MALIGNANT LYMPHOMAS IN SHEEP

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ABSTRACT

Malignant lymphoma is the general term applied to any neoplastic disorder of lymphoid tissue, including Hodgkin's disease and reticulum cell sarcoma. This group of neoplasms are among the most commonly occurring spontaneous neoplasms of sheep in New Zealand, being exceeded in frequency only by carcinomas of the small intestine and primary neoplasms of the liver. It has not been established whether malignant lymphoma is of sporadic or enzootic occurrence in this country, but from limited epidemiological observations in which on two occasions the disease was seen in two animals from the same property, it is possible that the latter distribution occurs. This and most other series indicate that mature sheep are most frequently affected but the disease does occur in younger sheep and lambs.

A study has been made of the pathology of ovine malignant lymphoma, based on 22 cases, most of which were collected from slaughter-houses and histological material from a further 18 cases filed previously at this laboratory. In nearly all cases nodular or diffuse lesions, consisting of accumulations of invasive neoplastic cells of lymphoid origin, were distributed widely throughout the body. Gross evidence of tumour in the lymph nodes was present in all except three cases of the disease. This involvement was usually multiple, with many of the carcass and visceral lymph nodes containing tumours. The spleen contained neoplastic lesions in 73 per cent. of the cases examined. Of the non-lymphoid organs affected by malignant lymphoma, the liver, kidney, bone marrow, heart, small intestine and abomasum predominated. Of the 40 cases examined, 37 were classified as lymphosarcomas and three as reticulum cell sarcomas. The degree of cellular differentiation in the lymphosarcomas varied from primitive lymphoblastoid to well differentiated lymphocytic cell types, with the less

differentiated forms being more common. Some nuclear and cytoplasmic abnormalities which have previously been described as being non-specific in other types of neoplasms were seen in specimens from 10 cases which were examined with an electron microscope.

Support for the hypothesis that "malignant lymphoma of sheep is transmissible" was sought by attempts to experimentally transmit this disease to lambs using intraperitoneal injections of cell-free tumour extracts during gestation or within 12 hr of birth. To date none of these sheep, which are only three years old, have developed overt neoplasia but 20 of them have developed elevated numbers of circulating lymphocytes. This has persisted for periods of two years or more and there is evidence to indicate that this should be interpreted as a preclinical phase of malignant lymphoma.

To investigate an hypothesis that malignant lymphoma of sheep is due to infection by an oncorna virus, electron microscopic examinations were made on specimens from fresh tumour as well as on preparations from tissue cultures which had been inoculated with various ovine lymphoid tumour homogenates. These studies were inconclusive and it was not until cultures of phytohaemagglutinin-stimulated lymphocytes derived from the sheep with experimentally transmitted lymphocytosis were examined that virus-like particles, consistent with the morphology of "type-A" oncorna viruses could be demonstrated. They were present in membrane-limited vacuoles and cytoplasm of these cells and less frequently extracellularly in the five experimentally inoculated sheep examined but were not present in any of the lymphocytes from an equal number of control sheep.

Lymphocyte cultures from 15 of the experimentally inoculated sheep showed sensitization to antigens in ovine malignant lymphoma homogenates when tested by a macrophage migration inhibition test. This test also

demonstrated the presence of common tumour specific antigens in five of the six tumour extracts used either for sheep inoculations or in testing for macrophage inhibitory factor production.

In an attempt to accelerate the development of overt neoplasia in three of the experimentally inoculated animals showing a persistent lymphocytosis, they were given a combined course of the immunosuppressive agents azathioprine and horse anti-sheep lymphocyte globulin. This resulted in a profound fall in circulating lymphocytes and while the cell mediated immunity was suppressed, as judged by the survival of skin allografts, macrophage migration inhibition in response to tumour antigens was not significantly altered in two of the three animals under treatment.

A close relationship was demonstrated in the experimentally inoculated sheep between the occurrence of lymphocytosis, the development of tumour specific cell mediated immunity and the presence of virus-like particles in phytohaemagglutinin-stimulated lymphocytes. This evidence combined with the demonstration of common apparently tumour specific antigens in malignant lymphoma extracts used in these studies provides strong support for the hypothesis"that this disease of sheep is transmissible and is most probably due to an infection with an oncogenic virus". The exploitation of the sheep model described is of potential value for the comparative study of preneoplastic events in the general field of cancer research.

CHAPTER 1

NEOPLASIA IN SHEEP AND COMPARATIVE ASPECTS OF THE EPIDEMIOLOGY OF MALIGNANT LYMPHOMA

I. NEOPLASIA IN SHEEP

The literature concerning neoplasia of sheep has previously been reviewed from this laboratory (Simpson, 1971) and is summarized in an updated form in Table 1.I. The majority of information has been obtained from sheep killed in slaughter-houses. With certain exceptions it would appear that there is a generally low prevalence of neoplasia in sheep. Of the more commonly reported neoplasms (Table 1.II) some are associated with known aetiological agents or environmental factors which imply actiological significance. This, coupled with the overall apparently low prevalence of ovine neoplasia, makes the sheep an interesting and valuable model for cancer research.

Rather than again review ovine neoplasia in depth, only the more important neoplasms of sheep and their significance will be outlined.

Carcinoma of the Small Intestine

Before 1960, carcinomas of the small intestine of sheep had been reported only occasionally. Since that time it has been shown that this neoplasm is commonly seen in sheep in New Zealand (Dodd, 1960, 1964; Cook, 1964; Webster, 1966, 1967; Simpson, 1971, 1972a, 1972b). The only other localities outside New Zealand in which relatively large numbers of carcinomas of the small intestine occur are Australia (McDonald and Leaver,1965). Scotland (Norval, 1968, cited by Simpson, 1971) and Iceland (Georgsson and Vigfússon, 1973). Simpson (1972a) examined the viscera of 35,300 ewes killed in 10 slaughter-houses in seven geographic areas of New Zealand during 1967 and 1968 and recorded

TABLE 1.I

The relative frequency of the more commonly occurring neoplasms of sheep diagnosed by various authors

Reference	Primary liver tumours	Malignant lymphomas	Cartilaginous tumours	Carcinoma of the small intestine	Squamous cell carcinoma	Total tumours examined
Sticker (1902)[1]	5	1	0	0	-	7
Feldman (1931)	5	4	2	0	2	40
Jackson (1936)	3	4	0	1	-	28
Monlux, Anderson and Davis (1956)	8	20	3	1[4]	1	66
Cotchin (1960)	12	10	2	1[4]	1	38
Smith and Jones (1961)	17	14	1	2	16	129
Brandley and Migaki (1963)	8	11	3	0	-	34
Manktelow (1963)[2]	38	19	1	20	3	100
Sastry and Twiehaus (1965)	0	3	2	2[4]	1	10
Webster (1966)[3]	27	23	2	63	-	120
Webster (1967)[3]	34	7	2	339	-	385
Anderson, Sandison and Jarrett (1969)	33	44	3	1	-	107
Cordes and Shortridge (1971)	12	39	0	143	3	256

(1) Cited by Feldman (1931)

(2) Personal communication

(3) The lesions described as "spleen tumours" are omitted from this table because they were shown subsequently to be hyperplastic rather than neoplastic (Jolly, 1967).

(4) The primary site of these lesions were not stated and they could have arisen in the large intestine.

TABLE 1. II

Miscellaneous Naturally Occurring Neoplasms of Sheep Diagnosed by Various Authors

Anatomical Region	Tumour Type	No of Cases	Reference
Mouth	Gingival fibroma	1	Lombard and Gaubert (1959)
Oesophagus	Papilloma	1	Anderson, Sandison and Jarrett (1969)
Reticulum	Squamous cell carcinoma	1	Georgsson (1973)
Rumen	Leiomyosarcoma	1	Sastry and Tweihaus (1965)
Abomasum	Leiomyosarcoma	1	Smith and Jones (1961)
Omasum	Acanthoma	1	Jackson (1936)
	Squamous cell carcinoma	1	Hanko (1947)
Small intestine	Adenomatoid hyperplasia of Brunner's glands	1	Jackson (1936)
Large intestine	Adenocarcinoma	1	Head (1953)
	Cystadenoma of rectum	2	Nobel, Neumann and Klopfer (1970)
	Squamous cell carcinoma	1	Cordes and Shortridge (1971)
Ducts of Liver/ Pancreas	Ductal Adenocarcinoma	15	Cordes and Shortridge (1971)
Pancreas	Carcinoma	1	Jackson (1936)
Liver	Angiosarsarcoma	5	Cordes and Shortridge (1971)
	Teratoma	1	Arora and Iyer (1969)
Peritoneum	Mesothelioma	1	Plummer (1956)
	Mesothelioma	1	Damodaran and Parthasarathy (1972)
	Carcinoma taken to be of peritoneal origin	1	Saini (1941) (1)

Table 1.II continued

Anatomical Region	Tumour Type	No of Cases	Reference
Digestive organs	Malignant fibroma or sarcoma	2	Brandly and Migaki (1963)
Lung	Carcinoma	1	Schutz (1880)(2)
		1	Eber (1891)(2)
		1	Besnoit (1895) (2)
		1	Sticker (1902) (3)
		1	Jackson (1936)
		2	Brandly and Migaki (1963)
	Multiple cystadenoma	1	Feldman (1931)
	Visceral mesothelioma	1	Feldman (1931)
	Rhabdomyoma	1	Day (1922)
	Haemangioendothelioma	1	Smith and Jones (1961)
Mammary gland	Fibrosarcoma	1	Plummer (1956)
	Carcinoma	1	Webster (1966)
	Adenoma	1	Manktelow (1963) (4)
Uterus	Leiomyoma	2	Feldman (1931)
	Smooth muscle tumour	1	Anderson and Sandison (1969a)
	Fibroma	1	Anderson and Sandison (1969b)
	Chondroma	1	Sastry (1959)
	Adenocarcinoma	1	Terlecki and Watson (1967)
Ovary	Granulosa cell tumour	1	Casarosa (1950) (1)
		5	Smith and Jones (1961)
		3	Cordes and Shortridge (1971)
Female reproductive system (unspecified)	Leiomyoma	1	Brandly and Migaki (1963)
Testis	Intratubular seminoma	5	Jensen and Flint (1963)
	Seminoma	2	Shortridge (1962)

Continued

Table 1.II continued

Testis (continued)	Sertoli cell tumour	1	Shortridge (1962)
	Haemangioma	1	Smith and Jones (1961)
Kidney	Adenoma	1	Flir (1952-53)
	Carcinoma	4	Sandison and Anderson (1968b)
		2	Smith and Jones (1961)
		1	Cordes and Shortridge (1971)
	Carcinoma (renal pelvis)	2	Webster (1966)
	Embryonal nephroma	2	Jackson (1936)
		1	Pamucku (1956)
		1	Brandly and Migaki (1963)
		2	Manktelow (1963) (4)
		3	Cordes and Shortridge (1971)
		1	Harcourt and Spice (1968)
		1	Feldman (1933)
		3	Flir (1952-53)
		1	Webster (1967)
		2	Smith and Jones (1961)
	Haemangioendothelioma	1	Flir (1952-53)
		2	Smith and Jones (1961)
Skin	Melanoma	1	Lund (1923) (1)
		1	Baxter (1960)
		1	Sastry and Tweihaus (1965)
		1	Anderson, Sandison and Jarrett (1969)
	Fibroma	1	Jackson (1936)
		1	Head (1953)
		1	Brandly and Migaki (1963)
		2	Cotchin (1960)

Continued

Table 1 II continued

Anatomical Region	Tumour Type	No of Cases	Reference
Skin (continued)	Fibropapilloma	5	Head (1953)
	Papilloma	1	Cotchin (1960)
		1	James, Rao and Sastry (1967)
		1	Manktelow (1963)(4)
		4	Cordes and Shortridge (1971)
	Cornu cutaneum	1	Jackson (1936)
	Lipoma	1	Manktelow (1963)(4)
		1	Smith and Jones (1961)
	Adenocarcinoma (cystic type)	1	Feldman (1931)
	Capillary haemangioma	1	Davis (1935)
Eye	Melanoma	1	Cotchin (1960)
Lacrimal	Adenocarcinoma of accessory lacrimal glands	1	Micheletto (1968)
		1	Smith and Jones (1961)
	Neurofibrosarcoma	1	James, Rae and Sastry (1967)
	Tumours (unspecified)	2	Davis, Leeper and Shelton (1933)
Nervous system	Lipoma of meninges (congenital)	1	Jackson (1936)
	Benign neurofibroma or neurolemmoma	1	Brandly and Migaki (1963)
	Meningioma	1	Pivnik (1968)
Anterior pituitary	Adenoma	1	Casarosa (1950b) (1)
	Adenoma of pars intermedia	1	Cordes and Shortridge (1971)
Thyroid	Carcinoma	1	Feldman (1931)
		3	Jackson (1936)
		1	Sandison and Anderson (1968a)
	Adenoma	1	Nobel and Neumann (1960) (5)

Continued

Table 1.IIcontinued

Anatomical Region	Tumour Type	No of Cases	Reference
Thyroid (continued)		8	Smith and Jones (1961)
	Tumour (unspecified)	1	Davis, Leeper and Shelton (1933)
Adrenal	Carcinoma of cortex	9	Feldman (1931)
		1	Sandison and Anderson (1968a)
	Adenoma of cortex	1	Feldman (1931)
		1	Cotchin (1960)
		1	Cordes and Shortridge (1971)
	Benign adrenal medullary tumour	1	Brandly and Migaki (1963)
	Pheochromocytoma	1	Sandison and Anderson (1968a)
	Carcinoma (unspecified)	8	Davis, Leeper and Shelton (1933)
	Adenoma (unspecified)	1	Davis, Leeper and Shelton (1933)
Bone	Osteoma	1	Jackson (1936)
	Osteosarcoma	1	Cotchin (1960)
		1	Feldman (1931)
		4	Webster (1966-1967)
		1	Manktelow (1963)(4)
		2	Cordes and Shortridge (1971)
Connective tissue	Fibrosarcoma	1	Jackson (1936)
		1	Feldman (1932)
		4	Cordes and Shortridge (1971)
	Fibroblastic tumour	3	Anderson and Sandison (1969b)
	Fibrolipoma	1	Jackson (1936)
	Lipoma	1	Anderson and Sandison (1969b)
	Liposarcoma	1	Cordes and Shortridge (1971)
	Mixed cell sarcoma	1	Jackson (1936)
	Myxoma	1	Jackson (1936)
		3	Cordes and Shortridge (1971)
	Rhabdomyoma	1	Feldman (1932)

Continued

Table 1.II continued

Connective tissue (continued)	Rhabdomyosarcoma	1	Collet, Collin and Flachat (1952-53) (6)
		1	Smith and Jones (1961)
		1	Manktelow (1963)(4)
		1	Harcourt and Spice (1968)
	Haemangioendothelioma	2	Anderson and Sandison (1969b)
	Haemangioma	1	Webster (1967)
	Lymphangioendothelioma	1	Anderson and Sandison (1969b)
	Synovioma	1	Cordes and Shortridge (1971)
Thymus	Thymoma	1	Santos and Costa Durao (1965)
		2	Brandly and Migaki (1963)
		1	Anderson, Sandison and Jarrett (1969)
		1	Cordes and Shortridge (1971)
	Carcinoma, (probably of Hassell's corpuscles)	1	Feldman (1931)
	Thymus tumour	1	Davis, Leeper and Shelton (1933)
	Malignant thymic tumour	1	Nyka (1929)
Heart	Sarcoma	1	Cotchin (1960)
	Fibrosarcoma	1	Misdorp (1962)
		1	Webster (1966)
		1	Smith and Jones (1961)
	Fibrolipoma	1	Webster (1966)
	Lipoma	1	Manktelow (1963)(4)
	Osteosarcoma	1	Misdorp (1962)
	Rhabdomyosarcoma	1	Manktelow (1963)
		3	Cordes and Shortridge (1971)
Brachiocephalic trunk	Chemodectoma	1	Ito et al (1971)
Hock Joint	Angiosarcoma	1	Cordes and Shortridge (1971)
Stifle Joint	Angiosarcoma	1	Cordes and Shortridge (1971)

Continued

Table 1 II continued

Anatomical Region	Tumour Type	No of Cases	Reference
Central nervous system	Astrocytoma	1	Cordes and Shortridge (1971)
	Cerebral meningioma	1	Pivnik and Frankhauser (1971)
Peripheral nervous system	Neurofibroma	1	Cordes and Shortridge (1971)
Unknown origin	Encephaloid tumour of chest	1	Crisp (1867)
	Carcinoma	1	Savage and Isa (1955)
	Adenoma	7	Nobel, Neumann and Kropfer (1970)

(1) Cited by Cotchin (1956)
(2) Cited by Goldberg (1920)
(3) Cited by Feldman (1931)
(4) Personal communication
(5) Cited by Anderson and Sandison (1968a)
(6) Cited by Harcourt and Spice (1968)

269 carcinomas of the small intestine. Analysis of his data showed that British breed ewes had a significantly higher prevalence of this neoplasm than did Merino, Merino-cross or Corriedale ewes. In the former group the prevalence varied from approximately 0.9 to 1.5 per cent., while the prevalence in the latter "finewool" breeds was 0.2 to 0.4 per cent. In Simpson's study a positive relationship was found between tumour prevalence and sheep stocking rates in areas from which the inspected ewes originated, but whether this was due to an effect of stocking rate per se, or whether it was due to some other factor related to intensity of farming such as soil type or fertility, climate, use of fertilizers, agricultural chemicals or animal remedies, pasture or weed species, could not be determined (Simpson, 1972b). The gross pathology of carcinomas of the small intestine based on the examination of a series of 450 cases (Simpson and Jolly, 1974) was of remarkable uniformity. The histological appearance of these neoplasms was also very similar, both in the morphology of the neoplastic cells and in the degree of formation of stroma. The appearance of two predominant cell types in each neoplasm was considered to reflect the origin of these tumours from a relatively undifferentiated cell type in the crypts of Lieberkuhn. Simpson and Jolly found that the primary site of tumour formation was in the jejunum and ileum, with a maximum frequency about the mid-portion of the small intestine and decreasing toward either end. In none of the tumours examined by them were primary tumours seen in the duodenum, whereas 10 per cent. of the primary lesions seen by Cordes and Shortridge (1971) were reported at that site. The latter authors considered that these carcinomas arise initially from luminal papillomas, but Simpson and Jolly argued that the polyps seen in their study were the result of growth of neoplastic tissue following invasion of the submucosa rather than the initial site of neoplastic transformation. Secondary spread of tumours was

most commonly *via* lymphatic vessels, by both metastasis and permeation and by transcoelomic dissemination of neoplastic cells or haematogenous spread in advanced cases.

Sheep Pulmonary Adenomatosis (Jaagsiekte)

This disease is primarily an adenomatous condition of the lungs of sheep which may metastasize to other organs of the body, such as the thoracic lymph nodes, pleura, peritoneal cavity, liver, spleen, kidney, myocardium and skeletal muscle. Clinically, affected sheep show dyspnoea and progressive wasting, leading to death. The disease may constitute a serious source of stock losses in affected flocks. Pulmonary adenomatosis of sheep has been the subject of a comprehensive review by Wandera (1971). It has been recognized at least since the turn of the century and reported from Peru, Chile, Iceland, United Kingdom, much of Europe, Southern Russia, Israel, India, Kenya, Tanzania, South West Africa, South Africa and the U.S.A.

The transmissible nature of sheep pulmonary adenomatosis was suspected by Robertson (1904) and Cowdry (1925a and b) and has since been proved in a number of laboratories (Sigurdsson, 1958; Markson and Terlecki, 1964; Enchev, 1966; Cuba-Caparo, de la Vega and Durand, 1967; Wandera, 1968) using cell-free and bacteria-free preparations injected into susceptible sheep. Microorganisms which have been detected in association with spontaneous cases of the disease or in tissue cultures inoculated with tumour material include *Mycoplasmas*, *Chlamydia* (Krauss, Wandera and Lauerman, 1971) and viruses. Recently Malmquist *et al.* (1972) demonstrated two types of virus in tumour tissue from sheep in Kenya. One was identified as Maedi virus and the other as a *Herpes* virus. The latter was morphologically similar in electron microscopic studies to that isolated by Smith and Mackay (1969), but gave rise to dissimilar cytopathic effects in fibroblastic

cell cultures. However the cytopathic effects reported in the work of Smith and MacKay were seen in cultures derived from alveolar macrophages and the morphological variation in response to the virus may have arisen from differing properties of the component cells in the two culture systems.

Hepatic Neoplasms

Primary tumours of the liver in sheep are amongst the most commonly reported ovine tumours. The most frequently reported histological form appears to be that derived from the hepatocyte and these have been recorded by various authors as liver carcinomas and hepatoblastomas. The latter name has been suggested by several authors (Frenkel, 1927; Nunes Pestica, Paz Ferreira and Carvalho Valera, 1961; Manktelow, 1965) largely on the basis of the presence of the foci of haematopoietic cells in these tumours. Manktelow found further support for an embryonic origin of these tumours by their demonstration in newborn lambs. Metastases are infrequently reported, but usually involve porto-hepatic lymph nodes or lungs. Tumours arising from bile duct epithelium form a smaller group of primary tumours of the liver.

In a survey of tumours affecting the liver conducted in this laboratory at the beginning of this present study (Johnstone, unpublished data), the livers of 136,164 mature sheep and 222,682 lambs killed in slaughter-houses were examined. A total of 26 neoplasms were found of which 15 were classified as hepatoblastomas, three as bile duct carcinomas, two haemangiosarcomas, four lymphosarcomas and two mastocytomas. Since the report of the two cases of mastocytoma (Johnstone, 1972) a further two cases of this neoplasm have been examined in this laboratory. In addition to the liver neoplasms, four vascular lesions believed to be hamartomas were noted in the survey.

Neoplasia of the Olfactory Mucosa

Neoplasms arising in the olfactory and ethmoid regions of the

nasal cavity, variously classified as adenopapillomas and adenocarcinomas, have occurred in outbreaks affecting several sheep in individual flocks of sheep in Europe, the U.S.A. and South Africa. The aetiology of these tumours and whether or not they represent a single biological entity is uncertain. In the cases investigated by Young et al. (1961) genetic factors did not appear to be of primary importance because several breeds of sheep were affected on the same property. Cohrs (1953, cited by Young et al., 1961) was able to transmit this tumour to other sheep using a cell-free preparation of the neoplasm. Similar attempts by Duncan et al. (1967) were unsuccessful. In two of five sheep fed ground nut meal containing aflatoxin for five years, neoplasms of the nasal cavity developed (Lewis, Markson and Allcroft, 1967). One of these was a chondroma and the other was described as a mucous adenoma with osteomatous differentiation in the stroma.

Squamous Cell Carcinoma of the Exposed Skin

Squamous cell carcinomas arising from the skin of the ears, muzzle and perineum are well recognized tumours of sheep. They are most frequently observed in geographical areas which experience high levels of exposure to sunlight, e.g. Australia. Lloyd (1961) considered that sunlight is the major aetiological factor in these cases and this seems reasonable in view of the known carcinogenic activity of ultra-violet light (Rueper, 1957). Other causes of chronic granulating lesions such as ear-mark wounds and penetration of ears by thorns have also been suggested as a cause of the tumour. Lombard and Magnol (1973) have reported increased frequency of this neoplasm in sheep of the Berrichon breed in France and have suggested that the degree of inbreeding in the particular flock examined was of aetiological importance.

Squamous Cell Carcinomas of the Wool Bearing Skin

These tumours are rare enough to contain [illegible]

sheep in Australia which show an inherited predisposition toward the development of dermoid cysts following the penetration of the skin by grass seeds (Carne, Lloyd and Carter, 1963). Borland (1966) demonstrated that intradermal implantation of autologous epithelial cells from the skin of these sheep resulted in the production of dermoid cysts identical to those occurring naturally and concluded that the latter were caused by transfer of epithelial cells into the dermis by grass seeds. A percentage of the cells lining these cysts undergo malignant transformation resulting in the development of squamous cell carcinomas. Cyst bearing sheep were more susceptible than normal sheep to the induction of papillomas following topical applications of dimethylbenzanthracene. This carcinogen also caused an increased frequency of neoplastic transformation in the lining cells of the implantation cysts when injected directly into these sites (Lloyd, 1966).

Tumours of Cartilage

Although claimed by Steiner and Bengston (1951) to be of relatively common occurrence, inspection of Table 1.II shows that cartilaginous tumours of sheep actually form a relatively small proportion of the total tumours in most surveys of ovine neoplasia. Contrary to most reports, Sullivan (1960) found 30 of the 32 cases he examined were malignant as judged by histological criteria.

Carcinoma of the Bladder

This tumour is of interest because of its increased frequency in sheep which have been fed on pastures infested with bracken fern (Harbutt and Leaver, 1969). Carcinomas in the bladder of cattle which have eaten this plant, have been reported in New Zealand (Smith and Beatson, 1970). However, Simpson (1971) was unable to demonstrate the carcinogenecity of New Zealand bracken fern (Pteridium aquilinum var. esculentum) in either rats or sheep which were fed diets containing 25

per cent. bracken fronds.

Malignant Lymphoma

As the study of this tumour of sheep forms the basis of this thesis, previous work reported by others will be discussed in its appropriate context in succeeding chapters.

II. COMPARATIVE ASPECTS OF THE EPIDEMIOLOGY OF MALIGNANT LYMPHOMA

Malignant lymphoma is the general term applied to any neoplastic disorder of lymphoid tissue, including Hodgkin's disease and reticulum cell sarcoma (see nomenclature, below). The literature pertinent to epidemiological aspects of malignant lymphoma in various species of animals is reviewed to provide a general background to the work presented in succeeding chapters of this thesis.

Malignant lymphomas are frequently occurring spontaneous tumours in a number of species. They are particularly common in the laboratory mouse, chicken and cat and are also of considerable significance in the dog and in cattle. Less is known about their occurrence in other domestic species but they appear to be an important tumour of the horse, pig and sheep.

Studies of the epidemiology of malignant lymphoma in domesticated animal species have particular advantages over those in the laboratory animals. The essentially random-bred nature of non-laboratory animals more closely reflects the situation in man than does that of the inbred laboratory mouse. They are also exposed to many of the environmental factors such as chemical pollutants which are of possible importance in carcinogenesis of man and the carnivorous household pets eat foods prepared in a manner very similar to those eaten by man. In short,

their habitat shows a closer similarity to that of man than does that of the animal adapted to laboratory conditions.

Much of the recent research into malignant lymphoma has been stimulated by the demonstration of the transmissible nature of this disease in the chicken and laboratory mouse and the subsequent purification and definition of a number of viruses believed to be causal agents. Evidence has been put forward to implicate viral aetiology in malignant lymphoma of several domesticated animal species, but to date only in the case of the cat is there unequivocal proof.

At present, intensive research programmes into malignant lymphoma are being carried out in cattle in several parts of Europe and the U.S.A. These have been prompted by the apparent endemic nature of the disease in these countries and the inference of a viral aetiology.

Nomenclature

The use of human disease nomenclature for similar conditions in animals is justified only when there is reasonable evidence that the conditions are in fact analagous. Morphological criteria alone are probably insufficient grounds to equate the diseases of the various species. This is especially valid in the case of the reticular neoplasms. For example, Forbus and Davis (1946), Bloom (1952), Simon, Small and Jaeschke (1964), Squire (1969) and Wells (1974) described certain of the malignant lymphomas of dog and pig as Hodgkin's-like, because some of the histological features of the well defined human condition were present. This is misleading and implies, unjustifiably, that an identical disease process is present. There is no doubt that a number of variants of malignant lymphoma do occur in animals but for the reasons stated, the broad term malignant lymphoma is used preferentially throughout this thesis when referring to otherwise undefined lymphoid neoplasms of animals.

Man

The reticuloses of man form a diverse disease spectrum of which malignant lymphoma is one of the major subgroups. Within the malignant lymphoma subgroup, follicular lymphoma, lymphosarcoma, reticulum cell sarcoma, reticulum cell sarcoma and Hodgkin's disease are the most commonly observed forms and constitute reasonably well-defined clinico-pathologic entities. For the purposes of this review, the collective title malignant lymphoma will refer only to these variants of the human disease. Lymphoid leukaemia is not considered a separate entity because it can be regarded as a haematogenous extension of a solid tumour affecting bone marrow, spleen or lymph nodes. Other leukaemias of myeloid and erythroid origin, multiple myelomas and mycosis fungoides will not be discussed.

In the present context it is considered impractical to review in any detail the vast bulk of material pertinent to malignant lymphoma of man. There is relatively little variation in the overall prevalence of malignant lymphoma in the major areas of the world. In the U.S.A. 55 people per million population developed one or other forms of malignant lymphoma in 1964, accounting for 0.014 per cent. of total deaths recorded in the vital statistics of the U.S., 1966 (cited by Lingeman, 1969). The rate in England and Wales was 42 per million (Symmers, 1966), while that for New Zealand was 85 per million (N. Z. Year Book, 1970). The reasons for these variations are unclear but may be explained in part by undefined factors of ethnic and geographic origin, variation in histological interpretation and recording, and availability or use of medical services.

The relative frequency of the various types of malignant lymphoma in a number of countries are shown in Table 1.III. Of interest is the relatively high proportion of reticulum cell sarcomas and the low proportion of Hodgkin's disease seen in Japanese people in comparison

TABLE 1.III

The Relative Prevalence of the Various Forms of Malignant Lymphoma Affecting Man in Several Different Countries

Author	Country	Numbers of Follicular Lymphoma	Numbers of Lympho-sarcoma	Number of Reticulum Cell Sarcoma	Numbers of Hodgkin's Disease	Total Number of Lymphoma (100%)
Lumb and Newton (1957)	Great Britain	61(17.5%)	63(18.2%)	29(8.4%)	194(55.9%)	347
Hilton and Sutton (1962)	Great Britain	39(10.7%)	87(24.0%)	85(23.4%)	152(41.9%)	363
Hancock (1958)	Great Britain	-	207(40.2%)	102(19.8%)	206(40.0%)	515
Symmers (1966)	Great Britain	90(8.5%)	222(20.8%)	269(25.2%)	486(45.5%)	1,067
Gall and Mallory (1942)	U.S.A.	42(6.7%)	220(35.6%)	127(20.6%)	229(37.1%)	618
Hellwig (1946)	U.S.A.	9(4.6%)	106(54.1%)	33(16.8%)	48(24.5%)	196
Jackson and Parker (1947)	U.S.A.	39(5.4%)	233(32.5%)	116(16.2%)	329(45.9%)	717
Williams et al. (1959)	U.S.A.	-	1733(46.6%)		1992(53.5%)	3,725
New Zealand Year Book (1970)	New Zealand	18(11.18%)	85(55.6%)		50(32.7%)	153
Gelpi (1970)	Saudi Arabia	-	23(53.4%)	5(11.6%)	15(35%)	43
Anderson et al.(1) (1970)	Japan	18(5.4%)	109(32.2%)	144(42.6%)	67(19.8%)	338
Davies (1964)	Uganda	-	193(46.4%)	124(29.7%)	100(23.9%)	417

(1) Atomic Bomb Casualty Commission Data

to other countries (Anderson et al., 1970). The Atomic Bomb Casualty Commission data from which their figures were compiled was chosen to exclude people immediately exposed to irradiation from atomic bombs. The causes for these differences are unknown, but they suggest that discrepancies in host reactivity presumably due to genetic factors, at present undefined predisposing or synergistic disease, or perhaps geographic influences may be responsible.

A peculiarly high prevalence of lymphosarcoma amongst children and young adults living in parts of Central Africa was first reported by Burkitt (1958).

Maps of tumour prevalence correlate closely to geographical zones and the distribution of certain malarial mosquitoes (Haddow, 1963). This so-called "Burkitt's tumour" of children is not, however, peculiar to these areas. Apparently identical or very similar undifferentiated lymphosarcomas with similar distribution of lesions have been reported from the U.S.A. (O'Conor, Rappaport and Smith, 1965), Jamaica (Bras, Murray and McDonnough, 1965), India (Desai, Meyer-Homji and Paymaster, 1965), New Guinea (ten Seldam, Cooke and Atkinson, 1966) and South America (Beltran, 1966; Fagundes, de Oliveira and Amaral, 1969).

Malignant lymphomas may occur at any age but periods of peak incidence are noted in the various forms of the disease. The follicular lymphomas characteristically develop at about 50 years of age and are only rarely observed in patients younger than 20 years (Symmers, 1966). Similarly, lymphosarcoma and reticulum cell sarcoma are most commonly encountered in middle or old aged patients (Gall and Mallary, 1942; Evans, 1966; Anderson et al., 1970). Evans found peak incidence in the fifth to seventh decades of life and Gall and Mallary showed that the poorly differentiated forms of lymphosarcoma tended to occur at an

earlier age. The exception, seen in certain geographic areas, of high incidences of childhood lymphosarcomas has already been mentioned. In most reports, Hodgkin's disease has age incidence peaks between 15 and 34 yr and above 45 yr. This pattern has been observed in the U.S.A., Netherlands, Sweden and Denmark (MacMahon, 1957). The early peaking observed in most Western surveys was not reported in Japanese cases by Anderson et al. (1970). Evans reported peaking between the ages of 20 to 40 yr and found that 20 per cent. of cases occurred in patients under 20 yr of age.

Malignant lymphomas show a constant predominance in males. Male to female ratios vary from 1.5:1 to 4:1 (Gall and Mallory, 1942; Symmers, 1966; Willis, 1967; Fagundes et al., 1969; N. Z. Year Book, 1973).

Racial differences in prevalence have been noted. With the exception of the childhood lymphomas of Africa and other tropical countries, people of Caucasian extraction show higher rates of malignant lymphoma than do other racial groups. (Vital Statistics of the U.S., 1966, cited by Lingeman, 1969; Anderson et al., 1970).

Laboratory Rodents and Lagomorphs

Of the commonly encountered laboratory animals the mouse eclipses all other species as a subject for investigation into malignant lymphoma. This emphasis on the mouse has developed with the availability of highly inbred laboratory strains showing a very high prevalence of spontaneously developing lymphoid tumours. There is little information on the prevalence of malignant lymphoma in feral mice, but it is believed to be low (Gross, 1970). Among the laboratory strains there is considerable variation in the frequency of occurrence of these neoplasms and data on some commonly used laboratory strains are given in Table 1.IV.

TABLE 1.IV

Prevalence of Spontaneous Malignant Lymphomas in Various Strains of Laboratory Mice

Strain	Approximate Lymphoma Prevalence per cent.
A	1
Ak	85
BALB	15
CBA	1
C3H	<1
C57 Brown	5
C57 Black	5
C58	85
DBA/2	30
I	5
R III	3
Swiss	5 - 11

Table reprinted from Gross (1970).

The demonstration of the viral aetiology of these tumours and the subsequent definition of a number of causal viruses has provided a background knowledge and stimulus for much of the research into malignant lymphoma of other animal species. The viruses are distributed widely throughout the various strains of laboratory mouse and are seen in low as well as high tumour frequency strains (Hilgers et al., 1972; Gross, 1970). The experimental study of malignant lymphoma in mouse has been extensively reviewed by Furth (1946), Kirshbaum (1951), Law (1954), Stansly (1963), Moloney (1964) and Gross (1970). Various aspects of this work in the mouse will be discussed in greater detail in later chapters.

The rat and rabbit are rarely affected by malignant lymphoma. Snell (1969) described two cases which occurred amongst several hundred rats of various strains maintained at the National Institutes of Health Laboratory, U.S.A. Both affected animals were of the Buffalo strain. Lopushinsky and Fay (1967) reported six cases of malignant lymphoma in cottontail rabbits in Michigan. Isolated reports of this disease in laboratory strains of rabbits have been made by Van Kampen (1968), Fox et al. (1970) and Ubertini (1972).

In the guinea pig it has been reported that virally induced malignant lymphoma occurs spontaneously with moderate frequency (Dunn, 1969; Gross et al., 1970). In the strains held at the National Cancer Institute, Bethesda, Congdon and Lorenz (1954) reported a prevalence of 3.3 per cent.

Chickens

Neoplastic diseases of the reticular and haematopoietic tissues of chickens are known as the avian leukosis complex. By far the most prevalent conditions in this disease spectrum are the virally

induced lymphosarcomas, lymphoid leukosis and Marek's disease (Hemboldt and Fredrickson, 1969; Gross, 1970). These tumours were first reported about the turn of the last century (Caparini, 1896, cited by Olson, 1941; Marek, 1907, cited by Hemboldt and Fredrickson, 1969). Until poultry rearing became a "mass-production" business the diseases were seen only sporadically but since 1920 their prevalence has been reported to increase steeply. Whereas the number of chickens slaughtered yearly in the U.S.A. between 1961 and 1968 did not change significantly, the number condemned due to leukosis rose from 100 to 2,000 birds per 100,000 slaughtered (Goldstein, 1968, cited by Purchase, 1972). The proportion of condemnations due to leukosis increased during this time from 5.7 to 48 per cent. (Calnek, 1972). Most of these losses are due to Marek's disease (Calnek, 1967; Purchase, 1972), while lymphoid leukosis tends to occur sporadically (Purchase and Burmester, 1972).

Marek's disease is of economic importance in all major areas of poultry production (Purchase, 1972). It is primarily a disease of birds between two and five months of age, although cases can occur as early as three weeks and as late as two years. All breeds and both sexes are affected (Jungherr and Hughes, 1965). Losses in individual flocks have been estimated to range from a few birds to 25 or 30 per cent. and occasionally as high as 60 per cent. (Jungherr and Hughes, 1965; Biggs, 1968). Lymphoid leukosis affects older birds, usually occurring between 14 and 30 weeks of age (Purchase and Burmester, 1972).

Lymphoid leukosis is caused by infection of birds with one of a number of recognized oncorna viruses (Gross, 1970).

In 1967 two groups of investigators (Churchill and Biggs, 1967; Nazerian et al., 1968; Solomon et al., 1968) isolated a herpes virus

from cases of Marek's disease and presented strong circumstantial evidence that this virus was the cause. Subsequently, the demonstration of cell-free virus in feather follicles and the transmission of Marek's disease by virus from this source (Nazerian and Witter, 1970) and the development of a successful vaccine (Churchill, Payne and Chubb, 1969) have established beyond doubt that the disease is due to a specific herpes virus. The infection of chickens by Marek's disease virus occurs with much greater frequency than is indicated by mortality or condemnation of meat as a result of this disease. Using immunological tests to detect the presence of precipitating antibodies to the virus in sera, Chubb and Churchill (1968), Witter et al. (1970) and Ianconescu and Samberg (1971) have shown a very high prevalence of Marek's disease virus infection in many commercial flocks. In some cases nearly 100 per cent. of the birds in a flock have shown serological evidence of infection. Marek's disease is the first naturally occurring neoplastic disease to have become controllable by vaccination.

Sheep

The prevalence of malignant lymphoma in sheep determined from surveys of animals killed in slaugher-houses is shown in Table 1.V. With the exception of the Netherlands and certain districts of France it is low. Estimates of the relative occurrence of malignant lymphoma compared to other neoplasms have also been obtained from slaughter-house and diagnostic laboratory surveys and are shown in Table 1.VI. It appears that malignant lymphoma is one of the most common types of tumour in sheep (Table 1.I.)

Most cases have been recorded in mature or aged animals. In the twenty cases reported by Monlux, Anderson and Davis (1956), one animal was one year old, three were five years old and the remainder were listed as "old". Fifteen of the seventeen cases in Migaki's (1969)

TABLE 1.V

The Prevalence of Malignant Lymphoma per Million Sheep Inspected at Slaughter-houses

Country	Author	Prevalence
Belgium	Mammerickx and Derzelle [1]	3.2 - 9.0 [2]
France	Lombard (1968) [1]	19 - 1400 [2]
Germany (Weisbaden)	(a) Woelke (1972) [1]	16.0
(West Berlin)	(b) Holzner (1972) [1]	0.4
Great Britain	Anderson and Jarrett (1968)	10.0
New Zealand	Webster (1966)	460.0 [3]
	Webster (1967)	22.0
Netherlands	Misdorp (1967)	160.0
U.S.A.	(a) U.S.D.A. Meat Inspection Division (1955) [4]	6.8
	(b) Monlux, Anderson and Davis (1956)	20.0
	(c) U.S.D.A. Meat Inspection Division (1960) [4]	6.8
	(d) Moulton (1963)	5.0
	(e) U.S.D.A. Consumer and Marketing Service (1965) [4]	5.0
	(f) Migaki (1969)	3.3

(1) Cited by Bostock and Owen (1973)

(2) Variations within this range according to district

(3) In "cull-for-age" ewes

(4) Cited by Migaki (1969)

TABLE 1.VI

The Numbers of Malignant Lymphomas Reported in Surveys of Sheep Neoplasms

Author	Country	No. of Neoplasms	No. of Malignant Lymphomas	Percentage Malignant Lymphomas
Sticker (1902)(1)	Germany	7	1	14
Feldman (1931)	U.S.A.	40	4	10
Davis, Leeper and Shelton (1933)	U.S.A.	34	7	2
Jackson (1936)	South Africa	28	4	14
Monlux, Anderson and Davis (1956)	U.S.A.	66	20	30
Cotchin (1960)	England	38	10	26
Smith and Jones (1961)	South Africa, England, U.S.A., Scotland	129	14	11
Manktelow (1963)(2)	N.Z.	95	19	20
Smith (1962)	U.S.A.	431	1	<1
Moulton (1963)	U.S.A.	163	77	47
Brandley and Migaki (1963)	U.S.A.	34	11	32
Sastry and Tweihaus (1965)	U.S.A.	10	3	30
Webster (1966)	N.Z.	120	23	19
Webster (1967)	N.Z.	385	7	2
Misdorp (1967)	Netherlands	32	16	50
Anderson, Sandison and Jarrett (1969)	Britain	107	44	41
Nobel, Neumann and Klopfer (1970)	Israel	114	3	3
Cordes and Shortridge (1971)	N.Z.	256	40	6

(1) Cited by Feldman (1931)

(2) Personal communication

series occurred in sheep described as "old", and nearly all of the 44 cases found in the series of Anderson, Jarrett and Crighton (1969) were listed as "mature".

Reports from New Zealand are conflicting. Of the 23 cases reported by Webster (1966) at least 15 were in "cull-for-age" ewes. The following year he recorded a further seven cases (Webster, 1967) of which six occurred in aged sheep and the other was in a lamb. However, Cordes and Shortridge (1971) examined 14 cases affecting lambs less than one year old and only six cases in older age groups.

The prevalence of malignant lymphoma in the U.S.A. is probably higher than that indicated by meat inspection statistics, because approximately 95 per cent. of the slaughtered population examined have not reached maturity (Brandley and Migaki, 1963). Due to the cost of transportation and slaughter, most range ewes are not killed in Federally inspected abattoirs.

Isolated reports of malignant lymphoma in sheep have come from Feldman (1926), Head (1953), Pallaske (1958), Lombard and Witte (1959), Enke (1964), Drawer (1965), Hilgenfeld and Krieg (1965), Nobel, Neumann and Klopfer (1967), Dukić, Stamatović and Durickovic (1969), Wittman and Urbaneck (1969), Ulbrich et al. (1970), Wittman (1970).

Preliminary epidemiological reports from Germnay indicate that the prevalence of malignant lymphoma in some areas and in some flocks of sheep reaches very high levels (Paulsen et al., 1971, 1972; Weiss et al., 1971).

Cattle

Malignant lymphoma is without doubt the most widely reported neoplastic disease of this species (Feldman, 1932; Monlux et al., 1956;

Plummer, 1956; Cotchin, 1960; Bendixen, 1965). Table 1.VII shows the frequency with which malignant lymphoma occurs in comparison with other bovine tumours in material reported by diagnostic laboratories and slaughter-houses. The reports of the prevalence of this disease from Colorado (Table 1.VII) appear to be lower than normal because of the high percentage of occular squamous cell carcinomas encountered in the Hereford cattle breed which comprise the majority of cattle slaughtered in this district. The same preponderance of occular squamous cell carcinomas was also observed in the series reported by Jackson (1936).

The prevalence of the disease reported from meat inspection statistics from various countries is shown in Table 1.VIII. The tumour is common in Germany, Sweden, Denmark, U.S.S.R. and the U.S.A. with most cases occurring in high-prevalence areas within these countries. Most cases in Germany have been reported in north-eastern areas and to the east of the Baltic Sea. Since the Second World War, the disease has been reported as spreading westward from the river Elbe and statistics indicate a gradual increase in the prevalence of this neoplasm in all areas (Bendixen, 1965). High tumour frequencies have been reported in middle and southern Sweden. Hjärre (1958, cited by Squire, 1964) observed that in many of these areas whole blood vaccination of calves against piroplasmosis had been carried out for many years. He suggested the increased prevalence of malignant lymphoma may have been partly due to the distribution of an oncogenic agent in the vaccine. Nearly all the cases reported from Denmark occurred on the islands of Zealand and Lolland-Falster (Bendixen, 1965). In the U.S.A. malignant lymphoma of cattle has been noted with greatest frequency in certain areas of California, the Midwestern States and New Jersey.

TABLE 1.VII

The Numbers of Malignant Lymphomas Reported in Surveys of Cattle Tumours

		Neoplasms	No. of Malignant Lymphomas	Percentage Malignant Lymphomas
Davis, Leeper and Shelton (1933)	U.S.A. (Colorado)	90	7	8
Jackson (1936)	South Africa	108	3	3
Monlux, Anderson and Davis (1956)	U.S.A. (Colorado)	908	20	<1
Plummer (1956)	Canada	447	142	32
Cotchin (1960)	England (London)	293	63	22
Smith and Jones (1961)	U.S.A. England Scotland South Africa	1371	369	27
Misdorp (1967)	Netherlands	231	20	9
Anderson, Sandison and Jarrett (1969)	Great Britain	302	40	13
Nobel, Neumann and Klopfer (1970)	Israel	500	283	57
Novloski and Santos (1970)	Brazil	36	11	28
Shortridge and Cordes (1971)	New Zealand	372	86	23
Prasad and Chandrasekharan (1971)	India	1163	13	1

TABLE 1.VIII

The Prevalence of Malignant Lymphoma per Million Cattle Inspected at Slaughter-houses

Country and District		Author	Prevalence
Germany Brandenburg		Welsch (1933)[3]	1,590-1,770[1]
Pomerania		Streigler (1933)[3]	5,000-6,000[1]
Berlin		Lockhau (1933)[3]	6,000
All States	(1938-42)	Fortner (1953)[3]	2,000
Berlin		Dobberstein and Seifried (1938)[3]	1,500-4,000[1]
Koenigsburg	(1938-42)	Fortner (1953)[3]	3,000
Tilsit	(1938-42)	Fortner (1953)[3]	15,000
Koeln	(1938-42)	Fortner (1953)[3]	90
Frankfurt	(1938-42)	Fortner (1953)[3]	70
Western Germany		Kruger (1962)[3]	200
Eastern Germany		Mieth, Schluter and Schwedler (1970)	4,800
Denmark		Bendixen (1965)	40 (10-100)[2]
Sweden	(1941)	Olsen (1961)[3]	800
	(1945)	Olsen (1961)[3]	3,100
	(1954)	Olsen (1961)[3]	6,000
	(1959)	Olsen (1961)[3]	3,400
	(1961)	Olsen (1961)[3]	500-800[2]
Netherlands		Misdorp (1967)	2.8
Belgium		Mammerickx (1969)	40
U.S.S.R.		Kudryavtseva (1972)	1,540
Great Britain		Anderson, Sandison and Jarrett (1969)	57
Brazil		Novloski and Santos (1970)	67
U.S.A. All States	(1922)	Sorensen et al. (1961)	40
	(1930)	Sorensen et al. (1961)	150
	(1935)	Sorensen et al. (1961)	90

TABLE 1.VIII (Contd.)

Country and District		Author	Prevalence
	(1938)	Sorensen et al. (1961)	150
	(1948)	Sorensen et al. (1961)	94
	(1950-52)	Sorensen et al. (1961)	90
	(1958)	Sorensen et al. (1961)	180
California		Theilen, Appleman and Wixom (1963)	80
California - San Francisco	(1959-60)	Reisinger (1963)	30
Los Angeles	(1959-60)	Reisinger (1963)	56
San Diego	(1959-60)	Reisinger (1963)	0
Pennsylvania-Philadelphia	(1959-60)	Reisinger (1963)	360
Scranton	(1959-60)	Reisinger (1963)	940
Minnesota	(1959-60)	Reisinger (1963)	600
Minnesota		Anderson et al. (1971)	148

(1) Variations within this range according to abattoirs in these districts.

(2) Variations within this range according to district.

(3) Cited by Bendixen (1965).

Peaking of malignant lymphoma prevalence in certain years has been observed in Swedish and American statistics (Table 1.VIII). These fluctuations were probably the result of marketing practices and economic and climatic factors which affected slaughter management Reisinger, 1963). Because of such factors it is extremely difficult to relate observed tumour frequency data with true tumour incidence at a particular time. Movement of cattle from one geographic area to another may also affect estimations of prevalence.

There are many reports of bovine malignant lymphoma occurring in different countries in an apparently casuistic manner (for example those of Jones, 1928; Lewis and Savage, 1930; Boyd, 1934; Frank and Thompson, 1940; Thompson and Roderick, 1942; Jasper, Sautter and Malmquist, 1946; Boyd et al., 1947; Sautter and Sellers, 1948; Rees, 1964).

Breed or sex have not been shown to influence the disease incidence (Hjärre, 1958; Reisinger, 1963; Bendixen, 1965; Conner et al., 1966; Anderson, Jarrett and Crighton, 1969; Migaki, 1969).

Usually, clinical lesions of malignant lymphoma do not develop under four years, with most cases occurring between five and eight years, although all age groups may be affected (Moulton, 1961; Bendixen, 1965; Migaki, 1969). The disease in immature animals occurs sporadically and is uncommon in most parts of the world (Monlux et al., 1956; Bendixen, 1959; Dungworth, Theilen and Lengyel, 1964; Theilen and Dungworth, 1965; Marshak et al., 1966) with the exception of Scotland where the large majority of cases reported by Jarrett and Crighton (1965) were seen in cattle less than three years of age.

Pigs

Malignant lymphoma is considered to be the most common neoplasm of this species (Cotchin, 1956; Sullivan and Anderson, 1959). Table

1.IX shows the prevalence compared with other neoplasms reported in series of neoplasms obtained from slaughter-houses and diagnostic laboratories. The prevalence of the disease per million pigs slaughtered in various slaughter-houses is shown on Table 1.X. With the exception of certain districts in France, rates appear fairly uniform throughout Europe and the U.S.A. There are numerous case reports in the literature, from many countries (Hodgson, 1903; Wyssmann, 1911; Lund, 1924; Biester and McNutt, 1926; Manegold and Machens, 1927; Feldman, 1930, 1932; Kernkamp, 1945; Bowler, 1948; Markson, 1955; Pyke, 1955; Salomon, 1955; Dimizio, 1959; Pavlovsky, 1962; Marienburg, 1963; Logger, Baars and Mouwen, 1966; Lombard and Granier, 1966).

All ages are affected by malignant lymphoma but it is most commonly a disease of young pigs. Anderson and Jarrett (1968) and Migaki (1969) showed 60 per cent. of affected pigs were younger than six to eight months. These results may be biased as 92 per cent. of pigs slaughtered were in this age group (Migaki, 1969). Two prevalence peaks occurred in Renier's et al. (1966) series, in which 80 per cent. of 45 cases were seen at about six months and the remainder at about 21 months of age.

There are no reports of a sex or breed influence on prevalence.

Dogs

Table 1.XI summarizes the reports showing the prevalence of malignant lymphoma relative to other canine neoplasms. Because of the lack of information regarding canine populations, it is difficult to determine the overall frequency of malignant lymphoma in dogs. Dorn, Taylor and Hibbard (1967) found a prevalence of 24 cases per 100,000 dogs in a defined canine population in two Californian counties.

It is usually reported (Bloom and Meyer, 1945; Smith, 1963;

TABLE 1.IX

The Numbers of Malignant Lymphomas Reported in Surveys of Pig Neoplasms

Author	Country	No. of Neoplasms	No. of Lymphomas	Percentage Lymphomas
Davis, Leeper and Shelton (1933)	U.S.A. (Colorado)	26	6	23
Jackson (1936)	South Africa	4	0	0
Plummer (1956)	Canada	28	14	50
Monlux, Anderson and Davis (1956)	U.S.A. (Colorado)	28	9	32
Cotchin (1960)	England	27	15	56
Smith and Jones (1961)	U.S.A. England Scotland South Africa	167	58	35
Smith (1962)	U.S.A.	1,824	6	<1
Brandley and Migaki (1963)	U.S.A.	187	67	35
Moulton (1963)	U.S.A.	2,005	1,093	57
Misdorp (1967)	Netherlands	32	12	38
Anderson and Jarrett (1968)	Great Britain	133	92	36
Nobel, Neumann and Klopfer (1970)	Israel	2	2	100

TABLE 1.X

The Prevalence of Malignant Lymphoma per Million Pigs Inspected at Slaughter-houses

Country and District	Author	Prevalence
Belgium	Mammerickx and Derzelle (1967)(1)	4
France (a) Many districts	Lombard (1968)(1)	1-640
(b) Brittany	Chevral et al. (1969)(1)	54
Germany (a) Hanover	Overbeck (1972)(1)	20
(b) Weisbaden	Woelke (1972)(1)	16
(c) West Berlin	Holzner (1972)(1)	3
Great Britain	Anderson, Sandison and Jarrett (1969)	25
Netherlands	Misdorp (1967)	20
U.S.A. (a)	U.S.D.A. Meat Inspection Division (1955)(2)	15
(b) Colorado	Monlux, Anderson and Davis (1956)	10
(c)	U.S.D.A. Meat Inspection Division (1960)(2)	14
(d)	Moulton (1963)	17
(e)	U.S.D.A. Meat Inspection Division (1965)(2)	21
(f)	Migaki (1969)	18

(1) Cited by Bostock and Owen (1973).

(2) Cited by Migaki (1969).

TABLE 1.XI

The Numbers of Malignant Lymphomas Reported in Surveys of Canine Tumours

Author	Country	No. of Neoplasms	No. of Malignant Lymphomas	Percentage of Malignant Lymphomas
Bloom and Meyer (1945)	New York City, U.S.A.	683	20	3
Cotchin and Douglas (1951)	London, England	1,121	13	1
Cotchin (1954)	London, England	2,361	88	3
Cotchin (1959)	London, England	4,187	159	4
Smith and Jones (1961)	England Scotland South Africa U.S.A.	5,315	181	3
Smith (1962)	Texas, U.S.A.	3,570	87	2
Mulligan (1963)	Denver & Los Angeles, U.S.A.	2,112	51	2
Howard and Nielsen (1965)	Connecticut, U.S.A.	953	41	4
Dorn, Taylor and Hibbard (1967)	California, U.S.A.	72	5	7
Murray (1968)	Nairobi, East Africa	139	34	24
Brodey (1970)	Pennsylvania, U.S.A.	2,917	155	5
Nobel, Neumann and Klopfer (1970)	Israel	70	6	9
Appleby, Hayward and Renyard (1971)	London, England	250	16	6

Squire, 1969; Altman and Squire, 1970) that malignant lymphoma affects mature or older dogs most frequently, although cases have been recorded at all ages. No sex prevalence has been found (Meier, 1957; Smith, 1962, 1963; Squire, 1964).

Certain breeds are said to be more susceptible to malignant lymphoma than others, Scottish terriers (Cotchin, 1954; Mulligan, 1963), boxers (Smith, 1963; Howard and Nielsen, 1965; Squire, 1969) and cocker spaniels (Squire, 1969) are examples. No breed susceptibility was observed by Meier (1957).

Cats

Malignant lymphoma is one of the most frequently occurring neoplasms in the domestic cat (Cotchin, 1952, 1957; Nielsen and Holzworth, 1953; Holzworth and Nielsen, 1955; Holzworth, 1960; Squire, 1964; Jarrett, 1966; Schmidt and Langham, 1967; Whitehead, 1967; Dorn et al., 1968; Crighton, 1969; Nielsen, 1969; Brodey, 1970). Table 1.XII lists reports of the relative frequency of this tumour compared to other malignant tumours in cats. The true prevalence of the disease is difficult to ascertain because of lack of accurate population denominators. Dorn et al. (1967) considered the incidence in two counties of California was 41.6 per 100,000.

Cats younger than six years of age are most frequently affected and within these limits the incidence peaks between six months and two years (Nielsen and Holzworth, 1953; Crighton, 1968; Nielsen, 1969; Jarrett, 1971; Meincke, Hobbie and Hardy, 1972).

Reports of breed and sex differences are scarce in the literature and difficult to assess. In the survey of Californian cats (Dorn et al., 1968) Siamese cats were more commonly affected than other breeds, but without statistical significance and the male to female

TABLE 1.XII

The Numbers of Malignant Lymphomas Reported in Surveys of Feline Neoplasms

Author	No. of Neoplasms	No. of Malignant Lymphomas	Percentage Malignant Lymphomas
Cotchin (1952)	226	33	15
Cotchin (1956)	200	39	19
Smith and Jones (1961)	174	17	10
Smith (1962)	404	13	3
Nielsen (1964)	254	82	32
Schmidt and Langham (1967)	256	57	22
Stunzi (1967)	248	69	28
Whitehead (1967)	165	46	27
Brodey (1968) (1)	413	109	26
Brodey (1970)	395	103	26
Schneider (1970)	1,154	306	26

(1) Cited by Nielsen (1969)

ratio of cases was 33:18. Cotchin (1957) reported a male to female ratio of 16:3. In the experience of Holzworth (1960), twice as many male as female cats are examined in veterinary clinics for all types of disease. She considers it likely that the observed male to female prevalence in malignant lymphoma is due to this fact.

Household clusters of high incidence have been reported (Jarrett et al., 1964; Loeb, 1964; Schneider et al., 1967; Schneider, 1970; Cotter, Gilmore and Rollins, 1973).

Transmission of malignant lymphoma has been demonstrated in cats following inoculation with cell free extracts of the tumour. Several strains of virus have been isolated from these cases and have been shown to be the causal agents by means of appropriate transmission experiments. Serological surveys have shown that many apparently normal cats have been in contact with or harbour these viruses (Hardy et al., 1969; Essex, Cotter and Carpenter, 1973; Jarrett et al., 1973).

Horses

Reports of malignant lymphoma in this species are infrequent. Table 1.XIII shows the prevalence of the disease in horses slaughtered in abattoirs in the U.S.A. and Netherlands. The observed variation in prevalence of malignant lymphoma in the horse population of the U.S.A. is probably explained in part by the irregularity in culling practices for this species, which has resulted in non-uniformity amongst the groups of animals submitted for slaughter (Migaki, 1969).

Table 1.XIV records the relative frequency of malignant lymphoma to other equine tumours.

Case reports in the literature include those of Danelius (1941), Runnells and Benbrook (1944), Tutt (1946), Foss (1958), Seils (1960),

TABLE 1.XIII

The Prevalence of Malignant Lymphoma per Million Horses Killed in Slaughter-houses

Country	Author	Prevalence
U.S.A. (1926-29)	Feldman (1930)	8
U.S.A. (1961)	Reisinger (1963)	506
U.S.A. (1958-1967)	Migaki (1969)	0-187(1)
U.S.A. (1967)	U.S.D.A. Consumer and Marketing Division (1967)(2)	172
Netherlands (1960-1965)	Misdorp (1967)	32

(1) Variation within this range over ten year period.

(2) Cited by Migaki (1969).

TABLE 1.XIV

The Numbers of Malignant Lymphomas Reported in Surveys of Equine Neoplasms

		No. of Neoplasms	No. of Malignant Lymphomas	Percentage Malignant Lymphomas
Plummer (1956)	Canada	14	2	14
Cotchin (1960)	London, England	90	2	2
Smith and Jones (1961)	England Canada South Africa U.S.A.	464	2	<1
Smith (1962)	U.S.A.	718	1	<1
Brandley and Migaki (1963)	U.S.A.	2	1	50
Misdorp (1967)	Netherlands	39	6	15

Theilen and Fowler (1962), Gillis (1965), Misdorp and Nijlan (1966), Dobin and Epstein (1969), Vaughan (1969), Bruere, Sutton and Davis (1970), and Conboy and Powers (1971). There is no known breed or sex prevalence.

Non-Human Primates

Reports of the disease are rare in these species. Lingeman, Reed and Garner (1969) reviewed 15 case reports from the literature of which only seven were satisfactorily diagnosed and confirmed by histological examination as malignant lymphoma. The majority of these were in Old World Monkeys, with only three reports concerning malignant lymphomas in the higher apes, all of these being in gibbons (Hylobates lar).

More recently Johnsen et al. (1971) reported four cases in a colony of 120 gibbons which had been splenectomised. In an interval of 25 months, 24 cases occurred in the colony of Rhesus monkeys (Macaca mulatta and Macaca arctoides) maintained at the U.S. National Centre for Primate Biology (Stowell et al., 1971). The prevalence of malignant lymphoma in this establishment was 6 cases per 1,000 for all non-human primates and 12 cases per 1,000 for Rhesus monkeys. Ten of these twenty four cases had received diagnostic head X-rays at three-monthly intervals for nine months.

Jungherr (1963) reported a lymphoma affecting a kidney from a Cynomolgus monkey. This was the only case recorded from 800,000 monkeys imported into the U.S.A. for the production and testing of polio vaccines.

No cases of malignant lymphoma were reported in the following series of necropsy examinations: Haberman and Williams (1957), 708 monkeys; Kennard (1941), 246 monkeys; Lombard and Witte (1959),

1,320 non-human primates; Lapin and Yakovleva (1963), 156 baboons and 214 Macaques.

The ages of monkeys affected in the series reviewed by Lingeman et al. (1969) were given in only four cases, all of which were pre-pubertal. Stowell et al. (1971) reported 21 of 24 cases affecting adults, while the four gibbons seen by Johnsen et al. (1971) were young adults (five to seven years).

Poikilothermic Animals

Neoplasia of reticular tissues of cold-blooded animals has been reported infrequently and in some cases doubt remains that the condition described was neoplastic.

Frye and Carney (1972, 1973) described neoplasms of haematopoietic cells of a terrapin (Pseudamys elegans) and a boa constrictor (Constrictor constrictor). These are the only reports of spontaneous neoplasia of such tissues in reptiles although sarcomas have been induced in turtles and serpents by inoculation with avian sarcoma virus (Svet-Moldavsky, Trubcheninova and Ravkina, 1967).

Lymphosarcoma of amphibians has been described and investigated by Inoue and Singer (1963), Balls (1962, 1964, 1965) and Balls and Ruben (1965, 1967). The possibility that the diseases described were granulomas of infectious origin needs to be excluded (Dawe, 1969).

There have been numerous examples of malignant lymphoma reported in bony fishes (teleosts). Schlumberger (1957) found the disease in 10 per cent. of the fish Esox masquinongy recovered from Lake Scugog in the vicinity of Toronto and Mulcahy (1963) reported that 0.12 per cent. of Northern pike (Esox lucius) inspected during a pike eradication programme in Ireland were affected. Cases in salmonid fish (Haddow and Blake, 1933), splake and brook trout (Dunbar, 1969)

and Mexican characins (Nigrelli, 1947; Friedman, 1962) are also recorded.

Neoplastic proliferation of haemocytes in five American oysters (Crassostrea virginica) and one Pacific oyster (Crassostrea gigas) was reported by Farley (1969), and a similar condition in another American oyster by Couch (1969). These are the only reports of haematopoietic neoplasia in molluscs.

No convincing reports of reticular neoplasia of insects were found by Dawe.

CONCLUSIONS

It is suggested from the preceeding consideration of neoplasia in sheep and of the epidemiological aspects of malignant lymphoma in various animal species that malignant lymphoma could be an important tumour in regard to comparative studies. Proof of a viral aetiology in murine, cavian, feline and avian species has stimulated parallel research in other species such as the ox, dog, pig, non-human primate and sheep. Of these, most progress has been made with studies of the disease in cattle and the apparent similarities between pathological and epidemiological aspects of malignant lymphoma in cattle and sheep in European countries has provided further incentive for the present investigation into this disease of sheep.

Relatively little informative data was available on the nature of malignant lymphoma of sheep at the inception of this study in 1970. Most reports were derived from surveys of tumours occurring in slaughter-houses or examined at diagnostic stations. The "gaps" in knowledge indicated by the literature at that time coupled with the investigations in other species suggested that an investigation of

this condition of sheep, with particular emphasis on its experimental transmission, could be of value in the field of comparative tumour research.

CHAPTER 2

THE PATHOLOGY OF MALIGNANT LYMPHOMA OF SHEEP

INTRODUCTION

Malignant lymphoma of sheep, although well recognized in surveys of tumours conducted in slaughter-houses and diagnostic stations (Tables 1.I, 1.V and 1.VI), has attracted little attention beyond this initial recognition. There is a scarcity of data concerning the pathology of this disease in sheep compared to the mass of published work in other species. It was therefore essential that, before proceeding to any experimental program, a study should be made of the main pathological features of the disease as it occurs in New Zealand. In this chapter the pathological details from a series of 40 cases of malignant lymphoma in sheep are described and discussed within the framework of existing reports of this disease in this and other animal species.

MATERIALS AND METHODS

In all except two cases, the specimens described were obtained from slaughter-houses during the routine inspection of carcasses for general fitness for human consumption. The other two cases were referred to us by a veterinarian who discovered them during a survey conducted to investigate causes of sheep mortality.

As a result of the method of carcass inspection employed in New Zealand slaughter-houses, it was not possible to examine the skin, feet, or uro-genital tract below the level of the kidneys in any animals. In some cases various parts of the carcass including the head were not available for inspection because of their premature disposal (Table 2.I).

TABLE 2.1

The Distribution of Histologically Confirmed Lesions in Spontaneously Occurring Cases of Malignant Lymphoma in Sheep Killed at Slaughter-houses or Found During a Survey of Sheep Mortality

Case No.	Age	General condition of carcass	Affected Organs: Lymph nodes	Spleen	Liver	Bone marrow	Kidney	Small intestine	Heart	Abomasum	Omentum	Lungs	Large intestine	Adrenal	Diaphragm	Reticulum	Rumen	Pancreas	Skeletal muscle	Genital organs and lower urinary tract	Spinal cord	Skin
401082	Mature	Poor	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	0	-	+
7286	Lamb	Good	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	0	+	0
6426	Mature	Poor	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	+	-	0	0	0
6186	Mature	Poor	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	0	0	0
6026	Mature	Good	+	-	+	0	+	+	+	-	-	+	-	-	+	-	-	-	-	0	0	0
5669	Mature	Good	-	-	-	0	+	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0
5651	Mature	Good	+	-	-	0	+	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0
5487	Mature	Poor	+	+	-	0	-	+	+	+	+	-	+	-	+	-	+	-	+	+	-	-
4858(2)	Mature	Poor	+	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4828(1,3)	Mature	Poor	+	0	0	-	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4827(3)	Mature	Poor	+	+	+	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0
4823(1)	Lamb	Good	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4811	Mature	Poor	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	0	0	0
4715	Mature	Good	+	+	+	0	-	+	+	+	+	-	-	-	-	-	-	-	-	0	0	0
4713	Mature	Good	+	+	+	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	0
4712	Mature	Poor	+	+	-	0	-	-	+	-	-	-	-	+	-	-	-	-	-	0	0	0
4699	Mature	Good	-	+	-	0	+	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0
4548	Mature	Poor	+	+	+	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4292	Unknown	Unknown	-	-	-	0	+	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0
4098(4)	Mature	Good	+	+	0	-	-	+	+	-	-	-	-	-	-	+	-	-	-	0	0	0
4097(4)	Mature	Good	+	-	+	-	+	+	-	-	+	-	-	+	-	-	-	-	-	0	0	0
3855	Mature	Unknown	+	+	+	0	-	-	-	+	-	-	+	-	-	-	-	-	-	0	0	0
Percent. affected organs in cases			86	73	68	50	48	47	47	37	21	16	11	11	11	5	5	5	5	20	20	33

\+ Lesions present.

\- Lesions absent.

0 Organ not inspected.

(1) Thoracic and abdominal viscera detached from carcass during routine inspection and were not examined.

(2) Thoracic and abdominal viscera detached , with the exception of a portion of liver attached to diaphragm, from the carcass and were not examined.

(3) Cases obtained from the same property.

(4) Cases obtained from the same property.

Material for histological study was fixed in 10 per cent. formol-saline and processed for paraffin sectioning by routine histological techniques. Sections were cut to approximately five micron thickness and stained by haematoxylin and eosin (HE), Gordon and Sweet's stain for reticulin and sometimes periodic acid-Schiff, Ziehl Neelsen, picro-Mallory, Giemsa and toluidine blue methods. Impression smears were fixed with 100 per cent. methanol and stained by the May-Grünwald-Giemsa method.

In 10 cases which were suitable for electron microscopy, one millimetre cubes of tumour tissue were fixed within 30 min. of slaughter in Karnovsky's solution at 4°C (Appendix I) and post fixed in 1 per cent. osmium tetroxide in 0.1M phosphate buffer. After processing and embedding in epoxy resin by routine methods (Appendix I), sections were cut on an LKB III ultramicrotome, stained with uranyl citrate and Reynold's lead solution (Reynolds, 1963) and examined in a Philips EM 200 electron microscope. Sections of epoxy resin embedded tissues one or two microns thick were examined by light microscopy after staining with haematoxylin and phloxine (Shires, Johnson and Richter, 1969), or toluidine blue.

In addition to the 22 cases from which fresh tissues were available, paraffin embedded material was examined from an additional 18 cases held in the files of the Department of Veterinary Pathology and Public Health, Massey University.

RESULTS

The Gross Pathology of Malignant Lymphoma in Sheep

The distribution of tumours and the age and general carcass condition in a series of 22 sheep affected by malignant lymphoma for which pathological details could be documented is shown on Table 2.I. All

gross lesions were confirmed histologically. Table 2.II shows the patho-anatomical form of the disease occurring in these cases, according to the classification of Anderson, Jarrett and Crighton (1969) (Appendix II).

Lymph nodes

Tumours in lymph nodes were found in all except three cases in this series. In two of these one or both kidneys only were involved and in the other both kidneys and spleen were affected. The regional lymph nodes of these kidneys were not personally examined and the possibility remains that they may have shown some evidence of neoplastic involvement or that small primary neoplastic foci elsewhere in the body may have been overlooked. It was common to see more or less generalized involvement of most lymph nodes by the disease process but lymph nodes of certain anatomic groups were more commonly affected than others. Gross tumourous enlargement of lymph nodes was detected in the sub-lumbar and iliac regions (Figure 2.3) in 14 of 19 cases, in the mediastinal region (Figure 2.2) in 13 of 19 cases and in the mesenteric (Figure 2.1) and cervical regions in 12 of 19 cases. In the majority of cases there was bilaterally symmetrical enlargement of paired carcass nodes (Figure 2.4). Affected nodes, despite even massive enlargement, usually retained their shape except in some instances where nodular masses produced a capsular irregularity (Figure 2.5) or where extra-capsular spread resulted in the fusion of two adjacent nodes (Figure 2.3). Their consistency was firm but if severely swollen they could be ruptured with relatively little pressure. The cut surface of grossly enlarged lymph nodes showed a bulging, glistening, grey-white, homogeneous mass of tissue in which the normal medullary and cortical features could not be distinguished (Figure 2.6a). In less severely affected cases lymphoid follicles appeared accentuated (Figure 2.6b). Petechial and ecchymotic

TABLE 2.II

The patho-anatomical classification of cases of spontaneously occurring malignant lymphoma in sheep killed at slaughter-houses or found during mortality surveys

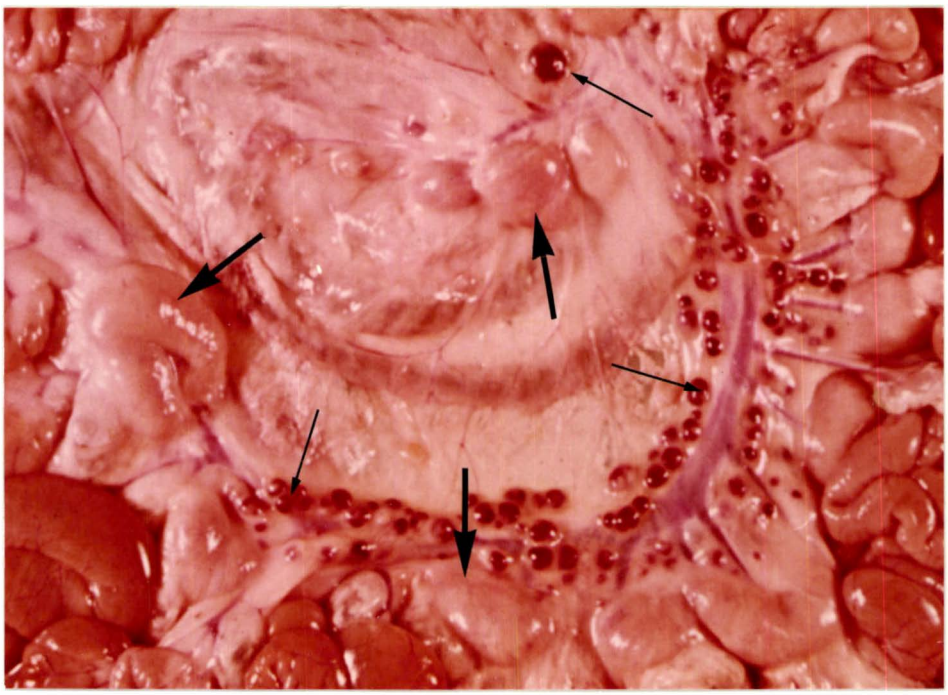
Multicentric form	Alimentary form	Thymic form	Skin form
Case No. 7286	Case No. 6026	Case No. 4713	Case No. 401082
" 6426	" 5669		
" 6186	" 4823		
" 5651	" 4699		
" 5487	" 4292		
" 4858	" 3835		
" 4828			
" 4827			
" 4811			
" 4715			
" 4712			
" 4548			
" 4098			
" 4097			
Totals 14 (63.6%)	6 (27.3%)	1 (4.5%)	1 (4.5%)

Figure 2.1

Malignant lymphoma causing enlargement of the mesenteric lymph nodes (large arrows) and haemal lymph nodes (small arrows) of a lamb.

Figure 2.2

Thoracic organs of a sheep with malignant lymphoma. There is neoplastic swelling of the incised posterior mediastinal lymphnodes (A), bronchial lymph nodes (B) and anterior mediastinal lymph nodes (C).



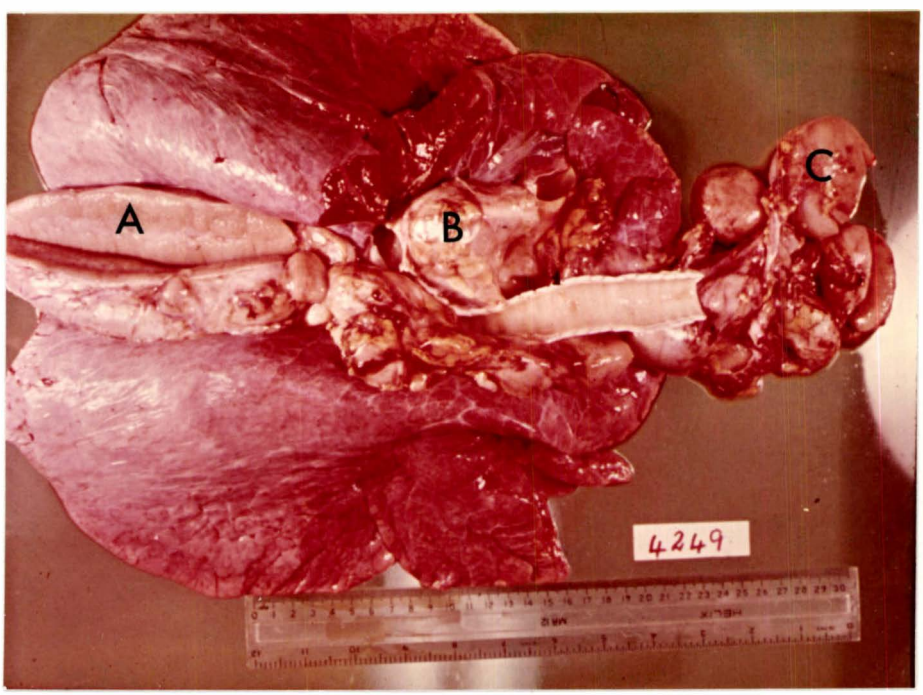

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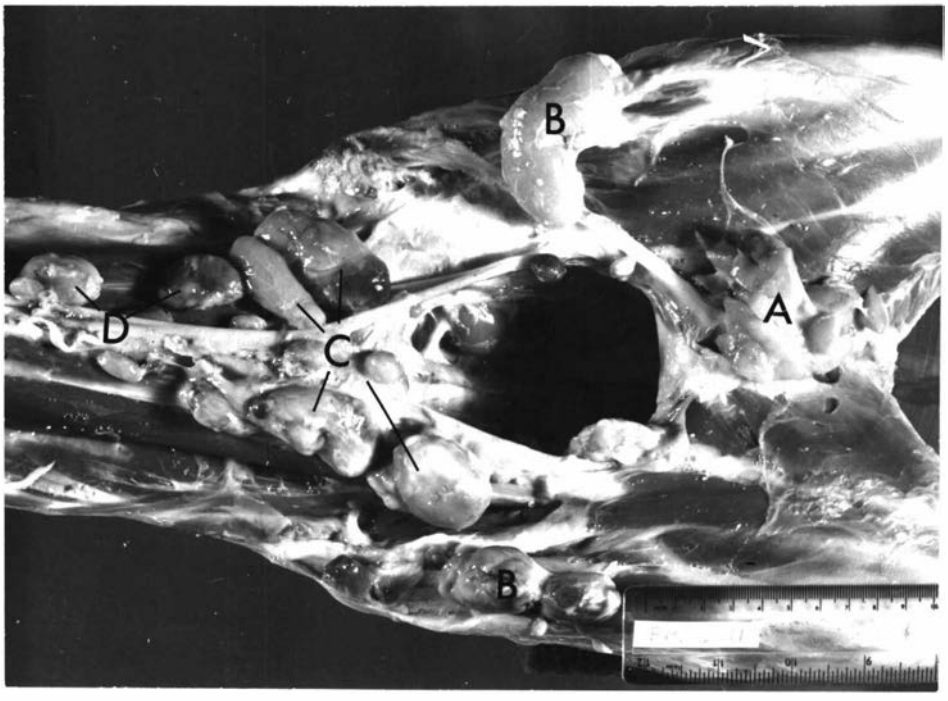
Figure 2.3

Enlargement of the superficial inguinal (A), precrural (B), iliac (C) and sub lumbar (D) lymph nodes in a sheep with malignant lymphoma. Adjacent lymph nodes (arrow) show fusion as a result of extra-capsular extension of the neoplasm.

Figure 2.4.

Bilateral involvement of the popliteal lymph nodes with tumour (arrows).

Same case as Figure 2.3.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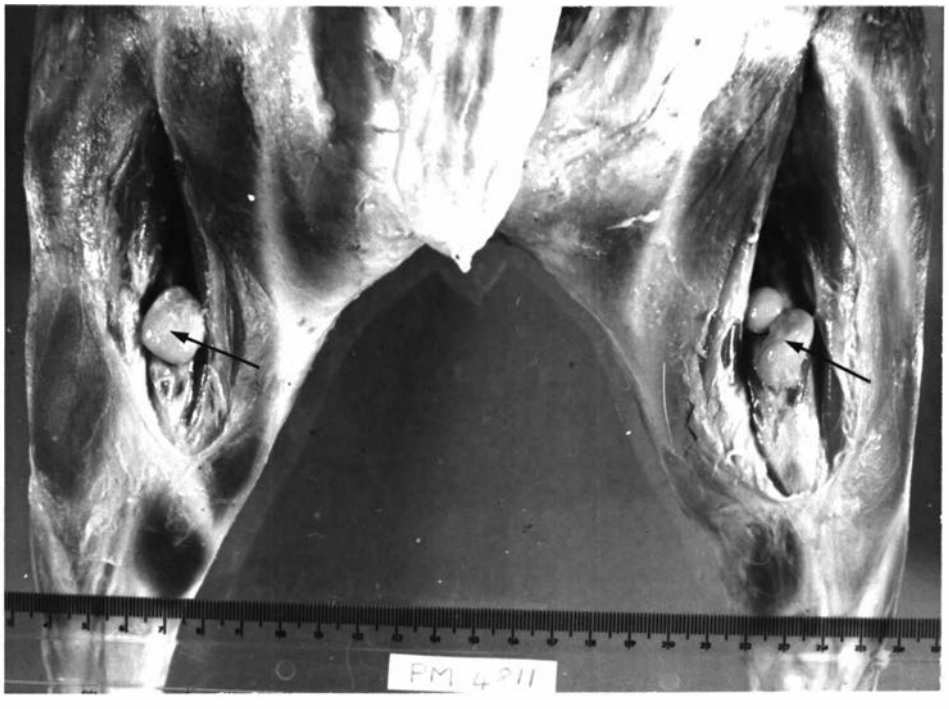
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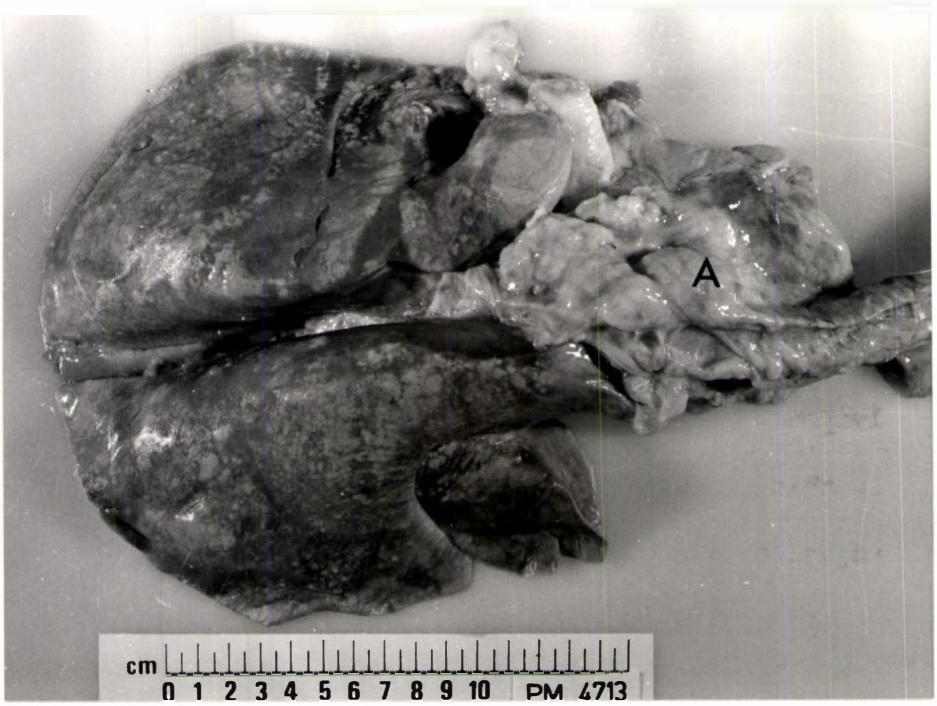
Figure 2.5

Tumourous enlargement of the anterior mediastinal lymphnodes (A) of a sheep with malignant lymphoma. In this case there is marked nodularity and fusion of adjacent lesions (compare with Figure 2.2).

Figure 2.6

Incised lymph nodes affected with malignant lymphoma. In (A) the spread of malignant cells have resulted in the obliteration of normal cortical, medullary and capsular features. In (B) the neoplastic process has accentuated follicular regions in the cortex (arrows).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cm
0 1 2 3 4 5 6 7 8 9 10
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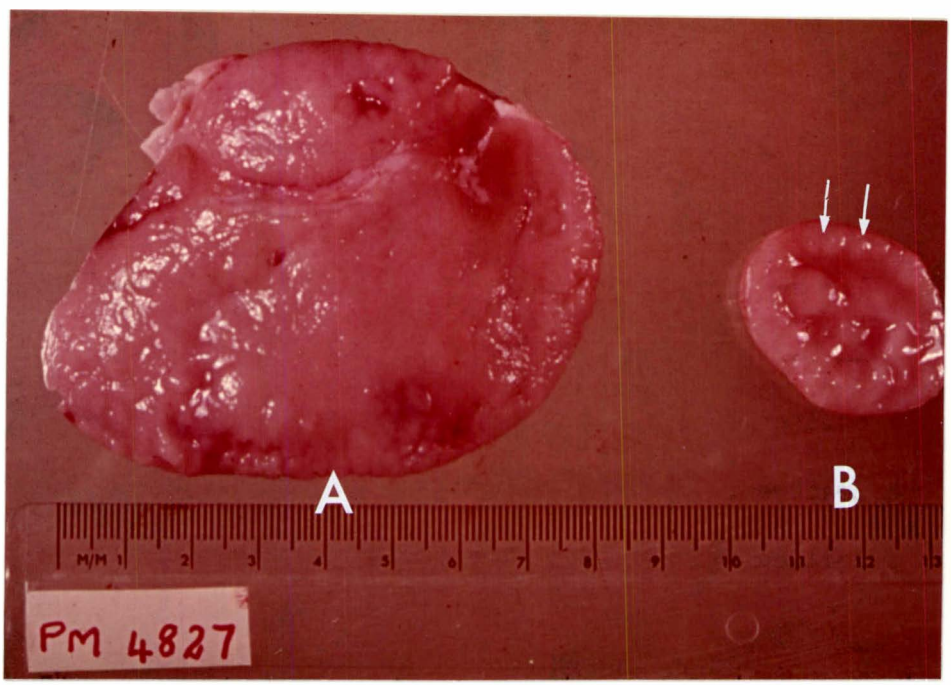
A
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haemorrhages and foci of necrosis were often present, particularly in the outer cortical regions (Figure 2.7). Fibrosis and calcification of such areas were occasionally encountered.

Neoplastic involvement of the haemal lymph nodes was observed in only one case. In this instance all the haemal nodes examined were spheroidal and swollen to a diameter of up to 0.5 cm (Figure 2.1). Their consistency was firm and the cut surface was bulging, red and homogeneous.

Spleen

The spleen was affected in 14 out of the 19 cases examined. Neoplastic involvement was diffuse in nine of these, with the organ weighing up to two and a half times more than expected. The cut surface was soft and bulging, with Malpighian corpuscles sometimes accentuated (Figure 2.8). In seven of the affected spleens, including two showing predominantly diffuse lesions, nodular tumours with sharply defined borders occurred which sometimes caused capsular distortion (Figure 2.9).

Liver

The liver was the most commonly affected non-lymphoid organ. There was either nodular or diffuse involvement with tumour in 13 out of 19 cases examined. The nodular form was usually multifocal with nodules of tumour being scattered throughout the liver substance, often causing distortion of Glisson's capsule (Figure 2.10). When involvement was diffuse the pale neoplastic tissue frequently showed indistinct borders with normal liver and emphasized the lobulation of the partially infiltrated hepatic parenchyma. Metastases to the porto-hepatic lymph nodes were seen in association with eight of ten affected livers in which these nodes were examined.

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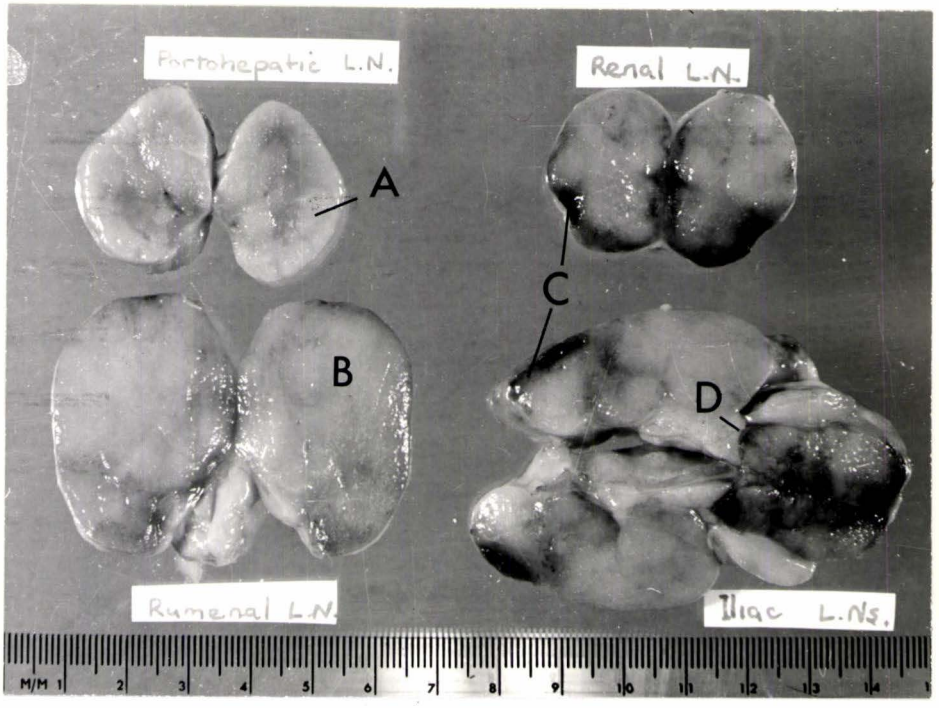
Figure 2.7

Incised lymph nodes(L.N.) from sheep with malignant lymphoma showing some of the commonly observed gross pathological features of the disease in these organs. These include: accentuation of follicular structure (A), obliteration of normal nodal architecture (B), haemorrhage and necrosis (C) and fusion of adjacent lymph nodes as a result of extra-capsular extension of the neoplasm (D).

Figure 2.8

Diffuse splenic enlargement in ovine malignant lymphoma with accentuation of Malpighian corpuscles (arrows).


Portohepatic L.N.
Renal L.N.
A
B
C
D
Ruminal L.N.
Iliac L.Ns.

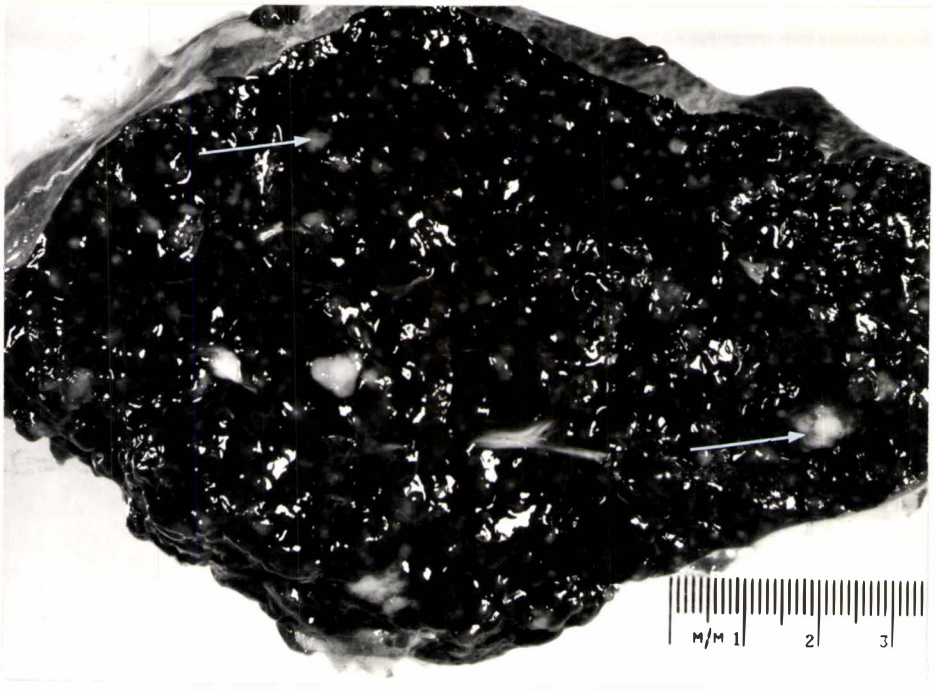
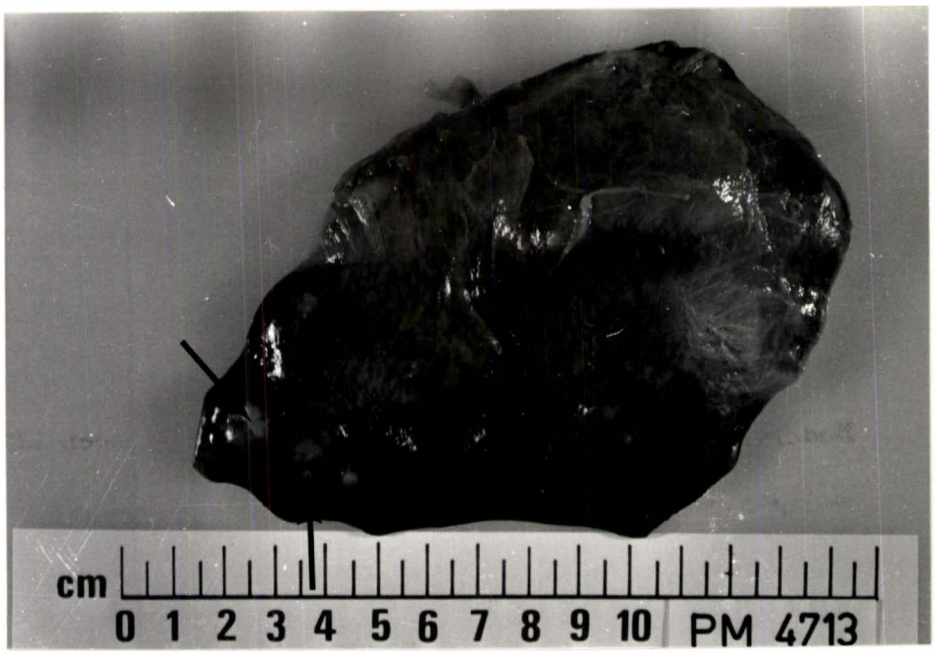

M/M 1
2
3

Figure 2.9

Nodular tumours (arrows) in the spleen of a sheep with malignant lymphoma.

Figure 2.10

Multiple nodular neoplastic lesions causing distortion of the capsule of the liver in a sheep with malignant lymphoma.


cm
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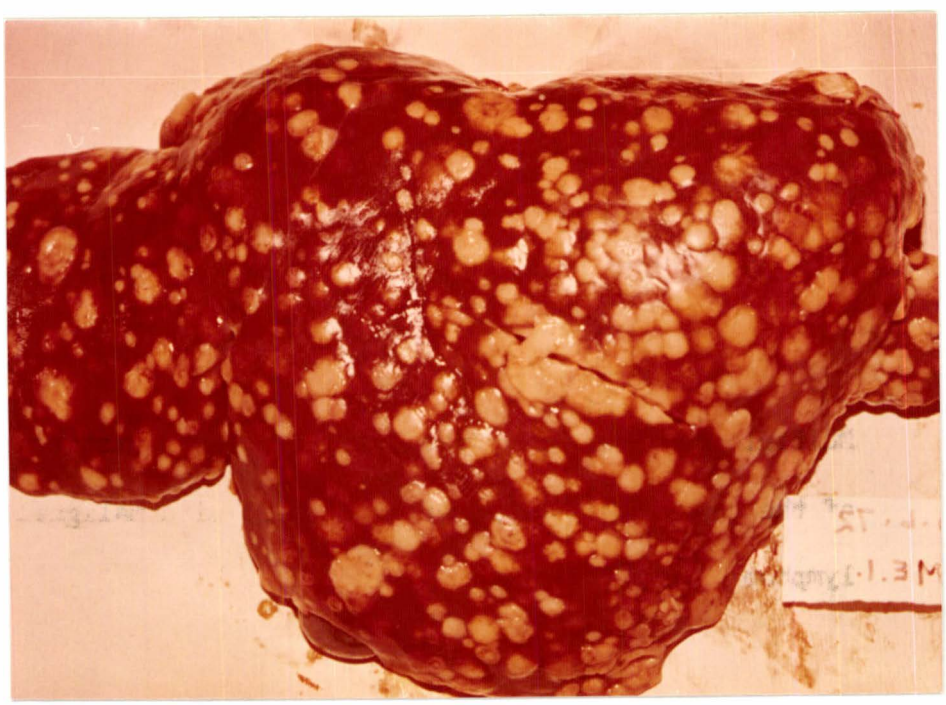

Kidneys

Tumour was present in the kidneys of 10 of 21 cases examined. Lesions affected both kidneys in eight of these and were restricted in all except one case to the cortices. The larger tumours were raised above the cortical tissue causing distortion of the renal capsule (Figure 2.11). These nodules of neoplastic tissue were frequently the site of focal haemorrhage and necrosis.

Heart

Invasion of the heart was recorded in 4 of 19 cases. In all instances the tumour involved cardiac muscle and adipose tissue in the region of the right auricle (Figure 2.12).

Rumen, reticulum, abomasum and intestines

Early lesions of malignant lymphoma found in tubular organs of the digestive tract were present in the submucosa. In more advanced lesions the muscle coats, peritoneal surface and mucosa were all thickened by the neoplastic tissue. When lesions were observed in the jejunum and ileum, Peyer's patches were the sites of greatest neoplastic involvement (Figure 2.13).

Other organs

Although involvement of bone marrow was diagnosed histologically in four cases, gross lesions were detected in one animal only. In this case, the red marrow of the proximal femur had been partially replaced by white, glistening, tumour tissue. A focus of malignant lymphoma was also present in the body of a thoracic vertebra and this had extended dorsally to invade the spinal cord and dura mater.

Plaques of neoplastic tissue were found in the omentum in four cases and the diaphragm of two (Figure 2.14). Nodular tumours affecting adrenals, lungs, pancreas, vagina, uterus and cervix were also

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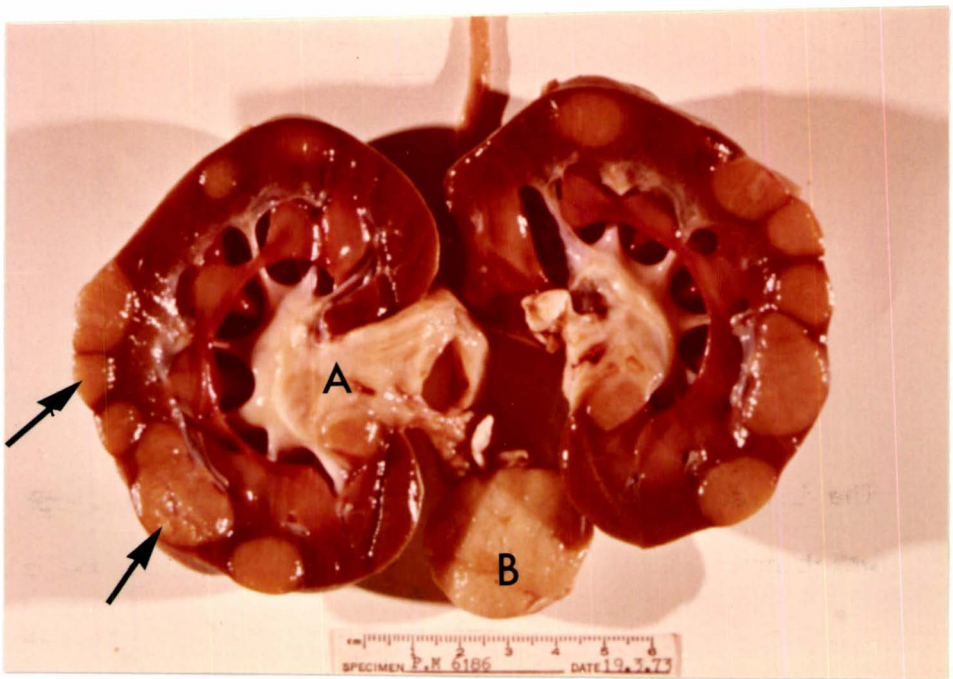
Figure 2.11

The incised kidney in a case of ovine malignant lymphoma in which nodules of tumour material have formed in the cortex (arrows). There is hydronephrosis resulting from tumourous occlusion of the ureter (A). The renal lymph node (B) is neoplastic.

Figure 2.12

The heart of a sheep with malignant lymphoma. The right atrium and right ventricle have been opened to show the tumour nodule in the atrial wall and cardiac adipose tissue (arrows).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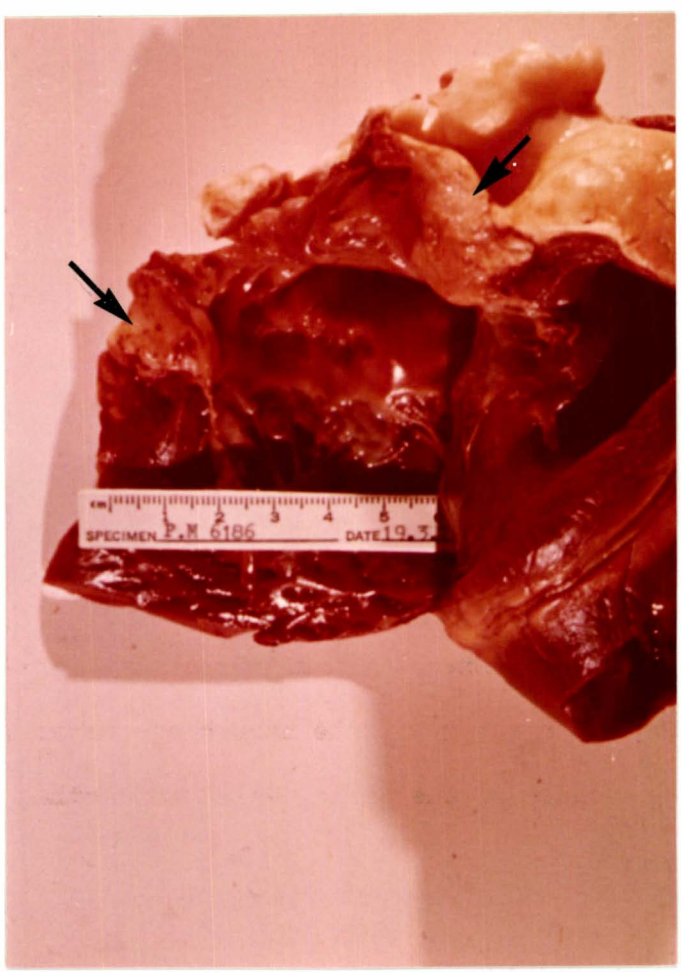
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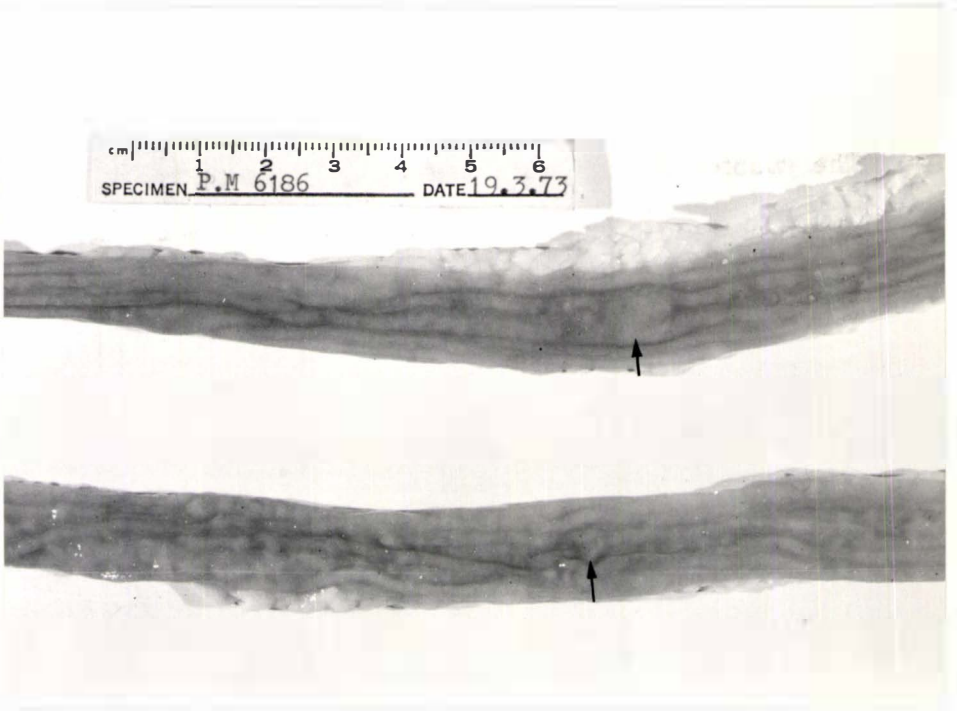
Figure 2.13

The mucosal surface of segments of ilium from a sheep with malignant lymphoma. Nodules of tumour tissue (arrows) are present in the submucosa.

Figure 2.14

A tumourous plaque (A) on the diaphragm of the case illustrated in the previous figure.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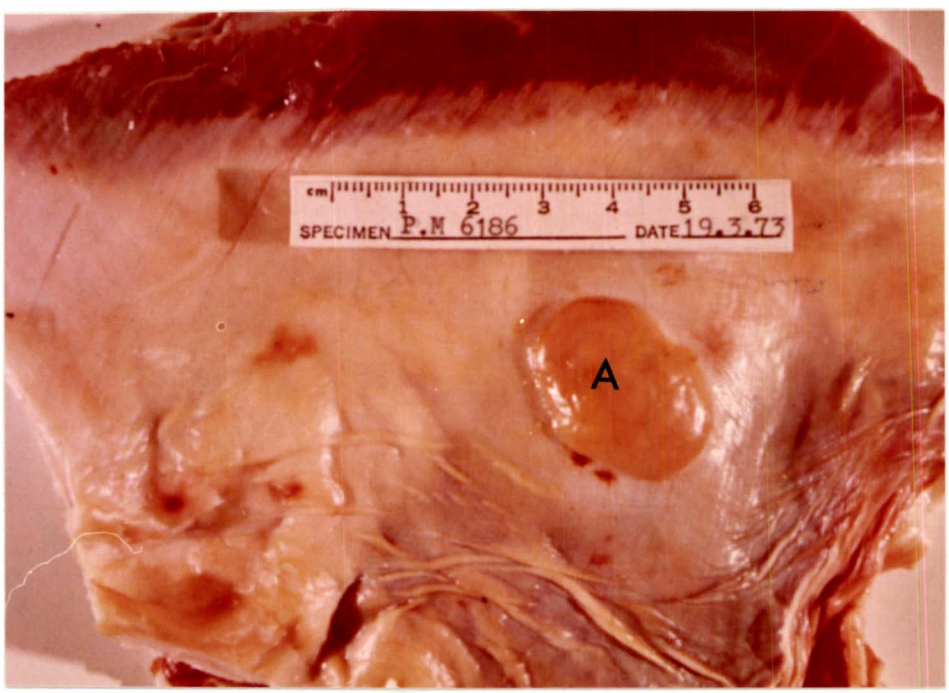
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The Histology of Malignant Lymphoma in Sheep

Lymph nodes

The histological appearance of the affected nodes varied considerably depending upon the degree of tumour infiltration which had occurred. Lymph nodes were seen in which some of the normal histological architecture remained, while in adjacent nodes the normal features were no longer evident. The afferent lymphatic vessels and subcapsular sinuses were the areas colonized first by the neoplastic cells (Figure 2.15). Clogging of the medullary sinuses and thickening of the trabeculae (Figure 2.16) occurred next and finally invasion of cortical structures with resultant obliteration of all normal architectural features (Figure 2.17). The destructive nature of this infiltrative process was well illustrated in sections stained by Gordon and Sweet's method in which distortion of the normal arrangement of reticulin fibrues was clearly demonstrable (Figures 2.18 and 2.19). Further extension of the neoplastic process involved massive capsular and extracapsular infiltration (Figure 2.20). Necrosis, usually focal and occasionally extensive, was commonly present in all infiltrated areas of the node and especially at the periphery. Focal haemorrhage, dystrophic calcification and fibrosis were common (Figures 2.21, 2.22, 2.23). Crystal shaped clefts, shown in frozen sections to contain cholesterol by means of the Romieu (1927) method, were noted occasionally in areas of chronic inflammatory reaction (Figure 2.23).

No Hassall's corpuscles or other evidence of thymic structure were seen in the anterior mediastinal lymphoid masses examined in case 4713 (Figure 2.5). It could not therefore be definitely established as being of thymic origin. A histological feature common to many of the tumours was the so-called "starry sky" appearance (O'Conor and Davies, 1960) imparted by occasional clear phagocytic histocytes among the masses of lymphoid cells (Figures 2.17, 2.24).

Figures 2.15 to 2.23 : Lymph node infiltration by neoplastic cells in ovine malignant lymphoma

Figure 2.15

Neoplastic lymphoid cells in the subcapsular sinus (arrows)

HE x 300

Figure 2.16

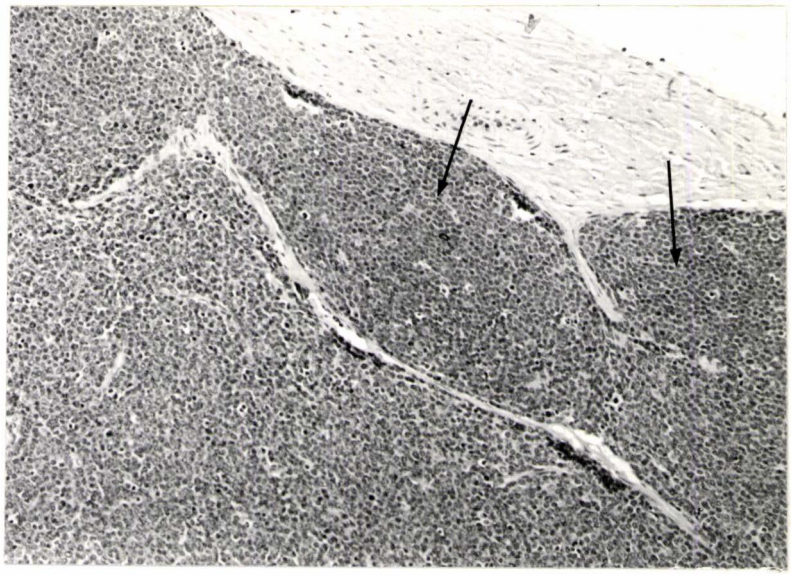
Neoplastic lymphoid cells in the medullary sinuses (arrows) adjacent to tumourous cortical tissue (C)

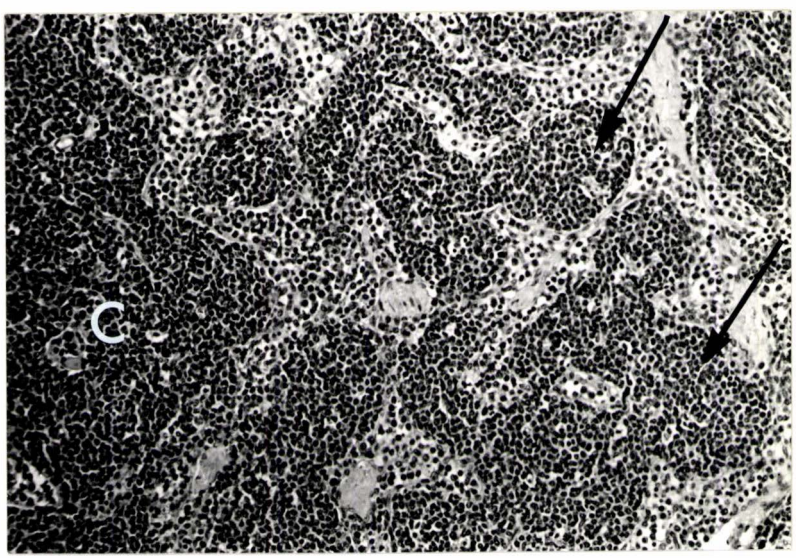
HE x 300

Figure 2.17

Cortical region showing obliteration of normal features and a "starry sky" appearance.

HE x 300




C

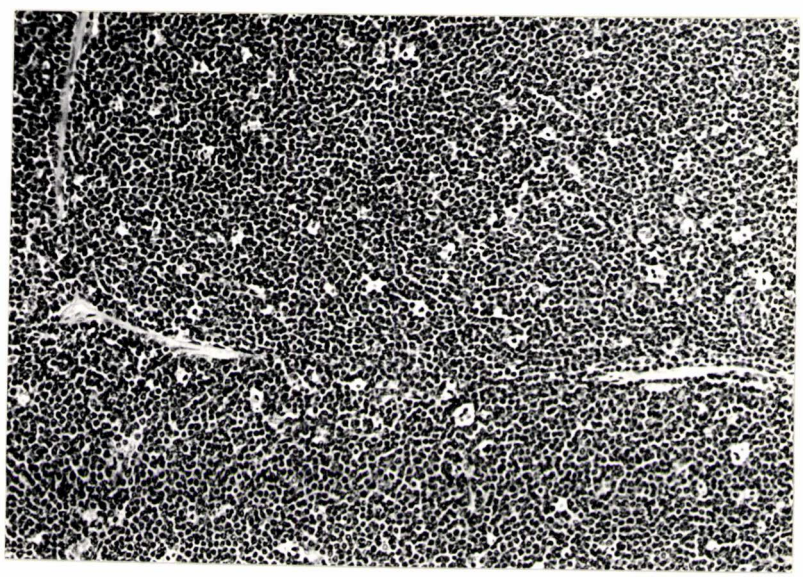


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Figure 2.18

Thickening and infiltration by neoplastic cells of the capsule of a lymph node.

HE x 120

Figure 2.19

Whole mount section of lymph node with advanced tumourous change showing distortion of reticulin network. Compare with normal node in figure 2.19 below.

Gordon and Sweet's x 4

Figure 2.20

Normal lymph node.

Gordon and Sweet's x 6

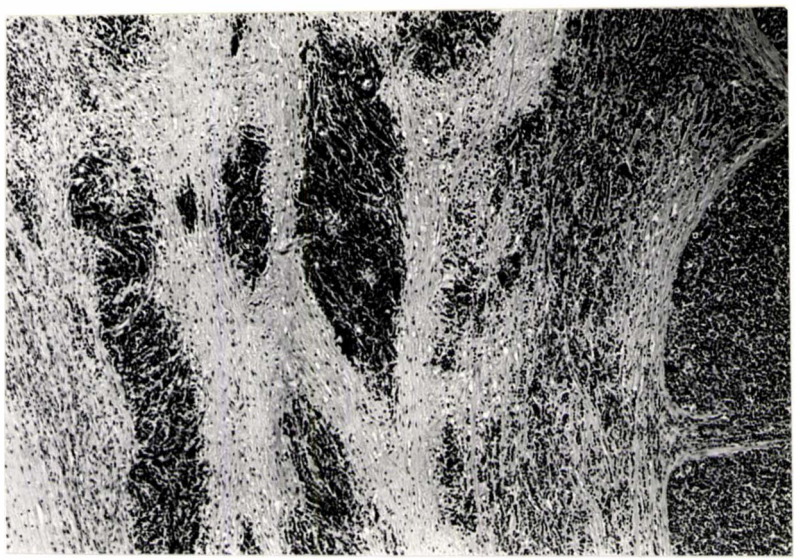

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Figure 2.21

Cortical region of tumourous lymph node showing an area of necrosis (N)

HE x 1,200

Figure 2.22

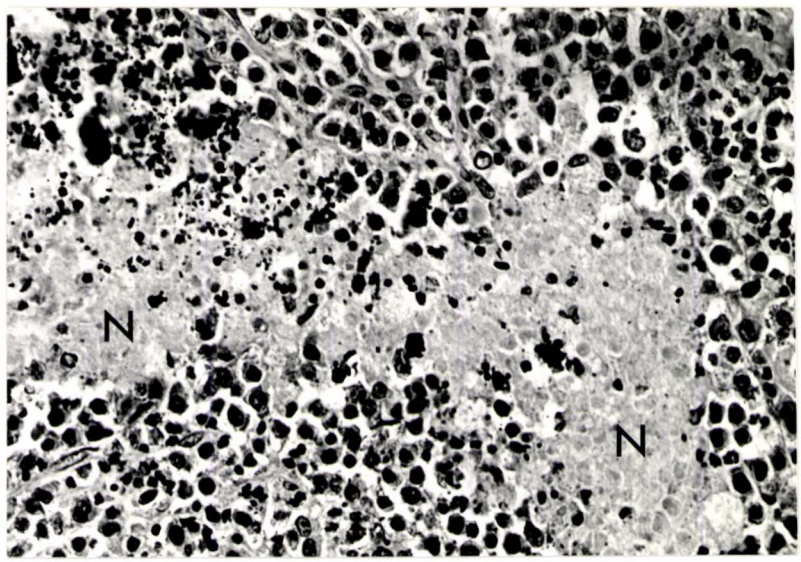
Cortico-medullary region of lymph node in malignant lymphoma showing an area of chronic damage with necrosis (N), fibrosis (F), calcification (large arrow) and cholesterol clefts (small arrows).

HE x 300

Figure 2.23

Multinucleated giant cells (large arrows) in an area of chronic inflammatory reaction associated with tumourous involvement of a lymph node. Note cholesterol clefts (small arrows).

HE x 1,200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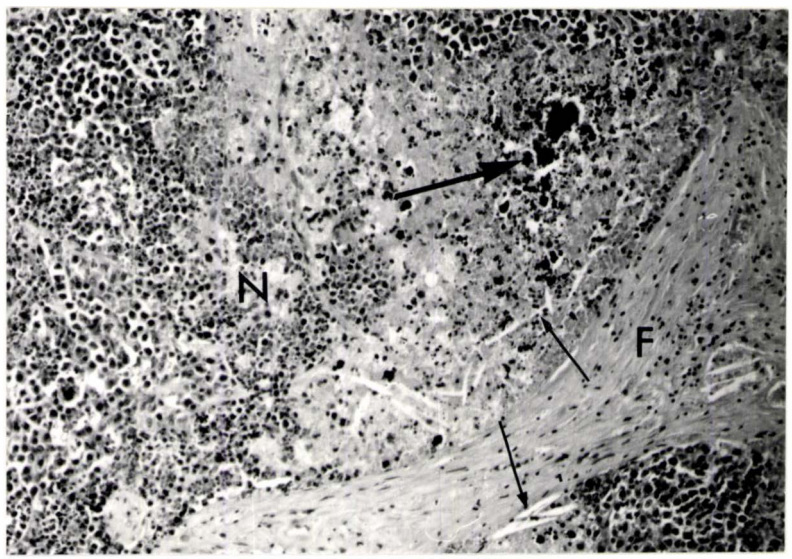
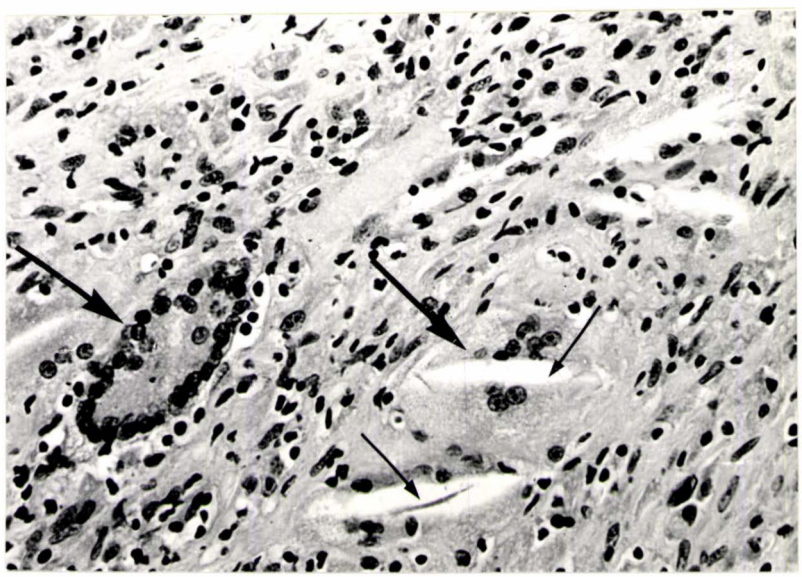
N


N
F



Spleen

Malignant lymphoma occurred in two histological patterns in this organ. In most cases the normal features shown by Malpighian corpuscles and sinusoids were obscured by their diffuse infiltration by neoplastic cells (Figure 2.24). Cases in which the gross appearance of the tumours was nodular or in which accentuation of Malpighian corpuscles had occurred, the outstanding histological feature was the focal accumulation of neoplastic cells at these sites (Figure 2.25). In other respects the changes observed were similar to those in the lymph nodes.

Liver

The first evidence of tumour infiltration in this organ was usually seen at the portal triads (Figure 2.26). Subsequent infiltration involved periacinar and centrilobular regions of the parenchyma and eventually resulted in the obliteration of normal hepatic architecture (Figure 2.27). However in three cases there was intravascular and intrasinusoidal accumulation of tumour cells without accompanying destruction of liver parenchyma. In sections from one of these cases, neoplastic cells were seen in particularly large numbers in almost all portal blood vessels (Figure 2.28). The blood vessels of lung and kidney in this case showed similar, although less spectacular, intravascular accumulations of lymphocytes suggestive of a true leukaemia.

Kidneys

The pathogenesis of the kidney lesions was remarkably constant from case to case. There was progressive infiltration of cortical interstitial tissues leading to the eventual destruction of the affected region (Figures 2.29, 2.30, 2.31). The extent of the disruption of normal renal architecture was well illustrated by the abnormal pattern

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Figures 2.24 and 2.25. Splenic changes in ovine malignant lymphoma

Figure 2.24

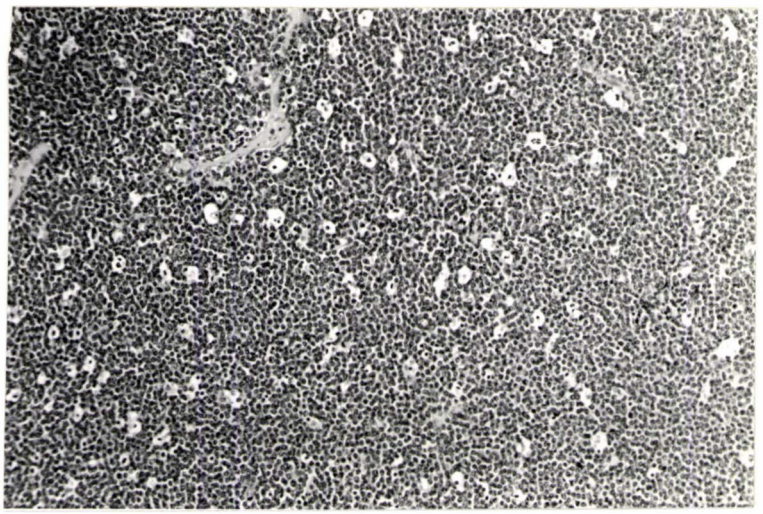
Total loss of normal histological detail in a diffusely infiltrated spleen. Note the "starry sky" appearance.

HE x 300

Figure 2.25

Accentuation of Malpighian corpuscles(arrows) in a case in which the neoplasm was nodular on gross examination.

HE x 300



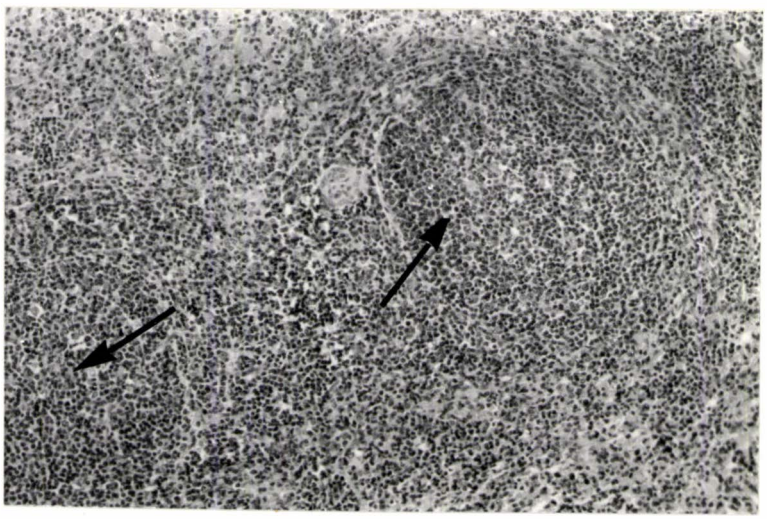


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Figure 2.26

Early stage of hepatic infiltration in malignant lymphoma with early focal tumour aggregates in portal and sinusoidal regions.

HE x 300

Figure 2.27

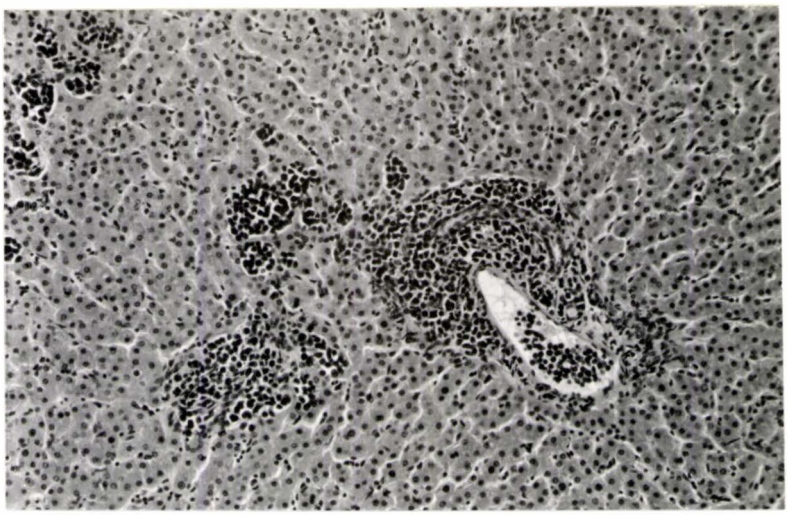
The lesion illustrated in figure 2.26 progresses through this stage and eventually obliterates entire hepatic acinar units.

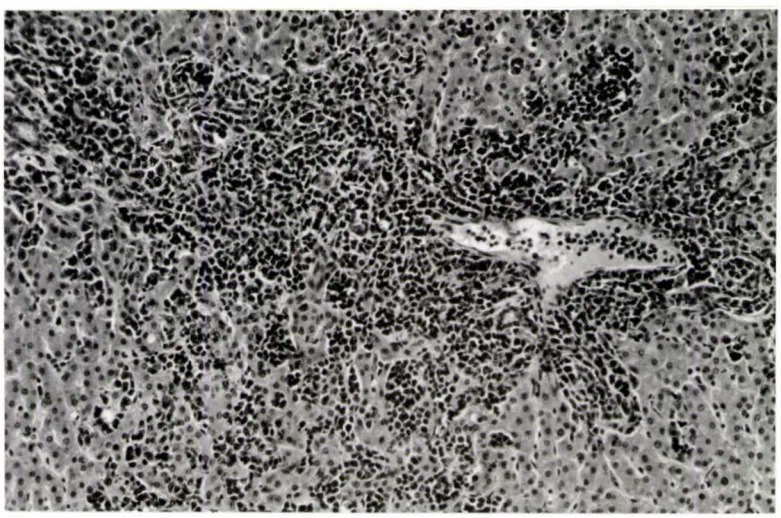
HE x 300

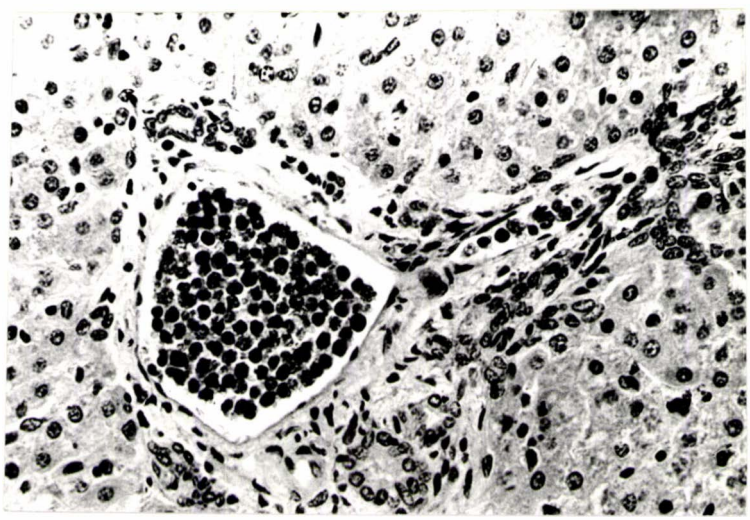
Figure 2.28

A portal region in case No. 7286 showing large numbers of lymphoid cells within the hepatic portal vein, suggestive of frank leukaemia.

HE x 1,200







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Figures 2.29 to 2.33. Progressive tumourous involvement in kidneys of sheep with malignant lymphoma.

Figure 2.29

Early periglomerular and interstitial infiltration about renal tubules.

HE x 300

Figure 2.30

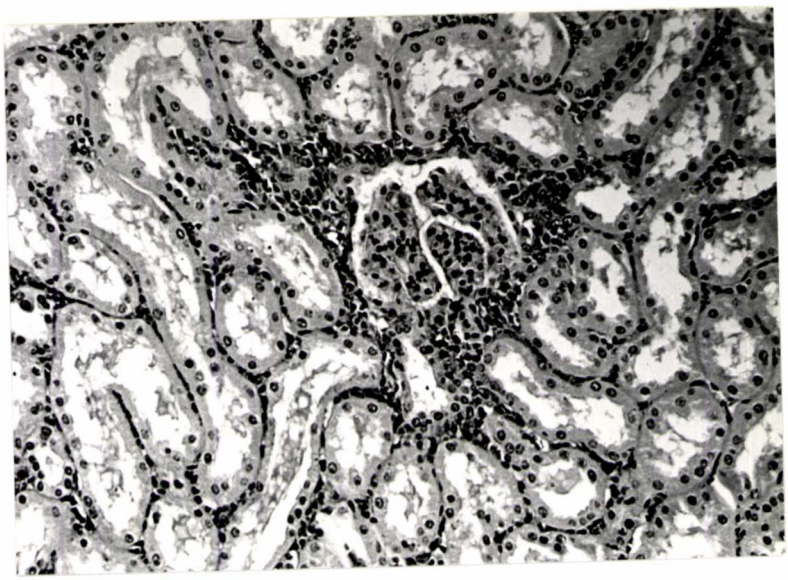
A more severe lesion than that illustrated in the previous figure. Degenerative changes are apparent in the tubular epithelium.

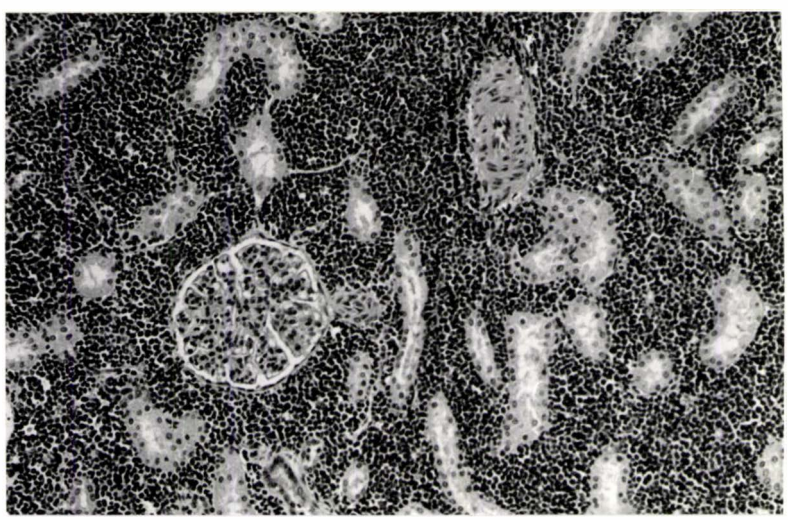
HE x 300

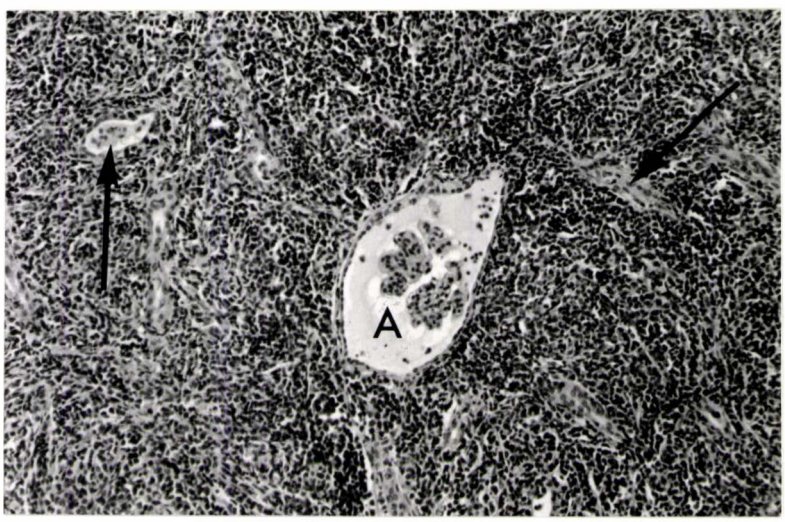
Figure 2.31

Lesion in which the invading cells have destroyed the usual histological features of the renal cortex. Tubular remnants (arrows) may be detected around the atrophied glomerulus (A).

HE x 300



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Following page 53

Figure 2.32

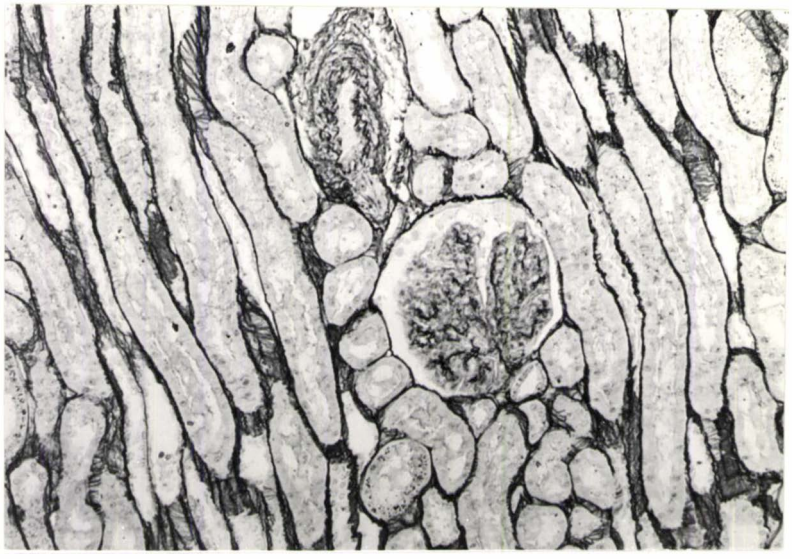
The normal appearance of the renal cortical reticulin fibres.

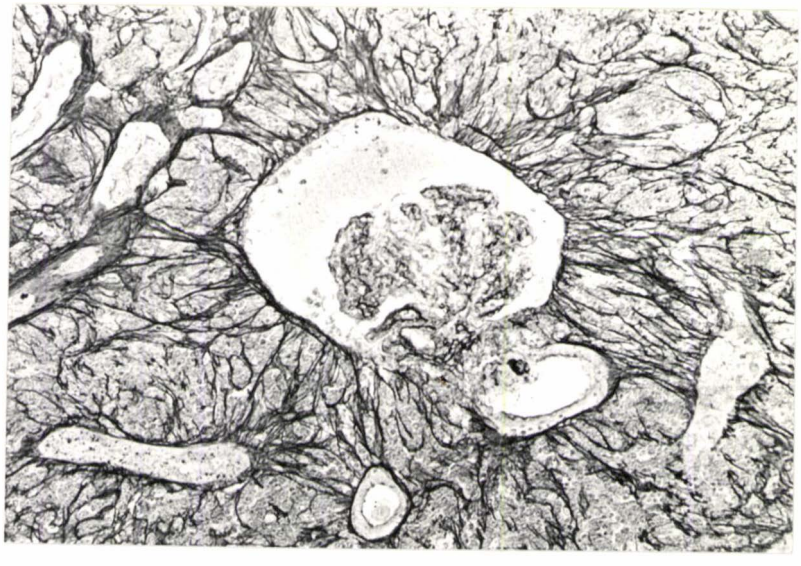
Gordon and Sweets' x 300

Figure 2.33

A similar field to that depicted above in the kidney of a sheep with advanced tumourous lesions in the kidney. Note the distortion of the normal arrangement of reticulin fibres.

Gordon and Sweet's x 300





of reticulin fibres (Figures 2.32, 2.33).

Focal haemorrhage and necrosis was commonly seen in the neoplasms and sometimes this was associated with focal calcification and fibrosis.

Heart

Considerable variation was present in the severity of lesions affecting this organ, but as already noted in regard to lymph node, liver and kidney, the disease was progressive. Spread was by infiltration between muscle fibres and into epicardial adipose tissue which led to the eventual destruction of these tissues (Figures 2.34, 2.35, 2.36). In 16 cases in which there was no gross evidence of malignant lymphoma sections of the heart were examined microscopically and in five of these animals neoplastic infiltration was recorded.

Rumen, reticulum, abomasum and intestine

In these organs there was initial colonisation of the submucosa by tumour cells followed by progressive infiltration of the mucosa and muscular layers (Figures 2.37, 2.38, 2.39). Neoplastic infiltration of enlarged Peyer's patches was also noted in those cases in which the jejunum and ileum was affected.

Lungs

The neoplastic lesions seen in the lungs of two of the three affected cases were similar histologically to peribronchial lymphoid hyperplasia associated with granuloma formation caused by the commonly occurring small lungworm of sheep Muellerius capillaris. However the proliferation of lymphoid cells was far in excess of that normally encountered in this parasitic condition and furthermore there were distinct subpleural drifts and nodules of lymphoid cells which were unrelated to parasitic granulomas. In the other case, parasitic lesions were not evident and extensive neoplastic lesions were present

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Figures 2.34 to 2.36. Progressive tumourous infiltration of myocardium in malignant lymphoma of sheep.

Figure 2.34

Light infiltration of lymphoid cells between the muscle fibre bundles.

HE x 300

Figure 2.35

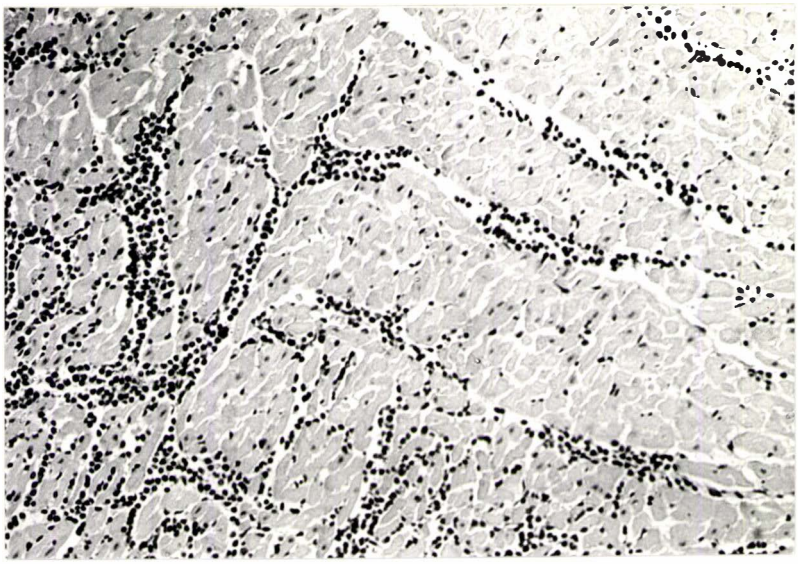
A lesion of increasing severity. The darker myocardial fibres to the right of the illustration show degenerative change. The Purkinje fibre (P) appears unaffected.

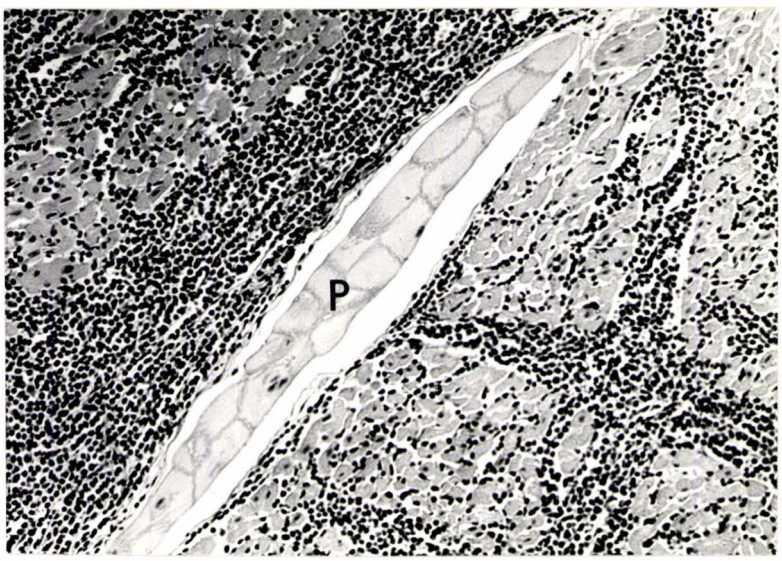
HE x 300

Figure 2.36

Heavily infiltrated myocardium

HE x 300

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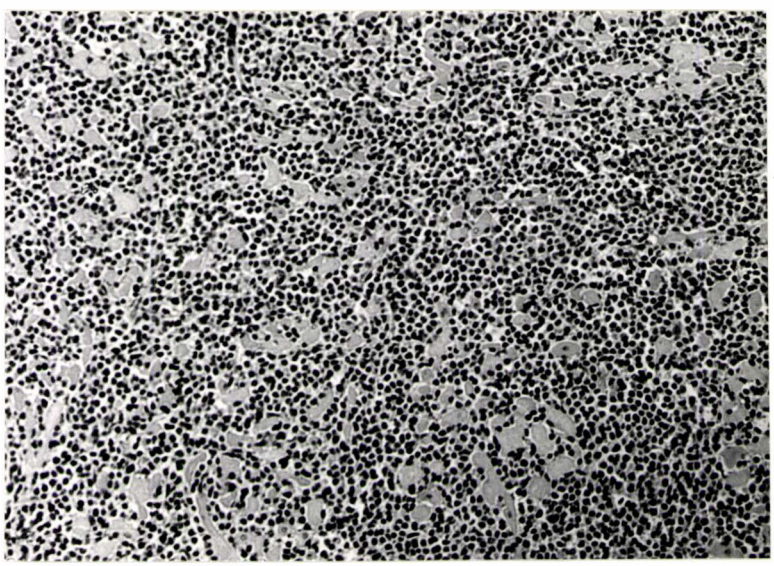
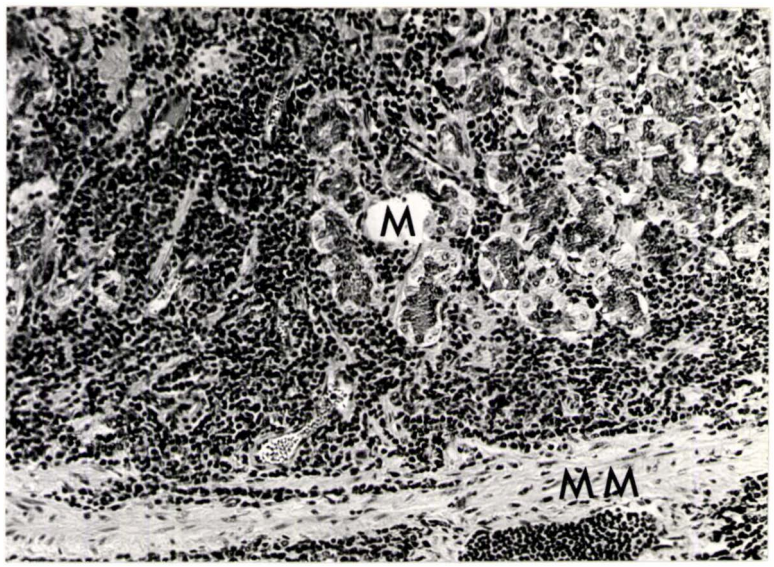


Figures 2.37, 2.38, 2.39

Diffuse infiltration of tumour cells in submucosa (S), muscularis mucosa (MM) and mucosa (M) of the abomasum in malignant lymphoma. Note the presence of neoplastic cells in a vein in Figure 2.39.

HE x 300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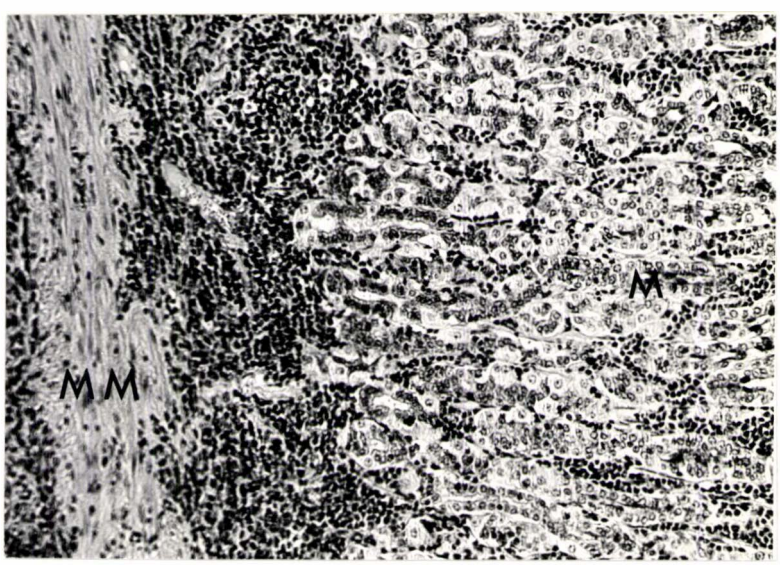
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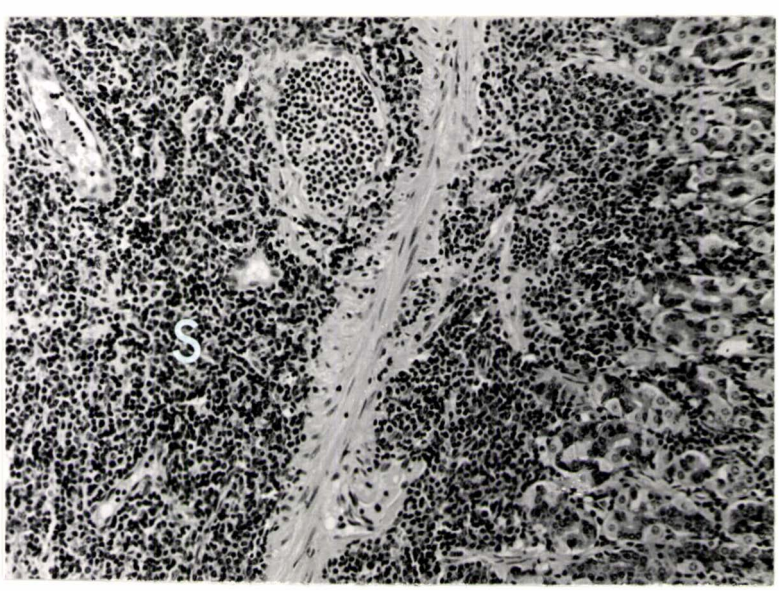
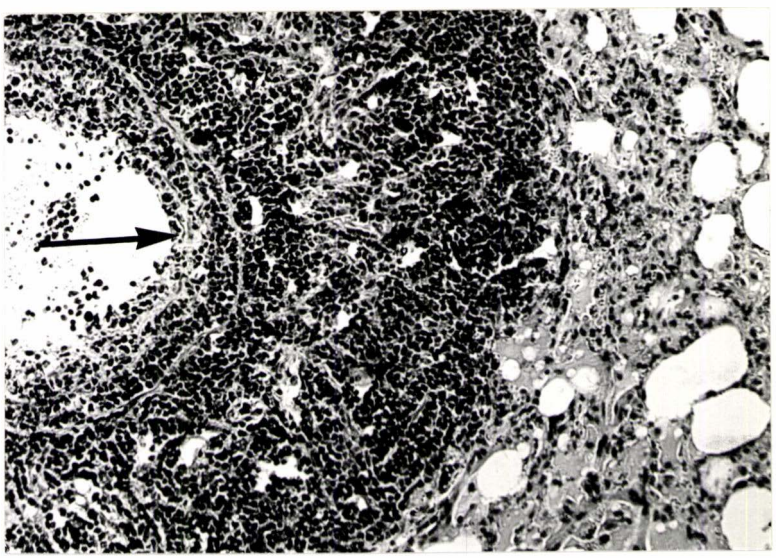

S

Figure 2.40

Peribronchial infiltration by cells in ovine malignant lymphoma affecting the lung. Bronchial epithelium (arrow) has been destroyed by the invasive lymphocytes and alveolar spaces adjacent to the neoplasm show accumulation of fluid.

HE x 300



throughout the lungs apparently centred on the peribronchial lymphoid tissue (Figure 2.40).

Other organs

All other tissues and organs examined (Table 2.I) showed the general features of malignant lymphoma already described. Invariably there was a progressive infiltration and replacement of normal tissues by neoplastic elements to give rise to plaques, nodules and diffuse tumours. The bone marrow from three of the seven animals in which there was no gross evidence of neoplasia at this site showed infiltration of the marrow and replacement of normal haematopoietic cells by neoplastic cells when examined microscopically.

Cytology of Malignant Lymphoma in Sheep

Table 2.III shows the classification of cases of malignant lymphoma in this series according to their cytologic features. There was usually cellular pleomorphism shown by the neoplastic cells in any one case and this was noted particularly in the more primitive forms. Consequently the cases have been classified on the basis of the predominant cell type present.

The lymphoblastoid type of cell was characterised by extreme immaturity resembling that of a stem cell. The cells were not associated with silver-positive fibres. The majority of nuclei were large, spherical or ovoid, although irregular forms were common. They were leptochromatic with marginated chromatin. Large, sometimes multiple nucleoli were present in a high proportion of cells. The cytoplasm was extensive and weakly to moderately basophilic (Figures 2.41, 2.42).

The prolymphocytic type was characterized by a spherical centrally situated nucleus which was smaller and relatively pachychromatic in comparison to the lymphoblastoid type. It was however frequently

TABLE 2.III

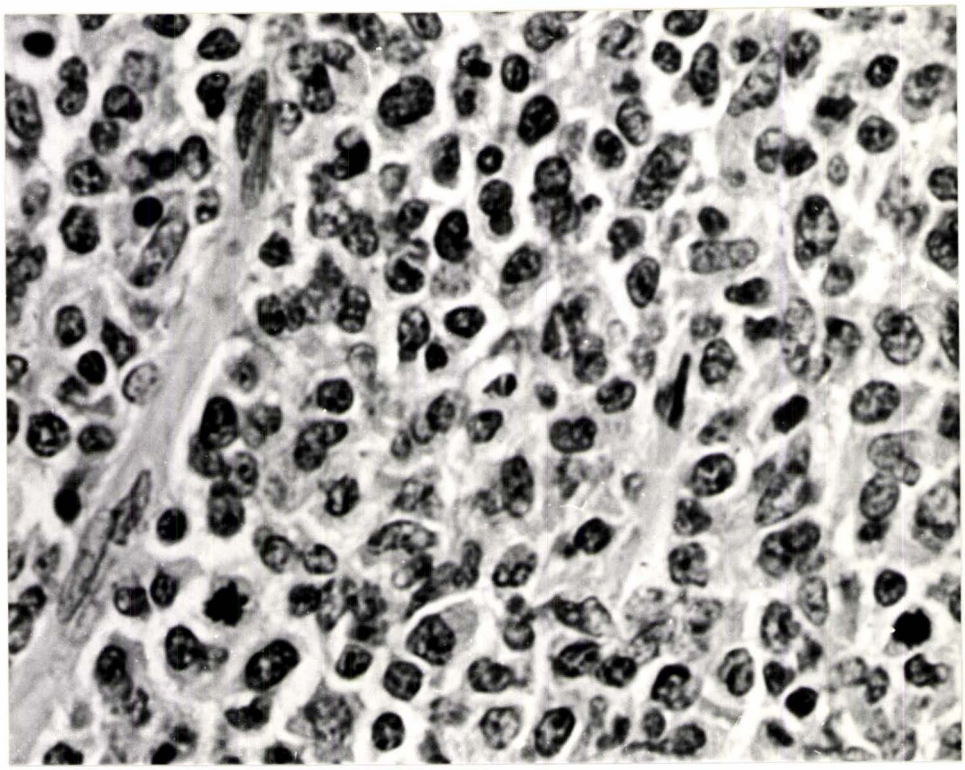
The cytological classification of 40 cases of spontaneously occurring malignant lymphoma in sheep killed at slaughter-houses or found during mortality surveys

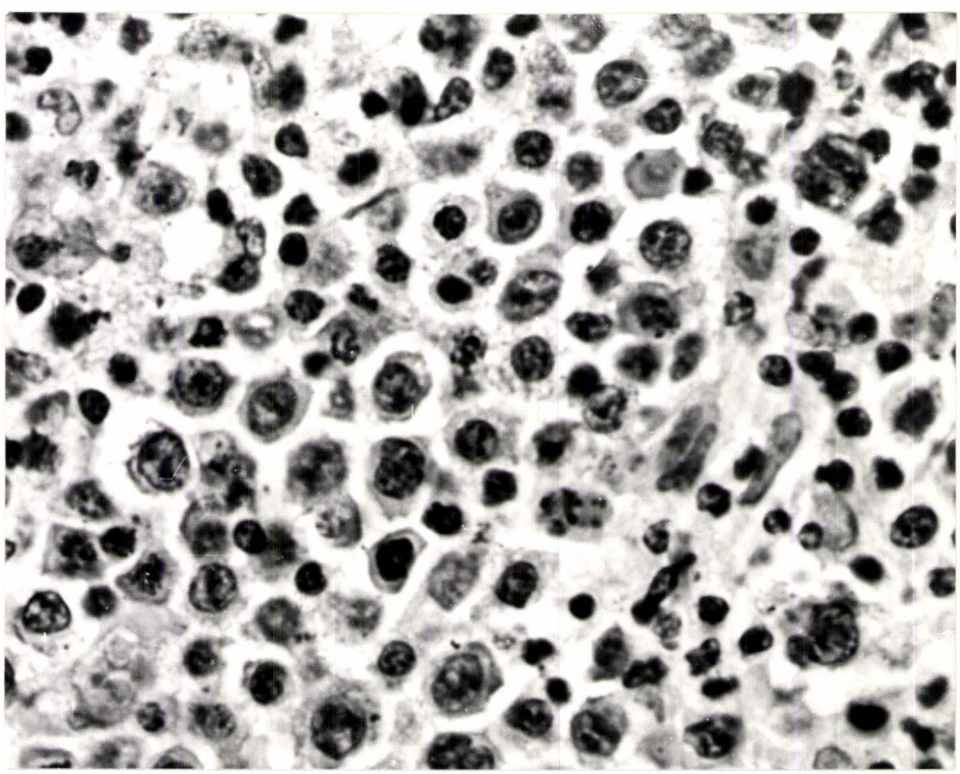
Predominantly lymphoblastic	Lymphoblastic/ prolymphocytic	Predominantly prolympholytic	Predominantly lymphocytic	Reticulum cell sarcoma
Case No. 6186	Case No. 4823	Case No. 7375	Case No. 4858	Case No. 4548
" 6026	" 4715	" 7286	" 4292	" 4303
" 5651	" 4097	" 6426	" 3845	" 401082
" 4828	" 3835	" 5669	" 3826	
" 4811	" 3807	" 5487	" 2739	
" 4713	" 2221	" 4827	" 400308	
" 4712		" 3101F		
" 4699		" 2136		
" 4098		" 691		
" 3873		" 534		
" 3858		" 400620		
" 639				
" 306075				
" 400802				
Totals 14	6	11	6	3
Percentage of all cases 35	15	27.5	15	7.5

Figures 2.41, 2.42

Lymphoblastic cell types in ovine malignant lymphoma showing extensive cytoplasm, irregularly shaped and leptochromatic nuclei, large and sometimes multiple nucleoli and numerous mitotic figures.

HE x 1,600





possible to distinguish the nucleolus. The cytoplasmic zone was fairly broad and moderately basophilic (Figures 2.43, 2.44).

Cells of the lymphocytic type were basically similar to normal mature lymphocytes but they were often larger and had more cytoplasm than normal cells. Nuclei were pachychromatic, centrally located, and nucleoli could not be seen (Figures 2.45, 2.46).

The reticulum cell sarcomas contained the only lymphomatous cells closely associated with silver positive fibres. The features of the cells were not uniform, showing considerable pleomorphism between different areas of tumour, and differing degrees of differentiation as judged by the production of reticulin. These cells varied in appearance from lymphoid stem cell to histiocytic types. Frequently whorled areas of fusiform cells were seen (Figures 2.47, 2.48, 2.49).

Ultrastructure

Electron microscopic studies were made of the tissues from 10 cases. Most showed many cells in which degenerative changes in the subcellular organelles were present which may have been the result of autolytic or necrotic change, or both. Due to the destructive infiltrative nature of the disease, large quantities of cellular debris were present which complicated the interpretation of findings (Figure 2.50). Tumour cells contained many ribosomes, polyribosomes and coarse mitochondria in the cytoplasm (Figure 2.51). The nuclei contained much heterochromatin and nucleoli were granular (Figure 2.51, 2.52) and indentations of the nuclear membrane were occasionally observed (Figure 2.50, 2.52, 2.53).

All sections were carefully examined for the presence of virus-like particles. Unequivocal virus particles were not seen, although in some cases structures resembling "C and A type" particles were seen

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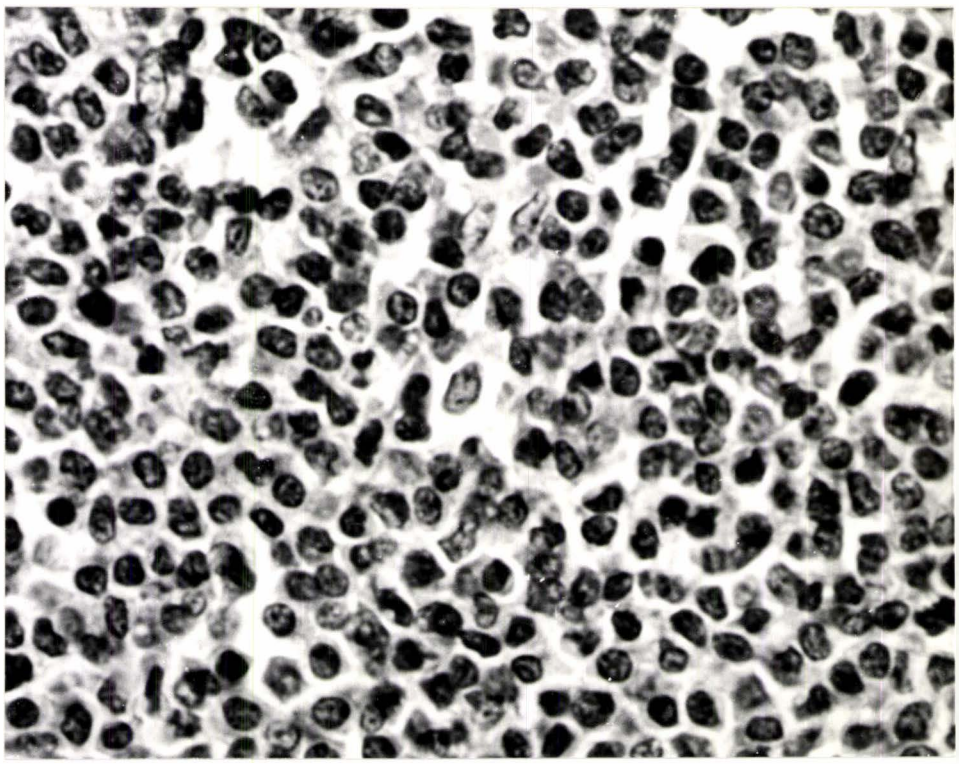
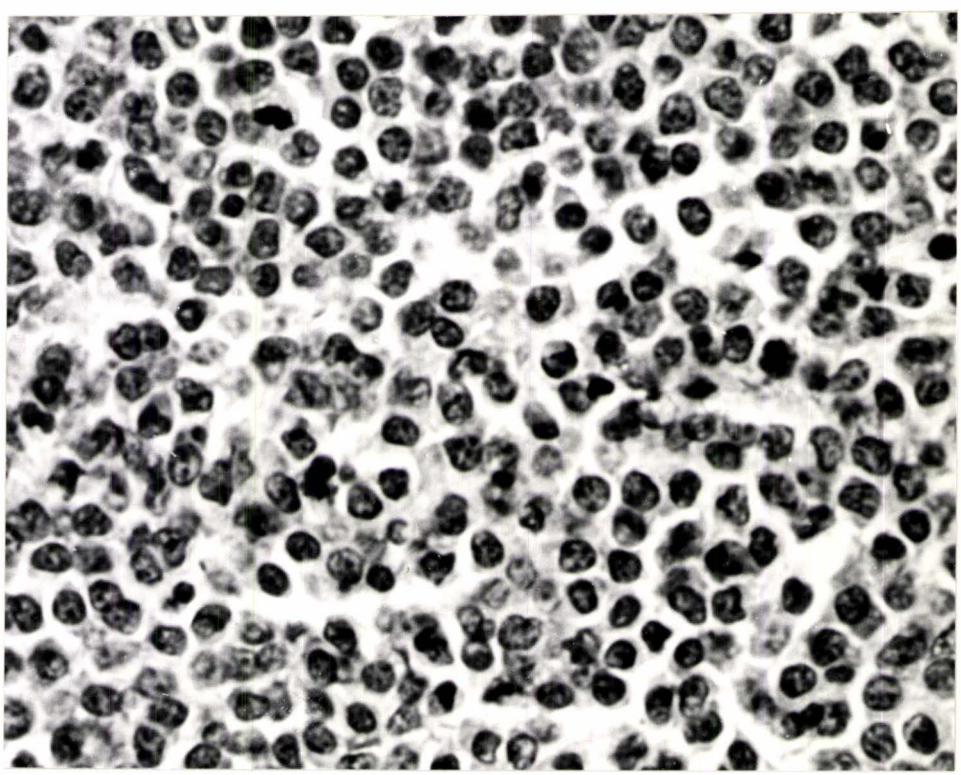
Figures 2.43, 2.44

Prolymphocytic cell types in ovine malignant lymphoma. The cells are smaller than those of the lymphoblastic cell type, show less extensive cytoplasm and increased density in nuclear staining.

HE x 1,600

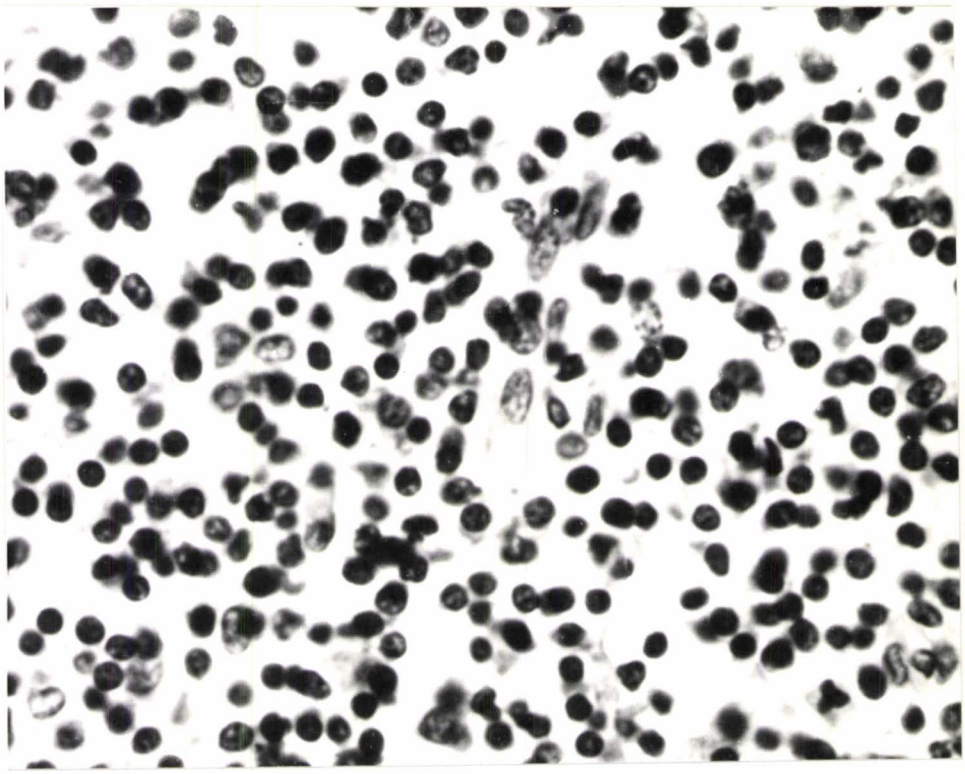


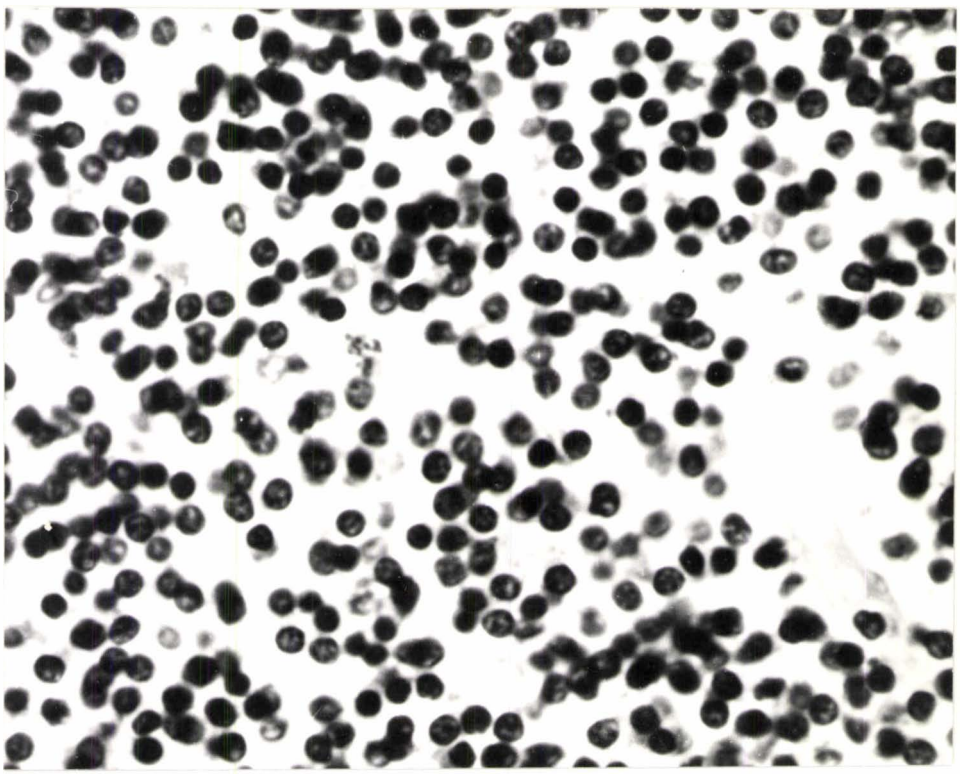


Figures 2.45, 2.46

Lymphocytic cell types in ovine malignant lymphoma. Morphologically they resemble normal lymphocytes, showing a dense pachychromatic nucleus and a small indistinct cytoplasmic region.

HE x 1,600

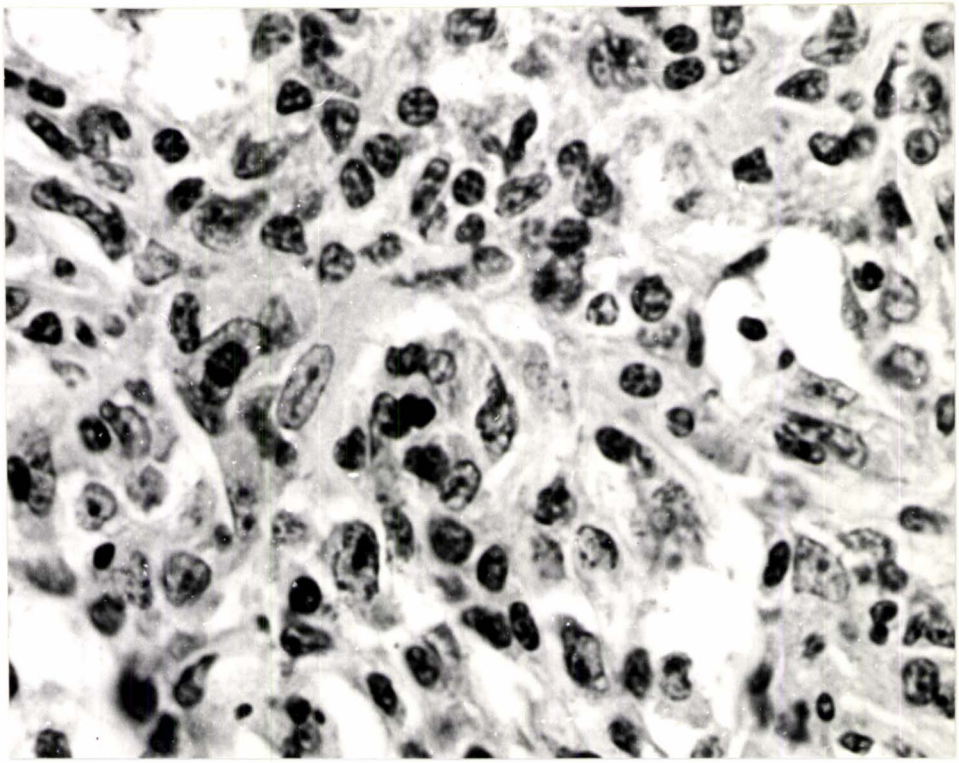




Figures 2.47, 2.48

Large pleomorphic cells in the lymph nodes of sheep affected with reticulum cell sarcoma.

HE x 1,600



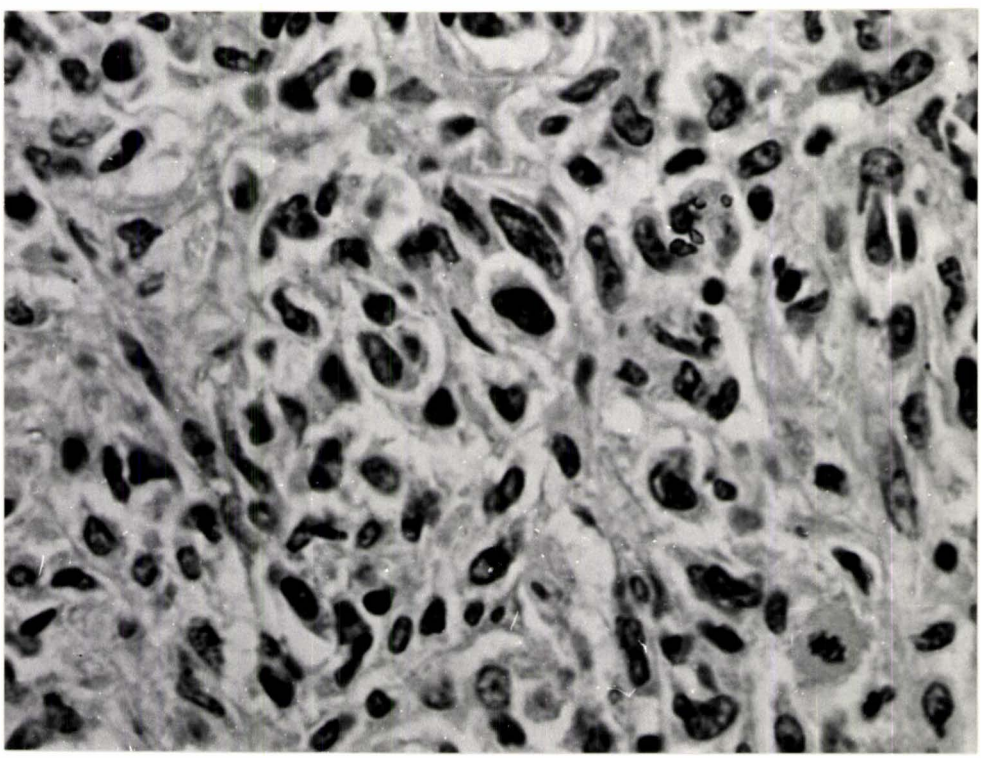
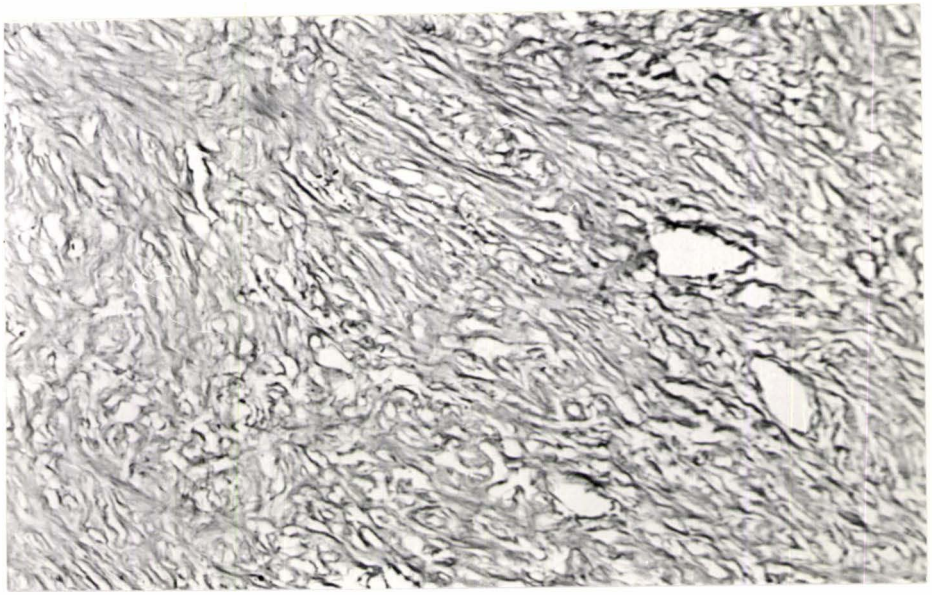


Figure 2.49

Production of fine reticulin fibres by neoplastic cells in a reticulum cell sarcoma.

Gordon and Sweet's x 400



Following page 57

Figure 2.50

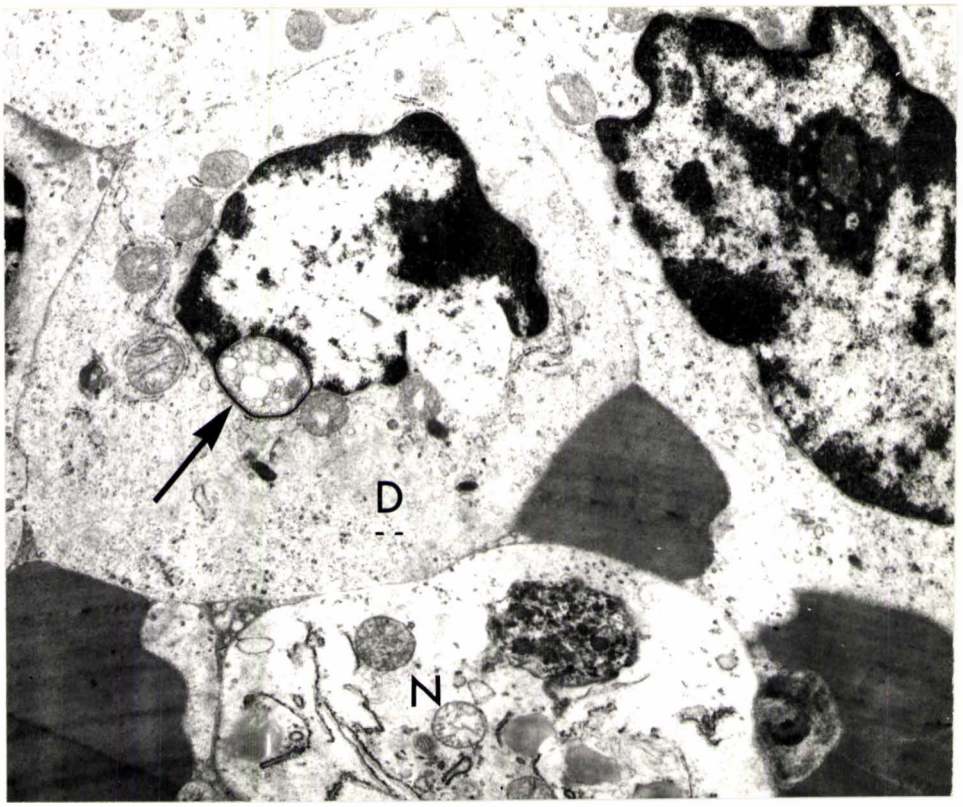
Neoplastic cells in the spleen of a sheep with malignant lymphoma showing a degenerate lymphocyte (D) and an adjacent necrotic cell (N). A "bleb" (arrow) has formed in the nucleus of the former.

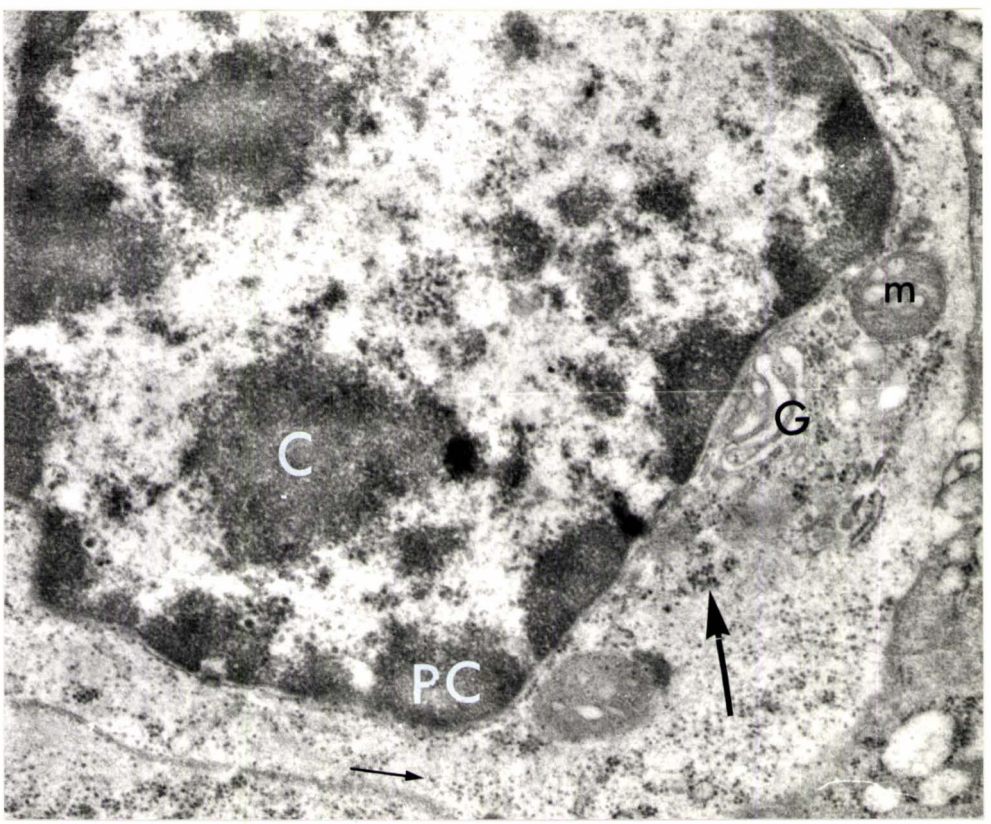
Electron micrograph x 8,400

Figure 2.51

The nucleus and cytoplasm of a neoplastic lymphocyte. The cytoplasm contains free ribosomes (small arrow) and polyribosomes (large arrow). A golgi apparatus (G) and mitochondria (M) are also present. Islands of chromatin granules (C) and peripheral chromatin (PC) can be seen in the nucleus.

Electron micrograph x 27,700


D
N


m
G
C
PC

Following page 57

Figure 2.52

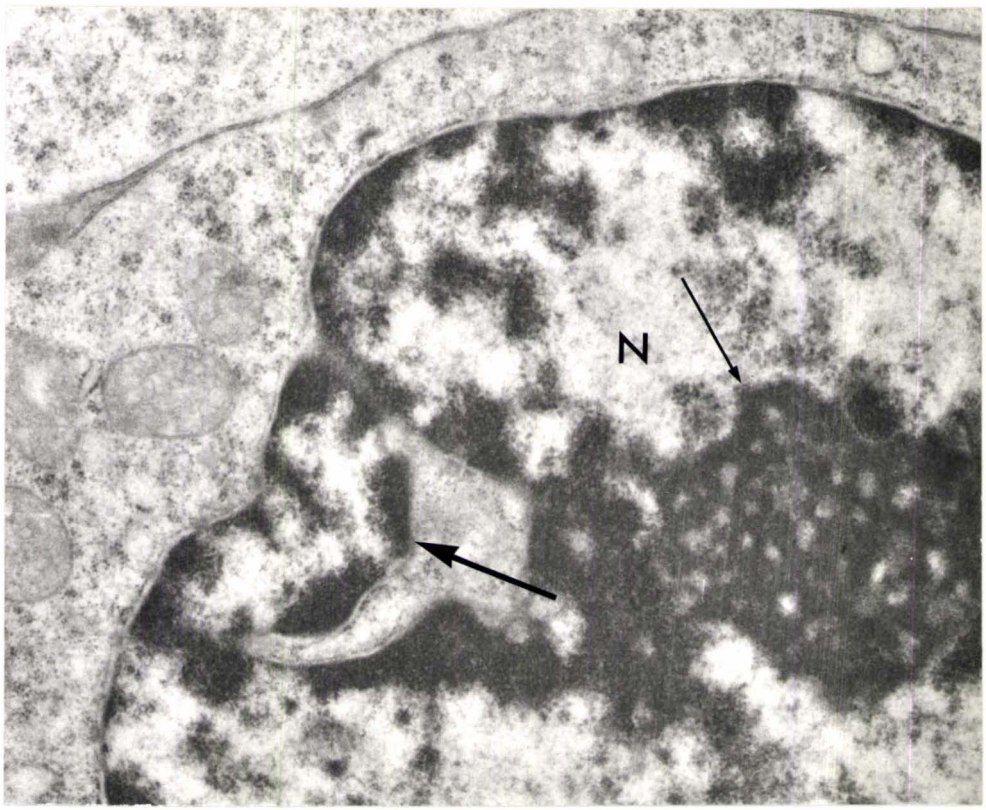
The nucleus (N) of a neoplastic cell in ovine malignant lymphoma in which an indentation in the nuclear membrane (large arrow) and a large granular nucleolus (small arrow) is present.

Electron micrograph x 24,300

Figure 2.53

Myelin-like bodies (M) within the cytoplasm of a neoplastic cell. The nuclear membrane shows a prominent indentation (arrow).

Electron micrograph x 12,300


N

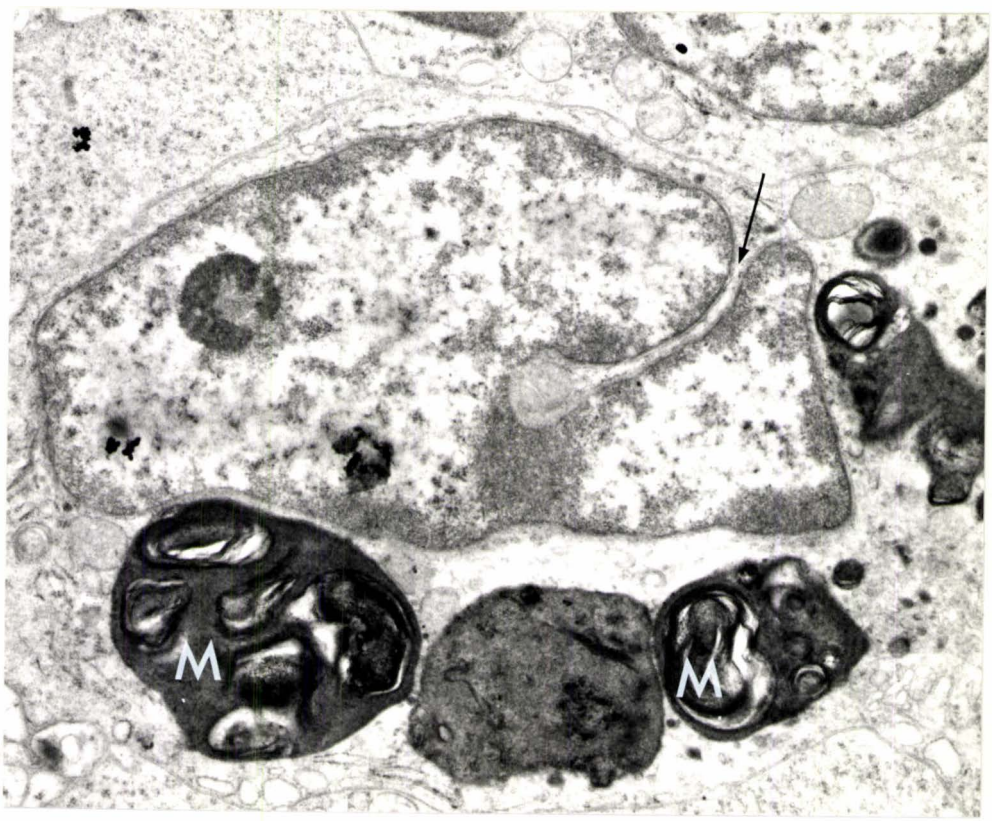

M
M

Figure 2.54

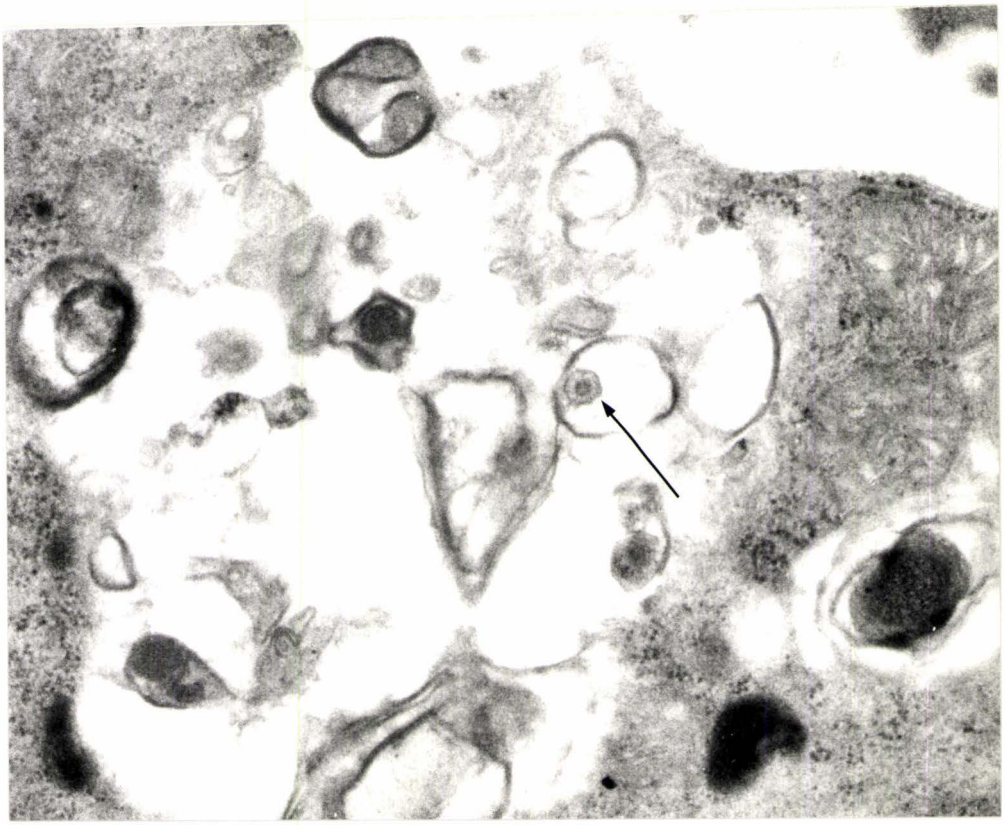
Distended endoplasmic reticulum in a tumour cell in which there is a structure resembling a budding "C-type" virus particle (arrow)

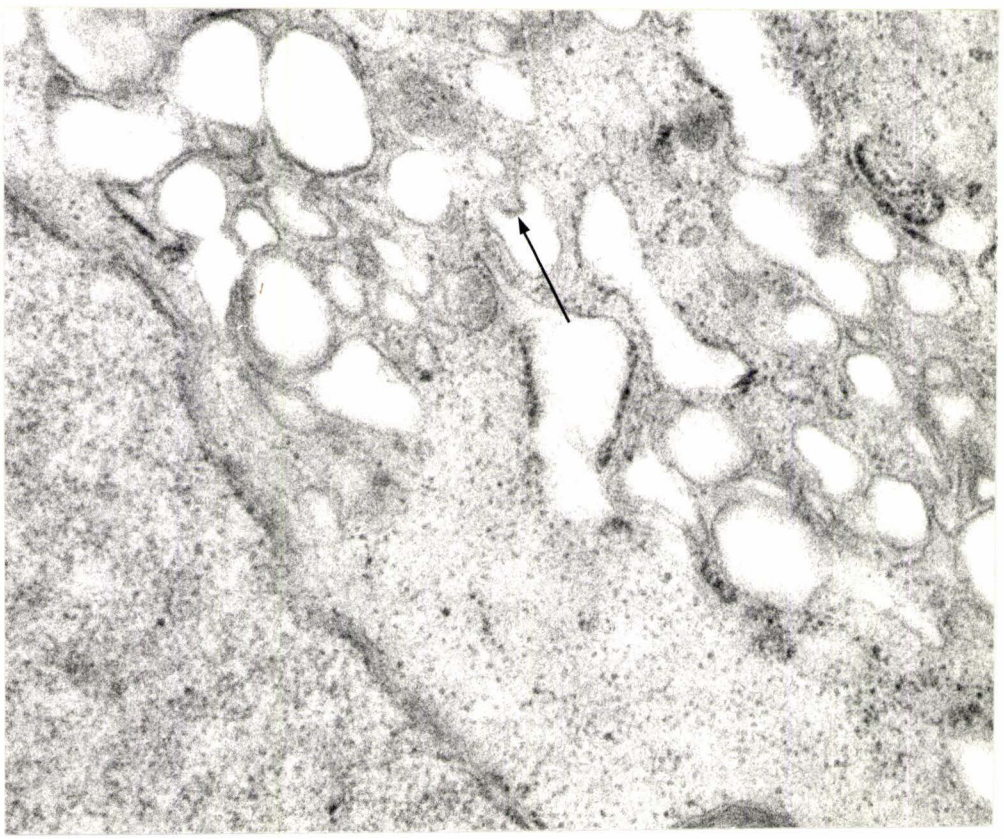
Electron micrograph x 40,000

Figure 2.55

Particle (arrow) resembling a "C-type" virus within a vacuole of a degenerate cell in a case of ovine malignant lymphoma.

Electron micrograph x 40,000





in the cytoplasmic vacuoles of cells (Figure 2.54, 2.55), and one of these sections a possible budding particle was present (Figure 2.55).

DISCUSSION

The most impressive feature of malignant lymphoma of all species is the enlargement of affected lymph nodes and the progressive spread of neoplastic tissue to other lymphoid and non-lymphoid organs. In this series, these changes were seen in nearly all cases. Gross enlargement of lymph nodes was absent in three animals only. In each of these cases the only lesions seen were present in the kidneys. The renal lymph nodes in these cases were normal on the gross post mortem examination, but absence of tumour was not confirmed histologically. It seems likely that had a histological examination of these nodes been performed, secondary lesions would have been present, because this was the case in the two other cases with apparently primary kidney lesions in which a histological examination of the renal lymph nodes was performed. Webster (1966) also noted gross lesions in sheep affecting only the kidneys. He suggested that they may be a separate anatomical variant of the disease, but it seems unlikely that primary lymphoid tumours would arise at this site. Lesions restricted to the kidneys have not been reported in sheep by other authors, but Migaki (1969) reported that solitary involvement of this organ was present in several pigs examined in his series. It seems probable that a small primary focus of tumour was located elsewhere in the body and which was overlooked during the gross examination.

Next to the lymph nodes, the spleen was the second most commonly affected organ in this series. Splenic involvement in malignant lymphoma has been frequently reported as a common lesion in many animal species (Monlux et al., 1956; Cotchin, 1960; Smith, 1962; Hugoson, 1967;

Dungworth, Theilen and Ward, 1968; Jarrett et al., 1968; Jarrett, 1971), whereas it is a relatively infrequent site, with the exception of Hodgkin's disease, in most malignant lymphomas of man (Rappaport, 1966).

Since practically any organ in the body may eventually become involved in the neoplastic process and because of errors of omission occurring during the collection of specimens, it is beyond the scope of this study to make detailed comparisons of the distribution of lesions in organs of the various species. The necessity of relying upon abattoirs as the major source of case material leads to some inaccuracies in the description of distribution of lesions and estimates of disease prevalence in the sheep population. Besides the errors of omission already mentioned, certain parts of the carcass are discarded before they are examined. For instance, the urogenital tract below the kidneys is not normally viewed in detail before its disposal. It is therefore impossible to accurately estimate from this series the frequency with which lesions of the uterus occur in the sheep. The uterus is a common site of tumour formation in cattle (Marshak et al., 1962; Bendixen, 1965; Smith, 1965; Hugoson, 1967; Migaki, 1969) and as the distribution of the lesions in sheep have much in common with those of cattle, it is possible that this organ may be a common site in this species too. In one of the two cases in which the complete urogenital tract was examined, extensive uterine lesions were present. Other parts of the sheep carcass not routinely examined in detail in New Zealand slaughterhouses include the skin and mammary glands, the feet, lower limbs, bone marrow, mucosal surfaces of the gastro-intestinal tract and, occasionally, the head.

The population of sheep slaughtered is not a true representation of the sheep population at large. Most are lambs and are therefore less likely to be affected by the disease. The older animals killed in

slaughter-houses are mainly fattened "cull-for-age" ewes. It is likely that many of the animals affected by malignant lymphoma are emaciated and therefore unsuitable for human consumption and consequently are not seen at abattoirs.

Although certain organs may not be examined during routine meat inspection at slaughter-houses, it is considered that the occasional absence of data regarding the presence or absence of tumours in these organs does not preclude the use of the broad patho-anatomical classification proposed by Anderson et al. (1969). This system of classification appears to have been of some value in regard to malignant lymphoma of cattle in that certain patho-anatomical forms of malignant lymphoma appear to have reasonably well defined epidemiological characteristics. For example, the enzootic form of the disease is almost always multicentric in distribution, whereas in sporadic cases a high incidence of the thymic form is seen. It should not however be assumed that this will necessarily hold true for the sheep. Neither can it safely be assumed that different forms are of differing aetiology. Table 2.IV shows the cases reported in the literature classified according to their patho-anatomical form. With the exception of Webster's (1966) series, the multicentric variety is the most commonly reported form in sheep. It is questionable whether or not the skin form constitutes a class of malignant lymphoma in sheep which is distinct from the multicentric form because all the cases of the skin form cited in Table 2.IV also had lesions elsewhere in the body which were typical of the multicentric form. Furthermore in sheep, in contrast to the situation in cattle, there is no apparent correlation of the skin form with any particular epidemiological pattern of the disease.

Included in the alimentary form in Table 2.IV are those cases in which the lesions were restricted to the organs of the abdominal and

TABLE 2.IV

The distribution of lesions in reported series of spontaneously occurring malignant lymphomas in sheep

	Anatomical form					
	Multicentric	Alimentary	Thymic	Skin	Other	Total
Jackson (1936)(1)	4	-	-	-	-	4
Head (1953)(1)	6	-	-	5	-	11
Monlux, Anderson and Davis (1956)(1)	13	-	1	6	-	20
Cotchin (1960)(1)	5	3	1	-	1	10
Webster (1966)(1)	3	14	6	-	-	23
Anderson, Jarrett and Crighton (1969)	33	-	5	6	-	44
Migaki (1969)(1)	15	-	-	2	-	17
Paulsen et al. (1971)(1)	4	2	-	-	-	6
Present series(1)	14	6	1	1	-	22
Total (percentage of total number of cases)	97(61)	25(16)	14(9)	20(13)	1(1)	157

(1) Classification provided by author after Anderson, Jarrett and Crighton (1969).

thoracic cavities. Approximately 30 per cent. of the sheep in the current series are included in this category. In Webster's series an even higher prevalence was reported. It appears from these results that this form of the disease is of much higher prevalence in New Zealand sheep than those in other countries from which malignant lymphoma has been reported. Involvement of the abdominal organs in the absence of peripheral lymphadenopathy are features of malignant lymphomas in cats (Holzworth, 1960) and Burkitt's lymphoma of African children (Burkitt and O'Conor, 1961; Wright, 1971).

Thymic involvement in cases of malignant lymphoma in sheep has previously been reported by Monlux et al. (1956), Cotchin (1960), Webster (1966) and Anderson et al. (1969). The difficulty of distinguishing between thymomas containing a preponderance of lymphocytes and lymphosarcoma of the thymus has been commented on by Castleman (1955) and Willis (1967). In advanced cases of the latter condition, the epithelial elements of the thymus are frequently obliterated (Anderson et al., 1969) resulting in difficulty in determining whether or not the neoplasm originated in the thymus or in anterior mediastinal or posterior cervical lymph nodes. Dungworth et al. (1964) reported that Hassal's corpuscles could not be demonstrated in any of their series of thymic lymphosarcomas in cattle. The pathological features of these tumours appear to be identical to the possible case of thymic lymphosarcoma seen in the present series. This form of the disease is by far the most commonly occurring manifestation of spontaneous malignant lymphoma of the laboratory mouse (Siegler, 1968), and has also been described in the cat (Holzworth, 1960; Anderson et al., 1969) and the pig (Anderson et al., 1969).

The most striking histological feature of the lesions seen in this series was the progressive infiltrative nature of the disease process

in the affected organ, leading to the eventual obliteration of its normal architecture.

The progressive colonization of lymph nodes by malignant cells followed a characteristic pattern. The afferent lymphatics and subcapsular sinuses were the first areas to show neoplastic involvement, followed by medullary and then cortical regions. This pattern has not been noted in other reports on sheep. Marshak et al. (1962) stated that the process in lymph nodes of cattle begins in the sinuses, particularly the medullary sinus, followed by the sequential infiltration of medulla and cortex. Krämer (1967) also demonstrated the initial lesions in the medullary sinus, while Squire (1964) observed neoplastic cells first either in the medullary sinus or in the follicles.

Several authors have observed early neoplastic changes in the primitive mesenchymal tissues of perivascular areas and in the right atrium of the heart. Jarplid (1964) considered that the presence of differentiating lymphocytes in the atrial wall of cattle in the preclinical phase of malignant lymphoma was indicative of an early neoplastic change, and the observations of Dungworth et al. (1968) tended to confirm this observation. In one series of seven sheep Enke, Jungwitz and Rössger (1961) found the heart to be involved in "most" animals. Paulsen et al. (1971) reported gross lesions of the heart in one of six cases examined and microscopic evidence of neoplastic infiltration in four of the remaining five. Involvement of the heart in the series reported here was seen grossly in four cases and microscopic evidence of tumour infiltration was present in a further five cases. In all of these the wall of the right atrium was affected.

Some difficulty was encountered in deciding whether or not lymphoid cell aggregates in the lungs of these cases were of neoplastic origin. Peribronchial lymphoid hyperplasia is a common incidental pathological

finding in sheep and may be associated with a variety of influences including various chronic inflammatory states such as parasitic infestation and chronic enzootic pneumonia. Because of the difficulty in differentiating peribronchial lymphoid hyperplasia from lymphoid neoplasia, the report of Swaen and Van Heerde (1973) of pulmonary reticulum cell sarcoma affecting 80 per cent. of Wistar rats from their laboratory must be viewed with reservation until confirmed by others. It is unusual for reticulum cell sarcomas to develop without metastasis to other organs as was reported in the majority of their cases. Furthermore, the lesions described by them were fairly typical of those seen in the commonly occurring chronic pulmonary disease of rats (Innes, Garner and Stookey, 1967).

In one case (No. 7286) large numbers of lymphoblastoid cells were present in the blood vessels of tissues examined and were suggestive of leukaemia. Cattle and sheep affected by malignant lymphoma frequently show a pretumourous lymphocytosis in the enzootic form of the disease (Chapter 3), but as is the case in dogs and cats, the majority of clinical cases are not accompanied by a frank leukaemia.

The cytology of the malignant cells constitutes an important basis of classification of malignant lymphoma in man. Thorough studies of the various cellular features and the clinical course of the diseases have been reported by Gall and Mallory (1942), Gall and Rappaport (1958) and Rappaport (1966) and a consistent relationship between the predominant cells type and the course and prognosis of the overt disease has been established. In contrast, few studies have been made of the cellular features of the malignant lymphomas in animals. Those of Bloom and Meyer (1945), Meier (1957), Squire (1965), Hugoson (1967) and Jarrett (1971) showed no correlations between cell type and clinical syndrome, however Holzworth (1960) reported that malignant lymphoma of cats in

which lymphoblastoid cell types predominated tended to be of shorter clinical duration and Smith (1965) considered that the stem cell malignant lymphoma commonly seen in cattle was associated with a rapid clinical course of the disease in this species. Squire considers that the cytologic details of this disease in animals may eventually be of value in indicating the clinical course, the effect of therapy and possibly of aetiological factors involved.

With the exception of the cases classed as the reticulum cell sarcomas, all malignant lymphomas in the current series are classifiable as lymphosarcomas of varying degrees of cellular differentiation. No lesions simulating the cytologic features of the follicular lymphomas or Hodgkin's disease of man were observed. The lymphosarcomas most commonly seen were composed of predominantly poorly differentiated cells. This is in contrast to those described by Enke et al. (1961), Hilgenfeld and Krieg (1965), Nobel et al. (1967), Anderson and Jarrett (1968) and Paulsen et al. (1971) who reported that the predominant cell type observed in the sheep with malignant lymphoma was well differentiated.

The "starry sky" pattern of cellularity reported as a constant feature of the Burkitt lymphoma of African children (O'Conor and Davies, 1960) has also been seen in malignant lymphoma of a number of animal species including the dog (Baskerville, Hunt and Lucke, 1966), cat (Squire, 1965, Baskerville et al., 1966, Nielsen, 1969), sheep (Paulsen et al., 1971) and cattle (Smith, 1965). Most authors believe that the "stars" are phagocytic macrophages responding to necrotic material resulting from tissue destruction occurring in areas of rapid neoplastic proliferation.

The ultrastructural abnormalities observed in the neoplastic cells of malignant lymphoma in sheep are probably of no particular significance. Similar changes have been reported frequently in malignant lymphomas and

other tumours affecting other animal species and have been generally interpreted as non-specific (Achong and Epstein, 1966; Parker et al., 1967; Fujimoto, Miller and Olson, 1969; Miller et al., 1969; Sonoda and Marshak, 1970; Rangan, Calvert and Vitols, 1971; Narang, 1973; Schumacher, Szekely and Park, 1973). The observation of virus-like particles in two of the cases must be interpreted with caution. Although similar structures have been described in and proved to be the cause of malignant lymphoma in avian, murine and feline species (Jarrett et al., 1964; Kawakami et al., 1967; Rickard et al., 1967; Laird et al., 1968; Hardy et al., 1969; Gross, 1970) and have been demonstrated in association with spontaneous cases of this disease in dogs (Chapman et al., 1967; Seman et al., 1967; Rudolph, 1971), cattle (Jarrett, 1962; Sorensen, 1962; Ueberschär, 1963; Dutcher et al., 1964 and 1967; Jensen and Schlidlovsky, 1964; Schulze, Wittman and Gralheer, 1966; Dutcher, 1968; Dutta et al., 1970; Estes, Coote and Noronha, 1970; and Sonoda and Marshak, 1970), sheep (Paulsen et al., 1971), pigs (Frazier, Ushijima and Howard, 1970) and man (Dmochowski and Grey, 1958; Braunsteiner, Fellinger and Pakesch, 1960; Dalton, Haguenau and Moloney, 1962; Anderson, 1965; Dmochowski et al., 1965; and Seman and Seman, 1968), the structures observed in the cases in this series may well have been unrelated to virus or to the neoplastic process. With the one possible exception described, "budding" forms were not seen in the sections examined and the morphology of the particles was not well enough defined to confirm their viral nature.

CONCLUSIONS

The majority of sheep affected with malignant lymphoma show generalized lymphadenopathy as a result of invasion and proliferation of neoplastic cells of lymphoid origin in lymph nodes. Non-lymphoid organs are also involved in most cases of the disease and in the

current series liver, kidney and heart were affected in 45 per cent. or more cases in which they were examined.

The general pathoanatomical form for classifying malignant lymphomas of domestic animals proposed by Anderson and co-workers may be applied to sheep. The multicentric form of the disease predominates but data from the present study and that of a previous series in New Zealand indicate that in this country there is a higher frequency of cases classifiable as being of alimentary type than that reported in other countries.

The disease is progressive and advanced lesions show total obliteration of normal tissue architecture by invasive neoplastic cells. Lymphosarcoma is the most common histological form of malignant lymphoma encountered but reticulum cell sarcomas also occur. The cytology of the lymphoid cells of the lymphosarcomas varies from predominantly lymphoblastic forms to reasonably well differentiated lymphocytic types. The less differentiated forms predominate. Non-specific ultrastructural alterations can be observed in the neoplastic cells but the presence of virus has not been unequivocally substantiated in the series reported here.

Malignant lymphoma in sheep has pathological features in common with that in other animal species. Of these it bears closest resemblance to this disease in cattle.

CHAPTER 3

ATTEMPTS TO TRANSMIT OVINE MALIGNANT LYMPHOMA TO SHEEP

INTRODUCTION

Malignant lymphoma and related haematopoietic neoplasms have been proved transmissible and of viral aetiology in several animal species including the chicken (Ellerman and Bang, 1908, cited by Gross, 1970; Rous, 1910; Furth, 1933; Burmester, Pricket and Belding, 1946; Nazerian and Witter, 1970), the laboratory mouse (Gross, 1951, 1970), the guinea pig (Congden and Lorenz, 1954; Jungeblut and Kodza, 1962; Opler, 1968) and the cat (Jarrett et al., 1964; Kawakami et al., 1967; and Rickard et al., 1967). Mastocytoma of the dog has been transmitted by cell-free extracts, but the virus observed in these cases has not been isolated (Lombard, Moloney and Rickard, 1963; Rickard and Post, 1968; Post, Noronha and Rickard, 1970). Transmission of malignant lymphoma in dogs using cell-free extracts has not been performed, although these tumours have been transplanted from affected to immune-suppressed animals (Moldovanu et al., 1966; Kakuk et al., 1968; Rickard, 1968).

It is likely that bovine leukosis is transmissible by cell-free extracts. In 1916 Knuth and Volkman (cited by Hugoson, 1967) reported transmission studies in which a lymphocytosis developed in the experimental animals. Transient or persistent lymphocytosis has been recorded following several other attempts to transmit the disease of cattle (de Toit, 1917; Dobberstein and Piening, 1934; Schlottler and Schlottler, 1934; Stasney et al., 1939, all of whom were cited by Hugoson, 1967; Hoflund, Thorell and Winquist, 1963; Bederke and Tolle, 1964; Theilen et al., 1967; Hatziolos, 1968; Weinhold and Straub,

1968; Rosenberger, 1968), and have been interpreted as a precancerous state by Bendixen (1965). Other transmission attempts in cattle have shown entirely negative results (Dutcher et al., 1963).

Wittman and Urbaneck (1969 and 1970) reported malignant lymphoma in 6 of 21 sheep occurring 10 to 23 mth after inoculation at one day of age with whole blood of cattle which had exhibited persistent lymphocytosis. Control animals were absent in this experiment. Olsen et al. (1972) reported the development of malignant lymphoma in five of 13 sheep inoculated between one and three days of age with cultured lymphocytes from a cow with overt lymphosarcoma and which were shown to contain "C-type" virus particles. Theilen (1971) observed the growth and regression of lesions diagnosed as fibrosarcoma in lambs which had been inoculated in utero with the virus of feline sarcoma.

There have been no reports in the literature of attempts to transmit malignant lymphoma to sheep using cell-free tumour extracts from sheep with this disease. The experiments in this chapter were designed to investigate the hypothesis that malignant lymphoma of sheep is a transmissible disease. For two major though possibly related reasons it seemed appropriate that the subjects for the transmission experiments should be either foetal or neonatal. Firstly, the success of transmission experiments involving the analogous disease in mice and chickens depended largely on the use of neonatal animals (Gross, 1951; Eckert, Beard and Beard, 1955; Graffi, 1957; Burmester, Fontes and Walter, 1960; Moloney, 1962; Beard, 1963; Buffet, Grace and Mirand, 1964; Rich, Geldner and Meyers, 1965). Secondly, the development of infection by the possible presence of an oncogenic agent appeared to be more likely if administered before the recipient reached full immunological maturity. Accordingly, lambs were inoculated with cell-free extracts of lymphoid tumours either during the intrauterine period of

development or neonatally. Following these treatments the lambs were monitored by haematological and clinical examinations.

MATERIALS AND METHODS

Experimental Animals

The sheep used in these experiments were the offspring of maiden New Zealand Romney ewes which had been mated to New Zealand Romney rams. Conception dates of the ewes were recorded by use of marking harnesses attached to the rams. At birth the lambs were identified with numbered brass ear-tags. Between the age of two and six weeks the lambs were vaccinated against commonly occurring clostridial diseases with a commercial vaccine;(1) had tails removed with a searing iron and the males were emasculated. At intervals of one month, between December and May, all animals were drenched with a broad-spectrum anthelmintic(2) for the first two years of age and thereafter when indicated by faecal counts of worm eggs. Weaning was at approximately 16 weeks of age. The animals were rotationally grazed as a single mob on mixed ryegrass and white clover pasture.

Inocula

Tumour tissue was collected fresh from slaughter-houses and transported to the laboratory in dry ice where portions were removed aseptically and homogenized in equal volumes of phosphate buffered saline (PBS) containing 200 IU of penicillin and 200 µg of streptomycin per ml of solution and using Sorval "Omnimixer" and a Kimax tissue

(1) Saffelin ATS, Glaxo Laboratories, New Zealand.

(2) Banminth II, Pfizer, New Zealand.

grinder. This homogenate was adjusted to a 50 per cent. volume per volume suspension with the same PBS antibiotic solution and stored in aliquots of one millilitre in sealed glass vials at -78.5°C. Prior to use, the homogenates were frozen and thawed twice. Normal lymph node tissue was prepared in an identical manner for inoculation into control animals. The various inocula used in these experiments are detailed in Table 3.I.

Inoculation of Animals

Lambs were inoculated intraperitoneally with one millilitre of tumour homogenate either within 12 hr of birth or between 41 and 68 days of gestation. Table 3.II shows the design of the various treatment groups.

For *in utero* inoculation the pregnant ewes were anaesthetized by intravenous injection of a five per cent. solution of thiopentone sodium and following intubation with a cuffed endotracheal tube were maintained in a surgical plane of anaesthesia with a halothane/oxygen mixture. With the ewe in right lateral recumbency the uterus was exteriorized through a laparotomy incision in the region of the left paralumbar fossa. The foetus was palpated through the uterine wall and guided by the lateral processes of the lumbar vertebrae, the tumour homogenate was introduced into the foetal peritoneal cavity using an 18 gauge (BSW) needle. The gravid uterus was then returned to the normal position in the abdominal cavity and the abdominal wound sutured. Ewes were kept under close observation for 24 hr and then returned to pasture where they were observed regularly. At birth the treated lambs were identified by numbered brass-eartags.

The lambs inoculated neonatally were given the same volume of inocula, by the same route and were identified in the same manner as those described above.

TABLE 3.I

Tumour Homogenates Used in Transmission Experiments

Inoculum designation	Histological form of malignant lymphoma	Organ from which tumour tissue obtained
OL_1	Lymphoblastic	Lymph node
OL_2	Lymphocytic	Lymph node
OL_3	Lymphoblastic	Thymus
Control	-	Lymph node(normal)

TABLE 3.II

Identification of Lambs Inoculated with Tumour and Control Extracts and the Time of Inoculation

Inocula	Time of Inoculation:- During Gestation	Neonatal	Total Lambs Inoculated
OL_1	401 404 408 412 413 419 431 433 (1)	402 420 421 425 426 427 428 432 434 435	20
OL_2	405 414 415 418 423 429 430 (2)	403 406 407 409 410 411 416 417 422 436	20
OL_3		437 438 439 440 441 443 442 444	8
Normal lymph node	424 (3)		4
Total	24	28	52

(1) Two inoculated lambs lost from this group during gestation

(2) Three inoculated lambs lost from this group during gestation

(3) Three inoculated lambs lost from this group during gestation

Control animals formed two groups. The contact controls which were bred from ewes in contact with the dams of the treated animals, were managed with the treated groups. At the beginning of the experiment this group comprised 15 lambs.

The population control group was made up of 20 sheep matched for age and breed and drawn at random from a Massey University commercial flock at the time the blood sampling was performed. Four lambs were inoculated *in utero* with cell-free extracts of normal lymph node tissue as a control group for the intra-uterine inoculation tumour group.

Details of identification, inoculation and age of experimental animals not noted in Table 3.II are shown in Appendix IV.

Haematology

After the first 10 months total and differential leucocyte counts were performed on all surviving animals at intervals of four to six months. Blood from the jugular vein was collected in 100 x 16 mm "Vacutainer"(3) vials containing 12 mg of sodium ethylene diamine tetra-acetic acid (EDTA). Total leucocyte counts were made using either an automatic particle-counter(4) or a Neubauer haemocytometer. The particle-counter was available for use during the examinations performed during December 1972 and April 1973. Differential leucocyte counts were performed on blood films stained by the May-Grünwald-Giemsa method (Darmody and Davenport, 1958). For total white cell counts less than 10,000 per mm^3, 100 cells were differentiated and for counts above 10,000 per mm^3, 200 cells were differentiated. Cell counting procedures were carried

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(3) Becton-Dickenson and Co., New Jersey.

(4) Celloscope III, Lars Ljunberg and Co., Sweden.

out "blind" to eliminate experimental bias. In order to test the degree of fluctuation in cell counts of animals in the interval between flock bleedings, a group of 10 sheep which had been inoculated with tumour material were selected for leucocyte counting at 10 to 12 day intervals over a four month period. Eight of the 10 animals chosen had shown lymphocytosis at the beginning of this period while two had been within the normal lymphocyte range.

Deaths in Experimental Animals

A post mortem examination was carried out on all animals dying during the course of the experiments. Particular attention was paid to examination of lymphoid tissues.

RESULTS

Clinical Examination

No clinical sign of overt malignant lymphoma development was seen in any sheep up to and including the last examination three years after inoculation.

Survival of Animals

The fate of the animals used in these experiments is shown in Appendix IV. No losses occurred as a result of neoplasia.

Leucocyte Analysis

The results of total and differential leucocyte counts performed on all animals in the flock are shown on Table 3.III. The general range and distribution of total lymphocyte counts within groups is illustrated in Figure 3.1. To test for significant differences between treatment groups, the multiple range test of Kramer (1956) for group means with unequal replications within each group was employed. The statistical method is shown in Appendix V and the significance of the

TABLE 3.III

Total and Differential Leucocyte Counts of Sheep Inoculated with Cell-free Extracts of Malignant Lymphoma from Spontaneously Occurring Cases in Sheep and Uninoculated Control Animals

August, 1972

Sheep No.	Leucocytes (per mm^3)	Lymphocytes (per mm^3)	Neutrophils (per mm^3)	Eosinophils (per mm^3)	Monocytes (per mm^3)
401	15,850	11,412	4,121	317	0
402	14,200	9,372	4,484	142	142
403	10,850	6,944	2,062	1,736	108
405	13,150	8,942	3,880	263	65
407	10,900	6,595	3,873	378	54
410	11,250	7,369	3,262	619	0
411	13,050	8,874	3,458	326	390
413	13,350	9,011	3,471	668	200
414	11,750	7,403	3,463	586	118
415	11,000	7,700	2,420	825	55
416	14,600	10,074	2,920	1,606	0
417	12,400	5,828	5,890	620	62
420	11,000	8,195	2,255	550	0
421	12,200	7,320	4,270	488	122
426	12,200	7,991	4,087	122	-
427	11,250	8,494	2,475	168	113
428	13,200	4,686	8,382	132	0
432	15,350	10,054	4,068	1,228	0
433	10,950	7,173	2,409	1,313	55
434	11,800	8,501	2,419	585	295
435	11,750	7,168	3,760	646	176
437	11,950	6,513	5,019	298	120
438	9,900	6,930	2,772	198	0
439	11,550	6,549	4,158	670	173
440	16,500	10,313	3,630	2,228	330
441	9,900	6,138	2,475	1,138	149
443	12,600	8,820	2,520	945	315
444	8,350	5,386	2,296	626	330
72	8,200	4,510	3,198	328	164
73	11,400	2,793	8,379	0	228
113	10,550	6,647	3,271	633	0
114	10,850	5,968	4,232	379	271
115	8,100	4,860	2,916	243	81
116	5,800	3,951	1,711	80	58
117	10,050	5,829	3,819	0	402
118	10,800	7,668	2,430	378	324
119	10,950	5,585	4,161	766	438
120	10,250	6,207	3,536	456	51
121	9,100	6,052	2,321	637	91
122	3,850	2,522	1,001	211	116
123	11,850	6,340	4,622	770	118
124	3,750	1,500	2,156	98	0
125	9,300	4,836	4,046	297	139
424	9,850	6,501	3,349	0	0
1	1,300	6,695	4,225	1,560	520
2	9,750	5,655	2,876	1,121	98
3	12,850	8,289	22,377	1,991	193
4	4,800	3,120	1,104	192	384
5	11,250	5,906	4,500	844	0

...Cont'd

TABLE 3.III

August, 1972

Sheep No.	Leucocytes (per mm^3)	Lymphocytes (per mm^3)	Neutrophils (per mm^3)	Eosinophils (per mm^3)	Monocytes (per mm^3)
6	9,700	4,996	3,735	872	97
7	8,650	5,234	3,114	259	43
8	5,090	3,283	1,451	178	178
9	3,900	2,555	1,248	97	0
10	7,600	4,636	2,356	570	38
11	11,200	6,832	1,960	1,792	616
12	12,000	7,800	3,720	420	60
13	6,900	4,105	1,760	966	69
14	10,650	7,085	2,663	849	53
15	10,400	6,448	2,440	1,460	52
16	14,400	9,216	4,464	576	144
17	11,300	6,215	3,842	1,243	0
18	10,300	6,386	3,296	618	0
19	9,800	5,194	3,381	1,225	0
20	11,950	6,214	4,541	1,195	0

Numbers 401 to 444 excluding 424 were inoculated with tumour extracts.
Numbers 72 to 125 were the contact control group, including 424.
Numbers 1 to 20 were the population control group.

TABLE 3.III Cont'd

December, 1972

Sheep No.	Leucocytes (per mm^3)	Lymphocytes (per mm^3)	Neutrophils (per mm^3)	Eosinophils (per mm^3)	Monocytes (per mm^3)
401	16,100	9,982	4,058	1,449	161
402	15,600	10,296	4,363	780	156
403	14,000	7,420	4,480	2,100	0
405	15,100	10,570	2,567	1,312	151
407	16,200	7,290	7,938	648	324
410	13,200	9,240	3,432	528	0
411	12,000	9,120	1,680	1,080	120
413	14,600	11,096	2,628	876	0
414	10,800	7,884	1,836	648	432
415	10,100	6,868	2,525	505	202
416	14,200	9,940	2,556	1,562	142
417	11,600	7,888	2,436	1,160	116
420	11,600	8,236	2,668	464	232
421	7,500	5,100	1,725	600	75
425	9,500	6,460	3,040	0	0
426	14,500	10,585	3,480	290	145
427	13,900	10,074	3,036	542	138
429	9,500	6,270	3,040	190	0
432	15,200	11,400	1,976	1,520	304
433	16,900	8,112	3,380	5,239	169
434	15,200	12,464	1,672	712	152
435	11,700	7,254	2,457	1,989	0
437	13,700	10,138	2,877	137	548
438	7,500	5,625	1,650	225	0
439	12,000	9,120	1,920	840	120
440	11,300	8,362	2,599	137	113
441	19,700	8,913	9,999	226	197
443	13,500	9,045	591	0	0
444	8,000	5,840	1,440	405	0
72	10,800	7,020	2,592	560	216
73	9,500	5,225	3,610	972	190
113	10,800	7,128	3,052	475	216
114	10,900	7,848	1,962	1,404	109
115	11,000	5,940	3,300	981	0
116	9,800	6,958	2,058	1,760	0
117	10,300	6,798	1,545	784	103
118	10,200	6,936	1,734	1,854	102
119	7,000	4,480	1,680	1,428	0
120	11,600	8,120	2,204	840	116
121	8,900	6,310	1,602	1,160	89
122	10,700	7,811	2,675	890	0
123	12,700	9,144	2,413	214	608
124	9,500	7,220	1,900	635	95
424	12,000	8,280	3,480	240	0
1	12,000	9,000	2,520	480	0
2	8,600	6,540	1,806	258	86
3	11,600	8,120	3,132	116	232
4	9,900	6,831	2,772	198	99
5	8,100	4,698	3,078	243	891

Cont'd...

TABLE 3.III

December, 1972

Sheep No.	Leucocytes (per mm^3)	Lymphocytes (per mm^3)	Neutrophils (per mm^3)	Eosinophils (per mm^3)	Monocytes (per mm^3)
6	7,600	5,244	1,520	684	152
7	9,500	6,460	2,945	0	95
8	11,500	7,705	3,105	230	460
9	7,100	5,512	1,633	213	142
10	9,000	5,220	3,240	450	90
11	10,600	6,996	3,180	318	106
12	9,700	6,596	2,910	97	97
13	11,500	8,280	2,530	460	230
14	6,400	3,456	2,816	0	128
15	10,100	5,959	3,737	0	404
16	9,600	4,896	4,608	0	96
17	7,900	5,293	2,370	158	79
18	8,000	6,160	1,760	80	0
19	9,700	7,275	2,328	97	0
20	7,700	5,621	1,925	77	77

Numbers 401 to 444 including 424 were treated with tumour extracts
Numbers 72 to 124 were the contact control group, including 424
Numbers 1 to 20 were the population control group

TABLE 3.III Cont'd

April, 1973

Sheep No.	Leucocytes (per mm^3)	Lymphocytes (per mm^3)	Neutrophils (per mm^3)	Eosinophils (per mm^3)	Monocytes (per mm^3)
401	16,700	11,690	4,175	668	167
402	14,800	11,988	2,220	148	444
403	14,300	9,165	3,948	846	141
405	9,700	4,947	4,559	97	97
407	17,200	12,556	4,300	344	0
410	14,700	8,232	6,174	294	0
411	8,900	6,764	1,869	267	0
413	16,000	11,040	4,640	160	160
414	10,200	7,956	2,142	0	102
415	10,400	7,488	2,184	624	104
416	14,700	9,114	4,263	882	441
417	9,900	7,326	2,376	198	0
420	11,100	8,214	2,553	333	0
421	16,300	9,454	6,194	652	0
425	8,800	6,688	1,936	88	88
426	14,200	9,656	3,976	142	426
427	12,600	8,190	4,032	378	0
428	16,400	10,168	5,412	656	164
429	8,700	6,873	1,653	174	0
432	17,500	12,075	4,725	700	0
433	11,600	7,772	3,596	232	0
434	15,700	7,693	7,379	471	157
437	12,700	9,525	2,667	381	127
438	10,200	6,936	3,060	102	102
439	12,800	8,960	3,584	256	0
440	9,700	6,208	2,619	679	194
441	9,800	5,782	2,646	1,176	196
443	11,100	7,659	3,108	222	111
444	4,900	3,773	1,029	49	49
71	10,200	7,242	2,856	102	0
73	12,800	5,504	7,296	0	0
113	9,900	7,029	1,980	794	297
114	10,700	6,634	3,852	214	0
115	10,700	6,848	2,782	749	321
116	7,200	4,536	2,736	72	216
117	9,400	5,640	3,196	470	94
118	10,300	7,313	1,648	824	515
119	8,100	5,184	1,944	648	324
121	8,700	6,596	2,134	776	194
122	9,100	6,188	2,366	182	364
123	8,200	6,314	1,230	410	246
124	14,000	6,580	6,860	420	140
424	9,900	7,524	1,188	1,188	0
1	10,000	6,300	3,400	0	300
2	9,600	6,528	2,304	768	0
3	7,900	5,056	2,370	316	158
4	11,900	6,307	5,355	238	0
5	10,300	5,047	4,841	206	206

Cont'd...

...Cont'd

TABLE 3.III

April, 1973

Sheep No.	Leucccytes (per mm^3)	Lymphocytes (per mm^3)	Neutrophils (per mm^3)	Eosinophils (per mm^3)	Monocytes (per mm^3)
6	8,700	5,742	2,784	0	174
7	9,200	6,164	3,036	0	0
8	8,800	4,840	2,816	264	0
9	9,500	6,460	2,090	855	95
10	6,700	4,020	2,613	67	0
11	12,300	7,749	4,059	246	246
12	8,900	5,162	3,204	445	89
13	9,700	5,432	3,686	485	97
14	11,300	6,677	3,390	1,130	113
15	7,300	4,891	1,971	292	146
16	12,300	6,396	4,920	984	0
17	8,900	5,251	3,293	267	89
18	6,100	3,904	1,891	244	61
19	12,400	4,836	6,944	496	124
20	10,000	6,900	2,400	700	0

Numbers 401 to 444 excluding 424 were inoculated with tumour extracts
Numbers 72 to 124 were the contact control group, including 424.
Numbers 1 to 20 were the population control group

TABLE 3.III Cont'd

September, 1973

Sheep No.	Leucocytes (per mm^3)	Lymphocytes (per mm^3)	Neutrophils (per mm^3)	Eosinophils (per mm^3)	Monocytes (per mm^3)
401	19,500	11,895	7,410	195	0
402	15,200	10,640	3,496	760	304
403	11,900	9,163	1,785	833	119
405	13,800	9,108	3,726	690	276
407	13,900	9,869	3,197	695	139
410	15,700	8,321	5,338	1,884	157
411	11,600	8,120	1,624	1,624	232
413	17,200	14,620	1,376	860	344
414	11,400	8,322	1,938	1,140	0
415	17,200	11,008	4,816	1,204	172
416	16,400	10,004	3,936	2,460	0
417	12,900	8,514	3,870	387	129
421	14,300	10,296	3,003	858	143
425	13,200	8,316	4,620	264	0
426	17,200	13,416	3,096	516	172
427	14,100	10,857	1,874	1,128	141
428	16,800	10,752	5,712	336	0
429	8,400	6,636	1,428	252	84
432	18,700	11,968	5,423	374	935
433	11,600	7,424	3,016	928	232
434	8,800	6,248	1,408	1,144	0
435	10,700	8,239	1,819	642	0
437	11,600	7,292	3,596	696	116
438	5,900	3,953	1,180	649	118
440	15,200	10,792	1,672	2,854	152
441	8,600	2,666	5,848	86	0
443	11,600	7,188	2,668	812	232
444	13,700	11,234	685	1,507	274
72	9,900	7,326	1,584	891	99
73	10,600	6,360	3,816	106	318
113	11,400	7,752	2,394	798	456
114	9,600	5,472	3,552	384	192
116	10,700	6,527	2,033	1,712	428
117	11,800	6,490	4,130	1,180	0
118	8,400	5,712	2,520	168	0
119	9,200	6,256	1,748	828	368
121	11,200	7,392	2,464	1,120	224
122	11,400	7,296	3,534	456	114
123	10,000	6,700	2,700	500	100
124	6,800	5,372	1,156	272	0
424	9,500	7,410	1,710	285	95
1	14,200	10,082	1,704	1,988	102
2	10,200	7,242	2,346	510	0
3	12,900	8,127	2,709	2,064	0
4	9,600	6,816	1,824	960	0
5	7,000	4,900	1,190	770	140

Cont'd...

...Cont'd

TABLE 3.III

September, 1973

Sheep No.	Leucocytes (per mm^3)	Lymphocytes (per mm^3)	Neutrophils (per mm^3)	Eosinophils (per mm^3)	Monocytes (per mm^3)
6	11,100	6,660	2,109	2,220	111
7	6,100	4,697	976	427	0
8	6,500	4,745	1,040	520	195
9	7,200	4,608	2,160	350	72
10	9,900	6,138	1,980	1,584	198
11	5,700	3,762	1,083	798	57
12	9,900	6,534	1,287	1,980	99
13	12,200	7,320	3,050	1,830	0
14	8,500	4,250	2,975	1,275	0
15	7,800	5,148	1,092	1,482	78
16	10,500	7,665	2,310	315	210
17	8,900	5,696	1,691	1,335	178
18	11,600	6,960	2,900	1,624	116
19	5,200	3,640	1,196	364	0
20	6,000	4,020	1,200	720	60

Numbers 401 to 444, excluding 424, were inoculated with tumour extracts
Numbers 72 to 124 were the contact control group, including 424.
Numbers 1 to 20 were the population control group.

TABLE 3.III Cont'd

March, 1974

Sheep No.	Leucocytes (per mm^3)	Lymphocytes (per mm^3)	Neutrophils (per mm^3)	Eosinophils (per mm^3)	Monocytes (per mm^3)
402	15,600	11,232	3,432	624	312
403	16,200	9,882	4,536	1,782	0
405	13,900	10,981	2,085	834	0
407	13,500	8,640	4,185	270	405
410	13,200	8,316	4,488	132	264
411	10,600	8,904	1,166	318	212
414	13,800	9,660	3,588	414	138
415	15,700	9,734	4,867	785	471
416	17,100	12,825	3,591	684	0
417	14,700	8,673	5,292	735	0
421	13,500	8,235	4,590	540	135
425	12,200	6,710	4,880	488	122
426	14,200	11,644	1,846	284	426
427	14,800	12,728	1,776	296	0
428	12,300	9,486	1,845	369	0
429	9,000	7,650	1,260	90	0
433	12,300	6,642	5,043	369	246
435	11,600	8,352	1,972	116	116
437	15,500	10,850	4,185	155	310
438	13,300	9,044	3,458	399	399
440	12,800	9,984	1,164	1,152	0
443	16,900	11,999	4,394	338	169
444	7,200	5,688	1,440	72	0
72	7,200	4,824	1,944	360	72
73	12,200	6,222	5,124	732	122
113	12,200	7,280	2,192	784	224
114	14,200	6,230	3,976	710	284
116	13,100	7,860	4,978	131	131
117	9,200	4,876	3,496	644	184
118	9,300	6,510	1,860	651	279
119	10,800	7,560	2,160	540	540
121	8,300	6,308	1,328	166	498
122	8,800	5,896	2,024	352	616
124	5,900	3,658	1,593	177	472
125	7,800	5,070	2,262	312	156
424	12,000	8,400	3,120	240	240
1	8,500	5,440	2,720	340	0
2	9,400	4,512	4,136	376	376
3	9,300	7,254	1,860	186	0
4	5,600	4,144	1,008	392	56
5	10,100	6,666	2,222	1,010	202
6	11,500	8,250	2,300	690	230
7	11,900	7,854	2,856	833	357
8	8,200	6,560	1,640	0	0
9	11,400	8,208	2,508	684	0
10	10,400	6,968	2,600	832	0
11	7,200	4,392	2,016	72	72
12	11,200	7,392	2,128	1,568	112
13	8,000	5,600	1,760	640	0
14	7,400	5,624	1,628	148	0

Cont'd...

... Cont'd

TABLE 3.III

March, 1974

Sheep No.	Leucocytes (per mm^3)	Lymphocytes (per mm^3)	Neutrophils (per mm^3)	Eosinophils (per mm^3)	Monocytes (per mm^3)
15	6,100	3,538	1,830	610	122
16	10,800	7,776	2,808	216	0
17	7,200	5,112	1,728	360	0
18	8,600	5,074	2,064	1,118	344
19	4,700	3,055	1,128	517	0
20	11,400	8,778	2,280	228	114

Numbers 402 to 444, excluding 424, were inoculated with tumour extracts
Numbers 72 to 125 were the contact control group, including 424.
Numbers 1 to 20 were the population control group.
Numbers 401, 413 and 432 were excluded because they were being treated with immunosuppressive drugs.

is shown on Table 3.IV. At this level of probability it is apparent that the lymphocyte counts of sheep inoculated with tumour extract OL_1 were significantly higher as a group than those of the control groups at all bleedings, that lymphocyte counts of sheep inoculated with tumour extract OL_2 were significantly higher than those of the population control group at all bleedings and those of the contact control group at all except the December 1972 bleeding. Sheep inoculated with tumour extract OL_3 were only significantly different from those of both control groups at the March 1974 bleeding. Otherwise, with the exception of the August 1972 bleeding in which the contact control group showed significantly lower levels of lymphocytes, there were no significant differences between OL_3 inoculated, contact control and population control groups. At no time were significant differences recorded between the two control groups. With the exception of the OL_1 inoculated groups in April and September of 1973, the groups treated with cell-free tumour extracts showed no significant differences between one another. The general trend indicated by these results is that the various treatment groups have higher levels of lymphocytes in the peripheral blood than the contact control or population control groups. Analysis of adjusted means of total white blood cells and lymphocytes show that the haematological effect of the treatments with tumour extracts was specifically on the lymphocytes.

Fluctuation of the total lymphocyte numbers in individual sheep over the period of the experiment is shown in Figure 3.2. The upper limit of the normal range of lymphocyte numbers in sheep blood has been assessed as approximately 8,500 per mm^3 (Coffin, 1953; Schalm, 1965). Table 3.V shows the animals in which this figure was exceeded during the course of the experiment. Tables 3.VI and 3.VII show those animals whose lymphocyte numbers were in excess of 2.5 and 3.0 standard deviations (SD), respectively, of the calculated lymphocyte means of

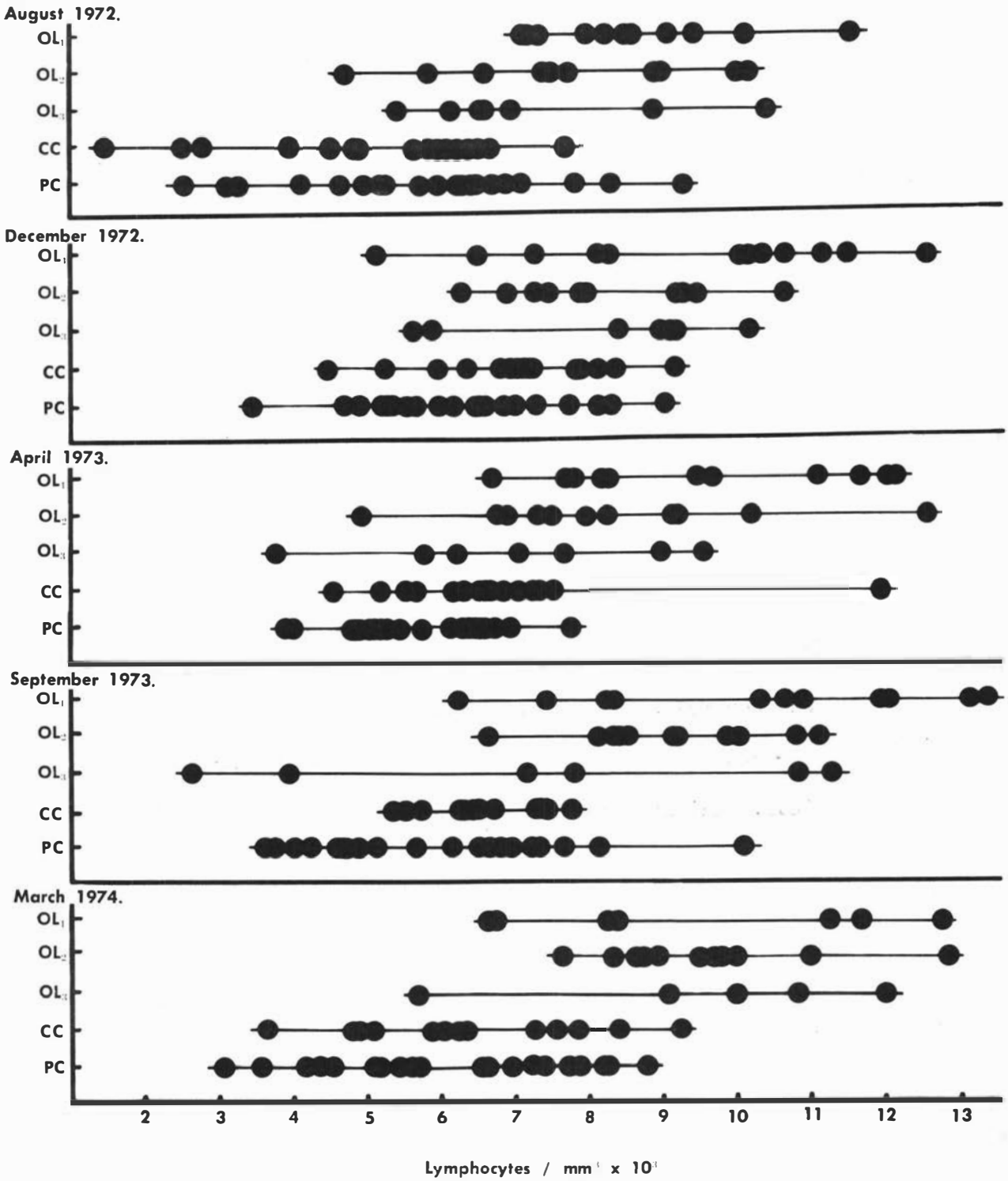
August 1972.
December 1972.
April 1973.
September 1973.
March 1974.
OL1
OL2
OL3
CC
PC
2
3
4
5
6
7
8
9
10
11
12
13
Lymphocytes / mm³ x 10³

TABLE 3.IV

Statistical Analysis Showing Effect of Different Tumour Extracts on the Total Circulating Lymphocytes of Sheep Inoculated in 1971

Time tested	Groups				
August 1972	$\bar{x}_{OL_1}$	$\bar{x}_{OL_2}$	$\bar{x}_{OL_3}$	$\bar{x}_{PC}$	$\bar{x}_{CC}$
December 1972	$\bar{x}_{OL_1}$	$\bar{x}_{OL_2}$	$\bar{x}_{OL_3}$	$\bar{x}_{CC}$	$\bar{x}_{PC}$
April 1973	$\bar{x}_{OL_1}$	$\bar{x}_{OL_2}$	$\bar{x}_{OL_3}$	$\bar{x}_{CC}$	$\bar{x}_{PC}$
September 1973	$\bar{x}_{OL_1}$	$\bar{x}_{OL_2}$	$\bar{x}_{OL_3}$	$\bar{x}_{CC}$	$\bar{x}_{PC}$
March 1974	$\bar{x}_{OL_1}$	$\bar{x}_{OL_2}$	$\bar{x}_{OL_3}$	$\bar{x}_{CC}$	$\bar{x}_{PC}$

The groups which are underscored by the same line show no significant differences at 95 per cent. confidence levels as estimated by the F test of Kramer (1956).

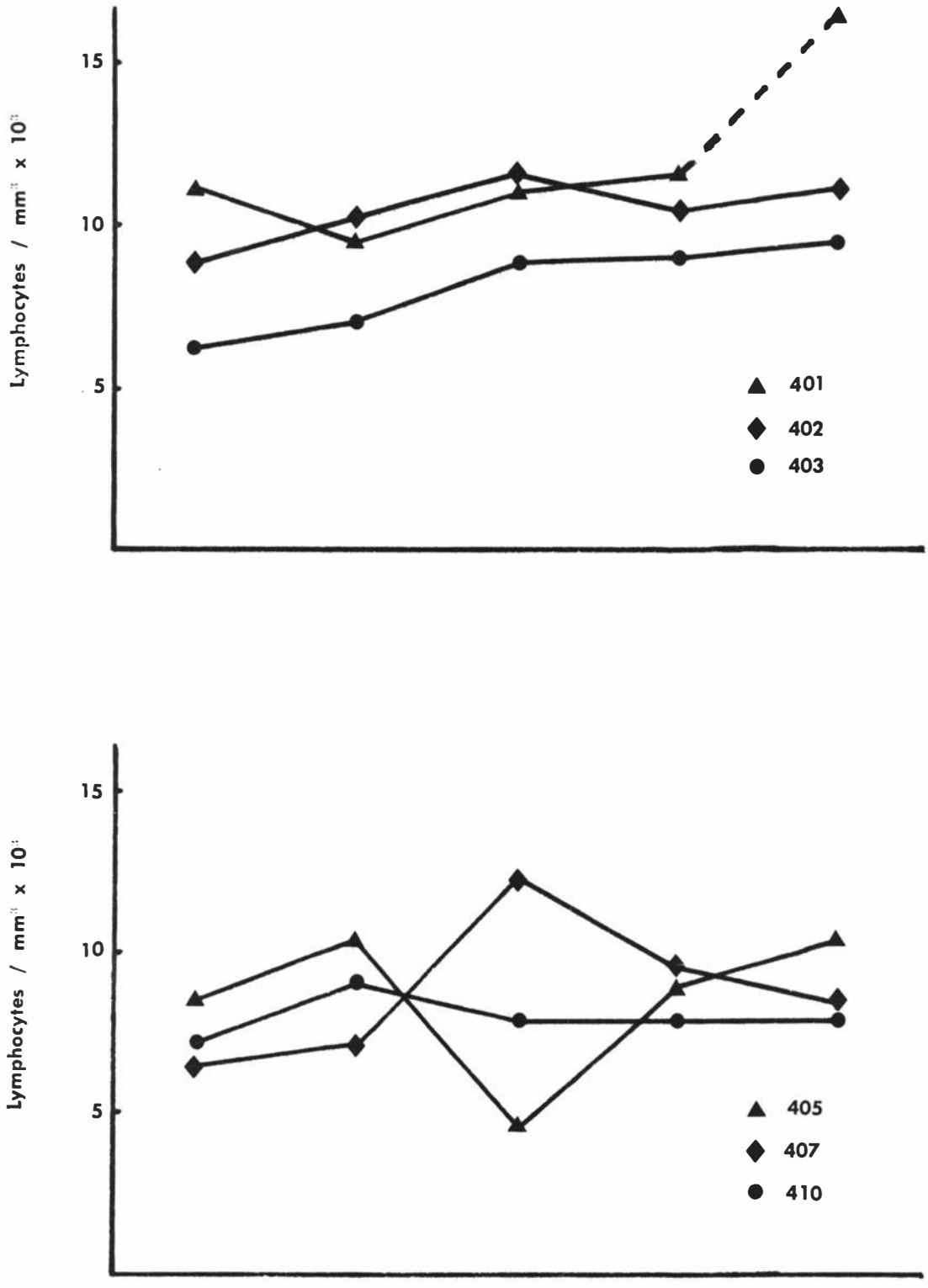

Figure 3.2

Total lymphocyte counts of experimentally inoculated and control sheep at intervals between August 1972 and March 1974.

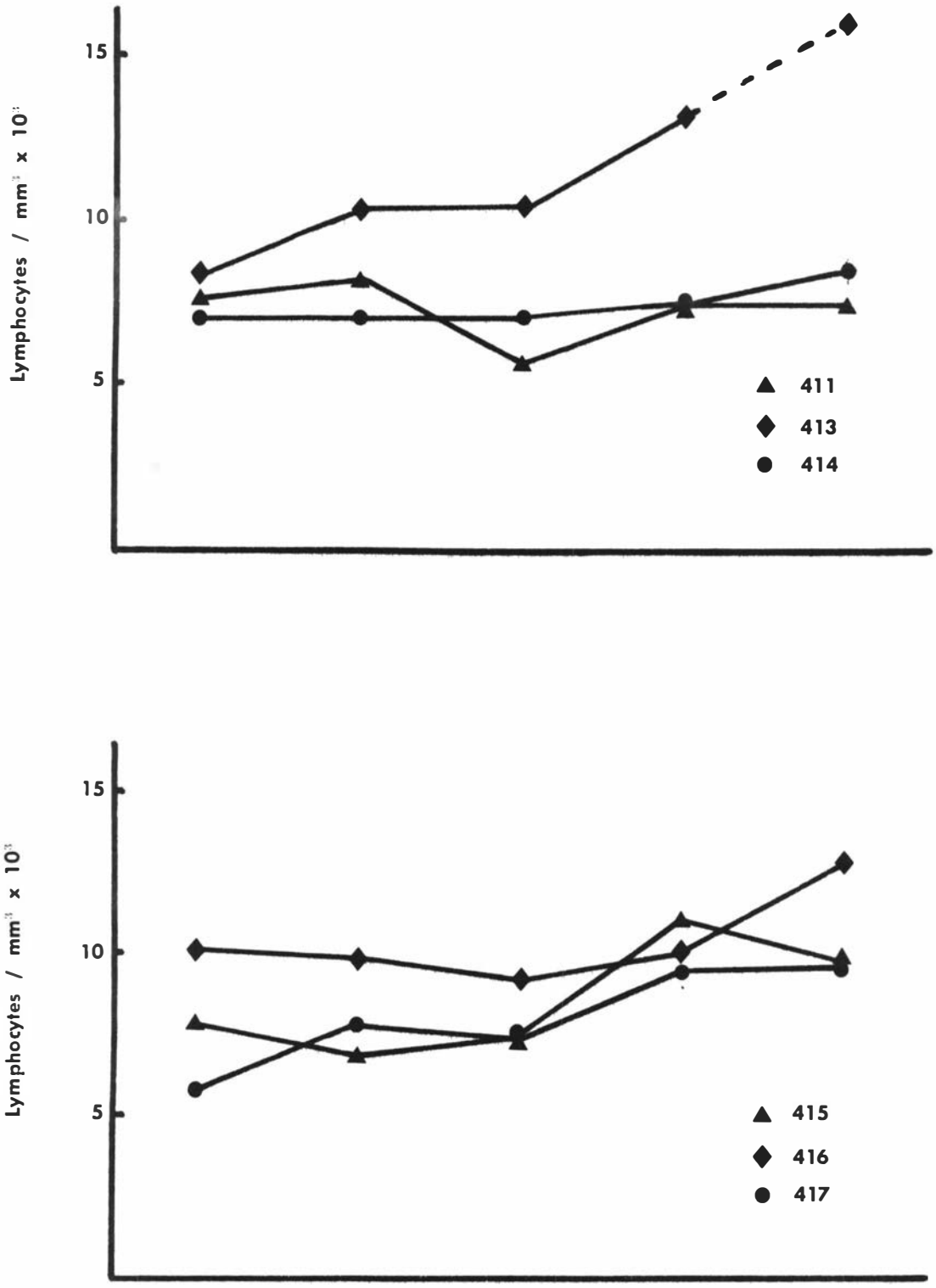

Figure 3.2 (continued)

Total lymphocyte counts of experimentally inoculated and control sheep at intervals between August 1972 and March 1974.

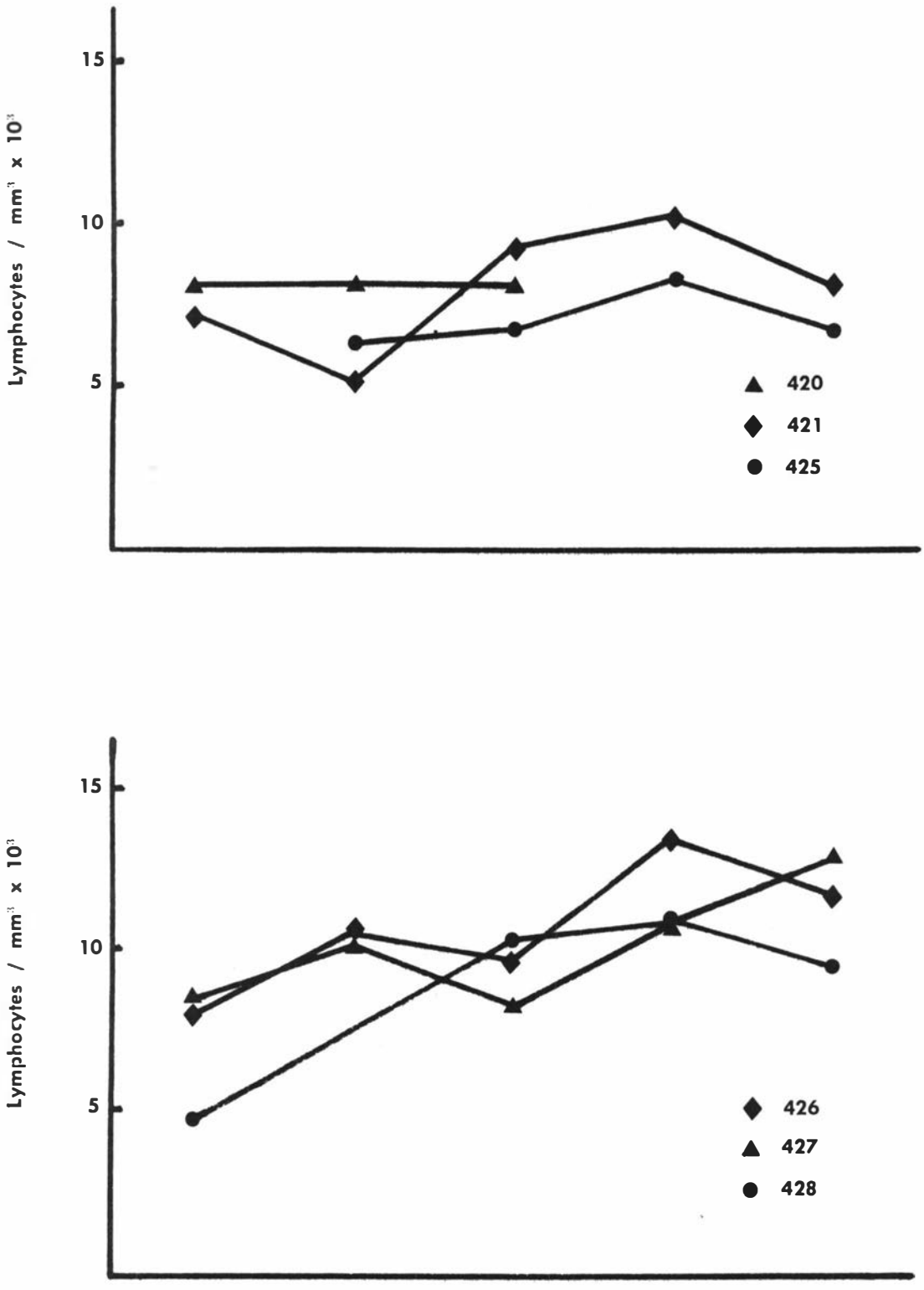

Figure 3.2 (continued)

Total lymphocyte counts of experimentally inoculated and control sheep at intervals between August 1972 and March 1974.

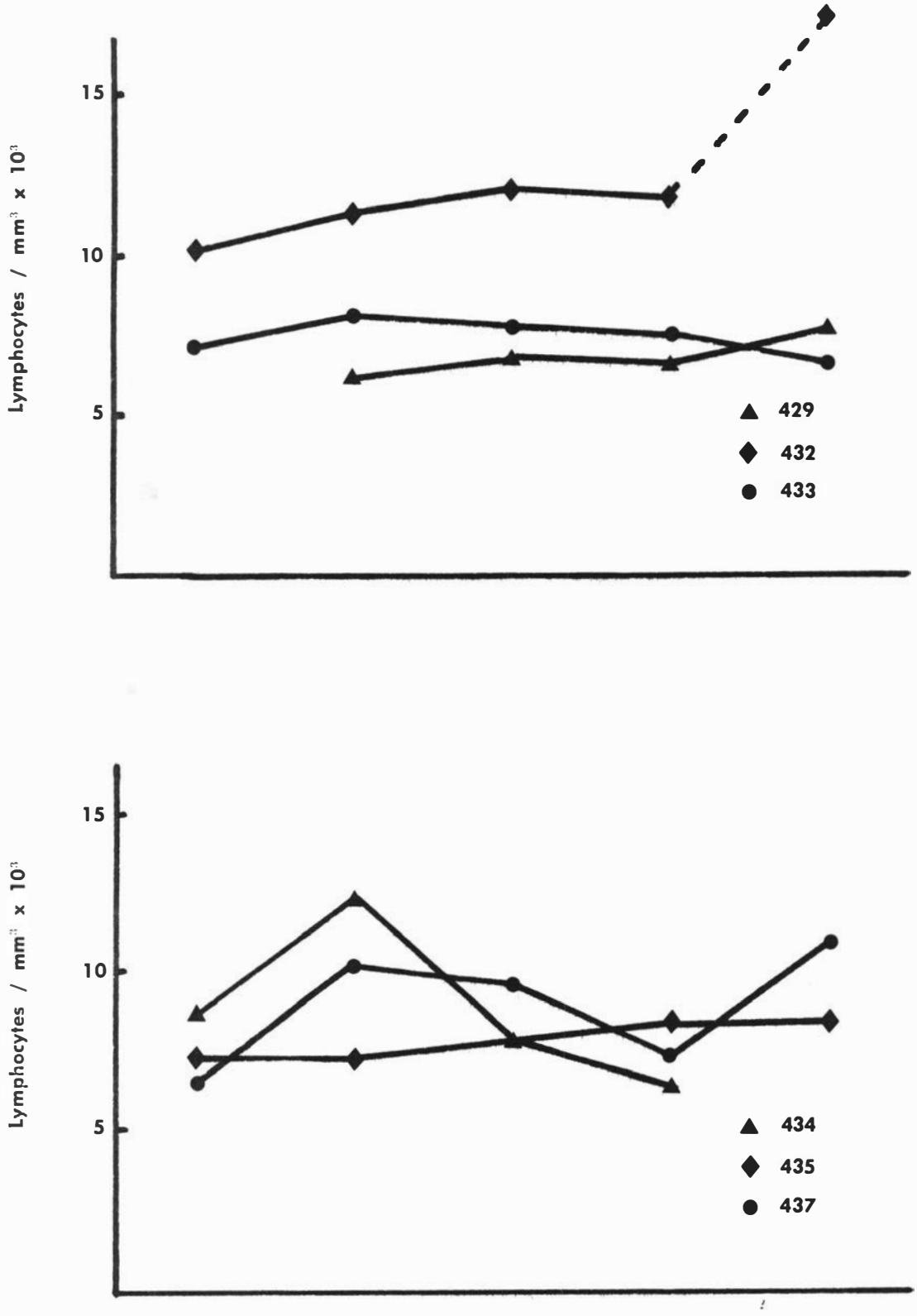

Figure 3.2 (continued)

Total lymphocyte counts of experimentally inoculated and control sheep at intervals between August 1972 and March 1974.

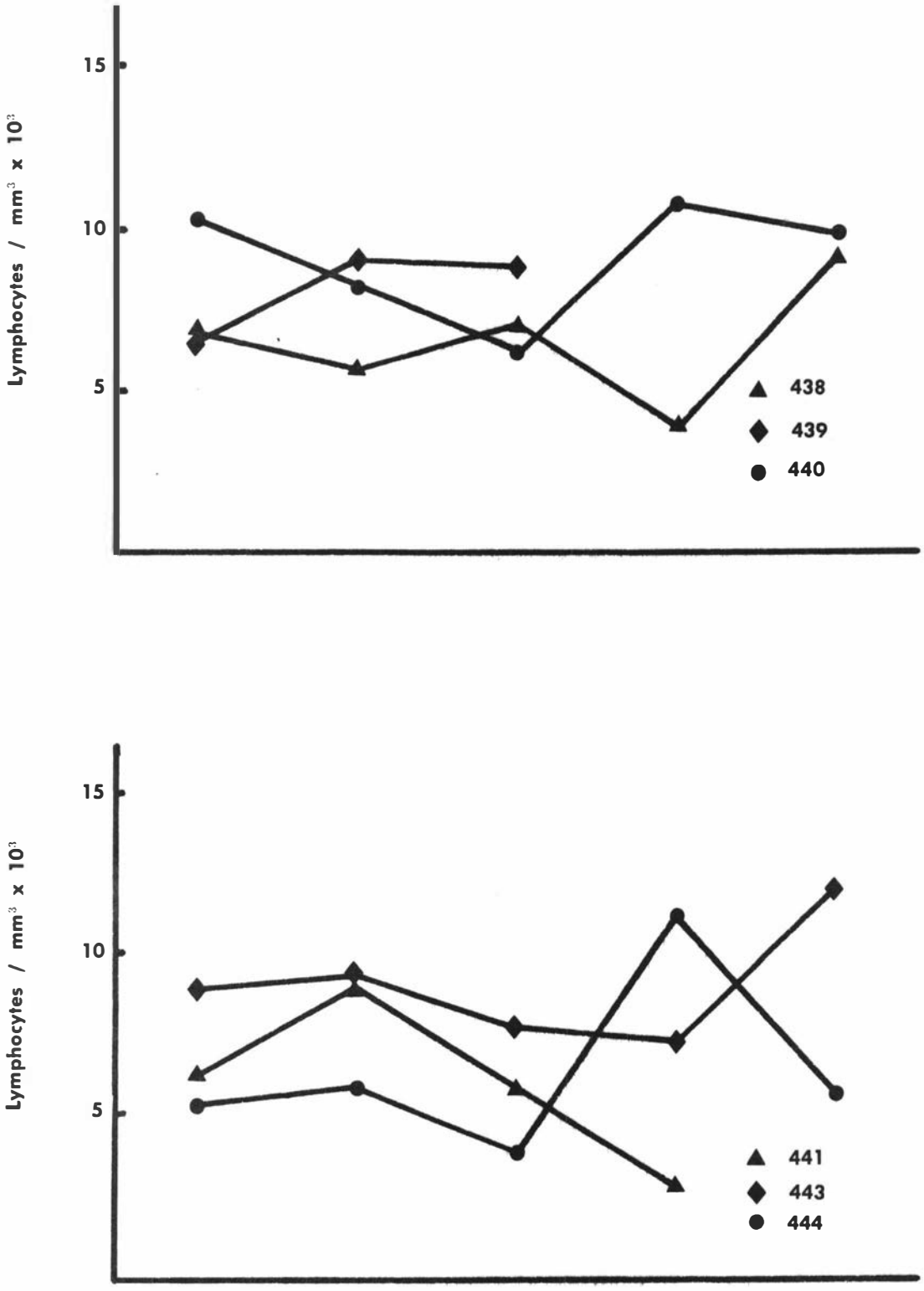

Figure 3.2 (continued)

Total lymphocyte counts of experimentally inoculated and control sheep at intervals between August 1972 and March 1974.

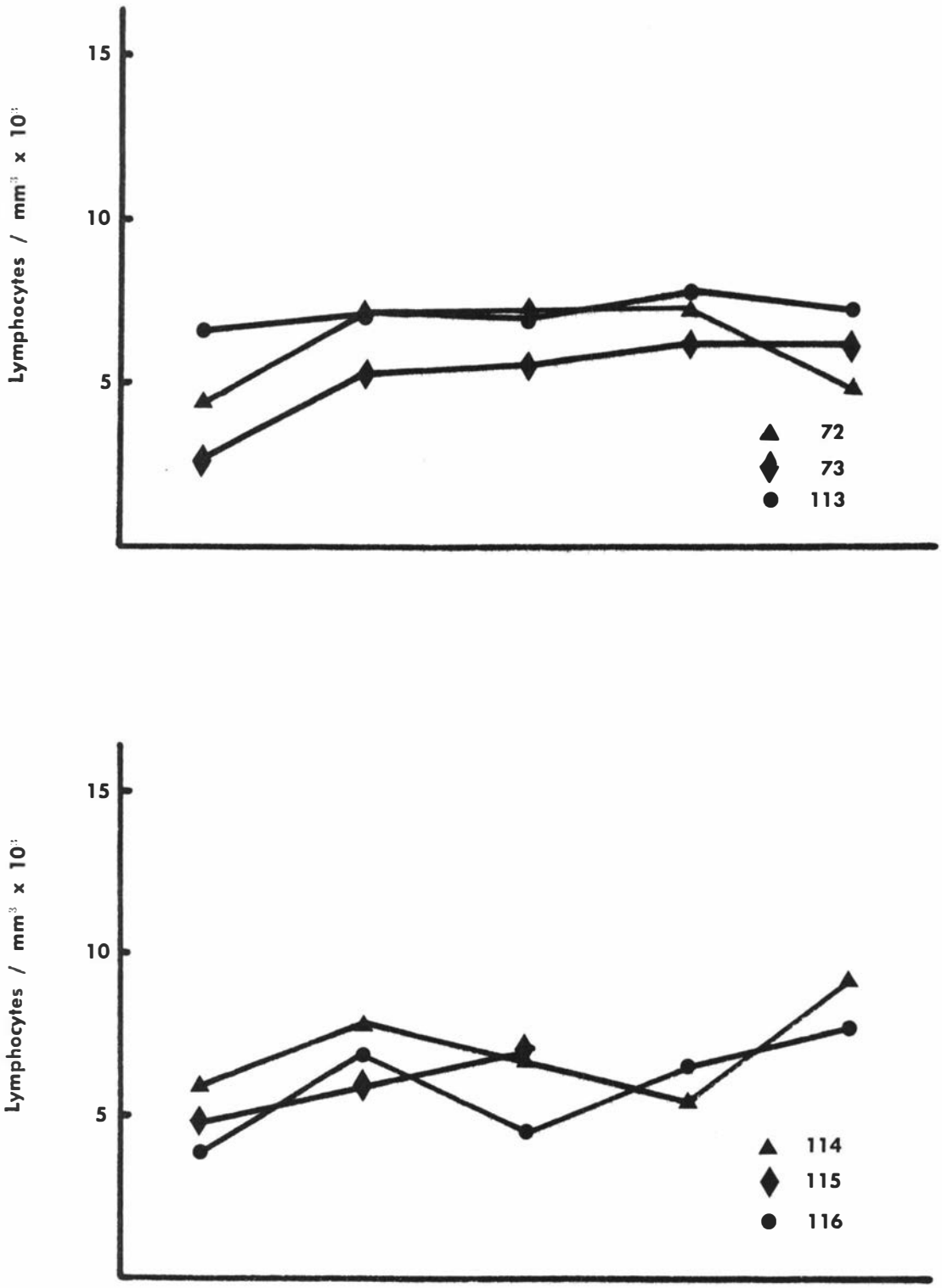

Figure 3.2 (continued)

Total lymphocyte counts of experimentally inoculated and control sheep at intervals between August 1972 and March 1974.

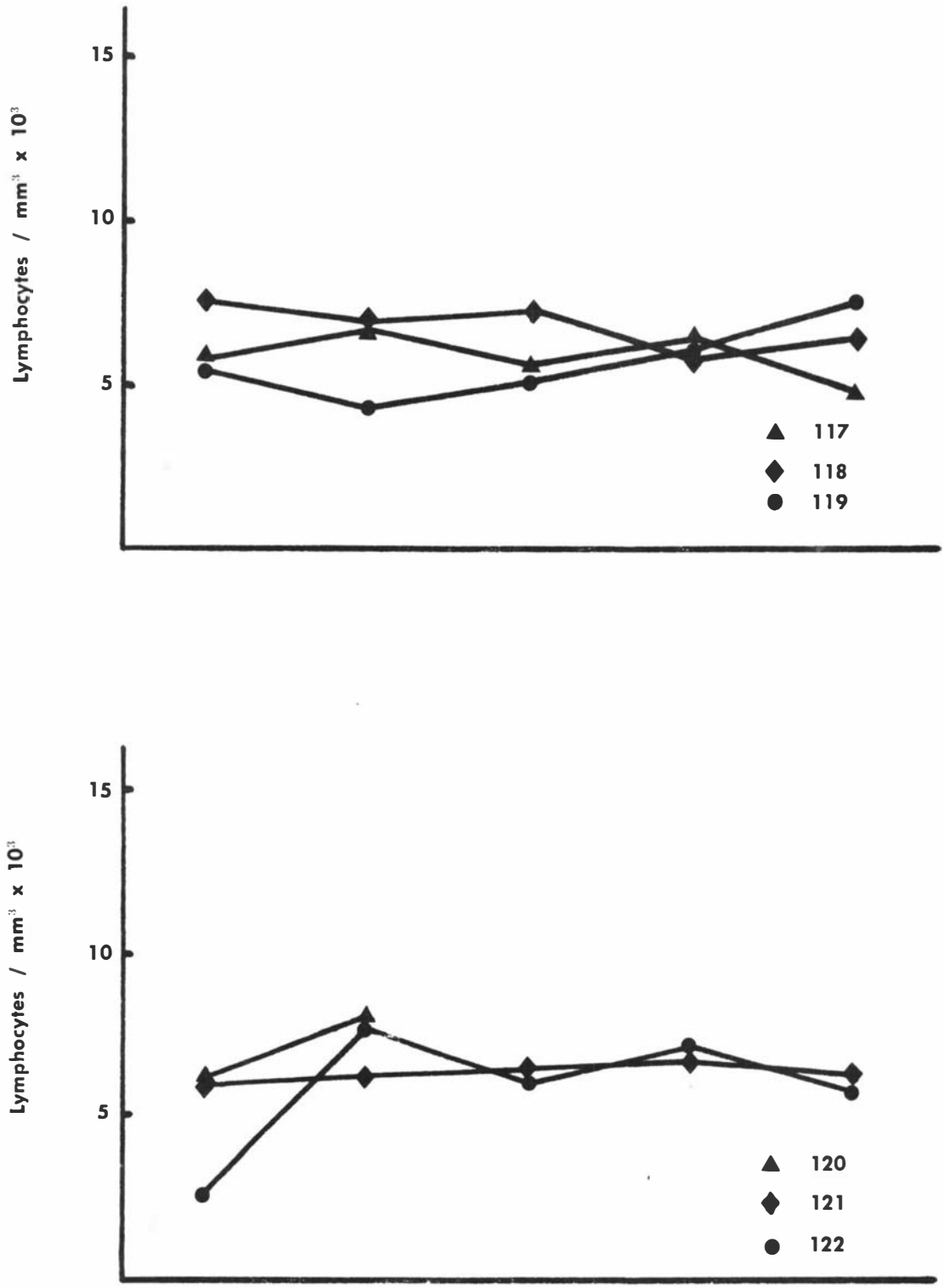

Figure 3.2 (continued)

Total lymphocyte counts of experimentally inoculated and control sheep at intervals between August 1972 and March 1974.

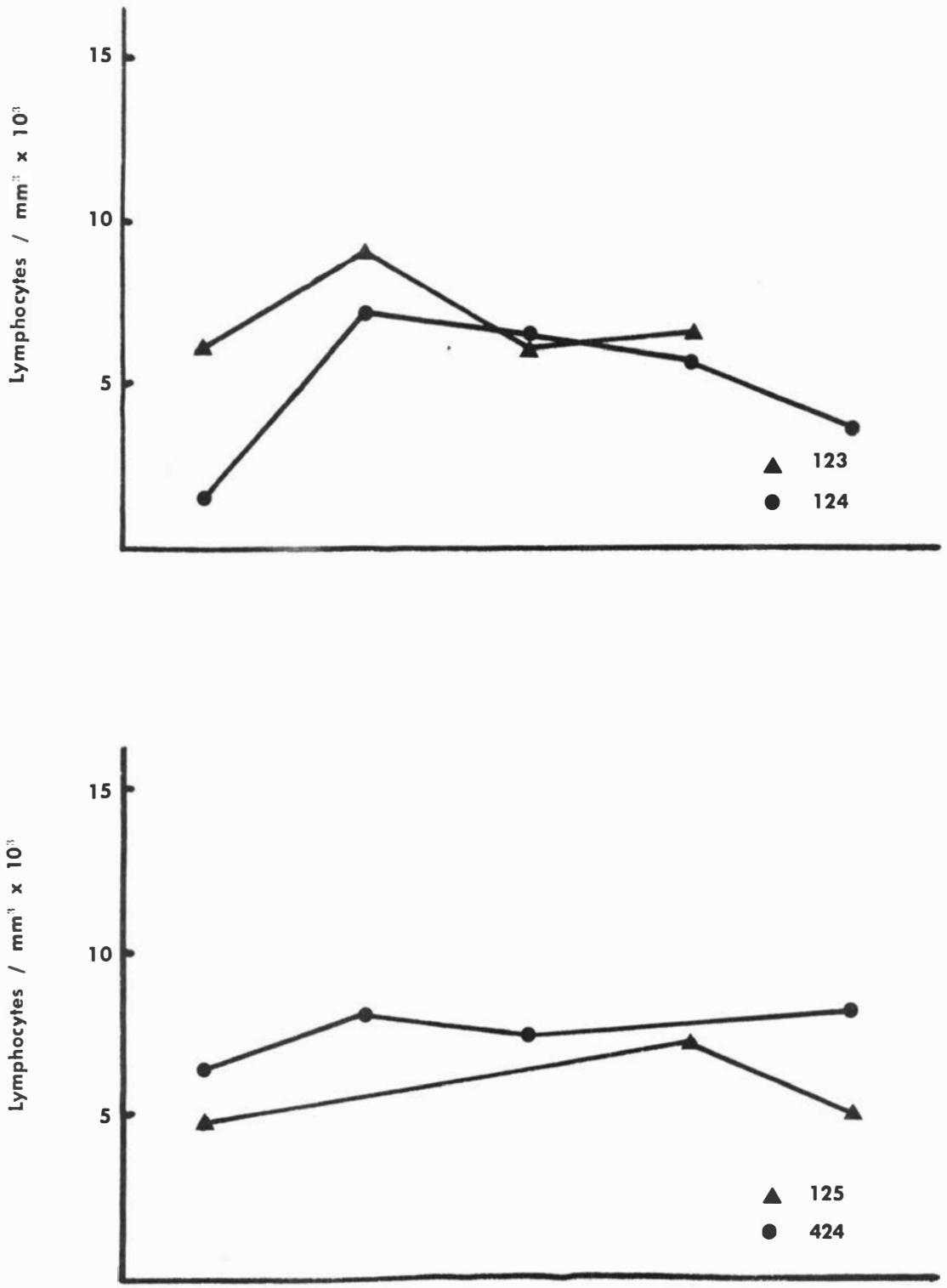

Figure 3.2 (continued)

Total lymphocyte counts of experimentally inoculated and control sheep at intervals between August 1972 and March 1974.

TABLE 3.V

Sheep Whose Total Lymphocyte Counts were in Excess of the Upper limit of Normal (8,500 per mm^3)

August 1972	December 1972	April 1973	September 1973	March 1974
401	401	401	401	NT
402	402	402	402	402
-	-	403	403	403
405	405	-	405	405
-	-	407	407	407
-	410	-	-	-
411	411	-	-	411
413	413	413	413	NT
-	-	-	-	414
-	-	-	415	415
416	416	416	416	416
-	-	-	417	417
-	-	421	421	-
-	426	426	426	426
-	427	-	427	427
-	-	428	428	428
432	432	432	432	NT
434	434	-	-	NT
-	437	437	-	437
-	-	-	-	438
-	439	439	NT	NT
440	-	-	440	440
-	441	-	-	-
443	443	-	-	443
-	-	-	444	-
-	-	-	-	114
-	123	-	-	-
Total 10	16	12	16	17

NT = Not treated. Animals 401, 413 and 432 were being treated with immunosuppressive agents after November 1973, and animals 434 and 439 died of intercurrent disease.

TABLE 3.VI

Sheep Whose Total Lymphocyte Counts were in Excess of 2.5 Standard Deviations (SD) of the Population Control (PC) Mean

	August 1972	December 1972	April 1973	September 1973	March 1974
PC mean	5,793	6,288	5,683	5,950	6,110
SD of mean x 2.5	2,745	2,780	2,500	2,873	2,815
PC mean 2.5 SD	8,538	9,068	8,183	8,923	8,925
Animals in	401	401	401	401	NT
excess of 2.5 SD	402	402	402	402	402
of PC mean	-	-	403	403	403
	405	405	-	405	405
	-	-	407	407	-
	-	410	410	-	-
	411	411	-	-	-
	413	413	413	413	NT
	-	-	-	-	414
	-	-	-	415	415
	416	416	416	416	416
	-	-	420	NT	NT
	-	-	421	421	-
	-	426	426	426	426
	-	427	427	427	427
	-	-	428	428	428
	432	432	432	432	NT
	-	434	-	-	NT
	-	437	437	-	437
	-	-	-	-	438
	-	439	439	NT	NT
	440	-	-	440	440
	443	-	-	-	443
	-	-	-	444	-
	-	123	-	-	-
	-	-	-	-	114
TOTAL	9	14	15	15	14

NT = Not tested. Animals 401, 413 and 432 were being treated with immunosuppressive agents after November 1973 and animals 420, 434 and 439 died of intercurrent disease.

TABLE 3.VII

Sheep Whose Total Lymphocyte Counts were in Excess of 3.0 Standard Deviation (SD) of the Population Control (PC) Mean

	August 1972	December 1972	April 1973	September 1973	March 1974
PC mean	5,793	6,288	5,683	5,950	6,110
SD of mean x 3.0	3,294	3,336	3,003	3,567	3,378
PC mean 3.0 SD	9,087	9,624	8,686	9,517	9,488
Animals in excess of 3.0 S.D of PC mean	401	401	401	401	NT
	402	402	402	402	402
	-	-	403	-	403
	-	405	-	-	405
	-	-	407	407	-
	-	413	413	413	NT
	-	-	-	-	414
	-	-	-	415	415
	416	416	416	416	416
	-	-	421	421	-
	-	426	426	426	426
	-	427	-	427	427
	-	-	428	428	-
	432	432	432	432	NT
	-	434	-	-	NT
	-	437	437	-	437
	-	-	439	NT	NT
	440	-	-	440	440
	-	-	-	-	443
	-	-	-	444	-
TOTAL	5	10	12	13	11

NT = Not tested. Animals 401, 413 and 432 were being treated with immunosuppressive agents after November 1973 and animals 434 and 439 died of intercurrent disease.

the population control group at these times.

From these data it can be seen that several sheep maintained high lymphocyte numbers which were well in excess of the normal range. In particular, sheep numbers 402, 416, 401 and 432,until the last two were removed from the experiment, were consistently 3.0 SD above the population control mean. In addition, several sheep were frequently in excess of 2.5 and 3.0 SD but appeared to show some fluctuation in levels of lymphocytes, i.e. 403, 405, 413, 426, 427, 428, 437 and 440.

Several animals have had relatively low lymphocyte counts throughout the experiment i.e. 420, 425, 429, 433, 435 , while others although generally low have on occasions shown high counts i.e. 407, 434, 438, 439, 441, 444, 114, 123 or a tendency toward a gradual increase over the experimental period, i.e. 414, 415, 417, 421, 427 and 435 . These trends are shown in Figure 3.2. The high lymphocyte count in sheep 441 at the December 1971 sampling was associated with a marked neutrophilia and is therefore most likely to represent a response to intercurrent bacterial infection. Animals in the contact control groups exceeded the normal limit of 8,500 lymphocytes per mm^3 on two occasions only. In both cases they were within 3.0 SD of the mean of the population control group.

Animals 401, 413 and 432 were subjected to a course of experimental immunosuppression after November 1973 and were not included in the March 1974 bleeding. Prior to this treatment the lymphocyte counts of these particular animals were 16,428 per mm^3, 16,170 per mm^3 and 17,402 per mm^3 respectively. Figure 3.3 illustrates the fluctuations in lymphocyte counts recorded in the 10 sheep which were sampled at 10 to 12 day intervals over a period of four months. All showed unpredictable fluctuations which in some instances were as much as 3,500 lymphocytes per mm^3 different from the previous recording. However,

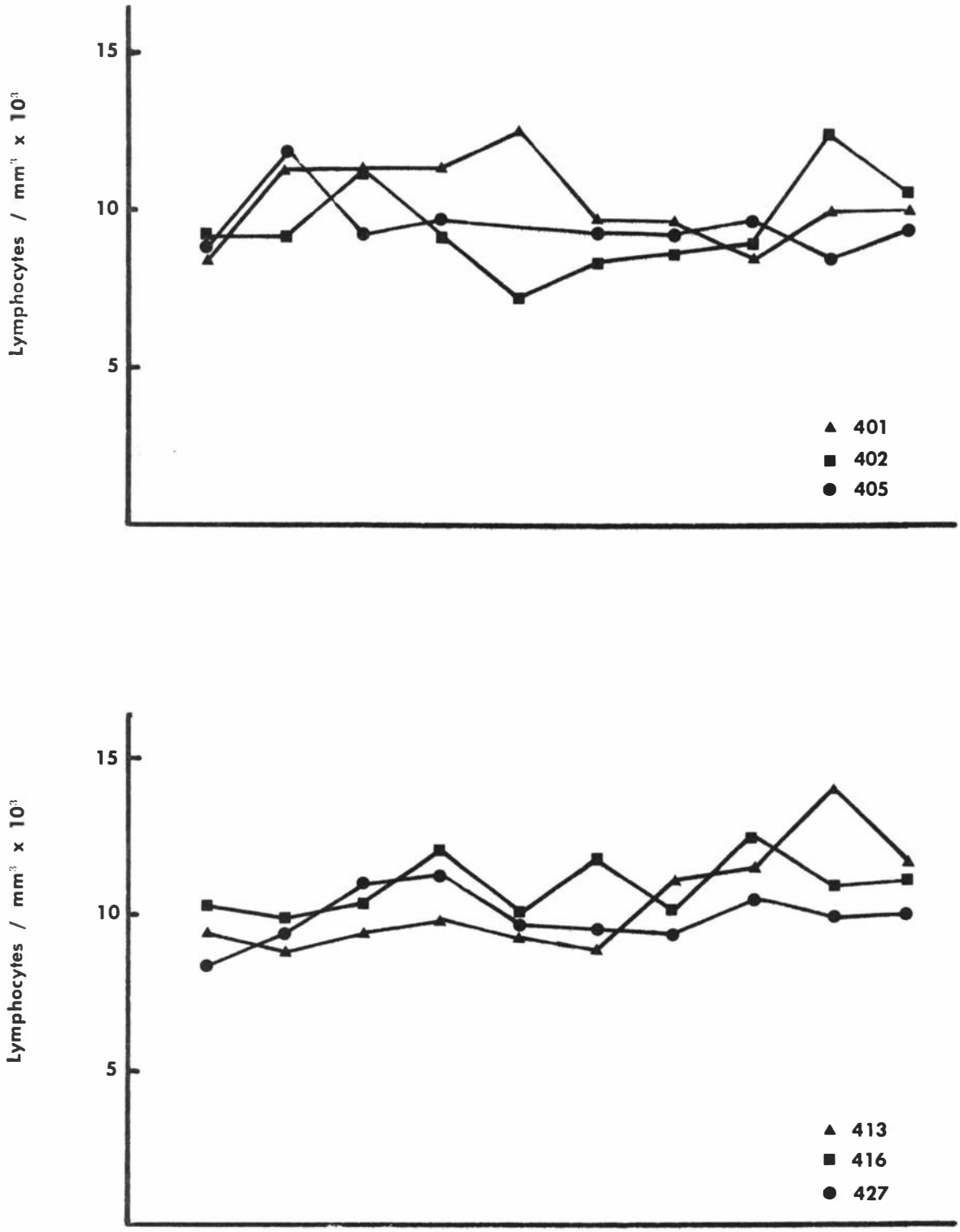

Figure 3.3

The fluctuation in lymphocyte numbers of sheep recorded at 10 to 12 day intervals over a period of four months.

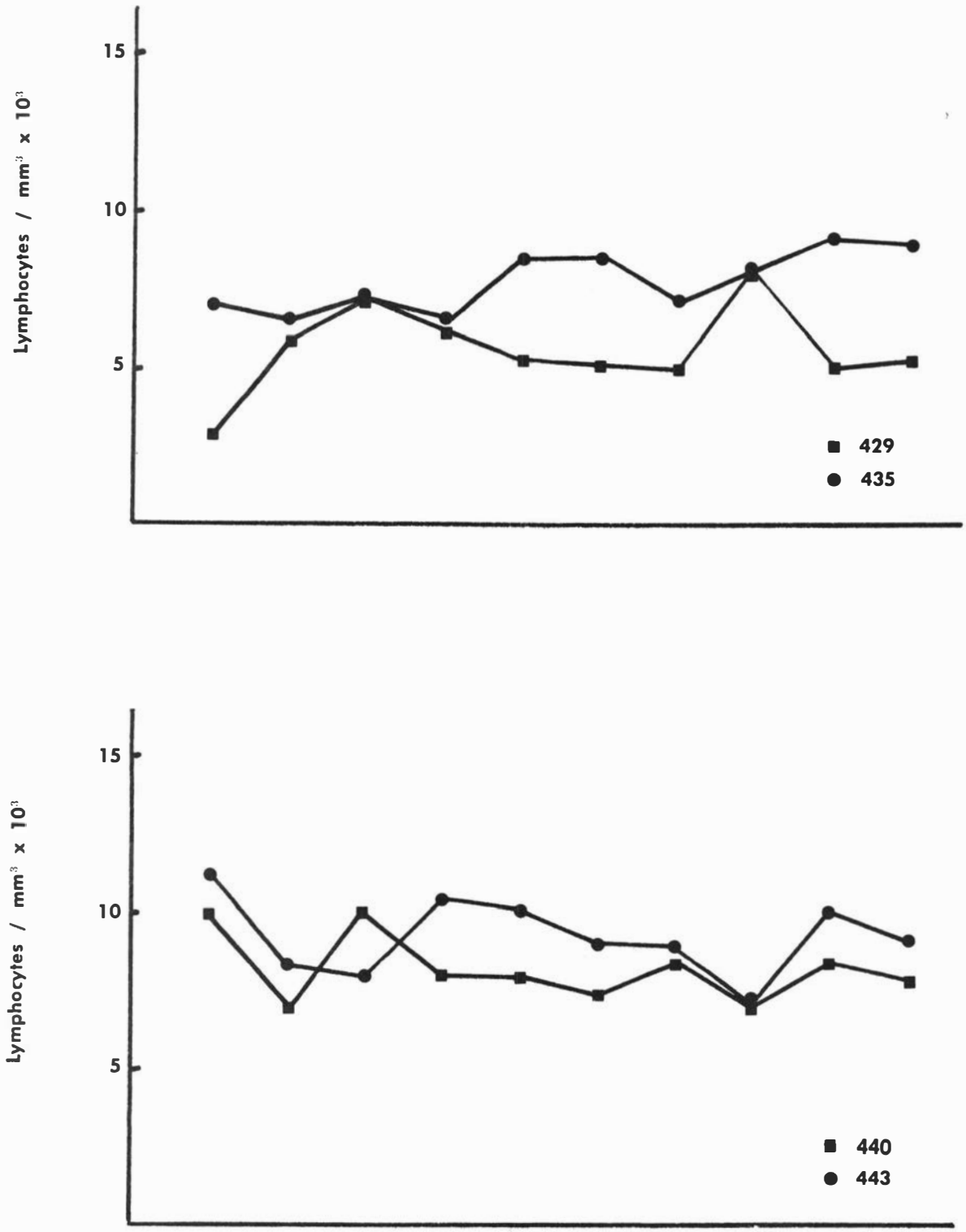

Figure 3.3 (continued)

The fluctuation in lymphocyte numbers of sheep recorded at 10 to 12 day intervals over a period of four months.

those whose lymphocyte numbers were above normal tended to remain elevated.

The development of lymphocytosis did not appear to be dependent on the time of inoculation. Abnormally high lymphocyte counts were recorded in sheep inoculated during gestation as well as neonatally. There was no apparent relationship between the sex of the animal and development of lymphocytosis (Table 3.VIII).

Morphology of Lymphocytes

The morphology of the lymphocytes as seen in stained blood smears was normal in all cases.

DISCUSSION

In the experiments reported above the most striking feature is the development of a lymphocytosis in approximately half the animals receiving inocula of cell-free material prepared from naturally occurring malignant lymphomas of sheep. Although there are no reports in the literature of other attempts to transmit the disease from sheep to sheep, observations made on sheep in Germany suggest that lymphocytosis occurs as a preclinical form of the spontaneous disease. For example Enke, Jungwitz and Rössger (1961) noted the phenomenon when they examined leucocyte numbers from a flock of sheep in which about 40 had been reported to die with malignant lymphoma.

More recently Paulsen et al. (1971) described the detailed haematological examination of 12 sheep from a flock of 384 of the German improved Landschaf breed. Nine of these had lymphocyte counts "suspicious" for malignant lymphoma and subsequently six of these nine sheep died with overt lymphoid neoplasms. The observed duration of lymphocytosis varied from 2 to 22 months and maximum numbers of

TABLE 3.VIII

The Sex of Sheep Whose Total Lymphocyte Counts were in Excess of 3.0 Standard Deviations of the Population at Some Time During the Course of the Experiment

Sheep No.	Sex
401	M
402	F
403	M
405	F
407	M
413	F
414	F
415	M
416	M
421	F
426	F
427	F
428	F
432	F
434	M
437	M
439	M
440	F
443	F
444	F
Total 20	M : F = 8 : 12
Total number of inoculated animals 29	M : F = 12 : 17

M = Male

F = Female

lymphocytes recorded in the animals dying with malignant lymphoma varied from 28,660 to 379,500 per mm^3. The exact ages of these animals was not known, but they were thought to be between four and a half and five and a half years old at the time of death.

A proportion of cattle dying with spontaneous malignant lymphoma also show lymphocytosis prior to death. The significiance of this change in relation to preclinical malignant lymphoma in this species has been the subject of considerable debate in the literature. Bendixen (1965) believes that this change occurs for a variable period of time in a high percentage of animals which subsequently develop overt malignant lymphoma. He found that this occurred in up to 95 per cent. of Danish cattle which subsequently died from the enzootic form of this disease. Other European workers are generally in agreement with his statement, but some American authors consider that lymphocytosis may not be such a reliable indicator of the disease in their country (Theilen et al., 1964; Marshak and Abt, 1968). However, Abt et al. (1970) report the occurrence of lymphocytosis in 65 per cent. and Olson et al. (1970) in 51 per cent. of cases in some herds in the U.S.A. Despite these differences there can be no doubt that in many cases of bovine malignant lymphoma a preclinical phase of lymphocytosis occurs and the same is probably true for the disease in sheep.

Lymphocytosis is defined as an increase in peripheral lymphocyte numbers above normal limits. In sheep the upper limit of the normal range is stated by Schalm (1965) and other authors to be approximately 8,500 per mm^3 of blood. Absolute estimates such as these do not account for fluctuations resulting from environmental or genetic variations which occur in different populations of sheep. To define the lymphocytosis observed in cases of preclinical malignant lymphoma of cattle, the levels of abnormality are best described in respect to

normal cattle in the appropriate age group and geographic area. On this basis levels for classification of lymphocytosis have been suggested to be in excess of 2.5 SD (Abt et al., 1970) or 3.0 SD (Marshak et al., 1968) of the normal population mean. In the present experiments in sheep all three of the above criteria were applied to the results and lymphocytosis was confirmed in individual animals as shown in Tables 3.V, 3.VI and 3.VII. It should be noted that as a group the animals that received tumour inoculum OL_3 did not show such a spectacular lymphocytosis. A possible explanation that should be considered is that the source tumour from which inoculum OL_3 was derived was a tumour which could be classified as being of the thymic type. In cattle, the thymic tumours are thought to represent a distinct epidemiological entity and might therefore be of different aetiology (Bendixen 1965; Dungworth et al., 1964; Hugoson, 1967).

Because the inoculations did not elicit a lymphocytosis in all animals in each of the treatment groups, the observation of significant differences between treated and control groups strongly reinforces the individual observations. The multiple range test used to evaluate statistical differences between the various groups is a conservative one. The calculation of specific significance and prediction levels would be extremely difficult in this situation where the number of replications within groups are variable.

Lymphocytosis has been reported in a number of non-neoplastic conditions affecting animals; for example, chronic debilitating conditions, adrenocortical insufficiency and during convalescence from some acute infections. In the experiments reported here there was no evidence that any of these factors were responsible for the observed changes. Furthermore, the persistence of lymphocytosis in individual animals and the magnitude of group differences between

sheep receiving tumour material and control sheep kept in an identical environment are such that they can only be satisfactorily explained as a response to the experimental procedure.

One cannot fail to note the similarities existing between the lymphocytosis which has developed in the experiments reported here and that occurring as a preclinical stage of spontaneously developing enzootic malignant lymphoma of cattle. It is reasonable to suppose that these sheep are showing a pretumourous phase of the disease. If this assumption is eventually proved correct by the appearance of overt tumours in these animals, then the value of lymphocytosis as an indicator of the disease may be of considerable value in early diagnosis, control and eradication programmes. Several European countries have instigated schemes aimed at the control and eventual eradication of bovine malignant lymphoma which rely on the detection of inspected animals by this method. Tested cattle are classified by lymphocyte numbers into leukosis "positive", "suspicious" and "negative" groups and the eventual fate of "positive" and "suspicious" animals then depends on the previous history of malignant lymphoma in the particular herd in question. Examples of "leukosis keys" devised for this purpose are those of Gotze et al. (1954, cited by Bendixen, 1965), Bendixen (1957) and Tolle (1964, cited by Bendixen, 1965). Although malignant lymphoma of sheep in New Zealand does not appear to be a problem of economic importance at present, it is probably important that we should be aware of possible means of detecting preclinical forms of the disease.

Analogy to malignant lymphoma of other species and the use of cell-free material derived from tumour material as the inocula indicate that the agent responsible for the persistent lymphocytosis is most likely a virus. The lack of clinical evidence of overt tumour develop-

the present time these sheep are only three years old and therefore have not reached the usual age at which spontaneous malignant lymphoma is most commonly seen.

CONCLUSIONS

A persistent lymphocytosis can be induced in sheep following inoculation *in utero* or at birth with cell-free material derived from ovine malignant lymphoma tissue. It is concluded that this is most likely caused by a virus and that it probably reflects a pretumourous phase of malignant lymphoma. It is postulated that more than three years is required for the development of overt neoplasia.

CHAPTER 4

ATTEMPTS TO DEMONSTRATE VIRUS IN TISSUE CULTURES

INTRODUCTION

The experimental evidence required to establish the aetiological relationship of a given microorganism to a given disease is exemplified by Koch's postulates. In many examples of virally induced neoplasms it has proved, for various reasons, impossible to fulfil these conditions. For instance, the behaviour of transforming DNA viruses is such that following tumour induction the infected cell does not replicate further virus. The presence of the virus in such cells can only be confirmed indirectly by immunological techniques, or by so called "rescue" techniques involving cell fusion (Watkins, 1971). The detection and characterization of the various oncogenic viruses has been greatly assisted by the development of techniques for their *in vitro* growth and cultivation in tissue culture systems. Such systems have permitted in some cases the production of virus divorced from the potentially suppressive effects of the host.

Under appropriate conditions of culture oncogenic viruses may show a variety of host-cell interactions. They may cause the following phenomena:-

(a) Cytopathy. For instance, the vacuolating agent, designated simian virus 40 (SV_{40}), of the rhesus monkey causes extensive vacuolation of the cells of the African green monkey kidney in culture (Goodheart, 1969).

(b) Loss of normal contact inhibition. This results in multilayered foci of "transformed" cells. Among the tumour viruses causing this effect are those of the Rous sarcoma of chickens (Manaker

and Groupé, 1956) erythroblastosis of chickens (Beaudreau et al., 1963), murine sarcoma (Hartley and Rowe, 1966), feline sarcoma (Kirsten, 1970) and parotid tumours of mice (Dulbecco, 1969). In addition, SV_{40} (Dulbecco) and various adenoviruses (Goodheart) will induce cell transformation under suitable conditions of culture.

(c) Replication in infected cells. Most of the RNA tumour viruses have been shown to do this (Gross, 1970), although with the exception of the sarcoma viruses of various animal species and the avian erythroblastosis virus, they do so without causing any visible cytopathic effects or cellular transformation. Epstein et al. (1965) demonstrated the replication of a herpesvirus from cultured cells of the Burkitt's tumour of African children.

This chapter reports experiments in which attempts were made to demonstrate a virus in malignant lymphoma of sheep by direct inoculation of cell-free extracts of tumour into tissue cultures.

MATERIALS AND METHODS

Cultures

I. Source of cells

Cultures were derived from foetal lamb kidneys obtained from pregnant ewes killed at slaughter-houses. These specimens were available during August, September, October and November, and the experiments were performed during these months in 1971 and 1972.

II. Preparation of primary cultures

The dispersal of kidney cells was achieved using essentially the same procedure described by de Oca (1973). Modifications of this method included:-

(a) the use of Hank's balanced salt solution (HBSS) (Appendix III) in place of Dulbecco's balanced salt solution.

(b) the use of 0.25 per cent. trypsin solution (Appendix III) rather than a mixture of 0.25 per cent. trypsin and 0.02 per cent. EDTA.

(c) the homogenization of tissue using a Sorvall Omnimixer rather than scissors to produce fragments of approximately one cubic millimetre prior to trypsinization.

After dispersal viable cells were counted using the trypan blue dye exclusion method (Ling, 1968) and concentration was adjusted to 5×10^5 viable cells per millilitre of growth medium (Appendix III) and cells dispensed in 100 ml aliquots into acid-washed 500 ml glass tissue culture flasks. Following "gassing" of the cultures with a four per cent. CO_2 and air mixture the culture flasks were securely stoppered and transferred to an incubator where they were maintained at $37^{\circ}C$ throughout growth.

Growth medium was replenished with fresh solution when the indicator in the medium registered an alteration in pH. Cultures were maintained until a dense and continuous monolayer of cells had formed. In most cases three to seven days were required to reach this stage.

III. Preparation of secondary cultures

(i) The primary cultures were washed gently with calcium and magnesium-free phosphate buffered saline (Appendix III).

(ii) Monolayers were detached from the culture flasks by agitation of the cultures after the addition of trypsin and EDTA (Appendix III) solution at $37^{\circ}C$. The free cells were transferred to 100 ml screw-top centrifuge tubes and further dissociated by pipetting.

(iii) The cells were then pelleted by centrifugation at 125 g for [illegible] supernatant fluid was discarded and the cells

were resuspended in growth medium by pipetting.

(iv) Total and viability counts of cells were performed as for the establishment of the primary cultures and the concentration of viable cells adjusted to 5×10^5 per ml of growth medium.

(v) "Seeding" of the secondary culture vessels was performed using a two millilitre Cornwall automatic syringe.

In 1971 all secondary cultures were grown in acid-washed 10 ml glass test tubes stoppered with red rubber bungs. To each tube one millilitre of the cell suspension was added, "gassing" was carried out as before, and incubation was at 37°C in tilted racks. In 1972 these cultures were established in screw-topped, acid-washed, 40 ml glass culture flasks and in Leighton tubes containing glass coverslips. Ten millilitres of cell suspension were added to each culture flask, and one millilitre to each Leighton tube. Cultures were "gassed" prior to incubation as before.

(vi) In all experiments, the medium of the secondary cultures was replenished following the inoculation of tumour extracts and thereafter at five day intervals until conclusion of the experiments at 20 days.

Culture Inoculation

I. Preparation of inocula.

(i) Inocula were obtained from cell-free extracts of malignant lymphoma of sheep prepared as detailed in Chapter 3. These were designated OL_1, OL_2, OL_3, OL_4, OL_5, and OL_6. The first three (1971 experiments) were from the same batches as those used in the lamb inoculations described in Chapter 3. The final three extracts (1972 experiments) were prepared from other spontaneous malignant lymphomas obtained from slaughter-

houses. All source tumours were classified histologically as being of lymphoblastoid type except OL_2 which was lymphocytic.

(ii) Before inoculation extracts OL_1, OL_2 and OL_3 were diluted 10 fold with growth medium. In the experiments performed in 1972 extracts OL_4, OL_5 and OL_6 were used as inocula in undiluted form.

II. Inoculation of secondary cultures.

(i) Inoculation was performed three days before the cells were expected to reach confluency.

(ii) Growth medium was decanted and cultures were gently washed with HBSS at 37°C.

(iii) Cultures in 10 ml test tubes and Leighton tubes were inoculated with 0.1 ml, while those in 40 ml culture flasks received 0.5 ml of the prepared inoculum.

(iv) The cultures were then reincubated for one hour at 37°C.

(v) After this time the appropriate quantity of growth medium was added to each culture, "gassing" was performed as before, and cultures returned to the incubator.

(vi) Control extracts derived from normal ovine lymph node were prepared, diluted and inoculated in the same manner as the tumour extracts. Tables 4.I and 4.II show the numbers and groups of cultures used in these experiments.

Examination of Secondary Cultures

I. General appearance.

The cultures were examined daily for evidence of contamination and change in pH of the medium. Obviously contaminated cultures were discarded. Less drastic pH changes in the absence of contamination were adjusted by the addition of appropriate amounts of either 0.1M hydrochloric acid or 7.5 per cent. sodium bicarbonate solution.

TABLE 4.I

1971 Experiments: Schedule of inocula and tissue cultures

Inoculum	Dilution of inoculum	No. of 10 ml cultures
OL_1	10^{-1}	9
	10^{-3}	9
	10^{-5}	9
OL_2	10^{-1}	9
	10^{-3}	9
	10^{-5}	9
OL_3	10^{-1}	9
	10^{-3}	9
	10^{-5}	9
Ovine lymph node control	10^{-1}	9
	10^{-3}	9
	10^{-5}	9

TABLE 4.II

1972 Experiments: Schedule of inocula and tissue cultures

Inoculum	Dilution of inoculum	No. of 40 ml cultures	No. of Leighton tube cultures
OL_4	10^0	4	20
OL_5	10^0	4	20
OL_6	10^0	4	20
Ovine lymph node control	10^0	4	20

II. Cytopathic effects and transformation.

(i) Daily examination of all cultures was made using an inverted microscope.

(ii) Stained coverslip preparations from Leighton tube cultures (1972 experiments) were prepared at five day intervals up to 20 days following inoculation. The cultures were gently washed in HBSS at 37°C and were fixed for five minutes in Bouin's fixative at room temperature. The coverslips were then removed from the culture tubes, placed in staining racks, and excess fixative was washed from them with gently running tap water. Staining was by HE. The stained coverslip cultures were mounted in DPX on glass slides for microscopic examination.

III. Electron microscopic examination of culture supernatants.

Examination of the culture supernatants for the presence of virus particles was performed on 10 ml test tube and 40 ml flask cultures at intervals of five days until 20 days after inoculation. The procedures were as follows:-

(i) The supernatant fluids of cultures in each group were pooled in 10 ml glass test tubes.

(ii) Cultures were washed with HBSS at 37°C and washings were pooled with the appropriate supernatant fluids.

(iii) These were centrifuged at 2,000 g for 10 min.

(iv) The supernatant fluid was transferred to methyl cellulose centrifuge tubes appropriate for the SW40 rotor of the Beckman Model L ultra-centrifuge, and were spun at 80,000 g for 120 min.

(v) The supernatant was discarded, and the resultant pellet was resuspended by pipetting in two drops of bovine serum albumin and two drops of two per cent. phosphotungstic acid (PTA).

(vi) The suspension was sprayed on to carbon-coated 200 mesh

copper electron microscope grids using an "atomizer".

(vii) When dry, the grids were examined in a Philips' EM 200 electron microscope for the presence of virus particles.

IV. Electron microscopic examination of cultures

Preparation of cultured cells for electron microscopy were performed at the same time intervals as the supernatant. In the 1971 experiments in which cells were cultured in 10 ml test tubes, two cultures were required from each group to provide sufficient cells for embedding and examination. In the 1972 experiments in which 40 ml flask cultures were inoculated with extracts OL_4, OL_5 and OL_6, the cells from a single culture were sufficient. The procedures were as follows:-

(i) The supernatants were decanted and cultures were washed to recover the supernatant fluid for examination as previously described.

(ii) Washing was repeated using calcium and magnesium-free phosphate buffered saline.

(iii) Trypsin and EDTA solution at 37°C was added to the cultures, and the cells dissociated from the glass by agitation.

(iv) The suspension was transferred to 10 ml centrifuge tubes and cells pelleted at 125 g for five minutes.

(v) The supernatant was decanted and the pellet resuspended by pipetting in 10 ml of calcium and magnesium-free phosphate buffered saline at 37°C. The cells were centrifuged at 125 g for five minutes.

(vi) Step (v) was repeated twice.

(vii) Chilled Karnovsky's fixative (Appendix I) was added to the tube and the cell pellet resuspended in the fixative for 120 min.

(viii) The fixed cells were pelleted by centrifugation at 125 g for

five minutes, and the supernatant discarded.

(ix) Ten millilitres of chilled 0.1M phosphate buffer (Appendix I) was added, and the cells resuspended and washed. After centrifugation at 125 g for five minutes the washing was repeated once.

(x) Post fixation in chilled one per cent. osmium tetroxide in 0.1M phosphate buffer was performed for 30 min.

(xi) The fixed cells were embedded in an epoxy resin (Appendix I), and sections cut and stained for electron microscopic examination as described in Chapter 2.

RESULTS

Examination of Cultures for Cytopathic Effects or Transformation

I. Microscopic appearance of unstained cultures.

Cells of sheep embryonic kidneys normally form monolayers in culture of variable cellular density. Dense foci of proliferating cells were seen where initial attachment occurred and the intermediate regions were gradually covered by the outgrowth of cells from these primary foci. The latter regions were much less densely populated. This form of growth was observed in all cultures, including the controls and no true areas of transformation could be recognized.

No cytopathic changes were regularly found within any groups of cultures, although various cultures showed degenerative foci and necrosis of cells in the monolayer. With aging the cultures became very dense and were easily damaged if agitated. In these cases the cells tended to lose adhesion to the glass surfaces and float off in sheets of varying size. Similar changes were found in the control cultures.

II. Appearance of stained coverslip preparations.

The sequential growth of the cultures were more clearly illustrated in these preparations. Dense primary foci of proliferating cells gave riaw to the thinner intermediate regions of the cultures. The cells in the primary foci were oval in cytoplasmic outline, while those in the "intermediate" region were elongated and fusiform (Figures 4.1, 4.2). By 10 days the cells in the "intermediate" areas showed loss of contact inhibition and were seen to cross and form areas in which cells were two layers thick (Figure 4.3). Pyknosis of nuclei and eventual necrosis of cells were fairly common in these crowded regions, and occurred in all cultures including controls. No multi-nucleate giant cells, cytopathic effects, transformed areas or other changes suggestive of virus interaction with the cultured cells were observed.

III. Examination of culture supernatant pellets in electron microscopic preparations.

In only one preparation was there negatively stained material suggestive of virus particles. This was seen in the preparation of supernatant from cultures inoculated with tumour extract OL_6 after 10 days of culture. The particles were spherical or ovoid and showed an outer limiting membrane approximately 12 nm thick. Their internal structure varied. In most the stain deposition was granular and appeared to outline internal structures (Figures 4.4, 4.5) while in others the heavy uptake of electron-dense stain within the outer membrane suggested the absence of internal structure (Figure 4.5). The external diameter of the particles varied from approximately 100 to 250 nm with most in the 120 to 170 nm range (Figures 4.4, 4.5, 4.6). Structures of similar morphology were not seen in the cultured cells from which this supernatant was obtained, nor were they found in similar examinations performed on material obtained from a further passage of this supernatant fluid in foetal lamb kidney cultures.

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Figure 4.1

A primary growth focus (F) in a "normal" foetal ovine kidney cell culture which has reached confluency.

HE x 750

Figure 4.2

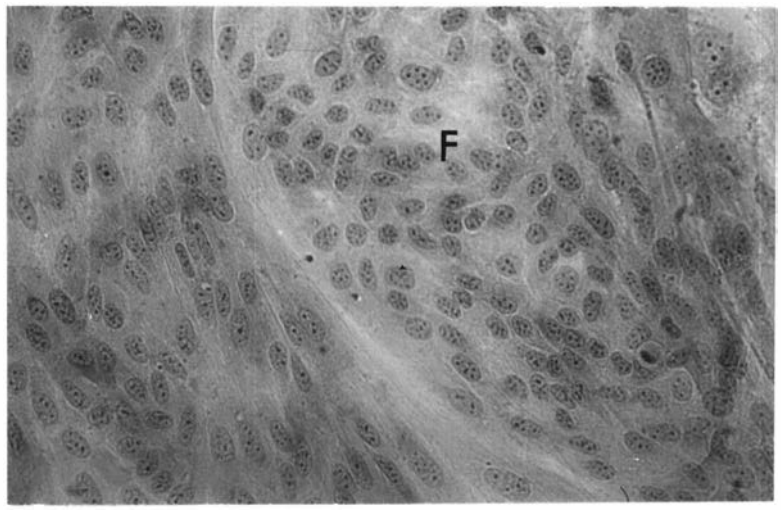
Elongated cells in an intermediate region of a "normal" foetal ovine kidney cell culture which has reached confluency.

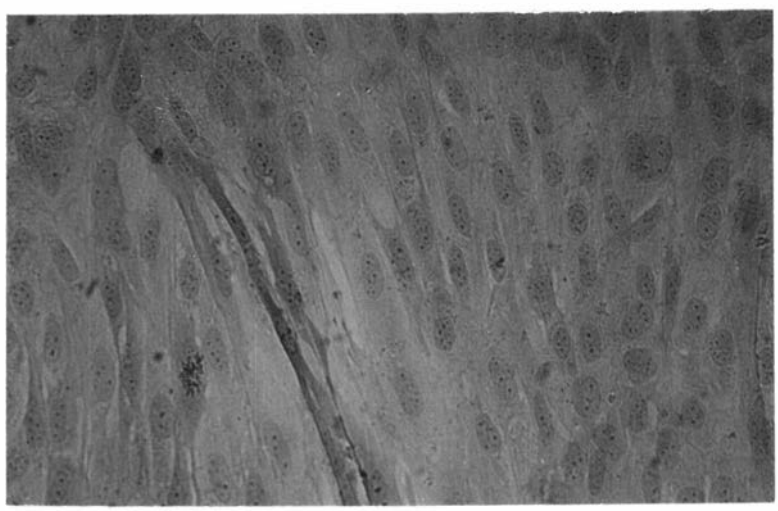
HE x 750

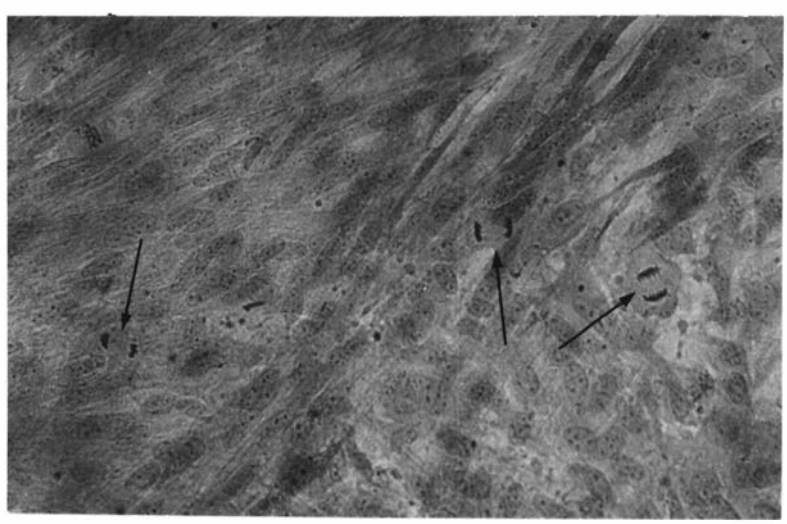
Figure 4.3

Multiple layers of cells in ageing "normal" foetal ovine kidney cell culture. Note the mitotic figures (arrows).

HE x 750


F

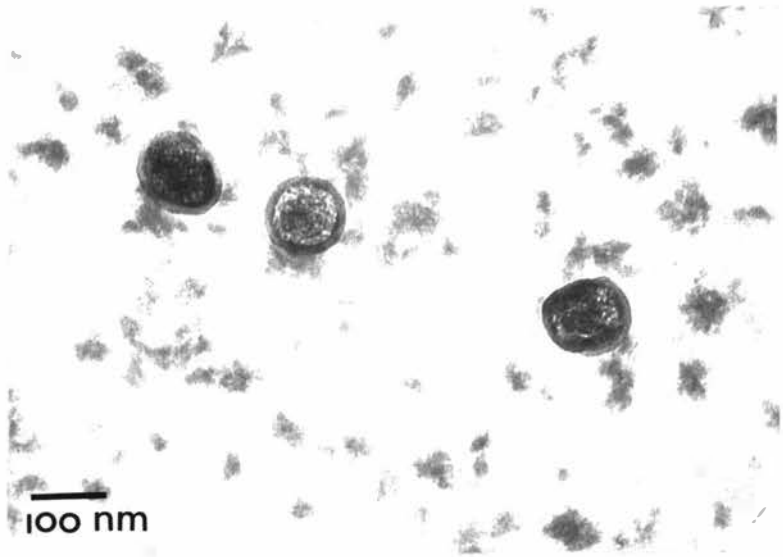


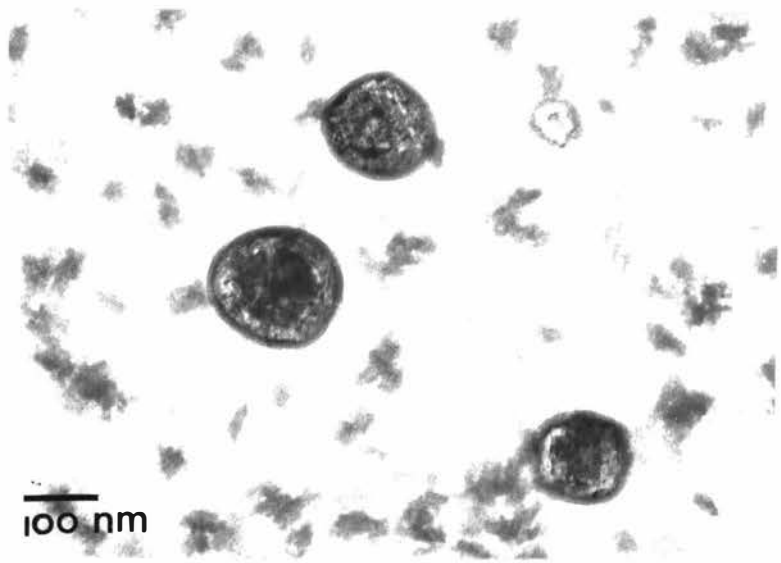


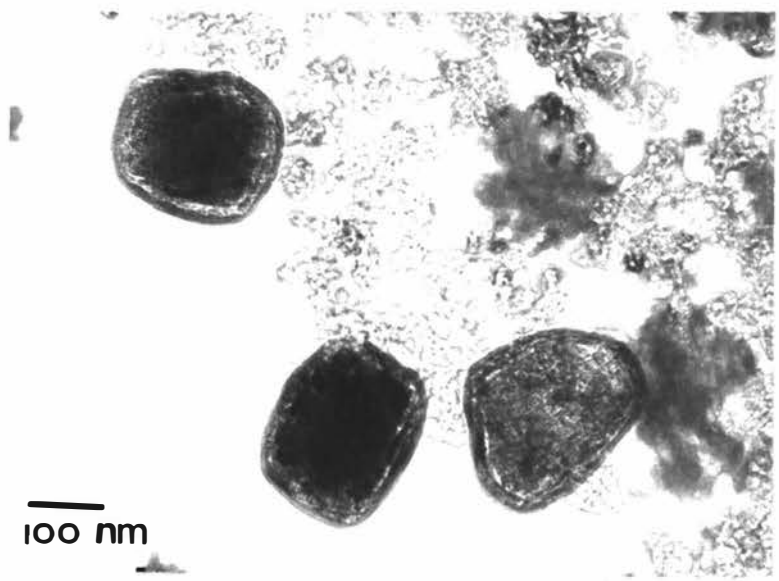
Figures 4.4, 4.5, 4.6

Virus-like particles in preparations of supernatant fluids of an ovine foetal kidney cell culture which had been inoculated with malignant lymphoma extract OL_6.

Phosphotungstic acid x 100,000


100 nm


100 nm


100 nm

IV. Examination of cultured cells in electron microscopic preparations.

No virus-like particles were seen in any of the cultures.

DISCUSSION

The failure of the extracts to produce cytopathic effects or transform the cells in these cultures may be interpreted in two ways. Firstly, if virus was present which had the potential to cause these changes, the concentration of virus may not have been high enough or the cultures may not have been suitable for their growth. Malmquist, Van der Maaten and Boothe (1969) described a virus isolated from cattle with lymphocytosis which induced cytopathic effects including formation of syncytia in bovine embryonic spleen cells. Cornefert-Jensen, Hare and Stock (1969) have described similar cultural changes in mixed cell cultures which were inoculated with material from bovine malignant lymphoma. However the virus particles observed by Cornefert-Jensen *et al.* in electron microscopic examination of these cultures differed in size from those reported by Malmquist *et al.* They encountered difficulty in reinfecting further cultures using supernatant fluids of the cultures and considered the virus to be strongly cell-associated. Viruses which have been proved as oncogenic and which are capable of inducing cytopathic effects or transformation in culture are relatively few in number. They include SV_{40}, various adenoviruses, polyoma virus, avian erythroblastosis virus, the papilloma group of viruses, and the chicken, mouse and cat sarcoma viruses. A second possibility is that the extracts may have contained virus which did not cause morphological change in the infected cultures. Such oncogenic viruses characteristically belong to the oncorna group. These are the RNA oncogenic viruses of the chicken leukosis complex, mouse leukaemia, guinea pig leukaemia, and malignant lymphoma of the cat. The particles observed in the supernatant fluid of foetal lamb kidney cell cultures 10 days after

inoculation with OL_6 show some features in common with these well characterized types.

De Harven (1968) has reviewed the morphological features of murine oncorna viruses and attempted to rationalize their classification on this basis. Two types are generally recognized at the ultrastructural level, and these are referred to as "type A" and "type C" particles. "Type A" particles can be further subdivided into two distinct forms. Within the cytoplasm of the cell they occur as spherical structures representative of the assembled viral nucleoid. These are the so-called "naked A particles" and they mature by a budding process at cell membranes to form "enveloped A particles". The latter differ from "naked A particles" in that they occur in extracellular situations or within cell vesicles, and are invested by a membrane derived from the host cell's plasma membrane. The nucleoid of both these forms has an electron-lucent centre. "Type C particles" are probably a later stage in viral maturation. They occur in the same situations as "enveloped A particles", but differ from these in nucleoid morphology. Their nucleoid is more irregular in outline and has an electron-dense centre.

The particles studied in the current experiments showed an external membrane of approximately 12 nm thickness surrounding the internal structures. Within this the uptake of PTA varied between particles. Some appeared empty because of the large amount of stain present, while others showed considerably less staining and internal structure was apparent. The smaller particles seen in these preparations are consistent in their general morphologic appearance to "type C" particles. Variation in the size of these particles has been reported in various species. De Harven states that in the mouse the diameter of particles may vary from 60 to 150 nm. In the chicken, variation has been reported from 64 to 82 nm (Dmochowski et al., 1959); in the guinea pig, from 70 to 90 nm (Opler, 1967); in the cat, from

100 to 115 nm (Kawakami et al., 1967; Laird et al., 1968); and in the cow 90 to 120 nm (Tumilowicz and Shirahama, 1969; Miller et al., 1969). Even taking into account the distortion which may result from PTA staining (Goodheart, 1969) it is unlikely that the larger particles observed in the current experiments were "C type" or "enveloped A" particles. In fact the interpretation of the identity of these structures must remain in doubt because of:-

(a) their irregularity of form and size

(b) the small numbers present

(c) the absence of similar particles in other cells or supernatant preparations in these experiments

(d) the inability to reproduce further evidence of these structures by passage of the supernatant fluid to subsequent cell cultures.

In view of the successful transmission of lymphocytosis to lambs using cell-free extracts OL_1, OL_2 and OL_3 of sheep lymphoid tumours, the failure to unequivocally demonstrate virus in tissue cultures inoculated with these and other tumour extracts is disappointing. If the hypothesis that the in vivo response in lambs inoculated with cell-free tumour extracts (Chapter 3) is due to the action of an oncogenic virus is valid, the failure to culture this agent may have been due to a number of factors. It is possible that the titres of postulated virus in the diluted extracts may have been too low for infection of the cultures. Relatively small amounts of the original tumour material from which the extracts OL_1, OL_2 and OL_3 were derived were actually inoculated on to the tissue cultures. It was demonstrated by Beard (1956, 1957) that plasma containing lesser numbers of avian myeloblastosis viral particles had a greatly reduced infectivity in chicken inoculation tests. Macpherson (1969) stated that high concentrations of virus were required to effect cellular transformation by polyoma

virus. Another possibility is that the agent may require longer than 20 days for it to induce cytopathic changes or be directly demonstrable. Todaro (1969) considered that the initial cell concentration was important in allowing the development of transformation in culture. In the case of SV_{40}, from four to six generations of cells, subsequent to infection, are required for the production of transformation. Cell division rate slows as the density of cells in the monolayer increases and he considers that an ideal concentration of cells after inoculation is 750 per cm^2 of growing surface. With this concentration, the confluent monolayer of cells is obtained in two to three weeks. The cell concentration in the current experiments was considerably higher and consequently the chance of obtaining transformation could have been lessened. Passage-A virus, which is the enhanced virulence form of the original Gross virus, required up to 24 days of culture before sufficient virus was replicated to perform successful bioassays in the mouse. In later passages of the agent through the same culture system the latency period was reduced to eight days. It is also possible that the cells used for the cultures on which the malignant lymphoma material was inoculated were unsuitable for viral replication, however, the viruses of avian and murine leukaemia replicate freely in a variety of epithelial and mesenchymal cell cultures (Gross, Dreyfuss and Moore, 1961; Ginsburg and Sachs, 1962; Ioachim, 1967). The cultures used in the present experiments contained both epithelial and fibroblastic elements and should therefore have supported growth of this type of virus in the absence of other inhibitory factors.

The experimental work reported in this chapter does little to support the hypothesis that malignant lymphoma in sheep is due to an oncogenic virus. However the circumstantial evidence obtained from the transmission studies reported in Chapter 3 and parallel work in some other animal species argues strongly toward a viral aetiology.

The failure to obtain evidence demonstrating the agent in these experiments, while disappointing, is not necessarily final.

Another possible approach might be to attempt the demonstration of virus-like particles in cell cultures derived directly from lymphoma tissue. Although not reported here attempts were made to do this but in spite of repeated efforts actively multiplying cell cultures could not be established.

CONCLUSIONS

Present experiments indicate that the inoculation of malignant lymphoma material on to tissue cultures does not result in any cytopathy or transformation; neither can unequivocal virus-like particles be demonstrated by electron microscopy of the cell cultures or culture fluids. However, the possibility of demonstrating virus by this method can not be entirely excluded especially if high titre inocula could be obtained.

CHAPTER 5

THE DEMONSTRATION OF VIRUS IN LYMPHOCYTES STIMULATED BY PHYTOHAEMAGGLUTININ

INTRODUCTION

After the failure to unequivocally demonstrate virus directly in tumour tissue or indirectly in cell culture systems, attention was turned towards the demonstration of virus in lymphocytes derived from sheep in which lymphocytosis had been induced by tumour material as described in Chapter 3. That this approach might be rewarding was indicated by the report of Miller et al. (1969) in which ultrastructural evidence of virus was demonstrated in lymphocytes of cattle either with malignant lymphoma or with presumed pretumcurous lymphocytosis. Lymphocytes from these animals were first stimulated with phytohaemagglutinin (PHA) and the resultant blast cells were shown to contain virus-like particles in almost all cases (Table 5.II). In the following experiments similar studies were made on lymphocytes from five sheep with induced lymphocytosis.

MATERIALS AND METHODS

Source of Lymphocytes and Preparation of Cultures

Blood lymphocytes were derived from five sheep which had developed persistent lymphocytosis (numbers 401, 413, 416, 426 and 432), from one contact control sheep (113) (see Chapter 3) and from four more control sheep of the same age which were selected at random from the Massey University commercial flock. The preparative steps were as follows.

Four 10 ml aliquots of venous blood were collected from each sheep using heparinized Vacutainers. These were centrifuged at 125 g for 15 min. Buffy coats were aspirated and run on to cotton wool separation

columns (see Appendix VI) which were left undisturbed for 15 min. Ten millilitres of HBSS at 37°C was run on to the columns and the eluted cells collected into 10 ml disposable plastic syringes. The washings, containing lymphocytes and erythrocytes, were transferred to 10 ml stoppered test tubes and centrifuged at 125 g for 10 min. The supernatant fluid was discarded and the pellet resuspended in 0.15M NH_4Cl to lyse remaining erythrocytes. The suspension was again centrifuged at 125 g for 10 min. and the supernatant discarded. The remaining lymphocytes were washed three times in HBSS. In this process the cells were suspended for five minutes and recovered by centrifugation at 125 g for 10 min. Total cell counts were made in a Neubauer haemocytometer and differential counts made on smears stained by MacNeal's tetrachrome.(1) Following addition of phytohaemagglutinin (PHA-P)(2) at a concentration of 20 µl per ml the cultures were gassed with a 4 per cent. CO_2 and air mixture and incubated at 37°C for 36 and 72 hr. All of the above manipulations were carried out with strict observance of aseptic technique.

Harvesting of Cells for Electron Microscopy

After incubation the cultures were transferred to 50 ml screw-top centrifuge tubes and centrifuged at 125 g for 10 min. The pellet was recovered and resuspended in chilled 0.1M phosphate buffer (Appendix I) for five minutes, and centrifuged at 125 g for 10 min. The cells were washed in this manner three times before fixation for 30 min. in chilled 1 per cent. osmium tetroxide in 0.1M phosphate buffer. After fixation the cells were repelleted by centrifugation as before and washed for five minutes in chilled 0.1M phosphate buffer. They were

(1) Searle Scientific Services, Chadwell Heath, Essex, London.

(2) Wellcome New Zealand Limited, Otahuhu, New Zealand.

again recovered by centrifugation at 125 g for 10 min. and embedded in an epoxy resin following dehydration in a graded alcohol and propylene oxide series (Appendix I). Electron microscopic examination was performed on sections cut and stained as described in Chapter 2.

RESULTS

Yield of Lymphocytes from Peripheral Blood

The procedure described above resulted in practically pure yields of lymphocytes from peripheral blood as determined by the examination of stained smears of cell preparations. Occasional granulocytes were seen, but these never exceeded 0.5 per cent. of the cells recovered. Harvests varied between 60 x 10^6 and 150 x 10^6 lymphocytes per 40 ml of blood, with the higher yields being obtained from animals with elevated lymphocyte counts.

Examination of Cultures by Electron Microscopy

Virus-like particles were detected in all PHA-stimulated cultures from sheep with induced lymphocytosis but not from those derived from control cultures (Table 5.I).

The particles were generally spherical or ovoid with electron-lucent centres and were surrounded by a unit membrane (Figures 5.1, 5.2, 5.3). Their external diameter ranged from 60 to 120 nm. Morphologically they fulfilled the criteria laid down by de Harven (1968) for "type A" oncorna viruses. They were most commonly located in groups within membrane-limited vacuoles in the cells (Figures 5.1, 5.2) but were also observed free in the cytoplasm as well as extra-cellularly (Figure 5.3).

Structures resembling particles budding into intracytoplasmic vacuoles were not conclusively observed. No particles of "C type" virus morphology were seen.

TABLE 5.I

The Occurrence of Virus-like Particles in Cultures of Phytohaemagglutinin-stimulated Lymphocytes of Sheep

Sheep No.	No. of cultures with virus-like particles		
	Number of cultures examined		
	36 hr cultures	72 hr cultures	total
401	...	1/1	1/1
413	1/1	2/2	3/3
416	1/1	2/2	3/3
426	1/1	1/1	2/2
432	1/1	2/2	3/3
113 (contact control)	0/1	0/2	0/3
Population control$_1$	0/1	0/1	0/2
Population control$_2$	0/1	0/1	0/2
Population control$_3$	0/1	0/1	0/2
Population control$_4$	0/1	0/1	0/2

Figure 5.1

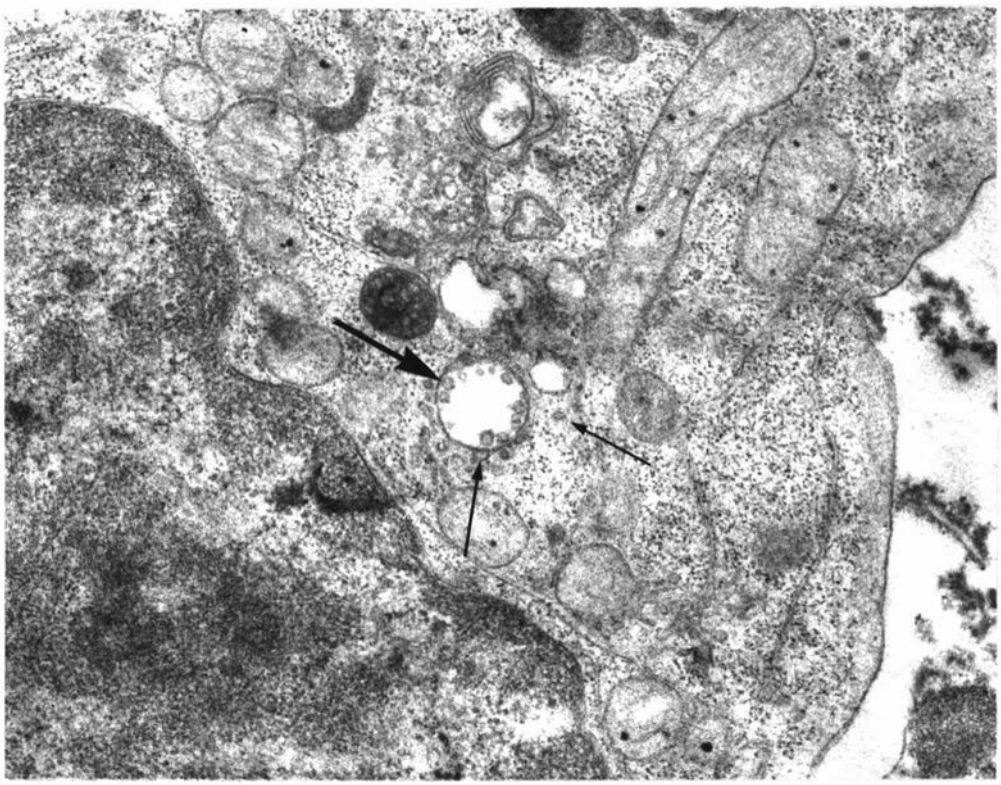
A vacuole within a phytohaemagglutinin-stimulated lymphocyte from sheep No. 413 containing numerous structures (large arrow) consistent in morphology with type A oncorna viruses. Similar particles are present in the cytoplasm (small arrows).

Electron micrograph x 20,000

Figure 5.2

"Type A" virus-like particles in a similar preparation from sheep No. 426 to that shown in the previous figure.

Electron micrograph x 50,000



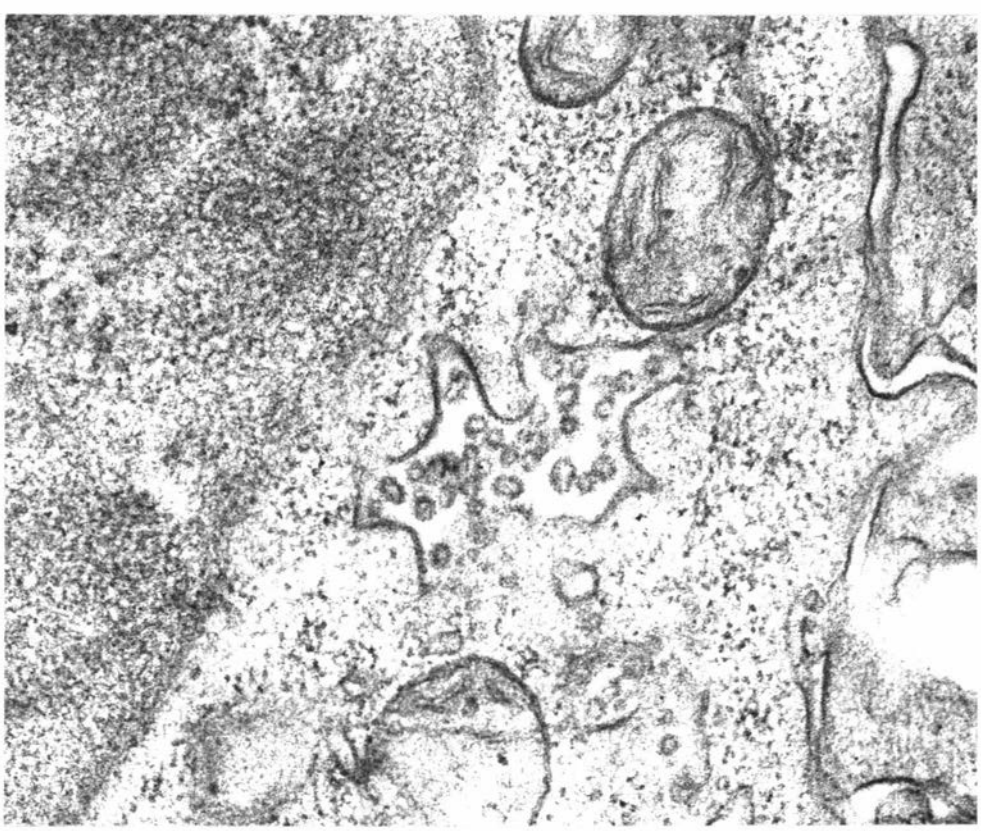


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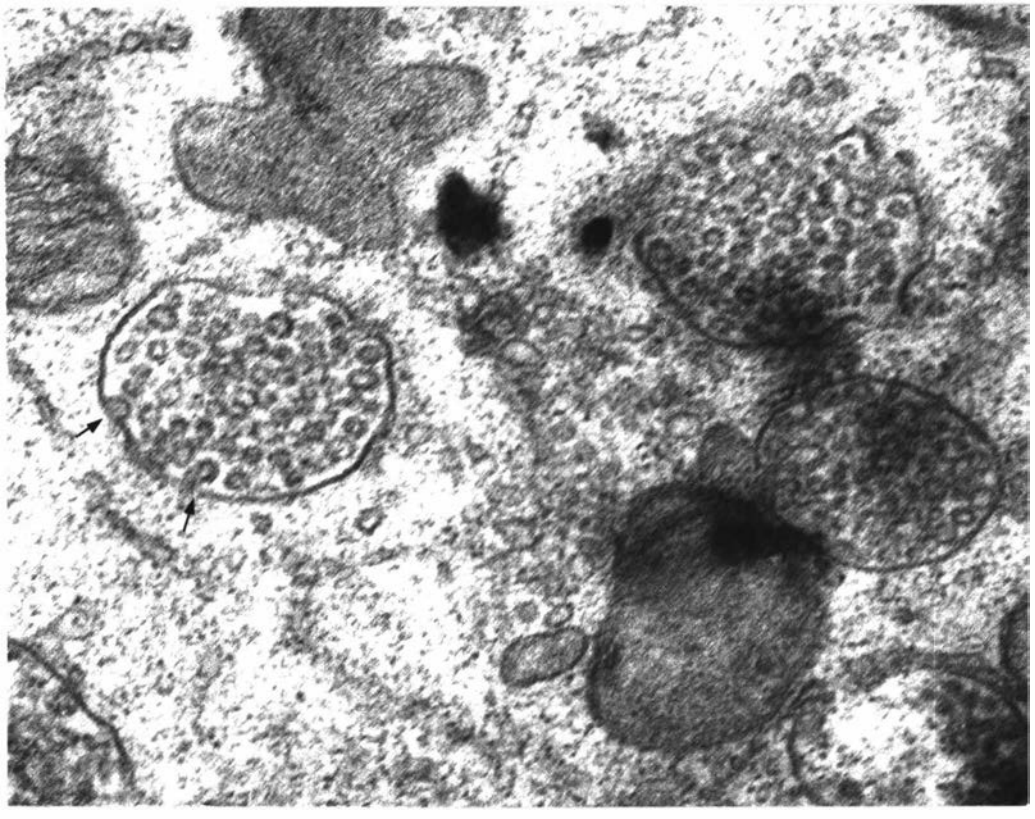
Figure 5.3

Large numbers of virus-like particles present in 3 vacuoles within a phytohaemagglutinin stimulated lymphocyte from sheep No. 432. Possible budding forms (arrows) are present.

Electron micrograph x 50,000



DISCUSSION

The presence of large numbers of particles in the vacuoles of cells in these cultures raises the question of their significance. Their situation, size and morphology indicate they are likely to be viruses belonging to the oncorna group. This is supported by similar observations by other investigators in this field. Weiss et al. (1971) reported that PHA-stimulated lymphocyte cultures from two sheep with malignant lymphoma contained oncorna virus-like particles when examined after 72 hr incubation. This group of workers (Paulsen et al., 1972) subsequently extended these investigations and demonstrated virus-like particles in seven sheep which showed persistent lymphocytosis or overt malignant lymphoma. Lymphocyte cultures stimulated by mitogen from the remaining 38 clinically normal animals in this flock showed no production of virus-like particles. The production of mixed "C type" and "vesicular" viruses was seen in these preparations in which the latter form were most abundant. In size and morphology the structures seen in the present experiments closely resembled the "vesicular" form of the virus Paulsen et al. described. In similar experiments with bovine lymphocyte cultures particles resembling oncorna virus were described (Miller et al., 1969; Dutta et al., 1970; Olsen et al., 1970; and Stock and Ferrer, 1972). These reported results, summarized in Table 5.II, strongly emphasize the association of the virus-containing cultures with malignant lymphoma and particularly with lymphocytosis. It seems likely that these viruses may be causally related to bovine lymphocytosis and/or malignant lymphoma. This association is further supported by the observation that the particles isolated from sheep in Germany showed a strong precipitation reaction in immunodiffusion tests with sera of 13 of 14 sheep with spontaneous malignant lymphoma, but in only 3 of 87 clinically normal sheep (Paulsen, pers. comm.) Budding forms of virus were not conclusively observed in the present cultures. Weiss et al. and Stock and Ferrer have also noted difficulty in

TABLE 5.II

The combined frequency of "C-type" virus particles in cattle as detected by examination of short-term, PHA-stimulated lymphocyte or buffy coat cultures

Clinical status of animals tested	Number of animals with "C-type" particles / Number of animals studied				
	Pennsylvania(1)	Wisconsin(2)	Minnesota(3)	Total	Per cent. positive
Malignant lymphoma	3/5	10/14	5/7	18/26	69
Persistant lymphocytosis	6/6	10/10	5/5	21/21	100
Non-lymphocytosis (in multiple case herd)	3/13	5/21	-	8/34	23.5
Normal (in malignant lymphoma free, non-lymphocytosis herd)	0/6	2/26	0/5	2/37	5.4
Normal cattle (inoculated with malignant lymphoma material in utero or neonatally)	-	15/26	-	-	57.7

(1) Stock and Ferrer (1972)

(2) Olson et al. (1970 a and b)

(3) Dutta et al. (1970)

demonstrating this phenomenon in culture. The latter reported that budding forms could not be seen even after the rapid cell fixation method of de Thé (1964) was utilized. Budding particles were eventually demonstrated in their cultures at 3, 12 and 18 hr but a total of only seven "buds" were present in 900 sections of cells examined. This rarity of "buds" and the difference in ages of the cultures when examined probably explain why no "buds" were conclusively demonstrated in our experiments. The fact that we demonstrated relatively few extracellular virus-like particles is probably due to the thorough washing of cells which was shown to be necessary by preliminary experiments. In these,plastic embedded material prepared from cultures was extremely difficult to section due to the presence of hard particulate material assumed to be due to crystalline material derived from the culture fluid.

It is emphasized that these results are preliminary in nature and should be repeated on a larger scale. However, against the background of other experimental work in this thesis and the results of other workers it seems probable that these viruses are related to persistent lymphocytosis, and thereby to malignant lymphoma of sheep.

CONCLUSIONS

Particles consistent in morphology with the "type A" oncorna viruses of mice, chickens and cats and those recognized in association with persistent lymphocytosis and malignant lymphoma of cattle and sheep can be detected in PHA-stimulated lymphocyte cultures from sheep with experimentally transmitted lymphocytosis and not in similar preparations from control sheep. They can be seen in the cytoplasm within cytoplasmic vacuoles as well as extracellularly. It is probable that these particles are aetiologically related to lymphocytosis and thereby to malignant lymphoma in sheep.

CHAPTER 6

STUDIES ON CELLULAR IMMUNITY

INTRODUCTION

It has been demonstrated that tumours are able to elicit an immune response in the host in which they arise. Recent reviews on this topic are those of Old and Boyse (1965) and Rich and Siegler (1967). Much of the earlier work concentrated on the humoral response to tumour antigens, but more recently emphasis has shifted to cell mediated immunity as an important factor in host response to tumours (Churchill et al., 1968; Nordin, 1972). Field and Caspary (1970) have indicated the possibility of the in vitro testing of lymphocytes for their ability to produce mediators associated with cellular immunity as a diagnostic and perhaps prognostic test for neoplasia in humans, but there has been little attention focussed on the detection of precancerous changes by such methods. It became apparent that the presumed pretumourous lymphocytosis induced in the sheep experiments (Chapter 3) represented a model for the immunological investigation of a precancerous state. In the work of Field and Caspary a type of macrophage migration inhibition test showed particular promise as an investigatory tool in human cancer research. These workers however employed what might be considered a non-specific antigen in the form of basic protein derived from normal central nervous system material and were able to demonstrate that lymphocytes of patients with malignant tumours frequently showed reactivity to it. The present experimental situation in sheep allows the use of specific antigens in the form of cell-free extracts of malignant lymphoma as well as extracts from appropriate control tissues.

Although the macrophage migration inhibition test is technically

involved, it represents one of the best of all currently available methods for the detection of specific cell mediated immunity (Herbert, 1970). Accordingly, the major emphasis of the experiments reported below is based on this technique. For comparative purposes, simple intradermal tests were also carried out.

MATERIALS AND METHODS

Animals

The sheep tested for the presence of cell mediated immune reaction to tumour and control antigens described later were those that had been inoculated with tumour extracts OL_1, OL_2 and OL_3 and the contact control animals as described in Chapter 3.

The Macrophage Migration Inhibition Test

The principle on which this test is based is simple and depends upon the ability of sensitized lymphocytes to respond when exposed to the sensitizing antigen, by the release of a factor which inhibits the migration of macrophages. This factor is known as migration inhibitory factor or MIF. As applied to the present work, the macrophages for the test were derived from the peritoneal cavity of normal sheep, the lymphocytes were derived from blood of the various sheep (see above), and the antigens were derived from ovine malignant lymphoma tissue or normal ovine tissue. The test is illustrated in diagrammatic form in Figure 6.1 and described in more detail below. Aseptic techniques were maintained with regard to preparation and handling of all materials used in these procedures.

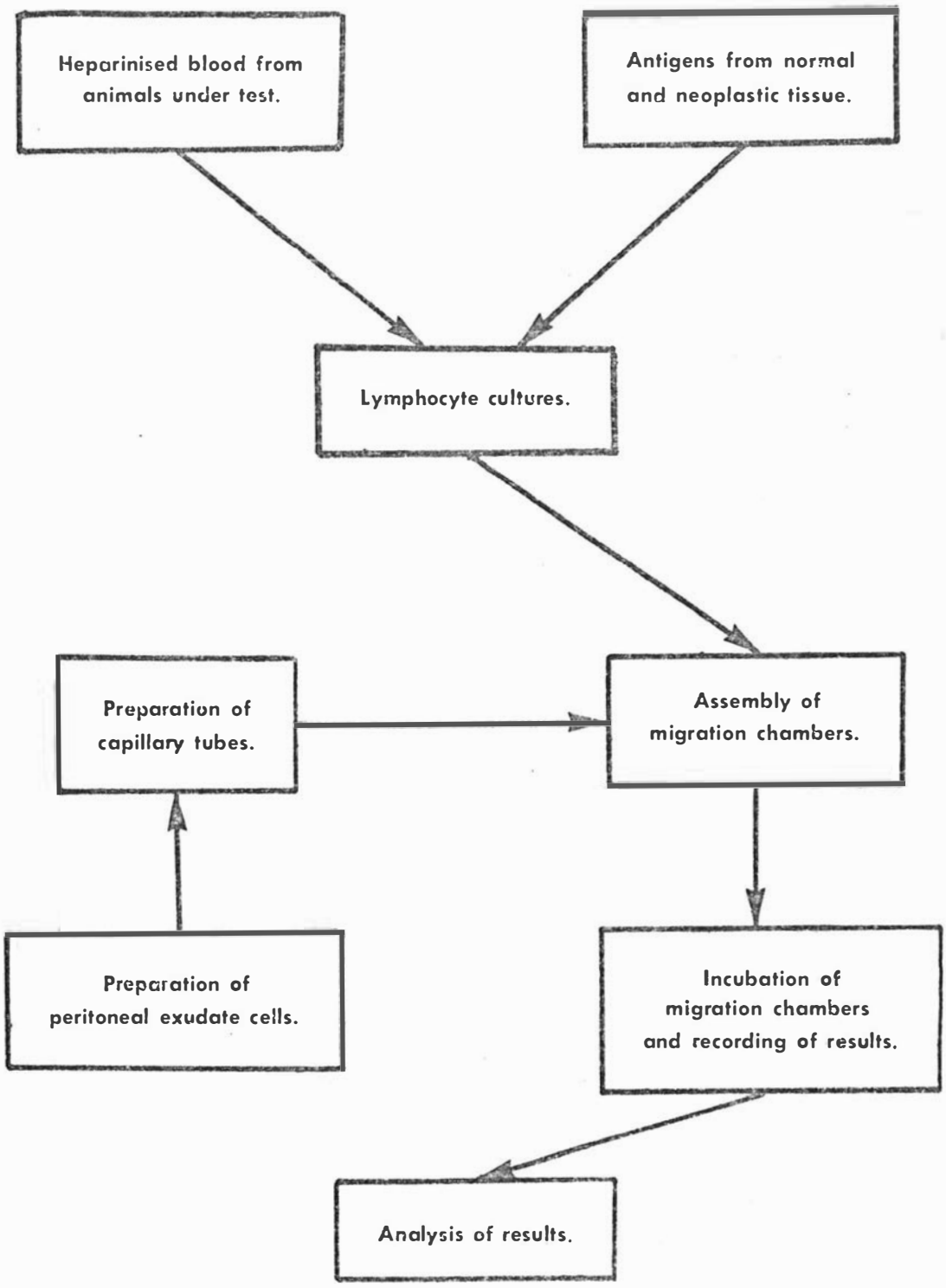


Figure 6.1

Schematic representation of the procedure used in the macrophage migration inhibition test.

I. Source of antigens

(i) Tumour antigens, designated OL_7, OL_8 and OL_9, were derived from tumour tissue of three cases of spontaneously occurring malignant lymphoma of lymphoblastoid type obtained from a local slaughter-house.

(ii) Normal lymph node antigen (NL) was obtained by surgical removal of the precrural lymph node of sheep 72, a randomly selected contact control.

(iii) Normal kidney antigen (NK) was obtained from normal renal cortex from a healthy animal killed at a local slaughter-house.

II. Preparation of antigens

The tissues OL_7, OL_8, OL_9, NL and NK were individually homogenized with an equal volume of HBSS with antibiotics using the same techniques as described in Chapter 3. Each homogenate was held at $4^{o}C$ for 24 hours in sterile 20 ml screw-top Universal containers before being centrifuged for 10 min. at 2,000 g. The supernatant was aspirated and stored in 20 ml Universal containers prior to inoculation of cultures. Each batch was tested for cytotoxicity in culture with lymphocytes. The cultures were established as described below and five per cent. antigen by volume of the culture was added. Cytotoxicity of control and antigen inoculated cultures was judged by the trypan blue dye-exclusion method (Ling, 1968) after 24 hours. At this concentration of antigen no differences in lymphocyte viability between the control and antigen inoculated cultures were detectable and therefore in subsequent experiments the various antigenic solutions were pipetted at this five per cent. level.

III. Lymphocyte cultures

(i) Lymphocyte cultures were prepared from inoculated and contact control sheep of the experimental flock by the same technique

as that described in Chapter 5.

(ii) Depending on the number of antigens to be used, either five or six 10 ml cultures were established from each sheep under test. One of these served as an uninoculated medium control, while to each of the others was added one of the tumour or normal tissue extracts. All sheep were tested with NL, NK, OL_8 and OL_9 antigens, but due to quantity limitations only 27 of the 41 sheep could be tested with OL_7.

(iii) The cultures were inoculated with the various antigens and incubated for 24 hours at 37°C.

(iv) Following incubation the cultures were centrifuged at 2,000 g for 10 min. and the supernatant fluid was dispensed into 10 ml screw-top glass bottles, which were labelled and stored at -20°C until tested for MIF activity.

IV. Preparation of peritoneal exudate cells (PEC)

The source of PEC for use in these experiments were rams culled from the Massey University commercial flock on various grounds including "ill thrift", testicular abnormalities and lameness.

(i) Intraperitoneal injection of 1,000 ml of sterile paraffin oil at 37°C was made on two occasions with a seven day interval between injections. For ease of administration the oil was dispensed in 500 ml blood collection packs[1] and the injection was made through a 12 gauge (BSW) needle. The cells produced by this stimulus were harvested 7 to 14 days after the second paraffin injection.

(ii) After slaughter by exsanguination and spinal cord transection

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(1) Tuta, Lane Cove, N.S.W., Australia.

the rams were skinned, care being exercised to avoid puncturing the abdominal wall.

(iii) The ventral abdominal wall from xiphisternum to pubis was swabbed with alcohol and the prepared area was isolated with sterile surgical drapes. Using aseptic techniques the abdominal cavity was opened at the region of the umbilicus with a mid-line incision to enable introduction of the hand. Through this incision 1,500 ml of cold HBSS with antibiotics and 5 IU of heparin BP per ml was introduced and gently washed around the organs of the abdomen to suspend the PEC.

(iv) The incision was then extended anteriorly to the xiphisternum and posteriorly to the region of the anterior margin of the preputial attachments, and the fluids present in the abdominal cavity aspirated by suction into a sterile 2,000 ml flask.

(v) The aspirated fluid was transferred to a large sterile separation funnel, and aqueous and oil fractions allowed to separate. The aqueous fraction containing the PEC was collected into sterile screw-top 100 ml centrifuge tubes and centrifuged at 125 g for 10 min.

(vi) The pelleted cells were pooled and washed twice in HBSS by centrifugation at 125 g for 10 min. and were finally resuspended in an equal volume of growth medium (Appendix III). Smears of these preparations stained by MacNeal's method were shown to contain a high percentage of macrophages, with a very few (total less than 0.5 per cent) lymphocytes and polymorphonuclear leucocytes.

V. Preparation of migration chambers

Plastic tissue culture petri dishes of 35 x 10 mm dimensions[2] were used as migration chambers. Six equidistant spots of sterile silicone grease, dispensed from a syringe into each dish approximately 0.5 cm from its perimeter, provided the anchorage for the capillary tubes. The previously

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(2) Falcon Plastics No. 3001, Becton Dickenson and Co., U.S.A.

prepared lymphocyte culture supernatants were thawed and five millitres of each added to each of two migration chambers. This step was performed while the PEC were being packed in the capillary tubes by centrifugation (see step VI (ii) below).

VI Preparation of capillaries

(i) Lengths of vinyl capillary tubing[3] were cut into approximately 100 cm lengths and irradiated overnight with ultra-violet light.

(ii) Peritoneal exudate cells were aspirated into one millilitre tuberculin syringes and injected through an 18 gauge (BSW) needle into the capillary tubing. The tubing was then divided into lengths of approximately five centimetres and one end of each piece was heat-sealed. These were wrapped in groups in sterile aluminium foil, placed in 10 ml test tubes and centrifuged at 625 g for five minutes.

(iii) Using a sterile razor blade the tubes were cut at the cell-media interface and the portion containing the cells was further divided into 0.5 cm lengths. Care was taken to make the cuts cleanly and at right angles to the long axis of the capillary tube.

(iv) The tubes were now ready for assembly in prepared migration chambers and this step was completed as rapidly as possible. Six were placed in each chamber taking care to position them flat on the chamber base with the outer end anchored in the silicone grease spot and the inner end directed centrally

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(3) SV61 Dural Plastics, N.S.W., Australia.

(Figure 6.2). The outer end was effectively sealed by the grease.

(v) The assembled migration chambers were placed on a level board and transferred to a humidified CO_2 incubator at $37^{o}C$ for 16 hr.

VII Measurement of macrophage migration

(i) After incubation the cultures were removed from the incubator with as little disturbance to the preparations as possible and the area of migrating cells from each capillary tube was photographed through an inverted microscope (Figure 6.3).

(ii) The processed films were printed on uniform paper using an identical enlargement for each.

(iii) The photographs were dried and glazed and the areas of migration were identified and cut out. Individual weights of migration areas were measured to the nearest milligram.

(iv) The migration areas were expressed as the percentage inhibition compared to the media control culture. This was calculated by the following formula:

$$\text{macrophage migration inhibition} = 100 - 100.\left(\frac{\bar{x}_a}{\bar{x}_c}\right)$$

where $\bar{x}_a$ = mean weight of migration areas in antigen stimulated culture

$\bar{x}_c$ = mean weight of migration areas in media control culture.

In each animal tested, this assumes that the media control has zero inhibition and therefore the other treatments within the group are expressed as percentage of inhibition with respect of their particular group control. Statistical analysis of the results was performed using the multiple range F test described in Chapter 3.

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Figure 6.2

Assembled macrophage migration chamber showing arrangement of capillary tubes (arrows).

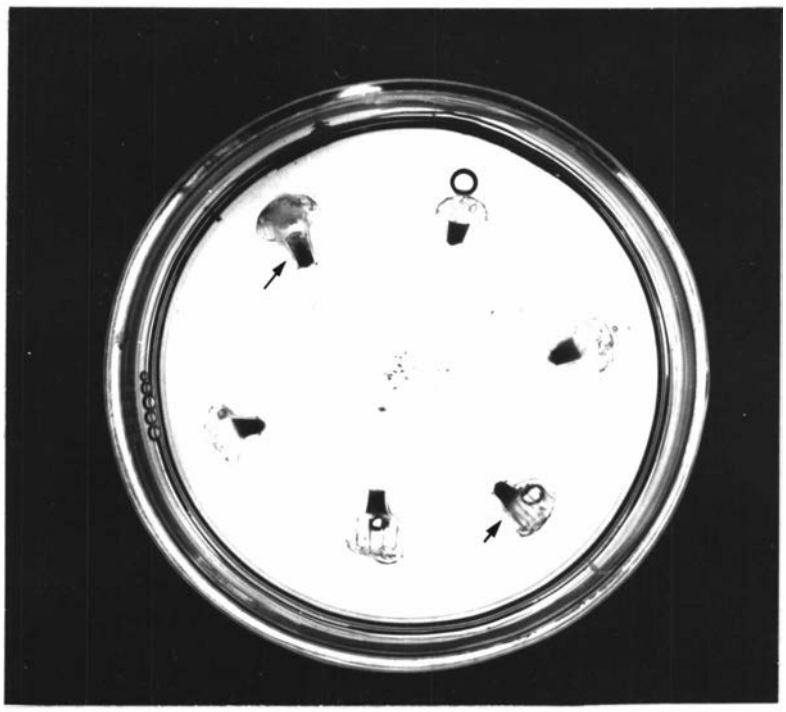


Figure 6.3a

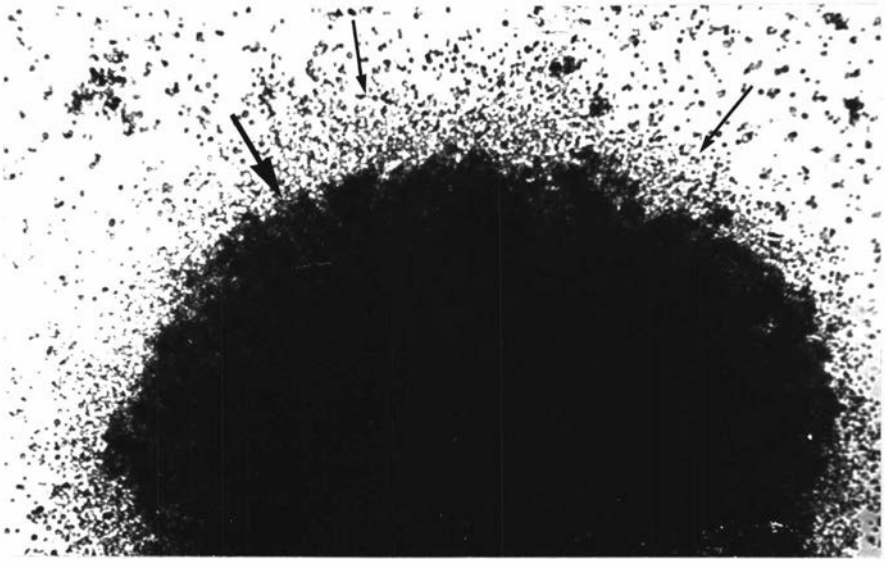
The uninhibited migration of ovine peritoneal exudate cells from a capillary tube after 16 hr in culture. The migration "fan" consists of a dense area (large arrow) visible to the naked eye and an outer (small arrows) zone of migration made visible by microscopy.

Unstained x 65

Figure 6.3b

Marked inhibition of peritoneal exudate cell migration caused by the presence of macrophage inhibitory factor in the culture medium.

Unstained x 65



Intradermal Testing of Sheep for Delayed-type Hypersensitivity to Tumour Antigens

I. Antigens

The antigens used in this test were OL_8, OL_9, NK and NL as detailed previously in this chapter.

II. Testing procedure

An intradermal injection of 0.1 ml of each antigen was made into the wool-free area of the inner flank in all sheep previously inoculated with tumour extracts OL_1, OL_2 and OL_3, as well as contact control animals. The injection sites of three contact control sheep and three sheep previously inoculated with tumour material were chosen at random from these and examined histologically at 48 hr after exposure to antigen.

RESULTS

Macrophage Inhibition Test

The percentage inhibition of macrophage migration for the various groups based on lymphocytes derived from contact control sheep and those originally inoculated with extracts of tumours OL_1, OL_2 and OL_3 are shown in Tables 6.I, 6.II, 6.III and 6.IV respectively. A summary of statistical analyses of results are shown in Tables 6.V, 6.VI and 6.VII. With one exception (sheep 72) there was no significant macrophage inhibition by supernatants from control tissue antigen stimulated lymphocytes whether or not lymphocytes were derived from control sheep or sheep previously inoculated with extracts of tumours OL_1, OL_2 or OL_3. A repeat test with sheep 72 gave a non-significant macrophage migration inhibition (Table 6.VII) thus being more in keeping with the results of other controls.

TABLE 6.I

Inhibition of Macrophage Migration Produced by Antigen-Stimulated Lymphocyte Cultures Derived From Contact Control Sheep

Sheep No.	C	Per cent. inhibition with:- NK	NL	OL_7	OL_8	OL_9
72 (1st test)	0	-9.0	32.6	-3.4	NT	NT
72 (2nd test)	0	NT	(12.5)	NT	12.5	-16.7
73	0	-9.6	-3.2	NT	0	-9.6
113	0	-13.4	4.5	-4.3	6.7	-6.6
114	0	-7.65	-9.3	-3.9	0	-8.7
115	0	9.83	8.1	3.5	-5.5	11.1
116	0	-18.7	-15.9	-22.7	-32.1	-22.1
117	0	-6.4	-6.5	6.5	-6.4	-9.6
118	0	-6.1	-9.4	NT	-9.4	-7.6
119	0	3.5	3.5	-3.4	3.5	10.4
121	●	-3.9	-13.2	-7.5	-5.6	11.3
122	0	-10.0	0	-13.3	3.3	3.3
123	0	9.5	-7.2	-1.5	13.6	5.4
124	0	-7.7	-12.3	NT	-19.2	-12.3
424	0	-2.8	-2.8	-2.8	-5.4	5.5

Key: NT = Not tested

C = Medium control - assumed to be nil

NK = Normal kidney antigen

NL = Normal lymph node antigen

OL_7, OL_8, OL_9 = Malignant lymphoma tumour extracts

TABLE 6.II

Inhibition of Macrophage Migration Produced by Antigen-Stimulated Lymphocyte Cultures Derived from Sheep Previously Inoculated with OL_1

Sheep No.	C	Per cent. inhibition with:- NK	NL	OL_7	OL_8	OL_9
401	0	-2.0	0.04	76.9	74.4	79.2
402	0	7.5	11.2	83.2	63.1	63.1
413	0	-13.3	-2.8	65.1	67.8	64.6
420	0	3.7	-3.7	NT	33.3	44.5
421	0	-11.3	-11.3	67.5	61.4	59.1
425	0	-11.1	-11.1	NT	5.6	5.6
426	0	9.5	0	71.5	85.7	62.0
432	0	-1.8	1.9	51.0	56.4	61.9
433	0	-20.0	-25.0	NT	-35.0	-25.0
434	0	-12.8	10.3	5.2	-12.8	15.4
435	0	3.6	-3.5	NT	3.6	10.7

Key: NT = Not tested

C = Medium control - assumed to be nil

NK = Normal kidney antigen

NL = Normal lymph node antigen

OL_7, OL_8, OL_9 = Malignant lymphoma tumour extracts

TABLE 6.III

Inhibition of Macrophage Migration Produced by Antigen-Stimulated Lymphocyte Cultures Derived from Sheep Previously Inoculated with OL_2

Sheep No.	C	Per cent. inhibition with:- NK	NL	OL_7	OL_8	OL_9
403	0	-13.0	8.7	NT	65.3	56.6
405	0	3.0	-2.9	NT	17.6	26.5
407	0	8.6	-4.6	27.5	24.2	12.6
410	0	-13.3	0	53.4	33.3	33.3
411	0	-2.8	5.8	25.8	37.2	57.2
414	0	0	-5.5	NT	16.7	16.7
415	0	5.9	5.9	26.5	64.8	26.5
416	0	-7.9	3.2	69.9	57.2	66.6
417	0	7.3	-7.3	-4.9	22.0	17.0
429	0	-2.6	2.7	6.6	-1.3	-14.0

Key: NT = Not tested

C = Medium control - assumed to be nil

NK = Normal kidney antigen

NL = Normal lymph node antigen

OL_7, OL_8, OL_9 = Malignant lymphoma tumour extracts

TABLE 6.IV

Inhibition of Macrophage Migration Produced by Antigen-Stimulated Lymphocyte Cultures Derived from Sheep Previously Inoculated with OL_3

Sheep No.	Per cent. inhibition with:-					
	C	NK	NL	OL_7	OL_8	OL_9
437	0	7.7	0	NT	-7.6	10.3
438	0	-1.4	-1.4	11.3	5.9	8.9
440	0	0	0	NT	-9.5	-9.5
441	0	-7.7	-11.5	NT	-7.7	-7.7
443	0	-3.0	12.2	75.8	69.7	54.6
444	0	-15.0	-15.0	NT	-15.0	-30.0

Key: NT = Not tested

C = Medium control - assumed to be nil

NK = Normal kidney antigen

NL = Normal lymph node antigen

OL_7, OL_8, OL_9 = Malignant lymphoma tumour extracts

TABLE 6.V

Analysis of Results of the Macrophage Migration Inhibition Test: Animals Whose Lymphocytes Showed Clear-cut Differences in Response to Control and Tumour Extracts

Sheep	Degree of Lymphocytosis	Inoculation Group	Antigens					
401	+ +	OL_1	NK	C	NL	OL_8	OL_7	OL_9
402	+ +	OL_1	C	NK	NL	OL_8	OL_9	OL_7
403	+ +	OL_2	NK	C	NL	OL_9	OL_8	
413	+ +	OL_1	NK	NL	C	OL_9	OL_7	OL_8
416	+ +	OL_2	NK	C	NL	OL_8	OL_9	OL_7
421	+	OL_1	NK	NL	C	OL_9	OL_8	OL_7
426	+ +	OL_1	C	NL	NK	OL_9	OL_7	OL_8
432	+ +	OL_1	NK	C	NL	OL_7	OL_8	OL_9

Antigens underscored by the same line show relationships which are not significantly different ($P > 0.05$) one with the other (as estimated by the F test of Kramer, 1956).

Key:

Scoring of degree of lymphocytosis:

+ + Persistent

\+ Sporadic

\- Absent

C Medium control - assumed to be nil

NK Normal kidney antigen

NL Normal lymph node antigen

OL_7, OL_8, OL_9 Malignant lymphoma tumour extracts

TABLE 6.VI

Analysis of Results of the Macrophage Migration Inhibition Test:

Animals Whose Lymphocytes Showed Less Clear-cut Overall

Differences in Response to Control and Tumour Extracts

Sheep	Degree of Lymphocytosis	Inoculation Group	Antigens
405	+ +	OL_2	NL C NK OL_8 OL_9
407	+	OL_2	C NL NK OL_9 OL_8 OL_7
410	+	OL_2	NK C NL OL_8 OL_9 OL_7
411	+	OL_2	NK C NL OL_7 OL_8 OL_9
415	+	OL_2	C NK NL OL_7 OL_9 OL_8
420	-	OL_1	NL C NK OL_8 OL_9
443	+	OL_3	NK C NL OL_9 OL_8 OL_7
72 (1)	-	CC	NL NK C OL_7 OL_8 OL_9

Antigens underscored with the same line show relationships which are not significantly different ($P > 0.05$) one with the other as estimated by the F test of Kramer (1956).

Key:

(1) 1st test

Scoring of degree of lymphocytosis: OL_7, OL_8, OL_9 = Malignant lymphoma tumour extracts

+ + Persistent

++ Sporadic

- Absent

CC Contact control

NK Normal kidney antigen

NL Normal lymph node antigen

C Medium control - assumed to be nil

TABLE 6.VII

Analysis of Results of the Macrophage Inhibition Test:

Animals Whose Lymphocytes Showed No Signficant Differences

in Response to Control and Tumour Extracts (P 0.05)

Sheep No.	Degree of Lymphocytosis	Inoculation Group	Sheep No.	Degree of Lymphocytosis	Group
414	+	OL_2	72	-	CC
417	+	OL_2	73	-	CC
425	-	OL_1	113	-	CC
429	-	OL_2	114	-	CC
433	-	OL_1	115	-	CC
434	+	OL_1	116	-	CC
435	-	OL_1	117	-	CC
437	+	OL_3	118	-	CC
438	+	OL_3	119	-	CC
440	+	OL_3	121	-	CC
441	-	OL_3	122	-	CC
444	+	OL_3	123	-	CC
			124	-	CC
			424	-	CC

Key:

Animals in which a trend of macrophage inhibition was observed in tumour extracts although not significant at P=0.05 as estimated by the F test of Kramer (1956).

Second test

CC Contact Control

Scoring of degree of lymphocytosis:

+ + Persistent

\+ Sporadic

\- Absent

The supernatants of lymphocyte cultures, exposed *in vitro* to tumour extracts OL_7, OL_8 and OL_9, from sheep inoculated during gestation or neonatally with cell-free tumour extracts OL_1, OL_2 or OL_3 caused significant ($P < 0.05$) inhibition of macrophage migration in 15 of the 27 tested animals. A further four of these cases showed a highly suggestive although less significant degree of inhibition. Of the 15 significant results, 7 were from the 11 animals which had been treated with OL_1, 7 were from the 10 which had been treated with OL_2 and only 1 from the group of 6 which had been treated with OL_3. There was no significant differences between reactions given by tumour antigens OL_7, OL_8 and OL_9.

Intradermal test for delayed type hypersensitivity

I. Examination of injection sites

No positive reactions in the form of erythema swelling or induration were detected in any of the sheep tested at 24 or 48 hrs after the intradermal injections.

II. Histological examination of injection sites.

No lesions consistent with delayed-type hypersensitivity were observed in any of the skin biopsy samples.

DISCUSSION

The results reported here demonstrate that the lymphocytes from a high proportion of sheep which had been experimentally inoculated with tumour material (Chapter 3) produce a factor which, when cultured in the presence of extracts derived from various ovine lymphoid tumours, is capable of inhibiting the normal migration of sheep peritoneal exudate cells. The phenomenon of inhibition of macrophage migration *in vitro* has been recognised since 1932 when Rich and Lewis (cited by Bloom, 1971) reported the depressed migration of macrophages from

splenic fragments of tuberculous rabbits and guinea pigs when cultured in the presence of tuberculin. Since this time, it has been the subject of considerable interest and research which has resulted in its development and quantitation to the stage where the macrophage migration inhibition test is generally recognized as having considerable potential as a technique for the investigation of cell mediated immunity. The test depends upon the ability of a proportion of lymphocytes to release a variety of substances, collectively known as lymphokines (Dumonde et al., 1969), when they are exposed to an antigen to which they have previously become sensitized. Migration inhibitory factor is one of these lymphokines. In guinea pigs the intradermal inoculation of lymphokines causes an erythematous reaction, with induration and a histological picture closely resembling that seen in a classical delayed-type hypersensitivity reaction (David, 1971). It therefore seems most probable that these substances, produced in vitro by sensitized lymphocytes in response to antigens, are the mediators of the delayed-type hypersensitivity reactions observed in vivo. They are therefore likely to be involved in any host immunologic response to cancer antigens which is of this nature.

The production of lymphokines by sensitized lymphocytes in response to subsequent antigenic stimulus is highly specific. David, Lawrence and Thomas (1964) showed that the migration of peritoneal cells from guinea pigs sensitized to dinitrophenol (DNP) - guinea pig albumin conjugate was inhibited by this particular antigen, but not by DNP - bovine gamma globulin, although the sera had comparable titers of anti-DNP antibodies in passive cutaneous anaphylaxis reactions against both. Other authors have similarly demonstrated the specificity of the reaction with bacterial products such as tuberculin (Bloom and Bennett, 1966), protein antigens (George and Vaughan, 1962), synthetic

polypeptides (David and Schlossman, 1968), carbohydrates (Gerety, Ferraresi and Raffel, 1970). This specificity has also been shown with viruses such as the fibroma virus (Tomkins, Adams and Rawls, 1970), transplantation alloantigens in the mouse (Al-Askari et al., 1965) and tumour antigens in the guinea pig and also with using whole tumour cells as a source of antigen (Kronman et al., 1969) or soluble tumour specific antigen (TSA) preparations in the guinea pig (Bloom, Bennett and Oettgen, 1969) in the laboratory mouse (Halliday and Webb, 1969). Nordin (1972) has stressed the importance in such experiments of detecting differences between tumour specific and histocompatability antigens. The latter are expressed on most tumour cells and the possibility that these may initiate production of MIF must be eliminated by adequate control cultures. In the present experiments both normal lymph node and kidney were employed as controls. Lymph node was chosen because this was considered to be the nearest normal tissue equivalent of a malignant lymphoma. Kidney was chosen, not only because of its usefulness in the expression of histocompatability antigens, but also because some of the malignant lymphoma tissue employed as antigen was derived from tumour nodules in the kidney and it was a possibility that some renal tissue might have been present as a contaminant. The only confusing result was that arising in the case of sheep 72, which apparently had developed hypersensitivity to the antigens of normal lymph node extract. Paradoxically, this extract was derived from a lymph node of this particular animal and it is possible that antigen to which the lymphocytes of this sheep had been previously sensitized was present in the preparation. In a repeat experiment with this particular animal's lymphocytes, the degree of macrophage inhibition was non significant and the first result may therefore have been due to some experimental error. With this one exception, none of the tests on animals showed significant levels of inhibition with any of the normal kidney or

lymph node extracts and none of the tumour extracts caused inhibition of migration when cultured with lymphocytes of uninoculated sheep.

In view of the specificity of the macrophage migration inhibition reaction it is clear that the lymphocytes from some sheep which had previously been exposed to malignant lymphoma antigens (OL_1 and OL_2) in the transmission experiments had become sensitized to at least one antigen which was common to all three tumour antigens (OL_7, OL_8 and OL_9) used in the test system. It seems likely, therefore, that the reaction of lymphocytes from sheep which had previously received tumour material was related to TSA(s) which were common to OL_1, OL_2, OL_7, OL_8 and OL_9. The antigenic relationship of tumour material OL_3 to the other tumours is less obvious. The lymphocytes of only one animal (sheep 443) in the group which had received OL_3 showed any notable reaction to the test tumour antigens and even then reaction with OL_9 was not significantly different from the reaction elicited by normal lymph node tissue (Table 6.VI). It is possible that inoculation of OL_3 material did not result in a positive "transmission". Evidence for this is seen in the small number of sheep showing lymphocytosis in this group when compared with those receiving OL_1 and OL_2 (Tables 6.V, 6.VI, 6.VII). The source material from which CL_3 was derived was a thymic form of malignant lymphoma and in cattle at least, this form of malignant lymphoma may be a distinct epidemiological and pathological entity (Bendixen, 1965; Dungworth et al., 1964; Hugoson, 1967). The same could be true for this form of malignant lymphoma in sheep.

Macrophage inhibition tests have not previously been described using sheep cells, and considerable preliminary experimentation was performed to establish the system described here. Most of the previous work has been carried out using small laboratory animals, in particular the guinea pig, and in such systems the recovery of PEC from sensitized

animals is relatively simple. In the present experiments the possibility of performing a direct MIF test utilizing PEC of experimentally inoculated animals was considered initially. Direct tests using PEC of sensitized animals have the advantage of providing both the lymphocyte and macrophage populations required in the assay in a single "operation". Without slaughtering these animals it was found impossible to recover sufficient PEC to perform the test by the direct method and the indirect method described here was used. Although an extra culturing step is involved there are certain advantages. The most important of these is that it is possible to achieve a degree of standardization of the batches of macrophages used for the test, because for any one day's experiments, all macrophages were derived from the same sheep. Nevertheless, slight variations in migrations areas occurred between tested sheep and within each test system with any particular batch of PEC. These variations were compensated for by the use of 2 x 6 replicates of capillaries for each antigen tested. This experimental design facilitated the statistical analysis of the results in such a way as to minimise the overall effect of small variations in the recorded migrations. The causes of variation which are intrinsic to the experimental method here have been examined by Hughes (1972) and are as follows:-

(a) Storage of PEC. The migration activity of guinea pig PEC is not significantly affected by up to four hours of storage in growth medium prior to capillary preparation. From four to five and a half hours a significant reduction of 9.7 per cent. occurs in control migration. This effect may have been observed in some of our cultures, because assembly of the chambers was performed between one and six hours after harvesting of the PEC.

(b) Preparation of capillaries: At least with guinea pig peritoneal cells, it is important to lead capillaries with equivalent cell numbers if even migration is to be obtained. Care should also be taken to cut the tubes cleanly and at right angles to their long axis. The latter procedure is subjective and errors may result in small fluctuations in recording of migration areas. After packing of the capillaries by centrifugation they should be mounted in chambers within 30 minutes to avoid deterioration. This factor should not have affected our results because chambers were assembled within five minutes of capillary centrifugation. The effect of air drying of cells before assembly is critical and consequently assembly of chambers must be made as soon as possible after cutting the capillary tube.

(c) Serum concentration: Variation in serum concentration in the culture medium causes considerable variation in capillary migration in the direct macrophage migration inhibition test in the system employed by Hughes. In current experiments, the concentration of foetal calf serum used in lymphocyte cultures was standardized, but its eventual concentration in the migration chambers could not be assessed, and variation due to this factor cannot be fully discounted.

(d) Hydrogen ion concentration: An increase in pH from 7.2 to 7.6 or 8.0 causes significant absolute reduction in both antigen inhibited and control PEC migrations but this does not influence the relative percentage inhibition between test and control preparations in the guinea pig system. For this reason the pH of the system employed here was carefully monitored. In the essentially open culture system employed it was found impossible to avoid some slight fluctuation in the pH of the medium and this probably accounted for some of the variation recorded between migration chambers.

(e) Anticoagulants: At certain concentrations, heparin may exert a deleterious effect on migration of macrophages. The low level employed

in these experiments and the effect of very thorough cell washing make it unlikely that the presence of heparin influenced the results.

(f) Concentration of antigen and duration of incubation: Hughes showed that differences in migration can be detected as early as two and a half hours after the beginning of incubation. He also found that higher concentrations of antigen accelerated the migration. As the antigen concentration employed in present experiments was unknown, but presumed to be low, tests were read at 16 hr, but sensitivity was not tested and the greatest expression of migration inhibition may have occurred earlier or later than this time.

(g) Incubation on a non-horizontal surface: The influence of gravity on migration may be considerable if the chambers are tilted. Providing cultures are arranged so that capillary tubes are radially opposed, this effect should be minimized when mean areas are computed. The effect does, however, lead to greater variance of within group computations and is best avoided. Placing the migration chambers on a level surface overcame much of this problem although exact level positioning of the capillaries within the chambers could not be guaranteed. Occasional tubes floated away from the "anchor" grease spots during incubation and were excluded from the experiment.

(h) Measurement of migration: During migration the macrophages form two definable areas. These comprise the primary zone (Figure 6) which is visible to the unaided eye, and an outer zone which is visible by transmitted light microscopy (Figure 6). Hughes reported that the outer limit seen by microscopy gave a more sensitive and accurate measure of migration and in the present experiments migration areas were estimated on this limit. In some cases it was difficult to define exactly because of cell-scatter from the tubes which resulted from movement of culture dishes and this factor probably accounted for some variation in recordings.

From the above considerations it is clear that the test results are potentially subject to a variety of errors arising from technical factors. In the present experiments those which appear most likely to have caused variation in results are the physical movement or tilting of the cultures, pH fluctuations and variation in the concentration of serum in the media. However, the possible presence of these variables has not presented any great difficulty in the evaluation of results. Many of these potential errors are likely to have affected controls as well as test preparations and those that do not come into this category have had their importance diminished by the 2 x 6 fold replicates of each test and the subsequent statistical analysis.

The use of vinyl tubing for this test has not previously been reported, although Hughes (1972) utilized polythene tubing in a similar fashion. Such plastic tubing has several advantages over the thin walled glass capillary tubing employed by others. Apart from being cheaper it is technically less difficult to cut accurately and is easily sealed and manipulated.

Tables 6.V, 6.VI and 6.VII show the relationship of the macrophage migration inhibition test to the degree of lymphocytosis induced by tumour extracts (Chapter 3). From inspection there is a relationship between animals with persistently elevated lymphocyte counts with those showing statistically confirmed positive tests and this suggests that the test may be an accurate indicator of the presumed pre-tumourous stage of malignant lymphoma. A growing body of evidence as surveyed by Burnett (1970), associates neoplasia with failure of adequate "immunosurveillance". It is tempting to suppose that the appearance of cells sensitized to tumour specific antigen and the development of the pre-clinical phase of malignant lymphomas may both stem from the failure of the sheep to eliminate a carcinogenic factor. The macrophage

migration inhibition test has been used to demonstrate cell mediated immunity to cancer in a variety of species. In man, Segal et al. (1972) have shown that tumour cells from 33 of 57 patients with malignant neoplasms, when cultured in the presence of autochthonous lymphocytes, caused the production of MIF as determined by the test. Of the tumours tested, 17 were malignant lymphomas and of these 10 caused the release of MIF from the patients own lymphocytes. Goldstein, Shore and Gusberg (1971) employing the leucocyte migration inhibition test, showed a similar situation in patients suffering from invasive squamous cell carcinomas of the cervix. Field and Caspary (1970) have reported sensitization to basic protein from the central nervous system in overt cases of tumours and suggest that detection of cell mediated immune reactions of this type may form the basis of a useful in vitro screening test for cancer. The present results indicate that at least in malignant lymphoma of sheep it may be possible by this means to detect a preclinical stage of this disease.

In view of the specificity of MIF production by sensitized lymphocytes, the cross reactivity of the tumour extracts tested here is of further significance. It has been observed on several occasions (Klein, 1971) that virally induced tumours share similar antigens while this is not the case for chemically induced cancers (Basombrió, 1970). The results obtained here may therefore be interpreted as further evidence for the viral aetiology of ovine malignant lymphoma.

The failure to demonstrate delayed type hypersensitivity in vivo in the experimental sheep by intradermal inoculation of tumour antigens used in the MIF test was not surprising. Tumour specific antigens are generally weak antigens and will not usually elicit strong reactions in such tests (Bartlett, 1972).

CONCLUSIONS

A modified macrophage migration inhibition test is extremely valuable for the investigation of specific cell mediated immunity in sheep previously exposed to tumour antigens. A proportion of sheep which receive cell free tumour material early in life show clear evidence of cell mediated immunity when tested *in vitro* with other malignant lymphoma antigens.

Present evidence based on six tumours indicates that malignant lymphomas of sheep share a common tumour specific antigen although there is some evidence that this may not be true of the thymic form of tumour.

Intradermal tests employing similar tumour antigens do not result in any measurable reaction in sheep which are positive to the macrophage migration inhibition test.

CHAPTER 7

IMMUNOSUPPRESSION OF SHEEP WITH PERSISTENT LYMPHOCYTOSIS

INTRODUCTION

The hypothesis that cancer results from a breakdown in the body's immunological surveillance mechanism was developed by Burnett (1970) and postulates that such a breakdown allows the proliferation of abnormal cells, with the eventual result of overt malignancy. The increasing use of powerful immunosuppressive drugs has provided an opportunity for testing the validity of this concept and there is now considerable evidence in the literature to suggest that the use of these agents can increase the incidence or accelerate the development of neoplasia in treated subjects. Investigations into tumour development in laboratory animals subjected to treatment with various immunosuppressive chemicals and antilymphocyte sera (ALS) have been reported by several workers (Allison and Law, 1968; Casey, 1968a, b; Hirsch and Murphy, 1968; Allison, 1970; Mellars, 1971; Zipp and Kountz, 1971). In humans undergoing immunosuppressive therapy for a variety of clinical and surgical conditions the observed frequency of tumours has also been higher than expected. This increase in frequency has been particularly notable in regard to the reticuloses. Penn and his colleagues (Penn, Halgrimson and Starzl, 1971; Schneck and Penn, 1971) discovered 17 cases of reticulum cell sarcoma and two cases of malignant lymphoma in a series of approximately 5,000 "transplant" patients who had been subjected to various immunosuppressive regimes. In all 17 cases azathioprine was used and in five of these ALS was also employed. This prevalence of tumours is approximately twice the expected level. Walder, Robertson and Jeremy (1971) found an increased

frequency of skin tumours in Australian patients treated with azathioprine and prednisone following kidney transplantation. In these cases hyperkeratotic changes were observed in the skin which were most likely the result of high levels of solar irradiation. Walder et al. considered the hyperkeratosis a preneoplastic lesion which subsequently could develop into overt neoplasia when potentiated by the immunosuppressive therapy. The significance of the numerous individual reports of neoplasia arising in small samples of patients treated with immunosuppressive agents such as those of Doak et al. (1969) and McKhann (1969), cannot be statistically confirmed. They do however lend general support to the larger series and the experimental work on laboratory animals.

The demonstration of specific cell mediated immunity in our own sheep showing presumed preclinical malignant lymphoma (Chapter 6) led us to consider the possible effects that suppression of the immune response of these animals may have. The possibility that this treatment could accelerate the development of overt neoplasia was the foremost consideration, although it was realized that the elimination of pre-malignant clones of lymphocytes was also a possible sequel. Consequently, three sheep showing a marked and persistent lymphocytosis as the result of malignant lymphoma transmission experiments (Chapter 3) were subjected to a course of treatment in which azathioprine and ALS were used in combination. This particular combination of drugs was utilized because of previous reports such as those of Penn and his colleagues indicating that overt neoplasia sometimes developed after their use.

MATERIALS AND METHODS

Animals

The sheep chosen for this experiment were three which had shown persistently high levels of lymphocytes following either antenatal or neonatal inoculation with extracts of tumour material. They were No's 401, 413 and 432 (see Chapter 3 for details of inoculation and lymphocyte counts).

Immunosuppressive Agents and Treatment Regime

I. Immunosuppressive Agents

Azathioprine and the globulin fraction of antilymphocyte serum (ALS) were used. The preparation of ALS by injection of foetal lamb thymocytes into a horse and its testing in healthy sheep is described in Appendix VII.

II. Treatment Regime

Azathioprine was administered orally in daily doses of 100 mg per sheep for the first 25 days of treatment. For the following 50 days, the daily dosage was reduced to 50 mg per sheep. This was equivalent to approximately 1 and 2 mg per kg respectively. Treatment with ALS was initiated at the same time as the azathioprine regime and continued at three day intervals for a total of 27 days. Each ALS treatment consisted of the intraperitoneal injection of the ALS globulin derived from 100 ml of "raw" ALS. Throughout the course of immunosuppressive treatment all sheep were given normal therapeutic doses of penicillin and streptomycin in order to protect them from any intercurrent bacterial infections.

Leucocyte Counts

Leucocyte counts were performed at intervals of three to five days throughout the period of immunosuppressive therapy and at less frequent intervals thereafter.

The methods were the same as those detailed in Chapter 3.

Skin Grafts

Full thickness skin grafts were made on each sheep one month after the beginning of immunosuppressive treatments to test the degree of suppression of allograft rejection. The skin over the right paralumbar fossa was shaved and swabbed with methylated spirits and tincture of benzalkonium chloride. This area was anaethetized with a subcutaneous injection of two per cent. lignocaine solution using an "L" block technique. Within this area two separate pinch grafts of approximately one centimetre diameter were made. One was sutured back into its original place using 4/0 monofilament nylon suture material with an attached atraumatic needle and the other was transferred to a similar site on one of the other animals. In its place was grafted skin from a different donor. As a result, each sheep had two skin grafts applied, one autograft and the other an allogeneic homograft from one of the other sheep. Graft survival was judged by the appearance of the grafted skin. In particular examination for evidence of necrosis, hardening and sloughing was made.

Macrophage Inhibition Test

Nine months after the cessation of immunosuppressive treatment the lymphocytes of the three treated animals were tested for MIF production in response to tumour specific antigens by the macrophage migration inhibition test described in Chapter 6. The tumour antigens used in this test were those previously designated OL_8 and OL_9.

Examinations for Neoplasia

The peripheral lymph nodes were examined by palpation at approximately monthly intervals. In addition approximately one month following the final azathioprine treatment, exploratory laparotomies were performed on the sheep and abdominal organs were examined visually

and by palpation for evidence of tumours. The surgical approach was made through the right paralumbar fossa, ventral to the skin graft sites.

RESULTS

The Effect of Immunosuppressive Agents on Leucocyte Numbers

During the three days following the initial treatment with immunosuppressive drugs there was a rapid and precipitous drop, of the order of 65 per cent., in the numbers of circulating lymphocytes. Thereafter the levels of these cells fluctuated slightly but remained within the accepted normal range for sheep. At the time of writing, nine months have elapsed since treatment was initiated. The mean lymphocyte counts of these sheep are shown on Figure 7.1

The Effect of Immunosuppressive Agents on Survival of Skin Grafts

There was no evidence of rejection at any time of either homografts or autografts in any of the sheep. After three weeks the boundary between host and graft skin could be determined only by the presence of the "ring" of sutures. All grafts were still intact after 10 months.

Macrophage Migration Inhibition Test

The percentage inhibitions and the summary of the statistical analyses of these are shown on Table 7.I. The degree of migration inhibition is less in all sheep when compared to the assays performed before the immunosuppressive treatments were started (compare with Table 6.I). This is most apparent in the case of sheep 401 in which the macrophage migration in the presence of lymphocyte culture supernatant fluids from cultures to which tumour extracts had been added were not significantly different from the medium control. In the test for MIF production by lymphocytes from the other two sheep however, a statistically significant inhibition of migration was still detectable

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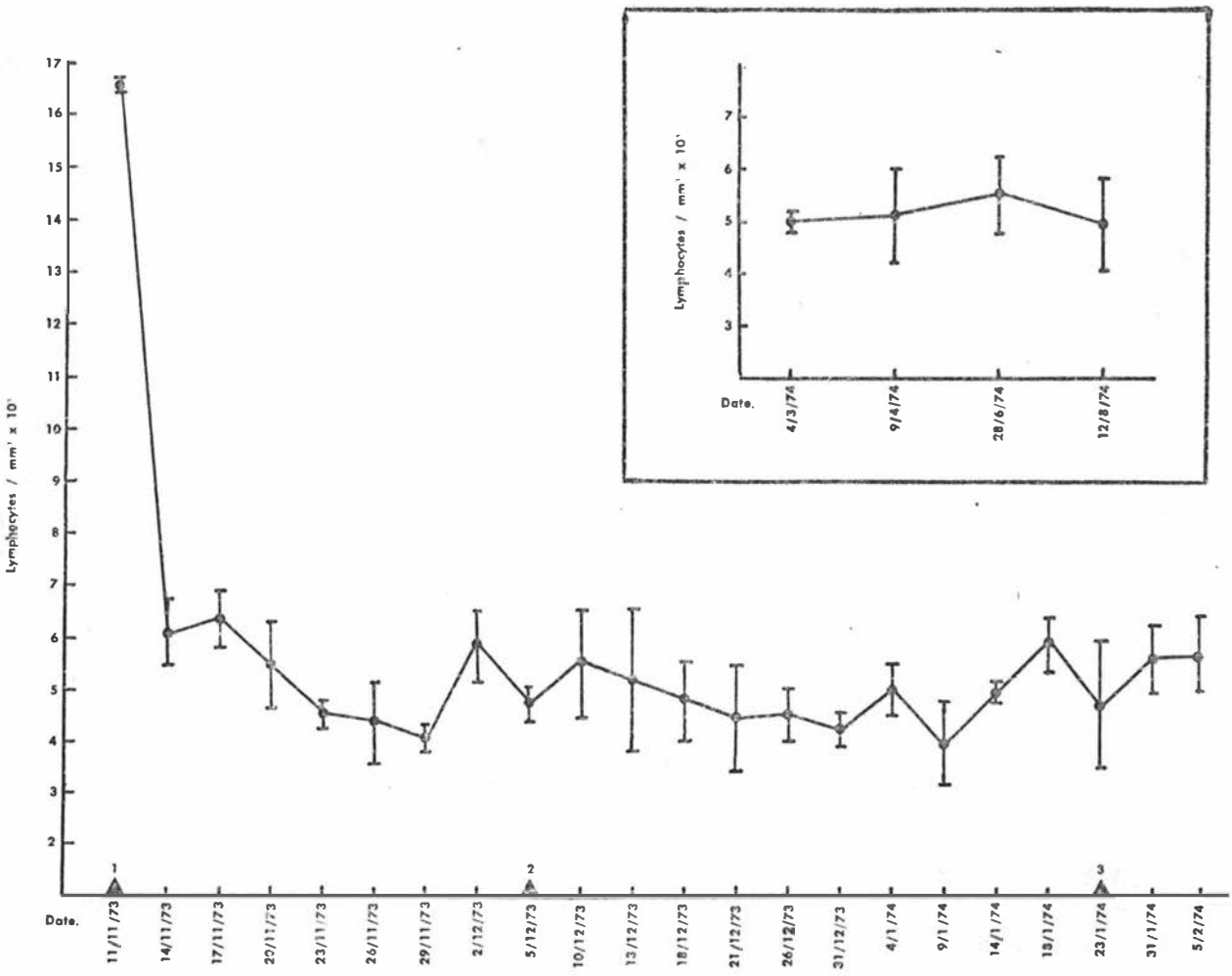

1 Commencement of immunosuppressive treatment.

2 Final ALS treatment.

3 Final azathioprine treatment.

Figure 7.1

The mean lymphocyte counts and standard errors of means of sheep treated with immunesuppressive agents. Results recorded at longer time intervals are shown in the inset.

TABLE 7.I

Results and Statistical Analysis of the Inhibition of Macrophage Migration Produced by Tumour Antigen-Stimulated Lymphocyte Cultures Derived from Immunosuppressed Sheep.

Sheep No.	Percentage inhibition with		
	C	OL_8	OL_9
401	0	25	25
413	0	45	55
432	0	53	48

Sheep No.	Significance of results of 95 per cent. confidence levels with antigens		
401	C	OL_8	OL_9
413	C	OL_8	OL_9
432	C	OL_8	OL_9

Antigen underscored by the same line showed no significant differences ($P > 0.05$) as assessed by the F test of Kramer (1956).

C = Medium control

following the course of immunosuppressive treatment.

Examination of Sheep for Overt Neoplasia

No diffuse or nodular enlargement was detected in any of the palpable peripheral lymph nodes at any time up to nine months after cessation of the immunosuppressive regime. No tumourous lesions were seen or palpated in the liver, kidneys, spleen, lymph nodes or other abdominal viscera at exploratory laporotomy one month after cessation of the immunosuppressive course.

DISCUSSION

The precipitous fall in circulating lymphocytes in the animals treated with immunosuppressive agents was the most outstanding feature of these experiments. In view of the pilot trials using ALS in sheep prior to this experiment (Appendix VII) and reports in the literature of the effect of ALS in other species (Gray et al., 1966; Taub and Lance, 1968; Denman and Frenkel, 1968) this profound and prompt decrease was not unexpected. However, cessation of treatment is usually associated with the gradual recovery of lymphocyte numbers to their pretreatment level (Lance, Medawar and Taub, 1973) although following closely spaced ALS treatments the lymphopenia may be persistent and take considerably longer than the three to four weeks usually required for recovery after single doses of ALS. In the present experiment the lymphocyte levels were reduced from abnormal to normal ranges and these have remained normal for at least nine months from the start of treatment. It is generally assumed that ALS selectively removes thymus dependent cells (Lance et al., 1973) and it might therefore be concluded that the cells responsible for the lymphocytosis are, in fact, thymus dependent cells (T-cells). However, the effect may have been confused by the action of azathioprine which suppresses both humoral (B-cell dependent) as well as cell mediated (T-cell

dependent) compartments of the immune system. No overt tumours developed in any of the sheep. This may have been due to insufficient lapse of time and this explanation has some support from the parallel situation of delayed tumour development in man subsequent to immunosuppressive therapy (Doll and Kinlen, 1970; Walder et al., 1971). Alternatively it is possible that the aetiological agent of malignant lymphoma is closely and exclusively associated with the lymphocytes responsible for the lymphocytosis. Their destruction by the immunosuppressive agents might therefore result in the removal of all or most of the malignant lymphoma agent from the sheep. This explanation seems very unlikely. In mice and cats for example, malignant lymphoma viruses have been shown to be present in a variety of cells including macrophages and megakaryocytes (Gross, 1970; Jarrett, 1966), and in the chicken the virus of Marek's disease can be found in feather follicles in the pretumourous stage of the disease (Witter et al., 1970).

The retention of the homografts for over nine months indicates that the treatment regime was highly successful in the suppression of cell mediated immunity as it is usually assessed. In spite of this, the macrophage migration inhibition tests indicate that there is still a degree of presumed tumour specific immunity in two of the three treated animals. This may provide some explanation for the failure, at this stage, of the experiment to enhance tumour development. On balance the most satisfactory explanation for the absence of signs of overt malignant lymphoma appears to be the time factor, and it would be premature to rule out the subsequent appearance of tumours consequent to the experimental immunological suppression.

CONCLUSIONS

Combined treatment with azathioprine and the gamma globulin of anti-lymphocytic serum of sheep with induced lymphocytosis causes a return from high to normal numbers of circulating lymphocytes. The treatment also results in total suppression of rejection of allogeneic homografts for at least nine months. From the limited data available it seems that tumour-specific cell mediated immunity may also be significantly suppressed in some but not all sheep. It appears that the elapse of nine months is insufficient time for overt tumour development to follow immunosuppressive treatment.

CHAPTER 8

GENERAL DISCUSSION

Models for experimental tumour research have been provided to a large extent in the past by conventional laboratory animal species such as the mouse. It should, however, be pointed out that the parallel tumours of outbred domestic animals may mirror their human counterparts more accurately. Experimental data derived from tumour models of conventional laboratory animals must be viewed in the context engendered by intensive inbreeding and strain development. The position arising from such manipulation, with its resultant genetic homogeneity and definition, can in certain experimental situations be of great advantage. However, the knowledge gleaned from such cancer research is often difficult to extrapolate to the field of human cancer. It is in this respect that outbred animal models offer much promise. The New Zealand environment in which sheep are farmed intensively provides an ideal situation for such comparative studies. Beside forming a large reservoir of animals in a comparatively natural environment, sheep may be relatively easily maintained under laboratory conditions for variable lengths of time, are of convenient size in comparison to other domesticated animals such as the cow and are relatively inexpensive to purchase and maintain.

The study of malignant lymphoma in sheep appeared to offer considerable advantages as an experimental model for cancer research. Evidence is mounting to show that certain lymphoid tumours in both animals and man may be related to infections with certain viruses. At the same time immunological factors associated with the development and progress of neoplasia have received increasing attention. The ovine model

offered a practical opportunity to study these two aspects in relation to one another in standard farm animals that were maintained in a conventional rural environment.

The pathological studies incorporated within this thesis were not intended to be exhaustive in nature. They do, however, confirm that malignant lymphoma of sheep as it occurs in this country, shows the basic morphological features reported by other workers studying malignant lymphoma in sheep overseas. In nearly all cases which were investigated, generalized lymphadenopathy was the outstanding feature. Other commonly involved tissues were spleen, liver, kidney, bone marrow, heart and intestines. Striking parallels were noted between the gross features of malignant lymphoma of sheep and those of malignant lymphoma in other species, in particular cattle. However, insufficient cases of ovine malignant lymphoma were available to allow any correlation of anatomical forms of the disease with epidemiological data as proposed by Anderson et al. (1969). Neither was it possible to determine whether or not the disease exists in this country in an enzootic form such as has been described in cattle and sheep in certain parts of Europe. The occurrence of two cases from each of two properties in this limited study may be indicative of clustering of cases similar to that reported in regard to malignant lymphoma in some bovine and feline populations.

Lymphosarcoma was the main type of malignant lymphoma recorded, although three cases of reticulum cell sarcoma were also seen. The former were most commonly composed of lymphoid cells showing poor differentiation and a variety of non-specific ultrastructural alterations which have been reported with some frequency in many other types of neoplasm. Particles consistent with viral morphology could not be convincingly demonstrated in freshly fixed tumour material but it is acknowledge that the chances of demonstrating virus in this way are poor.

With the exception of the work performed by Paulsen and his colleagues (Chapter 2, 3 and 5) investigation into malignant lymphoma of sheep has been restricted to somewhat superficial epidemiological and pathological studies derived largely from sheep examined in slaughter-houses or at diagnostic stations. Therefore, the bulk of our work has been concentrated on other experimental approaches to this problem. We have explored the hypothesis that this disease of sheep is transmissible, and is caused by an oncogenic virus. Consequently the experiments have been designed around a flock of sheep which were inoculated *in utero* or at birth with various ovine cell-free tumour extracts derived from spontaneously occurring malignant lymphomas. Perhaps the greatest personal disappointment in this project was the failure to conclusively demonstrate by the production of overt tumours that malignant lymphoma is transmissible. However the development of persistent lymphocytosis in a high proportion of these sheep as a result of this treatment was exciting, as this could indicate a preclinical phase of the disease. This hypothesis is justified on several grounds. In the spontaneously occurring form of the disease in both sheep and cattle, persistent lymphocytosis is frequently reported as a preclinical phase of the disease. It has also been shown to occur in experimentally induced cases of the disease in cattle. In sheep, lymphocytosis has been observed prior to development of overt neoplasia in animals inoculated with tissue culture material containing bovine "C-type" virus particles. In the experiments reported here futher support for this conclusion is afforded by:-

(a) The demonstration of a strong correlation between animals showing lymphocytosis, and those with lymphocytes sensitized to apparently tumour specific antigens as demonstrated by macrophage migration inhibition tests, and

(b) The presence of virus-like particles in lymphocytes stimulated by antigen which were derived from sheep showing lymphocytosis and

the absence of such virus-like particles in similar preparations from normal sheep. These virus-like particles also have a morphology similar to "A-type" particles in mice and those demonstrated by Paulsen et al. (1972) in lymphocytes of sheep with overt malignant lymphoma.

Although no overt tumours have as yet developed in these sheep, it should be noted that these sheep have not yet attained the age at which spontaneous cases of malignant lymphoma commonly occur . Furthermore, in the virally induced malignant lymphoma of chickens, mice and cats, infection by the virus in clinically normal animals has been shown to be common while the development of neoplasia is a comparatively rare event. Whereas the original hypothesis "that malignant lymphoma of sheep is a transmissible disease" has not yet been fully proved, the results obtained are still of considerable importance. Our current hypothesis is that the persistent lymphocytosis in sheep inoculated with malignant lymphoma extracts is a precancerous state and as it can be readily monitored haematologically and immunologically it provides an ideal model for the study of preneoplastic events in general.

The fact that the observed lymphocytosis apparently followed inoculation with cell-free tumour extracts suggests strongly that the aetiological factor is infectious and, in the light of evidence from other models of malignant lymphoma in chickens, mice and cats, that it is a virus. The search for direct virus involvement in spontaneously occurring malignant lymphoma in sheep has been pursued without success by attempts to visualize it in fresh tumour tissues and in cultured lamb kidney cells which were inoculated with tumour material. The possible reasons for the failure of these approaches were considered in Chapters 2 and 4. It was not until cultured phytohaemagglutinin-stimulated lymphocytes of experimentally inoculated sheep were examined (Chapter 5) that virus-like particles could be readily and regularly demonstrated by electron microscopy. Furthermore the demonstration of common

antigenic specificities between the various malignant lymphoma extracts used in the macrophage migration inhibition tests adds weight to our hypothesis that this disease is of viral aetiology.

The immunology of neoplasia is today one of the most rapidly developing areas of cancer research. Not only is the knowledge of the basic mechanisms involved in cancer immunity expanding at a great rate but already prophylactic vaccination against two spontaneously occurring neoplastic diseases, fibromatosis of cattle and Marek's disease of chickens, is a practical reality. The development of these vaccines represents a considerable advance and it is reasonable to suppose that experience and knowledge derived from their application may benefit human cancer therapy and prophylaxis in the future. In its simplest form, immune responses to various tumour antigens may be attributable to both humoral and cell mediated divisions of the body's immunological system. Because the latter appears to be the more important in tumour immunity we were particularly impressed by the demonstration of a specific cell mediated reaction toward various tumour antigens in our experimentally inoculated sheep (Chapter 6). In many ways the demonstration of MIF production by the sensitized lymphocytes raises more questions than it answers. Firstly, we have no precise idea of the nature of the antigen involved in this reaction. It certainly appears to be common, at least to five of the six tumours tested and considered against the inactivity of control tissues is therefore probably a tumour specific antigen. The negative results recorded in the in vitro situation raises questions as to its significance in the pretumourous state. For development of tumours, does this state of immunity need to be suppressed, or in other words is this an effective form of tumour immunity? The immunosuppression experiments described in Chapter 7 were attempts to clarify this position. Unfortunately in terms of tumour development they have to date been inconclusive. Perhaps time is the important

factor here. The identity of the cells responsible for the lymphocytosis is also in question. If these are potentially malignant and if they are T-cells as suggested by the effect of the treatment regime, then perhaps this is a pretumourous phase of that situation recorded by Catovsky et al. (1974) in certain human lymphoblastic leukaemias in which the malignant lymphoblastic cells were shown to exhibit the characteristics of T-cells.

Despite these uncertainties, an attempt must be made to assess the practical significance of the work recorded in this thesis. From strictly veterinary aspects the present evidence indicates that malignant lymphoma in sheep is of little immediate economic importance in New Zealand. However, as a cause of stock losses in certain other countries this disease is of greater consequence and environmental or other changes may induce a similar situation here. It is now realized that at least in Germany and France, malignant lymphoma of sheep exists in an enzootic form (Chapter 1). It is reasonable to suggest that if not controlled it could reach the epizootic proportions of malignant lymphoma in cattle seen in parts of Europe. For this reason it is important that the ovine disease is recognized and controlled here in New Zealand. This could entail appropriate quarantine regulations perhaps utilizing haematological or immunological tests on imported sheep suspected of this disease, as well as the examination of locally recorded cases.

Any disease occurring in food animals must be considered in the light of its public health importance. If, as seems reasonable, we assume that malignant lymphoma of sheep is caused by a virus, two questions immediately arise. Can it infect man and if so, what effects might such infection have? It has been demonstrated, that as a group, the oncorna viruses causing malignant lymphoma in the various animal

species are closely related. Cross reactivity has been shown in immunological tests between oncorna viruses of cats and laboratory rodents (Geering et al., 1968). This is also true for the bovine and ovine viruses tested (Paulsen, pers. comm). Cross species transmissibility has been shown with the Rous sarcoma virus of the chicken (reviewed by Gross, 1970), Moloney and Gross viruses of the mouse (reviewed by Gross, 1970), feline sarcoma viruses (Theilen et al., 1970; Theilen, 1971) and bovine lymphoma virus (Olson et al., 1972). Furthermore, the feline lymphoma viruses have been reported to replicate readily in cultured human, canine and porcine cells (Jarrett Laird and Hay, 1970). At this stage it would therefore seem imprudent to entirely discount the possibility of oncorna viruses from animals infecting man and perhaps even causing human tumours. Essex (1972) considered this possibility and suggested that under conditions of temporary immunological suppression, such as observed in many of the childhood viral diseases, that such infection with feline oncorna virus was worthy of consideration. This probability has not however been substantiated by epidemiological studies conducted in households from which cases of feline malignant lymphoma have been recorded (Dorn et al., 1968) but it cannot be completely discounted.

If these viruses are potentially pathogenic to man, then it is of importance to limit their spread from the animal reservoir. It is well recognized in the chicken, laboratory rodent and cat that infection by the viruses is widespread in clinically normal animals (Chapter 1). While the most important method of spread in the mouse has been shown to be by "vertical" transmission of the virus, "horizontal" infection has been demonstrated for the cat and chicken. The handling by humans of infected animals of the latter species could therefore constitute a health risk. As the route of virus transmission is unknown for the other domesticated animal species, the risk involved in regard to sheep is purely speculative. Our present experiments provide some information

on the transmissibility of the putative malignant lymphoma agent. In spite of the close association of contact control sheep with those that had developed lymphocytosis after receiving injection of cell-free tumour material, none of the contact control sheep developed either lymphocytosis or evidence of cell mediated immunity to specific tumour antigen. It therefore appears that natural transmission, even to the same species, is not easily achieved in a horizontal manner under natural field conditions. Vertical transmission from ewe to lamb appears to be the most likely natural route of transmission. Whether this is transplacental or via milk clearly requires further investigation.

Finally this project has seen the development of a potentially useful model for comparative research in neoplasia. Perhaps its most valuable contribution is in the provision of a transmissible presumed precancerous state. To realize this potential considerable work will be involved. One of the most pressing objectives must be the isolation and characterization of the virus-like particles already seen in lymphocyte cultures. Once this is achieved there is a wide field of research then open. Possible projects include the following:-

(a) Comparison of the isolates with other known oncogenic viruses.
(b) Determination of the distribution of the virus within sheep populations at risk using immunological techniques.
(c) Establishment of continuous cell culture lines which will support and allow replication of virus in sufficient quantity for further immunological studies and closer investigation of virus-cell relationships.

It would also seem appropriate even at this early stage, to investigate lymphocytes from humans and other animals with and without reticuloses

to see if they show the release of MIF when exposed to sheep tumour antigen.

Clearly the extension of this work to projects such as those suggested above is a large step, but it is nevertheless a logical one and consistent with the major goal of cancer research, namely the understanding and prevention of the cancerous state in man.

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APPENDIX I

Reagents and Procedures Used in Preparation of Tissues for Electron Microscopy

Fixation:

Modified Karnovsky's Fixative

Formaldehyde	2 ml
Glutaraldehyde	3 ml
$Na_2HPO_4.12H_2O$	2.51g
KH_2PO_4	0.41g
H_2O	95 ml

Dissolve $Na_2HPO_4.12H_2O$ and KH_2PO_4 in water to make 0.1M phosphate buffer, then add formaldehyde and glutaraldehyde. Store at 4°C.

Cut tissue for fixation into cubes of approximately $1mm^3$, add adequate fixative and allow the specimen to fix for at least four hours at 4°C.

Wash in chilled 0.1M phosphate buffer for 30 min. and then post-fix in 1 per cent. OsO_4 in 0.1M phosphate buffer for two to four hours at 4°C.

After post-fixation wash in chilled 0.1M phosphate buffer for 30 min and then briefly in distilled water at 4°C.

Dehydration:

Dehydration of fixed tissues is carried out in a graded alcohol/ propylene oxide series as follows:

25% ethanol/H_2O	15 min	at 4°C
50% ethanol/H_2O	15 min	
75% ethanol/H_2O + 1% Uranyl acetate	0.5 to 24 hr	
95% ethanol/H_2O	30 min	
100% ethanol	30 min	at room temperature
100% ethanol	30 min	
50% ethanol/propylene oxide	15 min	
100% propylene oxide	15 min	

Infiltration and Embedding with Resin: (1)

Dehydrated tissue specimens are placed in 25 per cent. resin with propylene oxide and left overnight at room temperature. The propylene oxide is gradually evaporated and remaining resin is discarded at the end of this time and replaced with fresh 100 per cent. resin in a gelatin capsule.

Polymerization of the resin is effected by heating at 60°C for 36 hr.

(1) Durcupan-ACM, Fluka, Switzerland.

APPENDIX II

Proposed Patho-Anatomical Classification of the Malignant Lymphomas of Animals

Following are the definitions of terms used in the proposed classification of Anderson, Jarrett and Crighton (1969):

"Multicentric lymphosarcoma: disseminated lymphosarcoma in which bilateral enlargements of superficial lymph nodes and variable infiltration of other organs are usual. The organs most commonly involved are the liver, spleen, kidneys, lungs, myocardium, gastro-intestinal tract and bone marrow.

Thymic lymphosarcoma: lymphosarcoma which appears to start or grow most rapidly in the thymus. The thymus may be the only obviously affected site, or the condition may be disseminated with lymph node involvement and infiltration of other organs. The major lesion is always in the thymus, which becomes totally replaced by the neoplasm.

Alimentary lymphosarcoma: lymphosarcoma in which the major lesions occur in the gastro-intestinal tract and mesenteric lymph nodes. One of these sites may be affected, though usually both are involved. The superficial lymph nodes are not affected and splenomegaly is unusual. The liver is infiltrated in 50% of cases, but other organs are only rarely involved.

The skin form: disseminated lymphosarcoma in which infiltration of the skin and subcutaneous tissue produces nodular thickening and loss of hair. There is usually an associated multicentric complex.

Leukemia: absolute lymphocyte count greater than 30,000/mm^3 with or without circulating lymphoblasts, or a count of less than 30,000/mm^3 with an abnormally high proportion of one cell type."

APPENDIX III

The Preparation of Reagents and Media for Use in Tissue Culture Experiments

1. Water (H_2O)

Water for use in solutions is deionised and passed through a bacteriological filter.

2. Sterilization

Solutions are sterilized by autoclaving for 15 minutes at a pressure of 15 lbs per square inch, or by passing through millipore filters of 22μ pore diameter.

3. Hank's Balanced Salt Solution (HBSS)

Materials

$CaCl_2$	1.4 g	Solution 1
H_2O	200 ml	
Glucose	15 g	Solution 2
NaCl	8.0 g	
KCl	4 g	
$MgSO_4.7H_2O$	2 g	
KH_2PO_4	0.6 g	
$Na_2HPO_4.2H_2O$	0.6 g	
Phenol red	0.2 g	
H_2O	800 ml	

Method

(i) Mix solutions 1 and 2 separately.

(ii) Combine solution 1 and 2.

(iii) Add 2 ml of chloroform.

(iv) Dispense in 100 ml screw-top bottles and store at $4^{o}C$.

(v) This solution is 10 times concentrated and is diluted before use.

To use

(i) Add nine parts of water to one part of concentrated solution.

(ii) Dispense in 100 ml screw-top bottles and autoclave.

(iii) Immediately before use add 0.2 ml of antibiotic solution (see below) and adjust pH to desired level by addition of either 0.1M. HCl or 7.5 per cent. $NaHCO_3$.

4. Phosphate Buffered Saline (PBS)

Materials

NaCl	8.0 g	Solution 1
KCl	0.2 g	
$Na_2HPO_4.2H_2O$	1.15 g	
KH_2PO_4	0.2 g	
Phenol red	0.2 g	
H_2O	800 ml	
$CaCl_2$	0.1 g	Solution 2
H_2O	100 ml	
$MgCl_2.6H_2O$	0.1 g	Solution 3
H_2O	100 ml	

Method

(i) Mix solutions 1, 2 and 3 separately.

(ii) Sterilize each by autoclaving.

(iii) When cool mix and dispense in 100 ml screw-top bottles.

(iv) Store at $4^{o}C$.

For Use

(i) Add 0.2 ml antibiotic solution per 100 ml solution.

(ii) Adjust pH to desired level by addition of either 0.1M HCl or 7.5 per cent. $NaHCO_3$.

5. Calcium and Magnesium Free Phosphate Buffered Saline

Materials

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	0.15 g
KH_2PO_4	0.2 g
H_2O	1000 ml

Method

(i) Dissolve reagents in water and dispense in 100 ml screw-top bottles.

(ii) Sterilize by autoclaving.

(iii) Store at $4^{\circ}C$.

For Use

(i) Add 0.2 ml antibiotic solution per 100 ml solution.

(ii) Adjust pH to desired level by addition of either 0.1M HCl or 7.5 per cent. $NaHCO_3$.

6. Trypsin Solution (0.25 per cent.)

Materials

Trypsin[1] (1:250)	1 g
PBS (Calcium and magnesium free)	400 ml

Method

(i) Dissolve trypsin in PBS.

(ii) Sterilize by filtration.

(1) Difco, Detroit, Michigan, U.S.A.

(iii) Dispense in 100 ml screw-top bottles.

(iv) Store at -20°C.

7. Trypsin and Versene Solution

Materials

Trypsin (1:250)	2.5 g
NaEDTA	0.2 g
NaCl	8 g
KCl	0.2 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15 g
NaOH	0.067 g
H_2O	1000 ml

Method

(i) Combine reagents.

(ii) Sterilize by filtration.

(iii) Dispense in 20 ml screw-top bottles.

(iv) Store at -20°C until use.

8. Trypan Blue

Materials

Trypan blue	0.1 g
PBS	100 ml

Method

(i) Dissolve trypan blue in PBS.

(ii) Store at 4°C.

9. Growth Medium (for foetal lamb kidney cultures)

Materials

Lactalbumin hydrolysate	5.0 g
HBSS	900 ml
Yeast extract	1.0 g
Bovine serum (filtered)	100 ml

Method

(i) Dissolve the lactalbumin hydrolysate and yeast extract in HBSS.

(ii) Dispense solution in 40 ml aliquots in 100 ml bottles.

(iii) Sterilize by autoclaving.

(iv) Store at $4^{o}C$.

For Use

(i) To each 90 ml of solution add 10 ml bovine serum and 0.2 ml antibiotic solution.

(ii) Adjust pH using 0.1M HCl or 7.5 per cent. $NaHCO_3$.

(iii) Warm to $37^{o}C$ before adding to cultures.

10. Growth Medium (for lymphocyte cultures)

Materials

Eagles minimal essential medium[2] (MEM)	10 g
H_2O	800 ml
Foetal calf serum[3]	150 ml

Methods

(i) Dissolve MEM in H_2O.

(ii) Add foetal calf serum.

(2) Wellcome Ltd., Otahuhu, N.Z.

(3) Flow Laboratories, Irvine, Scotland.

(iii) Sterilize by filtration.

(iv) Dispense in 100 ml screw-top bottles.

(v) Store at $4^{o}C$.

To Use

(i) Add 0.2 ml antibiotic solution per 100 ml media.

(ii) Adjust pH with 0.1M HCl or 7.5 per cent. $NaHCO_3$.

11. Antibiotic Solution

Materials

Benzyl penicillin(4)	10^6 IU
Dihydro-streptomycin(4)	1 g
H_2O	20 ml

Method

(i) Dissolve penicillin in 10 ml H_2O.

(ii) Dissolve streptomycin in 10 ml H_2O.

(iii) Combine both solutions.

(iv) Dispense in 5 ml bottles.

(v) Store at $-20^{o}C$.

To Use

(i) Add 0.2 ml of solution in 100 ml of fluid to give a final antibiotic concentration of 100 IU penicillin and 100 µg streptomycin per millilitre.

(4) Glaxo, Palmerston North, N.Z. Ltd.

APPENDIX IV

Identification of sheep in experimental flock with designation of tumour inocula, time of inoculation and cause of death in individual animals up to October 1974.

Lamb No	Inoculum	Time of Inoculation	Fate of Lamb
401	OL_1	68 days of gestation	Alive
402	OL_1	Neonatal	Alive
403	OL_2	Neonatal	Alive
404	OL_1	54 days of gestation	Death from Haemonchosis 12/3/72
405	OL_2	53 days of gestation	Alive
406	OL_2	Neonatal	Lost before July, 1972, cause unknown
407	OL_2	Neonatal	Alive
408	OL_1	53 days of gestation	Death from neonatal starvation and exposure
409	OL_2	Neonatal	Death from sinusitis and encephalitis following dog bite
410	OL_2	Neonatal	Alive
411	OL_2	Neonatal	Alive
412	OL_1	57 days of gestation	Death from pneumonia 10/11/71
413	OL_1	56 days of gestation	Alive
414	OL_2	45 days of gestation	Alive
415	OL_2	45 days of gestation	Alive
416	OL_2	Neonatal	Alive
417	OL_2	Neonatal	Alive
418	OL_2	45 days of gestation	Death from neonatal starvation and exposure
419	OL_1	55 days of gestation	Lost before July 1972, cause unknown
420	OL_1	Neonatal	Alive
421	OL_1	Neonatal	Alive
422	OL_2	Neonatal	Lost before August, 1972 cause unknown
423	OL_2	52 days of gestation	Died with septicaemia 10/10/71
424	Normal lymph node	52 days of gestation	Alive

Continued

Appendix IV continued

Lamb No	Inoculum	Time of Inoculation	Fate of Lamb
425	OL_1	Neonatal	Alive
426	OL_1	Neonatal	Alive
427	OL_1	Neonatal	Alive
428	OL_1	Neonatal	Alive
429	OL_2	41 days of gestation	Alive
430	OL_2	50 days of gestation	Death from neonatal exposure and starvation
431	OL_1	50 days gestation	Lost before August 1972, cause unknown
432	OL_1	Neonatal	Alive
433	OL_1	50 days of gestation	Alive
434	OL_1	Neonatal	Alive
435	OL_1	Neonatal	Alive
436	OL_2	Neonatal	Alive
437	OL_3	Neonatal	Death from neonatal exposure and starvation 5/9/71
438	OL_3	Neonatal	Alive
439	OL_3	Neonatal	Death from chronic facial eczema
440	OL_3	Neonatal	Alive
441	OL_3	Neonatal	Alive
443	OL_3	Neonatal	Lost between March and September 1972, cause unknown
444	OL_3	Neonatal	Alive
72	Control	Not inoculated	Alive
73	Control	" "	Alive
113	Control	" "	Alive
114	Control	" "	Alive
115	Control	" "	Lost between April and September 1973, cause unknown
116	Control	" "	Alive
117	Control	" "	Alive
118	Control	" "	Alive
119	Control	" "	Alive
120	Control	" "	Death from facial eczema March 1973

Continued

Appendix IV continued

Lamb No	Inoculum	Time of Inoculation	Fate of Lamb
121	Control	Not inoculated	Alive
122	Control	" "	Alive
123	Control	" "	Lost between December 1972 and April 1973, cause unknown
124	Control	" "	Alive
125	Control	" "	Alive

APPENDIX V

Multiple Range Test for Group means with Unequal Replication (Kramer, 1956)

Method

The means ($\bar{x}$) of groups A.....D are ranked in order and replications (n) in each are tabulated:

Groups	A	B	C	D
$\bar{x}$	$\bar{x}_A$	$\bar{x}_B$	$\bar{x}_C$	$\bar{x}_D$
n	n_A	n_B	n_C	n_D

The degrees of freedom (n_2) of the error mean square and the standard deviation (SD) are obtained from the analysis of variance of means. The appropriate significant studentized ranges (Zp,n_2) for a five per cent. Duncan multiple range test (Duncan, 1965) were listed and the significance range factors ($R'p = SD.Zp,n_2$) calculated.

p	2	3	4	5
Zp,n_2	(2)	(3)	(4)	(5)
R'p	$R'p_A$	$R'p_B$	$R'p_C$	$R'p_D$

Then in order for the differences between two means (eg. $\bar{x}_A$ and $\bar{x}_B$) to be significant at $p < 0.05$.

$$(\bar{x}_A - \bar{x}_B) \times \left(\frac{2.n_A.n_B}{n_A \; n_B} \right)^{\frac{1}{2}}$$

must exceed $R'p_B$

Duncan, D. B. (1955) Multiple range and multiple F tests. Biometrics, 11: 1-42.

APPENDIX VI

Cotton Wool Columns for Separation of lymphocytes from whole blood of sheep

Materials:

One 15 x 125mm Glass Test tube(1)
One 5 inch Pasteur pipette
Absorbent cotton wool
One 10ml plastic disposable syringe with 18g (BSW) needle
One inch length of surgical rubber tubing of 5millimetre internal diameter

Assembly of Columns

(i) The cotton wool is washed in two per cent. detergent(2) solution and rinsed overnight in tap water.

(ii) After cotton wool is dry it is packed in the test tube surrounding the pasteur pipette as shown in Figure VI .1.

(iii) The column is sterilized by autoclaving .

(iv) Buffy-coat cells from heparinized blood samples are placed on the cotton wool by pipetting and allowed to soak its entire length.

(v) The syringe is attached to the top of the Pasteur pipettes as in Figure VI.2.

(VI) HBSS is run onto the column and drawn through by syringe (Figure VI.3).

(VII) Pure lymphocyte suspensions are obtained following lysis of erythrocytes and washing of the cultures as detailed in Chapter 5.

(1) "Kimble", Owens - Illinois, U.S.A.

(2) "Decon 75", Medical Pharmaceutical Developments Ltd., Brighton, U.K.

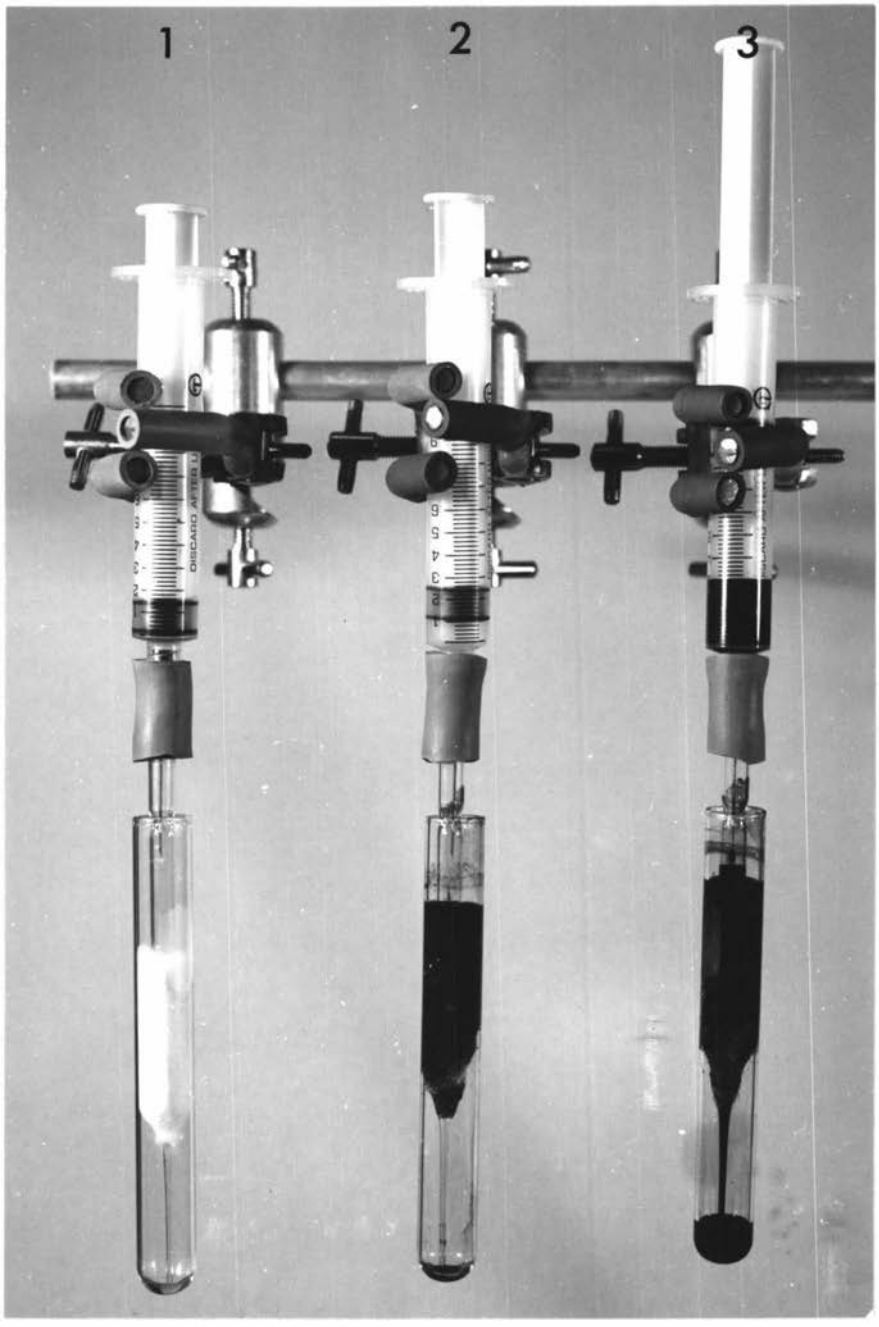

Figure VI

APPENDIX VII

Antilymphocyte Serum

Preparation

The thymus gland was removed aseptically from foetal lambs which had been recovered from the pregnant uteri of sheep at a local slaughter-house. Cells were teased from the thymus into phosphate buffered saline (PBS) at 4°C and washed three times in PBS by centrifugation at 600 *g* for 10 min. Adjustment was made to a 50 per cent. V/V cell suspension in PBS and 50 ml of this were immediately injected into a horse by the intra-venous route. Two further injections of similarly prepared suspensions of thymocytes were given by the same route of injection at seven day intervals. The horse was anaethetized and exsanguinated seven days after the third treatment. Blood was collected and allowed to clot in large conical pyrex flasks. It was then left at room temperature over-night to enable contraction of the clot to occur. Approximately 10 l of serum was decanted and passed through a filter of 22 μ pore diameter. The filtrate was dispensed into 100 ml glass bottles and stored until required at 4°C. Some of this "raw" antilymphocyte serum was tested in a sheep and in slide agglutinatin tests (see below). The remainder was pooled and the globulin fraction of the serum proteins precipated by treatment for three hours in 45 per cent. ammonium sulphate solution. This was collected by centrifuging at 2,000 *g* for 30 min and the resultant pellet resuspended and dissolved in saline. This solution was dialized against distilled water for 24 hr and the dialysate diluted with PBS to 20 per cent of the original volume of serum.

Testing

Slide agglutination test

Ten-fold dilutions of both the "raw" serum and globulin fraction

were tested for agglutinating ability with single-cell suspensions of ovine lymphocytes obtained from peripheral blood as described in Appendix VI. Two drops of the "serum" were added to an equal volume of the lymphocyte suspension on a clean glass slide. The slide was gently agitated and then examined for lymphocyte agglutination under a microscope at 30 min. A positive agglutination occurred with both serum and globulin at 1 in 10 dilution.

Response in injected sheep

Injections of serum or globulin were given at three day intervals to normal sheep which had received allogeneic homografts of skin as described in Chapter 7 for a period of three weeks. The initial two serum treatments were given by the intra-venous route, but because of adverse effects, all subsequent injections of both sera and globulin were administered intra-peritoneally. The dose of serum on each occasion was 100 ml and that of globulin 20 ml. Antibiotic cover to prevent intercurrent bacterial infection was given daily.

Both antilymphocyte preparations caused an immediate and profound fall in circulating lymphocytes which persisted throughout treatment.

Skin grafts of both tested sheep showed no gross evidence of rejection during the treatment course. Histologically they were normal except for a light infiltration of lymphocytes.

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Two Cases of Hepatic Mastocytoma in Sheep

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Abstract. Two mastocytomas of the liver of sheep are described. The specimens were obtained during a survey of hepatic neoplasia that was conducted on abattoir-slaughtered sheep. Although tissues other than the liver were not examined, both tumours had metastasised, one to the portohepatic lymph node, and the other intrahepatically. It is believed that this is the first report of mastocytoma in this species.

Mastocytomas are common neoplasms of dogs but are seen less frequently in other species of domestic and laboratory animals [1–7]. There appears to be no report in the literature of mastocytoma in sheep.

Materials and Methods

Specimens were collected at two local abattoirs during a survey of hepatic neoplasia in sheep.

Tissue blocks were fixed in 10% formol saline, embedded in paraffin, sectioned at 5μm and stained with haematoxylin and eosin, Giemsa, and toluidine blue.

Results

A total of 358,846 sheep livers, of which 222,682 were from lambs younger than 6 months, were examined. The two cases of mastocytoma reported here were found in a series of 40 neoplasms examined. Both were in adult sheep.

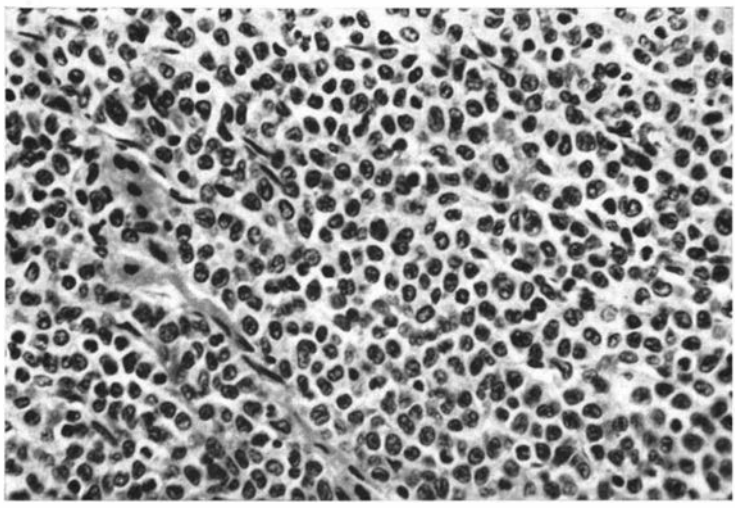

Fig. 1. A section of the hepatic tumour from the sheep in case I, showing mast cells and eosinophils within the collagenous stroma. HE.

Pathology

Case 1

A firmly encapsulated pale mass with external dimensions of $6 \times 6 \times 4$ cm was situated on the posterior margin of the left lobe of the liver. The cut surface exposed faintly green tissue divided into lobules by broad radially orientated bands of fibrous tissue. There was no fibrous boundary between the mass and the hepatic parenchyma. An area of necrosis 1 cm in diameter was present in the central area of the mass. The portohepatic lymph nodes were slightly swollen and contained several pale firm nodules about 2.0 mm diameter.

Tissue sections of the mass consisted of closely packed cells in a sparse collagenous stroma (fig. 1). The most frequent cell type in the HE-stained sections was large, with abundant finely granular cytoplasm and margins that were frequently poorly defined. The nuclei were round or ovoid, sometimes indented, and had marginated chromatin. A small nucleolus could be seen in most of the cells. Few mitotic figures were present. The cytoplasmic granules stained metachromatically with Giemsa and toluidine blue. In some areas of the tumour the mast cells were less typical and had smaller, more

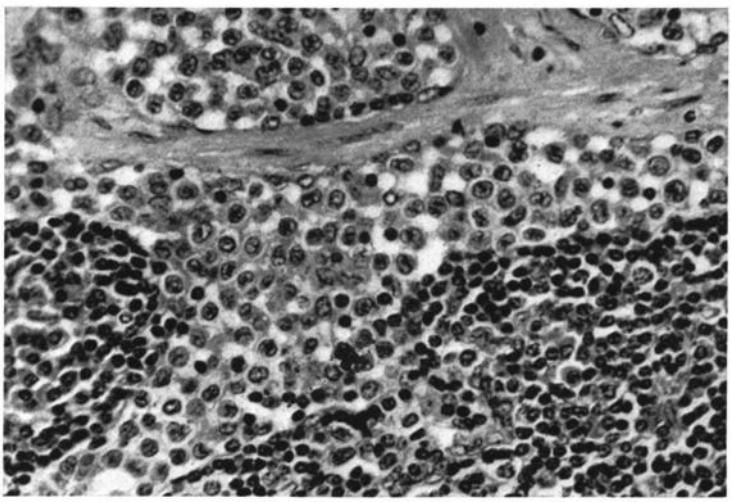

Fig. 2. A section of the portohepatic lymph node from the sheep in case I, showing infiltration of the cortex and germinal follicles by mast cells. HE.

deeply stained, irregularly shaped nuclei and fewer cytoplasmic granules. Large numbers of eosinophils and a few macrophages were dispersed between the mast cells. The hepatic sinusoids adjacent to the tumour were compressed, but there was little evidence of diffuse invasion of the hepatic parenchyma by the mast cells.

The lesions in the portohepatic lymph nodes (fig. 2) consisted of aggregations of mast cells morphologically similar to those in the liver, but lacking a collagenous stroma. Fewer eosinophils were seen in association with these mast cells.

Case II

The liver had multiple pale lesions 0.2–0.5 cm in diameter dispersed throughout the parenchyma. The portohepatic lymph nodes were normal.

Neoplastic mast cells were localised to the portal areas (fig. 3) and were similar to those in case I, except that the cytoplasmic granules were larger and more distinct. Eosinophils were present in large numbers. A few mast cells were seen in portal veins.

A diagnosis of hepatic mastocytoma was made in both cases on the basis of the histologic appearance of the tumours.

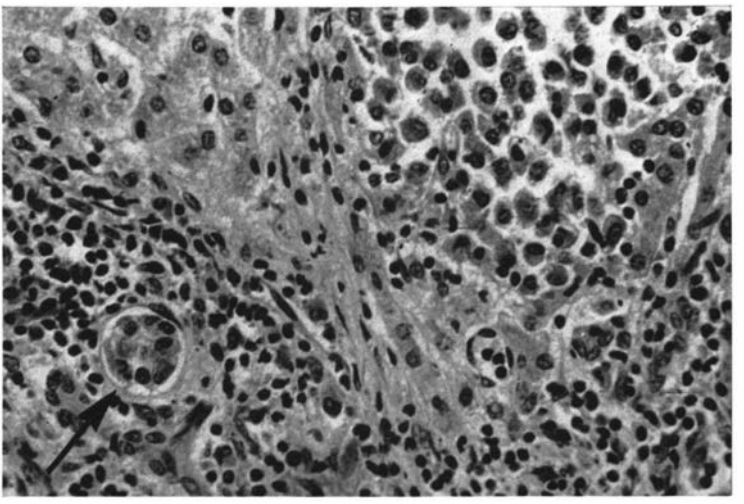

Fig. 3. Infiltration of hepatic portal region in the sheep in case II by lymphocytes and mast cells: several mast cells are present in one of the veins (arrow). HE.

Discussion

The appearance of the mast cells in these lesions conforms to the description of the morphology and staining reactions of ovine mast cells given by VEGAD [8]. Eosinophils are characteristic components of mastocytoma in other animal species [7].

The liver is a fairly common site of secondary localisation in cutaneous mastocytoma in the dog. HOTTENDORF and NEILSEN [5] reported that 37 of 91 dogs with mastocytoma had hepatic metastases. In the cases reported here, the livers were examined separately from the carcass, and it is not known whether the tumours were primary or metastatic. Both tumours had, however, metastasised, the first to the portohepatic lymph nodes, and in the second case intrahepatically.

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