in the mouse (for review Katz, 1977; McKenzie and Potter, 1979) and to a lesser extent, in man (Winchester and Kunkel, 1979). A group of antigens expressed preferentially on human B lymphocytes and monocytes has been detected by alloantisera from multiparous females (Winchester *et al.*, 1975), and these antigens may be analogous to the murine Ia antigens (Friedman

et al., 1977). It has been suggested that heteroantisera to B lymphocytes are also directed against Ia antigens (Niederhuber and Moller, 1972) and such sera have been used in the mouse (Raff, 1971) chicken (McArthur *et al.*, 1971), rabbit (Chou *et al.*, 1977) and man (Greaves and Brown, 1973).

Although Ia antigens have been found on both T and B cells (Frelinger *et al.*, 1974) they occur in highest concentrations on peripheral B lymphocytes (Shreffler and David, 1975; McDevitt *et al.*, 1976). Despite some criticism of their appropriateness, the B lymphocyte /Ia antigens have been useful in the classification of human acute lymphoblastic leukaemias (Mann *et al.*, 1979).

3. OTHER MARKERS OF T AND B LYMPHOCYTES

A number of other markers have been used to identify T and B cells of man. Of the T cell markers, spontaneous rhesus monkey E rosettes form with normal T and neuraminidase-treated non-T cells (Lohrmann and Novikovs, 1974). However, cells from some chronic lymphocytic leukaemia (CLL) patients, presumably B cells, were found to form rhesus E rosettes. Human erythrocytes will rosette with human thymocytes (Baxley *et al.*, 1973) and a proportion of human T cells (Kaplan, 1975). The lectin *Helix pomatia* A haemagglutinin binds to neuraminidase-treated T lymphocytes of man (Hammarstrom *et al.*,1973) and cattle (Morein *et al.*, 1979).

Human B cells have receptors for the Epstein-Barr virus in their cell membranes (Greaves *et al.*, 1975) and a number of heterologous E rosettes form with B cells of the rabbit (rat erythrocytes), rat and mouse (rabbit erythrocytes) (Braganza *et al.*, 1975) and human (mouse erythrocytes) (Gupta *et al.*, 1976a,b). The mouse E rosette that forms with human B lymphocytes identifies B cells early in their ontogeny (Gupta *et al.*, 1976a) and is used for differentiating CLL from certain nonrosetting lymphosarcoma cell leukaemias (Koziner *et al.*, 1977).

4. "NULL" LYMPHOCYTES

"Null" cells are a functionally and phenotypically

heterogeneous population of lymphocytes that cannot be classified as either typical T or B cells (Dickler, 1976a). They may or may not have a combination of Ia antigens and Fc and C receptors. Some "null" cells are involved in antibody-dependent cellular cytotoxicity, whilst others bearing Ia antigens probably represent different early stages of B cell differentiation (Chess and Schlossman, 1977).

B. CYTOCHEMICAL IDENTIFICATION OF T AND B LYMPHOCYTES

Lymphocytes contain lysosomal enzymes (Weissmann and Dukor, 1970) whose biological functions are undetermined. Attention has recently been focused on using the presence of these enzymes for the cytochemical identification of T and B lymphocytes in tissue sections and cell smears as a practical alternative to immunological methods. These enzymes include acid phosphatase, β glucuronidase, β glucosaminidase and α naphthyl acetate esterase (ANAE) (Table 3).

1. Acid Phosphatase, β Glucuronidase, β Glucosaminidase

Tamaoki and Essner (1969) applied cytochemical techniques for these 3 enzymes to sections of lymphoid tissues. They demonstrated a difference in distribution of cells showing enzymic activities and concluded that these enzymes may identify cells that were preferentially found in the thymus-dependent areas of the lymphoid organs. Dockrell *et al.* (1978), using the immunofluorescent detection of SIg to identify B cells, confirmed these results. However, the evidence that these cytochemical activities are unique to T cells is somewhat dubious as the thymus-dependent areas of lymphoid tissues also contain B lymphocytes (de Sousa, 1981). Furthermore, Wehinger and Mobius (1976) found that acid phosphatase and β glucuronidase activities were neither unique to, or invariably present in, E rosetting T cells, but may represent a functional state of the cell. The lack of correlation between cytochemical and biochemical activities was further demonstrated by Tanaka (1979) who found that B cell lysates had a higher activity of these enzymes than did T cell lysates.

Although the specificity of acid phosphatase for T lymphocytes has been challenged, this enzyme has been used to diagnose T acute lymphoblastic leukaemia (Catovsky *et al.*, 1974, 1978b) and convoluted T cell lymphoma (Stein *et al.*, 1976). However this marker is unsatisfactory with other lymphomas (Micheau and Belpomme, 1978) or with human lymphoblastoid cell lines (Parker *et al.*, 1978).

2. Acid α Naphthyl Acetate Esterase (ANAE)

Nonspecific esterases were first detected in human lymphocytes by Lake (1971) and recently, ANAE activity has been suggested as a specific cytochemical marker for both murine (Mueller *et al.*, 1975; Ranki *et al.*, 1976) and human (Kulenkampff *et al.*, 1977; Horwitz *et al.*, 1977; Grossi *et al.*, 1978; Knowles *et al.*, 1978; Manconi *et al.*, 1979; Ferrari *et al.*, 1980) T lymphocytes. Evidence for the specificity of ANAE activity for human T cells has come from lymphocyte fractionation (Horwitz *et al.*, 1977; Grossi *et al.*, 1978; Knowles *et al.*, 1978) and double marker studies (Ferrari *et al.*,1980). Furthermore, ANAE-positive cells are localised in the thymus-dependent areas of lymphoid organs (Horwitz *et al.*, 1977; Seymour *et al.*, 1978), are mature T cells in the peripheral circulation as few thymocytes show such activity (Kulenkampff *et al.*, 1977) and may be specifically T helper lymphocytes (Grossi *et al.*, 1978; Manconi *et al.*, 1979). However, Yang *et al.* (1979) demonstrated that nearly 50 percent of human C receptor-bearing lymphocytes were also ANAE-positive, a result at variance with other studies (Horwitz *et al.*, 1977; Knowles *et al.*, 1978; Ferrari *et al.*, 1980).

ANAE activity has not been found reliable as a T cell marker in species other than man and mouse. Yang *et al.* (1979) showed that a proportion of bovine SIg-positive, C receptor-bearing lymphocytes were ANAE-positive. Fossum (1978) found that rat B lymphocytes showed ANAE activity although Dockrell *et al.* (1978) had demonstrated ANAE-positive cells in thymus-dependent areas of lymphoid tissue. Finally, ANAE activity was shown not to be specific for T cells of the cat or gerbil (Dockrell *et al.*, 1978).

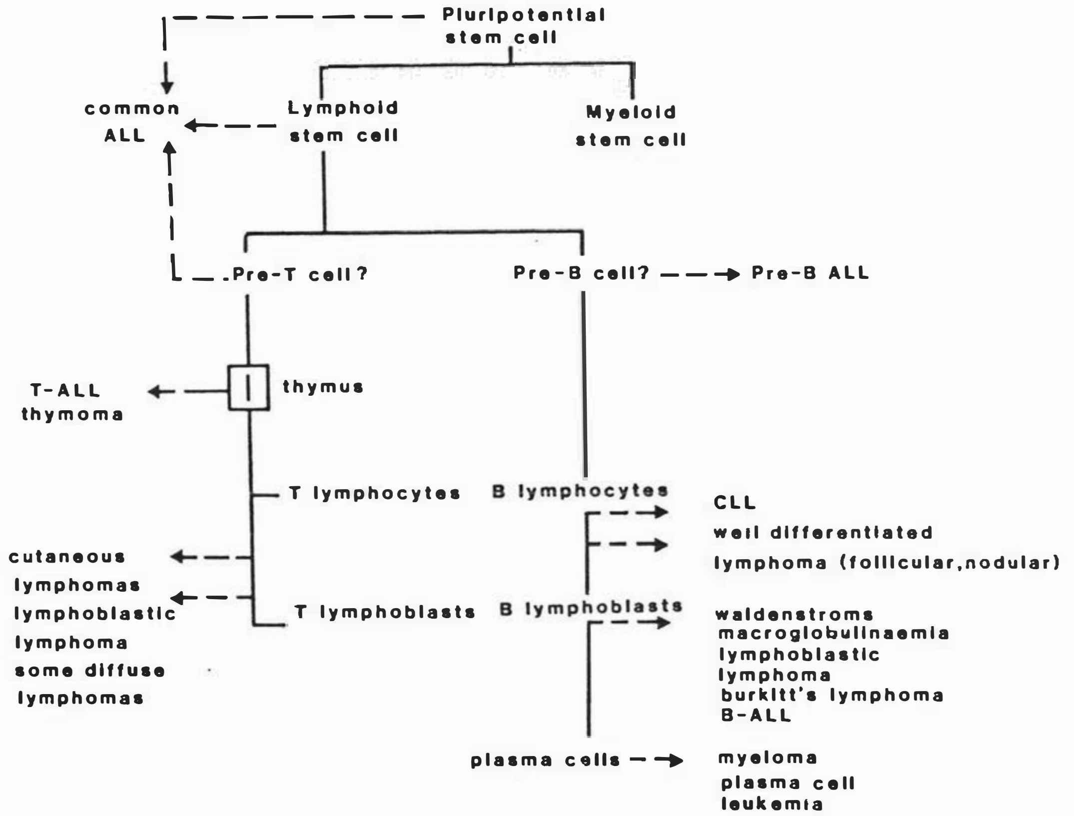
ANAE has been detected in human lymphomas and leukaemias (Kulenkampff *et al.*, 1977; Knowles *et al.*, 1978; Sundstrom *et al.*, 1978; Knowles *et al.*, 1979; Pinkus *et al.*, 1979). Whilst a single intense focal spot of activity appears to identify E rosetting cells, some B cell lymphomas show a granular distribution of activity (Pinkus *et al.*, 1979). However, not all T cell lymphomas would have been identified exclusively by ANAE activity (Knowles *et al.*, 1979; Pinkus *et al.*, 1979). Knowles *et al.*(1979) contend that the usefulness of ANAE staining may not be from the direct identification of a T cell lymphoma, but from the determination of the stage of T cell maturation at which the neoplastic process occurred.

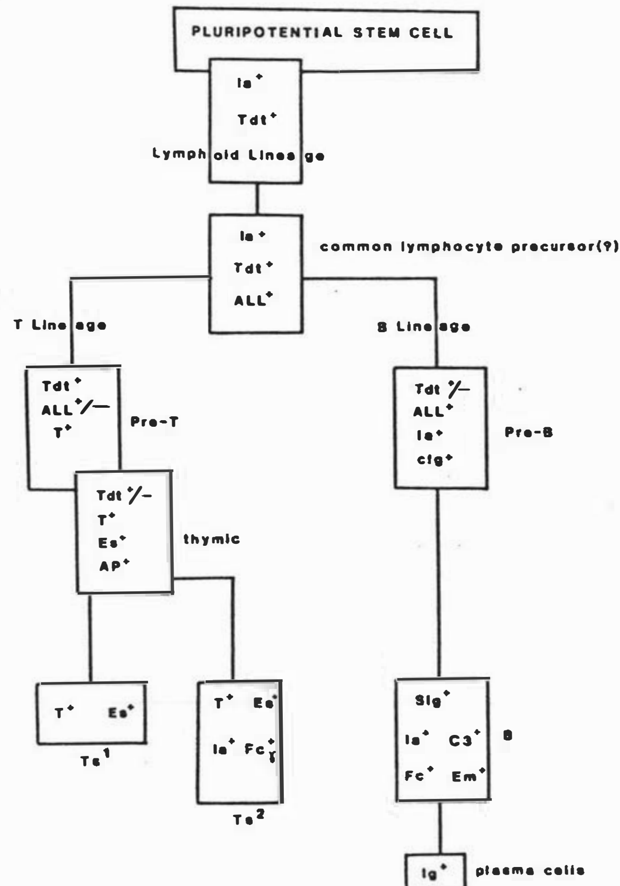
C. CONCLUSION

The results from the use of immunological and cytochemical markers for identifying the immunological origin of lymphomas and leukaemias have only been alluded to in this part of the review. The following part will examine in more detail the immunological characterisation of these neoplasms and conclusions are drawn at the end of this chapter about the pertinence of these markers to lymphoma research.

PART 2: THE IMMUNOLOGICAL CHARACTERISATION OF LYMPHOMAS AND LEUKAEMIAS

Lymphomas and lymphoid leukaemias are neoplastic, monoclonal expansions of cells that mimic the surface characteristics and ecotaxic behaviour of their normal progenitors. The immunological characterisation of these diseases using techniques previously outlined has been of benefit to the understanding of their pathogenesis and has given insights into the differentiation and maturation of the normal lymphoid cell (Catovsky *et al.*, 1978a; Greaves and Janossy, 1978; Seligmann *et al.*, 1978; Silverstone *et al.*, 1978; Greaves, 1979) (Figure 1). Therefore, studies in other species are relevant to the immunological characterisation of sporadic ovine lymphoma in that they may by comparison provide explanations for the behaviour of the neoplastic


Pluripotential stem cell
common ALL
Lymphoid stem cell
Myeloid stem cell
Pre-T cell?
Pre-B cell?
Pre-B ALL
T-ALL
thymoma
thymus
T lymphocytes
B lymphocytes
CLL
well differentiated
lymphoma (follicular,nodular)
cutaneous
lymphomas
lymphoblastic
lymphoma
some diffuse
lymphomas
T lymphoblasts
B lymphoblasts
waldenstroms
macroglobulinaemia
lymphoblastic
lymphoma
burkitt's lymphoma
B-ALL
plasma cells
myeloma
plasma cell
leukemia


PLURIPOTENTIAL STEM CELL
Ia+
Tdt+
Lymphoid Lineage
common lymphocyte precursor(?)
ALL+
T Lineage
B Lineage
Pre-T
Pre-B
clg+
thymic
Es+
AP+
Ts1
Ts2
SIg+
C3+
Fc+
Em+
B
Ig+
plasma cells

lymphocyte in the sheep.

Most of the literature on cell marker studies focuses on human lymphomas and leukaemias, but there are reports for the neoplastic lymphoid diseases of chickens (Cooper *et al.*, 1974), rats (Takeichi *et al.*, 1979), guinea pigs (Shevach *et al.*, 1972a; Rhim and Green, 1977), rhesus macaques (Holmberg *et al.*,1978) mice, dogs, cats, cattle and sheep. This section reviews the human data and evaluates the reports concerning the latter 5 species.

A. THE IMMUNOLOGICAL CHARACTERISATION OF THE NON-HODGKIN'S LYMPHOMAS AND LEUKAEMIAS OF HUMANS

The importance of cell marker studies of human lymphomas and leukaemias has not been confined to an appreciation of their pathogenesis, but is of practical relevance to their diagnosis, prognosis and classification (Berard *et al.*, 1976). In recent years the older histological classifications which were based on inaccurate theories of lymphocyte derivation and differentiation, have been re-evaluated. More rational classifications cognizant of the immunological character of these neoplasms have been proposed (Table 5) and include the Lukes-Collins, (Lukes and Collins 1974, 1977; Lukes, 1978; Lukes and Parker, 1978; Lukes *et al.*, 1978), Kiel (Gerard-Marchant *et al.*,1974; Lennert *et al.*, 1975; Lennert, 1978) and Memorial Sloan Kettering (Siegal *et al.*, 1978; Filippa *et al.*, 1978) classifications. These await the demonstration of their clinical relevance before wide acceptance is likely (Butler *et al.*, 1975; Lennert, 1978, pp101-106; Bloomfield *et al.*, 1979; Leonard, 1980). As most of the literature uses modifications of the Rappaport system (Mann *et al.*, 1979) (Table 6), this classification will be used in the following synopsis. Because Hodgkin's lymphoma has no exact clinical or pathological counterpart in domestic species (Moulton and Dungworth, 1978) and because of the apparent immunological complexity and uncertainty of this disease, it has been considered inappropriate to include it in the present review.

TABLE 5

COMPARISON OF THREE CLASSIFICATIONS OF HUMAN MALIGNANT LYMPHOMAS (FROM LENNERT, 1978)

Lukes and Collins, 1974	Kiel Classification, 1974	Rappaport Classification, 1966	
	Low-grade malignant lymphomas		
	Lymphocytic	ML lymphocytic, well differentiated, diffuse	
B cell: small lymphocyte (CLL)	CLL and others		
T cell: Sezary's syndrome (and mycosis fungoides)			
B cell: plasmacytoid lymphocyte	*Lymphoplasmacytoid* (immunocytic)	ML lymphocytic with dysproteinemia	
B cell: small cleaved FCC large cleaved FCC	*Centrocytic*	ML lymphocytic, well and poorly differentiated, nodular or diffuse	
	Centroblastic/centrocytic	ML lymphocytic, well differentiated	
follicular	follicular		
follicular and diffuse	follicular and diffuse	ML lymphocytic, poorly differentiated	nodular or
diffuse	diffuse		
with or without sclerosis	with or without sclerosis	ML lymphocytic-histiocytic	diffuse
		ML histiocytic	
B cell: large noncleaved FCC	*High-grade malignant lymphomas* *Centroblastic*	ML histiocytic ML undifferentiated	nodular or diffuse
B cell: small noncleaved FCC	*Lymphoblastic*	ML undifferentiated, diffuse; ML lymphocytic, poorly differentiated, diffuse	
Burkitt type	Burkitt type		
T cell: convoluted lymphocyte	convoluted-cell type		
U cell: (undefined) Unclassifiable	others		
B cell: immunoblastic sarcoma T cell: immunoblastic sarcoma	*Immunoblastic*	ML histiocytic, diffuse	

CLL: chronic lymphocytic leukaemia
ML: malignant lymphoma
FCC: follicular centre cell

TABLE 6

A MODIFIED RAPPAPORT CLASSIFICATION OF HUMAN MALIGNANT LYMPHOMAS (AFTER MANN *ET AL.*, 1979)

Nodular (follicular) lymphomas	Diffuse lymphomas
Lymphocytic, poorly differentiated	Lymphocytic, well-differentiated
Mixed lymphocytic-"histiocytic"	Lymphocytic, intermediate differentiation
"Histiocytic"	Lymphocytic, poorly differentiated
	Mixed lymphocytic-"histiocytic"
	"Histiocytic"
	Undifferentiated Burkitt's
	Undifferentiated, pleomorphic (non-Burkitt's)
	Lymphoblastic
	Unclassified

1. FOLLICULAR (NODULAR) LYMPHOMA

Follicular lymphomas are B lymphocyte neoplasms arising from the follicular centre cell (Lukes and Collins, 1974; Jaffe *et al.*, 1974). These cells bear monoclonal SIg (Aisenberg and Long, 1975; Leech *et al.*, 1975) often with K light chains (Pinkus and Said, 1979). C3 receptors usually are present (Jaffe *et al.*, 1974) but the occurrence of Fc receptors is variable (Pinkus and Said, 1979). The cell of origin does not play an immediate role in the secretory B cell system as serum immunoglobulin abnormalities are not a feature of follicular lymphomas (Moore *et al.*, 1970). The monoclonality of follicular lymphoma cells further indicates that they arise from a migratory B lymphocyte with the ability for ecotaxis to the B cell areas of the lymphoid system (Warnke and Levy, 1978). If a leukaemic phase develops, the resultant *lymphosarcoma cell leukaemia* has the same pattern of immunological surface markers on the neoplastic cells as that of the cells of the original follicular lymphoma (Jaffe and Green, 1977).

Although earlier reports suggested the possibility of a T cell follicular lymphoma (Peter *et al.*, 1974; Aisenberg and Ling, 1975) the current consensus is that the T lymphocytes present in follicular lymphomas are either remnants of the original tissue or are part of the immune response to the neoplasm (Jaffe *et al.*, 1974; Pinkus and Said, 1979).

2. DIFFUSE LYMPHOMAS

Most *chronic lymphocytic leukaemias* (CLL) and *diffuse well-differentiated lymphocytic lymphomas* are different clinical manifestations of the same population of circulating neoplastic B lymphocytes (Braylan *et al.*, 1976).

Dissimilarities in marker patterns of the 2 neoplasms suggest that they arise from different B cell subsets. The cells of both types, in contrast to those of follicular lymphoma, show faint SIg fluorescence (Braylan *et al.*,1976), do not display capping (Cohen, 1978) and form spontaneous rosettes with mouse erythrocytes (Gupta *et al.*, 1976b).

Cells of CLL have only C3d receptors (Ross *et al.*, 1973) and show a decreased affinity for C3 (Logue and Cohen 1977). From these differences Siegal (1978) concluded that CLL cells were early in B lymphocyte development. However, although their capacity to secrete immunoglobulin appears limited, Mann *et al.* (1979) believe that because the cells immunologically resemble the well differentiated lymphocytes of the medullary cords of normal lymph nodes, CLL should be considered a neoplasm of the secretory part of the B lymphocyte system.

There have been a few reports of T cell CLL (Brouet *et al.*, 1975; Siegal, 1978; Pinkus *et al.*, 1979; Crosier and Beard, 1981).

Lymphocytic lymphomas of intermediate differentiation are B cell neoplasms which are thought to arise from lymphocytes either of the primary follicles or of the mantle zones of secondary follicles of lymph nodes (Nanba *et al.*, 1977).

Most *diffuse poorly differentiated lymphocytic lymphomas* arise from B lymphocytes (Aisenberg and Long, 1975; Knowles *et al.*, 1979; Leong *et al.*, 1979; Pinkus and Said, 1979). Many of these derive from FCC follicular centre cells (Leech *et al.*, 1975) and consequently have a distinctive morphology. However, Pinkus and Said (1979) showed that the neoplastic cells had undergone a qualitative or quantitative loss of different markers and they considered that this was directly due to either the neoplastic transformation or the stage of differentiation represented by the malignant clone. Some of these lymphomas do not have the morphology of follicular centre cells and are either of T or non-T, non-B origin (Jaffe *et al.*,1975; Waldron *et al.*, 1977; Pinkus and Said 1979). The cell neoplasms often are infiltrated by epitheloid histiocytes and this has led to the suggestion that they may derive from lymphokine-producing mature peripheral T lymphocytes (Jaffe *et al.*, 1975).

Lymphomas composed of large cells, namely the *mixed lymphocytic - "histiocytic"* and *"histiocytic" lymphomas* are also immunologically heterogeneous and may be common

morphological end points of transformed cells of diverse origins. Although most of these lymphomas appear to arise from B cells (Habeshaw and Stuart, 1975; Morris and Davy, 1975; Brouet *et al.*, 1976; Taylor, 1976b; Knowles *et al.*, 1979; Pinkus and Said, 1979) which consistently bear monoclonal SIg, a variable expression of other markers has been shown (Mann *et al.*, 1979). Some large cell lymphomas bear T lymphocyte markers (Brouet *et al.*, 1976; Pinkus and Said, 1979).

Burkitt's lymphoma is of B cell origin (Fialkow *et al.*, 1973; Mann *et al.*, 1976), derived from the small noncleaved follicular centre cell (Lukes and Collins, 1974).

3. OTHER LYMPHOMAS

Hairy cell leukaemia is now considered to be of B cell origin as the synthesis of monoclonal SIg has been demonstrated (Debusscher *et al.*,1975; Utsinger *et al.*, 1977).

While a T cell variant has been described (Saxon *et al.*, 1978), the normal counterpart for the hairy cell has yet to be identified.

Mycosis fungoides and the related *Sézary's syndrome* are of T cell origin (Lutzner *et al.*, 1975; Zucker-Franklin *et al.*, 1974). Functional studies by Broder *et al.* (1976) have suggested that the Sézary syndrome is a neoplastic proliferation of helper T lymphocytes.

4. ACUTE LYMPHOBLASTIC LEUKAEMIA (ALL)

Acute lymphoblastic leukaemia is a proliferation of immature lymphoid cells and can be classified into 6 subtypes defined by a battery of lymphocyte markers (Table 7). Of the 4 widely recognised subtypes, common ALL accounts for approximately 65 percent of cases, T ALL 15 to 20 percent, unclassified (no T, B or C ALL markers) 15 percent and B ALL, 2 percent (Mann *et al.*, 1979). In addition, Vogler *et al.*(1978) described a pre-B ALL, and Greaves (1979) found some cases of pre-T ALL.

The variation of cell markers seen in ALL has led to the postulate that these markers indicate various levels of lymphocyte ontogeny at which maturation arrest has occurred

TABLE 7

MARKERS OF ACUTE LYMPHOBLASTIC LEUKAEMIA
(MODIFIED FROM MANN *ET AL.*, 1979)

Type	E	EAC	SIg	CIg	HTLA	HBLA	ALL-Ag	TdT	AP
ALL unclassified	-	-	-	-	-	±	-	±	±
Common ALL	-	-	-	-	-	+	+	+	-
Pre-T-ALL	+		-	-	+	-	±	+	-
T-ALL	+	±	-	-	+	-	-	+	+
Pre-B-ALL	-	-	-	+	-	+	+	±	±
B-ALL	-	±	+	-	-	+	-	-	±

E: rosette formation with sheep erythrocytes
EAC: rosette formation with erythrocyte-IgM antibody-complement complexes
SIg: surface immunoglobulin
CIg: cytoplasmic immunoglobulin
HTLA: human T-lymphocyte associated antigens
HBLA: human B-lymphocyte-associated antigens
ALL-Ag: common ALL antigens
TdT: terminal deoxynucleotidyl transferase activity
AP: acid phosphatase activity

(Greaves and Janossy, 1978; Greaves, 1979). Given that the markers represent the relevant phenotype appropriate for that developmental level (Greaves, 1979), then the early lymphoid lineage and sequence of gene expression can be mapped (Figure 2).

Although an understanding of the ontogeny of lymphocytes will aid in the determination of the basic defects in leukaemogenesis, the immunological refinements to diagnosis have had immediate practical benefits in the determination of prognosis and the selection of treatment (Tsukimoto *et al.*, 1976; Belpomme *et al.*, 1977; Chessells *et al.*, 1977).

B. THE IMMUNOLOGICAL CHARACTERISATION OF THE LYMPHOMAS AND LEUKAEMIAS OF ANIMALS

1. MICE

The majority of *murine leukaemias* and *lymphomas* are of T cell origin, irrespective of the mouse strain or the mode of leukaemia induction. These include most "spontaneous" leukaemias (Shevach *et al.*, 1972b; Old and Stockert, 1977; Haran-Ghera and Peled, 1979), those induced by radiation or the radiation leukaemia virus (Kaplan, 1967; Haran-Ghera and Peled, 1973; Old and Stockert, 1977; Haran-Ghera and Peled, 1979), other murine leukaemia viruses (MuLVs) (Shevach *et al.*, 1972b; Haran-Ghera and Peled, 1973; Dawson *et al.*, 1976) and a range of carcinogens (Shevach *et al.*, 1972b; Haimovich *et al.*, 1977).

Murine B cell neoplasms are less frequent. They include spontaneous leukaemias (Slavin and Strober, 1978) and some of the chemically induced neoplasms. The methylcholanthrene-derived L1210 leukaemia and a 7, 12-dimethylbenz (a) anthracene (DMBA) induced lymphoma (Bergman and Haimovich, (1977) are of B cell origin. However, the cells from another DMBA-induced lymphoma (Haran-Ghera and Peled, 1973) initially thought to be neoplastic B lymphocytes were found to bind immunoglobulin avidly via the Fc receptor (Haimovich *et al.*, 1977). This serves to illustrate the technical difficulties associated with using SIg as a marker.

Some retroviruses induce B cell lymphomas and leukaemias. Shevach *et al.* (1972b) reported a single B cell neoplasm in a C57/BL6 mouse induced by Rauscher MuLV and Reddy *et al.* (1980) using cloned Rauscher MuLV induced B cell lymphomas in 50 NIH/Swiss mice. However, Dawson *et al.* (1976) found this virus caused only T or non-T, non-B cell neoplasms in BALB/c mice. Finally, the Abelson MuLV which is a complex composed of replication defective virus and a helper, Moloney MuLV (Teich and Dexter, 1978), induces a leukaemia that does not involve the thymus, and where the earliest changes are found in the bone marrow (Abelson and Rabstein, 1970a,b). These leukaemias and their cell lines appear to be B lymphocytes early in ontogeny as they express Lyb-2 and Lyt-4 antigens (Rosenberg and Baltimore, 1976). The *in vitro* infection of lymphocytes by Abelson MuLV is dependent on the prior stimulation of cells by B lymphocyte mitogens (Raschke *et al.*, 1975) and some cell lines have intracytoplasmic IgM (Teich and Dexter, 1978; Warner, 1978). Only a few Abelson leukaemia cell lines have SIg (Premkamur *et al.*, 1975) but many non-SIg-bearing cells can be induced to do so with the addition of dimethyl sulphoxide (Weimann, 1976) or theophylline (Warner, 1978) implying that they are of the B cell lineage.

The ontogeny of murine lymphoid cells has been suggested by the extensive study of the expression of antigens and various markers including terminal deoxynucleotidyl transferase (TdT) activity (Katz, 1977; Old and Stockert, 1977; Silverstone *et al.*, 1978; Old, 1981). Silverstone *et al.* (1978) and Warner (1978) delineated the early development of the lymphocyte from the pluripotent stem cell, its commitment to either the T or B cell lineages and its early maturation stages. The placing of each leukaemic cell line into part of the sequence has suggested techniques for the identification of the equivalent normal cell. Moreover, as some B cell lines have detectable TdT activity (Silverstone *et al.*, 1978; Reddy *et al.*, 1980) this has been considered as further evidence for the role of this enzyme in the

generation of immunological diversity (Silverstone *et al.*, 1978).

2. DOGS

Canine lymphomas can be classified either as thymic, multicentric, alimentary or leukaemic according to the criteria of Jarrett *et al.* (1966). From a series of 39 cases including 5 thymic, 32 multicentric and one each of alimentary and leukaemic forms, Onions (1977) concluded that there could be a relationship between the immunological cell type and the pathoanatomical form of the disease. The 19 B cell neoplasms included 18 multicentric and the solitary alimentary lymphoma. The 6 T cell lymphomas consisted of one multicentric and 5 thymic cases, 2 of which were not E rosetting and may have been derived from immature thymocytes (Kelly, 1980). The skin was involved in 2 T cell lymphomas, a feature of certain human T cell neoplasms (Mann *et al.*, 1979). Onions (1977) drew attention to the need for caution in the interpretation of some of his data as SIg determinations were made in less than half the cases. In defining B cell neoplasms, heavy reliance was placed on the presence of C receptors although they are known to occur on some canine thymocytes, T cells and non-B lymphocytes (Kelly, 1980). Had SIg determinations been made in all cases and monoclonality demonstrated, then up to 6 cases with percentages of T and B lymphocytes approximating those found in normal lymph nodes, and 6 cases of "null" and unclassified lymphomas may have been allocated to either the T or B cell category.

Other reports of the immunological characterisation of canine lymphomas have been made although 2 of these are of limited value because of the few markers used. Dutta *et al.* (1978) monitored the presence of SIg and C receptors on blood lymphocytes from a leukaemic dog for 6 weeks and assessed cells from the lymph node of this and another case. The majority of cells bore both markers. Because the pathology of these 2 cases was described inadequately, no relationship between the anatomical distribution of the lesions and their immunological origin could be assessed.

Holmberg *et al.* (1976a) examined lymphoma cells for SIg only and found that the majority of cells in 11 of 16 multicentric lymphomas had detectable SIg. A single case was assessed using both E rosette and SIg markers and neither were found on a significant proportion of cells. In a subsequent report (Holmberg *et al.*, 1977) E rosette formation and the detection of SIg and C receptors were used on 5 multicentric cases, 3 of which were determined as being of B cell origin. In another case, the majority of cells had C receptors only and although the authors imply otherwise, this lymphoma could have originated from the T cell lineage. An important finding of Holmberg *et al.* (1976a, 1977) was that no association could be found between the immunological and cytological cell type of canine lymphoma and they contended that the morphological diversity reflected a maturation block at different stages of lymphocyte development.

3. CATS

Before the immunological characterisation of human and animal lymphomas and leukaemias became a widespread practice, Mackey and Jarrett (1972), predicted that multicentric and thymic *feline lymphomas* would have a T cell origin whereas the alimentary and leukaemic forms would be derived from B lymphocytes. This proposal was based on the colonisation pattern of malignant cells within the lymphoid system by histological examination of 45 spontaneous and virally induced feline lymphomas where the pattern of neoplastic development was constant within each of the 4 anatomical variants. Although feline lymphoma has been studied in other respects, there has been a paucity of complete data to test this hypothesis.

Mackey *et al.* (1975) and Cockerell *et al.* (1976a) found that the majority of cells from thymic lymphomas formed E rosettes. A further 36 cases of thymic, alimentary and multicentric lymphomas were described by Hardy *et al.*(1977) who reported that all thymic and 10 of 18 multicentric lymphomas were derived from T lymphocytes. Three of 4

alimentary lymphomas were of B cell origin. As their data was in summarised form, and they were vague about their criteria for describing 7 lymphomas as being "mixed", few conclusions can be drawn from their results which otherwise support the proposal of Mackey and Jarrett (1972). The suggestion that multicentric lymphomas were likely to be of T cell origin differs from the conclusions of Holmberg *et al.* (1976b). These authors, on the basis of one case, suggested that multicentric lymphomas may be undifferentiated as there were few cells positive for E rosette formation or for the presence of C receptors or SIg. Because they showed that only 35 percent of normal feline thymocytes form E rosettes, an alternative explanation is that this case may have been derived from nonrosetting thymocytes. Despite the incomplete range of markers used, the results of Holmberg *et al.* (1976b) from 10 other cases of thymic and alimentary lymphomas were in general agreement with other workers.

In single case reports, Madewell *et al.* (1979) and Holmberg *et al.* (1976b) failed to detect E rosette formation with, or C receptors on the majority of cells in renal lymphomas. Legendre and Becker (1979) described a skin lymphoma that had not only elevated percentages of E rosette-positive blood lymphocytes but also 44 percent of lymphoma cells formed E rosettes. If, as in man and dog, skin lymphomas are T cell neoplasms, then these cells could be representative of both the E rosette-positive and negative populations of the thymus.

Although exceptions occur, the balance of reports for the feline model indicate that the anatomical distribution of the lesions can be used to predict whether the cells are neoplastic T or B lymphocytes.

4. CATTLE

Bovine lymphoma (synonyms: leukosis, leukaemia, lymphosarcoma) can be divided into 2 categories on epidemiological grounds. The first, *enzootic lymphoma* is induced by the bovine leukaemia virus (BLV) (Theilen *et al.*,

1964; Ressang *et al.*, 1974; Burny *et al.*, 1978; Ferrer, 1980) and is found in geographical and herd aggregations in cattle older than 3 years. The second, *sporadic lymphoma* is of unknown aetiology (Olson, 1974; Callahan *et al.*, 1976; Ressang, 1976; Straub and Weiland, 1977; Burny *et al.*, 1978; Kettmann *et al.*, 1978) is of low prevalence and is found in animals under 3 years of age. This latter form of the disease can be further subdivided into a juvenile or calf form, a thymic form and a cutaneous or skin form (Grimshaw *et al.*, 1979). Adult cattle infected with BLV can alternatively develop a persistent lymphocytosis (Ferrer, 1980). Lymphocyte markers have been detected on cells from the blood of cattle with persistent lymphocytosis, and on blood lymphocytes and neoplastic cells of lymph nodes from both sporadic and enzootic forms of bovine lymphoma.

(i) Persistent Lymphocytosis

Early workers suggested that persistent lymphocytosis was a preneoplastic stage of enzootic lymphoma (Bendixen, 1965). As most authors now consider persistent lymphocytosis to be a separate manifestation of infection by BLV (Ferrer *et al.*, 1979), only a brief consideration of the cell marker studies of this response will be made here.

All reports agree that B cells are responsible for most of the increased numbers of circulating lymphocytes (Garcia de Lima and Mitscherlich, 1973; Muscoplat *et al.*, 1974a; Weiland and Straub, 1975; Kenyon and Piper, 1977a,b; Paul *et al.*, 1977a,b; Takashima *et al.*, 1977; Atluru *et al.*, 1979; Paul *et al.*, 1979). Opinions differ about whether T cells increase (Garcia de Lima and Mitscherlich, 1973) do not vary (Takashima *et al.*, 1977) or decrease (Paul *et al.*, 1979) and such differences may be a reflection of the lack of an adequate T cell marker in cattle (Paul *et al.*, 1979). In persistent lymphocytosis Kenyon and Piper (1977b) showed that the B lymphocyte expansion was not necessarily due to BLV-infected cells but may represent an immune response to infection.

Although Muscoplat *et al.* (1974a) suggested that it was necessary to demonstrate endogenous SIg synthesis of

cells to prove the B cell nature of lymphocytosis, no attempts have been made by others to do so, nor are cells routinely pretreated to remove labile SIg (Horwitz and Lobo, 1975; Lobo *et al.*, 1975). However, Kumar *et al.*(1978) reported unpublished results suggesting that unlike human lymphocytes, SIg was not readily dissociable from significant proportion of cattle lymphocytes. This question and whether persistent lymphocytosis is a monoclonal or polyclonal expansion of B lymphocytes have yet to be addressed.

(ii) Enzootic Lymphoma

Enzootic lymphoma is a neoplasm of B lymphocytes. Although only 3 of 6 Japanese cases of Onuma *et al.*(1979) had elevated percentages of SIg-positive cells, 9 of 10 cases in the American series of Onuma *et al.* (1978) had over 50 percent SIg-bearing cells. All of the 8 cases of Takashima *et al.*(1977) had elevated percentages of SIg-positive cells and 5 of these also had increased values for C receptor-bearing cells. In the latter cases, C receptor values were less than those for SIg-bearing cells, a situation also found in normal cattle blood (Paul *et al.*, 1979). The major criticism of all of these studies is that the monoclonality of SIg has not been demonstrated. Indeed, only an early study by Tsuyguchi *et al.* (1973) has made any attempt to analyse the nature of SIg on bovine lymphoma cells. They were unable to come to any conclusions due to the limitations of the ^{125}I labelled antibody technique and the artifacts demonstrated by nonspecific adsorption of serum IgM. However, they did find L chain activity and gammaglobulin determinants, but could not specify whether μ or γ chains were present. Blood lymphocytes from the lymphoma case appeared to have a preponderance of μ chain determinants.

In general, elevated B lymphocyte percentages occur if there are changes in the relative proportion of T and B cells in blood from cattle with enzootic lymphoma (Wilkie *et al.*, 1979; Onuma *et al.*, 1979). However, in some cases the percentages of B cells may be normal or depressed (Takashima *et al.*, 1977; Onuma *et al.*, 1978). Kumar *et al.*

(1978) reported 3 cases in which the percentages of SIg-positive cells were normal but had elevated percentages of Fc receptor-bearing cells. This indicated the possibility of a proliferation of a Fc receptor-positive SIg-negative subset of blood lymphocytes.

(iii) Sporadic Lymphoma

The percentages of cells bearing either T or B markers are usually reduced in blood and lymphoid neoplasms from animals affected with sporadic bovine lymphoma. Muscoplat *et al.* (1974b) could demonstrate SIg on less than one percent of blood lymphocytes from 2 calves with an acute lymphocytic leukaemia. Blood E rosetting, C receptor and SIg-bearing cells were reduced in 4 cases of calf and 2 cases of thymic lymphoma (Takashima *et al.*,1977). The same extent of reduction was found in these cases when lymph nodes or thymic neoplasms were examined. Similarly, the percentages of SIg-positive cells were reduced in both blood and involved tissues of 5 calf, 2 thymic and 2 skin cases of Onuma *et al.* (1978) and the case of calf and another of skin lymphoma from Japan (Onuma *et al.*, 1979).

5. SHEEP

The 2 reports describing the immunological characterisation of *ovine lymphoma* have been cases caused by experimental infection with BLV. Cell marker studies have not been done with sporadic ovine lymphomas.

Nemeth *et al.* (1979) described a single case that involved the peripheral lymphoid tissue and the thymus. The cells from both blood and lymph nodes were positive for both acid phosphatase and ANAE. Although no surface markers were employed, the involvement of the thymus and cytochemical results indicated to the authors that this was a T cell neoplasm. Takashima and Olson (1980) found in excess of 70 percent SIg-bearing cells in lymph nodes from 4 cases of lymphoma and determined elevated B lymphocyte percentages in the blood of these and 2 others, irrespective of whether they were leukaemic or not. More than 80 percent of cells retained their SIg after overnight incubation in

serum-free medium.

C. SUMMARY AND CONCLUSION

The recognition of T and B lymphocytes by their cell surface phenotype has advanced the understanding of the normal lymphoid system. However, the classical definition of T and B lymphocytes has become less distinct with subsets being identified that share common markers. Marker techniques have been refined and with the appreciation of technical difficulties and the application of standard approaches, reproducible results can now be achieved.

The E rosetting technique has been valuable in identifying human T lymphocytes. Similar techniques in domestic species have not been as successful. For humans, monoclonal antibodies now provide the most accurate means of identifying T cells to determine their level of differentiation and their functional role. Except in the mouse and man, anti-T cell antisera for other species do not approach the same level of refinement.

SIg is considered the hallmark of the B lymphocyte but its detection has been fraught with technical difficulties. Other markers often found on B cells such as Fc and C receptors can not alone define the B lymphocyte. In combination, they do identify subsets which may be important in defining specific types of lymphomas and leukaemias.

Finally, apart from specific uses, the cytochemical identification of lymphoid subpopulations is limited and the application of successful techniques to other species must be carefully assessed.

The immunological characterisation of lymphomas and leukaemias by these techniques has enhanced the understanding of their pathogenesis and has revealed the possible pathways of ontogenesis for the normal lymphocyte. The literature on the cell markers of human lymphomas and leukaemias is comprehensive. Diagnosis, classification and in some cases, treatment has improved from such studies. On the other hand, the lymphomas and leukaemias of other species have not been as completely investigated. Despite the

limitations of documentation some general conclusions can be made. In the mouse most lymphomas are of T cell origin. However, the intriguing Abelson MuLV-induced disease is a B cell lymphoma. The extensive knowledge of the murine lymphoid antigens has led to the delineation of the ontogeny of the lymphocyte in this species.

For dogs and cats a relationship appears to exist between the immunological cell type and the anatomical classification of the disease (Mackey and Jarrett, 1972; Onions, 1977). This may reflect the ecotaxic behaviour of the normal progenitor cell. However, marked differences are apparent between the 2 species. In dogs, multicentric lymphomas are B cell in origin, whereas in cats they are composed of neoplastic T lymphocytes.

Enzootic bovine lymphoma induced by BLV is a B cell neoplasm, and this appears to be the case for some of the experimentally BLV-induced lymphomas in sheep. However, one report indicates that ovine T cell lymphomas may also be caused by BLV. Sporadic bovine lymphoma on the other hand is unique, as all neoplasms so far reported are derived from cells that do not bear any of the usual lymphocyte markers.

Therefore cell surface markers have provided for a more rational approach to the study of the malignant lymphoid diseases, and in the case of humans have improved the classification of lymphomas and leukaemias. In research, the target cells for lymphomagenesis can be identified and the confounding effects of including neoplasms of dissimilar origins can be avoided. Finally, and most importantly in relation to this study of sporadic ovine lymphoma, a specific disease can be more closely matched in 2 species for comparative purposes.

CHAPTER 2
THE IDENTIFICATION OF OVINE T AND B LYMPHOCYTES

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CHAPTER 2

THE IDENTIFICATION OF OVINE T AND B LYMPHOCYTES

INTRODUCTION

Before lymphomas and leukaemias can be characterised as being either of T or B cell origin, it is essential to be able to identify these lymphocyte subpopulations. There have been few cell marker studies of both sheep T and B lymphocytes (Bohs *et al.*, 1976; Outteridge *et al.*, 1981a,b) and consequently there is a lack of confirmatory data for this species.

This chapter describes the application of standard techniques by which T lymphocytes were detected by anti-T cell antisera and B cells by the presence of SIg, Fc and C receptors. In addition, the usefulness of ANAE activity as a T cell marker was explored as reports suggest that this enzyme is a marker for human T lymphocytes (Knowles *et al.*, 1978). The abilities of these methods to identify T and B cells were assessed using lymphocytes from the blood and mesenteric lymph nodes of normal sheep.

MATERIALS AND METHODS

Sheep

The sheep used were clinically normal rams, ewes or ram castrates aged 8 months and older.

Harvesting of Blood Lymphocytes

Twenty ml of heparinised jugular blood were centrifuged at 1,500 g for 15 min. The buffy coat was removed, resuspended to 4 ml in phosphate buffered saline (PBS), pH 7.0 (Appendix), layered over 3 ml of Ficoll-sodium metrizoate (Lymphoprep, Nyegaard and Co.) of density 1.077 gm per ml and centrifuged at 800 g for 25 min (Boyum, 1968). The lymphocytes at the PBS/Lymphoprep interface were collected and washed twice by centrifuging at 700 g for 10 min in RPMI 1640 medium (Flow Laboratories) containing antibiotics (Appendix). The cells were incubated in plastic petri dishes at 37°C for 1 hour in an atmosphere of 5 percent CO_2-in-air to remove contaminating

monocytes (Horwitz and Garrett, 1977) and to free the lymphocytes of adherent immunoglobulin (Kurnick and Grey, 1975; Lobo *et al.*, 1975).

The nonadherent cells were harvested and resuspended to a concentration of approximately 20×10^6 per ml in RPMI 1640. At least 95 percent of the cells were lymphocytes and more than 95 percent of these were viable as estimated by trypan blue exclusion (Appendix).

Preparation of Mesenteric Lymph Node Cells

The mesenteric lymph nodes of 4 sheep were diced in RPMI 1640, pressed through a 40-mesh stainless steel sieve and the cells were washed in RPMI 1640 by centrifugation at 200 g for 10 min.

After resuspension in medium, the cells were then incubated in plastic petri dishes at 37°C for 1 hour before the nonadherent cells were harvested and resuspended in RPMI 1640 to approximately 20×10^6 per ml.

Nylon Wool Fractionation of Lymphocytes

Lymphocytes were fractionated on nylon wool columns by the method of Brown and Greaves (1974). Three hundred mg of nylon wool (Leukopak, Fenwal) were packed into the barrel of a disposable 10 ml plastic syringe and washed with 100 ml of 0.2 N HCl. A further wash with 100 ml of distilled water was followed by RPMI 1640 containing 20 percent foetal bovine serum (FBS, Laboratory Services) (RPMI 1640-FBS) until the eluting medium remained at pH 7.0 as judged by the colour of the neutral red indicator. The column was then saturated with RPMI 1640-FBS and incubated at 37°C for 30 min. Approximately 80×10^6 lymphocytes in 5 ml of RPMI 1640-FBS were loaded onto the column. After incubation at 37°C for 30 min the cells were eluted at this temperature in 20 ml of RPMI-FBS over a period of 20 min. Cells retained by the nylon wool were freed by teasing this material in RPMI 1640. Both the eluted and the freed lymphocytes were pelleted by centrifugation and resuspended to approximately 20×10^6 cells per ml in RPMI 1640 and were examined for the presence of membrane markers and ANAE activity.

Percoll Fractionation of Lymphocytes

Percoll (Pharmacia) of density 1.130 gm per ml was used as described by Gutierrez *et al.* (1979). Nine parts of Percoll were mixed with 1 part of tenfold concentrated PBS. This solution, designated 100 percent Percoll, was further diluted with normal PBS to give concentrations of 70, 60, 50 and 40 percent Percoll.

Approximately 80 x 10^6 blood lymphocytes, harvested as described above were centrifuged in RPMI 1640 at 200 g for 10 min. The supernantant was discarded and 2 ml volumes of each of the Percoll concentrations were layered over the cell pellet to form a discontinuous gradient beginning with 100 percent Percoll and continuing in decreasing concentrations. The gradient was then centrifuged at 1750 g for 10 min and the cells displaced to each concentration interface were collected. Those removed from the interface between the 40 percent and 50 percent Percoll solutions were designated "Fraction 1", those between the 50 percent and 60 percent solutions "Fraction 2", and those between the 60 and 70 percent solutions "Fraction 3". The cells in each of the fractions were washed twice in RPMI 1640 by centrifugation at 200 g for 10 min and were examined for membrane markers and ANAE activity.

Production of Antisera

(i) *Anti-T cell antisera*

The thymus from a caesarian-delivered lamb was removed aseptically and washed in Eagles' minimum essential medium (MEM, Wellcome) (Appendix). The organ was diced and pressed through a 40-mesh stainless steel tissue sieve. The cells were washed once in MEM by centrifuging at 700 g for 10 min and counted. Rabbits were each injected intraperitoneally with 10^8 thymus cells in 10 ml of MEM. The remaining cells were stored at - 70°C in MEM supplemented to 20 percent with FBS (MEM-FBS) and 10 percent dimethyl sulphoxide. Fourteen days later these were thawed and a second intraperitoneal injection of approximately 10^8 cells was made. The rabbits were anaesthetised with ether and exsanguinated 18 days after the second injection and the sera collected and stored

at $-20^{o}C$. After being inactivated at $56^{o}C$ for 30 min, each antiserum was absorbed twice for 30 min each with washed sheep erythrocytes (100 µl packed cells per ml of antiserum), twice for 30 min with sheep liver powder (Appendix) (100 mg of powder per ml of antiserum) and 4 times each for 30 min with lamb bone marrow (Appendix) (approximately 1 part of bone marrow to 1 part of antiserum); all absorptions were done at room temperature. These absorptions failed to remove completely the nonspecific activity of the antisera when it was tested against sheep lymphocytes. However when the antisera were diluted 1:7 with PBS (Johnson *et al.*, 1978) this activity was lost. The antisera were preserved with NaN_3 added to a final concentration of 0.01 percent.

(ii) Anti-ovine gamma globulin antisera

Ovine gamma globulin (OGG) was prepared from 40 ml of sheep serum by the method of Herbert (1976). Forty ml of a 70 percent saturated ammonium sulphate solution were added to the serum and mixed at room temperature for 2 hr. The mixture was centrifuged at 2,000 g for 15 min. The supernatant was then removed and the precipitate dissolved in distilled water to a volume of 40 ml. The addition of ammonium sulphate and subsequent centrifugation were repeated twice. After the final centrifugation the precipitate was resuspended in approximately 2 ml of distilled water and dialysed against 0.15 M NaCl at $4^{o}C$ for 20 hr, with a change of saline after 16 hours. The protein concentration was measured at 280 nm and the homogeneity of the preparation was assessed by cellulose acetate electrophoresis and immunoelectrophoresis against sheep serum.

Rabbits were each injected subcutaneously with an emulsion containing 70 mg of OGG in 1 ml of saline and 1 ml of Freund's complete adjuvant (Difco). Twenty one days later they received further subcutaneous injections of 70 mg of OGG in Freund's incomplete adjuvant (Difco). The animals were bled from their marginal ear veins 14 days after the second injection and the sera collected. After being heat inactivated the sera were absorbed with sheep erythrocytes

and liver powder as described above. The gamma globulin fractions of these antisera were obtained by ammonium sulphate precipitation and were conjugated with fluorescein isothiocyanate.

Conjugation of Antisera with Fluorescein Isothiocyanate (FITC)

The technique of Johnson *et al.*(1978) was followed. The immune gamma globulin was diluted to 10 mg per ml by the addition of 10 percent (final concentration) bicarbonate buffer (Appendix) and 0.15 M NaCl. Fifty μg of FITC (Isomer 1, BBL) was added for each mg of gamma globulin and the solution stirred for 1 hr at room temperature. Unconjugated FITC was removed on a Sephadex G-25 (Sigma) column (Appendix). NaN_3 was added to the FITC-gamma globulin conjugate at a final concentration of 0.01 percent.

Detection of Membrane Markers

(i) *Detection of T cell-specific antigens*

One hundred μl of anti-T cell antisera were added to approximately 2 x 10^6 lymphocytes in 100 μl of RPMI 1640 and incubated at 4^oC for 30 min. The cells were then washed 3 times in 2 ml amounts of PBS containing 0.03 M NaN_3 (PBS-NaN_3) by centrifuging at 200 g for 5 min. After the final centrifugation the lymphocytes were resuspended in 100 μl PBS-NaN_3, 25 μl of FITC-conjugated anti-rabbit IgG (Wellcome) were added and the cells were re-incubated at 4^oC for 30 min. The cells were then washed 3 times in PBS-NaN_3 and finally resuspended in a drop of 50 percent glycerol in PBS-NaN_3. They were examined under a Reichart Immunopan fluorescent microscope fitted with a darkfield condenser and a 500 nm wavelength exciter filter. Two hundred cells were examined from each preparation. Controls were lymphocytes incubated with 25 μl of FITC-conjugated anti-rabbit IgG.

(iii) *Detection of Complement Receptors*

Receptors for complement were detected by the methods of Kajdacsy-Balla amd Mendes (1976) and Sher and McIntyre (1977). Four hundred μg of zymosan A (Sigma) were added to each 1 ml of MEM-FBS. The suspension was sonicated to

(ii) *Detection of Surface Immunoglobulin*

Surface immunoglobulin was detected by the method of Cahill *et al.* (1978). One hundred *u*l of FITC-conjugated anti-OGG were added to 100 *u*l of a lymphocyte suspension containing approximately 2 x 10^6 cells. This mixture was incubated at 4^oC for 30 min, washed 3 times in PBS-NaN_3 by centrifugation at 200 g, mounted and examined as described above.

disperse the zymosan particles and then incubated with an equal volume of undiluted guinea pig serum (Wellcome) at 37°C for 1 hr. The complement-coated (ZC) particles were washed 3 times in MEM-FBS at 200 g for 5 min, resuspended in MEM-FBS to their original volume and sonicated.

Two hundred µl of ZC particles were incubated at 37°C for 1 hr with 200 µl of RPMI 1640 containing approximately 4 x 10^{6} lymphocytes. The cells were then centrifuged for 5 min and the pellet resuspended in 100 µl of MEM-FBS. One drop of Giemsa stain was added to aid detection of the lymphocytes. Lymphocytes with 3 or more adherent particles were considered positive for C receptors; 200 cells were counted for each preparation. Control preparations consisted of a similar concentration of untreated zymosan particles incubated with sheep lymphocytes.

(iv) *Detection of Fc receptors*

The method of Sjoberg and Inganas (1979) for the detection of receptors on human lymphocytes was followed. Latex beads (Sigma), approximately 0.8 µ diameter, were suspended at a concentration of 0.4 percent in 2 percent ovine gamma globulin (Fraction II, Hyland Laboratories) in PBS and incubated at 37°C for 24 hr. The IgG-coated latex (IgG-latex) particles were then washed 3 times in PBS by centrifugation at 800 g for 10 min and resuspended to 0.2 percent in PBS containing 2.5 percent bovine serum albumin and 0.02 percent NaN_3. The suspension was sonicated to disrupt aggregates.

Approximately 2 x 10^{6} lymphocytes in 100 µl of RPMI 1640 were added to an equal volume of the IgG-latex suspension and centrifuged at 200 g for 5 min. The pellet was incubated at room temperature for 15 min, resuspended in a drop of RPMI 1640 and examined. A lymphocyte was considered positive for Fc receptors if it had 3 or more adherent particles; 200 cells were counted for each estimation. Control determinations were made by incubating lymphocytes with 100 µl of 0.2 percent suspension of uncoated latex

beads.

Determination of Acid α Naphthyl Acetate Esterase (ANAE) Activity

ANAE activity was determined by the technique of Kulenkampff *et al.* (1977).

(i) *Preparation of the ANAE stain*

One gm of pararosaniline hydrochloride (Sigma) was dissolved by warming in 20 ml of distilled water and 5 ml of 35N HCl; this solution was filtered and stored at $4^{\circ}C$ in the dark. Hexazotised pararosaniline was prepared by adding 2 ml of freshly made 4 percent $NaNO_2$ to 2 ml of the pararosaniline hydrochloride solution and mixed for 1 min. Forty ml of 0.7 M phosphate buffer, pH 5.8 (Appendix) were added and the solution adjusted to pH 5.8 with 10 M NaOH. Finally, 10 mg of α naphthyl acetate (Sigma) was dissolved in 400 μl of acetone and added to the mixture.

(ii) *Staining of lymphocytes*

Lymphocytes were resuspended in 200 μl of FBS and cytocentrifuged onto glass slides at 1,500 rpm for 10 min. These preparations were fixed wet in 2.5 percent glutaraldehyde in PBS at $4^{\circ}C$ for 10 min and washed in water. The slides were incubated in the ANAE stain for 3 hr at $37^{\circ}C$. After washing in water and counterstaining with 0.2 percent light green in 0.2 percent acetic acid, the preparations were dehydrated in a series of alcohols, cleared in xylol and mounted in D.P.X. mountant (BDH).

(iii) *Preparation and staining of lymphoid tissues*

Pieces of thymuses, spleens, tonsils and lymph nodes collected from lambs at a slaughterhouse were fixed in formol-sucrose (Appendix) at $4^{\circ}C$ for 24 hr before being transferred to Holt's solution (Appendix) to be infiltrated at $4^{\circ}C$ for a further 24 hr. Frozen sections of these tissues, cut to approximately 8 μ thickness, were air-dried and placed in the staining medium within 30 min, stained, counterstained, dehydrated and mounted as before.

RESULTS

A. THE EVALUATION OF LYMPHOCYTE MARKER REAGENTS USING LYMPHOCYTES FROM NORMAL SHEEP

(i) *Blood Lymphocytes*

The mean percentages and the ranges of values of lymphocytes from blood expressing T cell antigens, SIg, C and Fc receptors are shown in Table 8. T cell antigens were detected on 68.7 percent of the cells while 25.3 percent bore SIg. ZC rosettes formed with 14.1 percent of the lymphocytes and IgG-latex particles adhered to 16.7 percent of cells.

Less than 1 percent of cells reacted in any of the control lymphocyte preparations.

(ii) *Blood Lymphocytes Fractionated by Nylon Wool*

Following nylon wool fractionation of blood lymphocytes the nonretained cells were enriched for T lymphocytes (94.4 percent) while 2.1 percent were SIg-positive. The retained cell population consisted of 53.9 percent T cells and 45.5 percent B cells (Table 9).

(iii) *Mesenteric Lymph Node Lymphocytes*

Lymph nodes from 4 sheep had a mean of 69.4 percent T and 27.3 percent B lymphocytes (Table 10).

B. THE EVALUATION OF ANAE STAINING USING LYMPHOCYTES FROM NORMAL SHEEP

(i) *Blood Lymphocytes*

A mean of 66.4 percent of blood lymphocytes showed either a single discrete paranuclear spot or several smaller granules of ANAE activity distributed throughout the cytoplasm of the cell (Figure 3; Table 11). Although a mean of 68.0 percent of lymphocytes from the same populations were T cells as judged by the detection of T cell antigens, regression analysis of the individual results revealed no relationship between the percentage values for ANAE-positive and T cells, as only 4.8 percent (r^2 = 0.484) of the variation was common to both cell subpopulations.

(iii) *Blood Lymphocytes Fractionated by Nylon Wool*

To further assess the relationship, if any, between ANAE activity and T lymphocytes identified by anti-T cell antisera, T cell-enriched populations were prepared by nylon wool fractionation of blood lymphocytes and stained

TABLE 8

THE PERCENTAGES OF NORMAL SHEEP BLOOD LYMPHOCYTES BEARING VARIOUS SURFACE MARKERS

	T	SIg	C	Fc
Mean[(a)]	68.7± 8.1	25.3± 9.2	14.1± 3.6	16.7± 3.1
n	21	29	8	10
Range	53.4-84.8	11.2-47.6	7.5-18.8	12.0-22.2

T: T lymphocyte antigens
SIg: surface immunoglobulin
C: C receptor
Fc: Fc receptor
n: number of determinations
(a) mean ± standard deviation

TABLE 9

NYLON WOOL FRACTIONATION OF NORMAL SHEEP LYMPHOCYTES: THE PERCENTAGES OF UNFRACTIONATED AND FRACTIONATED LYMPHOCYTES EXPRESSING EITHER T CELL ANTIGENS OR SURFACE IMMUNOGLOBULIN

	Before Fractionation		Nonretained cells		Retained cells	
	T	SIg	T	SIg	T	SIg
Mean[(a)]	74.0±10.4	24.1± 7.6	94.4±3.2	2.1±1.7	53.9± 7.0	45.5±10.2
n	6	7	6	7	3	7
Range	57.8-84.8	12.0-32.1	88.9-97.3	1.0-5.5	46.4-60.3	35.6-67.4

T : T lymphocyte antigens
SIg : surface immunoglobulin
n : number of determinations
(a) mean ± standard deviation

TABLE 10

THE PERCENTAGES OF NORMAL SHEEP MESENTERIC LYMPH NODE LYMPHOCYTES EXPRESSING EITHER T CELL ANTIGENS OR SURFACE IMMUNOGLOBULIN

	T	SIg
Mean[(a)]	69.4± 3.4	27.3±10.2
n	4	4
Range	66.1-73.8	12.4-35.3

T: T lymphocyte antigens
SIg: surface immunoglobulin
n: number of determinations
(a) Mean ± standard deviation

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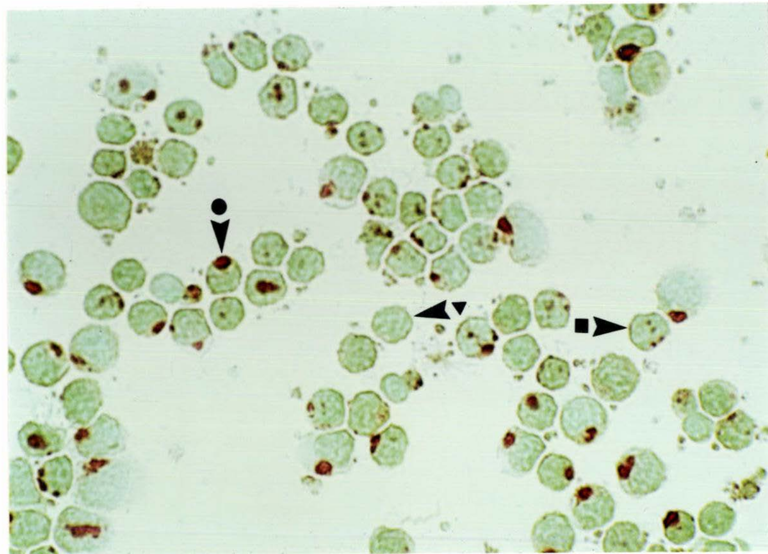
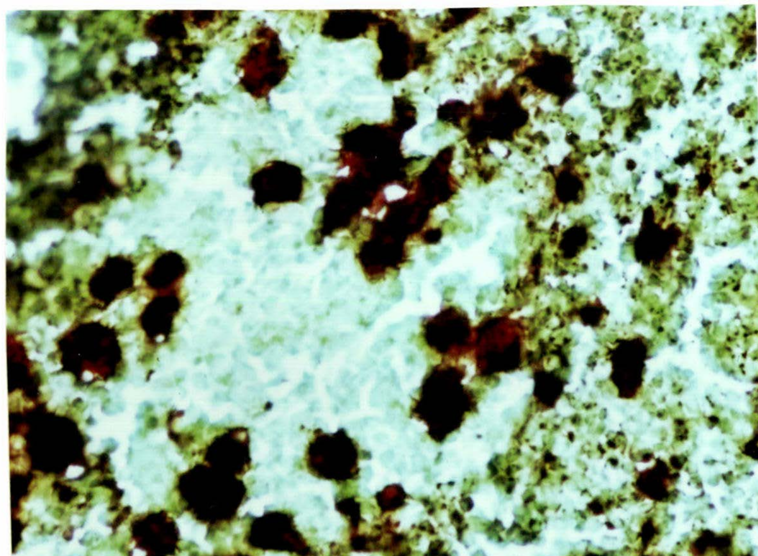
FIGURE 3: Normal sheep blood lymphocytes with ANAE-positive cells showing focal (●) or granular (■) activity, and ANAE-negative cells (▼). (Magnification 420 times)

FIGURE 4: Lymphoid follicle of a mesenteric lymph node from a sheep. The follicular centre cells are predominantly ANAE-negative and are surrounded by a cuff of cells that are ANAE-positive. (Magnification 150 times)

FIGURE 5: The paracortex of a mesenteric lymph node from a sheep. The paracortex shows a mixture of ANAE-positive (●) and ANAE-negative (▼) cells. (Magnification 660 times)





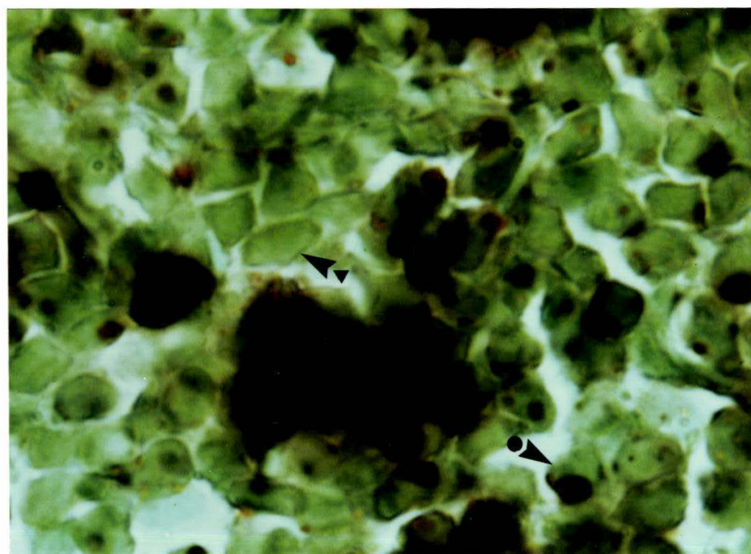


TABLE 11

THE PERCENTAGES OF NORMAL SHEEP BLOOD LYMPHOCYTES EXPRESSING T CELL ANTIGENS AND HAVING ALPHA NAPHTHYL ACETATE ESTERASE ACTIVITY

	T	ANAE
	77.8	61.0
	84.3	70.0
	57.8	72.3
	61.4	74.2
	60.0	67.2
	68.0	58.2
	73.8	54.1
	70.5	67.0
	64.8	66.4
	60.2	79.5
	75.1	62.8
	62.1	64.3
Mean[(a)]	68.0±8.41	66.4±7.1
n	12	12
Range	84.3-57.8	79.5-54.1
	r^2=0.0484	
	t=0.7132 ns	

T: T lymphocyte antigens
ANAE: alpha naphthyl acetate esterase
n: number of determinations
ns: not significant
(a) mean ± standard deviation

for ANAE activity. Table 12 shows that while the nonretained fraction is enriched for T cells (mean 94.7 percent) and depleted of B cells (mean 1.7 percent) a marked disparity occurs when the T cell value is compared with the ANAE-positive value (mean 43.2 percent). The ANAE-positive cells in the T cell-enriched fraction generally showed small granules of activity; cells bearing single spots of enzyme activity were found frequently in the adherent population.

A single fractionation of human blood lymphocytes was used to evaluate the behaviour of ANAE-positive cells in a nylon wool column. The percentages of ANAE-positive cells recovered from both the retained and nonretained fractions were in agreement with the expected results for human T cells (Brown and Greaves, 1974) although actual T lymphocyte numbers were not determined. The majority of ANAE-positive human lymphocytes in the nylon wool eluate had a single spot of activity in the cytoplasm. In contrast, most of the sheep lymphocytes in the eluate displayed granular ANAE activity in the cytoplasm.

(iii) *Blood Lymphocytes Fractionated on Percoll Gradients*

The distribution of T and B lymphocytes across a Percoll discontinuous density gradient was determined using anti-T antisera and SIg staining in conjunction with staining for ANAE activity. The values obtained for T and B cells were subjected to a Student's t test and are considered separately (Table 13). In Fraction 1 there was significant enrichment of B lymphocytes when compared to unfractionated cells ($t = 4.029$, $p = 0.01$); and in Fraction 3, T lymphocyte enrichment occurred ($t = 3.149$, $p = 0.05$). However, only about 9 percent of the total cells were recovered in each of these 2 fractions. The majority of cells were found in Fraction 2, where the proportion of the 2 subpopulations did not differ significantly from that found in unfractionated lymphocytes. Although enrichment for B cells occurred in Fraction 1 and for T cells in Fraction 3, these fractions were not significantly depleted of the other lymphocyte subpopulations.

The pairs of values for T and ANAE-positive cells from

TABLE 12

NYLON WOOL FRACTIONATION OF NORMAL SHEEP LYMPHOCYTES
THE PERCENTAGES OF UNFRACTIONATED AND FRACTIONATED
LYMPHOCYTES EXPRESSING T CELL ANTIGENS AND HAVING
ALPHA NAPHTHYL ACETATE ESTERASE ACTIVITY

	Before Fractionation		Nonretained cells		Retained cells	
	T	ANAE	T	ANAE	T	ANAE
Mean[a]	73.3±13.8	68.6± 5.1	94.7± 2.2	43.2± 9.3	46.4	69.1
n	3	4	3	4	1	1
Range	84.3-57.8	72.3-61.0	97.0-92.6	52.2-30.3		

T : T lymphocyte antigens
ANAE : alpha naphthyl acetate esterase activity
n : number of determinations
(a) mean ± standard deviation

TABLE 13

PERCOLL DISCONTINUOUS GRADIENT FRACTIONATION OF NORMAL SHEEP LYMPHOCYTES : THE PERCENTAGES OF UNFRACTIONATED AND FRACTIONATED LYMPHOCYTES EXPRESSING EITHER T CELL ANTIGENS OR SURFACE IMMUNOGLOBULIN

	Before Fractionation		Fraction 1		Fraction 2		Fraction 3	
	T	SIg	T	SIg	T	SIg	T	SIg
Mean[(a)]	65.1± 5.8	31.9±11.6	59.3± 4.5	56.9± 4.5	60.4±9.4	40.0±20.3	81.0± 9.7	72.2±19.2
n	5	5	4	4	5	5	5	5
Range	60.0-73.8	14.0-43.0	47.0-69.4	53.0-63.0	49.5-71.8	14.0-66.4	67.1-90.5	2.0-47.0
Yield[(b)]			9.2		44.0		9.3	
Range of Yield			2.1-24.4		21.9-84.7		2.5-20.0	
			t=1.220	t=4.029	t=0.9522	t=0.769	t=3.149	t=0.9659
			ns	p=0.01	ns	ns	p=0.05	ns

T: T lymphocyte antigens

SIg: surface immunoglobulin

n: number of determinations

ns: no significant

(a) Mean ± standard deviation

(b) Percentage of total lymphocytes recovered

the Percoll gradient fractionation were evaluated statistically by regression analysis (Table 14).

a) Unfractionated lymphocytes

The percentages of ANAE-positive and T lymphocytes were determined before each of 5 Percoll fractionations. When the 5 pairs of values obtained were analysed a negative regression was found. However, these values were part of the larger analysis of unfractionated lymphocytes (see (i) above) where no relationship could be found, hence this negative regression is likely to be a chance effect of sampling.

b) Lymphocytes from Fraction 1

Four pairs of values subjected to the same type of analysis did not show any significant relationship between ANAE-positive cells and T lymphocytes.

c) Lymphocytes from Fraction 2

When the results of Fraction 2 from 5 experiments were examined, a significant positive relationship between T cell and ANAE values was found. Eighty three percent of the variation of the ANAE values was common with that of the T values ($t = 3.855$; $0.1 < p < 0.5$; 3 degrees of freedom). Using the derived equation, it could be predicted that 31.4 percent of T lymphocytes would not have ANAE activity. Because of the small number of samples confidence levels were not calculated.

d) Lymphocytes from Fraction 3

No significant relationship was found between T lymphocytes and ANAE-positive cells in Fraction 3 from 5 experiments.

(iv) *ANAE Activity of Cells in Tissue Sections*

Sections from thymuses, spleens tonsils and popliteal and mesenteric lymph nodes were examined to determine the distribution of ANAE-positive and ANAE-negative cells.

The follicular centre cells of lymphoid follicles of tonsil, spleen and lymph nodes (Figure 4) contained predominantly ANAE-negative cells surrounded by compact cuffs of cells that were mostly ANAE-positive. The

TABLE 14

PERCOLL DISCONTINUOUS DENSITY GRADIENT FRACTIONATION OF NORMAL SHEEP LYMPHOCYTES: THE PERCENTAGES OF UNFRACTIONATED AND FRACTIONATED LYMPHOCYTES BEARING T CELL ANTIGENS AND HAVING ALPHA NAPHTHYL ACETATE ESTERASE ACTIVITY

	Before Fractionation		Fraction 1		Fraction 2		Fraction 3	
	T	ANAE	T	ANAE	T	ANAE	T	ANAE
	62.1	64.3	69.4	54.2	49.5	26.0	67.1	47.4
	73.8	54.1	55.2	63.8	71.8	66.5	90.5	44.8
	68.0	58.2	ND	ND	62.5	51.5	75.0	50.7
	60.0	67.2	47.0	63.5	52.0	34.1	84.5	30.8
	61.4	74.2	65.7	67.4	66.0	42.0	87.8	52.2
Mean[a]	65.1±5.8	63.6±7.8	59.3±10.2	62.2±5.6	60.4±9.4	44.0±15.7	81.0±9.7	44.2±8.6
r^2	0.7663		0.1657		0.8320		0.4471	
t	3.136 p>0.05		0.6302 ns		3.8546 <0.05 P>0.01		0.3747 ns	

T : T lymphocyte antigens

ANAE : alpha naphthyl acetate esterase activity

ND : not done

ns : not significant

(a) mean ± standard deviation

paracortex and medullary cords of the lymph nodes and the splenic arteriolar sheaths showed both ANAE-positive and ANAE-negative cells, (Figure 5) the former being the more numerous but not preferentially distributed to the thymus-dependent areas. There was no evidence of the specific compartmentalisation of cells bearing either a single spot or multiple granules of ANAE activity.

Sections from the thymus of a 6 months-old-lamb showed that most of the cells in both the cortex and the medulla had either a single spot or a granular distribution of ANAE activity.

DISCUSSION

The reagents and techniques used in these experiments allowed the identification of sheep T and B lymphocytes. Although it was not shown directly that T cell antigen-bearing and SIg-positive cells were mutually exclusive subpopulations, in most instances the total of the 2 values were less than 100 percent of the total lymphocytes, implying a lack of overlap between these 2 subpopulations. Further evidence to support the specificity of these immunological reagents came from the examination of nylon wool fractionated lymphocytes. The nonretained cells from the nylon wool column contained few SIg-bearing lymphocytes while over 94 percent of these cells were T cells. Conversely, the retained fraction was enriched for B cells and relatively depleted of T cells. Although double marker, depletion and functional studies may have further confirmed the specificity of these reagents, the limited range of markers available precluded such verification. Furthermore, Outteridge *et al.* (1981a) found that double rosetting interfered with the count of each marker and felt that physical separation data were more valid than those derived by double marker analysis.

E rosetting techniques for the identification of T cells have been successful in a number of species, and Binns (1978) has described the use of sheep erythrocytes in dextran for the detection of sheep T lymphocytes. However, in

preliminary studies not reported here, this technique was found to be unreliable in that less than 6 percent of sheep lymphocytes formed rosettes. Attempts to form E rosettes between sheep T lymphocytes and human, hedgehog, opossum, goat, cattle erythrocytes were also unsuccessful. Reliance was therefore placed on anti-T cell antisera to detect these cells and problems of reagent specificity were largely resolved by a number of absorptions with lamb bone marrow and by diluting the antisera with PBS.

The mean percentage of blood lymphocytes bearing T cell antigens was 68.7 percent. Using an anti-T cell antiserum, Bohs *et al.* (1976) found that approximately 55 percent of sheep blood lymphocytes were T cells. Studies using E rosetting techniques for sheep T cell identification (Binns, 1978; Outteridge *et al.*, 1981a,b) indicated that, at most 40 percent of sheep lymphocytes formed T cell rosettes. Therefore the use of anti-T cell antisera appears to detect more sheep T lymphocytes than does the use of E rosetting techniques implying that a large proportion of the non-E rosetting, non-SIg bearing cells are thymus-derived. In fact, Outteridge *et al.* (1981a) reported that *in utero* thymectomy led to the loss of "null" cells from sheep blood, and the use of heterologous anti-T cell antisera may more accurately detect the distribution of thymus-derived lymphocytes in the sheep than E rosetting techniques.

For B cells, 25.3 percent of blood lymphocytes had detectable SIg. Other studies using immunofluorescent techniques reported values ranging from 15 to 33 percent (Ey, 1973; Symons and Binns, 1975; Bohs *et al.*, 1976; Outteridge *et al.*, 1981a,b).

The value for C receptor bearing cells seen in this study (14.1 percent) was lower than that reported by Outteridge *et al.*, (1981a) who described 25.8 percent of cells being C receptor-positive. These authors used EAC instead of ZC complexes and found that the method of lymphocyte isolation influenced the values for both E

rosetting and SIg-bearing lymphocytes. As they did not determine the percentage of C receptor-bearing cells in Ficoll/hypaque-separated lymphocytes, direct comparisons with the results from the present study can not be made.

The value for Fc receptor-bearing lymphocytes (16.7 percent) in this study was lower than the approximate 36 percent detected by Outteridge *et al.* (1981b). Again differences in technique and lymphocyte separation could account for this discrepancy in results. However, in another report, Outteridge *et al.* (1981a) detected only 7.4 percent Fc receptor bearing lymphocytes; this difference in their 2 results, which is not alluded to in either of their papers, may be due to their various methods of lymphocyte separation.

There do not appear to be any reports defining lymphocyte subpopulations of peripheral lymph nodes using both T and B cell markers simultaneously. The results of the detection of SIg on mesenteric lymph node lymphocytes were in general agreement to those of Cahill *et al.* (1978) who found a range from 18 to 34 percent of SIg-positive cells.

The cytochemical identification of sheep T lymphocytes was attempted using ANAE activity as an alternative to immunological techniques. Although useful as a T cell marker in man (Knowles *et al.*, 1978), ANAE appears not to be a specific marker for sheep T cells. Whilst the values for both T cell antigen-bearing and ANAE-positive lymphocytes appeared to be in close agreement, regression analysis showed that no relationship existed between these 2 sets of data. This difference was emphasised by the lymphocyte fractionation studies which showed that although the cells eluted from the nylon wool column were enriched for T lymphocytes, the same fraction was not enriched for cells with ANAE activity. A reduction in ANAE-positive cells in this fraction was not expected and the possibility was considered that cellular interactions with nonphysiological materials and prolonged incubation at 37°C may have caused a redistribution of ANAE activity in the lymphocytes. This

was investigated by the use of Percoll discontinuous density gradient fractionation. Although the use of Percoll fractionation failed to provide purified sheep T and B cell subpopulations as reported by Guttierrez *et al.* (1979) for human lymphocytes, there was a statistically significant partitioning of B lymphocytes to the less dense fraction of the Percoll gradient and T lymphocytes to the more dense. Regression analysis found a positive relationship between T lymphocytes and ANAE-positive cells only in Fraction 2. The significance of the relationship is unknown. The gradient may have been specifically enriched for T cells that were also ANAE-positive; however, this needs further investigation to exclude the possibility that this was a chance result.

There was little evidence for a clustering of ANAE-positive or ANAE-negative cells in different compartments in lymph nodes, tonsils or spleens. Although follicular centre cells were mainly ANAE-negative no other preferential distribution of cells was observed.

The detection of ANAE activity has been used previously in the sheep. Nemeth *et al.*(1979) used ANAE to identify a thymic lymphoblastoma as being T cell in origin, but they did not use immunological markers to verify their observations. In experiments not reported in this thesis the ANAE technique was applied to a number of sporadic ovine lymphomas without any correlation being found between the cytochemical and immunological results. These combined observations indicate that ANAE activity is not specific for ovine T lymphocytes. This lack of specificity has been demonstrated also for cattle (Yang *et al.*, 1979), rats (Fossum, 1978), cats and gerbils (Dockrell *et al.*, 1978).

In conclusion, on the basis of the nylon wool fractionation studies, it was found that the anti-T cell antisera and anti-ovine gamma globulin antisera were reliable reagents for the detection of sheep T and B lymphocytes respectively. The use of these reagents in the immunological characterisation of sporadic ovine lymphoma is reported in the following chapter.

CHAPTER 3
THE IMMUNOLOGICAL CHARACTERISATION OF SPORADIC OVINE LYMPHOMA

Introduction
Materials and methods
    Lymphomas
    Preparation of cell suspensions
    Detection of lymphocyte markers
Results
Discussion

CHAPTER 3

THE IMMUNOLOGICAL CHARACTERISATION OF SPORADIC OVINE LYMPHOMA

INTRODUCTION

The characterisation of lymphomas or leukaemias as being of T or B cell in origin is essential for an understanding of their pathogenesis, for comparative studies and for the identification of the target cells in aetiological studies. Sporadic ovine lymphoma has not been characterised in this way. This chapter reports the use of anti-T cell antisera and the detection of SIg to determine the percentages of T and B lymphocytes in 17 cases of sporadic ovine lymphoma. Comparisons are made between the data obtained and the pathoanatomical and histological classification of the lymphomas (Johnstone and Manktelow, 1978).

MATERIALS AND METHODS

Lymphomas

The neoplasms examined were 17 ovine lymphomas detected during the routine inspection of carcasses at slaughterhouses. A histological diagnosis of lymphoma was made from selected portions of affected tissues fixed in 10 percent formol saline (Appendix), processed by routine histological techniques and stained with haematoxylin and eosin.

Classification of the lymphomas as multicentric or alimentary was based on the criteria of Johnstone and Manktelow (1978) and these were further classified on the morphology of the predominant cell type (Table 15).

Preparation of Cell Suspensions

Tissues from neoplastic nodules or lymph nodes whose normal architecture had been totally effaced by lymphoma, were diced in RPMI 1640 and pressed through a 40-mesh sieve. Cells were washed in medium by centrifugation at 200 g for 10 min, resuspended in RPMI 1640 and counted. Viability was assessed by trypan blue exclusion.

When the cells were to be stored for retrospective

examination, they were resuspended to approximately 2 x 10^6 per ml in RPMI 1640-FBS supplemented to 10 percent with dimethyl sulphoxide. One ml aliquots, in heat sealed ampoules, were cooled at approximately $1^{o}C$ per min for at least 2 hr and when frozen placed in a liquid nitrogen refrigerator. When required the cell suspensions were brought to $37^{o}C$ and washed in RPMI 1640 by centrifugation at 200 g for 10 min.

Detection of Lymphocyte Markers

Freshly prepared cells or cells recovered from liquid nitrogen storage, were layered over Lymphoprep and centrifuged at 800 g for 25 min to remove contaminating erythrocytes and dead cells (Davidson and Parish, 1975). The cells at the medium-Lymphoprep interface were collected, washed twice in RPMI 1640 by centrifugation and resuspended to approximately 20 x 10^6 per ml.

T cells and SIg-bearing B cells were detected as described in Section A, Chapter 2. Lymphocytes harvested from the blood of normal sheep were used as controls.

The lymphomas were judged as being either of T or B cell origin if more than 60 percent of cells examined expressed either marker.

RESULTS

The results of the pathological classification and the immunological data are presented in Table 15. Most lymphomas could be assigned to one of 2 groups (Figure 6). Seven lymphomas contained predominantly T cells, 6 were of B cell origin, one had neither T nor B cells in the majority (T=B), one had cells bearing both markers (T + B> 100 percent) and 2 had cells bearing neither marker ("Null" cells). There was no correlation between the immunological data and the morphology of the lymphoma cells noted in the histological examination.

When these results were compared with the anatomical classification of the lymphomas (Table 16a) it was seen that 4 of the 5 alimentary lymphomas were of B cell origin while one had cells with both markers. All 7 T cell lymphomas

TABLE 15

IMMUNOLOGICAL CHARACTERISATION OF SPORADIC OVINE LYMPHOMAS

Lymphoma	Anatomical[a] Classification	Histological[a] Classification	T[b]	SIg[b]	Immunological Classification	Comments
OL 1	multicentric	lymphoblastoid	67.6	2.5	T	Very faint fluorescence
OL 2	alimentary	prolymphocytic/ lymphoblastoid	81.9	91.1	T+B	Strong T fluorescence Intense SIg fluorescence smaller cells
OL 4	multicentric	prolymphocytic/ lymphoblastoid	64.3	12.7	T	T variable staining SIg very faint
OL 5	multicentric	stem cell	48.7	37.2	T=B	
OL 6[c]	multicentric	prolymphocytic	63.0	56.0	B	T very faint
OL 6[d]			27.2	62.2		SIg variable intensity
OL 7[e]	alimentary	lymphoblastoid	31.3	77.4	B	Some cells strong SIg staining, most
OL 7[e]			25.1	74.6		spotted pattern
OL 8	multicentric	lymphocytic	92.3	26.8	T	
OL 9	multicentric	lymphocytic	29.6	64.7	B	
OL 10	multicentric	lymphoplasmacytoid	84.5	(f)	(T)	
OL 11	alimentary	prolymphocytic	2.9	87.4	B	
OL 12	multicentric	lymphoblastoid	75.4	27.8	T	
OL 14	alimentary	prolymphocytic/ lymphoblastoid	4.5	84.6	B	SIg faint but evenly stained
OL 17	multicentric	stem cell	83.3	47.3	T	T very faint, evenly stained
OL 18	liver kidney spleen	lymphoblastoid	7.0	95.0	B	SIg faint and unevenly stained
OL 19	multicentric	lymphoblastoid	85.4	42.8	T	T fluorescence stronger than SIg
OL 20	multicentric	lymphoblastoid	8.0	<1.0	"NULL"	
OL 22[g]	multicentric	lymphoblastoid	3.3	4.5	"NULL"	
OL 22[h]			<1.0	<1.0		

T : T lymphocyte antigens
B : SIg-bearing cells
SIg : surface immunoglobulin
OL : ovine lymphoma

(a) according to the criteria of Johnstone and Manktelow (1978)
(b) percentage of cells expressing marker
(c) cells from kidney lesion
(d) cells from lung lesion
(e) different mesenteric lymph nodes
(f) insufficient cells
(g) cells from spleen
(h) cells from lymph node

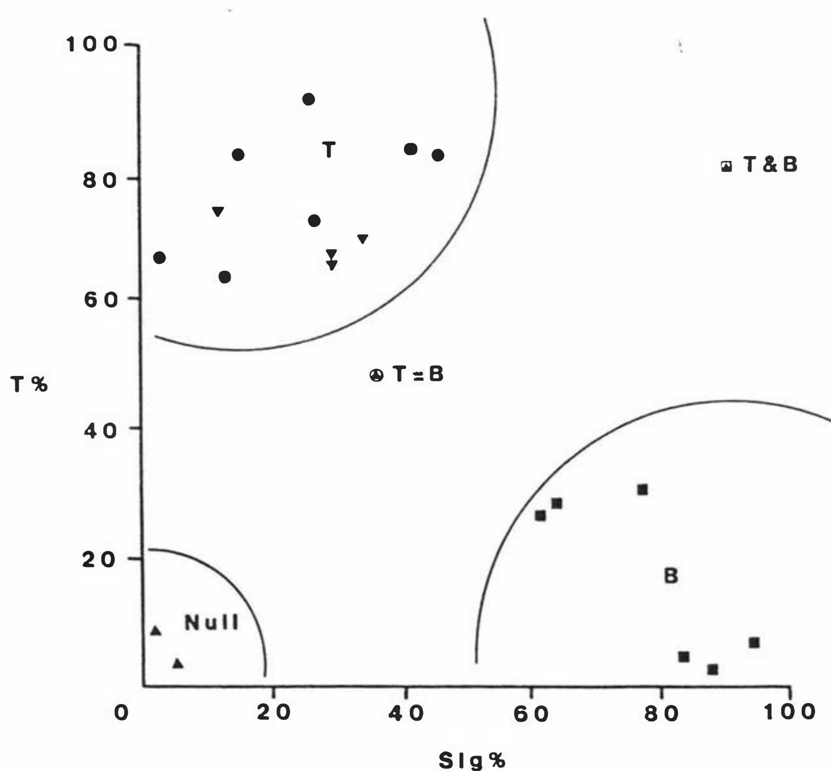


FIGURE 6: The distribution of 17 sporadic ovine lymphomas according to the percentages of cells bearing T cell antigens or surface immunoglobulin.
(●: T cell lymphoma)
(■: B cell lymphoma)
(▲: "null" cell lymphoma)
(◪: cells bore both T cell antigens and surface immunoglobulin)
(◬: unclassifiable)
(▼: normal mesenteric lymph node)

TABLE 16a

RELATIONSHIP OF THE IMMUNOLOGICAL CHARACTERISATION OF OVINE LYMPHOMAS TO THEIR PATHO-ANATOMICAL CLASSIFICATION

	T	B	T+B	T=B	Null
Alimentary	-	4	1	-	-
Multicentric	7	2	-	1	2

TABLE 16b

RELATIONSHIP OF THE IMMUNOLOGICAL CHARACTERISATION OF OVINE LYMPHOMAS TO THE ORGANS INVOLVED

	T	B	T+B	T=B	Null
Lymph Nodes:					
Alimentary	5	5	1	1	2
Lumbar	7	1	-	1	2
Thoracic	7	3	-	1	2
Distal	6	1	-	1	-
Gastrointestinal tract	1	4	1	-	-
Liver	1	2	1	-	2
Spleen(enlargement)	4	1	-	1	2
Kidney	-	3	1	-	1

were of the multicentric form. This latter classification also contained the 2 "null" cell, the single T=B and the 2 B cell neoplasms. Of these 2 B cell neoplasms, OL6 involved the abomasal, mesenteric and hepatic lymph nodes and the liver; in OL9, lymphomas were found in the wall of the abomasum, on the serosa of the rumen and involving the colon.

When individual organ involvement was examined (Table 16b) 4 of the 6 lymphomas involving the gastrointestinal tract were of B cell origin as were 3 of 4 renal lymphomas. All 7 T cell neoplasms affected the thoracic lymph nodes and 6 of these had also involved the distal lymphoid tissues, a corollary of the original finding of T cell predominance in multicentric lymphomas.

DISCUSSION

The detection of T cell antigens and SIg were used in the immunological characterisation of 17 cases of sporadic ovine lymphoma. It was assumed that as cell suspensions were prepared from neoplastic nodules of organs and totally effaced lymph nodes, then the majority of cells in each preparation were neoplastic. Because of this assumption, the results reported here are tentative, but it is nevertheless, a realistic one when considering the limitations of the markers used. Given that the majority of cells were neoplastic, then 7 lymphomas were found to be neoplasms of T lymphocytes, and 6 were of B cell in origin. The cells from 2 lymphomas bore neither marker, whilst those of one had both. One lymphoma (OL5), in which near equal numbers of T and B cells were identified, was considered unclassifiable. The morphology of the lymphoma cells did not correspond with their T or B cell nature. However, it was found that all T cell neoplasms were multicentric, whereas 4 of the 6 B cell neoplasms were confined to the alimentary tract and associated lymphoid tissue. The other 2 B cell lymphomas (OL6, OL9) also involved the gastrointestinal tract but had extended into other organs and lymph nodes. These results suggest that a relationship may exist between the immunological

origin of the neoplastic lymphocyte and the pathoanatomical classification of the lymphoma, a situation similar to that reported in dogs (Onions, 1977) and cats (Hardy *et al.*, 1977).

The confinement of neoplastic B cells primarily to the gastrointestinal lymphoid tissue, and the widespread dissemination of malignant T lymphocytes may reflect not only the difference between the recirculation tempos of T and B lymphocytes (de Sousa, 1978) but also differences in specific recirculation patterns by these 2 subpopulations. Recirculation patterns in adult (Cahill *et al.*, 1977) and foetal sheep (Pearson *et al.*, 1976) have been described. Scollay *et al.* (1976) suggested that certain sheep B lymphocytes may preferentially circulate through the gastrointestinal lymphoid tissue and the B cell nature of the alimentary lymphomas reported in this chapter may be considered as indirect evidence in support of such a pattern.

Given that T cells might also preferentially recirculate through the gastrointestinal lymphoid tissue (Cahill *et al.*, 1977) and that lymphomagenesis could be a random event, it then seems reasonable to assume that some alimentary lymphomas might be T cell in origin. Only one T cell lymphoma involved the gastrointestinal tract (OL4) but this was classified as multicentric as it had a limited extension into the thoracic and lumbar lymph nodes. Whether the finding of only a single case is due to the low numbers of lymphomas examined, or whether it reflects a difference in the susceptibility to transformation by this subpopulation of cells is not known.

The 3 other immunological categories of sporadic ovine lymphoma, namely T and B, "null" and unclassifiable, highlight the potential limitations of the 2 markers used. These limitations are compounded by the inability in this system to determine whether the cells bearing a particular marker are neoplastic or normal. Lymphomas having both T and B cell characteristics are infrequent (Sandilands *et al.*,1974; Shevach *et al.*, 1974; Onions, 1977; Foon *et al.*, 1980).

In the case of OL2, the immunofluorescent staining of SIg was intense on some cells only, and was variable on other larger and possibly neoplastic cells. It would be necessary to demonstrate the intrinsic and monoclonal nature of the SIg detected on these cells (Lukes *et al.*, 1978) before any conclusions can be made about the presence of both T and B cell markers. For OL5, neither T nor B cells were found in the majority, but this lymphoma may have been placed into either category had a marker been associated with neoplastic cells in cytocentrifuged smears, had monoclonal SIg been demonstrated or had immunoperoxidase techniques been applied to tissue sections (Borowitz *et al.*, 1981). Finally, the 2 "null" cell neoplasms (OL20, OL22) may have been classified into one or the other ontological pathway (Greaves, 1979) had more T and B cell markers been used. In these experiments, the use of only 2 markers was dictated by the limited number of cells available from all lymphomas, a result of the need to store cells in liquid nitrogen for retrospective cell marker studies.

The identification of T, B or "null" cell sporadic ovine lymphomas should enable a more accurate comparison of these neoplasms to be made with the lymphomas of other species. In this respect, and in its relationship to the pathoanatomical classification, sporadic ovine lymphoma is more akin to the feline lymphoma (Hardy *et al.*, 1977) than the enzootic or sporadic disease of cattle, (Takashima *et al.*, 1977). Direct comparisons can not be made with the experimental infection of sheep by BLV as only limited immunological and pathoanatomical data are available (Takashima and Olson, 1980).

Little can be inferred about the aetiology of sporadic ovine lymphoma from the immunological data obtained. Whilst many agents induce lymphomas of a single cell type, FeLV also causes lymphomas of varying cell types. However, it has yet to be clarified whether strain differences are partially responsible for this variation (Essex *et al.*, 1979).

The data presented here identify the target cells of

lymphomagenesis in the sheep which might enable more accurate studies between the lymphomas of sheep and those of other species (Johnstone and Manktelow, 1978). Further studies will require the additional use of other surface markers, and the application of immunoperoxidase techniques to tissue sections to allow the unambiguous interpretation of results (Borowitz *et al.*, 1981).

SECTION B:

INVESTIGATIONS INTO THE ASSOCIATION OF RETROVIRUSES WITH SPORADIC OVINE LYMPHOMA

CHAPTER 1

LITERATURE REVIEW: RETROVIRUS-LYMPHOCYTE INTERACTIONS: TARGET CELL SPECIFICITY, MECHANISMS OF TRANSFORMATION AND THE INHIBITION OF MITOGENIC RESPONSIVENESS OF LYMPHOCYTES

Introduction
Target cell specificity of retroviruses
The molecular basis of retroviral transformation of lymphoid cells
- (i) Early events
- (ii) Oncogenes of the transforming viruses
- (iii) Nontransforming leukaemia viruses
  - a. Viral recombination
  - b. Viral structural products
  - c. Promoter insertion

Retroviral inhibition of lymphocyte mitogenesis

CHAPTER 1

LITERATURE REVIEW: RETROVIRUS-LYMPHOCYTE INTERACTIONS: TARGET CELL SPECIFICITY, MECHANISMS OF TRANSFORMATION AND THE INHIBITION OF MITOGENIC RESPONSIVENESS OF LYMPHOCYTES

INTRODUCTION

Retroviruses have developed a symbiotic relationship with the cells they infect, permitting both the cells and the virus to survive. This symbiosis may have either a beneficial or deleterious effect on the total host organism depending on the infecting virus. Some of the genetically transmitted retroviruses (the endogenous retroviruses) which are integrated into the cellular genome of all somatic and germ cells as virogenes (Todaro, 1980), or proviruses (Temin, 1974), could be important in murine embryogenesis and differentiation (Levy, 1977; Pincus, 1980). Alternatively, other endogenous retroviruses which are not normally oncogenic may have a role in the development of spontaneous (Hartley *et al.*, 1977) or radiation-induced (Haas, 1978) murine lymphomas. Although the horizontally transmitted retroviruses (the exogenous retroviruses) infect only a limited number of cell types, their relationship is also symbiotic. Cell death does not follow infection and neoplastic transformation of the cell may occur after a long latent period (Wyke, 1981b).

The ultimate effects of infection by either endogenous or exogenous retroviruses are influenced by numerous factors and these include the host immune response, cellular and genetic restrictions on replication and transformation, the age of the animal and the presence of environmental carcinogens (Nowinski *et al.*, 1979; Pincus, 1980). Once infection of a cell is established there are 4 possible outcomes (Wyke, 1981b). There may be:

1. virus replication without cellular transformation.
2. virus replication and transformation of the host cell.
3. transformation of the host cell without virus replication.
4. neither replication nor transformation but the viral genome is maintained in the cell.

In addition to these effects there may be an alteration in the immune responsiveness of the host animal following infection. The depressed response observed in feline leukaemia virus (FeLV) infection in cats is not due to altered T and B subpopulations or to cellular transformation (Cockerell *et al.*, 1976b) but is a direct effect of the virus (Mathes *et al.*, 1979).

The following literature review serves as a background for the virological experiments reported in this section and places these experiments in perspective with the immunological studies reported in Section A. It examines the interaction between a retrovirus and a lymphocyte that determines the target cell specificity, that results in lymphomagenesis, and that alters the responsiveness of the immune system.

TARGET CELL SPECIFICITY OF RETROVIRUSES

Most exogenous retroviruses infect only a narrow range of cell types and may transform only a few of the cell types within this range. Infection is dependent on the interaction of viral envelope glycoproteins with specific glycoprotein receptors in the cell membrane (Delarco and Todaro, 1976). For murine leukaemia virus (MuLV) the envelope glycoprotein involved is designated gp70 and the host and cell range of the virus is determined by whether a cellular receptor is available for this glycoprotein (Elder *et al.*, 1977). The importance of these viral glycoproteins is illustrated by the existence of defective retroviruses that lack envelope (*env*) genes coding for these envelope glycoproteins. Infection and subsequent replication in other cells by these defective viruses is dependent on nondefective "helper" viruses to provide the envelope glycoproteins necessary for attachment (Bishop, 1978). Examples of detective leukaemia viruses are the Abelson MuLV (Baltimore *et al.*, 1979a) and the avian acute leukaemia viruses, namely, the avian erythroblastosis virus (AEV), the avian myeloblastosis (AMV) and the avian myelocytomatosis virus, MC29 (Beug *et al.*, 1979).

Once infection of an animal is established, replication may be widespread in a variety of cells but transformation is usually a rare event (Canaani and Aaronson, 1979) as evidenced by the long latent period preceding the development of leukaemias and lymphomas induced by most retroviruses. Furthermore, a narrow target cell specificity of transformation is indicated by the monoclonality within each neoplasm and between each case induced by a particular strain of virus. This is borne out for Moloney MuLV where DNA hybridisation studies showed that the target cells of this virus were T and B lymphocytes and of the nonlymphoid cells only mammary epithelia replicated the virus (Jaenisch, 1980). However, with respect to transformation, Moloney MuLV transforms only T cells (Baird *et al.*, 1977; Cerny *et al.* 1979).

FeLV infects a wider range of cells but transformation is also limited to a single subpopulation of lymphocytes. The sequential tissue distribution of an FeLV strain that induces T cell lymphomas has been studied by Rojko *et al.*, (1979) who showed that the virus had a wide tissue tropism. Early in infection FeLV could be detected in the epithelial cells of intestinal crypts, circulating mononuclear cells and the follicular B lymphocytes of the intestinal lymphoid tissue. Within a week the bone marrow cells and medullary thymocytes were infected and FeLV could be detected in circulating neutrophils and platelets thereafter. The virus did not appear to replicate in other T cell-dependent areas until the later stages of infection and then only in neoplastic lymphocytes. Rojko *et al.* (1979) did not determine which lymphocyte subpopulations were infected in blood but in a recent report (Rojko *et al.*, 1981) they demonstrated *in vitro* that all subpopulations of lymphocytes eventually become infected, although initially B lymphocytes replicated more virus. Azocar and Essex (1979), however, found that the majority of FeLV-infected cells in blood were T lymphocytes. This may reflect differences in the viral strains used by

both groups of authors as there is evidence to suggest that viral strains may differ in their target cells for transformation (Essex *et al.*, 1979). Nevertheless, Rojko *et al.* (1979) demonstrated that viral replication occurs mainly in B lymphocytes and that transformation may occur eventually in T lymphocytes. The mechanism of this dissociative phenomenon is unknown.

Other exogenous viruses display a more narrow target cell specificity. In chickens, studies have indicated that the target cell for transformation by the nondefective avian leukaemia viruses (ALVs) is an immature B lymphocyte in the bursa of Fabricius (Purchase and Gilmore, 1975). If bursectomised chickens are infected, lymphomas do not develop but the prevalence of osteopetrosis increases, indicating that not only is there immunological control of osteopetrosis but also ALV interacts with other target cells (Purchase and Gilmour, 1975; Graf and Beug, 1978). Cooper *et al.* (1974) could detect IgM but not IgG or IgA on the surface of lymphoma cells, suggesting that the target cell for ALV may be a lymphocyte prior to its transition from an IgM producer to a cell that produces either IgG or IgA.

A less mature B cell appears to be the target for Rauscher and Abelson MuLVs in mice. Reddy *et al.* (1980) established that Rauscher MuLV could infect and induce neoplastic transformation in pre-B lymphocytes. Analysis of the μ chains expressed by the lymphoma cells indicated that each neoplasm arose from a single transformed cell. A pre-B lymphocyte appears also to be the target cell of Abelson MuLV (Silverstone *et al.*, 1978). Rosenberg and Baltimore (1976) found that bone marrow had proportionally more target cells than did spleen and none were found in the thymus. These results are consistent with the target cell of Abelson MuLV being an immature cell not related to thymocytes. To further define the susceptible cell, Baltimore *et al.* (1979a) used athymic nude mice and CBA/N mice that have a defect late in B lymphocyte development to determine that both strains of mice have normal numbers of target cells. In

addition, they showed with monoclonal antibodies that the Abelson MuLV target cell is not the pluripotent stem cell.

The target cell of BLV has not been well defined. Paul *et al.* (1977a,b) suggested that only a small proportion of B cells were infected in cattle showing persistent lymphocytosis induced by BLV. Furthermore, Kenyon and Piper (1977a,b) demonstrated that a distinct subset of B cells were infected. Whether this is the same subset that transforms is unknown but DNA hybridisation studies have found homologous sequences only in lymphoma cells and not in other lymphoid or nonlymphoid tissues (Kettmann *et al.*, 1980).

The biological behaviour of a retrovirus may change if it infects a different species. BLV-induced lymphomas in cattle are invariably B cell in origin (Takashima *et al.*, 1977). When BLV is experimentally inoculated into sheep, however, some of the neoplasms induced involve the thymus and are presumed to be T cell in origin (Olson *et al.*, 1972; Nemeth *et al.*, 1979) while others are B cell lymphomas (Takashima and Olson, 1980). This difference in the biological behaviour of BLV within other hosts may be due to strain differences not detectable by standard *in vivo* or *in vitro* assays.

Therefore, in terms both of infection and transformation there can be marked differences in susceptibility of a cell to a particular virus. The susceptibility to transformation must be conferred on a cell at a genetic level, as infection of that cell does not necessarily result in its transformation (Nowinski *et al.*, 1979).

THE MOLECULAR BASIS OF RETROVIRAL TRANSFORMATION OF LYMPHOID CELLS

(i) Early Events

Before cellular transformation can occur, the DNA copy of the retroviral genome must be integrated into the cellular DNA. The events that lead to this integration are not completely understood and various explanatory schemes have been proposed or reviewed by Taylor (1977),

Weinberg (1977), Bishop (1978), Lerner (1978), Coffin (1979) and Taylor (1979).

Viral entry into a target cell occurs by either viropexis (Dales and Hanafusa, 1972) or the direct penetration of either whole virions (Aboud *et al.*, 1979) or viral cores (Miyamoto and Gilden, 1971). Following uncoating, the general consensus is that viral RNA-dependent DNA polymerase (RDDP) copies the virion RNA by synthesising a complementary strand of DNA (cDNA) to form a RNA-DNA hybrid. The ribonuclease activity of RDDP then removes the RNA genome processively, while at the same time copying the cDNA to form a linear duplex of double-stranded DNA. Several more steps are involved in which the double-stranded DNA is circularised and integrated into the cellular DNA (Bishop, 1978).

(ii) Oncogenes of the Transforming Viruses.

A number of molecular mechanisms for the retroviral transformation of cells have been discovered. Some viruses have a specific gene (or oncogene) responsible for transformation, whereas others do not. Rous sarcoma virus (RSV) carries an oncogene called *src* coding for a 60,000 dalton phosphoprotein which is responsible for inducing and maintaining the transformed state (Fischinger, 1980). A cellular gene of normal avian cells has a similar sequence to that of *src* and has been designated *c src* (Spector *et al.*, 1978).

Another group of avian retroviruses, the defective leukaemia viruses do not have a *src* gene (Stehelin *et al.*, 1976; Duesberg *et al.*, 1977; Sheiness *et al.*, 1978; Stehelin and Graf, 1978; Roussel *et al.*, 1979; Chen *et al.*, 1980) and do not seem to utilise the *c src* gene (Stehelin and Graf, 1978). However, these viruses do contain oncogene sequences which are specific for each subgroup of virus and are not found in the nondefective ALVs or RSV (Roussel *et al.*, 1979; Chen *et al.*, 1980). Identical, or at least similar, sequences can be detected, albeit in low numbers, in normal uninfected chicken cells (Sheiness *et al.*, 1978; Roussel *et al.*, 1979; Chen *et al.*, 1980) and their transcription in these cells is at low levels similar to that seen with the *c src* gene (Spector *et al.*,

1978; Roussel *et al.*, 1979). It has been suggested that the defective ALVs may have arisen by recombination between viral and cellular genes (Frisby *et al.*, 1979). Roussel *et al.* (1979) proposed that the 3 specific transforming genes *erb*, *mac* and *myb* found in AEV, MC29 and AMV respectively, were sequences that conferred upon a given virus its selective capacity to transform a specific cell. Why these viral oncogenes should cause leukaemia is uncertain but it is possible that transforming proteins may competitively inhibit the corresponding normal cellular protein function leading to a differentiation block (Roussel *et al.*, 1979).

The Abelson MuLV is unique as it is the only murine retrovirus known to transform lymphoid cells *in vitro* (Rosenberg *et al.*, 1975). In common with most of the other *in vitro* transforming viruses, it is a defective virus and requires Moloney MuLV as a helper virus for replication (Shields *et al.*, 1979b). Analysis of the defective Abelson MuLV genome reveals that it is smaller than that of its nondefective helper virus and contains about 25 percent of the Moloney MuLV sequences (Parks *et al.*, 1976). Shields *et al.* (1979a) found that the unique portion of the Abelson MuLV genome had no homology with the murine sarcoma viruses. Related sequences could, however, be found in both infected and uninfected cells (Baltimore *et al.*, 1979b) and hence it is possible that the virus has arisen from recombination between Moloney MuLV and normal cellular DNA. The central portion of the genome which is thought to come from the cellular DNA, codes for a single polyprotein of 90,000 to 100,000 daltons (Reynolds *et al.*, 1978; Witte *et al.*, 1978). This protein is similar to the transforming proteins of the sarcoma viruses (Collett and Erikson, 1978) as it is a phosphprotein and may be a kinase (Witte *et al.*, 1979b). Evidence suggests that it is a transmembrane protein and both this location and its kinase activity could induce cell division (Baltimore *et al.*, 1979a). As a similar protein may be expressed in normal T cells (Witte *et al.*, 1979a), particular proteins of this class could be of importance in normal cellular control.

There is little evidence for the existence of oncogenes in the nondefective leukaemia viruses and these viruses, with the possible exception of FeLV, do not code for transformation proteins. The FeLV-induced feline oncornavirus-associated cell-membrane antigen (FOCMA) occurs only on transformed cells infected by FeLV or the feline sarcoma virus. It is a 70,000 dalton protein (Snyder *et al.*, 1979) and antibody directed against it protects the animal from further replication of neoplastic cells (Essex *et al.*, 1975). It is not known if it is coded for by a viral gene, a viral recombinant gene or a cellular gene similar to *c src* but FOCMA may yet be shown to disrupt normal cell membrane-associated processes to lead to transformation (Kurth *et al.*, 1979).

(iii) Nontransforming Leukaemia Viruses

Although the nondefective retroviruses produce lymphomas and leukaemias *in vivo*, most do not transform cells *in vitro*. The way in which these viruses induce neoplasms is obscure, but a number of possible mechanisms have been proposed. These include viral recombination, the activity of viral structural products or the insertion of promoter sequences.

a. *Viral recombination*

Leukaemia viruses may acquire oncogenicity by recombination with cellular and/or viral genes (Hartley *et al.*, 1977; Nowinski and Hays, 1978). In the mouse a number of different endogenous viruses that show different tropisms for murine and nonmurine cells can be isolated from either spontaneous AKR (Hartley *et al.*, 1977) or radiation-induced (Haas, 1978) lymphomas. These isolates include ecotropic viruses which infect murine cells only, xenotropic viruses which infect nonmurine cells only and polytropic viruses which infect both murine and nonmurine cells. The polytropic viruses are the most oncogenic (Hartley *et al.*, 1977; Haas, 1978; Nowinski and Hays, 1978) and it appears that this polytropism is due to viral recombination between the ecotropic and xenotropic viruses at the level of the *env* gene (Elder *et al.*, 1977). Recombinant viruses have been found in several isolates (Hartley *et al.*, 1977; Devare *et al.*,

1978; Fischinger *et al.*, 1978; Haas, 1978) and generally emerge in the mouse just prior to the development of overt lymphoma. This suggests a relationship between the recombination and leukaemogenesis, however, the crucial genes and their products involved in oncogenesis have not been identified (Pincus, 1980). Recombination could alter virally coded surface differentiation molecules and induce neoplastic conversion through changes in the cell membrane (Elder *et al.*, 1977). Although opportunities may exist for a high frequency of recombination (Lerner, 1978), transformation, inevitable in some mouse strains, is still a rare event (Canaani and Aaronson, 1979). The mere generation of a recombinant virus does not confer on it oncogenicity and indeed many polytropic isolates are not oncogenic (Nowinski and Hays, 1978).

b. *Viral structural products*

Viral gene products may interact either directly with the host DNA (Todaro and Heubner, 1972) or indirectly with cell division control mechanisms operating via the cell membrane (Elder *et al.*, 1977; Lerner, 1978; McGrath and Weissman, 1979). Some researchers postulate that *env* gene products may be involved in leukaemogenesis. The receptor-mediated hypothesis for thymotropic MuLVs (McGrath and Weissman, 1978, 1979; McGrath *et al.*, 1979) postulates the existence of a rare subset of thymocytes which possess receptors for specific MuLVs. If a cell that is programmed to respond to that virus is infected then the continuous presentation of virus and viral glycoprotein at the cell surface would act as a perpetual mitogenic stimulus. Both the oncogenic exogenous and endogenous recombinant viruses could induce leukaemia by this mechanism. McGrath *et al.* (1979) argue that this hypothesis does not exclude the existence of oncogenes comparable to *c src* as it deals only with the external stimulus for transformation.

c. *Promoter insertion*

Integrated virogenes may be acting as passive mutagens or, as sugested by the promoter insertion hypothesis, virogenes may be able to subvert normal cellular control by placing certain cellular genes under

the influence of viral promoters (Hayward *et al.*, 1981). The development of this hypothesis followed the understanding of certain structural features of the integrated provirus or virogene (Hayward *et al.*, 1981; Payne *et al.*, 1981). The virogene appears to be flanked at both the 5' and 3' ends by sequences of approximately 350 nucleotides called long terminal repeats (LTRs) which are generated during integration and which contain a promoter sequence that could initiate transcription (Hughes *et al.*, 1979; Ju and Skalka, 1980; Yamamoto *et al.*, 1980). If this virogene was integrated close to, or directly adjacent to the 5' end of a cellular oncogene, then there would be enhanced expression of this cellular gene (Hayward *et al.*, 1981) as initiation of transcription could occur from both LTRs. Hayward *et al.* (1981) found evidence for increased transcription of a cellular homologue of the *myc* oncogene, the *mac* oncogene of Roussel *et al.* (1979), in avian lymphoma cells and these *myc* transcripts were covalently linked to viral U5 sequences similar to those found in the LTRs. Virogenes were located near or adjacent to the cellular homologue of *myc* and only a single virogene was necessary for lymphomagenesis. This genome did not need to have full replicative functions (Neel *et al.*, 1981; Payne *et al.*, 1981).

Before the promoter insertion hypothesis can be accepted, several anomalous observations need explanation. These, summarised by Wyke (1981a), include the absence of enhanced *myc* expression in a small proportion of avian lymphomas, the reason why *myc* causes carcinomas and myelocytomas in MC29 virus-infected birds and not a B cell lymphoma, and why in some cases transformation occurs with the virogene inserted at the 3' rather than the 5' end of the oncogene.

Further research is necessary to show whether this hypothesis has relevance to leukaemogenesis by other nontransforming viruses. In lymphomas of AKR mice, new viral DNA sequences emerge but there are no unique integration sites (Canaani and Aaronson, 1979), an important feature of the promoter insertion hypothesis. Feline lymphomas may be a useful model to clarify the

proposed mechanism as a proportion of these do not produce infectious virus (Francis *et al.*, 1979) although FOCMA is expressed (Essex *et al.*, 1979). If FOCMA is a transformation protein then the induction of the lymphoma in the absence of detectable virus may be explained by the promoter insertion hypothesis. Other "virus-negative" lymphoid neoplasms including those of cattle, dogs and man, may also be induced by a similar mechanism. The finding that ALV-induced neoplasia is accompanied by the expression of a cellular homologue of a viral oncogene without viral replication, nevertheless, appears to link the oncogenic mechanisms of the *in vitro* transforming and nontransforming retroviruses. The promoter insertion hypothesis may give clues to a common pathway for all carcinogens.

RETROVIRAL INHIBITION OF LYMPHOCYTE MITOGENESIS

The responses of lymphocytes to polyclonal activation by nonspecific mitogens including phytohaemagglutinin (PHA), Concanavalin A (Con A) and pokeweed mitogen (PWM), have been used as an *in vitro* measure of cellular immunocompetence. Altered responses have been found in a range of pathological conditions of man (Douglas, 1971; Matchett *et al.*, 1973; Schweitzer *et al.*, 1973; Mannick *et al.*, 1977) and lymphoproliferative disorders of animals (Dent, 1972; Muscoplat *et al.*, 1974b; Dent, 1975; Cockerell *et al.*, 1976b; Donovan *et al.*, 1977; Dutta *et al.*, 1978; Holmberg *et al.*, 1978; Atluru *et al.*, 1979; Paul *et al.*, 1979). Retroviral infections have been associated with a number of specific immunological defects (reviewed by Dent, 1972, 1975) including a depressed mitogenic responsiveness and a reduction in other *in vivo* and *in vitro* functional parameters depending on the nature of the infecting virus (Dent, 1975).

Recent studies have examined why a reduced mitogenic responsivess results from retrovirus-lymphocyte interactions. Virus-cell contact alone appears to be as effective in reducing mitogenic responsiveness as the more complete infection of a cell by a retrovirus (Wainberg and Israel,

1980). The immunosuppressive activity of retroviruses may reside in the viral envelope as disrupted Rauscher MuLV was inhibitory whereas the viral cores had no such immunosuppressive effect (Fowler *et al.*, 1977). Ultra-violet-inactivated FeLV or avian retroviruses were also shown to have a similar effect on the Con A-induced mitogenesis of feline and avian lymphocytes respectively, and this effect was not due to a viral blockade of Con A receptors (Hebebrand *et al.*, 1977; Israel *et al.*, 1979).

Mathes *et al.* (1979) isolated from FeLV a specific protein of 15,000 daltons (p15) that inhibited mitogenesis of feline lymphocytes. This protein was shown to suppress the responsiveness of human lymphocytes (Hebebrand *et al.*, 1979). The reduced responses in both cases were not due to any toxicity of p15, and further experiments indicated that the protein was not competing for Con A binding sites. However, p15 did cause alterations in cell membrane functions as evidenced by the interference with the normal capping process (Mathes *et al.*, 1979). *In vivo* injection of p15 reduced the normal cytotoxicity antibody response to FOCMA indicating that p15 may have a biological role in the establishment of FeLV infection and lymphomagenesis (Mathes *et al.*, 1979). The immuno-suppressive protein may explain the clinical signs of immunodepression such as the thymic atrophy often associated with acute FeLV infection in kittens (Jarrett, 1975).

The onset of immunosuppression resulting from *in vivo* infection with FeLV has been shown to occur at the time FeLV can be first detected in circulating leukocytes (Cockerell *et al.*, 1976b). This must be considered evidence for the direct effect of FeLV on the responsive-ness of lymphocytes because neither alteration in relative T or B cell numbers nor lymphocyte transformation had occurred at this early stage. Dunlap *et al.* (1979) found an *in vivo* defect in Con A receptor mobility on lymphocytes from cats infected with FeLV, similar to that found in the *in vitro* system of Mathes *et al.* (1979) which suggests that FeLV affects one function on numerous lymphocytes. The defect that prevents capping is unknown.

Other data suggest a different mechanism for the retroviral suppression of mitogenic responsiveness. Israel and Wainberg (1981) found that inhibition may occur directly from viral contact with macrophages rather than the lymphocyte target cell. The *in vitro* system they described used several inactivated retroviruses which induced macrophages to produce a protein that could inhibit mitogenesis. The mechanism of this inhibition was undetermined but as other workers have demonstrated the induction of suppressor cells in the spleen by retroviral infection of mice (Kirchener *et al.*, 1975) it is possible that suppressor T lymphocytes could be involved in this *in vitro* system. The importance of the role of macrophages in the *in vivo* immunodepression resulting from retroviral infection still remains uncertain.

Retroviral-induced immunodepression may allow the establishment of infection, but whether or not this is of direct importance in lymphomagenesis remains to be demonstrated.

CHAPTER 2

THE SEARCH FOR VIRUS IN SPORADIC OVINE LYMPHOMAS AND THEIR CELL SUSPENSION CULTURES USING ELECTRON MICROSCOPY

Introduction
Materials and methods
  Sheep
  Ovine lymphomas
  Suspension cultures
  Control lymphocyte cultures
  Electron microscopy
Results
  (i) Ovine lymphomas
  (ii) Suspension cultures of ovine lymphomas
  (iii) Control lymphocyte cultures
Discussion

CHAPTER 2

THE SEARCH FOR VIRUS IN SPORADIC OVINE LYMPHOMAS AND THEIR CELL SUSPENSION CULTURES USING ELECTRON MICROSCOPY

INTRODUCTION

Viruses have been demonstrated by electron microscopy in lymphomas and in material derived from tissue cultures of lymphoid neoplasms of several species of animals (Rich, 1968). Particles resembling type A and type C retroviruses have been reported in spontaneously occurring lymphomas of sheep (Johnstone, 1974), in sheep lymphomas experimentally induced by BLV (Olson *et al.*, 1972) and in material from mitogen-stimulated short term cultures of lymphocytes from enzootic ovine lymphoma (Weiss *et al.*, 1971; Paulsen *et al.*, 1972).

Although electron microscopy is considered to be a relatively insensitive method of demonstrating the presence of retrovirus (Lo and Howatson, 1978), it is, nevertheless, useful when employed in association with other techniques. In this chapter, the screening of sporadic ovine lymphomas and their cell cultures for virus using the electron microscope is described.

MATERIALS AND METHODS

Sheep

Tissue from 5 mature sheep with lymphoma that had been identified during the routine carcass inspection at a local slaughterhouse were collected and held at 0°C prior to subsequent processing. These 5 cases were designated OL1 to OL5 respectively. Lymph nodes and spleen from all animals and cells from suspension cultures of lymph nodes from OL1 and OL5 were screened by electron microscopy.

Ovine Lymphomas

Selected pieces of neoplastic tissue were diced in modified Karnovsky's fixative (Appendix) to approximately 1 mm cubes and placed into fixative at 4°C for at least

30 min.

Suspension Cultures of Ovine Lymphomas

Cell cultures from OL1 and OL5 were established and maintained as detailed in Section B, Chapter 3. PHA was added to a duplicate set of cultures to a final concentration of 10 μg per ml. After 24 and 48 hr in culture, cells were harvested by centrifugation at 500 g for 10 min and prepared for fixation in plain glass microhaematocrit tubes after the method of Doane *et al.* (1974). The pellet of cells was extruded into modified Karnovsky's fixative and held at $4^{o}C$ for at least 30 min.

Control Lymphocyte Cultures

Blood lymphocytes from 3 normal mature sheep were harvested and cultured as described in Section B, Chapter 4. At the time the cultures were established PHA was added to a final concentration of 10 μg PHA per ml. Harvesting and fixation of cells for electron microscopy was carried out as described for the suspension cultures of lymphoma cells.

Electron Microscopy

The fixed tissues and cells detailed above were post-fixed in 1 percent osmium tetroxide, dehydrated in a graded series of alcohols and embedded in epoxy resin (Durcupan ACM, Fluka) by routine methods. Sections were cut using an LKB III ultramicrotome, stained with uranyl acetate and lead citrate and examined in a Philips EM 200 electron microscope. Further details of reagents and methods used are recorded in the Appendix.

RESULTS

(i) *Ovine Lymphomas*

Virus-like particles numbering less than one per section in the intercellular spaces of a lymph node and the spleen from OL1 (Figures 7 and 8) and the spleen from OL2. The particles were spherical, of approximately 120 to 130 nm external diameter and had a spherical internal structure of 60 to 70 nm diameter. With one exception (Figure 9) all particles had electron-lucent centres. No particles were

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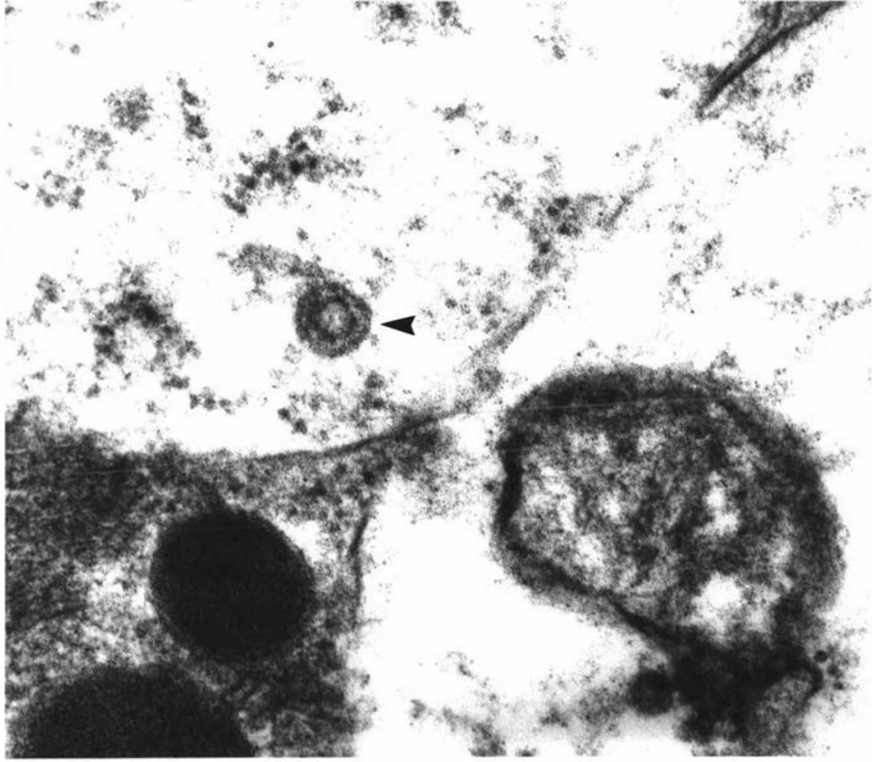
FIGURE 7: Virus-like particle in the intercellular space of the spleen from OL1. (Electron micrograph: magnification 82,500 times)

FIGURE 8: Virus-like particle in the intercellular space of the spleen from OL1. (Electron micrograph: magnification 82,500 times)



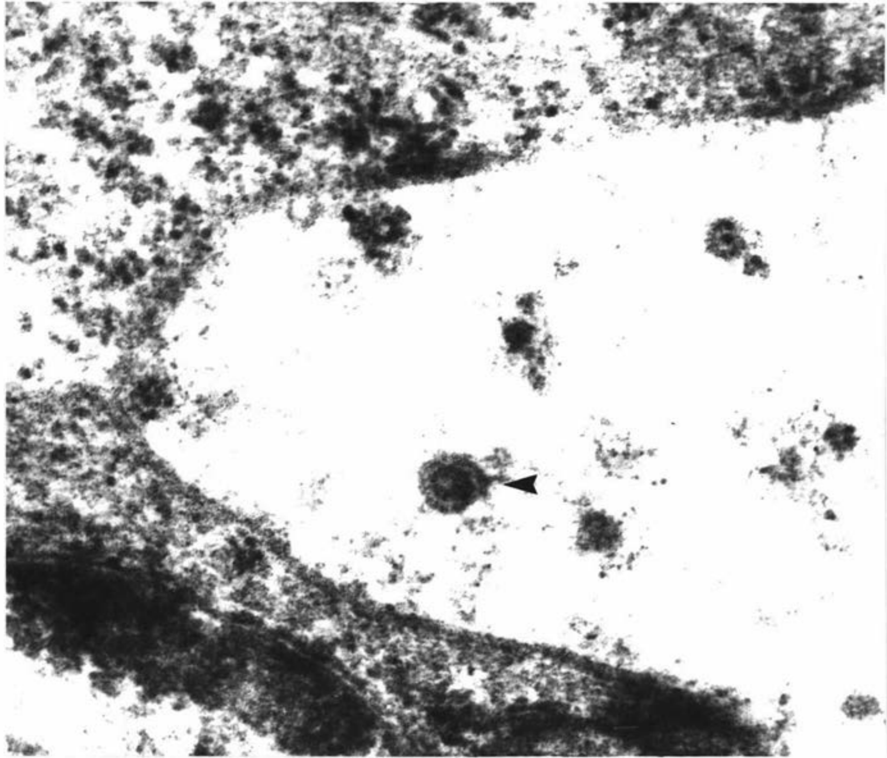


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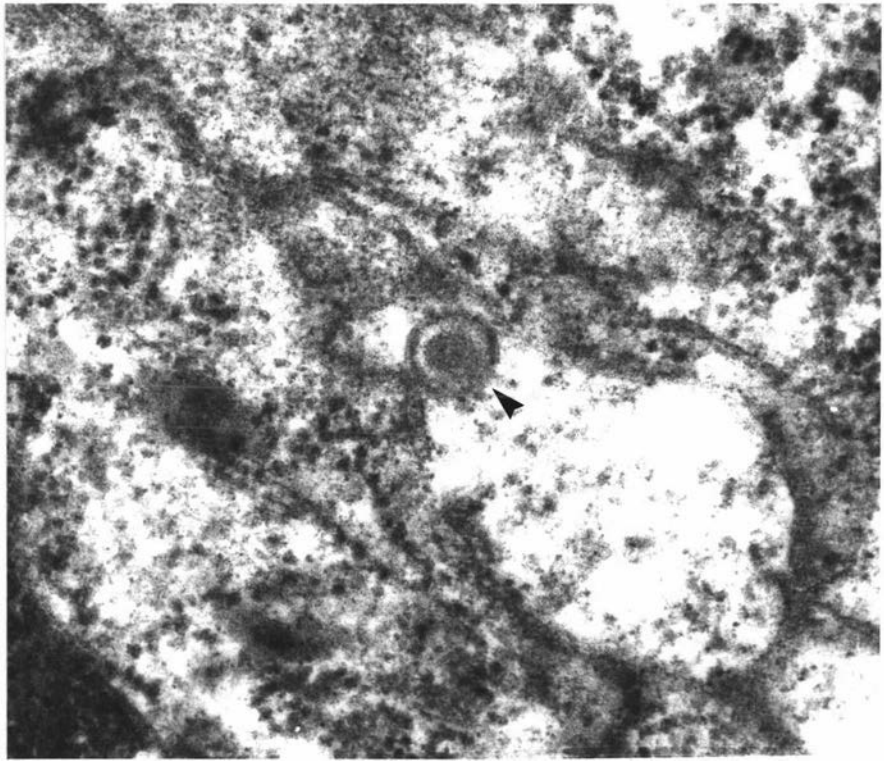
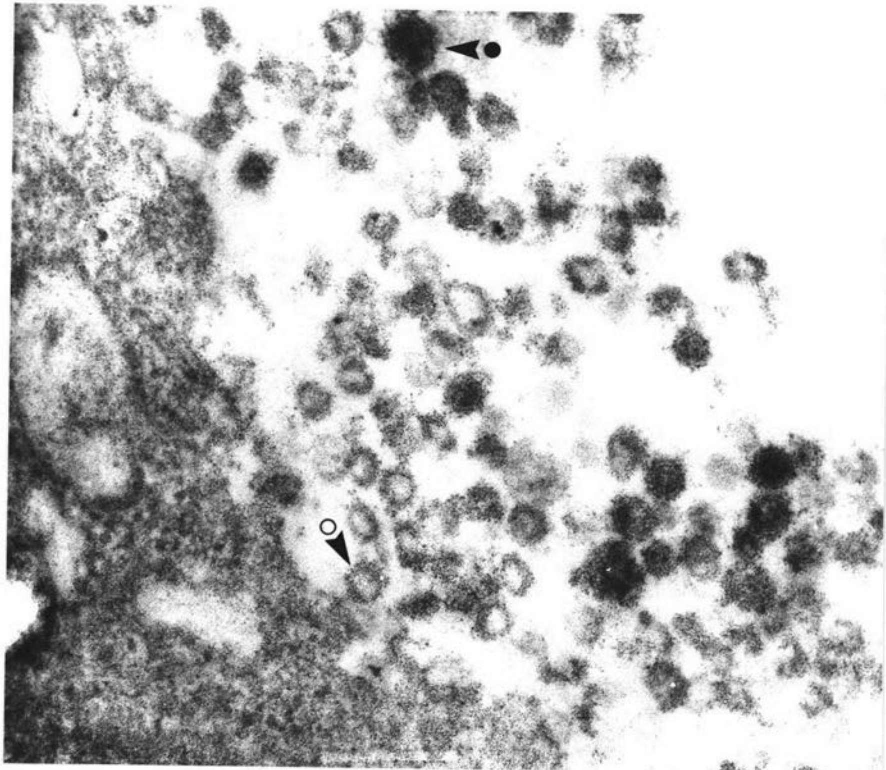
FIGURE 9: Virus-like particle with an electron-dense centre from the spleen of OL1. (Electron micrograph: magnification 82,500 times)

FIGURE 10: Virus-like particles (●) and vesicular structures (○) associated with cells from a suspension culture of OL5. (Electron micrograph: magnification 82,500 times)





observed budding from cell membranes. No virus-like structures were noted in tissues from OL3, OL4 or OL5.

(ii) *Suspension Cultures of Ovine Lymphomas*

Several virus-like particles with electron-dense centres were observed in the cellular debris of the cultures derived from OL5 whether treated or untreated with PHA (Figure 10). These particles measured up to 100 nm in external diameter with the electron-dense centre having a diameter of up to 80 nm. In addition, there were numerous vesicle-like structures with a diameter of 70 nm (Figure 10). No budding forms were seen.

(iii) *Control Lymphocyte Cultures*

Vesicular structures similar to the smaller bodies noted in the sections from the suspension cultures were seen in all sections from PHA-transformed lymphocytes from normal sheep. No particles resembling A or C type retroviruses were observed.

DISCUSSION

The particles noted in the neoplastic tissues are consistent in morphology with A and C type retroviruses. although an important morphological feature of retroviruses, namely budding from cell membranes, was not observed.

The failure to detect budding particles does not negate the possibility of a retrovirus being involved as low numbers of A and C type particles would imply even fewer budding forms, and viral-like particles are not found in normal lymphoid tissue (Rickard, 1968). Furthermore, retrovirus detection in lymphoma tissue using electron microscopy is considered by most authors to be a relatively insensitive technique and the results obtained by its use can be inconclusive (Haguenau, 1973; Dalton, 1975; Lo and Howatson, 1978). In BLV-induced lymphomas in cattle, virus particles are not readily detected in neoplastic tissue and budding viruses are always difficult to find (Dutcher, 1968).

Because of the differences in size and morphology of the so-called virus-like particles seen in the neoplastic tissues and the suspension culture, the question of whether

or not they represent the same structures is raised. Similar particles with variations in size and morphology have also been reported in lymphocyte cultures from sheep showing persistent lymphocytosis (Weiss *et al.*, 1971) and enzootic lymphoma (Paulsen *et al.*, 1972) and were interpreted by these authors as virus. Such particles were not observed in control cultures of normal PHA-transformed lymphocytes. PHA-stimulated lymphocytes were used in preference to unstimulated lymphocytes as controls because the latter do not undergo mitosis in culture and some retroviruses, including BLV, require cellular proliferation for viral replication (Miller *et al.*, 1969).

The smaller vesicular structures observed in the suspension culture of OL5 and in PHA-transformed control lymphocytes were also observed by Johnstone *et al.*(1979a). These authors examined PHA-transformed lymphocytes from sheep that had developed a persistent lymphocytosis following inoculation with cell-free extracts of sporadic ovine lymphomas. In contrast to those found in the present experiments these structures were in cytoplasmic vacuoles. Although Johnstone *et al.*(1979a) interpreted these to be virus-like they pointed out the need for care in such an interpretation, a need reinforced by the presence of similar structures in control lymphocytes found in the current experiments. However, vesicular structures considered virus-like have been described in association with enzootic ovine lymphoma (Weiss *et al.*, 1971) in which BLV has been positively identified (Ogura *et al.*, 1977) and in human and lymphoblast cell lines (Karpas, 1978; Karpas and Fischer, 1980). In none of the above reports is there good supporting evidence for their viral nature.

Other authors who have observed similar vesicular structures have considered them to be normal cellular organelles. Biberfeld (1971) described vesicles in vacuoles in PHA-transformed normal human lymphocytes and similar structures have also been recorded in the multivesicular bodies that are considered by some to be part of the lysosome system

of the cell (Smith and Farquhar, 1966). Furthermore, Dalton (1975) provides evidence that the dense and vesicular structures similar to those observed in the ovine lymphoma suspension culture are of cellular origin. He suggested that these structures were released from degenerating cells and that they had a high affinity for cellular surfaces. More information relating to the nature and genesis of the vesicles seen in the OL5 cell and transformed control lymphocyte cultures might come from determining the polarity of their membrane (Steck, 1974; Hochstadt *et al.*, 1975) as it has been suggested that these structures are pinocytotic vesicles (Biberfeld, 1971).

While it is likely that the vesicular structures do not represent retroviruses, the presence of low numbers of viral-like particles in 2 of 5 lymphomas examined reinforces the need for a more sensitive assay. Further experiments to examine retroviral involvement in sporadic ovine lymphoma are described in Chapter 4 of this section.

CHAPTER 3

CULTURE OF CELLS FROM SPORADIC OVINE LYMPHOMA

Introduction

Materials and methods

- Cells
  - (i) Foetal ovine skin (FOSK) cells
  - (ii) Foetal ovine kidney (ROK) diploid cell line
  - (iii) Lymphoma cells
- Cell cultures
  - (i) Establishment of FOSK primary cell cultures
  - (ii) Passage of confluent primary FOSK and ROK cell cultures
  - (iii) Establishment of ovine lymphoma cell cultures
    - a. Suspension cultures
    - b. Microcultures (media-screen feeder-cell cultures)
    - c. Plasma clot explant cultures

Results

- (i) Suspension cultures
- (ii) Microcultures (media-screen feeder-cell cultures)
- (iii) Plasma clot explant cultures

Discussion

CHAPTER 3

CULTURE OF CELLS FROM SPORADIC OVINE LYMPHOMA

INTRODUCTION

The development of continuous cell cultures derived from lymphomas and leukaemias have been important in the isolation of retroviruses from these diseases. Some viruses such as radiation MuLV may only replicate in lymphoid cells (Decleve *et al.*, 1976) and good evidence for a retrovirus associated with certain human lymphomas has come only after the establishment of continuous cell lines of these neoplasms (Kaplan *et al.*, 1977; Poiesz *et al.*, 1980). The particular advantages of such systems accrue from the relative stability and monoclonality of cultured cells in comparison to those obtained directly from lymphomas; from the large volume of readily available material they can provide ; and from the increased range and repeatability of examinations that can be made on the cells of any one neoplasm.

For sporadic ovine lymphomas, prior attempts at establishing long term cultures have been unsuccessful (Johnstone, unpublished data) and no assessment has been made of the growth requirements of cells from these neoplasms. This chapter describes the attempts at establishing continuous cell cultures by several conventional methods and the screening of a range of media and sera for ovine lymphoma cells.

MATERIALS AND METHODS

Cells

(i) *Foetal ovine skin (FOSK) cells*

Skin tissues were removed aseptically from ovine foetuses at approximately 90 days of gestation. These were obtained from a local slaughterhouse.

(ii) *Foetal ovine kidney (ROK) diploid cell line*

This continuous fibroblastic cell line was developed in this laboratory and the cells had been passaged in excess of 50 times before their use in these experiments

(iii) *Lymphoma cells*

Cells from either the neoplastic nodules in lymph nodes or other organs were obtained using aseptic techniques from 17 sheep with lymphoma identified during the routine inspection at a slaughterhouse and were kept on ice until culture establishment.

Cell Cultures

(i) *Establishment of FOSK primary cell cultures*

Skin tissues were washed twice in Hank's balanced salt solution containing antibiotics (Appendix) and were finely diced in MEM. The medium was removed and the cells were dissociated in a trypsin solution (Appendix) at 37°C for 30 min. The suspension was filtered through a 40-mesh tissue sieve to remove large fragments of tissue. The filtrate was centrifuged at 800 g for 10 min and the cells resuspended in Medium 199 and counted in a haematocytometer. Plastic culture flasks (3013, Falcon) were seeded with approximately 10^6 cells in 5 ml of Medium 199 supplemented to 20 percent with FBS (Medium 199-FBS) and incubated at 37°C. The medium was changed every 3 to 4 days and when the monolayers were confluent the cells were passaged.

(ii) *Passage of confluent primary FOSK and ROK cell cultures*

To passage confluent monolayers of primary FOSK and ROK cells, the monolayers were washed in PBS and detached at 37°C by the addition of 1 ml of a trypsin, versene and antibiotic mixture (ATV) (Appendix). The cells were used to establish new cultures in the appropriate medium and incubated as before.

(iii) *Establishment of ovine lymphoma cell cultures*

a. Suspension cultures

Six lymphomas were cultured in suspension (Table 17). Neoplastic tissues were diced in either Medium 199 or RPMI 1640 and pressed through a 40-mesh tissue sieve. The cells were washed in medium by centrifugation at 200 g for 10 min, resuspended and counted in a haematocytometer. Viability was assessed by trypan blue exclusion.

Approximately 10^7 viable lymphoma cells in 5 ml of

Medium 199-FBS or RPMI 1640 supplemented to 20 percent with FBS (RPMI 1640-FBS) were seeded into plastic culture flasks and incubated at 37°C. Media were changed as indicated by the pH of the culture media and in all cases cultures were maintained for over 3 weeks before being discarded.

b. Microcultures (media-screen feeder-cell cultures)

Cells from 6 lymphomas were screened for their *in vitro* nutrient requirements (Table 18) with the cultures being established by the method of Epstein and Kaplan (1979). Lymphoma cells from multiple organ sites (OL6, OL7, OL8 and OL10) or from single lesions (OL9 and OL11) were cultured over a feeder layer of ovine fibroblasts.

Normal FOS or ROK cells from confluent monolayers were resuspended by ATV and added to Medium 199-FBS to a final concentration of approximately 2 x 10^5 cells per ml. Two hundred µl of this cell suspension were added to each well of a microculture plate (Microtest II Tissue Culture Plate - 3040, Falcon) which was incubated in a humidified atmosphere of 5 percent CO_2-in-air for 1 hr. The medium was removed and replaced by a suspension of approximately 2 x 10^5 lymphoma cells in 200 µl of the specific medium/serum combination to be tested (Figure 11). The media tested included MEM, Medium 199, RPMI 1640, Ham's F10 and Waymouth's medium. The sera used included FBS, ovine serum (OS), equine serum (ES) or a combination of FBS and OS. Table 18 lists the media sera combinations for each lymphoma cultured. The lymphoma cell suspension was prepared in an identical manner to those for the suspension cultures. The microcultures were incubated in a humidified atmosphere of 5 percent CO_2-in-air and daily observation to check for the proliferation of cells was made using an inverted microscope.

In 3 cases (OL7, OL8 and OL10), following the death of the lymphoma cells, the cocultured ROK cells were detached by trypsinisation, transferred to plastic culture flasks and grown to confluency in Medium 199-FBS. Media from these cultures were tested for the presence of retroviruses as described in Section B Chapter 4.

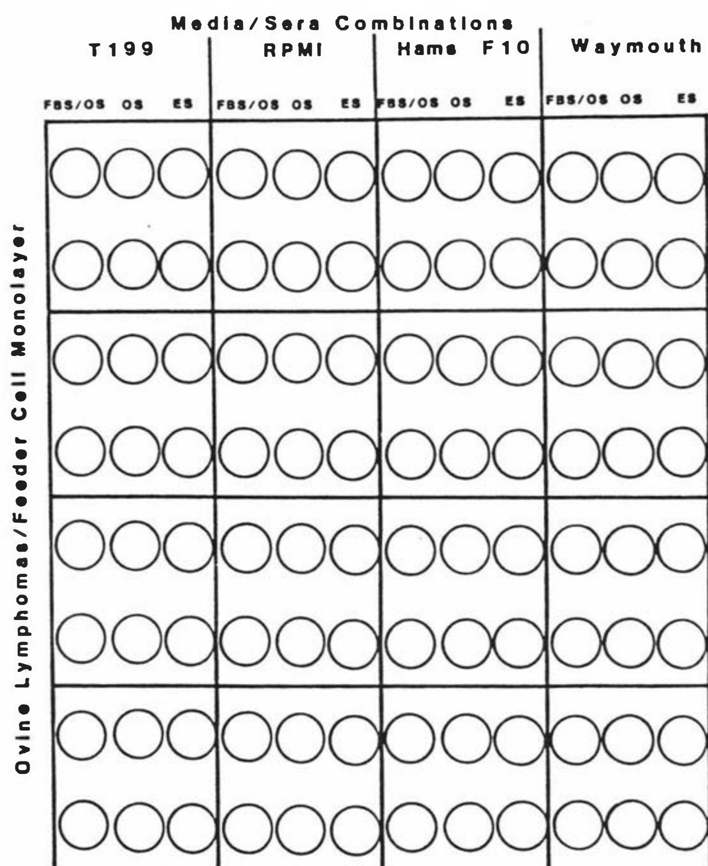


FIGURE 11: Schematic arrangement of different media/sera combinations for the media-screen feeder-cell microcultures.

c. Plasma clot explant cultures

Lymphoma tissues from 5 lymphomas (Table 17) were diced in RPMI 1640 and pieces approximately 1 mm diameter were placed into plastic culture flasks. After excess medium was removed each piece was covered by calcium-reconstituted ovine plasma (Appendix) which was allowed to clot. Five ml of RPMI 1640-FBS was then added and the flasks incubated at 37°C.

Before the lymphoma cells were passaged, reconstituted plasma was added to the new culture flasks and allowed to clot. Five ml of RPMI 1640-FBS was conditioned by its addition to the flasks overnight. New cultures were established by adding 2 ml of cell suspension from the primary culture to the medium in the new flasks.

Fibroblasts that grew from the tissue explants (designated ovine lymphoma fibroblasts - OLF cells) formed monolayers that were washed with PBS, detached with ATV and cultured as for other monolayer cultures but in RPMI 1640-FBS. These cell cultures were assessed for the presence of retroviruses as described in Section B, Chapter 4.

RESULTS

Despite the use of several techniques and the attempt at determining the optimal media/sera combination for growth, continuous cell lines were not established (Tables 17 and 18).

(i) *Suspension Cultures*

Of the 6 lymphomas cultured, only OL5 in Medium 199-FBS showed evidence of proliferation by the formation of morula-like cellular clusters. This proliferation had ceased by 7 days and the cells were never passaged. There was no evidence of cell replication in the other 5 lymphoma cultures (OL1, OL2, OL17, OL19 and OL20).

(ii) *Microcultures (Media-Screen Feeder-Cell Cultures)*

The results of screening 6 lymphomas for media requirements are summarised in Table 18. Although no cellular proliferation occurred in any of the wells it was observed that ovine lymphoma cells remained viable longer in certain media/sera combinations. The best media were

TABLE 17

TECHNIQUES FOR, AND RESULTS OF OVINE LYMPHOMA CELL CULTURES

OL	Culture Technique	Medium	Results
OL 1	Suspension	199/FBS	No proliferation observed
OL 2	Suspension	199/FBS	No proliferation observed
OL 5	Suspension	199/FBS	Brief proliferation 7 days
OL 6	Screen-feeder cell		
OL 7	Screen-feeder cell		
OL 8	Screen-feeder cell	*	*See Table 18
OL 9	Screen-feeder cell		
OL 10	Screen-feeder cell		
OL 11	Screen-feeder cell		
OL 11	Plasma clot explant	RPMI/FBS	Proliferation for at least 2 passages (18 days)
OL 12	Plasma clot explant	RPMI/FBS	Limited proliferation in some cultures
OL 14	Plasma clot explant	RPMI/FBS	No proliferation observed
OL 15	Plasma clot explant	RPMI/FBS	No proliferation observed
OL 16	Plasma clot explant	RPMI/FBS	No proliferation in node cultures; large motile cells accumulated in splenic cultures
OL 17	Suspension	RPMI/FBS	No proliferation observed
OL 19	Suspension	RPMI/FBS	No proliferation observed
OL 20	Suspension	RPMI/FBS	No proliferation observed

OL: ovine lymphoma, 199/FBS: Medium 199 + 20 percent foetal bovine serum, RPMI/FBS: RPMI 1640 medium + 20 percent foetal bovine serum

TABLE 18

MEDIA, SERA AND CELLS USED IN MICROCULTURES FOR THE SCREENING OF THE NUTRIENT REQUIREMENTS OF OVINE LYMPHOMA CELL CULTURES

Tissue of Origin	Media	Sera	Feeder Cell	Results
OL 6 Liver Kidney Lung	MEM 199 RPMI F10	FBS/OS OS ES	FOS ROK	(1) No difference between FOS/ROK (2) ES unsatisfactory (3) RPMI/F10-best lymphoma cell viability maintenence
OL 7 2x Node Spleen	199 RPMI F10 Waymouths	FBS/OS OS ES	ROK	(1) Waymouth's-not as satisfactory (2) ES unsatisfactory
OL 8 Node Spleen	199 RPMI F10 Waymouths	FBS/OS OS ES		As for OL 7
OL 9 Node	199 RPMI F10 Waymouths	FBS FBS/OS OS	ROK	(1) Number of wells contaminated (2) No observable differences in remainder
OL 10 2x Node Spleen	199 RPMI F10 Waymouths	FBS/OS OS Fresh ES	ROK	Poor viability of cultures from establishment
OL 11 Node	199 RPMI F10 Waymouths	FBS/OS OS ES	ROK	As for OL 7

OL: ovine lymphoma
MEM: Eagle's medium (Wellcome)
199: Medium 199 (Wellcome)
RPMI: RPMI 1640 medium (Flow)
F10: Ham's F10 medium (Flow)
Waymouths: Waymouth's MB 752/1 medium (Flow)
FBS: 20 percent foetal bovine serum
FBS/OS: 10 percent foetal bovine serum + 10 percent ovine serum
OS: 20 percent ovine serum
ES: 20 percent equine serum
FOSK: foetal ovine skin cells
ROK: diploid ovine kidney cell line

RPMI 1640 and Ham's F10 supplemented with FBS, OS or a combination of both sera. Cells survived in excess of 14 days in contrast to the less than 7 days observed for cells cultured in MEM, Medium 199, Waymouth's medium or if ES was a component of the medium. The fibroblast monolayer cells grew to confluency in all media and sera combinations. Following this screening only RPMI 1640-FBS was used in subsequent culture attempts.

(iii) *Plasma Clot Explant Cultures*

Twenty four hours following the establishment of OL11 in culture, small morula-like aggregations of up to 10 cells (Figure 12) were observed in the medium. These cellular aggregations increased in size (Figure 13) over the next 3 to 4 days and there was an increase in the number of single cells in suspension. Trypan blue exclusion counts showed more than 90 percent of cells in suspension were viable.

Seven days after establishment, the primary cultures of lymphoma cells were passaged. "Morulae" redeveloped in the secondary cultures and an apparent increase in cell numbers occurred. However, following the third passage after 10 days, the lymphoma cells failed to proliferate and eventually died after 21 days in culture.

A similar pattern was observed for some primary cultures of OL12, but the degree of proliferation was not as marked as for OL11 and the cells were never passaged. Most cells were dead within 14 days of initiating the culture.

No significant proliferation of lymphoma cells occurred from explant cultures of OL14, OL15 and OL16. However, large irregularly shaped motile cells possibly of monocytic origin appeared on the surface of the culture flasks of the explant cultures of spleen from OL16 after 24 hr. These were 2 to 3 times larger than lymphoma cells, did not increase in numbers after about 7 days and survived for over 3 weeks.

Approximately 5 days following explantation of all lymphomas, cells of fibroblastic morphology were seen growing out from the explanted tissues (Figure 14) under the plasma clot. These cells were adherent to the flask and were

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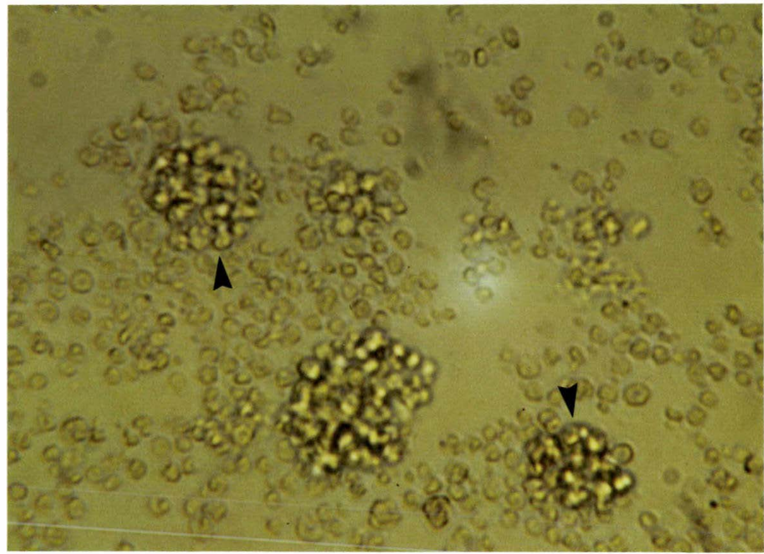
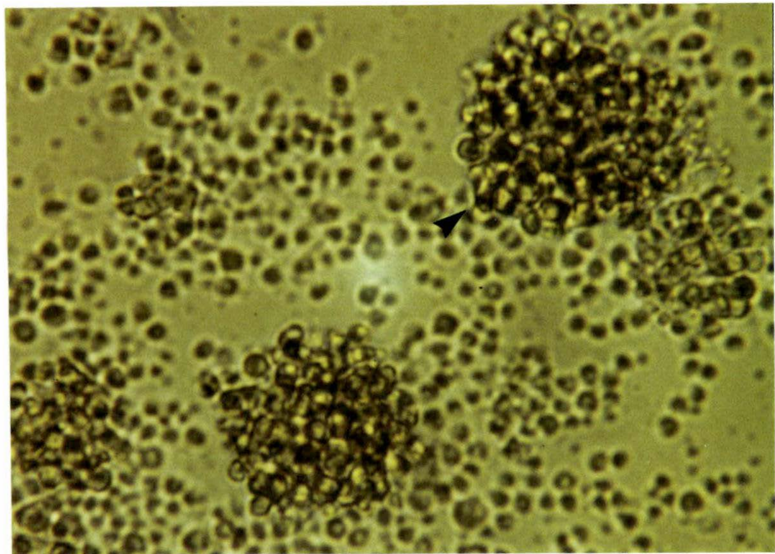
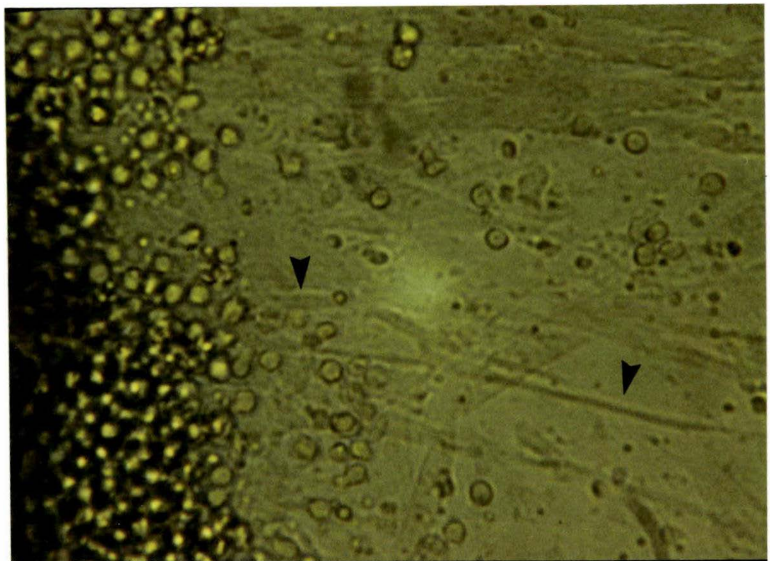
FIGURE 12: Small-morula-like clusters of cells 24 hours following the establishment of OL11 plasma clot explant cultures. (Magnification 250 times)

FIGURE 13: Larger cellular clusters several days after the establishment of OL11 plasma clot explant cultures. (Magnification 250 times)

FIGURE 14: Fibroblast-like cells (OLF cells) growing out of the lymphoma explants 5 days following the establishment of OL11 plasma clot explant cultures. (Magnification 250 times)







designated as ovine lymphoma fibroblast (OLF) cells. Only 2 lymphomas produced OLF cells that were cultured for more than 20 passages. These cells were OLF12P, OLF12L derived respectively from lymphomas of the popliteal and lumbar lymph nodes, and OLF15H, OLF15F derived respectively from a subcutaneous hindquarter and a forequarter lymphoma.

DISCUSSION

Earlier attempts in this laboratory using suspension cultures to establish cultures from sporadic ovine lymphomas had failed (Johnstone, unpublished data) and the attempts reported here using suspension cultures were also unsuccessful. As the nutritional requirements of ovine lymphoma cells in culture had not been defined it was decided to screen several different media/sera combinations over feeder fibroblast monolayers (Figure 11) to determine the most suitable media conditions for growth. Using a similar approach Epstein and Kaplan (1974, 1979) had successfully identified the specific growth requirements for the establishment of continuous cell cultures of human lymphomas. However, this technique was not successful with ovine lymphomas with the only conclusion drawn from the screening being that certain media/sera combinations allowed the longer survival of cells in culture. Equine serum, despite its usefulness in bovine lymphoma cultures (Hare *et al.*, 1968) was toxic to ovine lymphoma cells but not to the fibroblast monolayers. Although Epstein and Kaplan (1974, 1979) reported that particular feeder cells were of importance in the establishment of individual lymphoma cell cultures, Kaplan (pers. comm.) indicated that this was not as important as first thought. Therefore in the majority of the media screening experiments, the continuous ovine ROK cell line was used as a feeder layer. The choice of this particular line was one of convenience as these cells were readily available in contrast to other ovine cells such as FOSK cells which were passaged primary cells.

Utilising the information gained from the media screening experiments a plasma clot explant technique was tried (Paul,

1975). This approach was initially encouraging with OL11 being passaged twice and with OL12 showing some cellular proliferation with the formation of morula-like cellular aggregations. Romanowsky-stained smears of cells from OL11 cultures showed some cells in mitosis. However, cells from both OL11 and OL12 and the splenic explants of OL16 were motile and it is possible that the majority of the cells accumulating in the culture media had not proliferated but were viable cells migrating from the explanted neoplastic tissues. The OLF cell lines that were derived from the lymphomas by this technique provided additional lymphoma-associated material to screen for retroviruses (Section B, Chapter 4).

In all attempts efforts were made to minimise cell death before culture establishment. These included the reducing of the time lag before the preparation of the cultures, and the placing of lymphomas on ice within one hour and the cultures initiated within 3 hours of the death of the animal. Although these precautions may not have been adequate, there are other unknown factors that may be necessary for the establishment of continuous cell cultures of lymphomas and relatively few successes have been reported for either bovine (Burny *et al.*, 1978) or human lymphomas (reviewed by Epstein and Kaplan, 1979).

Whilst the results described here were discouraging, the nutrient screening technique may still be successful in defining the nutritional factors required for the establishment of ovine lymphoma cell cultures if different feeder cells, autologous sera and other nutritional combinations are used. Alternatively, the use of specifically conditioned media similar to those used in the establishment of human T cell lymphoma cell lines (Poiesz *et al.*, 1980) may be successful. In view of the difficulties encountered in later experiments to isolate and assay for a putative ovine lymphoma virus (Section B, Chapter 4), the development of such continuous cell lines is considered crucial for the complete evaluation of sporadic ovine lymphoma.

CHAPTER 4

ATTEMPTS AT THE BIOCHEMICAL DETECTION OF A RETROVIRUS ASSOCIATED WITH SPORADIC OVINE LYMPHOMA

Introduction

Materials and methods

- Cell cultures
  - (i) Ovine lymphoma cell suspension cultures
  - (ii) Control lymph node cell suspension cultures
  - (iii) Primary and continuous monolayer cell cultures
  - (iv) OLF cell cultures
  - (v) Retrovirus-infected monolayer cell cultures
  - (vi) Coculture of monolayer cultures with ovine lymphoma cells
- Virus isolation procedures
  - (i) Initial preparation
  - (ii) Sucrose density gradient centrifugation
  - (iii) Sedimentation velocity centrifugation
  - (iv) Attempted virus isolation from ovine lymphoma tissue homogenates
- ^{3}H uridine-labelling of cell cultures
- Assay for RNA-dependent DNA polymerase
  - (i) Exogenous primer-template assay
  - (ii) Exogenous primer-template assay with Actinomycin D
  - (iii) Endogenous assay
  - (iv) RNAase pretreatment of the ultracentrifuged pellet
  - (v) Assays across a sucrose density gradient
- Assay for radioactivity

Results

A. Assays for ^{3}H uridine-labelled RNA-containing virus particles
  - (i) Ovine lymphoma cell suspension cultures
  - (ii) Control lymph node cell suspension cultures
  - (iii) Continuous monolayer cell cultures
  - (iv) OLF monolayer cell cultures

B. Assays for RDDP activity in preparations from ovine lymphoma cell cultures and homogenates, OLF and cocultured fibroblasts
  - (i) Media from ovine lymphoma cell cultures

Results (*continued*)
(ii) Ovine lymphoma tissue homogenates
(iii) Media from OLF cell cultures
(iv) Media from monolayer cultures cocultured with ovine lymphoma cells
Discussion

CHAPTER 4

ATTEMPTS AT THE BIOCHEMICAL DETECTION OF A RETROVIRUS ASSOCIATED WITH SPORADIC OVINE LYMPHOMA

INTRODUCTION

Although electron microscopy can be a useful technique for the detection of retroviruses, it could not provide any information about the nature of the virus-like particles observed in 2 cases of sporadic ovine lymphoma (Section B, Chapter 2). Therefore it was necessary to use alternative methods to assay for the virus to further explore the hypothesis that a retrovirus was associated with this disease.

This chapter reports the use of 2 biochemical techniques in experiments with ovine lymphomas. Firstly, ^{3}H uridine-labelling of short term lymphoma suspension and OLF cultures was used in an attempt to detect high molecular weight RNA in particles having the bouyant density of retroviruses. Secondly, the retroviral enzyme, RDDP, was assayed for in preparations derived from suspension cultures and monolayer cocultures of ovine lymphoma, from OLF cultures and from lymphoma homogenates. Standard methods were used in the attempted viral isolations.

MATERIALS AND METHODS

Cell Cultures

(i) *Ovine lymphoma cell suspension cultures*

Cell suspensions were prepared from 6 cases of ovine lymphomas (Table 17), approximately 10^{7} cells in 5 ml of either Medium 199-FBS or RPMI 1640-FBS were seeded into plastic culture flasks as described in the previous chapter, and incubated at 37°C for 36 to 48 hr.

(ii) *Control lymph node cell suspension cultures*

The mesenteric lymph nodes of a sheep were collected from a slaughterhouse and washed twice in a Hank's balanced salt solution that contained antibiotics. The lymph nodes

were finely diced in RPMI 1640 and pressed through a 40-mesh tissue sieve. The cells were washed in medium by centrifuging them at 200 g for 10 min. Following resuspension and counting in a haematocytometer, approximately 10^7 viable cells per 5 ml of RPMI 1640-FBS were seeded into plastic culture flasks. Cultures were incubated at 37°C for 48 hr.

(iii) *Primary and continuous monolayer cell cultures*

Primary cell cultures from foetal bovine skin, lung and kidney, from foetal ovine skin, lung, kidney and testes and from feline embryos were established in Medium 199-FBS as described for FOSK cells in Section B, Chapter. These cells and the continuous line of ROK cells were passaged when confluent monolayers had formed. The cells were washed with PBS, detached at 37°C by ATV and the cells were used at approximately 2 x 10^5 per ml in Medium 199-FBS to establish new cultures.

(iv) *OLF cell cultures*

These cells were derived from the plasma clot explant cultures of ovine lymphomas and were described in Section B, Chapter 3. Media from 5 cultures (OLF11, OLF12, OLF14, OLF15 and OLF16) were collected over a number of passages and stored at -70°C. These were pooled according to each lymphoma irrespective of their tissue of origin.

(v) *Retrovirus-infected monolayer cell cultures*

BLV (New Zealand strain) infected foetal ovine spleen (BLV-FOS) cells were cultured in Medium 199-FBS and were passaged as for other monolayer cell cultures when confluent.

RSV (Prague C strain) infected chick embryo fibroblasts (RSV-CEF) were cultured in Vogt's medium (Appendix) supplemented to 20 percent with FBS (Vogt's-FBS). These cells were passaged as soon as they had become confluent. The cultures were washed once in PBS, 1 ml of ATV supplemented with 50 µl of 0.2 M EDTA was added and the cells incubated at 37°C until they had become detached. They were then resuspended in Vogt's-FBS, centrifuged at 200 g for 5 min and the supernatant removed. The cells were then resuspended

to approximately 2 x 10^5 cells per ml of medium and new culture flasks seeded. After 24 hr the medium was changed.

(vi) *Coculture of monolayer cultures with ovine lymphoma cells*

One lymphoma was cocultured with foetal bovine cells (OL1), one with foetal ovine cells (OL5) and 4 with ROK cells (OL7, OL8, OL10 and OL11). Foetal bovine and ovine monolayer cell cultures in their second to fifth passage were detached and new cultures established as described above, 24 hr prior to the addition of ovine lymphoma cells. These were added at approximately 10^6 cells per ml in either Medium 199-FBS or RPMI 1640-FBS. The bovine cocultures were passaged 5 to 6 days after establishment, with the lymphoma cells being harvested separately by centrifugation and then returned to the new cultures. The media from this and subsequent passages were collected and stored at -70°C.

The ovine cultures were not passaged, but the media were collected, pooled and also stored at -70°C.

The cocultured ROK cells were derived from the media-screen feeder-cell cultures used in an attempt to determine the nutrient requirements of ovine lymphoma cells (Section B, Chapter 3). These cells were passaged a further 4 or 5 times and the media were collected, pooled and stored at -70°C.

Other ROK cells cocultured with OL7, OL8, OL10 and OL11 were passaged and 24 hr later 5'bromodeoxyuridine (BrdU) was added at a final concentration of 40 µg per ml. Media was collected on 3 occasions over 12 days, pooled and stored at -70°C.

Virus Isolation Procedures

(i) *Initial preparation*

Media from cultures that were to be screened for retroviruses either by ^{3}H uridine-labelling or by the RDDP assay, were centrifuged at 800 g for 10 min to remove cells and then at 6,000 g for 30 min to remove subcellular debris. The resulting supernatants were then centrifuged at 80,000 g for 120 min in a SW 25.1 rotor in a Beckman L ultracentrifuge.

Each pellet was resuspended in 200 µl of a buffer (TNEM) containing 0.01 M Tris-HCl (pH 7.4), 0.1 M NaCl, 0.001 M EDTA and 0.05 percent mercaptoethanol and kept on ice until further processed by either (ii) or (iii) below.

(ii) *Sucrose density gradient centrifugation*

One hundred µl of resuspended pellet were layered onto a 5 ml 15 to 60 percent linear density gradient of sucrose in TNEM which was then centrifuged in a SW 50.1 rotor at 243,000 g for at least 4 hr at $4^{o}C$. Fractions were collected in 200 µl volumes using a peristaltic pump (Buchler Instruments) and densities were calculated from the refractive index measured with a refractometer (Abbe).

(iii) *Sedimentation velocity centrifugation*

Ten µl of 5 percent sodium dodecyl sulphate (SDS) in 45 percent ethanol (w/v) were added to 100 µl of resuspended pellet and incubated for 15 min at $37^{o}C$. The sample was then layered onto a 5 to 20 percent linear density gradient of sucrose (RNAase free, Sigma) in TNEM and centrifuged in a SW 50.1 rotor at 243,000 g for 150 min at $20^{o}C$. Gradient fractions of 200 µl were collected using a peristaltic pump and the acid-precipitable radioactivity of each determined.

(iv) *Attempted virus isolation from ovine lymphoma tissue homogenates*

The method of Nelson *et al.* (1978) was modified as follows. Neoplastic tissue (20 gm) from each of 6 lymphomas (Table 22) had been stored at $-70^{o}C$. This was thawed, minced and homogenised in twice its volume of TNEM in a Sorvall Omnimix at $0^{o}C$. The homogenate was then divided into 2 parts. One half was the test suspension whilst the other served as a positive mixing control. To the control half, 100 µl of RSV in TNEM was added. This RSV from RSV-CEF cells had been concentrated by preparative ultra-centrifugation until sufficient RDDP activity was present to incorporate 10,000 counts per min (cpm) of ^{3}H thymidine triphosphate (^{3}H TTP) after a 60 min incubation of the assay. Both halves of the lymphoma homogenate were then centrifuged at 2,000 g for 10 min at $4^{o}C$ and the resulting

supernatants centrifuged twice at 6,000 g for 30 min at 4°C. These supernatants were then layered onto a cushion of 20 percent sucrose in TNEM and centrifuged in a SW 25.1 rotor at 80,000 g for 120 min. The resulting pellets were resuspended in 200 µl of TNEM and kept on ice until assayed for RDDP.

^{3}H Uridine-Labelling of Cell Cultures

Cell cultures of 4 ovine lymphomas (OL16, OL17, OL19 and OL20) and of control mesenteric lymph nodes, OLF cells, uninfected ROK, foetal ovine testes and feline embryo fibroblast monolayer cells, and BLV-FOS and RSV-CEF cells were labelled with the addition to the medium of 8 µCi per ml of 5, 6 ^{3}H uridine (New England Nuclear) (Robinson, 1967). The media were harvested 36 to 48 hr later and processed by centrifugation and ultracentrifugation as described above. Each of the final ultracentrifuged pellets was resuspended in 200 µl of TNEM and held at 0°C.

One hundred µl of the resuspended pellet were layered over a 15 to 60 percent density gradient of sucrose (RNAase free) in TNEM. This was centrifuged from 4 to 22 hr according to the preparation at 4°C, 200 µl fractions removed and after the densities of each was measured, the acid-precipitable radioactivity was determined for each fraction including the pellet.

The second half of the resuspended pellet was treated with SDS and subjected to sedimentation velocity centrifugation as described above.

Assay for RNA-Dependent DNA Polymerase (RDDP)

The inital assay included the synthetic primer-template oligo dT:poly rA and was used to screen all material. This material included preparations from cultures of 15 lymphomas and 1 lymph node (Table 19), from 6 lymphoma and 2 lymph node homogenates (Table 22), from 8 OLF cell cultures (Table 23) and from 15 monolayer cultures cocultured with ovine lymphoma (Table 24). The assay was also performed using RSV and BLV as positive controls (Table 21).

If a preparation in this kinetic assay showed incorporation

of ^{3}H TTP into acid-insoluble material then it was necessary to test whether the activity was due to RDDP. Three additional assays were performed with each sample from 3 lymphomas and 1 lymph node to characterise the reaction (Table 20).

1. The addition of Actinomycin D to the primer-template reaction mix to inhibit DNA-directed DNA polymerases.

2. The removal of primer-template from the reaction mix to measure the endogenous activity of the preparation.

3. The RNAase pretreatment of the ultracentrifuged preparation followed by the endogenous reaction to determine the dependence of the activity on RNA template.

In all assays RSV and BLV preparations were used as controls (Table 21).

Finally, primer-template activity was measured across a sucrose density gradient to detect whether any activity was associated with a particle of known density with preparations from 7 lymphomas and 1 lymph node (Table 19), and of RSV and BLV.

In all kinetic assays, 2 criteria were applied to assess whether any activity was present in a preparation.

1. A sample was considered positive for enzyme activity only if there was an increase in ^{3}H TTP incorporation over 3 consecutive sampling times.

2. If an assay fulfilled the above criterion, then an *incorporation rate* was calculated using the formula;

$$\frac{\text{Final cpm} - \text{Initial cpm}}{\text{Total reaction time (min)}}$$

An incorporation rate of 10 cpm per min was chosen as the rate that had to be exceeded before a preparation was deemed positive for RDDP activity.

(i) *Exogenous primer-template assay*

When initially screening for RDDP activity the endogenous primer-template of oligo $(dT)_{10}$: poly rA was used to enhance sensitivity. To determine the kinetics of incorporation of ^{3}H TTP, each time point was assayed as follows:

Twenty µl of an ultracentrifuged pellet suspension in

TNEM prepared from either cell culture material or tissue homogenates, were mixed with 50 µl of reaction mix A containing 2 µM Tris-HCl (pH 8.5), 1 µM $MgCl_2$, 0.5 µM KCl and 0.6 µM dithiothreitol. This was incubated at $0^{o}C$ for 10 min in the presence of 0.1 percent NP40 detergent after which 43 µl of reaction mix B containing 0.01 µM adenosine triphosphate (ATP), 0.01 µM cytosine triphosphate (CTP), 0.01 µM guanidine triphosphate (GTP), 0.001 µM thymidine triphosphate (TTP), (all triphosphates, Sigma), 2 µCi ^{3}H TTP (New England Nuclear) and 0.005 units primer-template oligo $(dT)_{10}$:poly rA (Calbiochem) (1 unit yields an A_{260} of 1.0 in 1 ml of water) was added and the mixture incubated at $37^{o}C$ for the predetermined time. Assays were terminated in all cases by adding 1 ml of an aqueous solution at $0^{o}C$ containing 5 percent trichloroacetic acid and 1 percent (w/v) sodium pyrophosphate (TCA/NaPP). One hundred µl of a 5 mg per ml solution of bovine serum albumin was added as carrier.

(ii) *Exogenous primer-template assay with Actinomycin D*

The reaction conditions were the same as the above except that 10 µg Actinomycin D was added to reaction mix A.

(iii) *Endogenous assay*

The exogenous primer-template was excluded from reaction mix B with the other assay conditions remaining the same.

(iv) *RNAase A pretreatment of the ultracentrifuged pellet*

In these experiments 1 µg RNAase A (Sigma) was added to the mixture of resuspended pellet and reaction mix A. This was incubated for 60 min at $37^{o}C$ before the addition of reaction mix B. The assay then proceeded as (iii) above.

(v) *Assays across a sucrose density gradient*

Following centrifugation, the first 18 fractions were collected from the bottom of the gradient, were diluted in TNEM and recentrifuged at 243,000 g for 45 min at $4^{o}C$ in a SW50.1 rotor. After removing the supernatants the pellets were each resuspended in 20 µl of TNEM and reaction mix A added. The mixtures were incubated at $0^{o}C$ for 10 min after which reaction mix B was added. The final mixtures were

incubated at 37°C for 45 min. The pellet of the sucrose density gradient was also assayed for RDDP activity.

Assay for Radioactivity

Acid-precipitable radioactivity from the ^{3}H uridine-labelling experiments and the RDDP assays was measured following addition of 4 ml of TCA/NaPP to the sample. The precipitate was collected onto glass-fibre filter discs (GF/C, Whatman) by suction and the filters washed with 20 ml of TCA/NaPP followed by 30 ml of distilled water. After drying, the filters were placed into 5 ml of a toluene-based scintillation fluid (Appendix) and the levels of radioactivity measured in a Beckman LS 7000 liquid scintillation counter.

RESULTS

A. ASSAYS FOR ^{3}H URIDINE-LABELLED RNA-CONTAINING VIRUS PARTICLES

(i) *Ovine lymphoma cell suspension cultures*

Four lymphoma cultures (OL16, OL17, OL19 and OL20) were labelled with ^{3}H uridine and part of the ultracentrifuged preparations was centrifuged through a 15 to 60 percent sucrose gradient. A broad peak of radioactivity (Figure 15) at densities ranging from 1.18 to 1.14 gm per ml was found in sucrose gradients centrifuged for 4 hr (OL16 and OL20). In preparations centrifuged for more than 15 hr (OL17 and OL19) no discrete peaks (Figure 16) comparable to the RSV and BLV controls (Figures 17 and 18) were found. Most of the radioactivity was either in the gradient pellet or in fractions that had densities greater than those of the known retroviruses. Duplicate gradient centrifugations were not done for different times as there was insufficient ultracentrifuged pellet material from each lymphoma suspension culture.

SDS treatment of the other part of the ultracentrifuged pellet of OL20 was followed by sedimentation velocity centrifugation through a 5 to 20 percent sucrose gradient for 150 min. A discrete peak of radioactivity assumed to

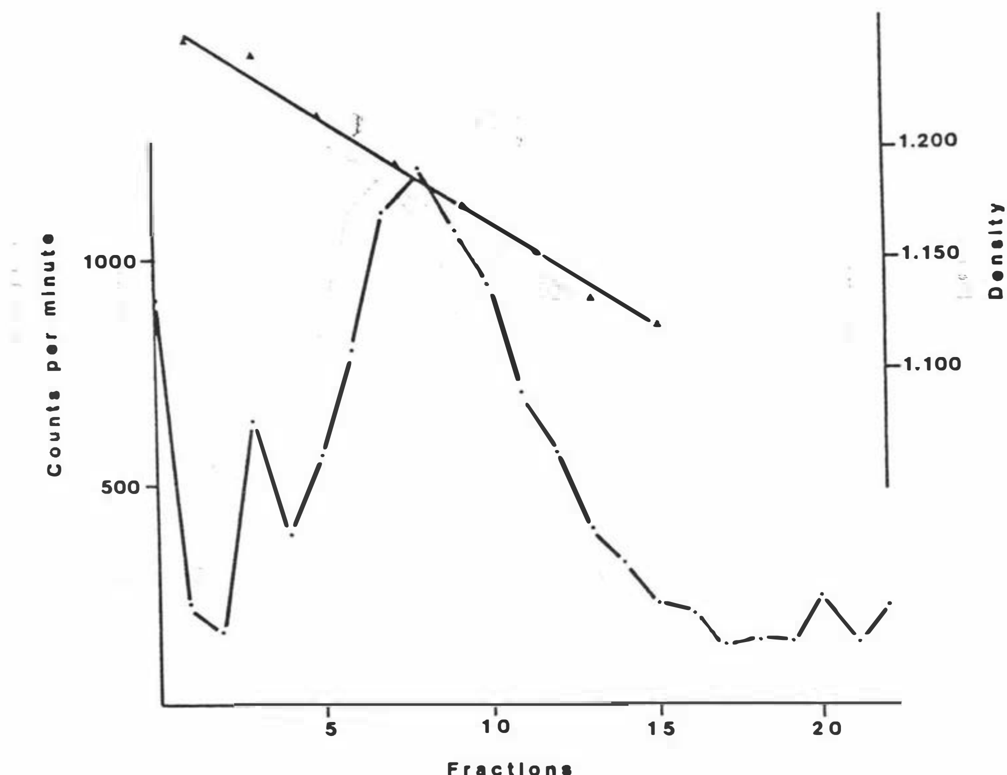


FIGURE 15: The distribution of radioactivity in a 15 to 60 percent sucrose density gradient after ^3H uridine-labelled material from cultures of OL16 was centrifuged at 243,000 g for 4 hours at $4^{\circ}C$. (▲: density in gm per ml)

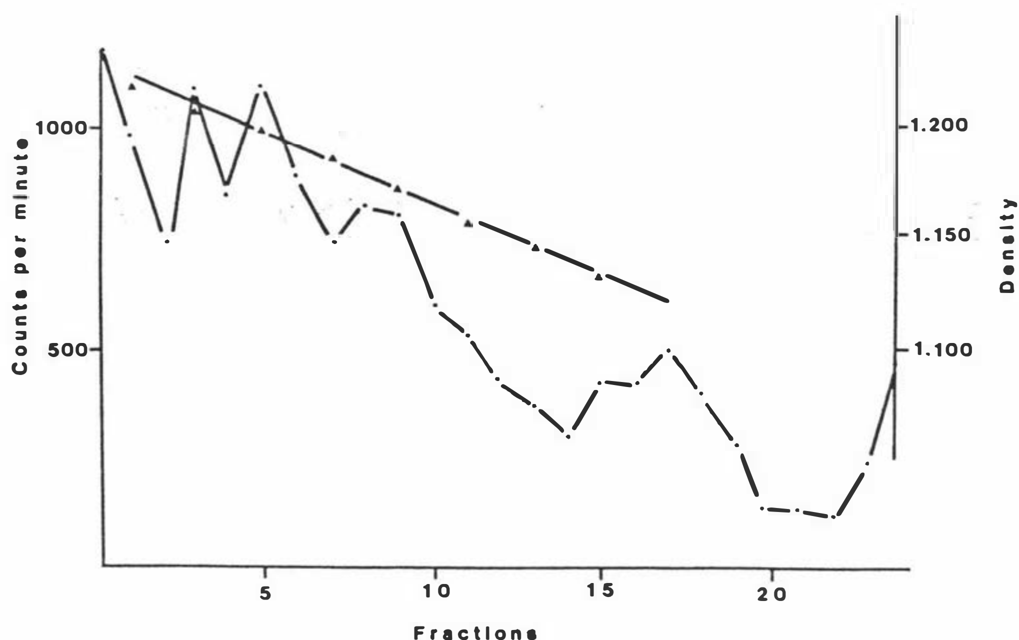


FIGURE 16: The distribution of radioactivity in a 15 to 60 percent sucrose density gradient after ^3H uridine-labelled material from cultures of OL19 was centrifuged at 243,000 g for 15 hours at $4^{\circ}C$. (▲: density in gm per ml)

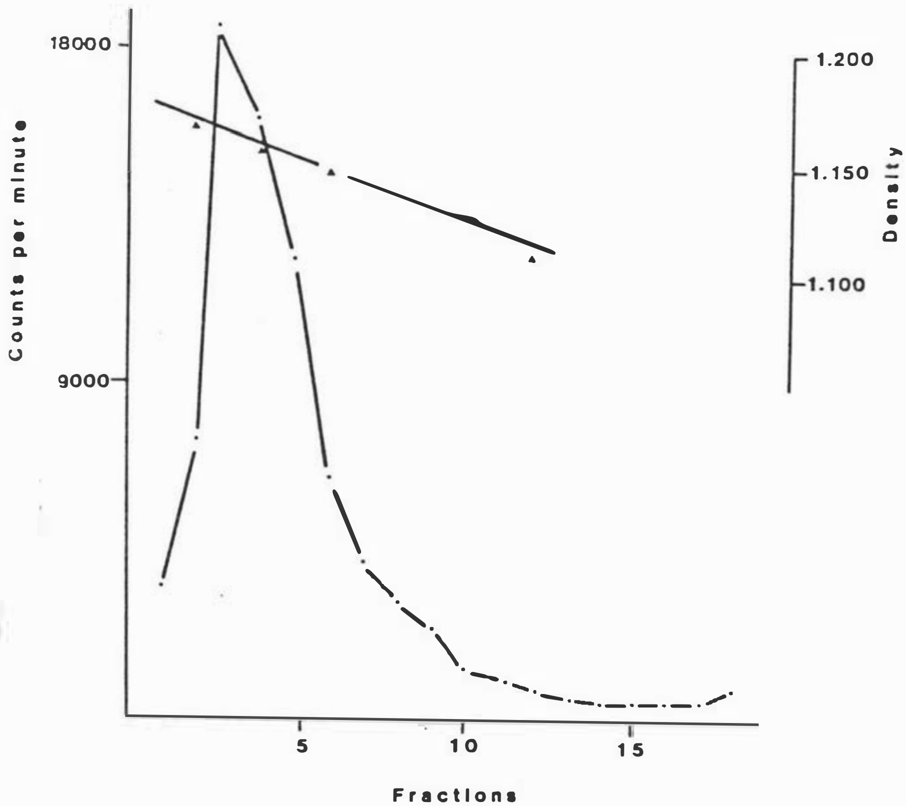


FIGURE 17: The distribution of radioactivity in a 15 to 60 percent sucrose density gradient after ^{3}H uridine-labelled Rous sarcoma virus was centrifuged at 243,000 g for 4 hours at $4^{O}C$. (▲: density in gm per ml)

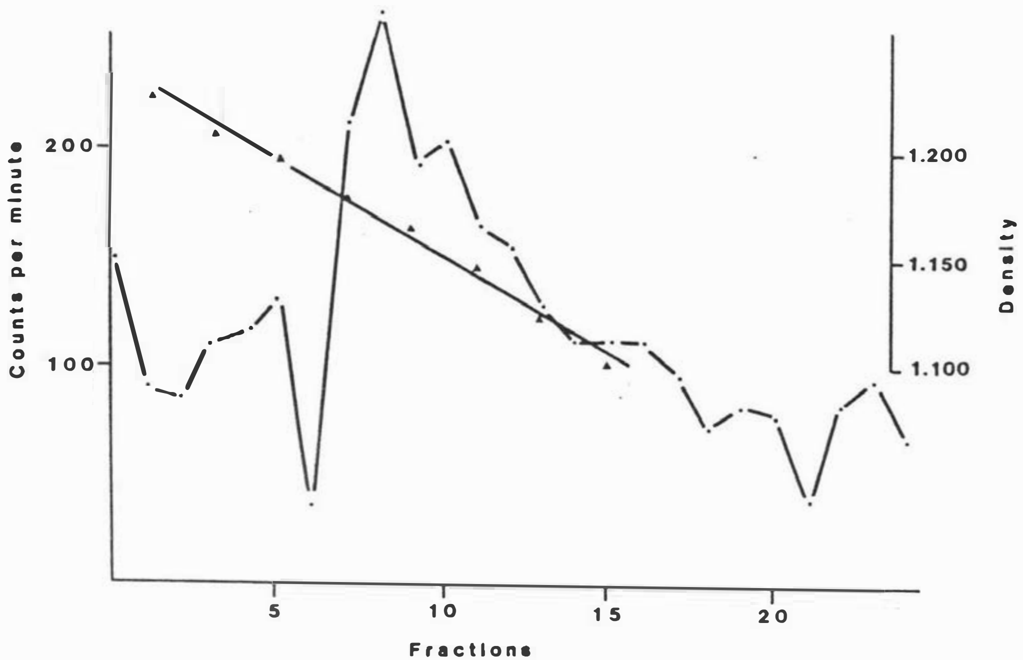


FIGURE 18: The distribution of radioactivity in a 15 to 60 percent sucrose density gradient after ^{3}H uridine-labelled bovine leukaemia virus was centrifuged at 243,000 g for 4 hours at $4^{O}C$. (▲: density in gm per ml)

be sedimenting was found (Figure 19) with a calculated sedimentation value of 7S. When ^3H uridine-labelled RSV was also subjected to sedimentation velocity centrifugation, a small yet discrete peak was observed with a sedimentation value of approximately 25S (Figure 20).

(ii) *Control lymph node cell suspension cultures*

In a control experiment using media from labelled lymph node cell suspension cultures, a peak of acid-precipitable radioactivity was found at a similar density as those for OL16 and OL20 after 4 hr centrifugation in a 15 to 60 percent sucrose gradient (Figure 21). SDS treated preparations showed a sedimentation value of approximately 7S.

(iii) *Continuous monolayer cell cultures*

To assess whether the peaks of radioactivity described in (i) and (ii) above were associated only with lymphocytes and lymphoma cells in culture, approximately 10^7 ROK, foetal ovine testes and feline embryo cells were labelled with ^3H uridine and the culture media processed for sucrose density gradient centrifugation. No peaks of radioactivity were found.

To test whether the radioactivity found in the lymphocyte and lymphoma preparations was from cellular RNA being released from degenerating cells, a simulation experiment was performed in which approximately 10^5 of the radiolabelled ROK cells were scraped from the flasks, resuspended in medium and ruptured by 20 strokes of a Dounce-type homogeniser. Ultracentrifuged pellets were prepared from this medium and duplicate sucrose density gradients centrifuged, one for 4 hr the other for 22 hr. As seen in Figure 22, a discrete peak at density 1.17 gm per ml was found after 4 hr centrifugation. This peak migrated to a density of approximately 1.22 gm per ml after 22 hr centrifugation, indicating that the radioactive material in the peak found after 4 hr was still sedimenting. When part of the initial ultracentrifuged pellet was treated with SDS and centrifuged through 5 to 20 percent sucrose a peak of radioactivity with a sedimentation value of 7S was found

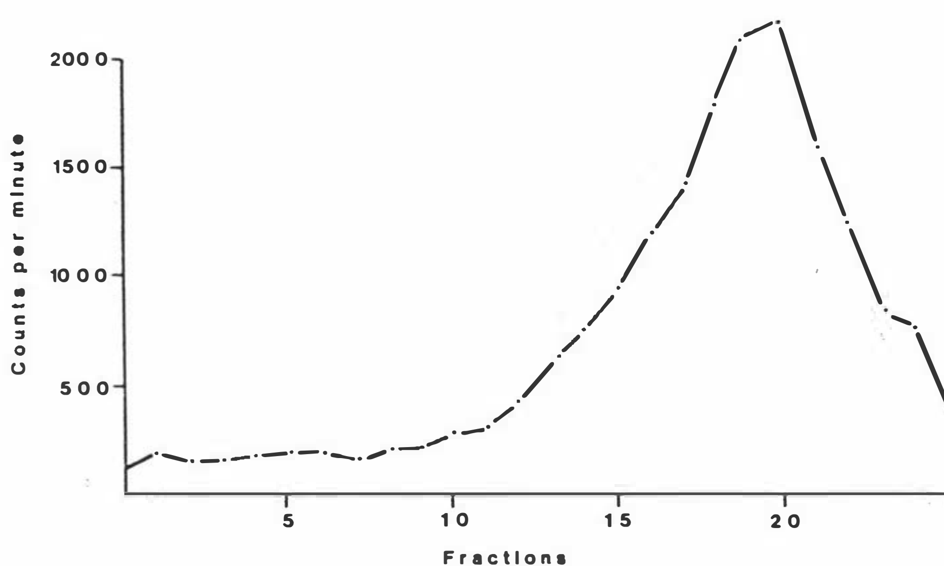


FIGURE 19: The distribution of radioactivity in a 5 to 20 percent sucrose density gradient after ^3H uridine-labelled material from cultures of OL20 was treated with sodium dodecyl sulphate and centrifuged at 243,000 g for 150 minutes at 20°C.

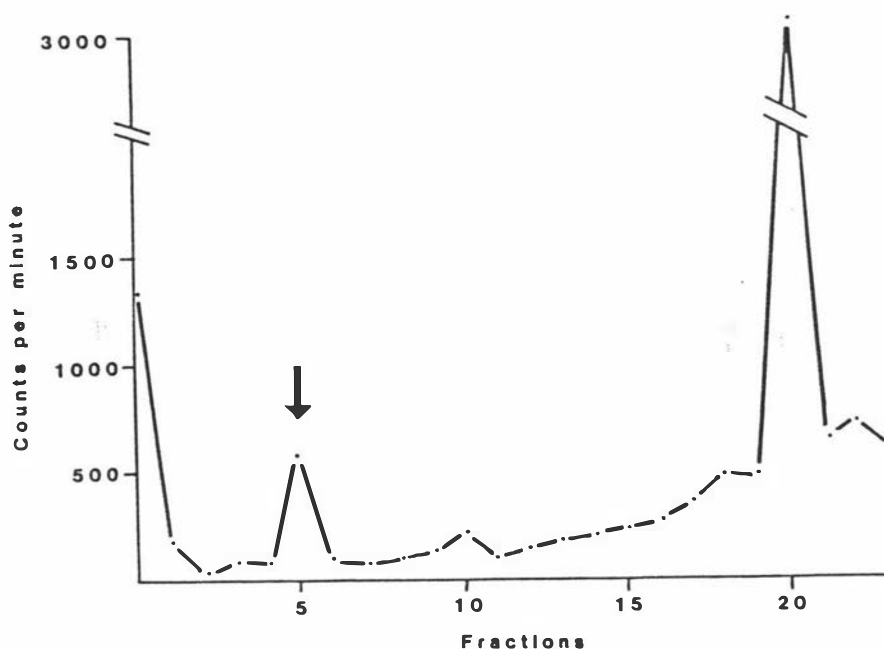
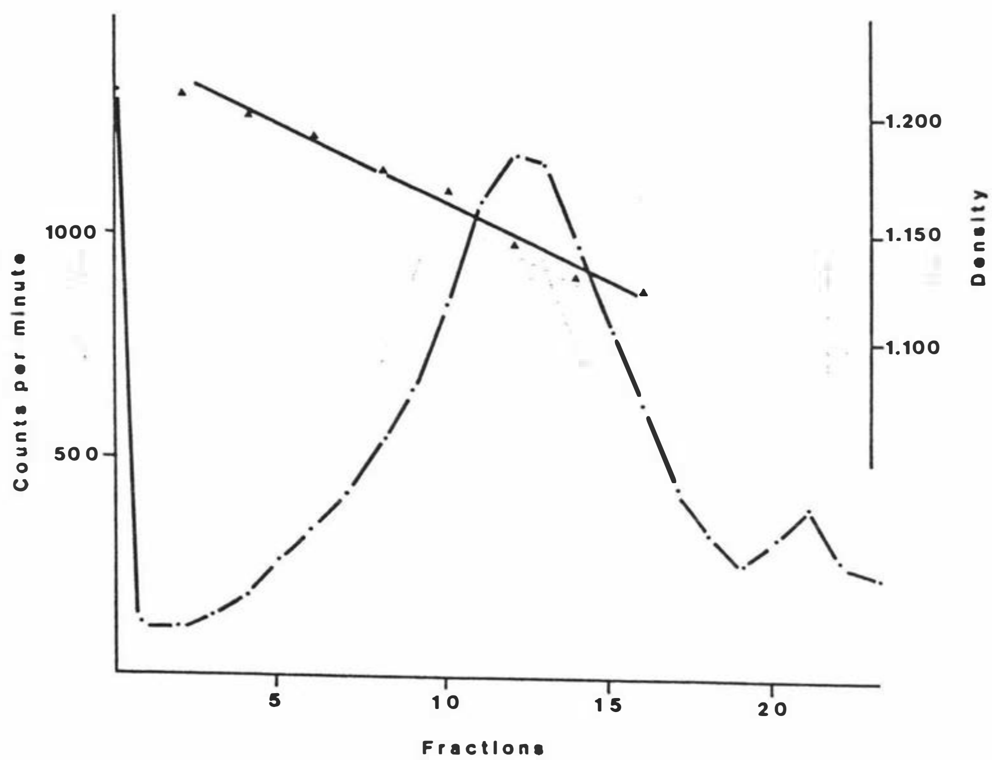
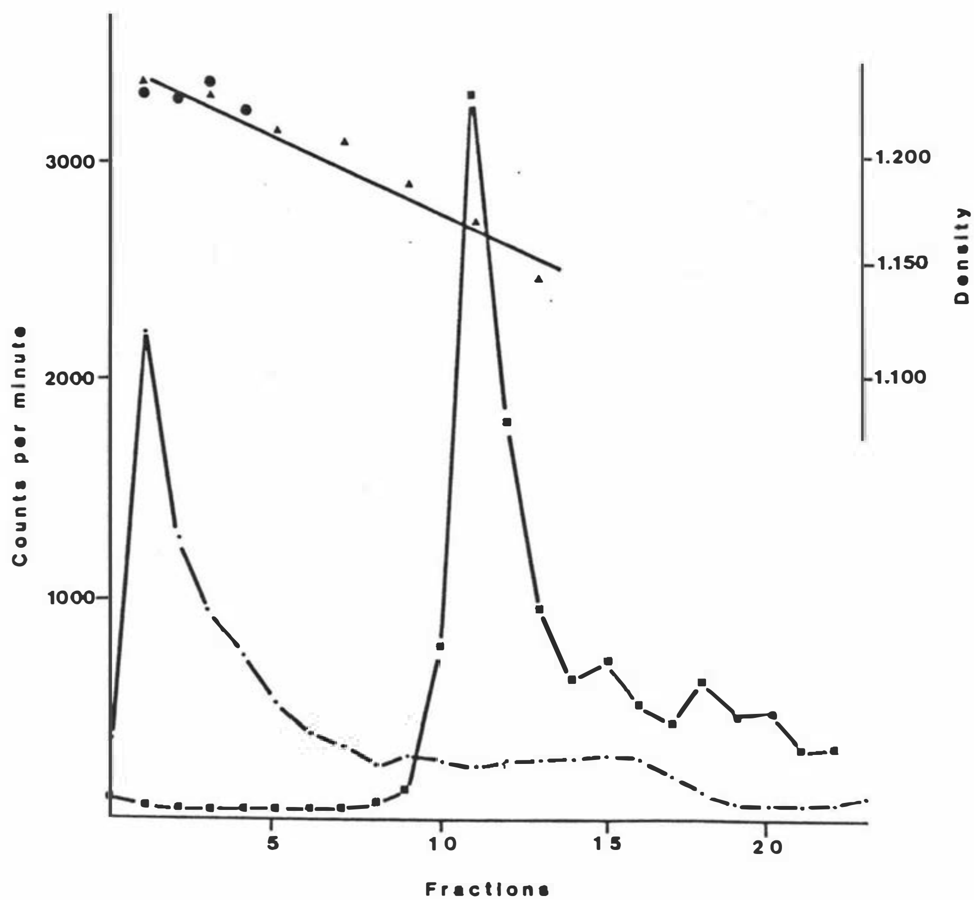


FIGURE 20: The distribution of radioactivity in a 5 to 20 percent sucrose density gradient after ^3H uridine-labelled Rous sarcoma virus was treated with sodium dodecyl sulphate and centrifuged at 243,000 g for 150 minutes at 20°C. (↓: peak of high molecular weight ^3H uridine-labelled material)

FIGURE 21: The distribution of radioactivity in a 15 to 60 percent sucrose density gradient after ^{3}H uridine-labelled material from cultures of normal mesenteric lymph node cells was centrifuged at 243,000 g for 4 hours at 4°C. (▲: density in gm per ml)

FIGURE 22: The distribution of radioactivity in duplicate 15 to 60 percent sucrose density gradients after ^{3}H uridine-labelled material from homogenised ROK cells was centrifuged at 243,000 g for either 4 hours (■—■) or 22 hours (•—•) at 4°C.
(▲: density in gm per ml of gradient centrifuged for 4 hours)
(●: density in gm per ml of gradient centrifuged for 22 hours)


Counts per minute
1000
500
5
10
15
20
Fractions
1.200
1.150
1.100
Density


Counts per minute
3000
2000
1000
5
10
15
20
Fractions
1.200
1.150
1.100
Density

similar to that from the lymphoma and normal lymph node cultures.

(iv) *OLF monolayer cell culture*

In another series of experiments ^{3}H uridine was added to OLF12P, OLF15F, OLF15H and OLF16 cell cultures between the fourth and seventh passages and the media processed as before. Discrete peaks in a 15 to 60 percent gradient were not found at the same densities from any of the cultures after 4 hr centrifugation in experiments repeated 4 times. When duplicate gradients from these 5 cultures were examined after 21 hr centrifugation, the profiles of radioactivity had altered, indicating that the radiolabelled material observed after 4 hr was still sedimenting.

In one experiment, part of the ultracentrifuged pellet prepared from OLF12P cultures was treated with SDS and centrifuged through a 5 to 20 percent sucrose gradient. The radiolabelled material had a value of approximately 7S.

There were no radioactive peaks found in sucrose gradients in repeat experiments involving OLF15F cells that had been passaged more than 15 times.

B. ASSAYS FOR RDDP ACTIVITY IN PREPARATIONS FROM OVINE LYMPHOMA CELL CULTURES AND HOMOGENATES, OLF AND COCULTURED FIBROBLASTS

(i) *Media from ovine lymphoma cell cultures*

Media from 15 ovine lymphoma cultures (Table 19) and medium from the single suspension culture of normal lymph node cells were screened for RDDP activity by the exogenous primer-template assay. Media from OL1 to OL15 were examined retrospectively after storage at -70°C. The types of ovine lymphoma culture from which the media were tested are listed in Table 17 and the results of the assays are presented in Table 19.

Only the preparations from OL11, OL12 and OL20 had incorporation rates exceeding 10 with OL19 having a marginal rate of 9.63. The others either demonstrated some incorporation with a rate less than 10 (OL1, OL2, OL5 and OL6) or failed to show a consistent increase in incorporation

TABLE 19

ASSAY FOR RNA-DIRECTED DNA POLYMERASE ACTIVITY IN OVINE LYMPHOMA CULTURE MEDIA

Source	Volume[a]	T0[b]	T60[c]	Consistent Increase	Incorporation Rate[d]	Activity in Sucrose Gradient
OL1	42	523	621	+	1.63	ND
OL2	30	207	563	+	5.93	ND
OL5	45	325	765	+	7.33	ND
OL6	40	210	540	+	5.55	ND
OL7	37	806	324	-	-	ND
OL8	35	387	505	-	-	ND
OL10	7	437	345	-	-	ND
OL11	60	40	553[e]	+	12.83	-(g)
OL12	128	521	3934[f]	+	75.84	-
OL14	40	492	675	-	-	ND
OL15	40	526	971	-	-	ND
OL16	50	798	1062	-	-	-
OL17	40	ND	ND	ND	-	-
OL19	100	296	874	+	9.63	-
OL20	200	1211	3524	+	38.55	-
Node[h]	200	237	1480	+	20.72	-

OL: ovine lymphoma

ND: not done

(a) volume in ml of culture media prepared for assay

(b) counts per min at beginning of assay

(c) counts per min after 60 min

(d) Incorporation rate= $\frac{\text{Final cpm - Initial cpm}}{\text{Total reaction time (min)}}$

(e) actually T40

(f) actually T45

(g) no activity found at density 1.15 to 1.18 gm per ml

(h) mesenteric lymph node culture from clinically normal sheep

and thus were considered negative.

Activity was also found in preparations from the control lymph node cell cultures with an incorporation rate of 20.72.

Experiments were carried out to test whether the activities observed in OL11, OL19, OL20 and lymph node cultures were due to RDDP (Table 20) with RSV and BLV being used as positive controls (Table 21). For both RSV and BLV, Actinomycin D did not inhibit the primer-template reaction; there was nearly a tenfold decrease in RSV activity (Figure 23) and over a threefold decrease in BLV activity when primer-template was removed (Figure 24); and for RSV, pretreatment of the virus with RNAase A virtually abolished incorporation. However, RNAase A did not affect the endogenous activity of BLV under the same conditions.

Compared with the controls, little variation in incorporation could be evinced by altering the assay conditions for OL11, OL19, OL20 and control lymph node preparations. This is clearly illustrated by the kinetic plots of OL20 (Figure 25) and control lymph node activities (Figure 26). Hence it was concluded that the observed activities from these 4 preparations were not dependent on primer-template and were not sensitive to Actinomycin D or RNAase A.

For 7 lymphoma and the control preparations activity was measured across sucrose gradients. No discrete peaks were found with most of the activity detected, if any, being located in the gradient pellet. This contrasted sharply with RSV and BLV controls (Figure 27 and 28).

(ii) *Ovine lymphoma tissue homogenates*

No significant activity could be detected in any of the 6 lymphomas, the 2 control lymph node preparations or recovered from material that had added RSV (Table 22). No difference was found between the reactions that included or excluded primer-template.

(iii) *Media from OLF cell cultures*

Ultracentrifuged preparations of media from 5 OLF cell

TABLE 20

ASSAYS FOR RNA-DIRECTED DNA POLYMERASE ACTIVITY IN OVINE LYMPHOMA CULTURE MEDIA USING DIFFERENT REACTION CONDITIONS AND Mg^{2+} AS THE DIVALENT CATION

Source	Volume[a]	Reaction Conditions	T0[b]	T60[c]	Consistent Increase	Incorporation Rate[d]
OL11	60	Primer/Template	269	156[e]	-	-
		No Primer/Template	361	335[e]	-	-
		No Primer/Template + RNAase A	764	655[e]	-	-
OL19	100	Primer/Template	149	419	+	4.50
		Primer/Template + Actinomycin D	190	380	+	3.17
		No Primer/Template	233	441	+	3.47
OL20	200	Primer/Template	312	1064	+	12.53
		Primer/Template + Actinomycin D	414	1798	+	23.07
		No Primer/Template	333	1238	+	15.08
		No Primer/Template + RNAase A	813	1650	+	13.95
Node[f]	200	Primer/Template	237	1480	+	20.72
		Primer/Template + Actinomycin D	409	1347	+	15.63
		No Primer/Template	430	1809	+	22.98
		No Primer/Template + RNAase A	651	2342	+	28.18

OL: ovine lymphoma
(a) volume in ml of culture media prepared for assay
(b) counts per min at beginning of assay
(c) counts per min after 60 min
(d) see Table 19
(e) actually T30
(f) mesenteric lymph node culture from clinically normal sheep

TABLE 21

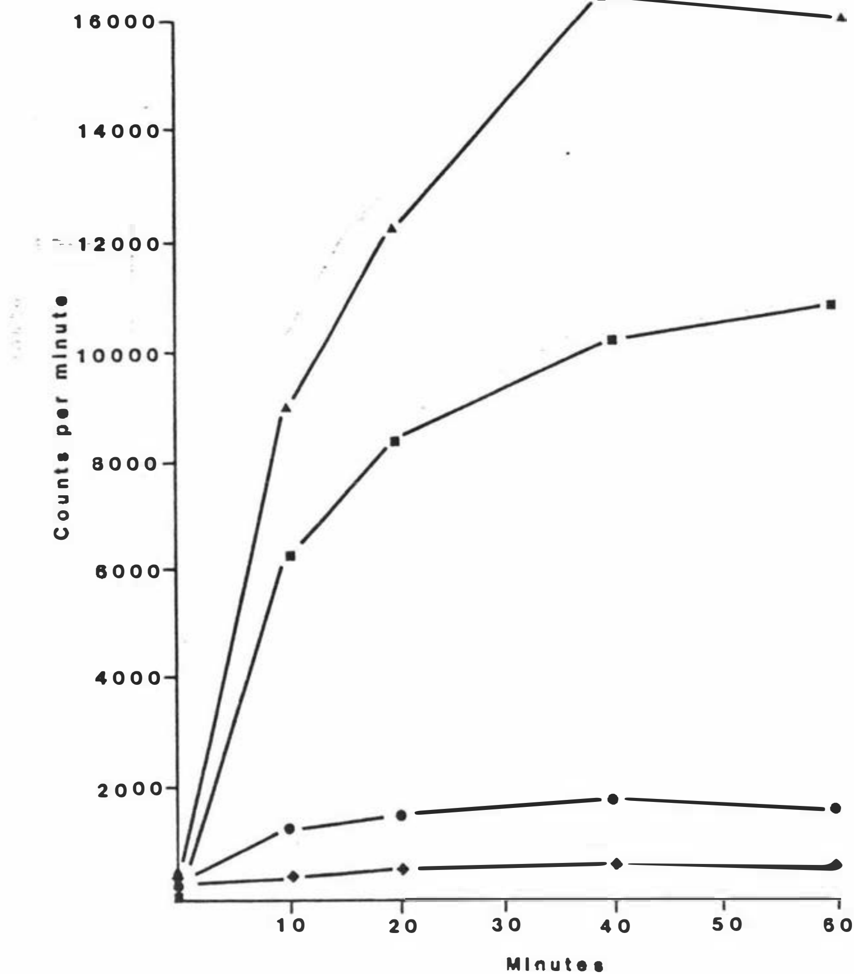
RNA-DIRECTED DNA POLYMERASE ACTIVITY OF ROUS SARCOMA VIRUS AND BOVINE LEUKAEMIA VIRUS USING DIFFERENT ASSAY CONDITIONS

Source	Volume[a]	Reaction Conditions	T0[b]	T60[c]	Consistent Increase	Incorporation Rate[d]	Divalent Cation
RSV	20	Primer/Template	138	2227	+	34.82	Mg^{2+}
RSV	20	Primer/Template	185	19051	+	314.43	Mg^{2+}
RSV	20	Primer/Template	52	4504	+	74.22	Mg^{2+}
	20	Primer/Template	83	915	+	13.87	Mn^{2+}
		Primer/Template	53	341	+	4.80	EDTA+ No cation
RSV	20	Primer/Template	86	10891	+	180.08	Mg^{2+}
	20	Primer/Template + Actinomycin D	274	16105	+	263.85	Mg^{2+}
	20	No Primer/Template	258	1673	+	23.58	Mg^{2+}
	20	No Primer/Template + RNAase A	212	568	+	5.93	Mg^{2+}
BLV	450	Primer/Template	895	2381	+	24.77	Mg^{2+}
BLV	1200	Primer/Template	601	4106	+	58.42	Mg^{2+}
		Primer/Template + Actinomycin D	566	3621	+	50.92	Mg^{2+}
		No Primer/Template	493	1550	+	17.62	Mg^{2+}
		No Primer/Template + RNAase A	737	2339	+	26.70	Mg^{2+}

RSV: Rous sarcoma virus
BLV: bovine leukaemia virus
(a) volume in ml of culture media prepared for assay
(b) counts per min at beginning of assay
(c) counts per min after 60 min
(d) see Table 19

FIGURE 23: The kinetics of incorporation of ^{3}H thymidine triphosphate into acid-precipitable material by Rous sarcoma virus in the RNA-directed DNA polymerase assay.
(■——■: assay with primer-template)
(▲——▲: assay with primer-template and Actinomycin D)
(●——●: assay without primer-template)
(◆——◆: pretreatment with RNAase A and assay without primer-template)

FIGURE 24: The kinetics of incorporation of ^{3}H thymidine triphosphate into acid-precipitable material by bovine leukaemia virus in the RNA-directed DNA polymerase assay.
(■——■: assay with primer-template)
(▲——▲: assay with primer-template and Actinomycin D)
(●——●: assay without primer-template)
(◆——◆: pretreatment with RNAase A and assay without primer-template)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12000
10000
8000
6000
4000
2000
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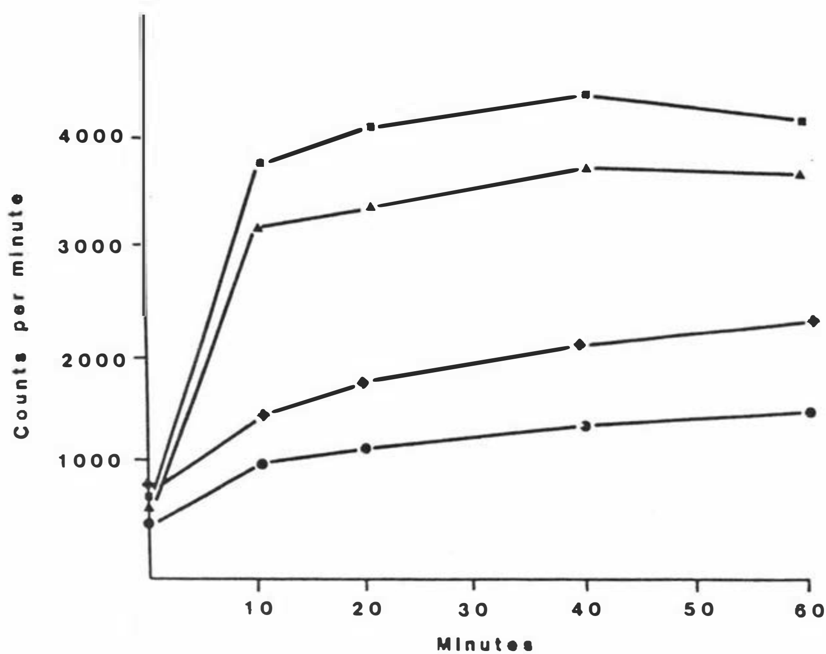
10
20
30
40
50
60
Minutes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40
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Minutes

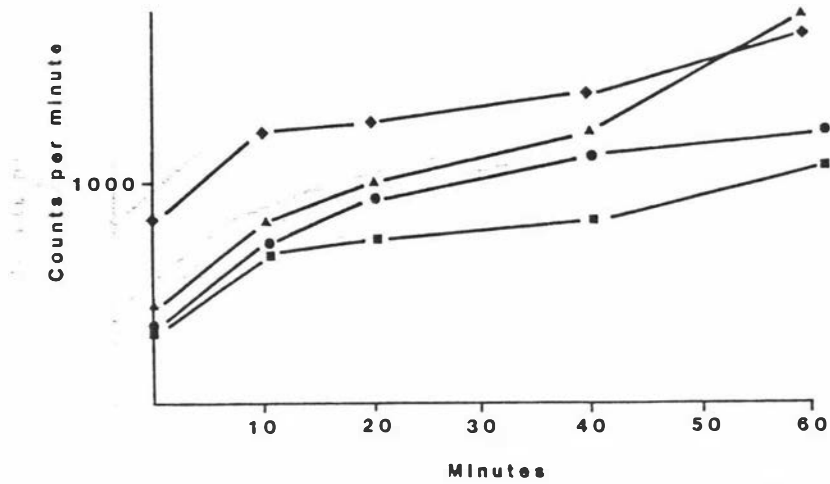


FIGURE 25: The kinetics of incorporation of ^{3}H thymidine triphosphate into acid-precipitable material by preparations from cultures of OL20 in the RNA-directed DNA polymerase assay.
(■——■: assay with primer-template)
(▲——▲: assay with primer-template and Actinomycin D)
(●——●: assay without primer-template)
(◆——◆: pretreatment with RNAase A and assay without primer-template)

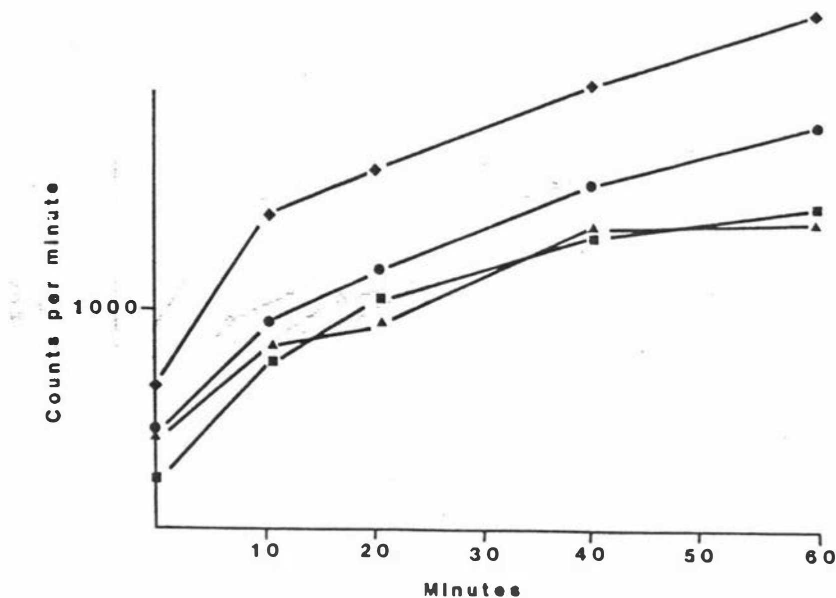


FIGURE 26: The kinetics of incorporation of ^{3}H thymidine triphosphate into acid-precipitable material by preparations from cultures of normal mesenteric lymph node cells in the RNA-directed DNA polymerase assay.
(■——■: assay with primer-template)
(▲——▲: assay with primer-template and Actinomycin D)
(●——●: assay without primer-template)
(◆——◆: pretreatment with RNAase A and assay without primer-template)

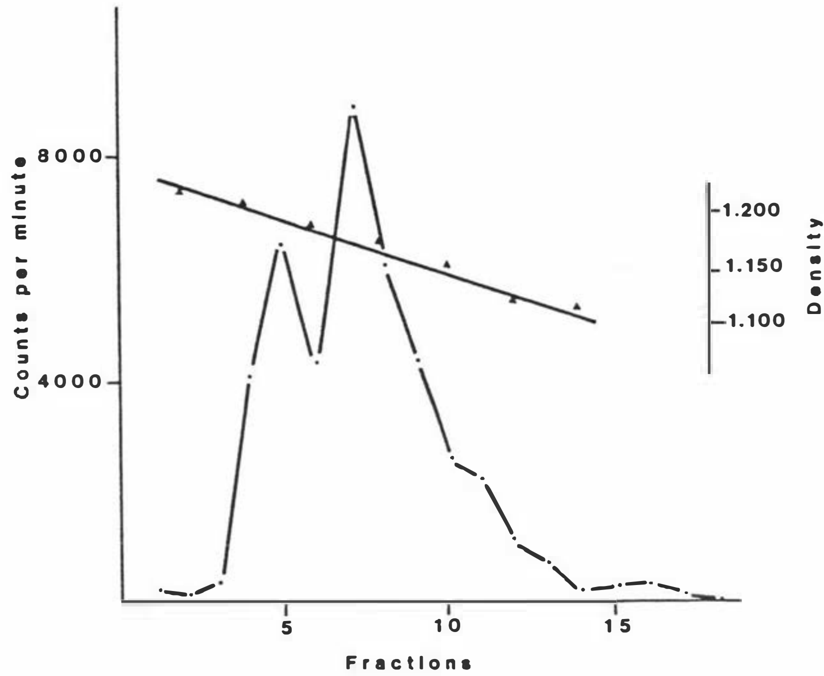


FIGURE 27: The distribution of RNA-directed DNA polymerase activity of Rous sarcoma virus in a 15 to 60 percent sucrose density gradient centrifuged at 243,000 g for 4 hours at 4°C. (▲: density in gm per ml)

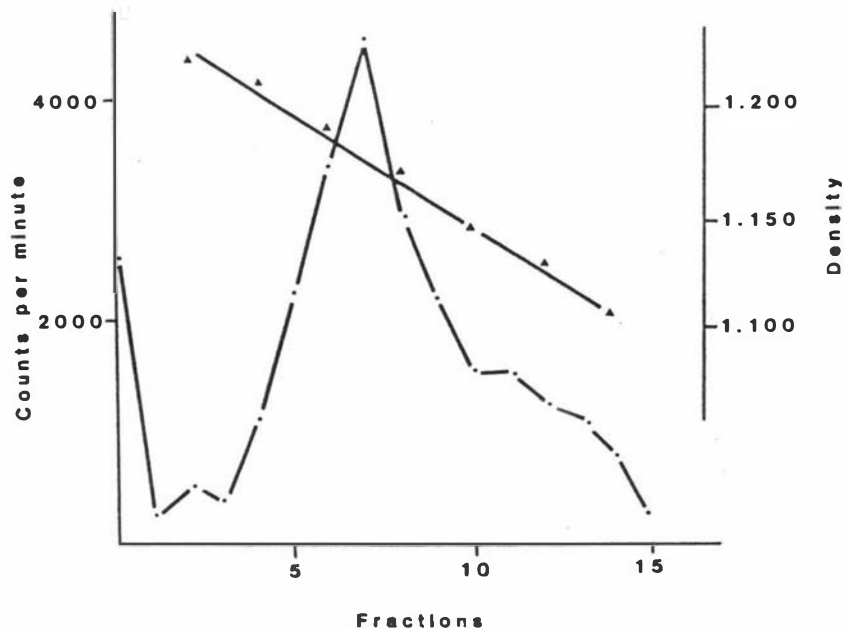


FIGURE 28: The distribution of RNA-directed DNA polymerase activity of bovine leukaemia virus in a 15 to 60 percent sucrose density gradient centrifuged at 243,000 g for 4 hours at 4°C. (▲: density in gm per ml)

TABLE 22

ASSAYS FOR RNA-DIRECTED DNA POLYMERASE
ACTIVITY IN OVINE LYMPHOMA HOMOGENATES

Source	Primer Template	T0[a]	T60[b]	Consistent Increase	Incorporation Rate[c]
Node 1[e]	-	717[d]	860	+	2.86
Node 1 + RSV[f]	-	202	628	+	7.10
Node 2	+	159	457	+	4.79
Node 2 + RSV	+	199	352	+	2.55
OL2	-	124	275	-	-
OL2 + RSV	-	75	295	+	3.67
OL11	-	55	256	+	3.35
OL11 + RSV	-	99	218	+	1.98
OL14	+	111	344	+	3.88
OL14 + RSV	+	73	364	+	4.85
OL16	-	235	568	+	5.55
OL16 + RSV	-	269	559	+	4.83
OL19	+	129	356	+	3.78
OL19 + RSV	+	106	495	+	6.48
OL20	+	156	382	+	3.76
OL20 + RSV	+	171	297	+	2.10

RSV: Rous sarcoma virus
OL: ovine lymphoma
(a) counts per min at beginning of assay
(b) counts per min after 60 min
(c) see Table 19
(d) actually T10
(e) mesenteric lymph nodes from clinically normal sheep
(f) RSV added in a mixing experiment

cultures did not show any significant incorporation of ^{3}H TTP (Table 23).

(iv) *Media from monolayer cultures cocultured with ovine lymphoma cells*

The lymphomas and monolayer cells used and the results of the assays are listed in Table 24. Significant activity was found in the foetal bovine lung cell culture media and in the pooled media from the foetal ovine cell cultures. Insufficient material was available to assess the nature of the activity observed as the bovine cells did not survive past the twelfth passage and the ovine cells did not passage at all.

No activity could be detected in the pooled media from ROK cocultured cells. The use of Mn^{2+} as an alternative divalent cation, or the use of EDTA without cations had little effect when compared to that of different cations on RSV activity (Table 22).

Finally, using BrdU an attempt was made to enhance retrovirus production from ROK cocultured cells. The media were pooled and assayed using either Mg^{2+} or Mn^{2+}. Activity was not detected (Table 24) nor was it found in control media from ROK cells not cocultured with ovine lymphomas.

DISCUSSION

The addition of ^{3}H uridine to cell cultures and the subsequent analysis of culture supernatants by density gradient centrifugation has been employed to detect RSV (Robinson, 1967), Rauscher MuLV (Barbieri-Weill *et al.*, 1979) FeLV (Sarma *et al.*, 1970), enzootic ovine lymphoma virus (Paulsen *et al.*, 1976) BLV (Ressang *et al.*,1974; Weiland *et al.*, 1974) and 2 other retroviruses, one associated with canine lymphoma (Onions, 1980) and the other with human leukaemia (Mak *et al.*, 1975). When this technique was applied to short term suspension cultures of sporadic ovine lymphomas, a radioactive peak in the density range of retroviruses was found in sucrose gradients through which preparations from OL16 and OL20 had been centrifuged for 4 hours. However, a similar peak was obtained from control preparations of normal lymph node

TABLE 23

ASSAYS FOR RNA-DIRECTED DNA POLYMERASE ACTIVITY IN MEDIA FROM OVINE LYMPHOMA FIBROBLAST CELL CULTURES

Source	Volume[a]	T0[b]	T60[c]	Consistent Increase	Incorporation Rate[d]	Divalent Cation
OLF11	160	201	347[e]	+	2.65	Mg^{2+}
		178	317[e]	+	2.53	Mn^{2+}
OLF11	200	91	439	+	5.80	Mg^{2+}
OLF12	60	279	157[f]	-	-	Mg^{2+}
OLF12	120	159	423	+	4.4	Mg^{2+}
OLF14	30	82	245[f]	+	4.08	Mg^{2+}
OLF15	60	289	168[f]	-	-	Mg^{2+}
OLF15	260	490	832	-	-	Mg^{2+}
OLF16	30	338	102	-	-	Mg^{2+}

OLF: ovine lymphoma fibroblast cells
(a) volume in ml of culture media
(b) counts per min at beginning of assay
(c) counts per min after 60 minutes
(d) see Table 19
(e) actually T55
(f) actually T40

TABLE 24

ASSAYS FOR RNA-DIRECTED DNA POLYMERASE ACTIVITY IN MEDIA FROM CELL CULTURES COCULTURED WITH OVINE LYMPHOMA

Cells	OL	Volume[a]	T0[b]	T60[c]	Consistent Increase	Incorporation Rate[d]	Divalent Cation
FBK	OL1	60	127	232[d]	-	-	Mg^{2+}
FBE	OL1	90	142	395[e]	-	-	Mg^{2+}
FBL	OL1	120	75	221[e]	+	3.65	Mg^{2+}
FBL	OL1		37	793[f]	+	12.60	Mg^{2+}
FOK, FOSK, FOL	OL5	307	978		+	11.18	Mg^{2+}
ROK	OL7	50	181	238	+	0.95	Mg^{2+}
ROK	OL8	50	388	240	-	-	Mn^{2+}
ROK	OL10	50	151	235	+	1.40	EDTA
ROK	OL11	180	376	344	-	-	Mg^{2+}
ROK + BrdU	OL7, OL8	100	576	406	-	-	Mg^{2+}
ROK + BrdU	OL10, OL11	100	539	250	-	-	Mg^{2+}

OL: ovine lymphoma
FBK: foetal bovine kidney
FBE: foetal bovine epithelial (skin)
FBL: foetal bovine lung
FOK: foetal ovine kidney
FOSK: foetal ovine skin
FOL: foetal ovine lung
ROK: diploid ovine kidney cell line
BrdU: 5' bromodeoxyuridine
(a) volume in ml of culture media prepared for assay
(b) counts per min at beginning of assay
(c) counts per min after 60 min
(d) see Table 19
(e) actually T40
(f) twice volume of ultracentrifuged pellet

cultures and of ruptured radiolabelled ROK cells after a similar period of centrifugation. The peaks derived from the ROK cells shifted to higher densities following a further 18 hours centrifugation. Insufficient material was available to repeat these experiments with OL16 and OL20, but there were no peaks found at the density of 1.5 to 1.18 gm per ml in similar preparations from OL17 and OL19 that had been centrifuged for more than 15 hours. It was concluded that the ^{3}H uridine-labelled material in these gradinets was unlikely to be retrovirus. Boone *et al.* (1969) showed that a significant amount of cellular RNA could be trapped with plasma membrane fragments and it is therefore likely that the peaks found in OL16, OL20 and control lymphocyte gradients were due to sedimenting cellular RNA. In support of this explanation was the observation that high molecular weight RNA could not be found in SDS treated pellets from any culture preparations.

Similarly, media from ^{3}H uridine-labelled fibroblast cultures derived from ovine lymphomas (OLF cells) showed no evidence of containing retroviruses or high molecular weight RNA although some sedimenting radiolabelled material was detected. The results can be interpreted in the same way as were those from the ovine lymphoma cultures. The validity of this explanation is supported by the number of experiments completed and by the duplicate gradients that were assayed after different times of centrifugation. The smaller peaks of radioactivity found in gradient preparations derived from later OLF cell passages could be due to the selection for cells more stable under culture conditions and therefore less likely to degenerate and release RNA. Support for this hypothesis came from experiments involving other labelled monolayer cell cultures including ROK cells in the fiftieth passage, and foetal ovine testes and feline embryo cells. Little or no radioactivity was found in sucrose gradients of preparations from these media, showing that some cell cultures do not readily release RNA into the culture media.

Although the low molecular weight RNA detected in ovine lymphoma and OLF cultures is likely to be of cellular origin, another possibility is that it is retroviral RNA not easily recoverable in high molecular weight form. Attempts to demonstrate 70S RNA directly in preparations of BLV have been unsuccessful (Weiland *et al.*, 1974; Mussgay and Kaaden, 1978) and it has required indirect methods such as a simultaneous detection assay (Schlom and Spiegelman, 1971) to demonstrate the presence of high molecular weight RNA (Kettmann *et al.*, 1975). This inability to directly detect 70S RNA may be due to virion instability. Barbieri-Weill *et al.*(1979) found that attenuated strains of Rauscher MuLV collected from cultures at 18 to 24 hour intervals were unstable as judged by the inability to detect 60-70S or 35S RNA, a reduction in RDDP activity and the loss of virion infectivity. However, if this MuLV strain was collected at 6 hour intervals then these properties were comparable to those of the nonattenuated strains. Virion instability may have contributed to the large amount of low molecular weight material found in the control preparations of RSV described in these experiments (Figure 20) but a more likely explanation is that the RSV was collected from aging, static cultures and the culture media may have contained cellular RNA. If the putative ovine lymphoma virus was intrinsically fragile then this could therefore cause difficulty in its detection and the release of lysosomal and cytoplasmic enzymes from dying cells in culture might further accomplish virion degradation. In these experiments, precautions taken to reduce the likelihood of virion breakdown were, of necessity, intuitive compromises. The time allowed for incorporation of ^{3}H uridine had to be sufficient to allow the accumulation of detectable quantities of virus but not long enough for significant degradation of the virus to occur. Standard methods such as the baking of glassware and the use of RNAase-free sucrose were used to reduce the exogenous introduction of RNAase into the assays and efforts

were made to reduce any residual RNAase activity by processing culture media at 0 to 4°C.

The second method used in an attempt to detect retroviruses involved assays for RDDP. Culture media from ovine lymphoma, OLF, and cocultured fibroblast cultures, and preparations from neoplastic tissue homogenates were assayed. The reagents and conditions chosen for the RDDP assay were similar to those described to detect the enzyme in the ovine maedi-visna virus (Schlom *et al.*, 1971), BLV (Gilden *et al.*, 1975; Wuu *et al.*, 1977) and RSV. Although methods that are suitable for one type of retrovirus may not be suitable for another, it was considered that conditions that could detect RDDP in an ovine retrovirus was a reasonable choice for initial investigation of a suspected virus in ovine lymphoma.

The use of a primer-template is essential for maximal sensitivity in the RDDP assay, but there is a lack of agreement as to which template is the most specific. The template used in these assays, oligo dT: poly rA, is the most sensitive for detecting RDDP in most retroviruses (Kiessling and Goulian, 1976; Sarngadharan *et al.*,1976). It can, however, be utilised by some cellular DNA polymerases (Knopf *et al.*, 1976; Robert-Guroff *et al.*, 1977). To confirm therefore, that any activity detected is RDDP, other criteria must be satisfied. This may entail the use of alternative primer-templates but should also include the use of an assay without synthetic primer-templates, to demonstrate that the enzyme detected can transcribe DNA from virion RNA. This endogenous reaction should be RNAase-sensitive and not be affected by Actinomycin D. Other criteria for the specificity of RDDP activity are listed by Sarngadharan *et al.* (1976) and Verma (1977).

Although a more standard measure of RDDP activity would be the calculation of the actual amount of ^{3}H TTP incorporated, the use of an incorporation rate was found to be a more convenient measure in these preliminary experiments. An incorporation rate

of 10 was arbitarily chosen as this appeared to reflect an overall increase in cpm from background to the final cpm by at least a factor of 3. A T_0/T_{60} ratio (Onions, 1980) was not a reliable indicator of this activity as background at T_0 varied from experiment to experiment.

Although there was significant incorporation of ^3H TTP into acid insoluble material by preparations from 3 ovine lymphoma cultures, none of the activities detected behaved like RDDP. When assay conditions were varied, incorporation was not enhanced by primer-template, the endogenous reaction was not abolished by RNAase A under conditions that did so for RSV and activity was not associated with particles of the bouyant density of retroviruses. In addition, similar activities were found in ultracentrifuged preparations from control lymph node cultures. The nature of this activity was not determined. In normal human lymphocytes there are DNA polymerase activities primed by small RNA molecules (Fox *et al.*,1973; Neubort and Bases, 1974). The endogenous activity from such molecules utilising cellular polymerases may be RNAase resistant if the RNA is bound to DNA, and would be independent of the primer-template.

RNAase-insensitive endogenous RDDP activity has been reported by Kiessling and Goulian (1979). The endogenous reactions of purified MuLV and AMV are 80 percent sensitive to RNAase (Kiessling *et al.*, 1972; Kiessling and Goulian, 1976, 1979). However, cell fractions of infected murine and avian cell cultures had reduced RDDP sensitivities to RNAase, and, in human leukaemic and normal cells, RNAase treatment enhanced incorporation (Kiessling and Goulian, 1979). This enhancement may have been due to the presence of alternative templates such as contaminating DNA fragments or the increase availability of incompletely hydrolysed RNA fragments. This enhancement phenomenon was noted in the experiments with ovine lymphomas reported here and may have been due to the contamination by cellular nucleic acids from both ovine lymphoma and normal lymphocyte cultures. It is

not understood why this also occurred for the BLV control (Figure 26). Assay conditions that were sufficient to demonstrate the RNAase-sensitivity of the endogenous RDDP activity of RSV were not effective with BLV. The enhancement following the treatment of BLV with RNAase was not a direct result of the preincubation for one hour as RDDP activity did not increase in a control experiment when RNAase was excluded from the preincubation mixture. These observations suggest that the presence of RDDP can not be excluded even if its endogenous activities are insensitive to RNAase.

The observed activity in cultured normal sheep lymphocytes needs further investigation considering that a similar activity found has been found in human lymphocytes (Kiessling and Goulian, 1979). This activity in sheep cells may be restricted to those of lymphoid derivation as whole cell homogenates of ROK cells did not show any significant incorporation (data not presented).

No significant activity with or without template was found in either ovine lymphoma or normal lymph node homogenates which was unexpected considering that activity was found in preparations from short term cultures of such material. Freezing may have destroyed this activity as unlike the culture preparations all tissue used had been stored at -70°C. The apparent sensitivity to freezing may be indirect evidence against the RDDP nature of the activity observed in these culture experiments, as Kiessling and Goulian (1979) found that retroviral RDDP was unaffected by long term storage at -20°C, or by several freeze-thaw cycles of infected cells.

Mixing experiments using RSV were done with node and lymphoma homogenates to assess the potential loss of RDDP activity due to the technique and from the possible presence of inhibitory substances. The amount of virus added could incorporate a total of 10,000 cpm of ^{3}H TTP after 60 min incubation. There was at least a 1,000-fold loss of RSV, a loss much higher than those reported in similar reconstition and recovery experiments. In experiments with parainfluenza

virus type 3, a 100-fold loss of virus occurred when it was mixed with lung homogenates (Dr J.K. Clarke, Department of Microbiology and Genetics, Massey University, pers. comm.). Kiessling and Goulian (1979) reported only a 30 percent loss in retroviral recovery experiments using cell cultures. The major implication of the mixing experiments involving ovine lymphoma was that a loss of the putative ovine lymphoma virus would also have occurred. Better recoveries may have resulted from continuous lymphoma cell lines but all efforts that were devoted to developing such lines were unsuccessful. The factors causing this loss could only be elucidated by the addition of RSV to parallel preparations and virus added to the final pellet would indicate the presence of inhibitors of the RDDP assay.

Significant incorporation of ^{3}H TTP was found in media from FBL and pooled media from FOS, FOK and FOL cells cocultured with ovine lymphomas, but insufficient material was available to assess whether the activity was due to RDDP. The search for a permissive cell line for a putative ovine retrovirus, an alternative to ovine lymphoma continuous cell lines, should continue. The RDDP assay may provide a sensitive technique to detect retroviral replication in any such permissive cell culture (Kellof *et al.*, 1972).

In summary, RSV and BLV were detected using biochemical methods, but conclusive evidence for the association of a retrovirus with sporadic ovine lymphomas was not obtained. Limitations may have been placed on the sensitivity of these techniques by the apparent interference by normal cellular components in the various assays or by the relatively small volumes of material tested. To detect significant amounts of a human retrovirus isolate, Poiesz *et al.*(1980) found it was necessary to use at least 20 litres of media from a human T cell lymphoma culture. A remaining avenue for further research using biochemical techniques would be to develop specific nucleic acid probes for retroviruses and use these in hybridisation studies.

CHAPTER 5

THE RESPONSES TO PHYTOHAEMAGGLUTININ BY LYMPHOCYTES FROM LYMPHOMA-INOCULATED, IN-CONTACT CONTROL AND POPULATION CONTROL SHEEP

Introduction
Materials and methods
    Animals
    Collection of lymphocytes
    Lymphocyte culture
Results
Discussion

CHAPTER 5

THE RESPONSES TO PHYTOHAEMAGGLUTININ BY LYMPHOCYTES FROM LYMPHOMA-INOCULATED, IN-CONTACT CONTROL AND POPULATION CONTROL SHEEP

INTRODUCTION

The results of Johnstone *et al.* (1979a) suggested that a viral agent might be responsible for the persistent lymphocytosis seen in sheep inoculated with cell-free extracts of sporadic ovine lymphomas. Since depressed responses to T and B cell mitogens have been reported in *in vitro* retroviral infections (Dent, 1972; Dent, 1975; Israel and Wainberg, 1981) then a similar finding for the lymphocytes from the inoculated animals might be taken as indirect evidence for a viral association with sporadic ovine lymphoma. In this chapter a comparison is made of the response to PHA by blood lymphocytes from these and control sheep.

MATERIALS AND METHODS

Animals

The animals used were 14 sheep that had been inoculated 7 years previously with cell-free extracts of ovine lymphomas either *in utero* or post-natally (Johnstone *et al.*, 1979a), 4 in-contact control sheep and 6 aged population controls. These animals were either ewes or ram catrates.

Collection of Lymphocytes

To avoid the effects of diurnal variations on PHA responsiveness (Fan *et al.*, 1977), each sheep was bled in the morning and afternoon of every third day for a total of 3 days. Blood lymphocytes were harvested as described in Chapter 2, Section A.

Lymphocyte Culture

Approximately 2×10^6 lymphocytes in 200 µl of MEM-FBS were added to the flat-bottomed wells of microculture plates PHA (PHA-P, Wellcome), diluted in MEM, was added in 5 to 20µg

volumes to give final concentrations of 5, 10 or 25μg PHA per ml of culture. Cultures lacking the mitogen served as controls. All cultures were established in triplicate and were incubated at 37°C in a humidified atmosphere of 5 percent CO_2-in-air. Seventy two hr later, 20 μl of ^3H thymidine (specific activity 0.2 μCi per ml, Radiochemical Centre) were added to each well. Following a further incubation for 18 hr the lymphocytes were harvested by a semiautomatic multiple cell harvester (Microtitre Minimash, Dynatech) onto glass-fibre discs (GF/C, Whatman). These were dried, placed in vials with 5 ml of scintillation fluid (Appendix) and the incorporated radioactivity measured in a liquid scintillation counter.

Means of the cpm for the triplicate cultures were calculated, from which mean background counts for the control cultures were subtracted. These adjusted means were transformed to square root values for analysis.

RESULTS

The optimal concentration of PHA was determined for the total experimental population and for each experimental group (Table 25). Analysis of variance showed that for the total population and for each group the maximum response was produced by 10 μg PHA per ml. Although there was a linear response to the increasing concentration of PHA used, there were highly significant deviations from linearity resulting from the maximum response occurring at 10 μg per ml. Therefore, in further analysis only the values for 10 μg per ml were considered. When the mean cpm responses at the 10 μg level (Table 26) were plotted (Figure 29)there was an apparent scatter of responses between the 3 groups of animals involved. This plot suggested that some of the mean responses from the lymphoma-inoculated group fell outside the ranges of both control groups. However, analysis of variance did not show any significant differences between the group mean responses ($p>0.05$). To compare the frequencies of different levels of response, separate frequency tables were constructed for each group (Table 27)

TABLE 25

MEAN RESPONSES[a] TO VARIOUS CONCENTRATIONS OF PHYTOHAEMAGGLUTININ BY LYMPHOCYTES FROM LYMPHOMA-INOCULATED, IN-CONTACT CONTROL AND POPULATION CONTROL SHEEP

	All Groups	Lymphoma-Inoculated	In-contact Controls	Population Controls
PHA concentration				
5μg/ml	128.87±44.13	131.97±43.06	117.47±34.65	131.18±46.72
10μg/ml	136.17±41.64	135.82±45.27	125.59±33.71	139.62±42.04
25μg/ml	110.13±39.71	100.45±34.62	102.36±31.11	116.28±42.83
		F Values		
Between concentrations	37.1[b]	17.8[a]	8.5[b]	15.4[b]
Linearity	35.5[b]	23.1[b]	7.0[c]	12.0[b]
Non linearity	38.6[b]	12.5[b]	10.0[c]	18.8[b]

PHA: Phytohaemagglutinin

(a) mean and standard deviation calculated as $\Sigma\sqrt{y}/n$ and $\Sigma(\sqrt{y} - \sqrt{\bar{y}})^2/(n - 1)$

(b) $p<0.001$

(c) $p<0.01$

TABLE 26

INDIVIDUAL RESPONSES TO PHYTOHAEMAGGLUTININ AT 10 UG PER ML BY LYMPHOCYTES FROM LYMPHOMA-INOCULATED, IN-CONTACT CONTROL AND POPULATION CONTROL SHEEP

Group	Sheep	Mean ± SD[a]	Response counts per min[b]
Population Controls	1	163.61±40.73	26769
	2	143.64±37.19	20636
	3	145.57±20.80	21191
	4	129.70±48.75	16822
	5	111.78±52.82	12493
	6	135.36±48.29	18322
In-contact Controls	7	135.51±32.36	18364
	8	134.62±32.18	18122
	9	104.92±40.01	11007
	10	123.78±23.18	15321
Lymphoma-Inoculated	11[c]	174.81±25.53	30559
	12[c]	167.76±18.22	28141
	13[c]	160.51±26.35	25763
	14[c]	88.32±17.59	7800
	15	160.98±18.04	25913
	16[c]	66.93±21.76	4479
	17[c]	105.68±27.76	11168
	18[c]	159.62±25.90	25479
	19[c]	102.49±33.14	10503
	20[c]	180.65±16.02	32632
	21[c]	127.55±19.20	16269
	22	170.45±19.85	29053
	23[c]	158.32±31.29	25064
	24[c]	126.62±28.60	16031

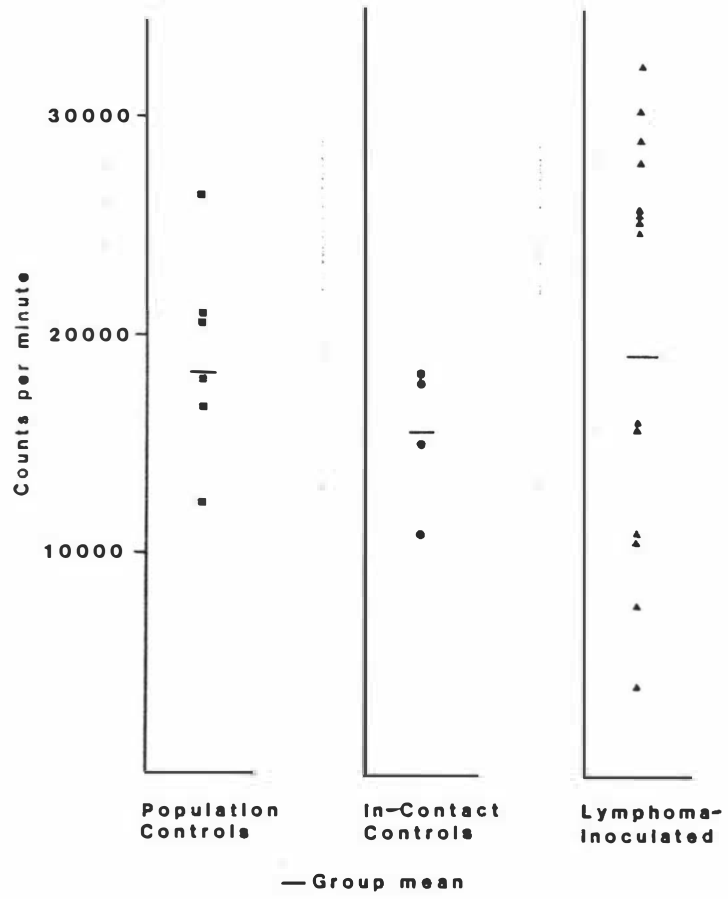
Analysis of variance: Between groups $F = 2.973$
$p \quad 0.05$ ns

SD: standard deviation
ns: not significant

(a) Mean and SD calculated as $\Sigma\sqrt{y}/n$ and $\Sigma(\sqrt{y} - \bar{\sqrt{y}})^2/(n - 1)$
(b) Mean converted to the original scale from the transformed values
(c) Lymphoma-inoculated sheep previously showing lymphocytosis

FIGURE 29: Scattergram of individual mean responses to 10 µg phytohaemagglutinin per ml by lymphocytes from lymphoma-inoculated (▲), in-contact control (●) and population control (■) sheep.

FIGURE 30: Plot of relative frequencies of optimal responses to phytohaemagglutinin by lymphocytes from lymphoma-inoculated (▲), in-contact control (●) and population control (■) sheep.


30000
20000
10000
Counts per minute
Population Controls
In-Contact Controls
Lymphoma-Inoculated
— Group mean

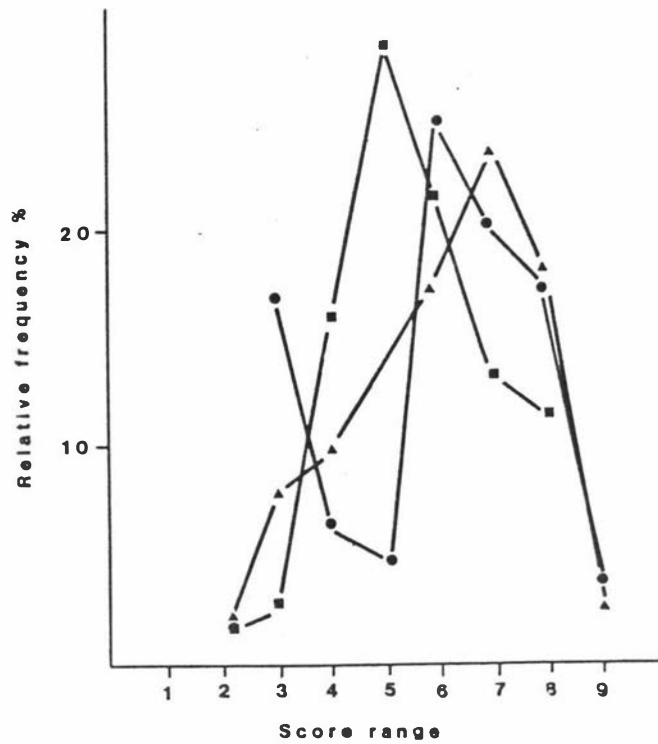

20
10
Relative frequency %
1
2
3
4
5
6
7
8
9
Score range

TABLE 27

FREQUENCY DIAGRAM OF OPTIMAL RESPONSES TO PHYTOHAEMAGGLUTININ BY LYMPHOCYTES FROM LYMPHOMA-INOCULATED, IN-CONTACT CONTROL AND POPULATION CONTROL SHEEP

Score		2	3	4	5	6	7	8	9
Range Root Response		26-50	51-75	76-100	101-125	126-150	151-175	176-200	201-225
Population Controls	Count	0	14	5	4	21	17	14	3
	Percentage	0	16.7	6.0	4.8	25.0	20.2	16.7	3.6
In-contact Controls	Count	1	2	11	20	15	9	8	0
	Percentage	1.5	2.9	16.2	29.4	22.1	13.2	11.8	0
Lymphoma-Inoculated	Count	4	18	22	30	38	52	37	6
	Percentage	1.8	8.3	10.1	13.8	17.5	24.0	18.0	2.8

and the relative frequencies plotted (Figure 30). The relative frequency distribution for each of the 3 groups did not show evidence of group differences in either mean or dispersion.

DISCUSSION

The lymphoma-inoculated and in-contact control sheep were the survivors of the flock described by Johnstone *et al.* (1979a) of which 12 of the inoculated sheep had previously shown a lymphocytosis. Although at the time of these experiments their individual lymphocyte numbers had returned to within the normal range, if the lymphocytosis had been induced by a retrovirus (Johnstone *et al.*, 1979a), then it is probable that the virus might have persisted and affected the mitogenic responses of lymphocytes from these sheep.

Whilst the responses to PHA of a number of lymphoma-inoculated sheep appeared to be outside the range of the 2 control groups no statistically significant differences were found between them. The apparent difference may not have occurred had more control sheep been examined.

The maximal response to PHA by the lymphocytes from the population control group occurred at the same PHA concentration, and was of the same order of magnitude, as those reported by Outteridge *et al.*(1981b) for sheep.

PHA was selected as the stimulating mitogen for 2 reasons. Firstly, it was in PHA-transformed lymphocytes of the lymphoma-inoculated sheep that the vesicular structures interpreted as virus-like by Johnstone *et al.* (1979a) were observed. Secondly, the lymphoma extracts inoculated by these authors were from 2 sheep with multicentric lymphomas and one sheep with a primary mass in the anterior mediastinum (Johnstone, unpublished data) which, by extrapolation of the data in Section A, were most probably T cell in origin. Therefore, the putative agent in these lymphomas might have been expected to infect a T lymphocyte target cell and to have altered its responsiveness to PHA. However, there was no statistical difference between the mitogenic

responsiveness to PHA of lymphocytes from lymphoma-inoculated, in-contact and population control sheep in these experiments.

CONCLUDING STATEMENT

CONCLUDING STATEMENT

Lymphomas and leukaemias are neoplasms of the immune system which are induced by retroviruses in some animal species. Both immunological and virological facets of sporadic ovine lymphoma were examined on this study to enable comparisons to be made between this disease and the lymphomas of other species.

The cells of sporadic ovine lymphoma appear to have diverse immunological origins, in contrast to those of bovine lymphoma (Takashima *et al.*, 1977). Furthermore, there appeared to be a relationship between the anatomical distribution of the lesions and whether the cells were T or B cell in origin. A similar relationship may exist for feline lymphoma (Mackey and Jarrett, 1972).

Retroviruses could not be conclusively demonstrated in experiments using biochemical techniques, although nonbudding particles were observed by electron microscopy in several lymphomas and in a suspension culture. The results of these experiments suggested that cellular enzymes and nucleic acids could interfere with the detection of virus in low titres, a problem also reported by other workers searching for retroviruses in human lymphomas (Poiesz *et al.*, 1980).

It has been customary to compare many of the diseases of sheep and cattle because of their ruminant physiology. However, while the low-prevalence and sporadic nature of ovine lymphoma is similar to that found in sporadic bovine lymphoma (Grimshaw *et al.*, 1979) and a retrovirus is not readily detectable in either disease (Burny *et al.*, 1978; Kettmann *et al.*, 1978; Ferrer, 1980), the syndromes occur in animals of different ages (Johnstone and Manktelow, 1978; Grimshaw *et al.*, 1979) and are composed of cells bearing different immunological markers (Takashima *et al.*, 1977). On the basis of the immunological and virological data derived from this study, sporadic ovine lymphoma may be more appropriately compared with the proportion of feline lymphomas which are apparently virus-negative (Francis *et al.*, 1979).

The use of sporadic ovine lymphoma for lymphoma

research is limited by the low overall prevalence of the disease in New Zealand (Webster, 1967) and this proved to be a major difficulty in the current studies. Inadequate numbers of affected sheep and limited quantities of material were a hindrance to the proper development and evaluation of the various techniques. Because of this restriction it was necessary in most experiments, and in particular, the assays for RNA-containing particles and RDDP activity, to extrapolate data obtained from one lymphoma to the next. In the circumstances this aspect of the experimental design was unavoidable and there is a need to develop systems which will provide more experimental materials over extended periods of time. These may be in the form of continuous cell lines of ovine lymphoma cells, monolayer cell lines permissive for a putative retrovirus, or live clinically affected sheep. The last possibility is least likely to be successful in view of the difficulty in detecting such animals.

Despite the lack of success in the present experiments to establish continuous lymphoma cell lines, the development of these lines must remain a priority in further studies. These cultures would aid the detection of retroviruses by providing large quantities of dividing cells and by reducing the contamination of preparations by cellular nucleic acids and enzymes that interfere with the biochemical techniques. Cell lines could provide information about the range of membrane markers present, about lymphoma-specific antigens, and about lymphoma cell transplantability and karyotype. Furthermore, these cells could be cultured with a large number of cell lines which could be screened for their permissiveness to retroviruses. Established techniques for retroviral rescue (Ressang, 1976) and treatment by the halogenated pyrimidines (Dixon, 1973) would have a greater expectation of successful virus recovery when applied to the lymphoma and cocultured cell lines. Finally, molecular probes for DNA hybridisation could be developed using the avian, murine, feline and bovine retroviruses and restriction enzyme analysis of DNA could be used to identify integration sites of any

homologous sequences (Koshy *et al.*, 1980; Neel *et al.*, 1981). If this information was in support of the promoter insertion hypothesis (Hayward *et al.*, 1981) for sporadic ovine lymphoma, then this disease would have added importance as a model for lymphomagenesis in the group of apparently virus-negative neoplasms and in particular those of man.

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APPENDIX

MISCELLANEOUS MATERIALS AND METHODS; SUPPLIERS OF PRODUCTS

MISCELLANEOUS MATERIALS AND METHODS

ALSEVERS SOLUTION (MODIFIED)

Glucose	20.5 gm
Sodium citrate	8.0 gm
Citric acid	0.55 gm
NaCl	4.2 gm
Water	to 1000.0 ml

Adjust pH to 6.1.
Sterilise by autoclaving.

ANTIBIOTICS (100 X CONCENTRATE)

Benzyl penicillin (Glaxo)	1×10^6 units
Streptomycin sulphate (Glaxo)	1.0 gm
Kanamycin sulphate (Sigma)	1.0 gm
PBS	to 100.0 ml

Sterilise by filtration.
Add 1 ml of concentrate to each 100 ml of medium.

ANTIBIOTIC-TRYPSIN-VERSENE (ATV)

Trypsin (Difco)	0.5 gm
Versene (EDTA)	0.2 gm
NaCl	8.0 gm
KCl	0.4 gm
Dextrose	1.0 gm
$NaHCO_3$	0.58 gm
Penicillin	2×10^5 units
Streptomycin	100 mg
Phenol red	0.02 gm
Water	to 1000.0 ml

Sterilise by filtration.

BUFFERS

0.5M Bicarbonate Buffer (pH 9.0)

Solution A

$NaHCO_3$	0.84 gm
Water	to 10.0 ml

Bicarbonate Buffer (*continued*)

Solution B

Na_2CO_3	1.06 gm
Water	to 10.0 ml

For buffer:

Solution A	7.7 ml
Solution B	0.8 ml
Water	8.5 ml

0.1M Phosphate Buffer (pH 7.2)

Solution A

Na_2HPO_4	28.39 gm
Water	to 1000.0 ml

Solution B

$NaH_2PO_4.2H_2O$	31.21 gm
Water	to 1000.0 ml

For buffer:

Solution A	36.0 ml
Solution B	14.0 ml
Water	50.0 ml

0.7M Phosphate Buffer (pH 5.8)

Solution A

Na_2HPO_4	9.9 gm
Water	to 100.0 ml

Solution B

$NaH_2PO_4.2H_2O$	10.9 gm
Water	to 100.0 ml

For buffer:

Solution A	4.0 ml
Solution B	46.0 ml

ELECTRON MICROSCOPY

Lead Citrate Stain

Lead citrate	0.02 gm
Water	10.0 ml

Add 0.1 ml of 10N NaOH to dissolve.

Modified Karnovsky's Fixative

Paraformaldehyde	2.0 gm
$Na_2HPO_4.12H_2O$	2.51 gm

Modified Karnovsky's Fixative (*continued*)

KH_2PO_4	0.41 gm
Glutaraldehyde (25 percent solution)	12.0 ml
Water	to 100.0 ml

Heat paraformaldehyde on 80 ml of water to 60°C.

Slowly add 1M NaOH until milky solution clears.

Add buffer salts, glutaraldehyde and make up to 100 ml with water.

Store at 4°C.

Uranyl Acetate Stain

Add uranyl acetate to 50 percent ethanol until it no longer dissolves.

Centrifuge to remove undissolved uranyl acetate.

Procedures Used in Preparation of Tissues

Tissue is cut into cubes of approximately 1 mm^3 and covered by Karnovsky' fixative for at least 4 hr at 4°C. After washing in 0.1M phosphate buffer, the tissue is post-fixed in 1 percent OsO_4 in 0.1M phosphate buffer for 1 hr at room temperature. A further wash in 0.1M phosphate buffer is followed by a brief rinse in water.

Dehydration of fixed tissues is carried out in a series of alcohols and propylene oxide according to the following protocol:

25 percent ethanol	15 min
50 percent ethanol	15 min
75 percent ethanol with 1 percent uranyl acetate	30 min to 24 hr
95 percent ethanol	30 min
100 percent ethanol	30 min
50 percent ethanol/ 50 percent propylene oxide	15 min
100 percent propylene oxide	15 min

Tissue infiltration is effected by 25 percent Durcupan-ACM (Fluka) in propylene oxide which is left overnight. The remaining resin is discarded and replaced with fresh resin for 6 hr. The tissue is then placed into moulds with a further volume of fresh resin and polymerisation effected at 60°C for at least 48 hr.

FORMOL SALINE

Formaldehyde (40 percent solution)	10.0 ml
NaCl	8.0 gm
Water	to 100.0 ml

FORMOL SUCROSE

Formaldehyde (40 percent solution)	50.0 ml
Sucrose	37.5 gm
$NaH_2PO_4.H_2O$	2.0 gm
Na_2HPO_4	3.25 gm
Water	450.0 ml

HANKS BALANCED SALT SOLUTION WITH ANTIBIOTICS

Solution A

NaCl	80.0 gm
KCl	4.0 gm
$MgSO_4.7H_2O$	2.0 gm
$CaCl_2$ (anhydrous)	1.4 gm
Water	to 1000.0 ml

Solution B

Na_2HPO_4 (anhydrous)	0.6 gm
KH_2PO_4	0.6 gm
Dextrose	10.0 gm
Phenol red	0.2 gm
Water	to 1000.0 ml

For final solution:

Solution A	250.0 ml
Solution B	250.0 ml
Water	500.0 ml

Add 5 x 10^6 units of penicillin and 5 gm of streptomycin. Sterilise by filtration.

HOLT'S SOLUTION

Sucrose	30.0 gm
Gum arabic	1.0 gm
Water	to 100.0 ml

LAMB BONE MARROW

Lambs less than 3 weeks of age are euthanased and their long bones removed, opened and the bone marrow flushed out with PBS. The collected marrow is pressed through a 40 mesh stainless steel tissue sieve. The cells are then washed 3 times in 15 ml of PBS by centrifuging at 700 g for 10 min.

MEDIA

Eagle's Minimum Essential Medium (MEM)

Eagle's MEM powder	10.0 gm
$NaHCO_3$ (4.4 percent solution)	50.0 ml
Water	to 1000.0 ml

Adjust pH with 1M NaOH or 1M HCl.
Sterilise by filtration.
Add antibiotics before use.

Ham's F10 Medium

Ham's F10 medium powder (with L-glutamine)	9.8 gm
$NaHCO_3$	1.2 gm
Water	to 1000.0 ml

Adjust pH with 1M NaOH or 1M HCl.
Sterilise by filtration.
Add antibiotics before use.

Medium 199

Medium 199 powder	10.0 gm
N-tris-(hydroxymethyl)methylglycine (tricine)	1.8 gm
Water	to 1000.0 ml

Adjust pH with 1M NaOH or 1M HCl.
Sterilise by filtration.
Add antibiotics before use.

RPMI 1640 Medium

RPMI 1640 Medium powder	10.4 gm
$NaHCO_3$	2.0 gm
Water	to 1000.0 ml

Adjust pH with 1M NaOH or 1M HCl.
Sterilise by filtration.
Add antibiotics before use.

Vogt's Medium

Ham's F10 medium	80.0 ml
Tryptose phosphate broth (Difco)	10.0 ml
$NaHCO_3$ (6.6 percent solution)	2.0 ml

Add antibiotics before use.

Waymouth's Medium

Waymouth's MB 752/1 Medium powder (with L-glutamine)	14.1 gm
$NaHCO_3$	2.24 gm
Water	to 1000.0 ml

Adjust pH with 1M NaOH or 1M HCl.

Sterilise by filtration.

Add antibiotics before use.

OVINE PLASMA FOR PLASMA CLOT EXPLANT CULTURES

Each 7 ml of jugular blood is collected asceptically into 3 ml of Alsever's solution. The blood is centrifuged at 1500 g for 10 min and the plasma removed.

For reconstitution, 0.2 ml of 2 percent $CaCl_2$ is added to each 1 ml of plasma, mixed and used before plasma coagulates.

PHOSPHATE BUFFERED SALINE (PBS)

NaCl	8.0 gm
KCl	0.2 gm
Na_2HPO_4	1.15 gm
KH_2PO_4	0.2 gm
Water	to 1000.0 ml

Adjust pH to 7.2-7.4.

Sterilise by autoclaving.

SCINTILLATION FLUID

2,5-diphenyloxazole (PPO)	20.0 gm
1,4-bis(2(4-methyl-5-phenyl-oxazolyl))benzene POPOP	100 mg
Triton X-100	1500.0 ml
Toluene (scintillation grade)	3500.0 ml

Scintillation Fluid (*continued*)

Add PPO, POPOP and toluene.

Mix overnight.

Add Triton X-100 and mix for another 3 hr.

SEPHADEX G-25 COLUMN

For each 5 ml of column volume, 1 gm of Sephadex G-25 is added to 0.15M saline containing 0.01 percent NaN_3 as a preservative. After the air in the mixture is removed by suction, the mixture is allowed to stand for 1 hr. The column is poured and allowed to settle for at least 2 hr.

SHEEP LIVER POWDER

Sheep livers are diced and washed twice with 0.15M saline prior to being homogenised. The homogenate is mixed with 4 volumes of acetone and centrifuged at room temperature. The precipitate is washed twice in acetone, filtered on a Buchler funnel and dried at 37°C. The dried powder is then ground in a mortar, sieved and stored at 4°C.

TRYPAN BLUE SOLUTION

Trypan blue	0.2 gm
Phosphate buffered saline	100.0 ml

Filter through filter paper.

TRYPSIN SOLUTION (0.25 PERCENT)

Trypsin (Difco 1:250)	2.5 gm
Hank's balanced salt solution	to 1000.0 ml
Penicillin	2×10^5 units
Streptomycin	100 mg

Adjust pH to 7.6 with $NaHCO_3$ solution (4.4 percent).

Sterilise by filtration.

TRYPTOSE PHOSPHATE BROTH

Tryptose phosphate broth (Difco)	29.5 gm
Water	to 1000.0 ml

Sterilise by autoclaving.

SUPPLIERS MENTIONED IN TEXT

Abbe, Bellingham and Stanley Ltd, London, England.
BBL, Division of Becton, Dickinson and Co., Cockeysville, Maryland, U.S.A.
BDH Chemicals Ltd, Poole, England.
Beckman Instruments Inc., Fullerton, California, U.S.A.
Buchler Instruments Inc., Fort Lee, New Jersey, U.S.A.
Calbiochem Ltd, San Diego, California, U.S.A.
Difco Laboratories, Detroit, Michigan, U.S.A.
Dynatech Laboratories Inc., Alexandria, Virginia, U.S.A.
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