An Electron Microscopic Study of Feline Lymphosarcoma

Summary of a Thesis
for the degree of Doctor of Philosophy
in the Faculty of Veterinary Medicine

by Helen M. Laird

University of Glasgow, 1968

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

SUMMARY

Lymphosarcoma, leukaemia or malignant lymphoma is a well recognised disease of the cat; its true incidence is not known. The aetiology of the disease has not been established but some experiments have suggested that a virus is involved. This study is concerned with electron microscopic examinations of material from field cases of feline lymphosarcoma and from animals and tissue cultures infected with feline leukaemic tissue extracts.

In Section 1 the examination of tissues from 9 randomly collected cats with spontaneous lymphosarcoma is described. Particles, morphological similar to the murine leukaemia viruses, were found to be present in 6 of these cats. The particles, approximately 100mμ in diameter were of 2 types The structure of Type 1 particles consisted of the outer bounding membrane within which there was an electron-dense membrane with a diameter of approximately 50 - 60mμ and an intermediate membrane with a diameter of approximately 80mμ. The structure of Type 2 particles consisted of the outer bounding membrane within which there was an electron-dense nucleoid with a diameter of approximately 60 mμ. Some particles appeared to have a "coating layer" on the outer surface of the bounding membrane. The particles were found in one or more of the following tissues, spleen, bone marrow, thymus, peripheral lymph nodes, and were found associated with lymphoid cells and megakaryocytes. Fully-formed particles were seen

extracellularly and in intracellular vacuoles; budding particles were observed at plasma membranes and vacuolar membranes and the various stages of particle replication at these surfaces were seen. In material from each of the other 3 cases of spontaneous lymphosarcoma a few virus-like particles were noted.

No correlation was established between the classification of a case according to the anatomical distribution of the lesions or the histopathological findings and the presence or absence of large numbers of particles. No particles were seen in tissues from a series of normal cats.

In Section 2 are described experiments with leukaemic tissue extracts from 3 of the particle-positive cases described in Section 1 and from 1 of the cases in which only a few virus-like particles were observed. Newborn kittens were infected with these inocula. Blood samples were taken from the kittens at various times, from 28 days to 1 year after inoculation, and tissue samples taken from kittens killed or dying at various times during the same period. It was found that particles could be demonstrated in the blood platelets and tissues. The inocula derived from the field case classified as most particle-positive induced particle replication in all of the kittens infected and both fully formed and budding particles were seen. Lymphosarcoma was histologically confirmed in some of the inoculated kittens. The second inoculum induced replication in some kittens but fewer particles were found associated with

the platelets and fewer tissues were found to contain particles. The third inoculum induced replication to a lesser extent. No particles were found in the kittens infected with the inoculum derived from the particle-negative case. Normal kittens and kittens infected with a normal lymph node extract were examined; in one kitten from each group a few virus-like structures were observed.

Section 3 describes further transmission experiments. It was found that a filtrate inoculum induced in kittens particle replication similar to that induced by the presumed cell-free extracts.

It was also shown that particle replication could be induced in adult cats which had been inoculated with a leukaemic tissue extract. Further, it was demonstrated that an inoculum from an infected kitten with no evidence of lymphosarcoma but with particles present in platelets could induce particle replication in kittens; this method of passage was successfully carried out through 4 passage generations.

Section 4 deals with the growth of the particles in tissue cultures. Two feline cell cultures were examined and inocula derived from 3 of the particle-positive field cases were tested in these cultures. It was shown that particle replication could take place in both types of cultures and that 2 of the 3 inocula investigated were effective in this respect. The 2 inocula which induced replication in tissue cultures were those which had been shown to be most active in inducing replication in newborn kittens and which had been derived from the field cases classified as the most particle-positive.

Section 5 describes attempts to isolate particles from leukaemic tissues from a field case, from plasma from inoculated kittens and from tissue culture fluid from cultures infected with leukaemic tissue extracts. Most preparations contained no particles; a few virus-like structures were seen in some tissue extracts and in a subsequent study, particles were found in tissue culture fluid.

Attempts to induce leukaemia in rats and mice, using feline leukaemic tissue extracts are described in Section 6. No evidence of particle replication could be detected in blood platelets or tissue and, in a 10 month period of observation, no animal developed leukaemia.

An Electron Microscopic Study of Feline Lymphosarcoma

Thesis for the degree of
Doctor of Philosophy
in the Faculty of Veterinary Medicine

by

Helen M. Laird

University of Glasgow

1968

ACKNOWLEDGEMENTS

It is a pleasure to thank most sincerely all those who have given help, advice and encouragement during the period of research recorded in this thesis.

In particular, I wish to thank -

Professor Sir William Weipers, who made it possible that this study be carried out at the Veterinary Hospital.

Professor W. F. H. Jarrett, whose initial experiments with feline lymphosarcoma provided the basis for the research described in this thesis and who, throughout the course of this study, gave guidance and encouragement, giving generously of his time for stimulating discussion.

Dr. G. W. Crighton, for assistance with the clinical aspects of this study and for the examination and interpretation of the pathological material.

Dr. J. C. Jarrett, for performing the feline cardiac punctures and for preparing the virus inocula and suspensions; also for many helpful discussions.

Dr. M. Stewart, for supervision of the cat colony and for careful clinical examination of the experimental cats.

Mr. D. Hay, for assistance with the tissue culture experiments.

Mrs. C. Burke, Mrs. C. McLay and Miss F. Douglas for technical assistance in the preparation of material for electron microscopy.

Miss A. Huntley and Mr. A. Finney for assistance with photography.

Miss J. Sommerville for excellent secretarial assistance and co-operation and for typing this manuscript.

This study was supported by a grant from the British Empire Cancer Campaign.

VOLUME 1

CONTENTS

Page No.

Note Volume 2 contains the Figures referred to in the text of Volume 1.

INTRODUCTION

Part 1

Leukaemia: the evidence for a viral aetiology for the disease in various species

Leukaemia is a well-recognised disease in many species, but its aetiology is only established conclusively for the fowl and for the mouse; both avian and murine leukaemias have been shown to be caused by viruses. There is strong evidence that feline lymphosarcoma (Jarrett, 1964a) and canine mast cell leukaemia (Rickard, 1967) are also of viral origin.

The original discovery of Ellerman and Bang (1908) that erythromyeloblastosis in chickens was transmissible with a cell-free inoculum and the later finding of Furth (1933) that chicken lymphomatosis could be transmitted with a filtrate have stimulated many investigators to try to identify viruses in other avian and mammalian leukaemias.

A well-defined, closely related group of viruses, inducing neoplastic disorders of the haematopoietic system of the fowl, have been identified and extensively investigated. It has been found that most of the avian virus strains are capable of inducing several distinct neoplastic diseases; the malignancies induced by these viruses are collectively termed "the avian leukosis complex". Although antigenic relationships and differences between the viruses can be demonstrated serologically, the viruses are morphologically indistinguishable. Diagram 1 shows the schematic representation of the virus morphology as presented by Vogt (1965) in an extensive review on the subject of the avian tumour viruses; in this, the physical, chemical and biological characterisation of the viruses are discussed fully and the way in which the

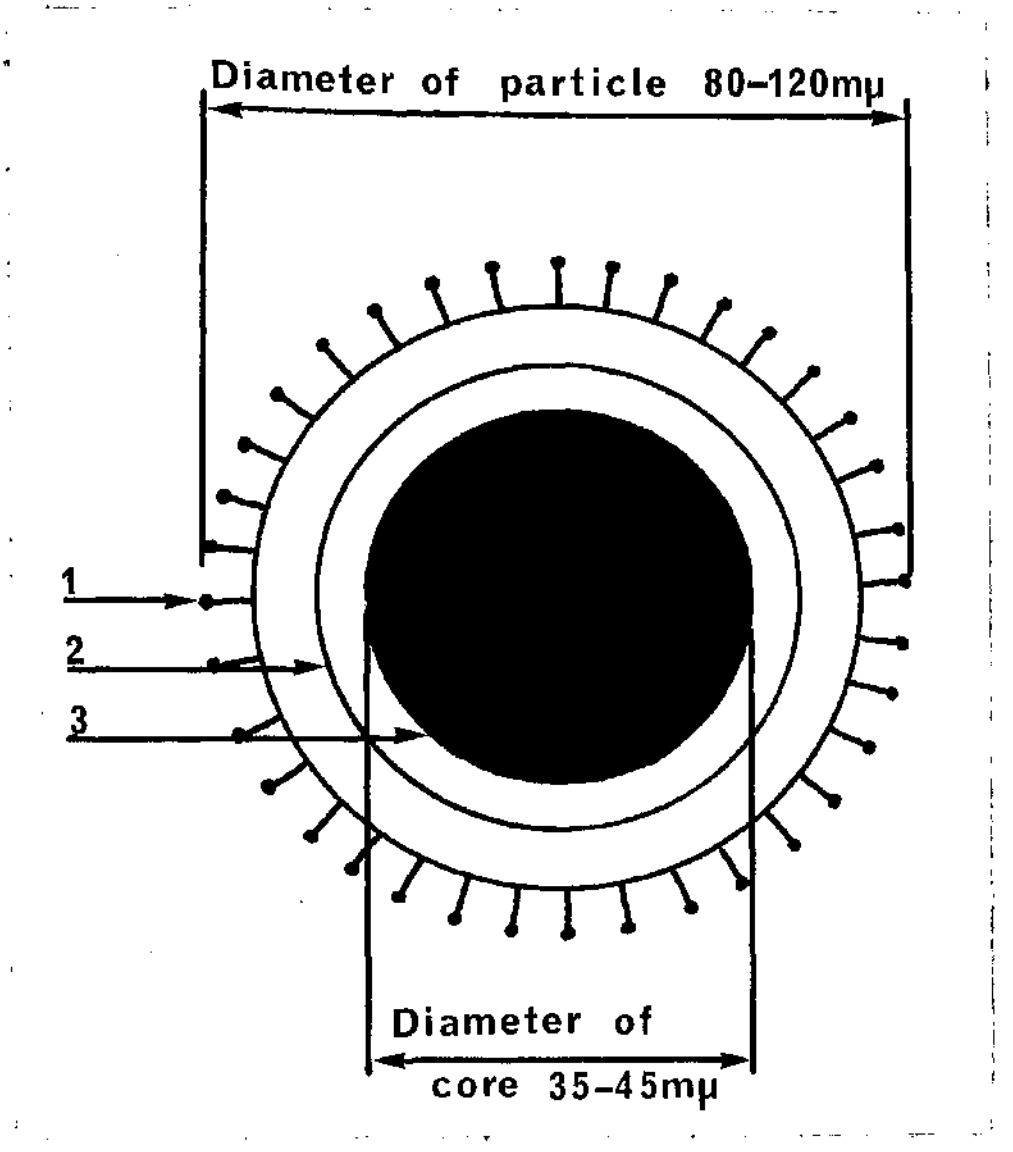


Diagram 1

Schematic representation of avian tumour viruses

1. Outer membrane with peripheral knobs.
2. Inner membrane
3. Nucleoid

(from Vogt, 1965)

viruses replicate and are released from cells by budding from the membrane is described.

The evidence of a viral aetiology for murine leukaemia was provided in 1951 by Gross when he produced leukaemia in mice by injecting them as newborns with filtered inocula prepared from leukaemic tissues from mice with spontaneous leukaemia. Later, a highly potent leukaemia virus "passage A" was derived by serial cell-free passage of leukaemic filtrates in newborn mice. Since these discoveries were made, many murine leukaemia viruses have been isolated from a wide variety of mouse tissues and tumours from many strains of mice; while some investigators consider that there is only one murine leukaemia virus (Gross, 1967), it has also been suggested that the viruses constitute a family of serologically related agents (Hartley et al., 1967).

A recent review of murine leukaemia (Rich and Siegler, 1967) deals with the pathological, immunological and biochemical aspects of the disease and a review by Kaplan (1967) deals with the natural history of murine leukaemia. The mode of virus replication and viral morphology have been described by many investigators (Dalton et al., 1961; 1964; Dmochowski et al.,1964; de Harven, 1965; Feldman and Gross, 1966; Yumoto et al., 1966). Structurally, the various murine viruses are identical to one another although, according to Dalton (1961) they may differ from one another slightly in size; Diagram 2 shows the schematic representation of the particles given by de Thé and O'Connor (1966) and Diagram 3 shows the various stages of virus replication and release which have been demonstrated in electron micrographs.

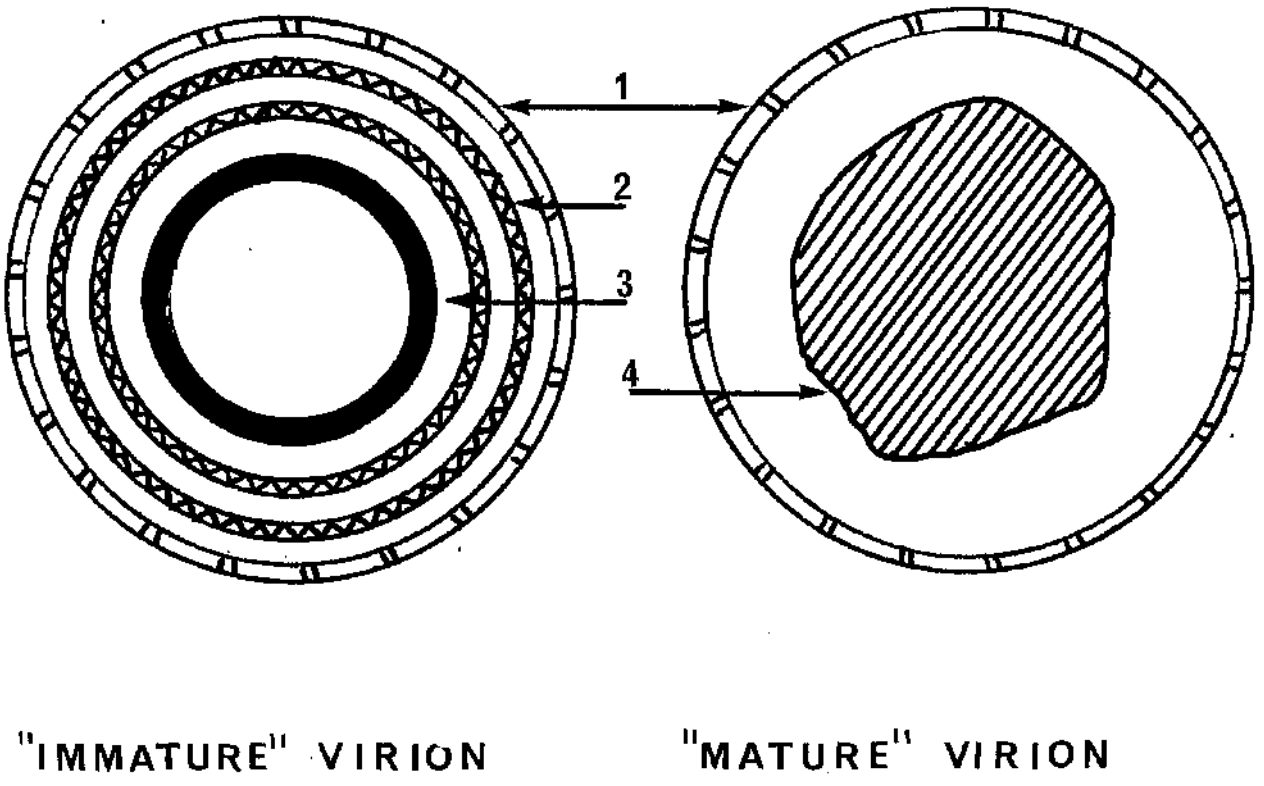


Diagram 2

Schematic representation of "immature" and "mature" virions seen in Rauscher leukaemia virus preparations.

"Immature" virion. The complete virion, 100 mμ in diameter, has: 1, the envelope; 2, the outer shell, 75 - 85 mμ in diameter, corresponding to a hollow coiled cylinder; 3, the inner shell, 55 - 65 mμ in diameter, possibly containing RNA (Type 1).

"Mature" virion. The complete virion, 100 mμ in diameter, has the two internal shells fused together to give a dense nucleoid, 4, of 65 - 70 mμ in diameter (Type 2).

(from de Thé and O'Connor, 1965)

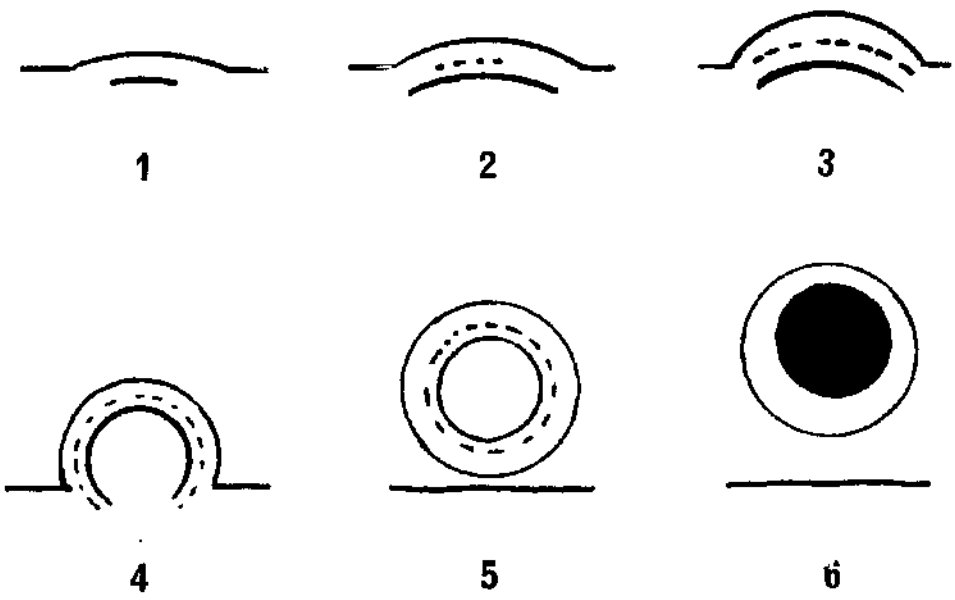


Diagram 3

Schematic representation of particle budding and release from the plasma membrane

1. Slight bulging of plasma membrane and formation of electron-dense inner membrane.
2. Further formation of inner membrane and formation of intermediate membrane.
3,4 Later stages of budding with more fully formed inner and intermediate membranes.
5. Complete particle of "immature" type free from the plasma membrane. (Type 1)
6. Complete particle of "mature" type. (Type 2).

There are few reports of experiments with leukaemias in other species; however, in some species attempts have been made to transmit the disease using cell-free inocula and electron microscope examinations have been made to try to identify a virus. The following paragraphs present the evidence available to suggest a viral aetiology for some mammalian leukaemias.

1. The human disease

Many attempts have been made to demonstrate the presence of viruses in human leukaemia. Some studies have investigated the effects of injecting human leukaemic tissue extracts into experimental animals; others have examined leukaemic tissues and tissue cultures derived from specimens from leukaemic patients and have attempted to show viruses electron microscopically. Lévy and Oppenheim (1966), de Harven (1966) and Dalton and Zeve (1967) have reviewed the literature on this subject.

Animal inoculation experiments have been inconclusive. One investigation (Herbut, 1967) suggested that a human leukaemia virus had been demonstrated but a further analysis of the virus indicated that it was probably of murine origin (Fink et al., 1967).

In vitro investigations have shown mycoplasma in some cultures of human leukaemic tissues (Dmochowski et al., 1967a), and herpes-like viruses have been seen in cells derived from Burkitt lymphomas (Epstein et al., 1964; O'Conor and Rabson, 1965; Zeve et al., 1966; de Harven, 1967a). de Harven found no "C" type particles in cultures of cells from cases of acute human leukaemia or in cultures derived from Burkitt tumours (de Harven et al., 1967; de Harven, 1967a), but Dmochowski (1967) has reported finding "C" type particles in 2

cultures from specimens from 2 patients with human leukaemia.

Electron microscopic investigations of plasma and tissues from leukaemic patients have shown virus-like structures in some specimens (Porter et al., 1964; Dalton et al., 1964b; Levine et al., 1967; Dmochowski, 1967; Dmochowski et al., 1967 a,b). Some of the particles are morphologically similar to the "C" type murine viruses and in tissues from a few cases the budding of particles from a plasma membrane has been demonstrated. Another type of virus-like particle has recently been seen in tissue from a patient with chronic lymphocytic leukaemia (Viola et al., 1967). In a recent study, Newell et al. (1968) examined plasma from 255 patients with leukaemia and related diseases. Although "virus-like" particles were found, they were not typical "C" type leukaemogenic viruses and they were found in plasma from control patients as well as in plasma from leukaemic patients. No correlation between the presence or absence of particles or between the numbers of particles found and the clinical status of the patients could be established.

Thus, although there is some evidence that virus-like particles can be found in some human leukaemia specimens, the results of the investigations made so far have been inconclusive.

2. The disease in cattle

Bovine leukaemia or lymphosarcoma is the commonest malignancy in cattle; recent papers by Jarrett (1966b) and by Jarrett et al. (1966) describe the disease and review the literature. The experimental transmission of the disease has been reported by several investigators using whole cell inocula derived from field cases of bovine lymphosarcoma (Rosenberger, 1963; Hoflund et al., 1963; Marshak et al., 1967; Theilen et al., 1967). However, attempts to transmit the disease with cell-free extracts from bovine leukaemic tissues have not succeeded (Jarrett, 1966b).

Electron microscope examinations have been made of bovine tissues and cow's milk with the aim of demonstrating viruses similar to the murine leukaemia viruses (Jarrett, 1962; Sorenson and Theilen, 1963; Ueberschär, 1963; Dutcher et al., 1964; Nazerian et al., 1968; Dutcher et al., 1967). A few intracytoplasmic virus-like structures have been seen in some specimens but no convincing evidence of budding of particles from membranes has been found. No particles morphologically characterisable as "C" type murine leukaemia viruses have been demonstrated extracellularly in tissue sections or in pellets derived from milk.

Thus, in bovine studies neither has a viral aetiology been demonstrated by cell-free transmission of the disease nor has a possible agent been identified in leukaemic animals.

3. The disease in the dog

Lymphosarcoma is a well recognised malignancy of dogs; a recent survey in California indicated an incidence of 24/100,000 dogs (Dorn et al., 1967); and that lymphosarcoma constituted 6.8% of all malignancies in males and 4.7% in females (Dorn et al., 1968a,b). Cellular transmissions of canine lymphosarcoma have been reported by several workers (Lombard et al., 1963; Moldovanu et al., 1965; Rickard, 1967) and cell-free passage of a canine mast cell leukaemia has been described by Rickard (1967). Lombard et al. (1963) demonstrated "viral-like" particles within the cytoplasm and Rickard (1967) found "suggestive virus-like particles" extracellularly. Virus-like structures seen in a single case of canine lymphosarcoma (Laird, unpublished result) are shown in Fig. 140. Chapman et al.(1967) found particles in tissues from

2 dogs with reticulum cell leukaemia; the illustration of the structures seen in sections of tissue from the 2 dogs show that they are not characteristic leukaemogenic-type particles; more convincing "C" type particles are shown in sections of a tissue culture established from cells from the pleural cavity of one of the dogs and particles in a pellet of the tissue culture fluid are morphologically similar to the murine viruses. In a later study (Chin et al., 1967) cells and cell-free filtrates from one of the dogs with reticulum cell leukaemia were injected into axenic mice; the mice all developed leukaemia and "C" type particles were found in their tissues. Whether the particles were of murine or of canine origin was not investigated.

None of these observations or experiments establishes a viral aetiology for the disease, but the results of the experiments of Rickard (1967) are strongly in favour of there being a virus associated with the disease process.

4. The disease in the guinea pig

One inbred strain of guinea pig, Strain 2, has been found to develop spontaneous acute lymphatic leukaemia (Congdon and Lorenz, 1954). Transplantable tumours have been derived from several spontaneous cases and studies with one of these, L2C, were reported by Nadel (1964). To provide "presumptive evidence" that a viral agent was associated with the tumour, Opler (1967a,b) showed that the disease can be transmitted with cell-free extracts of leukaemic tissues or with cell-free plasma preparations from guinea-pigs bearing L2C tumours. Electron microscopic examinations of tissues and plasma pellets revealed virus-like particles 80 - 90 mμ in diameter. Nadel et al. (1967) have also demonstrated virus-like particles, 62.5 - 75 mμ in diameter, in leukaemic cells.

The particles seen in the guinea pig tissues are associated with lymphoid cells and megakaryocytes and are found in the bone marrow, spleen and lymph node. Opler (1967c) produced ultracentrifuged plasma pellets from leukaemic guinea pigs and in these found 'type "C" particles and no evidence of cells or cell organelles'. Leukaemia developed in strain 2 guinea pigs inoculated with aliquots of the pellets examined electron microscopically. Thus, there is some evidence that the particles observed in the pellets may be associated with the leukaemogenic process and could be identified with a leukaemogenic agent involved in this particular guinea pig leukaemia.

Nadel describes the particles in his tumours as having a central core surrounded by a unit membrane and a "fuzzy outer coat". Opler describes the particles in his preparations as "mature Type C particles" and states that they "appear to be dissimilar to previously described viruses". They are smaller than the murine viruses and have a less dense intermediate membrane than them. Opler and Nadel both show particles within endoplasmic cisternae and perinuclear spaces. Budding from the plasma membrane has not been recorded but Opler has demonstrated budding from the membranes of the endoplasmic reticulum. Groups of free particles in intracellular spaces have been found. Thus, while there is some evidence that the disease may be transmitted experimentally by means of a virus inoculum, there appears to be no close correlation between the particles seen in the guinea pig and those seen in the mouse.

5. The disease in the rat

Spontaneous leukaemias in laboratory strains of rat appear to be rare and none have been observed in animals less than 1 year old (Bergs, 1967). The disease can be induced experimentally, however, by treatment with chemicals (Shay et al., 1951; Hartman et al., 1959; Huggins and Sugiyama, 1966; Gruenstein et al., 1966) and by inoculation with fractions of various rat tumours (Stresney, 1950; Bergs, 1967).

Murine leukaemia viruses have been shown to be leukaemogenic for rats. Moloney (1960) showed that the Moloney murine virus could infect rats and that both cell transplants and cell-free extracts prepared from the induced tumours could reproduce the disease in rats of the same strain. He also found that virus recovered from the rat tumours retained its infectivity for mice. Gross virus, Rauscher virus and Friend virus have also been shown to be capable of producing leukaemia in rats (Dmochowski et al., 1964; Rauscher, 1962; de Harven, 1965).

Thus, although these murine viruses show variations in their ongocenic capacity for particular mouse strains they have all been shown to be capable of inducing leukaemia in another species.

The induced disease in the rat has the same clinical and pathological characteristics as the spontaneous disease in mice (Moloney, 1960). Electron microscopic examinations of leukaemic tissues from rats with virus induced leukaemia show typical murine leukaemia viruses with the same size and morphology, being produced, by the same budding process, from the same cell types as in murine leukaemic tissues (Dalton et al., 1961; Dmochowski et al., 1964; Okano et al., 1963; Feldmen and Gross, 1966).

Dalton et al.(1961) showed further that viruses could be observed in blood

platelet pellets prepared from the peripheral blood of leukaemic rats. Fully formed particles were found in vacuoles in approximately 20% of the platelets and viruses were also observed lying free between the packed platelets. From this, Dalton et al. (1961) deduced that virus would be extractable from the plasma of leukaemic rats in the same way as the avian leukosis virus had been recovered from plasma (Sharp et al., 1952). This was later shown to be so (Moloney, 1962).

Since larger volumes of plasma may be collected from rats than from mice and since plasma extraction of virus avoids the problem of cellular contamination of the virus, plasma from leukaemic rats provides a rich source from which leukaemia virus, which retains its characteristic morphology and infectivity for mice, may be extracted. Thus, although the rat can contribute little to the study of spontaneous leukaemia it is of importance in providing a system for the production of large amounts of murine virus for quantitation and characterisation studies (Dalton, Huguenau and Moloney, 1964).

Conclusion

Thus, as was indicated at the beginning of this review, there is strong evidence for a viral aetiology for leukaemia in only 2 species, the avian and the murine, but there is some evidence to suggest that the canine mast cell leukaemia has a virus associated with it. Feline lymphosarcoma, the first leukaemia in a domestic animal for which a viral aetiology was proposed (Jarrett et al., 1964a) is now described in detail.

Part 2

Feline Lymphosarcoma: the description of the disease: the results of previous experiments and the object of the present study.

Feline lymphosarcoma may be studied from two slightly different angles; as a common disease in a domestic animal or from a comparative point of view, as a possible model for human leukaemia. The main model system studied in the past has been the murine one. Although longer-lived and consequently yielding results more slowly than mice, and although more difficult to obtain as experimental animals, cats offer certain advantages over mice for a model system since they are not syngeneic and they do live in small "family" groups. In the present experiments the general aim was to study the disease as it occurs in the cat, but the comparative aspect was the basic reason for the investigation.

The disease in the cat

Lymphosarcoma, leukaemia, or malignant lymphoma is a well recognised disease of the cat. A comparison between the feline, canine and bovine forms of the disease has been made by Jarrett et al. (1966a). In the cat, the true incidence is unknown due to the lack of accurate data on cat populations; however, a recent study by Dorn et al. (1967) showed that in one area of the United States (Alameda and Contra Costa counties, California) the incidence of malignant lymphoma was approximately 42 cases/100,000 cats. It has been found that the disease is the most common malignancy in cats (Cotchin, 1952;

Holzworth, 1960; Squire, 1964; Schmidt, 1967) and constitutes approximately 13% of all malignancies (Cotchin, 1957).

No particular age, sex or breed incidence was noted and cases were recorded in animals from 6 months to 17 years (Holzworth, 1960); in another study (Crighton, 1965) cases in animals less than 1 year old were very infrequent. Recently Dorn et al. (1968a, b) showed that lymphosarcoma represented 18.1% of all malignancies in female cats and 33.3% in male cats, and that the annual lymphosarcoma rates studied over a 3-year period were 58.4/100,000 for males and 27.9/100,000 for females. The epidemiological aspects of the disease have not been studied but there is one report in the literature of a household cluster of feline malignant lymphoma (Schneider et al., 1967).

The clinical and histopathological aspects of the disease have been described in detail (Cotchin, 1952, 1957; Nielsen and Holzworth, 1953; Holzworth and Nielsen, 1955; Holzworth, 1960; Crighton, 1965).

Three main forms of the disease are recognised and are classified according to the anatomical distribution of the lesions (Jarrett et al., 1966). The commonest form is the alimentary-mesenteric in which there is a grossly enlarged main mesenteric lymph node and one or more tumour masses present along the wall of the intestine; the liver is often affected and the spleen and kidneys may be involved. In the multicentric form all the superficial nodes are involved and the mesenteric lymph node is enlarged and often palpable: the liver, spleen and kidneys may be affected. The thymic form, which is less frequently observed, is characterised by a large mass in the anterior mediastinum.

Haematological examination shows that in the majority of cases there is no true leukaemia; there is neither an increased total nor percentage lymphocyte count; frequently, there is a lymphocytopaenia. Anaemia is common (Crighton, 1965).

Histological examination shows that all types of lymphoid cells can be found in the tumour lesions and that there are no distinctive morphological differences between tumour cells and normal lymphoid cells. Two main categories of case can be recognised; in one group the dominant cell types present in the various tumour lesions are "immature" and in the other group the tumour cells are classified as "mature" or "mixed" (Crighton, 1965).

Evidence suggesting a viral aetiology for feline lymphosarcoma

The aetiology of the spontaneous disease is not known. At the outset of this study, apart from a brief reference to a preliminary investigation by Jarrett (1966b), there were no reports in the literature of electron microscopic studies of spontaneous cat lymphosarcoma. Thus, there was no visual evidence for there being a virus involved in the natural history of the disease.

The presence of leukaemogenic type virus particles in leukaemic tissues from cats with induced lymphosarcoma was reported by Jarrett et al. (1964b); no particles were seen in 14 apparently normal, healthy cats. The inoculum used in these experiments was derived from leukaemic tissues from a cat with spontaneous lymphosarcoma; the tissue extract was subjected to high speed centrifugation and the presumed cell-free supernatant was used to infect kittens. No tumours arose at the site of inoculation. These results suggest that an

oncogenic virus was involved in the experimentally induced leukaemia and further, by analogy with the murine leukaemia viruses, that the particles observed in the tissues of the cats with induced lymphosarcoma might represent the causative factor. In these experiments, however, no study was made of the tissues from the field case of lymphosarcoma from which the leukaemogenic inoculum was derived, so it is not known whether viruses of the same type were present in the donor animal. (*See note at end of Section).

Thus, when this study began, it had been shown that feline lymphosarcoma could be transmitted with presumed cell-free extracts of leukaemic tissue and leukaemogenic-type particles had been demonstrated in tissues from induced cases; although an association between leukaemogenic-type particles and induced feline lymphosarcoma was suggested, no proof of a viral aetiology had been established.

The aims of the investigations and experiments described in the following study

The aims of the investigations and experiments were:-

1. to examine leukaemic tissues from cases of spontaneous feline lymphosarcoma and to discover if a virus-like particle could be identified.
2. to study the morphology of any particle found
3. to investigate the biological properties of the particle
4. to determine whether the appearance and behaviour of the particle were consistent with its being a leukaemogenic virus.

The results in all of the investigations and experiments were derived from electron microscopic examinations.

*

While the experiments to be described were in progress, other evidence in favour of a viral aetiology was provided by the experiments of Rickard et al., (Rickard et al., 1967; Rickard, 1967) who found "C" type particles in a single case of cat leukaemia and with a cell-free filtrate derived from tissues from this animal induced leukaemia in kittens. Further, Kawakami et al. (1967) observed type "C" particles in the plasma from a leukaemic cat and in the plasma of kittens with leukaemia induced with cell-free and whole cell extracts from leukaemic tissue from the cat.

MATERIALS AND METHODS

MATERIALS

Animals

1. Cats

(i) Lymphosarcoma field cases

The 8 cases of spontaneous feline lymphosarcoma examined were not specially selected for the study. The disease was diagnosed in the animals by veterinary practitioners and the cats were referred to the Veterinary Hospital. The cats in this series were all of those with lymphosarcoma admitted to the Hospital during a period of 18 months.

(ii) Normal adults

Ten healthy adult cats from the Dog and Cat Home, Corkerhill, Glasgow were used as control animals.

(iii) Kittens and adult cats used in transmission experiments

All of the kittens and cats used in the experiments belonged to the Animal Leukaemia Research Unit cat colony. This is a closed colony maintained in the country, several miles from the Veterinary Hospital. During the course of the experiments, the animals were housed at the colony, which is supervised by a veterinary surgeon, and were brought to the laboratory for blood examinations, to be killed, or for post-mortem examination. During the course of the experiments the kittens and cats being studied were not in contact with any animals from outwith the colony.

Breeding within the colony is random and the kittens in the experiments were obtained from the litters of 20 females. All of the animals were numbered and recorded individually and were identified

by physical characteristics of colour and coat markings. In cases of litters containing very similar kittens, ear-marking was used to distinguish between the animals.

2. Rats

The newborn and adult hooded Lister rats used in the experiments came from the rat colony at the Veterinary Hospital. The colony is maintained by brother-sister mating and skin grafting tests, performed by Dr. J. Anderson, have shown very few rejections indicating, that the rat line is approximately syngeneic.

3. Mice

Pure line C3Hf mice, maintained in the Veterinary Hospital colony by brother-sister mating, were used for the mouse experiment.

METHODS

1. Preparation of Tissue Blocks for Electron Microscopy

(i) Fixation

Small samples of tissue were excised from anaesthetised animals and were immediately immersed in one of the two fixatives routinely employed (see below). The samples were cut into pieces of less than 1 mm^3 and these were then placed in fresh fixative in bottles surrounded by ice.

Two fixation methods were used:-

(a) Osmic acid fixation

Tissues were fixed at 4°C for 1½ hours in 1% osmic acid at pH 7.4 in Zetterquvist buffer (Glauert, 1961) or pH 7.3 in Millonig buffer (Millonig, 1961, 1962).

(b) Glutaraldehyde-Osmic acid fixation

Tissues were fixed at 4°C for 1½ hours in 1.5% - 4.0% glutaraldehyde (Sabatini et al., 1963) at pH 7.3 in Millonig or Sorenson buffer. After fixation and 3 brief rinses of a few minutes each in the corresponding buffer the tissues were further fixed at 4°C for 1 hour in osmic acid as in (a).

The choice of fixation procedure used for each case was determined by the time of day at which the tissue sample was made available. Method (b) was preferred, but method (a) was used when necessary to ensure an uninterrupted fixation and embedding of the tissues.

(ii) Dehydration and embedding

Fixed tissues were dehydrated in a graded series of alcohols according to the following schedule:- 70% alcohol for 5 minutes (3 times), 90% alcohol for 5 minutes (2 times), absolute alcohol for 15 minutes (4 times). Specimens were then transferred to propylene oxide for 15 minutes and to fresh propylene oxide for a further 15 minutes. Tissues were embedded in the following Araldite mixture:-

Araldite embedding mixture

Stock mixture (kept in refrigerator) 1 part Araldite Resin CY212 (Ciba) and 1 part Araldite Hardener HY964 (Ciba).

Embedding Mixture 57 cc Stock Solution, 2.4 cc Di-n-Butyl Phthalate and 0.6 cc Araldite Accelerator DY064 (Ciba) mixed overnight with a magnetic stirrer.

Specimens were soaked in a 50:50 mixture of propylene oxide and Araldite for 30 minutes after which more Araldite was added to increase the Araldite to propylene oxide ratio to approximately 75:25. After 1-2 hours, the Araldite propylene oxide mixture was poured off and fresh Araldite added. Tissues were soaked overnight at room temperature in the Araldite. Specimens were embedded in fresh Araldite in gelatin capsules or Beem capsules (for very small specimens). The blocks were cured for 48 hours at 57°C.

2. Preparation of Tissue Culture Cells for Electron Microscopy

Culture medium was removed from a plastic dish containing a confluent cell sheet. The cells were washed twice with PBS (phosphate buffered saline) and then gently scraped from the plastic surface. The cells, in PBS, were spun in a plastic centrifuge tube at 500 x g for 5 minutes. The supernatant was removed and the cell pellet fixed in 1% osmic acid at 4°C (pH 7.3 in Millonig buffer) for 1 hour. The pellet was then cut into small pieces which were dehydrated and embedded in the same way as tissue samples.

3. Preparation of Blood Platelets for Electron Microscopy

Each individual or pooled blood sample was diluted with an equal volume of 0.3M potassium citrate and spun at 1,000 x g for 10 minutes. The sedimented large cell fraction was discarded and the supernatant was then spun at 10,000 x g for 10 minutes. The pellet thus formed consisted almost exclusively

of blood platelets and was prepared for electron microscopy. The supernatant was poured off and the centrifuge tube inverted to drain off any remaining fluid. A small volume of 1% osmic acid (pH 7.3 in Millonig's buffer) was added and the pellet was fixed at 4°C for 1½ hours. The fixative was then poured off and the pellet was rinsed with 70% alcohol. Fresh 70% alcohol was then added and the pellet was gently freed from the tube wall using a flat-ended orange-stick. Frequently the pellet broke up into small pieces at this stage but if this did not occur, the pellet was cut into ¼ mm^3 - ½ mm^3 pieces. The pellet specimens were then transferred to a glass container and were dehydrated and embedded in the same way as tissue samples (see Methods 1).

Note: In some instances, when only a small volume of blood had been obtained from an animal, no platelet pellet could be seen after the centrifugation procedure. Although no pellet was visible the fixation schedule was followed to the point of dehydration. Since osmic acid blackened any platelets present this procedure sometimes rendered visible previously invisible pellets. Such a pellet consisting of a thin layer of platelets was dealt with in the normal way. Only when no pellet was observed after fixation was a sample discarded.

4. Cutting, Mounting and Staining of Sections for Electron Microscopy

Sections were cut with either an LKB Mark 1 or an LKB Mark 2 Ultratome, using glass knives, and were mounted on uncoated Athene 483 copper specimen grids obtained from Smethurst High-Light Ltd., Bolton, Lancs.

Sections were stained for 10 minutes with uranyl acetate (Watson, 1958), rinsed successively in methanol, 50% methyl alcohol and distilled water and dried on filter paper, and further stained for 10 minutes with lead citrate (Reynolds, 1963), and rinsed with 0.02 NaOH and distilled water and again dried on filter paper. Sections were normally examined immediately after staining, but if this was not possible they were stored in Petri dishes which were kept in a dessicator.

5. Negative Staining of Samples for Electron Microscopy

A small sample of the suspension to be examined was placed in a glass Petri dish containing several drops of 1% osmic acid hanging from the lid. The suspension sample was fixed in the osmic acid vapour for 10 minutes at room temperature. A carbon coated electron microscope grid was placed on the surface of the suspension sample and after a few seconds' contact the grid was removed and the suspension fluid attached to the grid was blotted off with filter paper. The preparation was then stained with 2% uranyl acetate in 50% methanol. A drop of the stain was placed on a clean glass slide and the surface of the grid which had been in contact with the suspension sample was allowed to touch the stain for 5-10 seconds. Excess stain was immediately blotted from the grid surface with filter paper.

6. Electron Microscopic Examination of Specimens

(i) General operating procedures

The specimens were examined with an AEI electron microscope. Sections and suspension samples were prepared as described in the previous paragraphs.

Particle detection examinations were carried out by direct viewing without an optical magnifier at microscope magnifications of 20,000 x or 30,000 x. Under these conditions leukaemogenic-type particles were readily identifiable and sections and suspension preparations were easily scanned completely.

Electron micrographs were taken at various magnifications but for virus structure studies they were routinely taken at magnifications of 20,000 x, 30,000 x or 40,000 x.

A photographic enlargement of x $2\frac{1}{2}$ was used for making prints.

(ii) Selection of material to be studied and extent of examination

In the light of preliminary observations the following arbitrary procedures were adopted.

(a) Examination of tissues from field cases and from normal animals -

At least 2 tissues were selected from each case.

Sections from several blocks from each tissue were examined.

In all, at least 100 holes of grid mesh were scanned for each case.

Note: The area of each grid mesh hole of the Athene 483 electron microscope grids contained approximately 100 - 300 cell profiles.

The procedures outlined above allowed for the examination of approximately 10,000 - 30,000 cell profiles representing cells from several focal regions of several tissues from each case.

(b) Examination of tissues from inoculated kittens and from normal kittens.

Two or three tissues were selected from each case.

Sections from several blocks from each tissue were examined.

Ten holes of grid mesh were scanned for each tissue. These procedures allowed for the examination of approximately 3000 - 9000 cell profiles.

(c) Examination of blood platelet pellets

Ten holes of grid mesh were scanned for each pellet examination.

Note: The area of each grid mesh hole contained approximately 500 - 900 blood platelet profiles.

Each pellet examination therefore scanned approximately 5000 - 9000 platelet profiles.

(d) Examination of tissue culture preparations

Ten holes of grid mesh were scanned for each preparation.

Note: The area of each grid mesh hole contained approximately 100 cell profiles.

Each tissue culture examination therefore scanned approximately 1000 cell profiles.

(e) Examination of negatively stained preparations

Ten holes of grid mesh were scanned for each suspension preparation.

Terminology

Leukaemia or Lymphosarcoma

All of the forms of neoplasia of the lymphoid system of the mouse are referred to as leukaemia and the term is used generally to apply to the erythroleukaemias (Friend, Rauscher), the myeloid leukaemias (Rich, Graffi) and the lymphatic leukaemias (Gross, Moloney). The name 'leukaemia' is also the general one applied to the human disease in which the presence in the peripheral blood of large numbers of lymphocytes is a common manifestation.

Jarrett et al., (1966) have described the disease in the domestic animals and emphasised that although blood changes can occur the disease is often present without there being an increased absolute or percentage lymphocyte count even when there is severe and generalised involvement of the lymph nodes, spleen and other organs. For this reason, the term 'lymphosarcoma' is preferred and is used throughout this study.

Other terms commonly employed to describe the same disease include malignant lymphoma, adenosarcoma and leukosis, which is the one generally used of the avian disease and frequently used in bovine studies.

Particle Nomenclature

This study is concerned with the morphological identification of an agent possibly involved in the leukaemogenesis of feline lymphosarcoma. The leukaemogenicity of the particle has not been established. However, because the particle can be characterised as a virus by virtue of the demonstration of its replication in cells and tissue cultures and because this virus is found associated with feline lymphosarcoma, it will be referred to as a 'leukaemia virus particle'.

In the experiments to be described, this term is used to describe a particle which has the same morphology as the murine and avian leukaemia viruses. In tissue sections, particles, approximately 100 mu with the triple-membraned or the dense nucleoid structure, found at sites normally associated with the mouse and fowl viruses are referred to as "leukaemia virus particles". In negatively stained preparations of extracts from tissues, blood or tissue culture fluid particles which are of the same size and show the same staining characteristics as the murine leukaemia viruses are described as "leukaemia virus particles". When there is no possibility of misinterpretation, the term "particle" is sometimes used.

Virus-like particle and virus-like structure

These terms are used to describe a particle whose structure differs slightly from that characteristic of the leukaemia viruses but which is of the same order of size as the leukaemia viruses and is seen in tissue section at a site at which it is known that leukaemia viruses can be present.

Particle description

From a review of the literature, it can be seen that no consistent terminology is used in the description of the oncogenic RNA viruses. According to the original classification of Bernhard (1960) the murine and avian leukaemogenic viruses are classified as "C" type; however, different workers use a variety of terms to describe these viruses.

A recent attempt has been made to establish a universal terminology which would make it more possible to compare the findings of different workers without constant reference to electron micrographs (Classification of oncogenic RNA viruses, 1966). As already demonstrated in the General

Introduction there are two different morphological particles associated with murine leukaemia; the structure of one of the forms consists of three concentric membranes surrounding an electron-lucent core and the other form has an electron-dense core surrounded by one membrane. The relationship between the two forms is not known but since budding particles show a triple-membraned structure and the type of particle with an electron-lucent core is frequently observed extracellularly close to a cell membrane this form is described as the "immature 'C'" type and the electron-dense nucleoid form is termed the "mature 'C'" type. Despite the apparent implications of this terminology, those using it stress that "the term 'immature' or 'mature' in these contexts is in reference only to time after formation and is not meant to imply anything in regard to activity". This nomenclature is further confused by the fact that while some workers consider the particle with the electron-lucent core to be the infective particle and the dense core form to represent a degenerating virion (de Harven, 1965), other workers consider the reverse to be true and suggest that the particle with the electron-dense core is the infective virus (Dalton et al., 1961). So far, the sophisticated experiments which would be required to establish conclusively which of these hypotheses is correct have not been performed, although preliminary attempts have been made to establish the relationship between infectivity and the presence of one or other or both forms of particle in an inoculum (de Harven and Friend, 1966). Thus, despite the recommendation that the terms "mature and "immature 'C'-type particle should be employed it is felt that they should be avoided because of the confusion which can arise from their use.

Another form of nomenclature also currently used is that of de Harven (1965) who employs the terms "A1" to describe the particle with the electron-lucent core and "C" to describe the particle with the dense nucleoid. Although this terminology removes the confusion between "mature" and "immature" particles it introduces the character "A" normally only ascribed to viruses found intracytoplasmically or in intracytoplasmic cisternae.

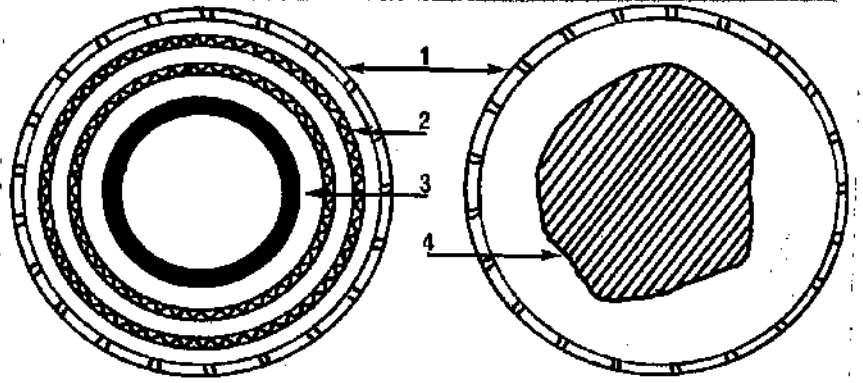
Many other workers use descriptive terms to distinguish between the two particle forms. Although descriptive classification is clumsy it has the advantage of being immediately understood.

Diagram 4 shows the structure of the 2 types of particle and gives some of the most commonly used descriptions.

In the present study the "triple-membraned form" is designated Type 1 and the "electron-dense nucleoid form" is designated Type 2.

Diagram 4

Terminology used in the description of murine leukaemia viruses and corresponding nomenclature used for the feline leukaemia virus



"IMMATURE" VIRION "MATURE" VIRION

Description of Particle and Particle Structure		Some authors using these terms
immature "C"	mature "C"	Classification of oncogenic RNA viruses, 1966.
A_1	C	de Harven
	C-1 and C-2 (1)	Yumoto
vesicular	particle-containing nucleoid (2)	Feldman and Gross
doughnut	target-like	Feldman and Gross
electron-lucent core electron-lucent nucleoid	(electron) dense nucleoid	Dalton Dmochowski
"triple-membraned form" Type 1	"electron-dense nucleoid form" Type 2	this study (3)

(1) Yumoto et al. (1966) use these terms, C-1 and C-2, to describe 'mature' viruses and transitional stages of development.

(2) This description is very misleading since many workers describe the other form as having an electron-lucent nucleoid.

(3) Feline leukaemia virus.

* from de Thé and O'Connor, 1965

Preliminary Studies

INTRODUCTION

The structure of the murine leukaemogenic viruses has been described in great detail by many investigators studying both tissue sections and negatively stained virus suspensions and many electron micrographs have been published showing very clearly the characteristic morphological features of the viruses (Dalton et al., 1961; Dmochowski et al., 1964; Dalton et al., 1964a; de Thé and O'Connor, 1966; Hall et al., 1967a). However, before making a study of field cases of feline lymphosarcoma it was considered advisable to examine mouse tissues known to contain large numbers of viruses in order to become familiar with their appearance in specimens prepared and examined according to the methods to be used in the cat studies. Unexamined material from the induced case of feline lymphosarcoma (Jarrett et al., 1964 a,b) was also available for examination. The aims of the preliminary studies were -

1) to ensure that the methods used in the preparation of tissues and tissue extracts were suitable for virus demonstration

2) to discover the best electron microscope magnifications to use when scanning sections or suspension preparations

3) to gain experience in identifying fully formed viruses and early budding stages seen in tissue sections and the characteristic particles seen in suspension preparations.

Experiment 1

The identification of Moloney mouse leukaemia virus in tissues from a mouse with leukaemia induced by the virus and in tissue cultures infected with the virus.

Materials and Methods

(i) Passage of Moloney leukaemia virus induced leukaemia

Newborn mice from a C3Hf litter were inoculated intraperitoneally with 0.1 ml of a 1/10 gm. equivalent suspension of Moloney leukaemia virus. After 4 - 5 months leukaemia developed in all of the mice producing palpable peripheral lymph nodes and mesenteric lymph nodes. Leukaemic tissue from one mouse was removed and an extract MLV1 produced as described below. 0.1 ml of the cell suspension of MLV1 was injected intraperitoneally into a litter of newborn mice and leukaemia developed in the mice after 10 - 20 days; tissue from one of these animals was used for preparing an extract MLV2 in the same way as MLV1. A litter of mice was inoculated with 0.1 ml of MLV2 and leukaemia developed in 10 - 20 days. The passage thus instituted was maintained in this way for 50 generations.

(ii) Preparation of leukaemic tissue extracts MLV1 - MLV49

Leukaemic spleen and mesenteric lymph node were removed from a single mouse and a 10% weight/volume suspension was made by adding the tissue, roughly minced with pointed scissors, to tris-saline (TS). This crude suspension was passed through a fine stainless steel sheet and the resulting suspension was washed three times by gently centrifuging the mixture, discarding the supernatant, and resuspending the solid material in TS. A 10% suspension of

the washed material in TS was then homogenised in the Sylverson grinder at full speed for 2 minutes at 4°C. The homogenate was spun at 1,000 x g for 10 minutes and the supernatant was removed for recentrifugation at 1,000 x g for 10 minutes. This procedure was repeated and the resulting supernatant was spun at 10,000 x g for 10 minutes. The supernatant from this spinning was used to inoculate mice or tissue cultures or was stored at -65°C.

(iii) Preparation of virus suspension from tissue culture fluid

The method used is based on the method of Duesberg and Robinson (1966). Culture fluid was collected from the bottles in which the infected cells were growing and was spun at 10,000 x g for 15 minutes to remove cells and cell debris: the supernatant was used immediately for virus extraction or was stored at -65°C. Stored fluid was thawed rapidly before use by standing the storage bottle in a water bath at 37°C. Centrifugation procedures were carried out at 4°C. For particle extraction, 3 Spinco SW 25-2 tubes were prepared by adding to each 6 ml of 60% sucrose in TS and on top of this 10 ml of 15% sucrose in TS. A volume of approximately 45 ml of the fresh or stored tissue culture cell-free fluid was layered on top of the sucrose column and the tubes were spun at 75,000 x g for 45 minutes. The supernatant was removed and a fresh volume of tissue culture cell free fluid was added; the centrifugation was repeated and the whole procedure repeated once more. A band formed at the interface between the 60% sucrose and 15% sucrose; the bands from the 3 tubes were collected and combined to give a volume of approximately 6 ml which was then diluted to 40 ml with TS. This suspension was layered on to a single tube prepared with 60% and 10% sucrose as already

described and the tube was spun at 75,000 x g for 1 hour. The band which formed (approx. 2 ml) was removed and diluted with 2 ml of TS and layered on to 0.6 ml of 60% sucrose in TS and spun at 100,000 x g for 45 minutes in the SW39 rotor. The resulting band (approx. 0.1 ml) was diluted to 0.5 ml with TS and layered on 4.5 ml of a 15% - 60% sucrose (in TS) gradient and spun for 2 hours at 100,000 x g. The band which formed in this gradient was removed for electron microscopic examination.

RESULTS

1. Tissue examinations

Samples of leukaemic spleen, mesenteric lymph node and thymus were taken from a mouse from the 13th passage of the Moloney virus induced leukaemia; these specimens were prepared for electron microscopy according to the routine procedure.

Virus particles, showing the well characterised morphology of the Moloney virus, were found in all of the spleen, lymph node and thymus sections examined. Budding, Type 1 and Type 2 particles were found associated with lymphoid cells and megakaryocytes; complete viruses were seen in extracellular spaces and intracellular vacuoles. Figures 1 - 11 show typical examples of virus formation and structure.

2. Tissue culture examinations

Approximately 50 x 10^6 cells from a primary culture of whole mouse embryo were suspended in 14 ml. of the leukaemic tissue extract MLV27 diluted 1:7 with growth medium. The cells and virus were incubated with stirring for 90 minutes at room temperature. The cells were then washed and grown in 8 oz. bottles.

(i) Examination of tissue culture fluid

Tissue culture fluid was collected from the cultures every 2 or 3 days between 14 and 28 days after infection of the cells; these samples were pooled and a virus suspension was prepared and negatively stained for electron microscopic examination.

Viruses were easily recognised by their characteristic structure which distinguished them from the granules and membranes in the small amount of contaminating cell debris in the suspension. Viruses with 3 membranes and others with electron-dense nucleoids were identified. Figure 12 shows groups of particles in a typical field from a suspension preparation; the uranyl acetate stain has penetrated to the core of the particles and surrounded them and thus the membrane structure is clearly defined. Figure 13 shows a group of 4 particles at higher magnification; one particle shows the three membrane layers very distinctly and in it there is an indication that the inner and intermediate membranes have a regular substructure; the adjacent particle has a more amorphous appearance with the membranes less clearly defined and with the core less densely stained; the remaining two particles are examples of disrupting viruses with semi-intact bounding membranes surrounding broken-up elements of the internal structure.

(ii) Examination of cells

Eight weeks after infection of the cultures, cells were harvested from the cultures by trypsinisation and a pellet of the cells was prepared using the standard procedures already described (see Materials and Methods section).

Large numbers of virus particles were found associated with the cells;

budding viruses were frequently observed at the cell membranes and complete particles, both Type 1 and Type 2 particles, were seen extracellularly, either singly or in a band round part of the cell or in large groups in the intercellular spaces. Figures 14 - 16 give typical examples of the virus distribution and structure.

Experiment 2

The examination of leukaemic tissue from a cat with lymphosarcoma induced with a feline leukaemic tissue extract.

Materials and methods

Previously unexamined, stored tissue blocks were available from the case of induced lymphosarcoma examined by Jarrett et al. (1964b) and shown by them to contain virus particles. Sections of mesenteric lymph node, which had been embedded in methacrylate, were cut and mounted on Formvar coated grids and stained with lead citrate for 10 minutes (Reynolds, 1963).

Results

Virus particles were found in most of the sections of mesenteric lymph node examined. The structure of the particles and the sites at which they were found were as had already been described by Jarrett et al. (1964b). Particles budding from the cell surfaces were easily identified and fairly frequently observed; occasionally two particles were found budding close to one another from a short length of plasma membrane (Figs. 17, 18). The detail of virus formation could be seen very clearly in some budding particles

demonstrating the continuity between the plasma membrane and the outer particle membrane and showing the partially formed intermediate membrane lying between the bounding membrane and the electron-dense inner membrane (Fig. 19). Most of the particles observed were of Type 1, a few Type 2 particles were observed and occasionally a "tailed" particle was seen. Single particles and groups of particles were found extracellularly and in intercellular vacuoles (Figs. 19, 20, 21).

DISCUSSION

These preliminary studies have shown that the methods of preparation and examination used were satisfactory for the demonstration of murine leukaemia viruses in tissues and suspension preparations; the micrographs of the murine viruses show the characteristic particles identical to those illustrated by other investigators. The study of sections of lymph node from the field case of feline lymphosarcoma confirmed the observations of Jarrett et al. (1964b) and provided an opportunity of examining tissue sections in which there were relatively few particles. Budding particles and complete particles, in vacuoles and in extracellular spaces, were readily identified at the magnifications of 20,000 x and 30,000 x.

On the basis of these findings, the material obtained from field cases, from inoculated kittens and cats and from tissue cultures were prepared and examined according to the methods used in these preliminary studies.

Section 1

The association of leukaemia virus particles with tissues from cases of spontaneous feline lymphosarcoma

INTRODUCTION

At the outset of this study, there were no reports in the literature of electron microscopic investigations of cases of spontaneous feline lymphosarcoma, apart from one brief comment that a very preliminary examination of a few cases had failed to reveal virus particles (Jarrett, 1966b).

Electron microscopic studies of spontaneous leukaemias in other species, reported before and during the present investigations, have been reviewed in the general introduction; it has been noted that while there is some evidence to support the theory that a leukaemogenic virus is involved in leukaemia in the guinea-pig, and in the dog, there is no convincing evidence to suggest that this is the case for bovine lymphosarcoma and only very slight evidence that a leukaemogenic-type virus is present in certain human leukaemias.

Most studies of the murine leukaemias have been concerned with induced cases of the disease or with tissue cultures infected with one or other of the murine leukaemia viruses; only a few studies have been concerned with the spontaneous disease (Dmochowski and Grey, 1957; Bernhard and Guérin, 1958; Dalton et al., 1961). Dalton et al. examined spontaneous leukaemias in two mouse strains with a high leukaemia incidence, C3Hf/FgLN with an incidence of approximately 80% at 10 months and the AKR strain with a similar incidence; they reported that in each strain particles could be found although these were present in smaller numbers than in cases of leukaemia induced with any one of the 4 viruses which they tested. The report did not state in what percentage of the cases of spontaneous leukaemia examined particles were found.

It was reported that bone marrow and spleen megakaryocytes were the cells with which particles were most frequently associated. Particle formation within specific granules of megakaryocytes appeared to be a characteristic of the spontaneous leukaemias and of the Gross virus induced leukaemia. The particles in the spontaneous leukaemias were morphologically similar to those in the virus induced cases; slight differences of particle diameter were noted in comparing particles found in the different leukaemias and it was reported that the particles in the two spontaneous leukaemias and those found in two of the virus-induced leukaemias (Friend and Gross) were slightly smaller (90 mμ) than those found in the other two virus-induced leukaemias (Moloney and Manaker C-60 - Schwartz) which were 100 mμ.

Thus, as far as the presence of characteristic leukaemia virus particles is concerned, the main difference between the spontaneous and the virus-induced murine leukaemias is that tissues from the latter contain many more particles than those from the former; this is perhaps not unexpected in view of the fact that the inocula used in the virus-induced leukaemias are highly concentrated virus preparations derived either from serially passaged leukaemias in which the virus titre has been increased or from infected tissue cultures which were producing large quantities of virus.

The aims of the investigations described in this section were

1) to determine whether or not leukaemia virus particles could be demonstrated in tissues from cases of spontaneous feline lymphosarcoma and if so, to study their morphology and mode of replication.

2) to study the organ and cell distribution of any particles observed.

3) to determine whether or not leukaemia virus particles could be demonstrated in tissues from normal healthy cats.

4) to collect and store tissues from field cases; this material would be available for transmission studies.

MATERIALS AND METHODS

1. Animals

Eight cases of spontaneous feline lymphosarcoma were examined. These were numbered CL1 - CL8.

Ten normal adult cats were studied as controls. These were numbered NA1 - NA10.

2. Tissues for electron microscopy

(i) from lymphosarcoma cases.

Samples of spleen were taken from each of the cases CL1 - CL8 and samples of mesenteric lymph node from CL2 - CL8. In addition, samples were taken from each of the animals CL1 - CL8 from one or more of the following: the peripheral lymph nodes, thymus, bone marrow. Blood samples for platelet examinations were taken from cases CL7 and CL8.

(ii) from normal animals.

Samples of spleen and mesenteric lymph nodes were taken from each of the animals NA1 - NA10 and of thymus from those animals in which this organ was present.

3. Tissues for histological examination

Samples were taken from each of the cases CL1 - CL7 from one or more of the following tissues: spleen, mesenteric lymph node, thymus, bone marrow, the peripheral lymph nodes. No material was available from cat CL8.

4. Tissues for storage

Leukaemic nodes and spleens from cases CL1 - CL7 were removed and stored separately in sealed containers at -65°C.

RESULTS

Histological examination of the tissues from each of the cats CL1 - CL7 confirmed the diagnosis of lymphosarcoma. Electron microscopic examinations revealed the presence of leukaemia virus particles in one or more of the tissues from 5/8 of the cases; in tissues from each of the other 3 cases only one or two virus-like particles were observed. No particles were found in any of the tissues from the normal cats.

1. Classification of cases

At post-mortem the cases were classified according to the anatomical distribution of the lesions; the classification system used was that described by Jarrett et al. (1966). Table 1 indicates the type of lymphosarcoma found in each of the 8 cases studied and shows that the series consisted of 3 cases of multicentric lymphosarcoma (CL1, CL4, CL6) and 5 cases of alimentary lymphosarcoma (CL2, CL3, CL5, CL7, CL8). Grossly, each case was typical of the class to which it was assigned.

2. Histological findings

An examination of tissues from each of the cases CL1 - CL7 confirmed the diagnosis of lymphosarcoma; no tissue was available from cat CL8. The main histological features of each case are described below and the dominant cell types found in the tumour of each animal are given in Table 1. (See also Figs. 22 and 23).

Table 1

Leukaemia virus particles in tissues from cases of spontaneous feline lymphosarcoma

Case No.	Histopathological Diagnosis	Dominant Cell Type	No. of Tissues Examined	No. of Blocks Examined	No. of Grid Squares Examined	Presence of Particles (1) A	B	C
CL1	Multicentric Lymphosarcoma	Mixed	3	7	50	Bone marrow; Iliac lymph node		Spleen
CL2	Alimentary Lymphosarcoma	Mixed	4	13	150		Prescapular lymph nodes	Spleen; Mesenteric lymph nodes
CL3	Alimentary Lymphosarcoma	Mixed	2	8	100	Spleen; Mesenteric lymph node		
CL4	Multicentric Lymphosarcoma	Immature	5	19	200	Spleen; Mesenteric lymph node; Iliac lymph node; Thymus; Bone marrow		
CL5	Alimentary Lymphosarcoma	Immature	4	7	100	Spleen; Mesenteric lymph node; Thymus; Bone marrow		
CL6	Multicentric Lymphosarcoma	Immature	4	10	100	Mesenteric lymph node; Bone marrow		Spleen; Anterio mediastinal lymph node
CL7	Alimentary Lymphosarcoma	Immature	5	8	100		Mesenteric lymph node (2)	Spleen; Pre-scapular lymph node; Thymus; Bone marrow
CL8	Alimentary (3) Lymphosarcoma	No histology available.	4	6	130		Spleen (2)	Mesenteric lymp node; Prescapul lymph node; Bon marrow

(1) A - tissues in which groups of particles and budding particles were found
B - tissues in which a single particle or a single group of particles was found and no budding particles were observed
C - tissues in which no particles were found

(2) A single particle found associated with blood platelet from this case.

(3) This diagnosis was based on clinical and gross anatomical examinations.

CL1 The lymph node and the spleen contained a wide spectrum of tumour cells ranging from medium lymphocytes to bizarre stem cells. In the liver there were a few small focal areas of immature lymphoid tumour cells.

CL2 Sections of lymph node from this case showed a wide range of cell types from medium lymphocytes to stem cells. Splenic involvement included the replacement of the Malpighian bodies with malignant cells and heavy invasion of the red pulp. The liver showed infiltrating masses of lymphoblasts.

CL3 A section of the main abdominal lymph node mass showed tumour cell sheets with a variety of cell types ranging from small lymphocytes to stem cells; the stem cells were focally distributed and in these focal areas there was a high mitotic rate.

CL4 Sections of lymph node showed fairly uniform tumour cell sheets with a high mitotic rate. Lymphoblasts were the predominant cell type. In the spleen there was infiltration of lymphoblasts and large lymphoid cells into the red pulp. The bone marrow was also heavily infiltrated.

CL5 The main tumour masses in the intestinal lymph nodes and along the ileum were composed almost entirely of lymphoblasts with a moderately high mitotic rate. The spleen was not markedly involved but the Malpighian bodies were enlarged and contained abnormal numbers of lymphoblasts.

CL6 The main tumour cell types in the lymph nodes were lymphoblasts and stem cells with a moderate mitotic rate. The splenic pulp was almost totally replaced with massive foci of lymphoblasts and stem cells. Similar cells were focally distributed throughout the bone marrow.

CL7 The mesenteric lymph node and other lymph nodes of the abdomen were heavily infiltrated with immature lymphoid cells with a low mitotic rate. The pancreas, liver and Peyer's patches of the small intestine were infiltrated with similar lymphoid tumour cell types.

3. Electron microscope findings

Table 1 shows the tissues examined and in which of these leukaemia virus particles were identified.

Particles were found singly and in small groups, in extracellular spaces, in intracellular vacuoles and budding from plasma membranes and vacuolar membranes (Figs. 24 - 27).

Extracellularly and in the intracytoplasmic vacuoles, the particles were circular or elliptical in profile, membrane-bound and had a mean external diameter of approximately 100 mμ. Two types of particle were found; morphologically, these were identical to the triple-membraned form and the electron-dense nucleoid form of the murine leukaemia viruses.

In sections of cat tissues the structure of the one type of particle (Type 1) was seen to consist of an external bounding membrane inside which there was an electron-dense inner membrane (Fig. 28). This membrane, denser than the outer membrane, had a mean diameter of approximately 50-60 mμ. Between these two membranes there was a third, intermediate membrane less dense than both the inner and outer membranes, with a diameter of approximately 80 mμ. Most of these particles had an electron-lucent core but a few had a slight condensation of electron-dense material in the core. On the outer surface of some particles there was a "coating layer". (Fig. 29) This was not a membrane but appeared to consist of numerous small discrete components or a diffuse layer a few mμ thick in close association with the external surface of the particle.

The second type of particle (Type 2) had an electron-dense nucleoid and an outer bounding membrane which frequently presented an irregular profile with a wrinkled appearance (Fig. 30). Individual diameter measurements ranged from 80 mu - 115 mu. The electron-dense nucleoid, approximately 60 mu in diameter, was centrally located in some particles but was frequently observed to be eccentrically situated within the outer membrane. This type of particle was not often found to have the "coating layer" associated with it. It was more usual to find this type of particle in extracellular spaces than in vacuoles.

In addition to observing individual and isolated particles in vacuoles and in intercellular spaces, groups of particles were found at both of those sites (Figs. 25, 31 - 33). Within vacuoles, 2, 3 or 4 particles were observed and extracellularly groups consisting of 2 - 7 particles were found in close association with individual cells. No large aggregates were seen. In the intercellular spaces and in the vacuoles, particles of both types could be seen within the same group; some groups, however, consisted exclusively of one of the types of particle.

At the plasma membrane incomplete particles at various stages of development were noted (Figs. 26, 34). Partially-formed particles were also observed projecting into intracytoplasmic vacuoles (Figs. 35 - 38). The first stage of particle development which could be recognised showed a short length of electron-dense membrane lying in the cell behind, close to, and parallel to the plasma membrane or vacuole-bounding membrane (Figs. 34, 39). Later stages of particle budding showed a slight bulging of the membrane and an extended projection of the membrane behind which the inner electron-dense

membrane was more fully developed (Fig. 39). At this stage, a short length of intermediate membrane could be seen forming between the plasma and inner membranes. Some cells showed only one projection or bud on the cell profile or distending into a vacuole (Figs. 35, 36); other cells showed 2 or 3 buds forming along a short length of plasma membrane (Fig. 34) and in some vacuoles several budding particles could be seen (Fig. 27). Almost complete particles, in which the inner and intermediate membranes were fully formed and circular in profile were observed attached to the plasma membrane or vacuole membrane by a thread of membrane (Figs. 40, 41, 42). Occasionally, two particles were seen attached to one another and one of the pair attached to the cell (Fig. 43). A few particles were observed with the outer membrane drawn out to form a "tail" (Fig. 44).

At all stages it could be seen that the cell membrane provided the outer membrane for the particle and that the inner and intermediate membranes were being formed within the cell (Figs. 27, 34). In the region of a budding particle the cytoplasm showed no characteristic features; the ribosome distribution was similar to that found along the periphery of the cell where no budding was seen; no cell organelles were found preferentially located near a budding particle. A "coating layer" could be seen on some complete and incomplete particles (Figs. 45, 46).

In general, particles were associated with two cell types. Megakaryocytes showed particles budding into intracytoplasmic vacuoles and complete particles, of both types, were found singly and in groups, in vacuoles and in intracellular channels (Fig. 25); budding from the plasma membrane was not observed

frequently. Complete particles of both types were found near the surface. Lymphoid cells showed particles budding from the plasma membrane and Type 1 particles were frequently found extracellularly near the cell surface; Type 2 particles were also, although less frequently, seen extracellularly (Figs. 24, 26). Occasionally lymphoid cells were observed with vacuoles containing one or two particles.

Table 2 gives a summary of the main observations made with respect to the types of particle found in individual tissues from the individual cases.

In cases CL3, CL4 and CL5, particles were observed in almost every section of each of the tissues examined. In cases CL1 and CL6 particles were found in only some of the tissues. Although no quantitative studies were made it appeared that particles were as frequently observed in the particle positive tissues from case CL1 and CL6 as in the tissues from the previous three cases. Particles were particularly abundant in the iliac lymph node sections from CL1 and in the bone marrow sections from CL6.

No budding particles or groups of particles were found in any of the tissues from cases CL2, CL7 and CL8. However, in each case one or more of the sections from one or more of the tissues showed particles with some of the morphological characteristics of the murine leukaemia viruses.

In case CL2, a single virus-like particle was found in a broken-up cell seen in a section of the prescapular lymph node (Fig. 47). The particle was approximately 100 mμ and had a layered membrane structure. No other virus-like particles were found in the examination of tissues from CL2. In case CL7 virus-like particles were observed in two tissues; in a section of a blood platelet pellet a single particle was found outside and close to a

Table 2

Types of particles observed in tissues

from cases of spontaneous feline lymphosarcoma

Case Number	Tissues examined and types of particle observed *			
	Spleen	Bone Marrow	Thymus	Other Tissues
CL1	-	Bd/TM		Bd/N iliac lymph node
CL2	-			(TM) (a) prescapular lymph node - mesenteric lymph node
CL3	TM			Bd/TM mesenteric lymph node
CL4	Bd/TM/N	Bd/TM/N	N	Bd/TM mesenteric lymph node N iliac lymph node
CL5	Bd/TM	Bd/N	N	Bd/TM mesenteric lymph node
CL6	-	Bd/TM		Bd/TM mesenteric lymph node - mediastinal lymph node
CL7	-	-		(N) (b) mesenteric lymph node (N) cat blood platelets - prescapular lymph node
CL8	(N) (b)	-		(N) (b) blood platelets - mesenteric lymph node - prescapular lymph node

The following symbols are used to denote the principal particle types observed:-

Bd - budding particle; TM - triple-membraned (Type 1); N - electron-dense nucleoid (Type 2). (a) one particle only; (b) one group of particles only

- indicates no particles found.

platelet membrane (Fig. 48) and a few particles with electron-dense cores were found in a section of mesenteric lymph node (Fig. 49).

In case CL8, in one section of a spleen megakaryocyte, a group of particles was seen; some particles had electron-dense centres (Fig. 50). In a blood platelet pellet section a single particle, similar to that found in CL7, was observed in a platelet vacuole.

Table 3 shows the examinations made of tissues from the normal cats and indicates that no leukaemia virus particles were found in any of the tissues from any of the cats.

Table 3

The examination of tissues from normal adult cats for the presence of leukaemia virus particles

	Tissues examined *		
Cat	Spleen	Mesenteric Lymph Node	Thymus
NC1	-	-	-
NC2	-	-	-
NC3	-	-	ntp
NC4	-	-	ntp
NC5	-	-	ntp
NC6	-	-	-
NC7	-	-	-
NC8	-	-	ntp
NC9	-	-	-
NC10	-	-	-

\+ particles found

\- no particles found

ntp no thymus present in these animals

DISCUSSION

Presence of particles

This study has established the existence of leukaemia virus particles in tissues from several cats with spontaneous lymphosarcoma and has failed to identify them in tissues from normal animals. After examining the first 4 cases in this series and finding numerous particles in 3 of them, a brief report of this observation was published (Laird et al., 1967). At the same time, Rickard et al. (1967) recorded a single case of spontaneous feline leukaemia in which "C" type particles were found and de Harven (1967b) mentioned a similar finding in a single case. Later, Kawakami et al. (1967) demonstrated virus in the plasma from a cat with spontaneous leukaemia. The particles demonstrated by de Harven and by the other 2 groups of workers are morphologically identical to those found in the animals in this series; in the cases investigated by Kawakami et al. and by de Harven, it was found that mature "C" type particles predominated while in the case examined by Rickard et al. particles with electron-lucent cores were found. In none of these reports is it stated how many cases were studied; thus, although in this investigation it has been shown that 5/8 cases were clearly particle-positive and that in 8/8 some particles were identified, the total number of cases examined in the 4 laboratories is not known and the percentage of particle-positive cases cannot be calculated. However, from the data available, it is established that 4 groups, working totally independently, have demonstrated the presence of leukaemia virus particles in at least 8 randomly selected cases; this suggests that the association of these particles with spontaneous feline lymphosarcomas may not be uncommon.

Particle morphology

The particles found in the feline tissues have the same morphology and mode of formation by budding as the murine leukaemia viruses (see General Introduction and Preliminary Studies). At electron microscope magnifications of 10,000 x and 15,000 x the particles were distinguished by their characteristic electron density; at the standard examination magnifications of 20,000 x and 30,000 x, the morphological details were clearly resolved.

With the exception of those particles with a "coating layer", the two types of particle seen in this study correspond completely with the descriptions and electron micrographs of immature and mature "C" type murine particles; the size and membrane osmophilia of the Type 1 feline particle make it morphologically indistinguishable from the immature "C" type and the size, membrane irregularity and nucleoid structure of the Type 2 feline particle makes it morphologically identical to the mature "C" type murine virus. The 'tailed' appearance and the eccentric position of the nucleoid within a wrinkled limiting membrane seen in some cat particles have been described in studies of the murine viruses (Dalton et al., 1961; Dalton et al., 1964; Feldman and Gross, 1964).

The "coating layer" seen on the surface of some particles, most frequently Type 1, has not been commented on in reports of the morphology of the murine leukaemogenic viruses as seen in tissue sections. However, an examination of some of the published electron micrographs reveals that in some instances such a layer appears to be present (Dalton et al., 1961; Dalton et al., 1964; Kajima and Pollard, 1967); it can also be seen on the surface of some

leukaemia viruses grown in tissue culture (Zeigel et al., 1966; Hall et al., 1967). Morphologically identical structures are seen on the surface of some murine sarcoma virus particles (Dalton, 1966) and a similar appearance noted on the surface of herpes-like viruses in Burkitt cells in tissue cultures was described by Toshima et al. (1967) as "irregular material attached to the virus" and it was suggested that this consisted of "materials carried by the virus when emerging from the cytoplasm". In negatively stained preparations of Rauscher mouse leukaemia virus some particles are described as having "peripheral knobs" or "spines" on the surface (Zeigel and Rauscher, 1964). Very clearly defined knob-like structures have been demonstrated on the outer surface of avian viruses (Bonar et al., 1963).

The appearance of the "coating layer" on the surface of the mouse viruses is similar to that on the cat particles and the discrete stud-like structures are clearly seen on some murine particles. It might be thought that this surface layer represents an artefact introduced during the fixation, dehydration and embedding processes and that the particles adsorb on to their outer surface material from the vacuole or extracellular spaces. A close examination of the cell or vacuole membranes in close proximity to a particle with a "coating layer" shows that the plasma membrane does not have this appearance and that the stud-like structures are restricted to the particle or to the membrane budding the particles. If the plasma membrane and particle surfaces were identical the preparative processes would produce similar artefacts on both; that this is not so implies either that the surface "coating layer" is an integral component of the particle structure or that the particle outer membrane differs in some way from the plasma membrane causing preferential adsorption, in a fairly regular manner, on to the

particle surface. The possibility that plasma membranes possess inherent chemical differences along specific regions has been commented on by Zeigel and Rauschor (1964).

The function of the various structured elements of the leukaemia viruses are not known but the "coating layer", if a true component of the particle, might be a significant factor in the initial virus-cell interaction which takes place when a virus infects a cell; in particular, it might indicate the location of a specific virus antigen. If, on the other hand, the "coating layer" is adsorbed by the particle, this might reflect a specific membrane affinity involved in the initial virus-cell interaction. How the localised budding area of membrane in continuity with the cell or vacuole membrane can develop the "coating layer" cannot be determined from this study; nor has it been established why only a certain proportion of the particles display this feature.

The recognition of particle budding in the feline tissues establishes that there is active production of particles by the cells; the budding process in the cat has been shown to follow the same steps as in the mouse (Dalton et al., 1961). This study has not revealed the presence of specific morphologically characterisable cell surface sites of particle production and has not been able to show how the inner and intermediate membranes are formed.

In some murine studies, in addition to the finding of Type 1 and Type 2 particles in vacuoles and extracellular spaces, 'A' type particles have been demonstrated at intracytoplasmic sites (Dalton et al., 1961; Kobayashi et al., 1967). In no case in this series were intracytoplasmic particles seen.

Cell association

The particles in the feline tissues were found, as in the murine leukaemias, associated with lymphoid cells and with megakaryocytes. No particular study was made of the cells; particles were found extracellularly and in intracytoplasmic vacuoles. In murine studies, Dalton et al. (1961) reported finding particles in megakaryocyte specific granules; particles were not seen at this site in the cat tissues.

The role of the different cell types in the development of lymphosarcoma is not known but in this study it was observed that budding particles were more commonly associated with lymphoid cells than with megakaryocytes and that, whereas budding from the former cells was from the cell surfaces, in the megakaryocytes the budding was into vacuoles. This has also been observed with the murine viruses and Dalton has suggested that the megakaryocytes "store" the virus released into vacuoles and intracytoplasmic channels, and that only when delamination of platelets from the megakaryocytes takes place are the viruses released. The association of particles with megakaryocytes is of particular importance in examining sections; these very large cells are immediately recognised and particles are readily identified in the vacuoles and channels. Individual free particles or budding particles associated with lymphoid cells are much more difficult to locate in tissue sections. Thus, sections in which megakaryocytes are found are more easily classified as particle-positive or particle-negative than those in which these cells are not present. In the cases studied in this series, whereas megakaryocytes were frequently found in tissue sections from CL3, CL4, CL5

and CL6, no megakaryocytes were observed in the spleen of CL1 and only a few seen in any of the tissues from CL2, CL7, and CL8; in these cases, small numbers of particles associated with lymphoid cells might have been overlooked and the tissues wrongly classified as particle-negative.

A comparison between the histological findings and the electron microscopic results shows that there is no correlation between the dominant cell type (immature or mixed) and the identification of small or large numbers of particles in a given case (see Table 1). It should be noted, however, that in cases CL4, CL5 and CL6, in which the dominant cell type was immature, particles were apparently more abundant than in the other cases.

The inclusion of blood platelet examinations for cases presented after CL6 resulted from the preliminary observations which were available from the transmission experiments described in Section 2. The full results of these experiments are given in Section 2 but the observation relevant to this section was that particles could be identified in the platelets of recently inoculated kittens. This observation was made in mouse leukaemia studies by de Harven and Friend (1966); Dalton et al. (1961) showed that particles could be found in platelets from mice with fully developed leukaemia and that 20% of the platelets from rats with induced leukaemia had associated particles. No figures are available for spontaneous or induced murine leukaemias.

Platelets from 2 cats with lymphosarcoma (CL7 and CL8) were examined; in each case only a single virus-like particle was observed. Examinations of the tissues from these cats showed a single group of characteristic particles in CL7 and a single isolated particle in CL8. The significance of these

observations cannot be assessed although it can be concluded that platelets from cats with lymphosarcoma do not necessarily contain large numbers of particles. Studies of platelets from cats with large numbers of particles in their tissues should help to establish whether or not the feline system resembles the murine one with respect to platelet particle association. Kawakami et al. (1967) showed virus in plasma from a leukaemic cat, but they did not study the platelets or tissues.*

If it were found that all cases in which particles were demonstrated in the tissues also had particles associated with platelets a blood platelet examination would provide a simple test to determine, without killing an animal, whether it was particle-positive or not. Such a test would be of value in the selection of cases for further studies. Obviously, before using this test as a diagnostic marker in suspected cases of lymphosarcoma, it would be necessary to study not only platelets from animals with lymphosarcoma, but also platelets from randomly selected normal animals some of which would be killed to confirm that they had no histopathological evidence of lymphosarcoma and others kept to discover whether or not this would develop the disease. Such an investigation was beyond the scope of the present study.

Anatomical classification and organ involvement

An examination of Table 1 shows that in this small series no correlation can be established between the findings of large numbers of particles in a case and the anatomical classification of the case. Because the same tissues were not examined from each animal, no direct comparisons can be made between cases, but it has been shown that whereas the histological examinations

* see note at end of this section

of GL5 showed that the spleen was not markedly involved and the electron microscopic examinations revealed particles, the spleens of GL1, GL2 and GL6 showed severe splenic involvement and in them no particles were found.

From the data presented in Table 2 it can be seen that neither the Type 1 particle nor the Type 2 particle seems to be preferentially associated with any specific organ, nor does one of the types predominate in any individual case. Until more is known of the relationship between the 2 types of particle, no further conclusions can be drawn from these preliminary observations.

Classification of cases as "particle-positive"

In this discussion the use of the term "particle-positive" is relative; although the absence of particles cannot be demonstrated, tissues which could not be classified with certainty as "particle-positive" (A) are described as B or C (see Table 1).

In the foregoing discussion it has been shown that there appears to be no correlation between any of the factors of anatomical classification, organ involvement or cell type classification and the classifications A, B or C. The basis for and the reliability of this classification must be considered.

A case was classified as particle-positive if in tissues from it budding particles and groups of characteristic particles were readily found. The method of examining cases has been described in the section dealing with materials and methods. In deciding which tissues should be taken from each case consideration was given to the murine findings; samples were taken from

grossly enlarged lymph nodes and from one or more of the following, bone marrow, thymus and spleen, whether or not gross changes in these tissues were evident. The requirement of very small tissue blocks and ultra thin sections for electron microscopy introduce the possibility of serious sampling errors and the subsequent misinterpretation of observations made on non-representative sections; for this reason several blocks were taken from each tissue and numerous sections from each block were studied. The number of "grid squares" surveyed in each examination was determined by 2 factors; the preliminary investigations with the mouse leukaemia tissue had shown that many particles were found in each grid square of tissue section and the study of the induced feline lymphosarcoma tissues had shown that particles were found in most grid squares of tissue section. Thus, the examination of 100 - 200 grid squares per case made it theoretically possible to detect particles present at approximately $\frac{1}{100}$ - $\frac{1}{200}$ of the concentration of the particles in the mouse or induced cat tissue.

Cat CL3 was the first case examined and in it particles were found in many sections. There was no way of determining whether or not this case was typical, but on the basis of the findings, the arbitrary criterion of classifying cases on the examination of 100 - 200 sections was adopted. In cases CL1, CL3, CL4, CL5 and CL6, fewer than 10 grid squares had to be examined to demonstrate particles in the particle positive tissues; in CL4, for example, particles were found in almost every section of every block; in CL1, on the other hand, while particles were found in almost every section of bone marrow and of the iliac lymph node, no particles were found in the spleen sections. From the data available, there is no way of assessing

whether failure to find particles was due to tissue selection and the examination techniques used or reflected inherent differences of particle numbers from organ to organ and from case to case. The failure to find convincing evidence of particles in any of the tissues from 3 of the cases may be due to the techniques employed. An alternative method of examining cases might be to study for each tissue as many sections as were necessary to include a predetermined number of megakaryocyte profiles; this would ensure more strictly comparable examinations basing the classification on the association of particles with the cell type most frequently found to contain particles. However, based on the techniques used in the present study, 5/8 of the cases appear to differ significantly from the other 3 in their having particles easily found and widely distributed throughout the tissue blocks and the organs of the animals whereas the others could not be conclusively classified as particle-positive. As noted in the introduction, particles were not easily demonstrated in spontaneous murine leukaemias so it is particularly interesting to have found large numbers of particles in so many cases in a species with a spontaneous leukaemia incidence much lower than that of the mouse strains studied (42/100,000 according to one estimate - Dorn et al., 1967 - compared with 80%).

In this investigation, no quantitative studies were made of particle-positive cases. Various investigations have used different methods of estimating particle frequencies; either particles per grid square or particle cell ratios have been used as comparison standards (Hagueneau et al., 1958; Feldman and Gross, 1966; Chapman et al., 1966a; Hagueneau and Hanafusa, 1968).

While these methods are valuable in systems in which large numbers of particles are present, they are of little use in a study such as the present one. Without making particle counts, but based on the relative ease with which particles were found in sections, it appeared that the cases CL3, CL4 and CL5 had increasing numbers of particles associated with them; this observation may be of importance in the interpretation of the results of the experiments described in Section 2.

In this study, no particles were found in tissues from 10 normal cats. No bone marrow samples were available, but careful examination of lymphoid cells and megakaryocytes in spleen and mesenteric lymph node sections showed no virus-like structures. Small thymuses were present in 6/10 of the cats; in the other animals the regression of the gland was complete. No particles were seen in the thymuses.

It might have been expected that particles would be observed in normal feline tissues if in fact the particles are vertically transmitted leukaemogenic viruses. Particles have been demonstrated in non-leukaemic mice of high and low incidence strains; viruses have been found in embryonic tissues (Feldman et al., 1967; Carmes, 1967) as well as in adults (Seaman and Dmochowski, 1965; Chapman et al., 1966b; Feldman and Gross, 1967; Kajima and Pollard, 1967, 1968; Swartzendruber et al., 1967; de Harven, 1963, 1964). One study of newborn and young AKR mice, 6 - 8 weeks old, failed to show particles in the spleen and thymus (Dirksen and Cailleau, 1967). Particles have also been shown in chemically induced and radiation induced leukaemias (Gross, 1958; Lieberman and Kaplan, 1959; Haran-Ghera, 1967 a,b), and in NZB/BL mice which develop autoimmune disease (Yumoto and Dmochowski, 1967; Prosser, 1968). Thus, there is good electron microscopic evidence

for the vertical transmission of the murine leukaemia viruses, although the number of particles seen in individual examinations is often small. (This subject was recently reviewed by Kaplan (1967)).

The failure to demonstrate particles in the normal cats does not preclude the possibility that small numbers of particles were widely distributed or focally situated in the tissues examined or were present in organs not studied. A more detailed investigation of normal feline tissues might reveal further similarities between the murine and feline leukaemias and in particular a study of feline embryonic tissues might provide valuable information for the understanding of the natural history of the disease in the cat.

SUMMARY

This investigation showed in spontaneous feline lymphosarcoma tissue particles which were morphologically identical to the particles previously demonstrated by Jarrett *et al.* (1964b) in the induced feline lymphosarcoma; the majority of cases examined had readily identified groups of particles and particles budding from cells. The particles in the cat tissues are structurally identical to the murine leukaemia viruses.

Note

Since this study was completed another case of spontaneous lymphosarcoma, CL9, has been investigated. At post-mortem a large thymic mass was found and the case was classified as a thymic lymphosarcoma after histological confirmation. The main cell type was the lymphoblast.

A blood sample for a platelet examination and specimens of spleen, bone marrow, thymus and a superficial node, were obtained from this animal.

An examination of the platelet pellets showed numerous particles, some in vacuoles and many between the platelets (Fig. 51); in vacuoles, only single particles were seen. Most of the particles had electron-dense nucleoids. No budding particles were observed.

In the spleen, no megakaryocytes were found and no lymphoid cells were seen with budding particles. However, many particles were noted in intercellular spaces. No Type 1 particles were seen. Most particles showed the typical electron-dense nucleoid although some appeared to have lost some of the electron-dense core material.

In the bone marrow many particles were found; some particles were seen budding from lymphoid cells and free particles of both Types 1 and 2 were found in intercellular spaces and cell vacuoles (Fig. 52). No megakaryocytes were seen in the sections examined.

In the thymus, many Type 1 particles were found extracellularly and in vacuoles in lymphoid cells and epithelial cells (Fig. 53).

Particles budding from lymphoid cells and free in intercellular spaces were observed in sections of the lymph node (Fig. 54).

Thus, in this case, many particles were found in the tissues and in the blood platelets and a blood platelet examination could have been used to determine the 'particle status' of the animal.

Section 2

Transmission studies with leukaemic tissue extracts from 4 cases of spontaneous feline lymphosarcoma

INTRODUCTION

The transmissibility of feline lymphosarcoma was established by Jarrett et al. (1964a). During the course of the present investigations Richard (1967) reported the development of leukaemia in kittens inoculated with a filtrate of a leukaemia tissue extract derived from a cat with spontaneous leukaemia and Kawakami et al. (1967) transmitted the disease using a whole cell or cell-free inoculum prepared from a field case.

In the experiments of Jarrett et al., 3/4 kittens died at 9, 12 and 18 months with palpable lymph nodes and histopathological evidence of the disease; the fourth cat developed typical multicentric lymphosarcoma at 15 months and a second passage with an inoculum derived from the leukaemia tissues of this animal produced extensive lymphosarcoma of the marrow cavities in 2 animals 2 months after inoculation. A second experiment with an inoculum derived from a different field case resulted in the development of lymphosarcoma in 2/7 animals after 23 and 43 months (Jarrett, 1966a).

From these results it is evident that the disease can be induced in kittens genetically unrelated to the cat with lymphosarcoma from which the inoculum is derived and that there is a long latent period between the time of inoculation and the development of the disease.

The mode of action of the leukaemogenic viruses is unknown. de Harven and Friend (1966) in a study of mice inoculated with Friend virus, failed to identify the target cells and the primary site of virus replication. However, they showed that virus replication took place as early as 4 days after inoculation and that budding virus could be seen in spleen, thymus,

bone marrow and blood platelets. Silvestre et al. (1966) showed that viruses could be identified in tissues from Rauscher virus infected mice from 4 - 12 days post infection to the end of their study at 16 - 35 days; in studies with another strain of mice, viruses were seen from 5 - 11 days through to 29 days. They further showed that a one-hundred-fold and a one-thousand-fold dilution of the inoculum produced similar effects and increased the period of failure to find virus in the spleen by only 2 days. The infectivity of virus produced in the early post infection stages has been demonstrated (Rauscher and Allen, 1964; Dalton and Moloney, 1962; de Harven, 1965; Chenaille et al., 1964).

In the murine studies the demonstration of plasma and platelet virus involved the pooling of blood samples from several inoculated animals which were killed at the time of blood sampling. While large volumes of blood are required for plasma virus preparations of sufficient concentration for electron microscopic identification of virus particles, a relatively small volume of blood, 2 - 5 ml, can be used to obtain a platelet preparation. This volume of blood can be withdrawn by cardiac puncture from a kitten and the animal kept alive; thus, in studies with kittens it is possible to make a series of blood platelet pellets from individual inoculated animals and to examine samples from each animal several times during the latent period.

Leukaemic tissues from the field cases examined in Section 1 were stored at -65°C and were available as source material for the preparation of inocula for transmission studies. Following the finding of leukaemia virus particles in tissues from some of the cats, 3 cases in which many particles

were found (CL3, CL4 and CL5) and 1 case in which only a single particle was observed (CL2) were chosen for further investigation as sources of inocula for the transmission experiments described in this section.

The aims of the investigation described in this section were -

1) to determine whether particles were associated with platelets from kittens at short times after inoculation and if so to make sequential studies of platelet samples from individual animals.

2) to examine tissues from pre-leukaemic inoculated kittens and to compare the tissue findings with the results of the platelet examinations made on the same animal.

3) to compare the early response of kittens to several inocula derived from different field cases.

4) to compare the results of the particle examinations with the histopathological findings.

5) to assess the leukaemogenic activity of the inocula as evidenced by the development of lymphosarcoma in infected kittens.

MATERIALS AND METHODS

1. Inocula Used in Transmission Experiments

(i) Inocula prepared from leukaemic tissues from field cases of lymphosarcoma

Extracts were prepared from the stored leukaemic lymph nodes from CL5, CL4 and CL3 and from the spleen and lymph nodes from CL2. The

following cell-free suspensions (CFS)were produced.

(a) CL5/CFS The stored leukaemic lymph node from CL5 was thawed and a 10% extract of the tissue made by adding 10 gms. of node to tris-buffered saline and homogenising at full speed in a Sylverson Grinder (Sylverson Machines Ltd., 55-57 Tower Bridge Road, London, S.E.1) for 2 minutes at 4°C. The resulting suspension was spun at 1,000 x g for 15 minutes and successive supernatants were spun at 1,000 x g for 15 minutes and 10,000 x g for 10 minutes. The final supernatant was stored at -65°C in 3.5 ml aliquots and a 50 ml aliquot.

(b) CL4/CFS A 10% extract of 20 gms. of stored leukaemic lymph node from CL4 was prepared in the same way as described for CL5/CFS. The CL4 suspension was spun at 1,000 x g for 5 minutes and successive supernatants were spun at 1,000 x g for 15 minutes (twice) and 10,000 x g for 10 minutes. The final supernatant was stored at -65°C in 4 ml and 15 ml aliquots.

(c) CL3/CFS A 10% extract of 45 gms. of stored leukaemic lymph node from CL3 was prepared in the same way as described for CL5/CFS. The CL3 suspension was spun at 1,000 x g for 20 minutes and successive supernatants were spun at 1,000 x g for 20 minutes and 10,000 x g for 10 minutes. The final supernatant was stored at -65°C in 4 ml aliquots and 30 ml aliquots.

(d) CL2/CFS A 10% extract of 10 gms. of stored leukaemic lymph node and spleen from CL2 was prepared in the same way as described for CL5/CFS. The CL2 suspension was spun at 1,000 x g for 10 minutes and successive supernatants were spun at 1,000 x g for 10 minutes and 10,000 x g for 10 minutes. The final supernatant was stored at -65°C in 2 ml aliquots.

(ii) Inoculum prepared from lymph nodes from a normal cat

LNE (Lymph node extract) A 10% extract of 5 gms. of fresh lymph node from a healthy cat was prepared in the same way as described for CL5/CFS. The suspension was spun at 1,000 x g for 10 minutes and successive supernatants at 1,000 x g for 10 minutes and 10,000 x g for 10 minutes. The final supernatant was stored at -65°C in 2 ml aliquots.

A mesenteric lymph node sample and a blood platelet pellet from this animal were prepared for electron microscopy.

2. Blood sampling for platelet examinations

Animals were anaesthetised and blood removed by cardiac puncture. Approximately 5 ml of blood from each animal were taken into a small volume of 0.3 M potassium citrate, pH 7.0. Individual and pooled blood samples were prepared for electron microscopy.

Experiment 1.

The examination for the presence of leukaemia virus particles of blood platelets and tissues from kittens inoculated with a leukaemic tissue extract (CL5/CFS) from the spontaneous feline lymphosarcoma CL5.

Materials and Methods

(i) Inoculation of kittens

Thirteen kittens from 4 litters were each inoculated intraperitoneally within 15 hours of birth with 1.0 ml of the inoculum CL5/CFS. The animals were numbered CL5/1 - CL5/13.

(ii) Blood sampling for platelet examinations

Twenty-eight days after inoculation, 8 of the kittens from 2 litters were bled and the individual blood samples were pooled to produce a single platelet pellet. Platelet pellets were also prepared from individual blood samples from kittens taken 42, 56 and 70 days after inoculation and at various times after 70 days from surviving animals.

(iii) Tissue Sampling

Samples of bone marrow, spleen and thymus were taken from 1 or 2 randomly chosen kittens killed 28, 56 and 70 days after inoculation and from the remaining animals when they were killed at later times *in extremis*.

Results

One kitten died less than a week after inoculation; no examinations were made of blood platelets or tissues from this animal.

1. Clinical and histological findings

None of the animals killed between 28 and 70 days showed any clinical signs of lymphosarcoma. The examination of tissues from the animals CL5/1, CL5/3-6 showed histological abnormalities of the bone marrow and spleen (Fig. 55); large numbers of focally arranged blast cells were a feature of the bone marrow and in both the spleen and marrow there was a megakaryocyte hyperplasia. No histological examination was made of tissues from CL5/2.

One kitten, CL5/7 had enlarged nodes and was in poor bodily condition 85 days after inoculation, and it was then killed; the gross lesions were similar to those found in cases of the alimentary form of spontaneous feline lymphosarcoma. Histological examination confirmed the diagnosis of lymphosarcoma; bone marrow involvement was severe and showed marked hyperplasia with mature and immature megakaryocytes widely distributed. In the spleen sections examined, no megakaryocytes were observed; the Malpighian corpuscles were enlarged and composed of blast cells which were invading the red pulp (Figs. 56, 57).

Kitten CL5/8 had enlarged superficial lymph nodes first palpable about 70 days after inoculation; the animal was found dead on day 86. The material available for histological examination showed severe post-mortem changes; however, the bone marrow was found to be very hyperplastic.

The kittens CL5/9 - CL5/12 were killed at different times in extremis, emaciated and in generally poor condition; all of the kittens showed slight to severe node enlargement. Histological examination showed the bone marrow to be hyperplastic (Fig. 58); the spleens were similar to the spleen of

CL5/7 and in the lymph nodes the cortical follicles were condensed and surrounded by lymphoblasts with a high mitotic rate. The dominant cell, scattered throughout the tissues and also forming distinct clusters, was an immature cell indistinguishable from a lymphoblast. These changes were similar to changes seen in spontaneous case tissues and were considered to indicate an early stage of malignancy.

2. Electron microscope findings

(i) Blood platelet examinations

Table 4 summarises the results of the examination of sections of pellets of blood platelets produced from individual or pooled blood samples taken at various times from 28 days to 1 year after the inoculation of the kittens. Typical leukaemia virus particles were found in all of the specimens.

It was found in this experiment, as in the succeeding ones, that the methods used to produce platelet pellets gave consistently good preparations for electron microscopic examination; the pellets consisted almost exclusively of platelets; a few red blood cells were found in a very few of the sections examined. In most pellets the platelets were clearly separated from one another, making it possible to examine platelet surface membranes with ease and thoroughness (Fig. 59); in the few pellets which had closely packed platelets such examinations were much more difficult to carry out and much more careful observation was required to discern early stages of particle budding from the external platelet membrane.

Table 4

The association of leukaemia virus particles with blood platelets from kittens inoculated as neonates with CL5/CFS

Kitten	Litter Number	Time from inoculation to blood sampling *				
		28 days	42 days	56 days	70 days	Later Times
CL5/1	2	≠				
CL5/2 (∅)	4					
CL5/3	1	≠	+			
CL5/4	3			+		
CL5/5	2	≠	+		+	
CL5/6	1	≠		+	+	
CL5/7	2	≠	+	+	+	
CL5/8	2	≠	+		+	
CL5/9	2	≠	+		+	+ (89 days)
CL5/10	4		+	+		
C15/11	2	≠		+	+	+ (22 weeks)
CL5/12	4		+	+	+	+ (27 weeks) (9 months) (1 year)

*

\+ leukaemia virus particles observed

\- no particles found

≠ Pooled blood sample from these kittens positive for leukaemia virus particles.

∅ No platelet pellet obtained from this animal bled and killed at 28 days.

Figure 60 shows in greater detail the typical appearance of the platelets seen in the blood samples from kittens in this experiment; there was considerable morphological variation between platelets in any one sample and between platelets from different samples but the general features observed in the kitten platelets were noted to be similar to those described for the platelets of other species (Dalton et al., 1961; Haguenau et al., 1963; David-Ferreira, 1964; French, 1967).

The particles found associated with the platelets were morphologically identical with the murine leukaemia viruses, with the particles found by Jarrett et al. (1964b) in an induced case of feline lymphosarcoma and with the particles found in the spontaneous feline lymphosarcomas described in Section 1 and in those reported by the other investigators studying single cases of spontaneous cat leukaemia (Rickard et al., 1967; de Harven, 1967). The particles were found budding from the surface of platelets and into platelet vacuoles (Figs. 61 - 64); completely formed particles were seen in vacuoles and in the spaces between platelet profiles (Figs. 65, 66). The fully-formed particles were approximately 100 mμ in diameter; both Type 1 and Type 2 particles were observed in vacuoles and free between platelets. In some instances the outer particle membrane showed the "coating layer" previously noted on the surface of some of the particles in spontaneous cases (Fig. 66).

Various stages of particle development, either at the platelet surface or at a vacuole surface could be distinguished (Figs. 67 - 70); the early stages were characterised by a slight thickening and "bulging"

outwards of the platelet outer membrane; the partially formed inner electron-dense membrane was seen lying behind and close to the bulging outer membrane. At a later stage the "protrusions" were more pronounced and formed hemispherical projections into the vacuole or inter-platelet space with the partially formed intermediate membrane visible between the outer and inner more electron-dense membranes. In many sections could be seen further stages of particle development, ranging from "buds" in which the 2 internal membranes were more fully formed to fully formed particles attached by a thread of membrane to the platelet surface or vacuole surface. At all stages of the development process the continuity between the outer particle membrane and the membrane of the platelet could be seen in favourably orientated sections.

The examination of the pooled sample taken at 28 days showed many particles; budding particles were frequently seen and there were many Type 1 particles but only a few of Type 2. Not many particles were found in the spaces between platelets but single particles in platelet vacuoles were frequently observed.

At 42 days large numbers of particles were found in all of the individual samples examined (Fig. 71). Some budding particles were seen; there were fewer than in the previous sample and most frequently the buds were forming within vacuoles. Complete particles, predominantly Type 2, were found in inter-platelet spaces and in vacuoles; some vacuoles contained more than one particle.

At 56 and 70 days and at later times, fewer particles were found than at 28 and 42 days. Very occasionally a budding particle was observed; Type 2 particles predominated and the intra-vacuolar site was

more commonly involved than the inter-platelet site.

The percentage of platelets with which particles were associated was estimated to vary from approximately 5% to 10% for the 28 and 42 day samples and from 1% to 5% for samples taken at later times.

(ii) Tissue examinations

Table 5 shows the results of the tissue examinations. It can be seen that leukaemia virus particles were present, in one or more of the selected tissues from each of the animals, at all of the times of examination between 28 days and 1 year; these particles, like those associated with platelets had the characteristic murine leukaemia virus morphology. Completely formed particles of both types were found associated with megakaryocytes and lymphoid cells, extracellularly and in vacuoles, and budding particles were seen at the plasma membranes and vacuolar surfaces of the same cell types.

(a) Spleen examinations

Most of the particles were found to be associated with megakaryocytes; occasionally, lymphoid cells were observed with a single particle budding from the surface. It was noted that no megakaryocytes had been seen in the sections from those spleens which were recorded as particle-negative.

At all of the times of examination the megakaryocytes were found to have many fully-formed particles in vacuoles and intercellular channels; many of the vacuoles contained 2 or 3 particles and the channels were frequently distended and in them groups of 2 - 5 particles were seen (Figs. 72, 73). The majority of the

Table 5

The association of leukaemia virus particles with tissues from kittens inoculated as newborns with CL5/CES

Kitten	Litter Number	Time from inoculation to killing (k) or death (d)	Tissue examined *		
			Bone marrow	Spleen	Thymus
CL5/1	2	28 days (k)	+	+	+
CL5/2	4	28 days (k)	+	+	+
CL5/3	1	42 days (d)	nta	nta	nta
CL5/4	3	56 days (k)	+	-	-
CL5/5	2	70 days (k)	+	-	-
CL5/6	1	70 days (k)	-	+	-
CL5/7	2	85 days (k)	+	-	nta
CL5/8	2	86 days (d)	+	nta	nta
CL5/9	2	89 days (k)	-	+	nta
CL5/10	4	15 weeks (k)	+	+	-
CL5/11	2	22 weeks (k)	+	+	+
CL5/12	4	52 weeks (k)	+	-	nta

*

+ leukaemia virus particles observed

\- no particles found

nta - no tissue available.

particles were of Type 1; some of these particles showed the external "coating layer" and frequently the apparent sub-structure of discrete "studs" was very distinctly seen. Type 2 particles were infrequently observed (Fig. 74); it was noted that in many of these particles the electron-dense core was less condensed than in particles of that type seen in the tissues from the spontaneous lymphosarcomas (Fig. 75). Several unusual forms of particle were observed (Fig. 76); cylindrical forms with clearly defined inner and intermediate membranes were noted; cylindrical sections were seen joining two elliptical or circular profiles (Fig. 77); two particles forming at right angles to one another were occasionally noted (Fig. 78). Particles were found budding into the vacuoles and channels (Fig. 79) and occasionally 2 or 3 particles attached to one another were seen apparently budding into an intercellular space.

The buds, forming at the surface of lymphoid cells, were similar to those seen at similar cell surfaces in the spontaneous lymphosarcoma tissues (Fig. 80).

While some of the infrequently observed extracellular particles were of Type 2, others were of Type 1.

The number of particles present and the predominant type of particle observed in each of 10 spleen specimens examined appeared to be independent of the time between inoculation and tissue examination.

(b) Bone marrow examinations

In two cases, CL5/6 and CL5/9, no megakaryocytes were observed, but in the bone marrow samples from each of the other kittens the megakaryocytes were found to contain a few budding particles and many completely formed particles. Both forms of particle were seen in the megakaryocytes; Type 2 particles were commonly found (Fig. 81). No atypical particles were noted in the bone marrow megakaryocytes but in all other respects the association of particles with these cells was the same as in the spleen. The megakaryocytes seen in sections of bone marrow from cases CL5/5 and CL5/7 had unusually large numbers of particles in vacuoles and in intercellular channels (Fig. 84).

Typical buds were seen at the surface of lymphoid cells (Figs. 82, 85); the partially formed inner and intermediate membranes could be discerned and the outer "coating layer" was seen on the surface of some particles. Fully formed particles were found free in the extracellular spaces near the lymphoid cell surface (Fig. 83). No particles were found associated with lymphoid cells in the cases CL5/6 and CL5/9 in which no megakaryocytes were seen.

Apart from the failure to find particles in cases CL5/6 and CL5/9 and the observation that cases CL5/5 and CL5/7 had very large numbers of particles no differences were noted between cases with respect to particle numbers or predominant particle type.

(c) Thymus examinations

In only 6 of the 12 animals was the thymus found to be present at post-mortem; particles were found in 3/6 of these cases. Fully formed and budding particles were found associated with lymphoid cells (Figs. 86, 87). The majority of the complete particles were of Type 1 and frequently showed the extra "coating layer" (Figs. 88, 89).

(d) Mesenteric lymph node examinations

Typical particles were found in the case CL5/6; the particles were free in the extracellular spaces and were mainly of Type 1.

No particles were found in the case CL5/12.

Discussion

In this experiment the leukaemogenic activity of the inoculum has been suggested by the development of the full clinical syndrome in the kitten CL5/7 and by the histopathological evidence of lymphosarcoma in kittens CL5/9-12. The development of lymphosarcoma in CL5/7 followed a latent period of 85 days which was shorter than that in the original passage of Jarrett et al. (1964a) and only slightly longer than the time taken for the marrow lesions to develop in their second passage experiment. In Kawakami's experiments (Kawakami et al., 1967) leukaemia developed in 5 - 11 weeks in 4/5 kittens inoculated with a whole cell extract from a case of spontaneous leukaemia and in 5 - 8 weeks in 3/4 kittens inoculated with a

cell-free extract from the same donor cat. The histopathological evidence of lymphosarcoma in inoculated kittens with no clinical symptoms of the disease was noted by Jarrett et al. in their studies and has been confirmed in this experiment. The significance of non-malignant abnormalities seen in tissues taken 1 month to 3 months after inoculation cannot be assessed; a detailed pathogenesis study would be required to investigate the significance of the changes observed.

In this experiment particles were observed in all of the platelet preparations examined and in one or more of the tissues from each of the inoculated kittens. Particles were more numerous in the kittens in this series than in the field case cats and sections of kitten tissues were very similar to sections of tissues from mice with induced leukaemia (Dalton et al., 1961).

The particles were morphologically identical with the particles in the spontaneous cases; both Type 1 and Type 2 particles were seen associated with megakaryocytes and lymphoid cells. The "coating layer" was particularly clearly demonstrated in many of the particles in many of the kittens.

No correlation was noted between the numbers of particles or the type or types of particle present in any given kitten and the length of time it had been infected; no differences could be determined between the association of particles with the bone marrow from CL5/1 taken 28 days after inoculation and bone marrow from CL5/12 taken 52 weeks after inoculation. Budding particles were observed in tissues taken from 28 days through to 1 year. No peculiar features of particle appearance or distribution could be discerned in the tissues from CL5/7 which had fully developed lymphosarcoma.

Thus, particles were demonstrable in the tissues during the post inoculation period, from 28 days onwards, whether or not lymphsarcoma had developed. As noted in the introduction to this section, similar observations have been made in the studies of murine leukaemias.

No blood samples were taken before 28 days since it was considered inadvisable to perform cardiac punctures before that time if the animals were to be kept alive and healthy. All of the platelet preparations contained particles. At 28 days a pool of blood samples was used to prepare a pellet; thus, the particle 'status' of individual kittens at that time cannot be assessed, although from the numbers of particles observed it is evident that either all of the kittens had considerable numbers of platelet associated particles or that some of the kittens had very large numbers of particles and others had fewer or none. There appeared to be more budding particles at early examination times than at later times; this observation is in agreement with the findings of de Harven and Friend (1966) who reported platelet budding from 4 days in mice inoculated with Friend virus and of Dalton et al. (1961) who reported platelet associated particles in leukaemic rats and made no mention of budding which, however, he reported in tissue examinations.

As mentioned in the General Introduction, Dalton et al. (1961) found 20% of leukaemic rat platelets to have associated particles; de Harven and Friend (1966) did not give an estimate of platelet involvement in preleukaemic states in mice. There are no data from experiments with Moloney or Friend murine viruses to indicate whether decreasing percentages of platelets are

involved as the time from inoculation to examination increases. The examination of kitten platelets in this experiment showed that there were fewer platelets with particles at later times; the failure to find convincing evidence of platelet associated particles in the field cases CL7 and CL8 could be related to this observation.

A comparison between the findings from the platelet examinations and those from the tissue examinations show that while there were fewer platelets involved in successive platelet examinations, there did not appear to be a similar reduction in the numbers of particles found in tissues; budding particles were found at all times from 20 days to 1 year.

Purified virus preparations from plasma from leukaemic mice have been examined by de Harven (1965). In Experiment 1, since only a small blood sample was taken from each kitten there was insufficient plasma available from which to attempt to prepare a concentrated particle pellet for negative staining examination or for embedding and sectioning; thus, there is no direct evidence that a viraemia developed in the inoculated kittens although the demonstration of free particles between platelets in the pellets suggests that this did occur. Recently, Kawakami et al. (1967) demonstrated particles in plasma taken from a leukaemic cat.

In studies with murine leukaemia viruses the association of particles with megakaryocytes was first noted by de Harven and Friend (1958) and later by Dalton et al. (1961) and by Dmochowski (1963); the association with platelets was noted by Dalton et al. (1961). The roles of megakaryocytes and platelets in the leukaemogenic process are not known. In both the murine

and the present feline studies, it has been shown that particles replicate in megakaryocytes and that large numbers of particles can be seen in megakaryocyte vacuoles and channels. When platelets detach from the megakaryocytes the particles in the delamination channels will be released and may either infect target cells near the megakaryocyte or enter the free circulating virus pool. Particles budded off from other cell types may also enter this pool. The contribution of the platelets is not known, but de Harven (1965) suggests that they also add to the plasma viraemia. Particles in megakaryocyte vacuoles will become platelet particles when the platelet detaches from the megakaryocyte; whether or not such particles are released directly into the plasma or remain within the platelet till the platelet dies, is not known; in either case the platelet acts as a 'transporter' of particles from the producing cell to some other site.

The appearance of the particle budding seen in the kitten platelets suggests that particles were being replicated in the platelets; such a dynamic interpretation cannot be substantiated but the finding of all stages of budding from platelet membranes and into vacuoles makes this seem possible. If this interpretation is correct, it implies that one of the following processes takes place: either particles actively budding in a platelet segment of a megakaryocyte continue to bud when the platelet detaches and thus that the platelet carries the necessary information for the continuing synthesis of particles; or platelets are capable of synthesising particles completely and can continue to do this throughout the lifetime of the platelet, in which case, particle budding could start after platelet detachment. Alternatively, particle formation could be arrested when platelets detach from the

megakaryocytes and the buds seen on the platelets would then be incapable of completion. From the electron microscopic evidence available this latter possibility seems the most unlikely; which of the former two hypotheses is the more likely to be correct is not possible to decide without further knowledge of the synthetic capabilities of blood platelets.

How long a virus can survive in the plasma is not known. de Harven (1965) suggested that the level of viraemia results from an equilibrium being established between virus production and virus clearance, either through the viruses infecting cells or by their being phagocytosed. No evidence of virus phagocytosis was observed in the kitten studies.

In this experiment with inoculum GL5/CFS it has been shown that particle replication occurs in megakaryocytes and lymphoid cells in inoculated kittens and that this can take place from 28 days to 1 year; it has also been shown that particles can be found in platelets throughout this period. The relationship, if any, between particle replication and the development of lymphosarcoma has not been established.

Experiment 2

The examination for the presence of leukaemia virus particles of blood platelets and tissues from kittens inoculated with a leukaemic tissue extract (CL4/CFS) from the spontaneous feline lymphosarcoma CL4.

Materials and Methods

(i) Inoculation of kittens

Seventeen kittens from 4 litters were each inoculated intra-peritoneally within 24 hours of birth with 0.7 - 1.0 ml of the inoculum CL4/CFS. The animals were numbered CL4/1 - CL4/17.

(ii) Blood sampling for platelet examinations

Twenty-eight days after inoculation a pooled platelet preparation was obtained from 8 kittens from 2 litters and platelet pellets were made from individual blood samples taken from the 7 kittens from the other 2 litters. Platelet pellets were also prepared from individual blood samples from kittens taken 42, 56 and 70 days after inoculation and after 13 weeks, 27 weeks and approximately 9 months from surviving animals.

(iii) Tissue sampling

Samples of bone marrow, spleen and thymus were taken from 1 randomly selected kitten killed at 28, 42 and 56 and 70 days after inoculation and from 8 animals killed *in extremis* after 13 weeks, 27 weeks and approximately 40 weeks.

Results

One animal died 2 months after inoculation; the cause of death was not established. Histological examination of tissues from this animal showed no signs of lymphosarcoma; no material was available for electron microscopy.

Four animals are still alive with no clinical signs of lymphosarcoma 16 months after inoculation.

1. Clinical and histological findings

None of the 12 animals killed in this experiment had developed clinical signs of lymphosarcoma; some of the animals had slight peripheral lymph node enlargement but at post-mortem no animal was found to have grossly enlarged abdominal nodes. The animals killed *in extremis* were emaciated and not eating.

Histologically 2 cases showed evidence of neoplasia; CL4/3 killed at 56 days, showed malignant changes in the bone marrow and CL4/7 killed *in extremis* at 13 weeks showed malignant changes in the spleen (Figs. 90, 91). The spleens from cases CL4/1 and CL4/2 killed at 28 days and 42 days respectively showed changes which, by comparison with changes seen in spontaneous cases of feline lymphosarcoma, were thought to be indicative of early malignancy.

2. Electron microscope findings

(i) Blood platelet examinations

Table 6 summarises the results of the examination of a pooled blood sample and of individual blood samples taken 28 days after

Table 6

The association of leukaemia virus particles
with blood platelets from kittens inoculated as newborns with GL4/OF3

Kitten	Litter	Time from inoculation to blood sampling *				
		28 days	42 days	56 days	70 days	Later
GL4/1	1	∅				
GL4/2	4	-				
GL4/3	2	≠	+	+		
GL4/4	2	≠	+ slight			
GL4/5	1	≠	+	+ slight	+	+ (13 weeks)
GL4/6	1	≠	+ slight	+	+ slight	+ (13 weeks)
GL4/7	2	≠	+	+ slight	+ slight	+ (13 weeks)
GL4/8	4	+ slight		?+	+ slight	
GL4/9	4	-		+ slight		+ (13 weeks) + (27 weeks)
GL4/10	2	≠	+ slight	?+	+	+ (27 weeks) - (9 months) + (10 months)
GL4/11	4	-	+ slight	+	+	+ (27 weeks) + (9 months) + (10 months)
GL4/12	3	-	+	+	+	+ (27 weeks) ?+ (9 months) + (12 months)
GL4/13	1		?+	+	+	+ (27 weeks) ?+ (9 months)
GL4/14	3		+	+	?+	?+ (9 months)
GL4/15	3	-		+		+ (9 months)
GL4/16	4				+	+ (27 weeks) ?+ (9 months)

* + leukaemia virus particles observed; - no particles found

≠ Pooled blood sample from these kittens positive for leukaemia virus particles

∅ No platelet pellet from this animal bled and killed at 28 days

inoculation and of individual examinations made at various times from 42 days to 48 weeks. Characteristic leukaemia virus particles were found in many of the preparations (Figs. 92 - 95), in some no leukaemia virus particles were seen and in several samples atypical virus-like structures were observed in platelet vacuoles; samples of this latter category are marked "?+" in the table.

In no pooled or individual platelet sample examined in this experiment were particles as abundantly present as in any of the preparations from Experiment 1. It was estimated that the percentage of platelets with associated particles was of the order of 0.1% - 4%. Morphologically the particles in the samples marked "+" were identical with those found in Experiment 1; budding particles were not found frequently but appeared to be most common in the samples taken at 42 days; at all times the Type 2 particles were more often seen than were Type 1 particles.

Those results recorded as "+ slight" indicate that the platelet examinations revealed clearly defined characteristic leukaemic virus particles but that there were found in approximately less than 0.5% of the platelets.

The atypical particles found in the samples marked "?+" were seen in platelet vacuoles; in only a few platelets from any one sample were such particles seen. The particles were of the same size as the typical leukaemia virus particles, approximately 100 mμ in diameter, but they had a homogenous electron-dense material filling almost the entire core of the particle; no internal membrane structure could be discerned.

(ii) Tissue examinations

Table 7 shows the results of the tissue examinations. It can be seen that leukaemia virus particles were found in one or more of the selected tissues from 8/10 of the animals.

(a) Spleen examinations

Typical particles were found in spleen sections from only 2 of the animals CL4/3 and CL4/11; the particles, seen in megakaryocytes, were mainly Type 1. Budding particles were not observed. Megakaryocytes were not seen in sections from any of the other cases. No particles were seen budding from or in close association with lymphoid cells in any of the cases.

(b) Bone marrow examinations

(b) Bone marrow examinations

Typical particles were found in 7/8 of the bone marrow samples; virus-like particles were seen in the remaining sample. Particles were observed budding from lymphoid cells; fully-formed particles of both types were seen free between cells and in the vacuoles and intracellular channels of megakaryocytes (Figs. 96, 97).

Large numbers of Type 2 particles were found singly and in groups in the megakaryocyte vacuoles (Fig. 98); in sections from each case Type 1 particles were frequently observed, usually singly, in vacuoles (Fig. 99).

Extracellularly, single particles and groups of particles were found in close association with lymphoid cells; the majority of these were Type 2 particles.

Table 7

The association of leukaemia virus particles
with tissues from kittens inoculated as newborns with CL4/CFS

Kitten	Litter	Time from inoculation to killing (k) or death (d)	Tissues Examined *		
			Bone Marrow	Spleen	Thymus
♯ CL4/1	1	28 days (k)	nta	-	-
CL4/2	4	42 days (k)	+	-	-
CL4/3	2	56 days (k)	+	+	-
CL4/4	2	70 days (k)	+	-	+
CL4/5	1	13 weeks (k)	+	-	+
CL4/6	1	13 weeks (k)	+	-	nta
CL4/7	2	13 weeks (k)	?+	-	nta
CL4/8	4	13 weeks (k)	+	-	nta
∅ CL4/9	4	27 weeks (k)			
♯ CL4/10	2	40 weeks (k)	nta	-	-
CL4/11	4	41 weeks (k)	+	+	nta
∅ CL4/12	3	48 weeks (k)			

* + leukaemia virus particles observed
?+ virus-like particles observed
\- no particles found

♯ Extra spleen sections examined from these cases from which no bone marrow was available.

∅ No material for electron microscopy taken from these cases

nta No tissue available

Note: 4 kittens inoculated with CL4/CFS still alive and healthy 16 months after inoculation.

(c) Thymus examinations

Particles were found in the thymus from only two of the 12 animals in which this organ was found to be present at post-mortem. In case CL4/5, particles, mainly of Type 1, were found extracellularly; some particles were found in lymphoid cell vacuoles. No budding particles were observed. In case CL4/4, only a few particles were observed; the particles were found in intercellular spaces and were of Type 1.

Discussion

In this experiment no animal which was killed had developed clinical signs of lymphosarcoma. However, 4 animals are still alive 16 months after inoculation, which is a shorter time than the latent period recorded for tumour development in some of the animals in the experiments of Jarrett (1966a); these animals are being examined frequently for any signs of deterioration.

The histopathological evidence of malignancy is less marked in this experiment than in Experiment 1 and only 2 animals showed convincing evidence of early lymphosarcoma.

Of the 3 tissues taken from each of the kittens in this experiment, only the bone marrow was consistently particle-positive; the bone marrow sections were very similar to those seen in Experiment 1, and they appeared to contain numbers of particles of the same order as were seen in the Experiment 1 sections.

The results of the spleen examinations are comparable with those of the spleen examinations made in Experiment 1, in that in both experiments the

some number of randomly chosen grid squares of tissue section were scanned; however, whereas megakaryocytes were found in sections of most of the spleens from the kittens in Experiment 1, this cell type was seen in sections from only 2 of the kittens in Experiment 2. The 2 kittens in which spleen megakaryocytes were found were the only 2 which had particle-positive spleens; the particles were associated with the megakaryocytes and were not found budding from or associated with lymphoid cells. Thus, the failure to find particles in the spleens of the other kittens may be due to the fact that no megakaryocytes were examined in these cases and the classification of some animals as particle-positive and others as particle-negative may result from differences in the degree of widespread megakaryocyte hyperplasia in the spleens. The histological findings would tend to support this. As discussed earlier, sampling difficulties present serious problems for the interpretation of results from electron microscopical examinations; the above result may be a case in point. It might be of value to investigate the particle negative spleens using the alternative method of specimen scanning based on megakaryocyte examinations (see Section 1, Discussion).

In this experiment the results of the platelet examinations were much less consistent than those made in Experiment 1. The peak budding time appeared to be at 42 days compared with 28 days in Experiment 1. Fewer particles were found at all times; in many instances only a few particles were seen and in some examinations particles could not be identified with certainty. By comparison with the findings from Experiment 1, these results suggest that in this experiment either few megakaryocytes were producing platelets with particles or that the megakaryocytes themselves had smaller

numbers of particles to contribute to the delaminating platelets. Since neither the precise origin of the particle-positive platelets nor the kinetics of platelet production is known the possible significance of these observations cannot be assessed. Particles seen lying free between platelets suggest that a viraemia developed in the kittens; no quantitative study was made to try to compare the levels of viraemia in this experiment with those in Experiment 1.

Experiment 3

The examination for the presence of leukaemia virus particles of blood platelets and tissues from kittens inoculated with a leukaemic tissue extract (CL3/CFS) from the spontaneous feline lymphosarcoma CL3.

Materials and Methods

(i) Inoculation of kittens

Nineteen kittens from 5 litters were each inoculated intraperitoneally within 24 hours of birth with 0.75 - 2.0 ml of the inoculum CL3/CFS. The animals were numbered CL3/1 - CL3/19.

(ii) Blood sampling for platelet examinations

Platelet pellets were prepared from individual blood samples taken from kittens 28, 42, 56 and 70 days after inoculation and at 6 months from surviving animals.

(iii) Tissue sampling

Samples of bone marrow, spleen and thymus were taken from 1 randomly chosen kitten killed 28, 31, 42 and 56 and 80 days after inoculation.

Results

Four of the kittens died within 28 days of inoculation. The cause of death was not established, but grossly there were no signs of lymphosarcoma; no material was taken for histological or electron microscopical examination.

Ten of the cats CL3/6 - CL3/15 were killed after 46 weeks during an outbreak of panleukopoenia in one section of the cat colony. No clinical signs of lymphosarcoma had been observed in any of these animals and at post-mortem no gross evidence of lymphosarcoma was found; no histological examinations were made of tissues from these animals.

1. Clinical and histological findings

No clinical signs of lymphosarcoma were observed in any of the animals CL3/1 - CL3/7 killed between 28 and 30 days. Histologically, none of the tissues showed evidence of lymphosarcoma; the spleens from CL3/3 and CL3/4 showed reactive changes and the bone marrow from CL3/4 and CL3/5 showed general hyperplasia. No abnormalities were observed in the thymuses.

2. Electron microscope findings

(i) Blood platelet examinations

Table 6 summarises the results of the platelet examinations; the presence of leukaemia virus particles in, or associated with, platelets was established in only a very few of the samples studied (Figs. 100, 101); in those samples in which particles were identified, the number of platelets found to contain particles was extremely small and it was estimated that less than 0.1% of the platelets were involved.

Table 8

The association of leukaemia virus particles with blood platelets from kittens inoculated as newborns with CL3/CFS

Kitten	Litter Number	Time from inoculation to blood sampling *				
		28 days	42 days	56 days	70 days	later times
CL3/1	1	-				
CL3/2	4	-				
CL3/3	1		-			
CL3/4	2					
CL3/5	4	-	-	+	+	+(80 days)
CL3/6	1	-	-	+	-	+(6 months)
CL3/7	1	-	-	-	-	?+(6 months)
CL3/8	1	-	-	-	-	?+(6 months)
CL3/9	2	-	?+	?+		+(6 months)
CL3/10	2			+		
CL3/11	2	?+		?+	+	?+(6 months)
CL3/12	3	-		+	+	?+(6 months)
CL3/13	3	-	?+	+	?+	
CL3/14	4	-	-		+	?+(6 months)
CL3/15	4	-	-		+	+(6 months)

*

\+ leukaemia virus particles observed

?+ virus-like particles observed

\- no particles found

In the samples recorded as "?+", the same kind of atypical particles as were found in platelets from Experiment 2 were seen in a few platelet vacuoles. As in Experiment 2, the morphology of these particles was not sufficiently characteristic of the leukaemogenic-type viruses to enable the samples to be classified as particle-positive.

(ii) Tissue examinations

Table 9 shows the results of the tissue examinations; no leukaemia virus particles were found in any of the tissues from any of the cases. No megakaryocytes were found in any of the spleen sections examined but this cell type, with no associated particles, was commonly seen in bone marrow sections. The thymus was present at post-mortem in 4/5 of the animals; no particles were found in any section of thymus.

Discussion

In this experiment, no clinical or histopathological evidence of lymphosarcoma was found; thus, the inoculum was not demonstrated to be leukaemogenic.

No leukaemia virus particles were found in any of the tissues; from the previous experiments it might have been thought that this could have resulted from the electron microscope sections not including megakaryocytes; however, while this may have been true of the spleen examinations, it was not so for the bone marrows in which megakaryocytes were observed and no particles were found associated with them.

Table 9

The association of leukaemia virus particles with tissues from kittens inoculated as newborns with CL3/CFS

Kitten	Litter Number	Time from inoculation to killing (k) or death (d)	Tissues examined *		
			Bone Marrow	Spleen	Thymus
CL3/1	1	28 days (k)	-	-	-
CL3/2	4	31 days (k)	-	-	-
CL3/3	1	42 days (k)	-	-	-
CL3/4	2	56 days (k)	-	-	-
CL3/5	4	80 days (k)	-	-	nta

* + leukaemia virus particles observed

\- no particles found

nta no tissue available.

Note: Kittens CL3/6 - CL3/15 killed at 46 weeks
No tissues available for electron microscopy

The results of the platelet examinations are difficult to interpret; no particles were found at the early times of examination and only a few at later times; some platelets contained structures which could not be classified with confidence as leukaemia virus particles.

The origin of the particles seen in the platelets is not known. No particles were found in bone marrow megakaryocytes; however, since the percentage of platelets with particles was very low it is possible that only a small number of megakaryocytes, each containing only a few particles, were involved, and that the electron microscope examination was not sufficiently extensive to include even one such megakaryocyte.

Experiment 4

The examination for the presence of leukaemia virus particles of blood platelets and tissues from kittens inoculated with a leukaemic tissue extract (CL2/CFS) from the spontaneous feline lymphosarcoma CL2.

Materials and Methods

(i) Inoculation of kittens

Six kittens from 2 litters were each inoculated intraperitoneally within 24 hours of birth with 1.0 ml of the inoculum CL2/CFS. The animals were numbered CL2/1 - CL2/6.

(ii) Blood sampling for platelet examinations

Platelet pellets were prepared from individual blood samples taken from kittens 28 days, 42 days and 10 weeks after inoculation.

(iii) Tissue sampling

Samples of bone marrow, spleen and thymus were taken from 1 animal 28 days after inoculation.

Results

Three animals from 1 of the litters died with pneumonia less than 28 days after inoculation; one animal CL2/2 was found dead, due to unknown causes, 56 days after inoculation; one kitten, CL2/3 died with panleukopoenia 7½ months after the start of the experiment.

1. Clinical and histological findings

No clinical signs of lymphosarcoma were seen in kitten CL2/3 observed for 7½ months.

Tissue for histological examination was taken from only one animal, CL2/1; apart from slight hyperplasia and very active erythropoesis of the bone marrow, no abnormalities were observed.

2. Electron microscope findings

Tables 10 and 11 record the examinations made and show that no leukaemia virus particles were seen associated with tissues, and only one virus-like particle was seen in the platelet examinations (Fig. 102).

Discussion

In the field case CL2, only a single virus-like particle was observed in the prescapular lymph node. The inoculum derived from this case was used to infect kittens in order that its activity might be compared with that of the inocula prepared from the 3 cases in which many particles were found.

Table 10

The association of leukaemia virus particles with blood platelets from kittens inoculated as newborns with CL2/CFS

Kitten	Litter Number	Time from inoculation to blood sampling *		
		28 days	42 days	later times
CL2/1	2	-		
CL2/2	1	-	?	- (10 weeks)
CL2/3	1	-	-	

*

\+ leukaemia virus particles observed

\- no particles found

? one virus-like structure observed.

Table 11

The association of leukaemia virus particles
with tissues from kittens inoculated as newborns with CL2/CFS

Kitten	Litter Number	Time from inoculation to killing (k)	Tissues examined *		
			Bone Marrow	Spleen	Thymus
CL2/1	2	28 days (k)	-	-	-

*
- \+ leukaemia virus particles observed
- \- no particles found

Note: Kittens CL2/2 died at 56 days and kittens CL2/3 killed at 31 weeks.
No tissues were available for electron microscopy.

The scope of the experiment was limited by the shortage of kittens and was carried out with the last two available litters. The only conclusion which can be drawn from the few observations which were made is that this inoculum CL2/CFS did not induce the same particle production responses in tissues and platelets as were produced in Experiments 1, 2 and 3.

Experiment 5

Experiment 5

The examination for the presence of leukaemia virus particles of blood platelets and tissues from kittens inoculated with an inoculum (LNE) prepared from lymph node tissue from a healthy cat.

Materials and Methods

(i) Inoculation of kittens

Eleven kittens from 4 litters were each inoculated intraperitoneally within 24 hours of birth with 1.0 ml of the inoculum LNE. The animals were numbered LNE/1 - LNE/11.

(ii) Blood sampling for platelet examinations

Platelet pellets were prepared from individual blood samples taken from kittens 28, 42, 56 and 70 days after inoculation.

(iii) Tissue sampling

Samples of bone marrow, spleen and thymus were taken from 2 animals killed at 28, 42 and 70 days and from 1 killed at 56 and 61 days.

Results

Three kittens from 2 of the litters died within a few days of inoculation; no material was taken for histological or electron microscopical examination.

1. Clinical and histological findings

During the course of the observations, 28 - 70 days, the animals remained healthy and showed no clinical signs of lymphosarcoma. Histological examinations showed reactive changes in the spleens and that the bone marrows, which were slightly hyperplastic, contained many fat cells. No thymic abnormalities were seen.

2. Electron microscope findings

Table 12 shows the results of the blood platelet examinations and Table 13 gives the findings of the tissue examinations. No leukaemia virus particles were observed in any of the tissue sections; in one platelet examination (L7 at 70 days) two single virus-like structures were seen (Fig. 103).

An examination of the mesenteric lymph node and of platelets from the normal cat from which the inoculum LNE was derived failed to reveal leukaemia virus particles.

Discussion

This experiment was performed to discover whether an extract of normal lymph node tissue would produce the same particle replication response in kittens as inocula prepared from leukaemic tissues. While the examination of donor tissues may fail to reveal particles it cannot prove that they are not present; thus, inocula from apparently normal, "particle-negative" animals

Table 12

The examination for the presence of leukaemia virus of blood platelets from kittens inoculated as newborns with normal lymph node extract LMF

Kitten	Litter Number	Time from inoculation to blood sampling *			
		28 days	42 days	56 days	70 days
L1	2	-			
L2	2	-			
L3	1	-	npp		
L4	2	-	-		
L5	1	-	npp	-	
L6	2	-	-	-	
L7	1	-	npp	-	?+
L8	2	-	-	-	npp

*

+	leukaemia virus particles observed
?+	2 single virus-like particles observed
-	no particles found
npp	no platelet pellet

Table 13

The examination for the presence of leukaemia virus particles of tissues from kittens inoculated as newborns with normal lymph node extract LNE

Kitten	Litter Number	Time from inoculation to tissue sampling	Tissues examined *		
			Bone Marrow	Spleen	Thymus
L1	2	28 days	-	-	-
L2	2	28 days	-	-	-
L3	1	42 days	-	-	-
L4	2	42 days	-	-	-
L5	1	56 days	nta	-	-
L6	2	61 days	-	-	-
L7	1	70 days	-	-	-
L8	2	70 days	-	-	-

*

\+ leukaemia virus particles observed

\- no particles found

nta no tissue available.

may contain particles present, latently, in low concentration in the donor cats. Such particles might induce replication in recipient kittens. It is also possible that inocula prepared from normal cats, not containing particles, might stimulate a virus present latently in the recipient kittens. This experiment has shown virus-like structures in only one kitten. Whether or not these structures could be characterised as typical leukaemia virus particles and if so, whether or not they are of donor or recipient origin, the conclusion which can be drawn from this experiment is that this inoculum, tested in randomly selected kittens, specimens from which were examined in the standardised way, did not produce the same particle production response as was produced by CL5/CFS, CL4/CFS or CL3/CFS.

Experiment 6

The examination for the presence of leukaemia virus particles in blood platelets and tissues from normal kittens.

Materials and Methods

(i) Kittens

The control group of normal, uninfected kittens consisted of 7 animals from 2 litters.

(ii) Blood sampling for platelet examinations

Platelet pellets were prepared from individual blood samples taken from kittens aged 28, 42, 56 and 70 days.

(iii) Tissue samples

Samples of bone marrow, spleen and thymus were taken from kittens aged 28, 42, 56 and 70 days.

Results

1. Clinical and histological findings

The spleen sections from some of the kittens showed reactive changes; bone marrow sections from all of the kittens contained early forms of all of the cell types and many fat cells were present; no abnormalities were found in the sections of thymus.

2. Electron microscope findings

In two platelet examinations of one kitten, virus-like structures were seen - see Table 14 (Figs. 104, 105, 106). No leukaemia virus particles were observed in any of the tissues (see Table 15).

Discussion

It has already been mentioned in the discussion in Section 1 that while in some murine studies, leukaemia viruses have been demonstrated in tissues from normal mice other investigators have failed to reveal viruses. The kittens in this small survey showed no evidence of typical leukaemia virus particles in tissues and in only one kitten were a few virus-like structures found in the platelets. The findings from this preliminary study can neither substantiate the hypothesis nor preclude the possibility that a latent leukaemogenic virus is carried, possibly in low concentration in all of or in a proportion of the general cat population. However, this

Table 14

The examination for the presence of leukaemia virus particles of blood platelets from normal kittens

Kitten	Litter Number	Age at time of platelet examinations *			
		28 days	42 days	56 days	70 days
NK1	1	-			
NK2	1	-	-		
NK3	2	-	npp	npp	
NK4	1	-	-	-	npp
NK5	2	-	-	-	-
NK6	1	?+	-	?+	-
NK7	1	-	-	-	-

*

+ leukaemia virus particles observed
?+ virus-like particles observed
- no particles found
npp no platelet pellet

Table 15

The examination for the presence of leukaemia virus particles of tissues from normal kittens

Kitten	Litter Number	Age at time of tissue sampling	Tissues examined *		
			Bone Marrow	Spleen	Thymus
NK1	1	28 days	-	-	-
NK2	1	42 days	-	-	-
NK3	2	56 days	-	-	-
NK4	1	70 days	-	-	-
NK5	2	70 days	-	nta	-

*

+ leukaemia virus particles observed
- no particles found
nta no tissue available

Note: Kittens NK6 and NK7 were transferred to another experiment not described in this study.

experiment has demonstrated a distinct difference between the kittens in Experiment 1 and the normal kittens in that while all of the former had particles associated with them, none of the uninfected animals, apart from the one with virus-like structures, showed particles in tissues or platelets examined in the same way as the specimens from Experiment 1. The experiment has also shown that the inocula tested in Experiment 2 and Experiment 3 produced responses in kittens involving morphological patterns not apparently universally found in normal kittens of the same ages.

GENERAL DISCUSSION

The three inocula from particle-positive field cases have been shown to induce different responses in the inoculated kittens; the inocula were similarly prepared and the recipient kittens in each experiment were randomly selected from the closed colony. Thus, the different responses must reflect inherent differences between the inocula; these differences might be qualitative or quantitative.

As shown in Table 2 in Section 1, both morphological types of particle were seen in case CL4 and also in case CL5; Type 2 particles were rarely found in case CL3. Another qualitative difference between these three cases was that whereas the dominant cell type in the cases CL4 and CL5 was "immature" that in case CL3 was "mixed" (see Table 1).

No particle counts were made to compare the 3 cases quantitatively but, as was noted in Section 1, there appeared to be increasing numbers of particles in cases CL3, CL4 and CL5.

The significance of these qualitative and "quantitative" observations is difficult to assess. The summarised results of the experiments 1 - 3 are as follows:-

CL5/CFS derived from a tumour with predominantly immature cells, with particles of both types in reasonable numbers, induced particle replication in platelets and tissues from 28 days; lymphosarcoma developed by 86 days in two kittens and there was histopathological evidence suggestive of lymphosarcoma in several other kittens.

CL4/CFS derived from a tumour with predominantly immature cells, with particles of both types but fewer of both than CL4, induced particle replication in platelets and in tissues from 28 days to such a degree that it could be demonstrated in some, but not all of the specimens examined; there was histological evidence strongly suggestive of lymphosarcoma in tissues from 2 of the kittens and in another 2 kittens changes were observed which were slightly suggestive of lymphosarcoma.

CL3/CFS derived from a tumour with "mixed" tumour cells, with Type 1 particles in smaller numbers than CL4, produced a slight response in kittens evidenced by small numbers of particles associated with some platelets, at some times of examination.

Whether or not the dominant cell type in the donor is an important factor in determining the effectiveness of an inoculum cannot be judged from the present experiments. Clearly, since the inocula were cell-free any influence the cell type may have had was of a secondary nature. It might be that the dominant cell type in the donor reflects or controls either the rate of particle production or the "infective" nature of particles produced.

A high rate of particle production would result in there being large numbers of "fresh" particles in the tissues from which an inoculum was derived whereas a slower rate might lead to an inoculum with a higher percentage of "inactivated" particles. As yet very little is known about the kinetics of particle replication and what happens to the particles produced in the mouse leukaemias so that the present results cannot be interpretated by comparison with those obtained from murine studies.

Which of the two morphologically distinct particles, Type 1 or Type 2, is the infective virus of murine leukaemia is not yet established; de Harven (1965) has put forward the hypothesis that the triple-membraned type (A1 type) is the biologically active particle and that the low biological activity of his preparation reflects the low "A1/C" particle ratio, 4/1,000. In the feline studies Type 1 particles were found in each of the spontaneous cases CL3, CL4 and CL5, thus according to de Harven's theory each contained active particles; the differences in degree of response in the 3 experiments might be explained by the numbers of particles present in each of the inocula. Since CL5, CL4, CL3 were "graded" in that order as most to least particle-positive, the results of the experiments could agree with this theory. If, however, the Type 2 particle is the infective agent the inocula CL5/CFS and CL4/CFS would have contained many active particles whereas CL3/CFS in which this form of particle was not so frequent, would have been a less effective inoculum. The slight response of kittens to CL3/CFS, by comparison with that to CL4/CFS and CL5/CFS, might be explained by this hypothesis. The present results cannot determine which of these 2 theories is correct.

Not enough data are available from Experiment 4 for any conclusion to be drawn except that the inoculation of kittens with leukaemic tissue extracts does not necessarily result in demonstrable particle replication in platelets and tissues at early times after inoculation.

The significance of the finding of a few virus-like structures in kittens inoculated with LME and in the untreated animals is difficult to assess. This might indicate that some or all normal kittens naturally have small numbers of particles present in their tissues. Further studies with normal animals are required to study this but it is clear from the present experiments that CL5/CFS, CL4/CFS and CL3/CFS infected kittens are distinctly different from normal and control experiment kittens.

These experiments have not determined at what sites and in which cells particle replication first takes place in the inoculated kittens; attempts to establish the origin of the viraemia in Friend leukaemia were made by de Harven and Friend (1966) but they were not able to find which cells were the primary target cells for virus; particles were seen 1 and 2 days after inoculation in the thymus and by day 4 viruses were found in many tissues. In the present experiments particles were less frequently observed in the thymus than in the other tissues examined. It should be emphasised that no kitten examinations were made before 28 days; examinations of tissue and blood platelets taken at earlier times might help to determine how and where the particle replication is initiated.

In the mouse it is suggested that viral proliferation and the subsequent steps in leukaemogenesis are separate although dependent processes (Rich et al., 1967; Kaplan, 1967). In this study no attempt has been made to relate particle production to leukaemogenesis.

SUMMARY

These experiments have shown that the replication of leukaemia virus particles can be demonstrated in kittens infected with inocula derived from leukaemic tissues from cats with spontaneous lymphosarcoma in which the same type of particles had been observed. Particles were frequently seen in the blood platelets and tissues from kittens inoculated with one or other of 2 of the inocula tested; in kittens infected with a third inoculum occasional particles were found. Some of the inoculated kittens showed histological evidence of lymphosarcoma.

As controls for these experiments some kittens were inoculated with an extract of lymph nodes from a normal, healthy cat and blood platelets and tissues from them and from normal untreated kittens were examined. Although one or two particles were observed in a very few control animals no response was observed similar to that induced by the leukaemic tissue extracts.

Section 3

Further transmission studies

(1) with a filtrate inoculum

(2) in adult cats

(3) in serial passage

INTRODUCTION

The inocula used in the experiments of Jarrett et al. (1964a) and also those used in the experiments described in Section 2, were prepared from leukaemic tissues by subjecting lymph node extracts to a series of high speed centrifugation procedures: the inocula were presumed to be cell-free. No tumours arose at sites of inoculation and randomly chosen litters of kittens responded in varying degrees to the inocula. No chromosome studies were carried out to determine the origin of the tumour cells in recipients developing tumours. To obtain further evidence that feline lymphosarcoma can be virally transmitted it was of interest to study the effects of a filtered inoculum. In the present investigations no kittens were available for a complete pathogenesis experiment but as a preliminary investigation the early responses of kittens to a filtered inoculum were tested.

It has been shown that some of the murine leukaemogenic viruses will infect adult mice although the latent periods for tumour development in them are much longer than for mice inoculated as newborns (Moloney, 1962). It has also been demonstrated that virus can be extracted from the plasma of inoculated adult mice (Rauscher and Allen, 1963). In the feline experiments described so far, kittens were inoculated, within 24 hours of birth, before they had developed full immunological competence. Whether or not lymphosarcoma could be induced in adults with a given leukaemic tissue extract it was of interest to investigate the early response of adults by examining blood platelets; if particles were being replicated the adults could then be used as a source of plasma from which particles could be purified for biophysical and biochemical characterisation and for further bioassay experiments.

The titre of virus extracted from the plasma of leukaemic mice can be increased by serial passage in newborn animals (Moloney, 1960). From the experiment with GL5/CFS described in Section 2, it was found that particles were most frequent in platelets and free in the plasma at 28 days and 42 days after the inoculation of kittens, although the percentage of platelets involved was smaller than that when rats were inoculated with Moloney leukaemia virus. With the eventual objective of producing a purified, concentrated, pool of particles for particle characterisation and a pathogenesis experiment, an attempt was made to increase the number of particles produced in the kittens and to estimate this by the examination of platelet pellets.

The aims of the preliminary experiments described in this section were

1) to compare the early responses of kittens to a filtrate inoculum GL5/CFSF with those already observed to result from inoculation with a presumed cell-free inoculum GL5/CFS.

2) to investigate the early response of adult cats to an inoculum GL5/CFS already tested in kittens.

3) to discover whether it was possible to maintain particle replication in serial passage and if so whether increased particle numbers were present in successive passages.

MATERIALS AND METHODS

1. Inocula

(a) GL5/CFS (See Section 2, Materials and Methods)

(b) CL5/CFSF (Cell-free suspension filtrate)

(b) CL5/CFSF (Cell-free suspension filtrate)

The stored 50 ml volume of CL5/CFS was thawed and was passed through a series of Millipore filters of porosity 0.8 μ, 0.65 μ and 0.3 μ. The filtrate was stored at -65°C in 3.5 ml aliquots.

(c) CL5/CLV1 (Cat leukaemia virus 1)

This inoculum was prepared from the blood of inoculated kittens. Three newborn kittens were inoculated with CL5/CFS and each kitten was bled 8 times, at 3 days intervals, starting 43 days after infection. Blood samples collected in EDTA were spun at 1,000 x g for 10 minutes and the supernatants, consisting of plasma and platelets, were stored at -65°C. A platelet pellet was obtained from 150 ml of the stored material by spinning at 10,000 x g for 10 minutes. The pellet was re-suspended in 15 ml of the supernatant and homogenised in a Potter homogeniser. The resulting plasma platelet homogenate was made up to 100 ml with additional supernatant and the final suspension was stored at -65°C.

(d) CL5/P2 (Passage 2)

This inoculum was a plasma platelet suspension prepared from pooled bloods taken 36 - 39 days after infection of 4 newborn kittens inoculated with CL5/CFS. Approximately 15 ml of blood from each animal were taken in EDTA and the pooled sample was spun at 1,000 x g for 10 minutes. A platelet pellet was obtained by spinning the supernatant at 10,000 x g for 10 minutes. The pellet was resuspended in 10 ml of the supernatant and the platelets were homogenised with a Sylverson grinder at half-speed for 2 minutes.

(e) CL5/P3 (Passage 3)

This inoculum was a plasma platelet suspension prepared from pooled bloods taken 23 days after infection of newborn kittens with CL5/P2. The suspension was prepared in the same way as CL5/P2. . The inoculum was used at once and not stored.

(f) CL5/P4 (Passage 4)

This inoculum was a plasma platelet suspension prepared from pooled bloods taken 48 days after infection of newborn kittens with CL5/P3. Preparation and usage were as described for P2 and P3.

2. Inoculation and Specimen Preparation for Electron Microscopy

Newborn or adult cats were inoculated with one of the inocula CL5/CFS, CL5/CFSF, CL5/CLV1, CL5/P2-P4. Blood samples and tissue samples were taken from individual animals at various times after inoculation; individual or pooled platelet preparations and tissues were prepared for electron microscopy.

Experiment 1

The examination of blood platelets and tissues from kittens inoculated with the leukaemogenic tissue filtrate CL5/CFSF.

Results

Three newborn kittens were each inoculated intraperitoneally with 1.0 ml of the cell-free filtered inoculum CL5/CFSF. One kitten was bled 26 days after inoculation and all three were bled 42 days after infection. The four blood samples were processed individually to produce platelet pellets. One kitten

was seen to be in extremis at 9 weeks and blood was taken from this animal prior to killing, at which time a sample of spleen was obtained for electron microscopy and samples of spleen, mesenteric lymph node (which was grossly enlarged) and bone marrow were taken for histological examination.

Table 16 gives the results of the examinations and shows that particles were found in all 4 of the platelet pellets.

At 26 days, particles of both types were found in the vacuoles of platelets from kitten 2; Type 2 particles were more commonly seen than Type 1 particles. No budding from the external platelet membranes was observed but a few platelets were noted to have particles budding into vacuoles. Some platelets had more than one vacuole containing a budding or complete particle and some vacuoles contained 2 or 3 particles. Figs. 107 and 108 show typical examples of the platelets from kitten 2.

At 42 days, particles were found in platelets from each of the 3 kittens; more particles were present in each of these platelet samples than in the sample obtained from kitten 2 at 26 days. A few particles budding from platelet surfaces and into vacuoles were observed and complete particles, mainly Type 2 were seen in vacuoles. In these preparations Type 2 particles were also found free in the inter-platelet spaces (Fig. 109).

The platelet sample taken from kitten 1 at 9 weeks contained many particles; most of these were Type 2 and they were found singly or in groups of 3 or 4 in platelet vacuoles. The examination of the spleen did not reveal particles in the megakaryocytes or budding from lymphoid cells; however, particles were found in the spleen blood platelets. Particles were also seen free in the intercellular spaces but not closely enough associated with individual cells or platelets to determine from which cells they had been produced.

Histological examination of tissues from kitten 1 showed some lesions

Table 16

The association of leukaemia virus particles with blood platelets from kittens inoculated with a cell-free preparation CL5/CFSF from the spontaneous feline lymphosarcoma CL5

Kitten	Time from inoculation to blood sampling * 26 days	42 days
ø 1		+
2	+	+
3		+

* + leukaemia virus particles observed

\- no particles found

ø Animal killed at 9 weeks. Blood platelets positive and particles found in the spleen.

which were similar to those observed in the field cases and which in the latter were thought to be evidence of early malignancy; in particular, in the spleen, some of the Malpighian corpuscles showed a peripheral accumulation of early lymphoblasts.

No further examinations were made of kittens 2 and 3 which were transferred to another experiment outwith the scope of this study.

Discussion

This preliminary investigation established that the filtered inoculum CL5/CFSF could induce effects in kitten platelets and tissues similar to those produced by the non-filtered inoculum CL5/CFS; thus, since filtration did not alter the capacity of the inoculum to induce particle replication it can be deduced that this response was initiated either by a virus or by a non-particulate component of the inoculum. The porosities of the Millipore filters used to prepare the inoculum were selected to extract cell debris, but to allow large particles of approximately 100 mμ to pass through the filter together with smaller particles. No attempt was made to determine the size of the active agent. It was not possible to determine accurately whether equal volumes of the inocula CL5/CFS and CL5/CFSF (derived from CL5/CFS by filtration) were equally effective in inducing particle replication in kittens; however, since particles were found in all of the CL5/CFSF inoculated animals at the same times as particles were found in the kittens inoculated with CL5/CFS (see Section 2) it can be deduced that filtration did not markedly affect the capacity of the inoculum to induce particle production.

The leukaemogenic activity of the inoculum CL5/CFSF was not investigated but it was noted that the histological examination of tissues taken from the

kitten killed at 9 weeks revealed changes similar to those observed in tissues from kittens inoculated with the non-filtered inoculum CL5/CFS.

Experiment 2

The examination of blood platelets and tissues from adult cats inoculated with CL5/CFS or CL5/CLV1

Results

Five healthy adult cats were bled to obtain individual pre-experiment platelet samples; each of 4 of the cats was then inoculated intraperitoneally with 2 ml of the inoculum CL5/CFS and 1 cat was similarly inoculated with 1 ml of CL5/CLV1; 26 days after inoculation, individual platelet pellets were prepared from blood samples from each of the cats and at 39 and 49 days after infection blood platelet pellets were obtained from cats 1, 2 and 3.

Cat 4 died accidentally at 26 days after the blood sample was taken; cat 3 was found to be *in extremis* at 15 weeks and at that time a blood sample was taken and the animal killed; samples of spleen and bone marrow were obtained for electron microscopy and for histological examination. No further examinations were made of cats 1, 2 or 5.

An examination of the 5 pre-experiment blood platelet pellets revealed no particles in the platelets or interplatelet spaces.

Table 17 shows that particles were found in 3/5 of the animals at 26 days, in 1/3 at 39 days and 3/3 at 49 days after inoculation. At 26 days, only a few particles were observed in cat 4, but particles were found in cat 1 in the platelets from which a few budding particles were seen; Type 1 and Type 2 particles were found singly in the platelet vacuoles in platelets from both cats (Fig. 110); cat 5, inoculated with CL5/CLV1, showed particles

Table 17

The association of leukaemia virus particles with blood platelets from adult cats inoculated with CL5/CFS or CL5/CLV1

Cat	Inoculum	Time from inoculation to blood sampling *		
		26 days	39 days	49 days
1	CL5/CFS	+ ø	-	+
2	CL5/CFS	- ø	-	+
3 ##	CL5/CFS	- ø	+	+
4	CL5/CFS	+ ø		
5 **	CL5/CLV1	+		

* + leukaemia virus particles observed

\- no particles found

Platelet sample taken at 10 weeks also +ve
Animal killed at 15 weeks and particles found in the bone marrow

ø A preparation from these pooled bloods inoculated into kittens induced particle replication in the kittens.

** This cat died accidentally at the time of blood sampling.

similar in type and distribution to those found in cat 1. At 39 days, only a few Type 1 particles were noted in cat 3. At 49 days, larger numbers of particles than had previously been seen were found in the platelet samples from each of the cats; in all three cats, Type 2 particles were found free in vacuoles and between platelets; budding into vacuoles was seen in platelets from cat 2, which had the greatest number of complete particles.

The examination of the platelet pellet and tissues taken from cat 3 at 15 weeks showed complete Type 2 particles in vacuoles and free between platelets and in the bone marrow megakaryocytes. No particles were seen in the spleen; the sections examined contained no megakaryocytes.

Although histological examination of spleen and bone marrow from cat 3 showed reactive changes there was no evidence of malignancy in these tissues.

A cell-free inoculum was prepared from the pooled platelets and plasma from blood samples from the cats at day 26. Kittens inoculated with this showed typical particles in platelets by day 28; the numbers of particles present appeared to be similar to those found in kittens inoculated with the original inoculum GL5/GFS.

Discussion

The results of this experiment show that particle replication can be induced in adult cats and that this could be detected in at least one animal 26 days after inoculation. In neither this experiment nor in those using kittens were blood samples taken earlier than 26 or 28 days after inoculation; thus, although at these times particles have been demonstrated in both adults and kittens, it is not known whether differences of response existed in the early post-inoculation stages.

The reason for the fluctuation between "particle-positive" and "particle-negative" recorded for successive examinations of samples from cat 1, is not known; it is possible that this finding is apparent rather than real and results from the way in which the platelet examinations were made and from the fact that the percentage of platelets with particles associated with them may have been close to the recognition level for "particle-positive" classification. Alternatively, this result may reflect real variations in particle numbers present at different times after inoculation.

Since the aim of this experiment was to examine early responses in cats, the animals were not retained to determine whether the particle replication phase was followed by the development of lymphosarcoma. However, the animals were bled several times between 49 days and 10 weeks in connection with experiments not described here; particles were demonstrated in the platelets at all times during this period. In the animal killed at 15 weeks the platelet and bone marrow examinations showed particles structurally identical and similarly distributed to the particles found in kitten platelets and tissues. The reactive changes observed in the spleen and bone marrow from this animal were similar to those seen in some of the kittens inoculated with the same inoculum. From this preliminary investigation, it would appear that, whether or not the inoculum GL5/CFS was leukaemogenic, during the post inoculation period of observation 26 days to 15 weeks, the particle production in adult animals was similar to that in kittens at the same times after inoculation. Until the relationship between particle replication and tumour development is understood, the significance of this observation, particularly in relation to latency, cannot be assessed.

In this experiment no attempts have been made to extract particles from cat plasma; however, from the platelet pellet examinations it was clear that particles were present in the plasma and a plasma platelet pool was collected and stored for later examination (the method of collecting and storage is described in Section 5).

Thus, it has been shown that 5 genetically unrelated cats, in which no particles were found before inoculation, were stimulated to replicate particles which were structurally identical to those seen in kittens and that an inoculum derived from the cats was capable of inducing kittens to replicate particles. This latter result indicated that it might be possible to carry out a serial passage experiment.

Experiment 3

The examination of platelets and tissues from kittens from 4 serial passages with particle-containing inocula.

Results

Table 18 summarises the procedures for and the results from the serial passages. The passage was initiated by inoculating several kittens with GL5/CFS and thereafter the passage was maintained, as indicated, by inoculating kittens or cats with plasma-platelet particle-containing preparations from kittens of the previous passage. It was found that characteristic leukaemia virus particles were present in all of the pooled and individual blood platelet samples taken from each of the passages. Particles were also found in all of the tissues examined with the exception of the spleen specimen obtained from a passage 2 kitten 13 weeks after inoculation (Figs. 111 - 113).

Table 16

The association of leukaemia virus particles with blood platelets and tissues from passage kittens and cats

Passage	Animals Inoculated	Inoculum	Time from inoculation to sampling	Type of Sample	Evidence of particles in inoculated animals *
1	Newborn kittens	CL5/CFS	36 days	Pooled bloods	Platelets +
			46 days	Pooled bloods	Platelets +
			47 days	Tissues from 1 kitten	Bone marrow + Spleen +
2	Newborn kittens	CL5/P2	34 days	Pooled bloods	Platelets +
			42 days	Pooled bloods	Platelets +
			13 weeks	Tissue from 1 kitten	Spleen -
			14 weeks	Tissue from 1 kitten	Bone marrow + Spleen + Thymus +
3	Newborn Kittens	CL5/P3	35 days	Individual blood samples from 2 kittens	2/2 Platelet Samples +
4	8 week old cats	CL5/P4	28 days	Individual blood samples from 5 kittens	5/5 Platelet Samples +

* + leukaemia virus particles observed

\- no particles found.

No obvious differences were noted between the numbers of particles present in the platelets in samples taken at comparable times from the different passages: no quantitation was attempted but it appeared that approximately the same proportion of particle associated platelets was found in each of the samples taken at 35 days in passage 3 as was found in the samples taken at 36 days in passage 1, and at 34 days in passage 2.

Table 19 shows the predominant types of particle present in the individual and pooled platelet pellets; budding particles were seen in very few preparations. The sites at which particles were seen are also recorded and it can be seen that particles were observed both free and in platelet vacuoles.

All of the young adult cats inoculated in passage 4 showed platelet particles at 28 days. One of these animals died immediately after the blood sample was taken. The animal had been in good condition, bright and eating well. At post-mortem the mesenteric lymph nodes were grossly enlarged and the peripheral lymph nodes were also enlarged; the spleen was not unusually large but the cut surface showed prominent Malpighian corpuscles typically seen in cases of spontaneous lymphosarcoma. The liver and thymus were grossly involved. The post-mortem findings suggested a diagnosis of lymphosarcoma; histological examination confirmed this diagnosis. The 4 other cats were killed during the outbreak of panleukopoenia 12 weeks after inoculation; none of these animals had shown clinical signs of lymphosarcoma; no post mortem examinations were performed and no material was taken for histological examination.

Discussion

This experiment has established that particle-containing inocula consisting of plasma and platelets from inoculated kittens which have not developed

Table 19

Types and sites of particles in pooled and individual preparations of platelets from passage kittens and cats

Passage	Time from inoculation to sampling	Predominant types of particle *	Main sites of particles
1 ϕ	36 days	TM/N	free and in vacuoles
	46 days	Bd/TM	multiple in vacuoles
2 ϕ	34 days	Bd/TM/N	free and in vacuoles
	42 days	N	in vacuoles
3 (1) ≠	35 days	TM/N	free and multiple in vacuoles
(2) ≠	"	TM/N	free and multiple in vacuoles
4 (1) ≠	28 days	TM	in vacuoles
(2) ≠	"	TM/N	in vacuoles and free
(3) ≠	"	N	in vacuoles
(4) ≠	"	TM	in vacuoles
(5) ≠	"	TM/N	in vacuoles and free

* Bd - budding particle
TM - triple-membraned - Type 1
N - electron dense nucleoid - Type 2
ϕ - pooled samples
≠ - individual kittens or cats.

lymphosarcoma can induce particle replication in newborn kittens and young adult cats similar to that induced by inocula derived from field case leukaemic tissues.

No obvious increase in particle numbers with passage was discerned. It was estimated that in most examinations approximately 1% - 5% of the platelets were involved; thus, the response was similar to that observed in the experiments with CL5/CFS and CL4/CFS.

Precise particle titration experiments are required to establish conclusively whether or not particle titre has been increased with passage. The activity of a murine leukaemia virus inoculum is normally assessed by bioassay (Moloney, 1960); often a spleen colony assay is used to compare inocula (Plusnik and Sachs, 1964; Axelrad and Steeves, 1954). In the present experiment with kittens in order to compare the particle titre in successive passages it would have been necessary to infect kittens with standard doses of inocula prepared from passage kittens in a standard way and to quantitate the particle production in the inoculated kittens at a given time; large numbers of kittens would have been required for this experiment and these were not available. Thus, since no <u>in vitro</u> tests have yet been developed the particle titres in successive passages could not be compared.

In the murine studies, increased titre of virus after serial passage was demonstrated (Moloney, 1960), but no examinations were made of platelets and tissues. It is possible that a highly infective population of particles is selected during the process of passage. The result of the passage 4 experiment, demonstrating particles in all of the animals at 28 days suggests that the response of these adults to P4 may have been greater than the response to CL5/CFS of the adults in Experiment 2. Since the adults in this

experiment were 8 weeks old and those in Experiment 2 were 6 months old, the differences in response may be due to the age of the animals rather than to differences between inocula. However, the histological diagnosis of lymphosarcoma in one animal of this experiment at 9 weeks may indicate a significant difference between the inoculum P4 and other non-passaged inocula.

Since the number of particles per dose of inoculum was not known for any of the inocula CL5/CFS, P2-P4, no quantitative comparisons could be made between the responses observed in the animals in the 4 passages. The results of the examinations seem to indicate that there were no qualitative differences between successive passages; both types of particle and both intravacuolar and free particles were observed in all of the passages, whether or not passage increased the particle titre.

The inoculated kittens and cats provided a source of particles; plasma and platelets for storage and subsequent investigation were obtained from the animals each time platelet or tissue samples were prepared for electron microscopy. Thus, a pool of platelets and plasma was obtained and it was established that each sample added to the pool contained particles and was capable of inducing particle replication. Attempts to isolate and purify particles from the plasma-platelet pool are described in Section 5.

SUMMARY

The experiments described in this section have shown that a filtered inoculum can produce early responses in kitten platelets and tissues similar to those induced by a non-filtered, presumed cell-free inoculum, that adult cats can replicate particles and that particle replication can be induced in kittens with inocula derived from inoculated, non-leukaemic animals.

Section 4

Replication of feline leukaemia viruses in tissue culture

INTRODUCTION

In a recent publication, Boiron et al. (1967) have extensively reviewed in vitro investigations on murine leukaemia viruses. Many of the murine viruses have been successfully grown in tissue culture; both cultures of cells taken from inoculated mice and cultures of normal mouse cells infected with murine leukaemia viruses have been shown to allow the viruses to replicate. The viruses produced in tissue culture are morphologically indistinguishable from those found in the animal tissues and plasma; they have been shown to be infective for mice, although studies with tissue culture grown Rauscher virus have shown it to have a decreased leukaemogenic activity in comparison with plasma derived virus (Lévy et al., 1966).

Malignant transformations caused by murine leukaemia viruses have been reported (Osato et al., 1966; Ioachim and Benrick, 1968; Sinkovicks et al., 1968), but because the leukaemia viruses produce no short-term cytopathic effect or transformation effect in cultures, indirect methods have to be used to detect the viruses. The most frequently used methods of establishing the presence of viruses in the cultures have been bioassays and electron microscopic examinations of sectioned tissue culture cells or of negatively stained culture fluid extracts; fluorescent antibody techniques and the complement fixation test have also been used to detect murine leukaemia viruses in vitro (Boiron et al, 1967).

Studies of virus-producing tissue cultures in which no cell transformation takes place have so far contributed very little information towards the understanding of the leukaemogenic process in animals; however, such cultures do provide a source of virus from which large quantities can be collected over

a period of time avoiding the necessity of killing animals to obtain small yields of virus. In studies with the feline lymphosarcoma extracts the difficulty of obtaining large numbers of kittens and the cost and space involved in maintaining them made it of particular importance to discover whether the particles in the donor animals would replicate in tissue culture and it would be possible to establish a particle-producing system from which particles could be purified for biophysical and biochemical characterisation.

The aims of the preliminary investigations described in this section were -

1. to discover whether a spontaneous feline lymphosarcoma derived inoculum could induce tissue culture cells to replicate leukaemia virus particles similar to those which were found in the donor cat.

2. to compare the effects of different inocula derived from 3 field cases of cat lymphosarcoma.

MATERIALS AND METHODS

1. Cell Cultures

(i) NK cells

This cell line was derived from combined spleen, thymus, liver, kidney and heart from newborn kittens. The cells were grown in 8 oz. bottles containing 14 ml of Eagle's medium supplemented with 10% tryptose phosphate broth and 10% foetal bovine serum or in 50 mm. plastic dishes with 4 ml of medium. The cells were fed every 2-3 days and subcultured every 4-5 days.

(ii) FL cells

This cell line, derived from the lungs of kitten embryos, was obtained from Dr. K. J. O'Reilly of the Wellcome Research Labs., Beckenham. Starting from a monolayer culture of the cells in their 40th passage, the cells were grown in Eagle's medium supplemented with 5% foetal bovine serum and the culture was maintained in the same way as the NK culture.

(iii) Control cultures

Uninfected NK and FL cultures were maintained in the same way as the infected NK and FL cultures and cells were harvested from them in the same way and at the same times as from the infected cultures.

2. Inocula

NK and FL tissue cultures were infected with inocula CL5/CFS, CL4/CFS and CL3/CFS (Section 2, Materials and Methods), CL5/CFSF (Section 3, Materials and Methods) and also with the following inoculum.

CL5/CLV5 (Cat leukaemia virus 5). Four newborn kittens were inoculated with a plasma platelet preparation from 3rd passage kittens (see Table 18). The animals were bled 37 days after infection. Approximately 15 ml of blood from each animal were taken in EDTA and the pooled sample was spun at 1,000 x g for 10 minutes. A platelet pellet was obtained by spinning the supernatant at 10,000 x g for 10 minutes. The pellet was resuspended in 10 ml of the supernatant and the platelets homogenised with the Sylverson grinder at half-speed for 2 minutes. The suspension was stored at -65°C in 1 ml aliquots.

Experiment 1

Materials and Methods

Each of the 3 NK cultures was infected with one of the inocula CL5/CFS, CL4/CFS and CL3/CFS.

To 20 ml of a suspension of NK cells containing approximately 2 x 10^6 cells /ml were added 3 ml of the appropriate inoculum. The cells and inoculum were mixed for 2 hours at room temperature, then the cells were spun from the mixture and resuspended in growth medium and grown in 8 oz. bottles. Cells were harvested from each of the infected cultures at 4 or 7 days and at 11, 15, 20, 25 and 29 days after inoculation; cells from the control cultures were harvested at the same times. Pellets of cells were prepared for electron microscopy.

Results

Table 20 summarises the results of the examinations. Particles were found in the culture infected with CL5/CFS and the culture infected with CL4/CFS, but not in the culture infected with CL3/CFS or in the control culture. The earliest times at which particles were identified were at 11 days after inoculation for the CL5/CFS infected cultures and at 20 days for those inoculated with CL4/CFS (Figs. 114 - 119).

1. CL5/CFS infected culture

Sections of the pellet of cells harvested after 11 days' infection with CL5/CFS showed typical Type 1 and Type 2 particles. In extracellular spaces, the particles were present singly, but not in groups; particles were not seen in intracytoplasmic vacuoles. The majority of the fully formed particles

Table 20

The association of leukaemia virus particles with NK cell cultures infected with tissue extracts from 3 cases of spontaneous feline lymphosarcoma

Inoculum	Time from culture infection to cell examination *						
	4 days	7 days	11 days	15 days	20 days	25 days	29 days
CL5/CFS	-		+	+	+	+	+
CL4/CFS	-		-	?+	+	+	+
CL3/CFS		-	-	-	-	-	-
Uninfected control culture	-		-	-	-	-	-

* + leukaemia virus particles identified

?+ virus-like particles found

\- no particles found

were Type 2 particles and as in the tissue sections showed greater variations in their diameter measurement than the Type 1 particle; the mean diameter of the particles was 110 mμ and that of the nucleoid was 65 mμ. Many of the particles had wrinkled and irregular external membranes and eccentrically placed nucleoids. A very few particles were seen budding from plasma membranes.

At 11 days only a few of the cells examined had particles associated with them. The type of cell producing the particles could not be identified; all of the cells in the sections examined electron microscopically were large with large nuclei which had coarse, highly osmophilic nucleoli; the cytoplasm was very sparse with degenerating mitochondria, vacuoles and granules of various shapes and sizes in a matrix of fine fibrils. Short strands of endoplasmic reticulum and Golgi regions could be seen in some cells. No characteristic morphological differences could be discerned between the cells in the infected culture and the normal control culture cells. Between 11 and 29 days the infected cells showed increasing evidence of degeneration and the control culture showed similar changes.

Increasing numbers of particles were found in the sections of cells taken at 15, 20, 25 and 29 days; more budding particles were seen, particularly at 25 and 29 days, and some cells showed multiple buds forming along a short length of plasma membrane. At all of these times there were many more Type 2 particles than Type 1 particles. At 25 and 29 days, compact groups of 2 - 10 particles were found in extracellular spaces; larger diffuse groups were also seen. No particles were observed in intracytoplasmic vacuoles.

2. CL4/CFS infected culture

Apart from a single virus-like particle seen in an intracytoplasmic vacuole, at 4 days, no particles were seen in the cells taken 4 days and 11 days after inoculation. At 15 days a few virus-like particles were seen in the extracellular spaces between cells; the structure of those particles was not clearly enough defined to enable them to be positively identified as leukaemia virus particles. Characteristic particles, mainly of Type 2, were found associated with the cells harvested at 20 days; in intercellular spaces, particles were present singly and in small groups of 2 - 3. No budding from membranes was observed and no particles were found in vacuoles. Larger numbers of particles were found at 25 days and 29 days; at 25 days, some budding particles were seen, a few Type 1 particles were observed, but the majority of the particles were of Type 2; at 29 days the number of particles present was similar to that found at 25 days and the electron-dense nucleoid form predominated. At both 25 days and 29 days, extracellular groups of from 2 to 7 particles were found but no particles were found in intracellular vacuoles. Budding particles were infrequently observed. As with the CL5/CFS infected cells, no specific morphological features were found associated with particle-producing cells. The general morphology was similar to that in the control culture and in the CL5/CFS infected culture and showed extreme degenerative changes at 29 days.

3. CL3/CFS infected culture

No particles were seen in any of the preparations from the CL3/CFS infected culture. The cells in this culture were similar to those previously described in the other cultures.

Experiment 2

Materials and Methods

Each of 2 FL cell cultures was infected with one of the inocula CL5/CLV5 and CL5/CFSF.

Monolayers of FL cells in 8 oz. bottles were infected with 1 ml of the appropriate inoculum diluted 1 in 4 in growth medium. After 5 hours of incubation at 37°C the inoculum-medium mixture was replaced by fresh medium and the cells were grown in the usual manner.

Cells were harvested at 7 days and at 14 days after inoculation and control culture cells were harvested at the same times. Pellets of cells were prepared for electron microscopy.

Results

Table 21 shows the results of the examinations. No particles were observed in cells from the control culture (Fig. 120). Virus-like particles were seen in both infected cultures after 7 days, but the particles were not sufficiently well defined to characterise them as leukaemogenic-type particles. At 14 days, however, leukaemia virus particles were easily found in both cultures; the size and morphology of those particles were typical of the murine leukaemogenic viruses (Figs. 121 - 128).

1. CL5/CLV5 infected culture

At 14 days there was some evidence of particle budding from the plasma membranes; some Type 2 particles were seen but the majority of particles were of Type 1. Particles were found singly in intracellular vacuoles and in extracellular spaces.

Table 21

The association of leukaemia virus particles with FL cell cultures infected with a passage kitten platelet preparation (CL5/CLV5) or a filtered lymphosarcoma extract (CL5/CFSF)

Inoculum	Time from culture infection to cell examination *	
	7 days	14 days
CL5/CLV5	?+	+
CL5/CFSF	?+	+
Uninfected control culture	-	-

* + leukaemia virus particles identified
?+ virus-like particles found
\- no particles found

2. CL5/CFSF infected culture

At 14 days many particles were found associated with the cells; groups of particles were found in extracellular spaces and near the cell surface intracytoplasmic vacuoles were seen containing one particle, or, less frequently, two particles. Both Type 1 and Type 2 particles were found; some of the former type of particles had a "coating layer" on the external membrane. Budding particles were observed at plasma membranes and occasionally in intracytoplasmic vacuoles.

As in the previous experiment, no morphological features were identified to distinguish the cells from the infected culture from those of the control culture, nor the particle-producing cells from other cells of the infected culture. The cells in the control culture and in both of the infected cultures were generally similar to those of the MK cultures.

Experiment 3

Materials and Methods

A culture of FL cells was infected with CL5/CFSF and the infected cells were maintained in tissue culture for 37 days.

To a monolayer of FL cells in a 4 oz. bottle was added 1 ml of CL5/CFSF diluted 1 in 10 in growth medium. The cells were incubated at 37°C for 24 hours after which time the inoculum-medium mixture was removed and replaced by fresh growth medium. The culture was maintained in the usual way.

Cells were harvested at 8, 15, 22, 29 and 37 days and cells from control cultures were harvested at the same times. Pellets of cells were prepared

for electron microscopy.

Results

Some unidentified technical error in the preparation of the cells harvested from the infected culture at days 15 and 22 resulted in the cells being insufficiently well preserved for particle examinations; thus, infected culture cells were examined at 8, 29 and 37 days only.

Table 22 summarises the results of the examinations which showed particles from 8 days in the infected cultures and no particles at any time in the control culture (Figs. 129 - 132).

At day 8 the infected culture contained large numbers of particles and most cells had particles associated with them; groups of particles were found extracellularly. Some buds were seen at cell membranes and complete particles of both types were observed near the cell surfaces. As in the previous experiments, Type 2 particles were more frequently seen than Type 1 particles.

At 29 days, very large groups of Type 2 particles were found; the particles were exclusively extracellular; budding was rare and Type 1 particles were observed very infrequently.

The findings at 37 days were similar to those at 29 days; the number of particles present appeared to be of the same order at both examination times and the predominance of Type 2 particles and infrequent budding seen at 29 days were also noted at this later time of examination. The morphology of the cells in both the infected and control cultures was similar to that seen in the previous experiment.

Table 22

The association of leukaemia virus particles with FL cell cultures infected with CL5/CFSF and maintained for 5 weeks

Inoculum	Time from culture infection to cell examination *				
	8 days	15 days	22 days	29 days	37 days
CL5/CFSF	+	∅	∅	+	+
Uninfected control culture	-	-	-	≠	-

* + leukaemia virus particles identified
- no particles observed
∅ poor fixation of cells: no examination possible
≠ no cells for examination

DISCUSSION

These experiments have shown that extracts from leukaemic tissues from cats with spontaneous feline lymphosarcoma could induce cells in tissue culture to replicate leukaemia virus particles similar to those which were found in the donor cats.

Particles were easily identified in the cultures infected with CL5- and CL4-derived inocula; the particles were morphologically the same as those found in the leukaemic donor tissues and in the inoculated kittens studied in the experiments described in Section 2. The same budding process for particle production was observed in the cells in culture as was seen in the *in vivo* studies. The Type 2 particles varied considerably in size and had a mean diameter of approximately 110 mμ. The same type of particle seen in tissue section had a mean diameter of 100 mμ. A similar observation was made by Zeigel *et al.* (1966) with Rauscher virus infected cultures. The ratio of Type 2 particles to Type 1 particles was much higher in the *in vitro* examinations than in the tissues from field case cats or from experimental kittens; budding particles and Type 1 particles were relatively difficult to find. Some particles showed a "coating layer" seen on the particles in the *in vivo* studies; this has also been demonstrated, without comment, in studies of mouse leukaemia virus-infected cultures (Duc-Nguyen *et al.*, 1966; Hall *et al.*, 1968). The general findings of the examinations of the kitten cultures are similar to those with the mouse culture described in the section dealing with preliminary investigations; more particles were present in the 8 week established mouse culture than were seen in any of the kitten cultures examined.

Both of the cell cultures, NK and FL, could be stimulated to produce particles; no attempt was made to quantitate particle production or to discern whether the NK and FL cultures were equally effective in producing particles. It was noted that in both culture systems degeneration occurred in the control and in the infected cells; particles were found budding from degenerating cells in the culture inoculated with CL5/CFS or CL4/CFS. Morphological changes associated with the infection of mouse cultures with Rauscher virus have been described by Chopra and Shibley (1967) and by Tyndall et al. (1965): no specific cellular changes were noted in the infected kitten cultures.

In murine studies the period between the time of infection and the appearance of particles was found by Chopra and Sibley (1967) to be approximately 10 days for Rauscher virus and by Osato et al. (1964) to be 5 days for Friend virus. By comparison, the results with the inocula CL5/CFS and CL4/CFS suggest that the particle titres of the cat-derived inocula were of the same order as the virus titres of the murine inocula if the rates of particle production were the same.

Although examinations were made for a limited period only it was evident that the cultures responded differently to each of the 3 inocula CL5/CFS, CL4/CFS and CL3/CFS; whereas infection with either of the first two induced particle replication no particles were seen in the cultures infected with CL3/CFS. The experiments testing these inocula in vivo (described in Section 2) also showed that CL3/CFS was either quantitatively or qualitatively different from the other 2 inocula. The demonstration of

the effectiveness of the 3 CL5-derived inocula to induce particle replication *in vitro* agrees with the findings from *in vivo* experiments described in Section 3 (Experiments 1 and 3); in particular, the filtrate inoculum, CL5/CFSF has been shown to induce particle production *in vivo* and *in vitro*.

No particles were observed in the control cultures. In studies with mouse tissue culture, leukaemia viruses have been observed both in normal cultures and in cultures of malignant but non-leukaemic tissues. Kindig and Kirsten (1967) listed several investigations which had shown this and they themselves showed typical particles in 6 lines of murine L cells and in one line of murine liver cells, but neither in control C3H or Swiss mouse embryo cultures, nor in the established 3T3 murine line during a 4 - 6 month period of examination. Hall *et al.* (1967a, b) have also shown particles in many cultures of normal and non-leukaemic tissues, some maintained with chemically defined media; some of these cultures underwent spontaneous neoplastic change. All of these cultures had been maintained for long periods (10 months or more) before examination. As yet, kitten cultures have not been studied for periods longer than 5 weeks, but in the light of murine studies the possibility must be considered that the uninfected kitten cultures contained particles activated by the inocula; however, since the different inocula produced different responses it seems more probable that the particle replication in the kitten cultures was directly attributable to the particles in the inocula.

From these experiments, it is clear that inoculated tissue cultures can provide a source of particles; such an *in vitro* system, free from the problems of obtaining kittens and the expense and space required for maintaining

them, could offer a standardised method of producing particles for biophysical and biochemical characterisation.

Attempts to demonstrate particles in tissue culture fluid collected from infected cultures are described in Section 5.

Note

Subsequent studies with the same inocula and another feline embryo culture have shown large numbers of particles associated with cells through 45 days and 10 passages (Fig. 133). Kittens have been injected with an inoculum derived from tissue culture fluid. This experiment is designed to discover whether or not the tissue culture derived particles will replicate in kittens and to test the leukemogenicity of the inoculum. No leukaemias have developed during the 2 months period since inoculation.

SUMMARY

The replication of leukaemia virus particles has been demonstrated in tissue cultures infected with inocula derived from 2 field cases of feline lymphosarcoma; the particles were morphologically identical to those seen in the leukaemic tissues from which the inocula were derived. The results of these experiments are similar to those obtained with murine leukaemia viruses grown in culture.

Section 5

Attempts to isolate and purify leukaemia virus particles from feline tissues, blood and tissue cultures

INTRODUCTION

For the biophysical and biochemical characterisation of a virus it is necessary to have a purified, concentrated pool of the virus.

The three sources from which such pools of murine leukaemia virus have been obtained are (1) leukaemic tissues from virus-inoculated mice or rats or from mice with spontaneous leukaemia; (2) plasma from inoculated mice or rats in the preleukaemic or the leukaemic phase, or from leukaemic mice which have developed the disease spontaneously; (3) tissue cultures of mouse cells in which the virus is replicating.

Each source has certain advantages and certain disadvantages in providing starting material from which to derive a virus pool.

The extraction of viruses from leukaemic tissues involves freeing the viruses from cells within which they might be present in vacuoles or to which they may be adsorbed, and at a later stage separating the viruses from the cellular debris produced in disintigrating the cells to free the viruses; the component of the cellular debris which is of approximately the same size and buoyant density as the viruses presents a contaminant which it is difficult to remove from the virus pool without seriously diminishing the virus titre. Rauscher and Allen (1964) used cell-free concentrates of leukaemic mouse spleen to obtain a virus pool for bioassay. No electron microscope examinations were made so the morphological homogenicity of the pool was not determined.

Chicken leukosis virus can be recovered from plasma and purified (Sharp et al., 1952; Bonar et al, 1963). This finding, together with the observation of Dalton et al (1961) that mouse leukaemia virus was present extracellularly in platelet preparations, led to attempts being made to isolate murine leukaemia

virus from plasma from rats and mice. Like leukaemic tissues, plasma provides a natural source of virus by comparison with *in vitro* sources; however, large numbers of rats and even larger numbers of mice are required to obtain enough plasma to prepare a large pool of virus. Twenty to twenty-five mice were used by de Harven and Friend (1964) in one experiment; mice were chosen in preference to inoculated rats since in de Harven's experiments (1965) it was found that the level of viraemia varied considerably from rat to rat and consequently that a pool of rat plasma would provide a non-homogeneous sample. Dalton *et al.* (1964) used inoculated rats to obtain plasma virus for morphological examination but no bioassays were performed.

Tissue cultures producing viruses and releasing them into the culture medium offer a constant source of virus; virus can be harvested and purified with less difficulty from this source than from tissues or plasma. However, the tissue culture grown viruses may differ biophysically, biochemically or biologically from those produced *in vivo* and this possibility must be considered in all analyses involving tissue culture derived virus. For example, Lévy *et al.*(1965) isolated Rauscher virus from tissue culture and from blood from leukaemic mice; no structural differences were found between viruses derived from these 2 sources. Later, however, Lévy *et al.* (1966) showed that the quality of the tissue culture grown virus was "more than 1000 times lower than the quality of plasma virus", defining 'quality' as "the number of particles corresponding to 1 effective dose".

Two methods may be used to examine viruses in tissue, plasma or tissue culture extracts; either a suspension of the virus can be examined using the negative staining technique or the viruses can be centrifuged from the suspension

into a pellet which is then fixed, dehydrated, embedded and cut in the same way as a tissue specimen.

The experiments described in Sections 1 - 4 have shown that leukaemia virus particles were present in leukaemic tissues from field cases of feline lymphosarcoma, that they could be seen lying free between platelets, which was presumed to indicate that they were freely circulating in the plasma of inoculated kittens, and that they were present extracellularly in infected culture preparations. Thus, particles have been shown to be present in the 3 starting materials from which murine leukaemia viruses have been extracted and purified.

The aim of the experiments in this section was to demonstrate leukaemia virus particles in extracts from feline tissues, blood and tissue cultures.

MATERIALS AND METHODS

Preparation of Virus Particle Suspensions for Electron Microscopy

1. From leukaemic lymph node

A 20% extract in tris-buffered saline (TBS) was made from stored lymph node tissue and a filtrate prepared in the same way as CL5/CFSF (see Section 3). The filtrate was spun in 3 x 12 ml tubes in the 40 rotor of a Spinco centrifuge at 30,000 x g for 60 minutes. The 3 pellets obtained were combined and resuspended in 1.5 ml of TBS and 0.5 ml of this suspension was layered on to a 4.5 ml 15% - 60% sucrose, in TBS, gradient. The gradient was spun at 39,000 r.p.m. for 18½ hours in the Spinco SW39 rotor. The base of the tube was punctured and the total gradient was collected as 12 x 8 drop fractions. Each fraction was made up to 5 ml with TBS and spun at 30,000 x g for 60 minutes in the Spinco 40 rotor.

The 12 pellets produced were fixed in 1% osmic acid, pH 7.4 in Millonig buffer, for 1 hour at 4°C and then dehydrated and embedded in the same way as the blood platelet pellets (see Materials and Methods).

2. From blood

The method used to produce a virus particle suspension is based on the method described by Moloney (1962) for the extraction of virus from leukaemic rats and mice.

Blood was taken from kittens and cats by cardiac puncture and individual blood samples were added to equal volumes of 0.3 ml potassium citrate (pH 7.0). Each blood sample was spun at 1,000 x g for 15 minutes at 4°C and the resulting supernatant was spun at 10,000 x g for 15 minutes at 4°C. The final plasma supernatant was used immediately for particle extraction or was stored separately or in a pool at -65°C. Stored plasma was thawed rapidly, immediately before the particle extraction procedure, by standing the storage bottle in a water bath at 37°C. Fresh or stored plasma was spun at 40,000 x g for 45 minutes in the 40 rotor of a Spinco centrifuge. The pellet formed was resuspended in 0.5 ml tris-saline (TS) and layered on to a 4.5 ml 15% - 60% sucrose (in TS) gradient and spun at 100,000 x g for 2 hours in the Spinco SW39 rotor. The gradient was then examined with a strong light in a darkened room; the position of the band was found and it was collected by puncturing the base of the centrifuge tube and collecting the 3 - 4 drops (approximately 0.15 ml) which constituted the band. This fraction was diluted to 5 ml with TS and was spun at 40,000 x g for 30 minutes in the Spinco SW39 rotor. The resulting pellet was resuspended in 0.1 ml TS and drops of this particle suspension were taken for negative staining and electron microscopic examination.

3. From tissue culture fluid

See Preliminary Studies.

Experiment 1

The examination of a leukaemic tissue extract

Materials and methods

Stored lymph node tissue from the spontaneous case CL3 was thawed and an extract made according to the method described in paragraph 1 of Materials and Methods in this Section.

Results

The examination of sections from each of the pellets 1 - 12 showed that each fraction from the gradient contained membranes, vesicles, amorphous material and a heterogeneous population of particles varying in size, shape and electron density. In general, the "lighter" fractions from the gradient contained less amorphous material and fewer electron-dense particles than the "heavier" fractions. No fraction was found to contain groups of characteristic leukaemia virus particles; a few structures, thought to be particles, were found widely scattered throughout the sections from fractions 10 and 11 (Figs. 134 - 137).

Discussion

The failure to concentrate virus particles from the leukaemic tissue may be due to several factors. From the studies with CL3/CFS described in Sections 2 and 4, it seems possible that CL3 tissues may not have contained very large numbers of particles although particles were found relatively easily

in tissue sections: the method of isolation, although suitable for murine leukaemia viruses may not have been the most suitable for concentrating the feline particles; the fractionation procedure may likewise have been less effective for these particles than for the murine viruses. Tissue was not available from the cases CL5 and CL4 to make comparative examinations.

Experiment 2

The examination of plasma concentrates from cats and kittens inoculated with leukaemic tissue extracts.

Materials and Methods

Plasma concentrates were prepared according to the method described in paragraph 2 of Materials and Methods in this Section. Plasma was obtained from cats and kittens as shown in Table 23.

Results

The band formed in the centrifugation procedure was in the region of 1.16 gm/cm^3, the buoyant density of the murine leukaemia viruses (O'Connor et al., 1964). From Table 23 it can be seen that in no examination were particles found which could be positively identified as of the leukaemogenic type. Many structures of the size of the leukaemia virus particles were found, but none with a recognisable morphology.

Discussion

The demonstration of virus particles in negatively stained preparations depends on there being large numbers of particles present in the suspensions and on the particles being morphologically distinguishable from other structures

Table 23

The examination for the presence of leukaemia virus particles of plasma from kittens and cats inoculated with leukaemic tissue extracts

Animals Inoculated	Inoculum	Times from inoculation to examination of plasma concentrate	Presence of Particles *
Kittens	CL5/CFS	28, 42, 56, 70 days	-
Kitten	CL4/CFS	28, 42, 56, 70 days	-
Kitten	CL3/CFS	28, 42, 56, 70 days	-
Young adult cats	CL5/CFS	3, 7, 14, 21, 56 days	-
Adult cats	CL5/CFS	26 days	-

* + Leukaemia virus particles identified

\- no particles found.

present in the drops examined. Murine leukaemia viruses, unlike, for example, Herpes viruses, with their distinct and characteristic capsomere arrangement, are difficult to identify unless present in high concentration. Their structure has been shown in purified preparations by many workers (Zeigel and Rauscher, 1963; Lévy et al., 1965; de Harven and Friend, 1964; de Harven, 1965; de Thé and O'Connor, 1966).

Since the murine viruses and feline particles are morphologically identical in tissue sections it would be expected that they should be structurally similar in negatively stained preparations. The failure to demonstrate feline particles in the plasma preparation may be due to low particle titres in the cats and kittens or may result from the method of preparation being unsuitable for the feline particles. Structures resembling disrupted particles were seen in some of the cat specimens. In a recent publication Kawakami et al. (1967) have shown "C"-type viral particles in plasma from a leukaemic cat and in plasma from experimentally infected leukaemic kittens; the particles were morphologically the same as murine leukaemic viruses. It may be that plasma particle titres are much higher in leukaemic cats and kittens than in experimentally infected animals in the pre-leukaemic phase such as were examined in this study.

Experiment 3

The examination of tissue culture fluid from cultures infected with leukaemic tissue extracts

Materials and Methods

Tissue culture fluid was collected from the following sources -

(i) NK cultures 11, 15, 20 and 29 days after infection with CL5/CFS or CL4/CFS, or CL3/CFS (See Section 4).

(ii) FL infected cultures 15 and 29 days after infection with CL5/CFSF. (See Section 4).

The individual samples were prepared according to the method given in paragraph 3 of Materials and Methods of this Section.

Results

As in the previous experiment no structures conclusively identifiable as leukaemia virus particles were found. The samples contained many membranes and a heterogeneity of structures; these contaminants were thought to be due to the degenerating cells in the culture contributing particles and cellular debris to the culture medium. The finding of particles in sections of the cells from the tissue cultures from which the fluid was taken and not in the fluid itself, may, in part, be caused by the cells adsorbing the particles and not releasing them into the fluid.

GENERAL DISCUSSION

Whereas characteristic leukaemia virus particles have been found easily in tissues from spontaneous cases of lymphosarcoma and from inoculated kittens and also in infected tissue culture cells, these have not been found in the extracts in these 3 experiments. The reason for this is not known but it may be that feline particles differ in some specific ways from the murine viruses which make it more difficult to free them from cells or that the particles are more fragile than the murine viruses and are damaged in the

concentration procedures, or that the procedures used to isolate murine viruses are not suitable for the extraction of the feline particles.

SUMMARY

No leukaemia virus particles were observed in any of the tissue, plasma or tissue culture extracts.

Note

In a subsequent study typical leukaemia virus particles were found in the tissue culture fluid from a kitten embryo culture 45 days after infection with a CL5-derived inoculum (Fig. 138). Sections of cells from this culture showed many more particles than had been seen associated with the cells from the cultures examined in Experiment 3.

Section 6

The effects on rats and mice of tissue extracts from a spontaneous feline lymphosarcoma

INTRODUCTION

It is clearly of interest and importance to investigate the host range of oncogenic viruses; at the fundamental level such studies are concerned with the examination of virus-cell interactions and consequently revealing what hazard a given virus may present to a species other than its normal host species. The cat, as a domestic animal, is a potential source of virus infection to the human. No investigations have been reported attempting to relate feline lymphosarcoma cases and cases of human leukaemia but one survey has been made in which a correlation between dog bites, canine lymphosarcoma and human leukaemia has been attempted (van Noosier et al., 1966). No definite conclusions can be drawn from the study.

Because of the difficulty of obtaining kittens for experiments and because of the apparently long latent period for the development of experimentally induced feline lymphosarcoma, it was of interest to discover whether the feline-derived inocula might be active in some other species. No test animal closely related to the cat is available for laboratory experimentation so preliminary investigations were made with the 2 available species, rats and mice. The negligible incidence of spontaneous leukaemia in the rat has been referred to in the general introduction and the induction of leukaemia in rats using murine viruses has already been discussed; thus, the rat would seem to be a reasonable test animal to use. Since almost all mouse strains are suspected of carrying latent leukaemia viruses the mouse is a less suitable test animal than the rat; however, if adequate control animals are available and the response in the test animals is significant, valid results can be obtained

using mice. One attempt to induce lymphosarcoma in cats using Moloney leukaemia virus resulted in one of the cats dying with histological evidence of the disease (Jarrett, unpublished); whether the lymphosarcoma was spontaneous or induced by the inoculum could not be established.

Two different aspects of the effects of feline derived inocula in the rats and mice were examined; the short-term effects were assessed by the same types of examinations of blood platelets and tissues as were used in the kitten experiments described in Section 2 and the long-term effects were assessed by keeping inoculated rats and mice under observation for 10 months.

The aims of the experiments described in this section were -

(1) to determine whether inocula derived from a case of feline lymphosarcoma would induce the replication of leukaemia virus particles in rats and mice.

(2) to determine whether these inocula were leukaemogenic for rats and mice.

MATERIALS AND METHODS

1. Animals

(i) Rats Adult male and female rats and baby rats 2 - 7 days old were used as test animals

(ii) Mice Baby mice 2 - 6 days old were used as test animals.

2. Inocula

The inocula CL5/CFS and CL5/CLV1 were both tested in rats and CL5/CLV1 was tested in mice (see Sections 2 and 3).

3. Blood sampling for platelet examinations

(i) Adult Rats

The collection of blood samples was carried out according to the method described for the cats (see Section 2). Approximately 2 ml of blood were obtained from each animal. For each examination 4 - 6 rats were bled. The individual blood samples were spun at 1,000 x g for 10 minutes, and then the supernatants were combined and spun at 10,000 x g for 10 minutes; the pellets produced were prepared for electron microscopy.

(ii) Baby Rats and Mice

Animals were anaesthetised. The axillary artery was exposed and cut and blood was collected into a plastic syringe with no needle. Approximately $\frac{1}{2}$ ml - 1 ml of blood was obtained from each animal. For each examination 6 - 10 rats or mice were bled. Platelet pellets were prepared in the same way as those from adult rats.

Note: The procedure of combining the samples after the initial spinning was adopted in some later examinations after it was found that the pooling of the diluted whole blood samples frequently gave rise to lysis and severely damaged the platelet morphology.

Experiment 1

The examination for the presence of leukaemia virus particles of blood platelets and tissues from rats inoculated with the leukaemic tissue extract (CL5/CFS) from the spontaneous feline lymphosarcoma CL5.

Materials and Methods

(i) Inoculation of Animals

(a) 10 adult male and 11 adult female rats were each inoculated intraperitoneally with 0.1 ml of the inoculum CL5/CFS

(b) 48 baby rats, 2 - 7 days old, were each inoculated as above.

(ii) Blood sampling for platelet examinations

Platelet pellets were prepared from pooled blood samples taken from adult rats 7, 14, 21, 28, 42, 56 and 70 days after inoculation. Similar preparations were obtained from baby rats.

(iii) Tissue sampling

Samples of bone marrow, spleen and thymus were taken from 1 adult rat and 1 baby rat killed at 28, 42, 56, and 70 days after inoculation.

Results

Tables 24 and 25 summarise the results of the examinations of the platelet pellets and the tissues and show that no leukaemia virus particles were observed in any of the preparations from the adult rats or from the baby rats inoculated with CL5/CFS.

Three female rats (inoculated as adults) and 7 female rats (inoculated when 2 - 7 days old) not used in the course of the experiment, were kept for further observation. These animals were examined each week for palpable lymph nodes. The animals are still alive 10 months after inoculation and show no signs of node enlargement.

Table 24

The association of leukaemia virus particles with blood platelets from rats inoculated with CL5/CFS

Pooled blood platelet sample	Age at time of inoculation	Time from inoculation to blood sampling	Presence or absence of particles *
1	2-3 days	7 days	npp
2	2-3 days	14 days	-
3	2-3 days	21 days	-
4	2-3 days	28 days	npp
5	2-3 days	42 days	-
6	2-3 days	56 days	-
7	2-3 days	70 days	-
8	2-4 months	7 days	-
9	2-4 months	14 days	-
10	2-4 months	21 days	-
11	2-4 months	28 days	-
12	2-4 months	42 days	npp
13	2-4 months	56 days	-
14	2-4 months	70 days	npp

* + leukaemia virus particles observed

\- no particles found

npp no platelet pellet

Table 25

The association of leukaemia virus particles with tissues from rats inoculated with CL5/CFS

Rat	Age at time of inoculation	Time from inoculation to tissue sampling	Tissues Examined *		
			Bone Marrow	Spleen	Thymus
1	2-3 days	28 days	-	-	-
2	2-3 days	42 days	nta	-	-
3	2-3 days	56 days	-	-	-
4	2-3 days	70 days	-	-	-
5	2-4 months	28 days	-	-	-
6	2-4 months	42 days	-	-	-
7	2-4 months	56 days	-	-	-
8	2-4 months	70 days	-	-	-

* \+ leukaemia virus particles observed

\- no particles found

nta no tissue available

Experiment 2

The examination for the presence of leukaemic virus particles of blood platelets and tissues from rats inoculated with the leukaemic tissue extract (CL5/CLV1) from the spontaneous feline lymphosarcoma CL5.

Materials and Methods

(i) Inoculation of animals

(a) 30 adult male rats were each inoculated intraperitoneally with 0.1 ml of the inoculum CL5/CLV1

(b) 75 baby rats, 2 - 7 days old, were each inoculated as above.

(ii) Blood sampling for platelet examinations

Platelet pellets were prepared from pooled blood samples taken from adult rats 7, 14, 21 and 28 days after inoculation. Similar preparations were obtained from the baby rats.

(iii) Tissue sampling

Samples of bone marrow, spleen and thymus were taken from 2 adults killed 28, 42, 56 and 70 days after inoculation. No tissue samples were taken from baby rats.

Results

Tables 26 and 27 give the results of the examinations and show that no leukaemia virus particles were found associated with tissues or platelets from rats inoculated with CL5/CLV1.

Sixteen male rats, inoculated as adults, and 9 female and 7 male rats, inoculated

Table 26

The association of leukaemia virus particles with blood platelets from rats inoculated with CL5/CLV1

Pooled blood platelet sample	Age at time of inoculation	Time from inoculation to blood sampling	Presence or absence of particles *
1	2-3 days	7 days	-
2	2-3 days	14 days	-
3	2-3 days	21 days	-
4	2-3 days	28 days	npp
5	2-4 months	7 days	-
6	2-4 months	14 days	-
7	2-4 months	21 days	-
8	2-4 months	28 days	-

* + leukaemia virus particles observed

\- no particles found

npp no platelet pellet

Table 27

The association of leukaemia virus particles with tissues from rats inoculated with GL5/GLV1

Rat	Age at time of inoculation	Time from inoculation to tissue sampling	Tissues examined *		
			Bone Marrow	Spleen	Thymus
1	2-4 months	28 days	-	-	-
2	2-4 months	28 days	-	-	nta
3	2-4 months	42 days	-	-	-
4	2-4 months	42 days	-	-	-
5	2-4 months	56 days	-	-	-
6	2-4 months	56 days	-	-	-
7	2-4 months	70 days	-	-	-
8	2-4 months	70 days	-	-	-

* + leukaemia virus particles observed

\- no particles found

nta no tissue available.

when 2 - 7 days old, were retained for further observations. Ten months after infection, all of the animals are still alive with no clinical symptoms of lymphosarcoma.

Experiment 3

The examination for the presence of leukaemia virus particles of blood platelets from mice inoculated with the leukaemic tissue extract (CL5/CLV1) from the spontaneous feline lymphosarcoma CL5.

Materials and Methods

(i) Inoculation of animals

67 baby mice, 2 - 5 days old, were each inoculated intraperitoneally with 0.1 ml of the inoculum CL5/CLV1.

(ii) Blood sampling for platelet examinations

Platelet pellets were prepared from pooled blood samples taken from mice 7, 14, 21 and 28 days after inoculation.

Note: No mice were killed for tissue sampling.

Results

Table 28 shows that the examination of the 7, 21 and 28 day platelet pellets failed to reveal the presence of leukaemia virus particles. Twenty mice were kept for further study and 10 months after inoculation are still alive and showing no signs of lymphosarcoma.

Table 28

The association of leukaemia virus particles
with blood platelets from mice inoculated with CL5/CLV1

Pooled blood platelet sample	Age at time of inoculation	Time from inoculation to blood sampling	Presence or absence of particles *
1	2-6 days	7 days	-
2	2-6 days	14 days	npp
3	2-6 days	21 days	-
4	2-6 days	28 days	-

* + leukaemia virus particles observed

\- no virus particles found

npp no platelet pellet

DISCUSSION

From the results of the experiments with rats it is clear that the 2 inocula tested did not induce the same responses in these animals as they had produced in kittens and cats. No leukaemia virus particles, replicating or free, were found in the platelet preparations or in the tissues from the rats. It was shown in the experiments described in Sections 2 and 3 that GL5/GLV1 induced particle replication in an adult cat by day 26 after infection (see Table 17) and that GL5/CFS induced particle production in cats and kittens by day 28 after infection (see Tables 17, 4). An examination of sections of a pellet of the inoculum GL5/GLV1 confirmed that particles were present in the inoculum (Fig. 139) and further tests with GL5/CFS in kittens have shown that the inoculum did not deteriorate with storage at -65°C. Thus, the failure to find particles in the rat tissues was not due to the inocula.

These results suggest, therefore, that either the particles were unable to penetrate rat cells and replicate or that the particle production rate was so low in the rats that replication could not be detected. The failure of the rats to develop leukaemia in the 10 month post inoculation period of observation indicates that the inoculum tested was not highly leukaemogenic for these rats.

The examinations in the mouse experiments were very limited but they did show that no extensive early particle production took place. The failure to induce leukaemia in any of the mice during the 10 month post inoculation period shows that the inoculum was not highly leukaemogenic for the mice and also that it did not stimulate in them any latent tendency to develop leukaemia.

While failure to demonstrate particle replication in rat and mouse tissues does not preclude the possibility that such a process is occurring, it indicates that neither the rat nor the mouse is a suitable animal for bioassays of inocula derived, directly, or indirectly via passage or tissue culture, from cases of feline lymphosarcoma.

Since it is known that the rats and mice used in these experiments are sensitive to mouse leukaemia viruses, the negative results support the claim that the feline particles are essentially different from, though morphologically identical to, the murine viruses.

SUMMARY

Neither of two feline leukaemia tissue extracts induced particle replication in blood platelets or tissues in rats inoculated as newborns or as adults and examined at various times from 7 days to 70 days. No leukaemias developed in the inoculated rats during a 10 month period of observation. One of these inocula was also tested in newborn mice; no particle production was seen and no leukaemias developed in the 10 month period of observation.

General Summary and Conclusions

General Summary and Conclusions

The investigations described in Section 1 demonstrate that leukaemia virus particles were present in large numbers in some cases of spontaneous feline lymphosarcoma in a randomly selected series.

The results of the experiments reported in Sections, 2, 3 and 4 show that morphologically identical particles could be found replicating in tissues from kittens and cats inoculated with cell-free extracts or a filtrate derived from leukaemic tissues from some of the field cases and that particles could be produced *in vitro* by infecting tissue cultures of normal feline cells with the same extracts and filtrate.

The findings from the experiments recorded in Section 5 indicate that, for some unknown reasons, it was more difficult to isolate feline virus particles than murine leukaemia viruses from tissue, blood or tissue cultures, although a later attempt to concentrate particles from tissue culture medium was successful.

In Section 6, the investigations of the effect of feline leukaemic tissue extracts in rats and mice are described; no evidence of particle replication could be demonstrated in the inoculated animals and the inocula were not leukaemogenic for either species in a 10 month period of observation.

This study has demonstrated the presence of a particle, morphologically identical to the murine leukaemia viruses, in field cases of feline lymphosarcoma and in kittens inoculated with leukaemic tissue extracts; it has shown that the cat and the mouse particles are found at the same cell sites, replicate in the same way and are present in the same organs and associated with the same cell types in these organs. These findings indicate that there

is a great morphological and biological similarity between the feline leukaemia virus particle and the murine leukaemia viruses.

The histological evidence of lymphosarcoma in some of the experimental kittens emphasises the similarity between the findings in this study and those in the earlier studies of Jarrett (Jarrett et al., 1964a; and Jarrett, 1966) and the recent experiments of Rickard (1967).

The results obtained in the present investigations, together with the previous results of Jarrett et al. (1964 a,b) provide evidence of a strong association between the presence of the feline leukaemia virus particle and feline lymphosarcoma and suggest that the particle found in the kittens and cats may be a feline leukaemogenic virus.

REFERENCES

Axelrod, A. A., and Steevos, R. A. Virology, 24, 513-518, 1964.

Bernhard, W. Cancer Res., 20, 712-727, 1960.

Bernhard, W. and Guérin, M. C. R. Acad. Sci. (Paris), 247, 1802-1805, 1958.

Bergs, V. V. J. Nat. Cancer Inst., 38, 481-490, 1967.

Boiron, M., Lévy, J. P. and Périès, J. In Progr med. Virol., Vol 9. pp 341-391 (Ed. J. L. Melnick) Karger, Basel/New York, 1967.

Bonar, R. A., Heine, U., Beard, D., and Beard, J. W. J. Nat. Cancer Inst., 30, 949-997, 1963.

Carnes, W. H. Federation Proc., 26, 748, 1967.

Chapman, A. L., Bopp, W. J., Brightwell, A. S., Cohen, H., Nielsen, A. H., Gravelle, C. R. and Werder, A. A. Cancer Res., 27, 18-25, 1967.

Chapman, A. L., Nielsen, A., Cohen, H., Larsen, W. E. and Werder, A. Proc. Soc. Exptl. Biol. Med., 122, 1022-1024, 1966a.

Chapman, A. L., Bopp, W., Brightwell, A. S., Nielsen, A. and Werder A. Proc. Soc. Exptl. Biol. Med., 123, 742-746, 1966b.

Chenaille, P., Lévy, J. P., Boiron, M. and Bernard, J. C. R. Acad. Sci. (Paris), 258, 3129-3132, 1964.

Chin, T. D. Y., Chapman, A. L., Gravelle, C. R., Werder, A. A. and Nielsen, A. H. In Comp. Leuk. Res., Vol 5. (Ed. J. H. Bendixen) Karger, Basel/New York, 1968.

Chopra, H. C. and Shibley, G. P. J. Nat. Cancer Inst., 39, 241-255, 1967.

Classification of Oncogenic RNA Viruses. J. Nat. Cancer Inst., 37, 395-397, 1966.

Congdon, C. C. and Lorenz, E. Am. J. Path., 30, 337-359, 1954.

Cotchin, E. Proc. Roy. Soc. Med., 45, 671-674, 1952.

Cotchin E. Vet. Rec., 69, 425-434, 1957.

Crighton, G. W. Ph.D. Thesis, University of Glasgow, 1965.

Dalton A. J. Nat. Cancer Inst. Monograph 22, 143-168, 1966.

Dalton, A. J., Law, L. W., Moloney, J. B. and Manaker, R. A. J. Nat. Cancer Inst., 27, 747-791, 1961.

Dalton, A. J. and Moloney, J. B. In Symposia Int. Soc. Cell Biol. I The Interpretation of Ultrastructure (Ed. R. J. C. Harris). New York Academic Press Inc., pp 385-392, 1962.

Dalton, A. J., Haguenau, F. and Moloney, J. B. J. Nat. Cancer Inst., 33, 255-275, 1964a.

Dalton, A. J., Moloney, J. B., Porter, G. H., Frei, E. and Mitchell, E. Trans. Assn. Am. Physicians, 77, 52-64, 1964b.

Dalton, A. J. and Zeve, V. H. Cancer Res., 27, 2465-2470, 1967.

David-Ferreira, J. F. Int. Rev. Cytol., 17, 99-148, 1964.

de Harven, E. J. Appl. Physics, 34, 2520, 1963.

de Harven, E. J. Exp. Med., 120, 857-868, 1964.

de Harven, E. Path.-Biol., 13, 125-134, 1965.

de Harven, E. Med. Clins. N. Am., 50, 887-900, 1966.

de Harven, E. Cancer Res., 27, 2447-2464, 1967a.

de Harven, E. Personal communication, 1967b.

de Harven, E., Clarkson, B. and Strife, A. Cancer, 20, 911-925, 1967.

de Harven, E. and Friend, C. J. Biophys. Biochem. Cytol., 4, 151-156, 1958.

de Harven, E. and Friend, C. Virology, 23, 119-124, 1964.

de Harven, E. and Friend, C. Nat. Cancer Inst. Monograph 22, 79-105, 1966.

de Thé, G. and O'Connor, T. E. Virology, 28, 713-728, 1966.

Dirksen, E. R. and Cailleau, R. Cancer Res., 27, 568-577, 1967.

Dmochowski, L. Progr. Exp. Tumor Res. Vol 3. (Ed. F. Hamburger) Karger, Basel/New York. pp 35-147, 1963.

Dmochowski, L. In Subviral Carcinogenesis. Monograph of the 1st Internat. Symp. on Tumor Viruses. pp 362-407, 1967.

Dmochowski, L. and Grey, C. E. Ann. N.Y. Acad. Sci., 68, 559-615, 1957

Dmochowski, L., Padgett, F. and Gross, L. Cancer Res., 24, 869-899, 1964.

Dmochowski, L., Dreyer, D. A., Grey, C. E., Hales, R. L., Langford, P. L., Pipes, F., Rocher, L., Seman, G., Shivley, J. A., Shullenberger, C. C., Sinkoviçs, J. G., Taylor, H. G., Tessmer, C. F. and Yumoto, T. Ann. N.Y. Acad. Sci., 143, 578-607, 1967a.

Dmochowski, L., Yumoto, T., Grey, C. E., Hales, R. L., Langford, P. L., Taylor, H. G., Friereich, E. J., Shullenberger, C. C., Shivley, J. A. and Howe, C. D. Cancer, 20, 760-777, 1967b.

Dorn, C. R., Taylor, D. O. N., Frye, F. L. and Hibbard, H. H. J. Nat. Cancer Inst., 40, 295-305, 1963.

Dorn, C. R., Taylor, D. O. N. and Hibbard, H. H. Am. J. Vet. Res., 28, 993-1001, 1967.

Dorn, C. R., Taylor, D. O. N., Schneider, R., Hibbard, H. H. and Klauber, M. R. J. Nat. Cancer Inst., 40, 307-318, 1968.

Duc-Nguyen, H., Rosenblum, E. N. and Zeigel, R. F. J. Bacteriol., 92, 1133-1140, 1966.

Duesberg, P. H. and Robinson, W. S. Proc. Nat. Acad. Sci., 55, 219-227, 1966.

Dutcher, R. M., Larkin, E. P. and Marshak, R. R. J. Nat. Cancer Inst., 33, 1005-1064, 1964.

Dutcher, R. M., Larkin, E. P., Tumilowicz, J. J., Nazerian, K., Eusebio, C. P., Stock, N. D., Guest, G. B. and Marshak, R. R. Cancer, 20, 851-856, 1967.

Ellerman, V. and Bang, O. Centralbl. f. Bakt., Abt. 1 (orig) 40, 595-609, 1908.

Epstein, M. A., Achong, B. G. and Barr, Y. M. Lancet, 11, 702-703, 1964.

Feldman, D. G. and Gross, L. Cancer Res., 24, 1760-1783, 1964.

Feldman, D. G. and Gross, L. Cancer, Res., 26, 412-426, 1966.

Feldman, D. G. and Gross, L. Cancer Res., 27, 1513-1527, 1967.

Feldman, D. G., Dreyfuss, Y. and Gross, L. Cancer Res., 27, 1792-1804, 1967.

Fink, M. A., Herbut, P. A., Sibal, L. R. and Wivel, N. A. J. Nat. Cancer Inst., 30, 1253-1255, 1967.

French, J. E. Br. J. Haemat., 13, 595-603, 1967.

Furth, J. J. Exp. Med., 58, 253-275, 1933.

Glauert, A. M. In Techniques for Electron Microscopy. p.172 (Ed. D. Kay) Blackwell, Oxford, 1961.

Gross, L. Proc. Soc. Exp. Biol. Med., 76, 27-32, 1951.

Gross, L. Acta Haematol., 19, 353-361, 1958.

Gruenstein, M., Meranze, D. R. and Shimkin, M. B. Cancer Res., 26, 2202-2205, 1966.

Haguenau, F., Dalton, A. J. and Moloney, J. B. J. Nat. Cancer Inst., 20, 633-649, 1958.

Haguenau, F., Hollman, K-H, Lévy, J. P., Boiron, M. J. Microbiol., 2, 529-538, 1963.

Haguenau, F. and Hanafusa, H. Virology, 34, 275-281, 1968.

Hall, W. T., Andresen, W. F. and Evans, V. J. J. Virol., 1238-1254, 1967a.

Hall, W. T., Andresen, W. F., Sanford, K. K. and Evans, V. J. Science, 156, 85-88, 1967b.

Hall, W. T., Hartley, J. W. and Sanford, K. K. J. Virol., 2, 238-247, 1968.

Haran-Ghera, N. Proc. Soc. Exp. Biol. Med., 124, 697-699, 1967.

Haran-Ghera, N. and Peled, A. Brit. J. Cancer, 21, 730-738, 1967.

Hartman, H. S., Miller, E. C., Miller, J. A. and Morris, F. K. Cancer Res., 19, 210-216, 1959.

Hartley, J. W., Rowe, W. P., Capps, W. I. and Huebner, R. J. Proc. Nat. Acad. Sci. (U.S.A.), 53, 931-938, 1965.

Horbut, P. A. Arch. Path. (Chicago) 83, 123-131, 1967.

Hoflund, S., Thorell, B. and Winquvist, G. Internat. Symp. Comp. Leukaemia Res. Hanover, 1963.

Holzworth, J. J. Am. Vet. Med. Assn. 136, 47-69, 1960.

Holzworth, J. and Nielsen, S. W. J. Am. Vet. Med. Assn., 126, 26-36, 1955.

Huggins, C. B. and Sugiyama, T. Proc. Nat. Acad. Sci (U.S.A.), 55, 74-81, 1966.

Ioachim, H. and Berwick, L. Int. J. Cancer, 3, 61-73, 1968.

Jarrett, W. F. H. In W.H.O. Report. 1st Anm. Symp. on Domestic Animal Leukaemia, Philadelphia, 1962.

Jarrett, W. F. H. In Some Recent Developments in Comparative Medicine. Symp. Zoo. Soc. London, No. 17. pp 295-301. Academic Press, London/New York. 1966a.

Jarrett, W. F. H. Proc. Roy. Soc. Med., 59, 661-662, 1966b.

Jarrett, W. F. H., Martin, W. D., Crighton, G. W., Dalton, R. G. and Stewart, M. F. Nature (London), 202, 566-567, 1964a.

Jarrett, W. F. H., Crawford, E. M., Martin, W. B. and Davie, F. Nature (London), 202, 567-568, 1964b.

Jarrett, W. F. H., Crighton, G. W. and Dalton, R. G. Vet. Rec., 79, 693-699, 1966.

Jarrett, O., Laird, H. M., Crighton, G. W., Jarrett, W. F. H. and Hay, D. In Comp. Leuk. Res., Vol. 5. (Ed. H. J. Bendixen). Karger, Basel/New York, 1968.

Kajima, M. and Pollard, M. Cancer Res., 27, 980-993, 1967.

Kajima, M. and Pollard, M. Nature (London), 218, 188-189, 1968.

Kaplan, H. S. Cancer Res., 27, 1325-1340, 1967.

Kawakami, T. G., Theilen, G. H., Dungworth, D. L., Munn, R. J. and Beall, S. G. Science, 158, 1049-1050, 1967.

Kindig, D. A. and Kirsten, W. H. Science, 155, 1543-1545, 1967.

Kobayashi, H., Kodama, T. and Sendo, F. Gann, 58, 395-396, 1967.

Laird, H. M., Jarrett, W. F. H., Jarrett, J. O. and Crighton, G. W. Vet. Rec., 80, 606, 1967.

Levine, P. H., Moross zewicz, J. S., Grace, J. T. and Lee, S. Chai. Cancer, 20, 1563-1577, 1967.

Lévy, J. P., Boiron, M., Silvestre, D. and Bernard, J. Virology, 26, 146-150, 1965.

Lévy, J. P. and Oppenheim, E. Path.-Biol., 14, 966-975, 1966.

Lévy, J. P., Silvestre, D., Leclerc, J. C. and Boiron, M. Virology, 29, 681-683, 1966.

Lieberman, M. and Kaplan, H. S. Science, 387-388, 1959.

Lombard, L. S., Moloney, J. B. and Rickard, C. G. Ann. N.Y. Acad. Sci., 108, 1086-1105, 1963.

Marshak, R. R., Hare, W. C. D., Dodd, D. C., McFeely, R. A., Martin, J. E. and Butcher, R. M. Cancer Res., 27, 498-504, 1967.

Millonig, G. In Proc. 18th Ann. Meeting Electron Microscope Soc. of America. pp 15-16, 1961.

Millonig, G. J. Appl. Phys., 32, 1637-, 1961.

Moloney, J. B. Nat. Cancer Inst. Monograph 4, 7-37, 1960.

Moloney, J. B. Fed. Proc., 21, 19-31, 1962.

Moldovanu, G., Moore, A. E., Friedman, M. and Miller, D. G. Nature (London), 210, 1342-1343, 1966.

Nadel, E. M., Proc. Amer. Assoc. Cancer Res., 5, 47, 1964.

Nadel, E., Banfield, W., Burstein, S. and Tousimis, A.T. J. Nat. Cancer Inst., 38, 979-982, 1967.

Nazerian, K., Dutcher, R. M., Larkin, E. P., Tumilowicz, J. J., Musebio, C. P. Am. J. Vet. Res., 29, 387-395, 1968.

Newell, G. R., Harris, W. W., Bowman, K. O., Boone, C. W. and Anderson, N. G. New England J. Med., 278, 1185-1191, 1968.

Nielsen, S. W. and Holzworth, J. J. Am. Vet. Med. Ass., 122, 189-197, 1953.

O'Connor, T. E., Rauscher, F. J. and Zeigel, R. F. Science, 144, 1144-1147, 1964.

O'Conor, G. T. and Rabson, A. S. J. Nat. Cancer Inst., 35, 899-904, 1965.

Okano, H., Kunii, A., and Furth, J. Cancer Res., 23, 1109-1175, 1963.

Opler, S. R. J. Nat. Cancer Inst., 38, 797-800, 1967a.

Opler, S. R. Sci. Proc. Amer. Assoc. Path. Bact. March. 1967b.

Opler, S. R. Sci. Proc. European Cancer Congress (Vienna). 1967c.

Opler, S. R. Am. J. Path. 51, 1135-1151, 1967d.

Osato, T., Mirand, E. A. and Grace, J. T. Nature (London) 201, 52-54, 1964.

Osato, T., Mirand, E. A., Grace, J. T. and Price, F. Nature (London), 209, 779-821, 1966.

Porter, G. H., Dalton, A. J., Moloney, J. B. and Mitchell, E. Z. J. Nat. Cancer Inst. 33, 547-556, 1964.

Pluznik, D. H. and Sachs, L. J. Nat. Cancer Inst. 33, 535-546, 1964.

Rauscher, F. J. J. Nat. Cancer Inst. 29, 515-543, 1962.

Rauscher, F. J. and Allen, B. V. J. Nat. Cancer Inst. 32, 269-275, 1964.

Reynolds, E. S. J. Cell Biol., 17, 208-212, 1963.

Rickard, C. G. In Comp. Leuk. Res. Vol. 5. (Ed. J. H. Bendixen). Karger, Basle/New York, 1968.

Rickard, C. G., Barr, L. M., Moronha, F., Dougherty 3rd, E. and Post, J. E. Cornell Vet., 57, 302-307, 1967.

Rich, M. A., Karl, S. C. and Clymer, R. Nature (London), 216, 270-271, 1967.

Rich, M. A. and Siegler, R. In Ann. Rev. Microbiology. 21, 529-572, 1967.

Rosenberger, G. Internat. Symp. Comp. Leukaemia Res. Hanover, 1963.

Sabatini, D. D., Bensch, K. and Barrnett, R. J. J. Cell Biol., 17, 19-58, 1963.

Schneider, R., Frye, F. L., Taylor, D. O. N. and Dorn, C. R. Cancer, Res., 27, 1316-1322, 1967.

Schmidt, R. E. J. Am. Vet. Med. Assn., 151, 1325-1328, 1967.

Seaman, G. and Dmochowski, L. Med. Rec. and Annals. 58, 400-405, 1965.

Sharp, D. G., Eckert, E. A., Beard, D. and Beard, J. W. J. Bacteriol., 63, 151-161, 1952

Shay, H., Gruenstein, G. T., Marx, H. E. and Glazer, L. Cancer Res., 11, 29-34, 1951

Silvestre, D., Lévy, J. P. and Leclerc, J. C. Path.-Biol., 14, 559-564, 1966.

Sinkovics, J. G., Györkey, F., Groves, G. F. and Györkey, P. Arch. ges. Virusforsch., 23, 169-174, 1968.

Sorensen, G. D. and Theilen, G. H. Ann. N.Y. Acad. Sci., 108, 1231-1240, 1963.

Squire, R. A. Cornell Vet., 54, 97-150, 1964.

Stasney, J. S., Canterow, A. and Pashkis, K. E. Cancer Res., 10, 775-782, 1950.

Swartzendruber, D. C., Boone Il Ma and Murphy, W. H. Proc. Soc. Exp. Biol. Med., 126, 731-734, 1967.

Theilen, G. H., Dungworth, D. L., Harrold, J. B. and Straub, O. C. J. Am. Vet. Med. Assn., 20, 373-386, 1967.

Tyndall, R. L., Vidrine, J. G., Teeter, E., Upton, A. C., Harris, W. W. and Fink, M. Proc. Soc. Biol. Med., 119, 186-189, 1965.

Toshima, S., Tagaki, N., Minowada, J., Moore, G. E. and Sandberg, A. A. Cancer Res., 27, 753-771, 1967.

Ueberschär, S. Dresch. tierärztl. Wschr. 70, 417, 1963.

Van Hoosier, G. L., Stenback, W. A., Mumford, D. M., Hill, W. A., Dunn, S. C., Macdonald, E. J., Macdonald, M. C., Taylor, H. G. and Trentin, J. J. Int. J. Cancer, 3, 7-16, 1968.

Viola, M. V., Dalton, A.J., Mitchell, E. and Moloney, J. B. New Engl. J. Med., 277, 503-506, 1967.

Vogt, P. K. In Advances in Virus Research, 11, 294-385, 1965.

Watson, M. L. J. Biophys. Biochem. Cytol. 4, 475-478, 1950.

Yumoto, T., Recher, L., Sykes, J. A. and Dmochowski, L. Nat. Cancer Inst. Monograph 22, 107-138, 1966.

Zeigel, R. F. and Rauscher, F. J. J. Nat. Cancer Inst. 30, 207-219, 1963.

Zeigel, R. F. and Rauscher, F. J. J. Nat. Cancer Inst., 32, 1277-1307, 1964.

Zeigel, R. F., Tyndall, R. L., O'Connor, T. E., Teeter, E. and Allen, B. V. Nat. Cancer Inst. Monograph 22, 237-263, 1966.

Zevo, V. H., Lucas, L. S. and Manaker, R. A. J. Nat. Cancer Inst., 37, 761-773, 1966.

An Electron Microscopic Study of Feline Lymphosarcoma

Thesis for the degree of

Doctor of Philosophy

in the Faculty of Veterinary Medicine

by

Helen M. Laird

University of Glasgow

1968

CONTENTS

This volume contains Figures referred to in the text of Volume 1.

Inoculum CL2/CFS

Platelet examination. Fig. 102.

Inoculum LNH

Platelet examination. Fig. 103.

Normal kittens

Platelet examinations. Figs. 104 - 106.

Section 3 Further transmission studies

(1) with a filtrate inoculum. Figs. 107 - 109.

(2) in adult cats. Fig. 110

(3) in serial passage Figs. 111 - 113

Section 4 Replication of feline leukaemia viruses in tissue culture

NK Cell Cultures

Figs. 114 - 119

FL Cell Cultures

Figs. 120 - 133

Section 5 Attempts to isolate and purify leukaemia virus particles from feline tissues, blood and tissue cultures

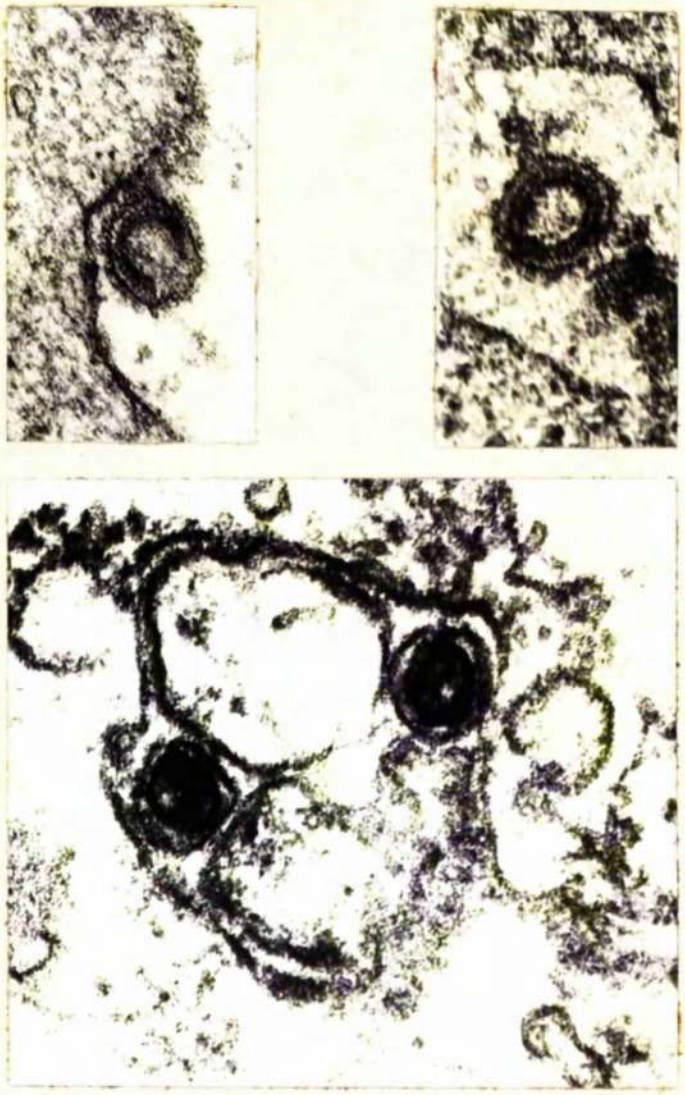
Particles in leukaemic tissue extract. Figs. 134 - 137

Particles extracted from tissue culture fluid. Fig. 138.

Section 6 The effects on rats and mice of tissue extracts from a spontaneous feline lymphosarcoma

Pellet of inoculum. Fig. 139.

Additional Figure - Canine lymphosarcoma. Fig. 140.

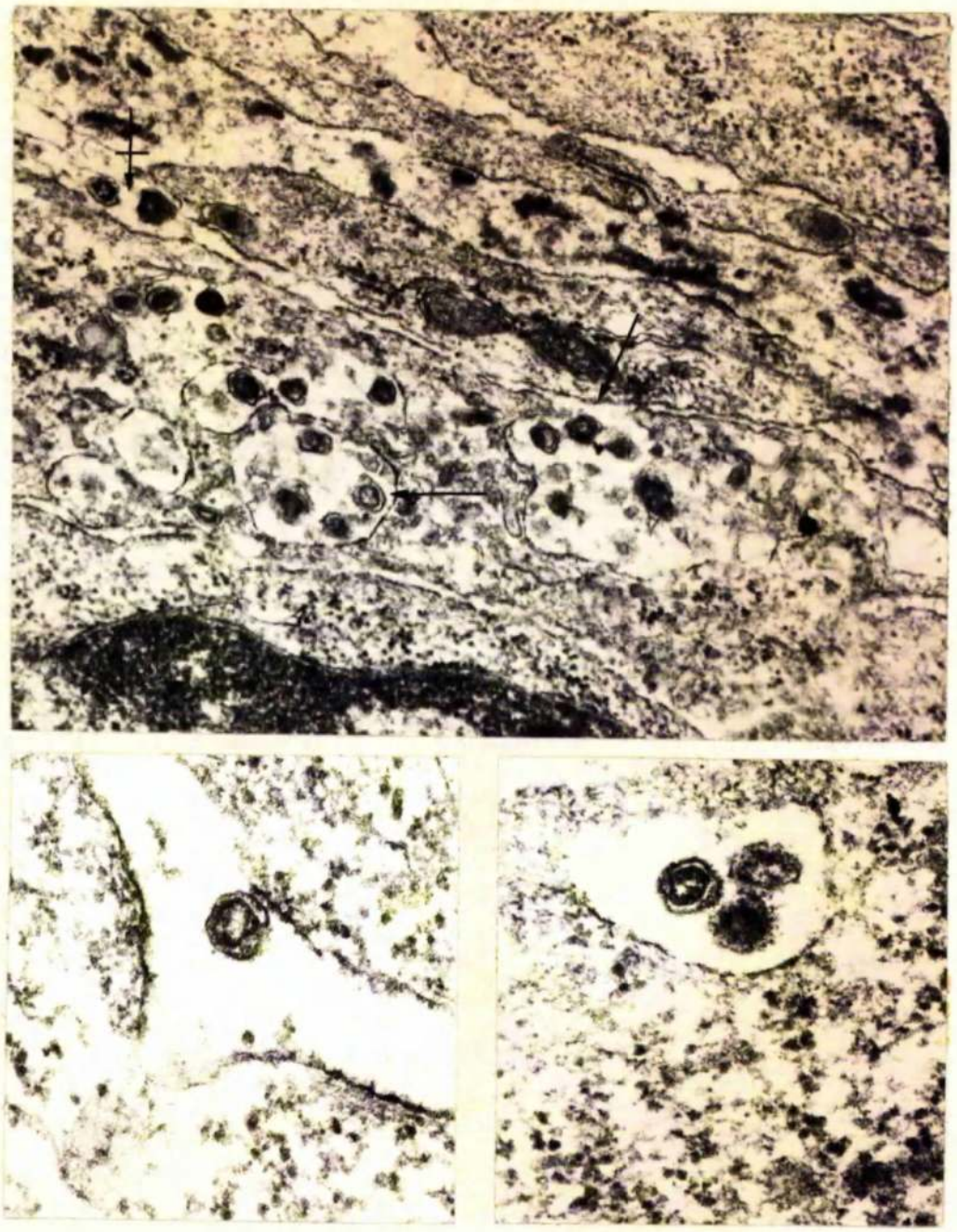


Moloney Mouse Leukaemia Virus

Fig. 1. (Top left) Incomplete Type 1 particle with "coating layer". x 120,000.

Fig. 2. (Top right) Type 1 particles. x 120,000.

Fig. 3. (Bottom) Type 2 particles. x 120,000.

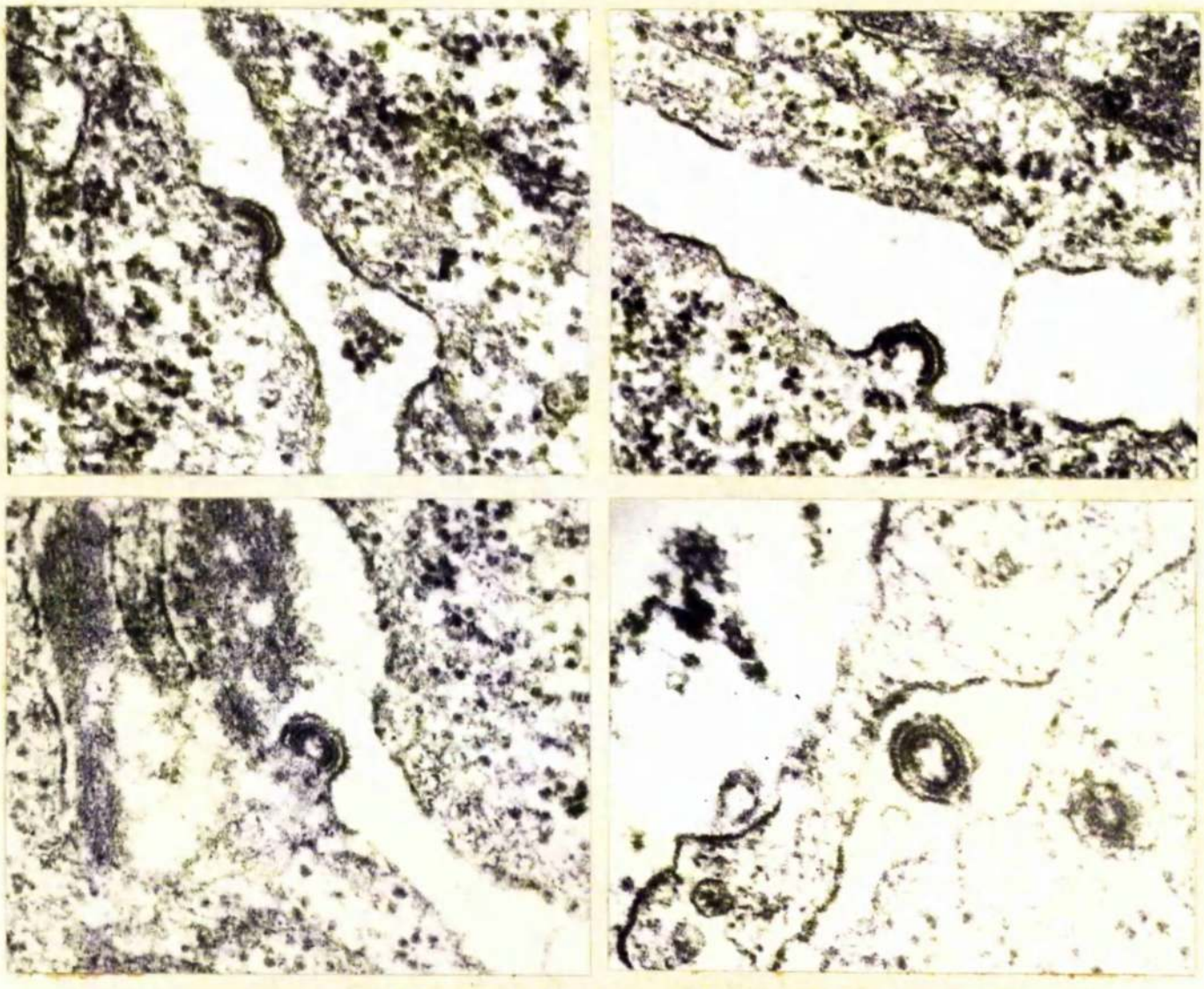


Spleen from leukaemic mouse

Fig. 4. (Top) Numerous particles in vacuoles (⟶) and intercytoplasmic spaces (⟶|⟶). x 50,000.

Fig. 5. (Lower left) Extracellular Type 2 particle. x 75,000.

Fig. 6. (Lower right) Group of 3 particles in intracellular vacuole. x 75,000.



Spleen from leukaemic mouse showing various stages of particle budding

Fig. 7 (Top left) Early bud. Inner and intermediate membranes are clearly defined. x 75,000.

Fig. 8 (Top right) Later stage. Continuity between outer particle membrane and plasma membrane is seen. x 75,000.

Fig. 9 (Lower left) Inner and intermediate membranes almost completely formed.

Fig. 10. (Lower right) Complete particle detaching from the plasma membrane. x 100,000.

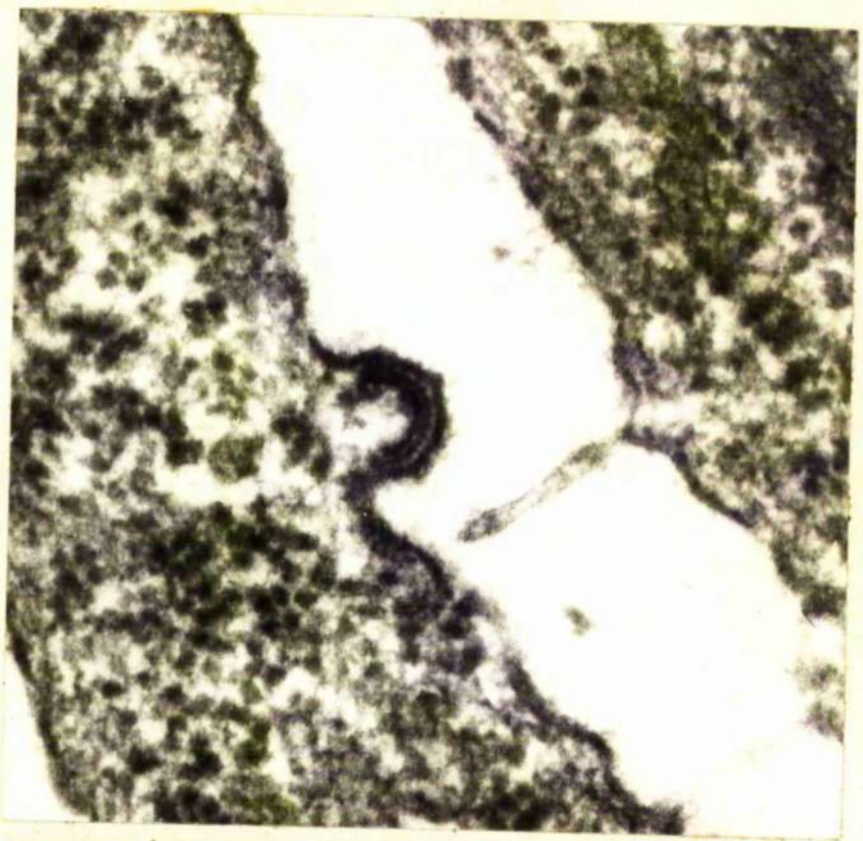


Fig. 11 High magnification of budding particle shown in Fig. 8. The triple-membraned structure is demonstrated. x 120,000.

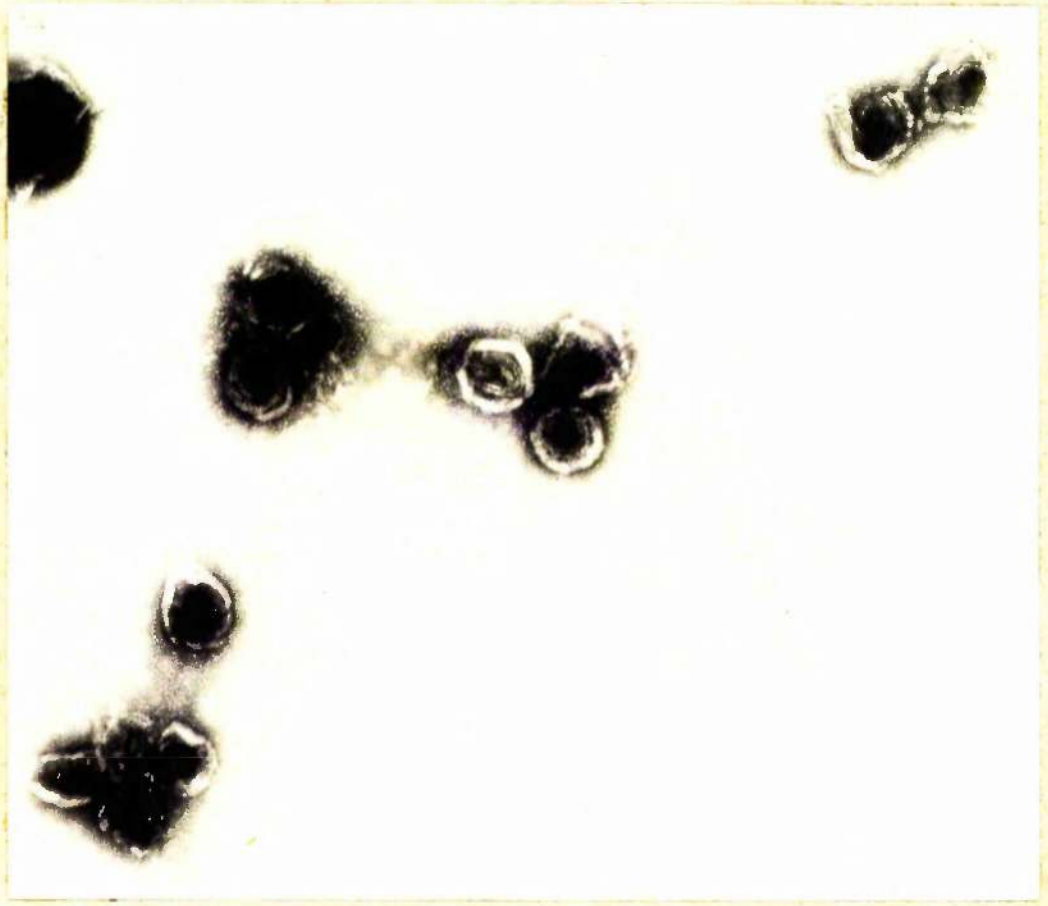


Fig. 12 Murine leukaemia virus extracted from tissue culture fluid. The triple-membraned structure of the virus can be seen in several particles. Negative staining with uranyl acetate. x 75,000.

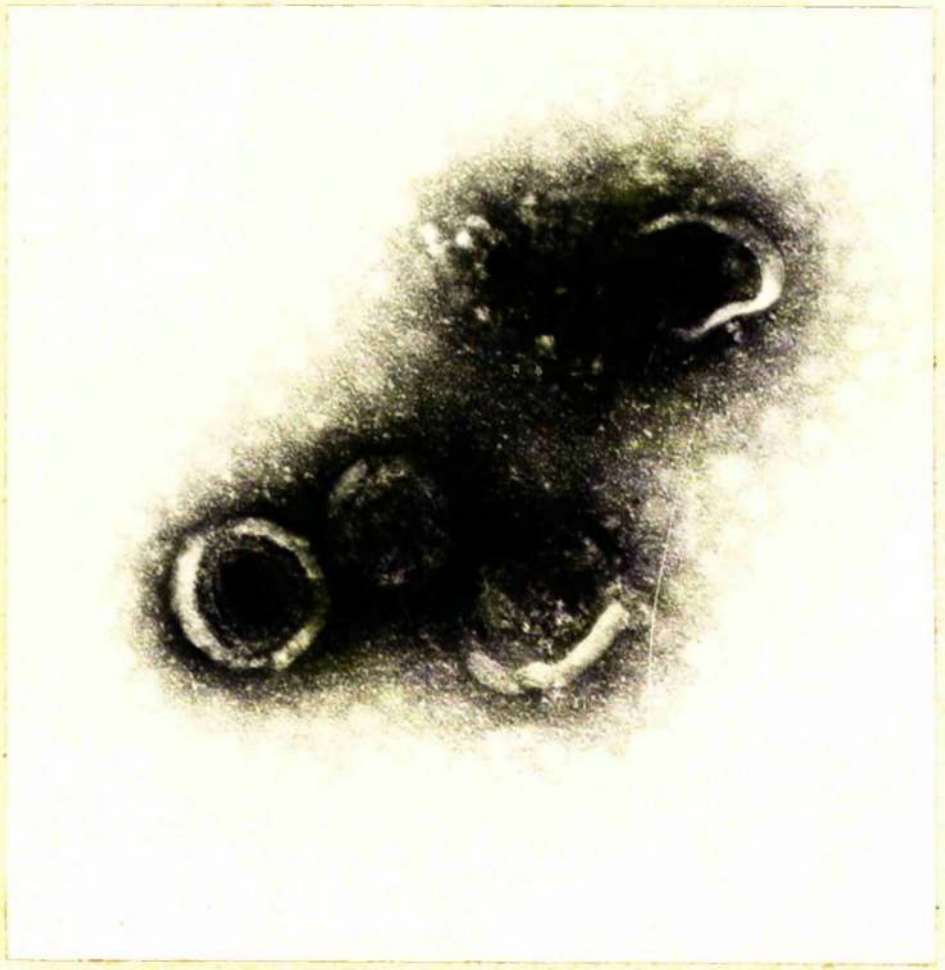


Fig. 13 Murine leukaemia virus extracted from tissue culture fluid. The triple-membraned structure is clearly defined in the particle at the lower left; the particle in the centre has the electron-dense nucleoid structure; 2 disrupting particles are seen at the right. x 150,000.

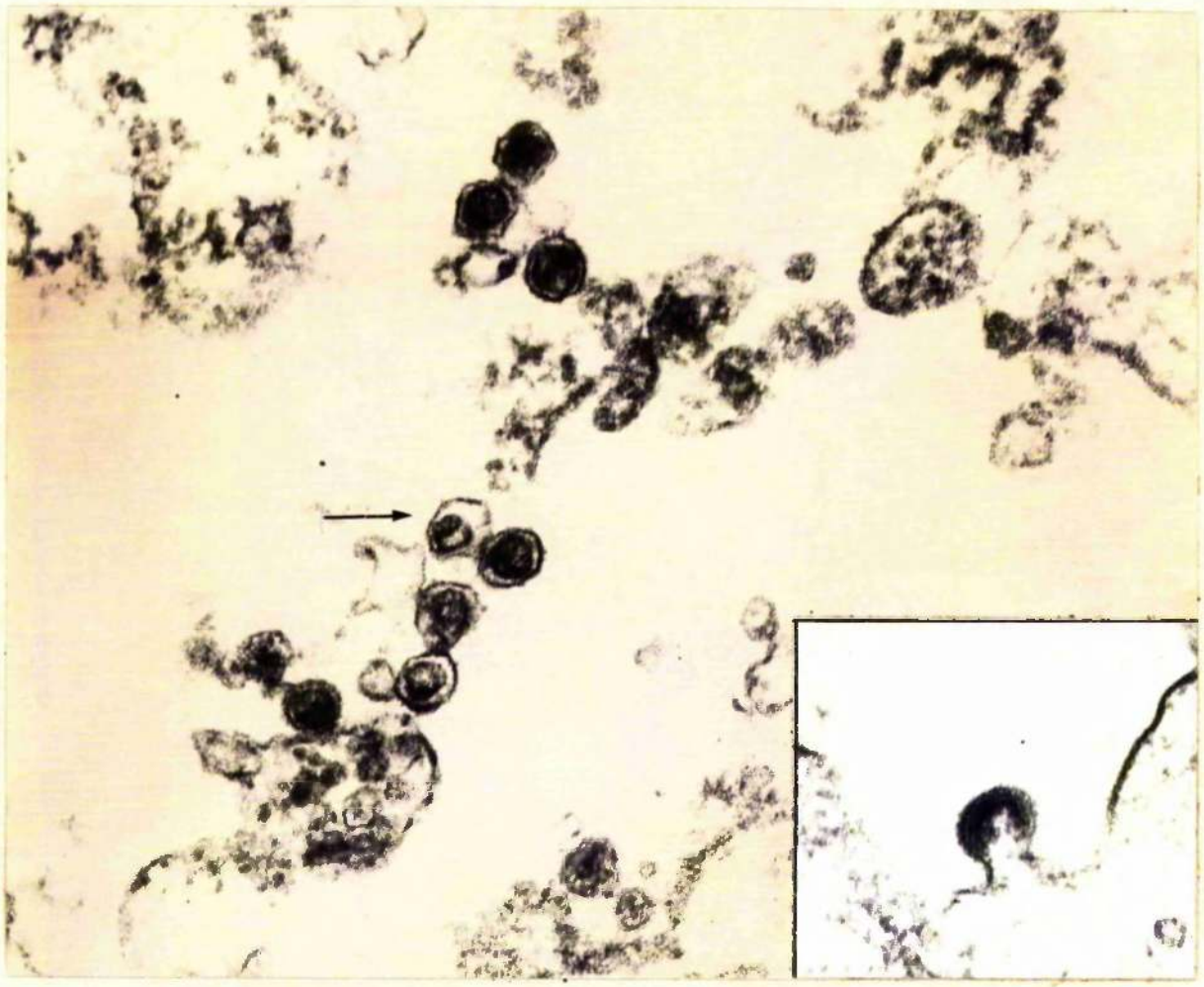
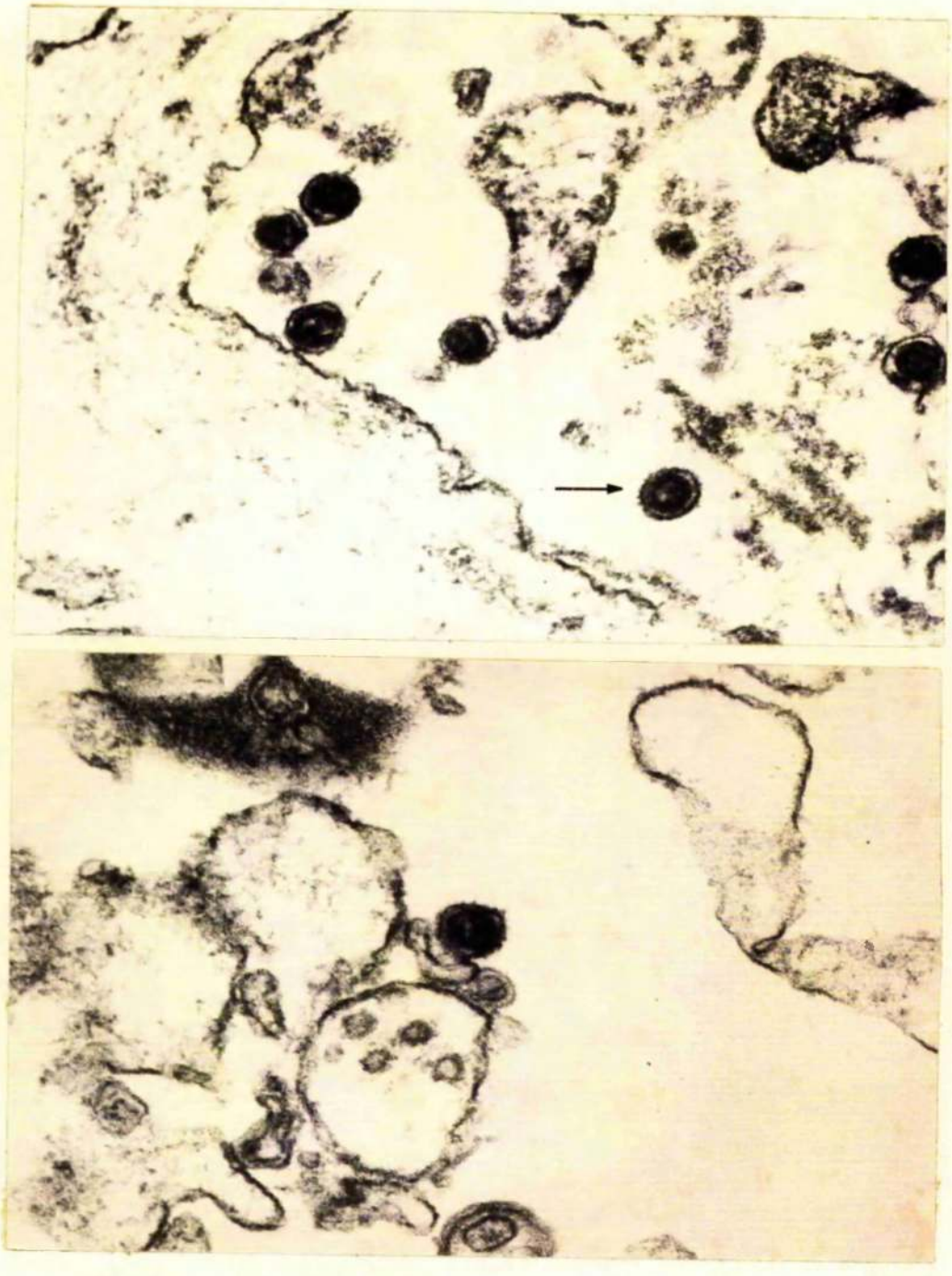


Fig. 14 Murine leukaemia virus in tissue culture. Group of particles, with electron-dense nucleoids, in an extracellular space. Some particles have an irregular outer membrane and in one particle the nucleoid is eccentric (⟶). x 75,000.

Inset shows detail of a budding particle. x 100,000.

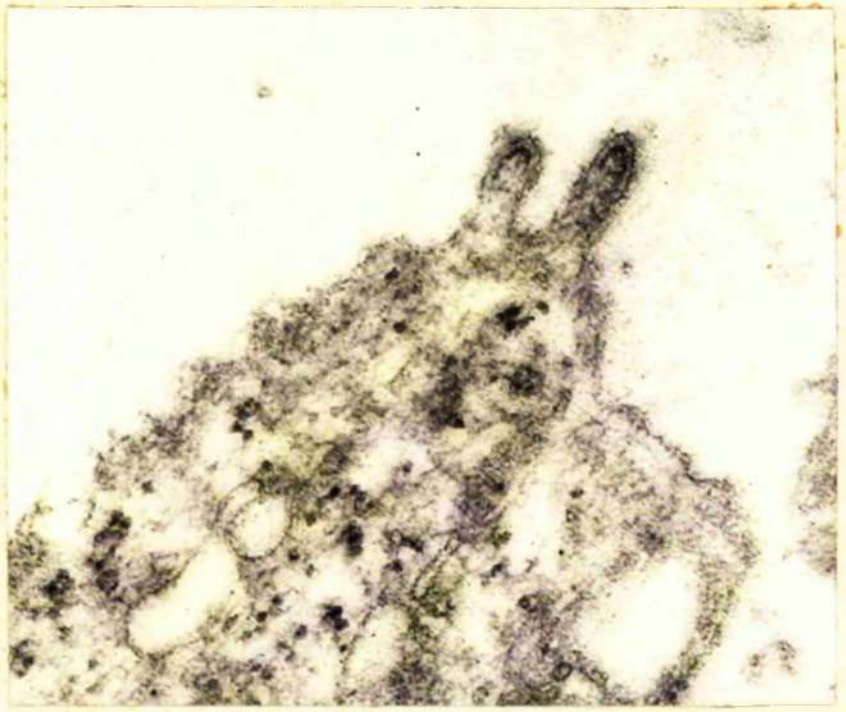


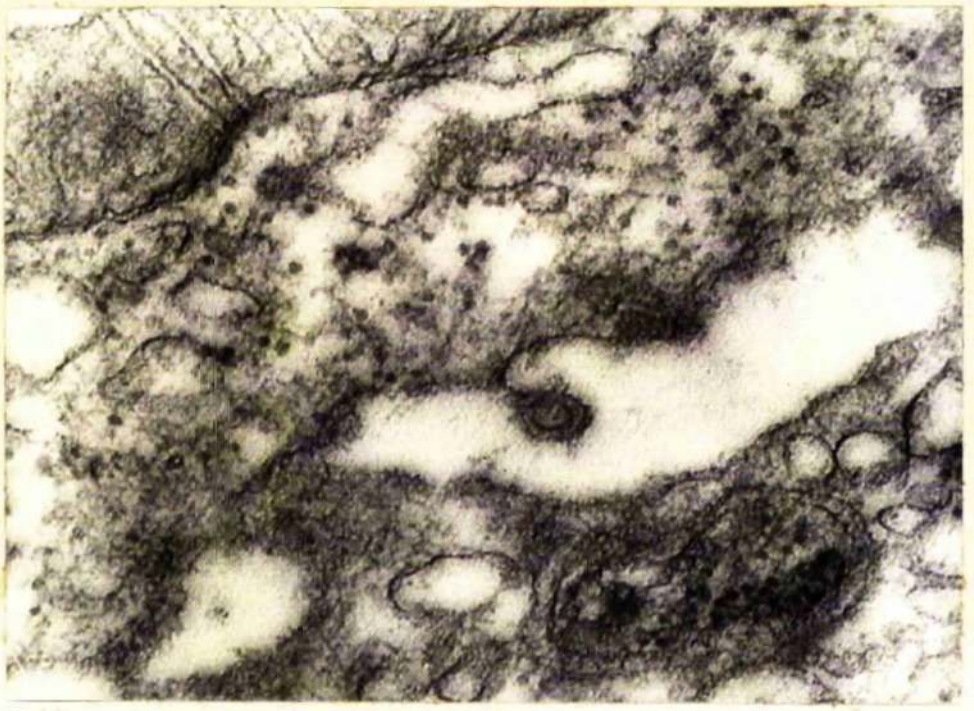
Murine leukaemia virus in tissue culture

Fig. 15 (Top) Extracellular particles near the plasma membrane. A Type 1 particle is shown (⟶). x 75,000.

Fig. 16 (Bottom) Single extracellular particle. The "coating layer"* is seen on some parts of the particle surface. x 75,000.

* See Section 1 discussion





Inoculated kitten, 15 months after infection. Mesenteric lymph node

Fig. 17 (Top) 2 particles budding from a cell surface. The inner electron-dense membranes are well developed. x 100,000.

Fig. 18 (Bottom) Type 1 particle detaching from the plasma membrane into an intracellular vacuole. x 100,000.

Fig. 19 Inoculated kitten, 15 months after infection. Mesenteric lymph node. Particle budding from plasma membrane. The 3 membrane layers are clearly defined and continuity between the outer particle membrane and the plasma membrane is shown. x 120,000.

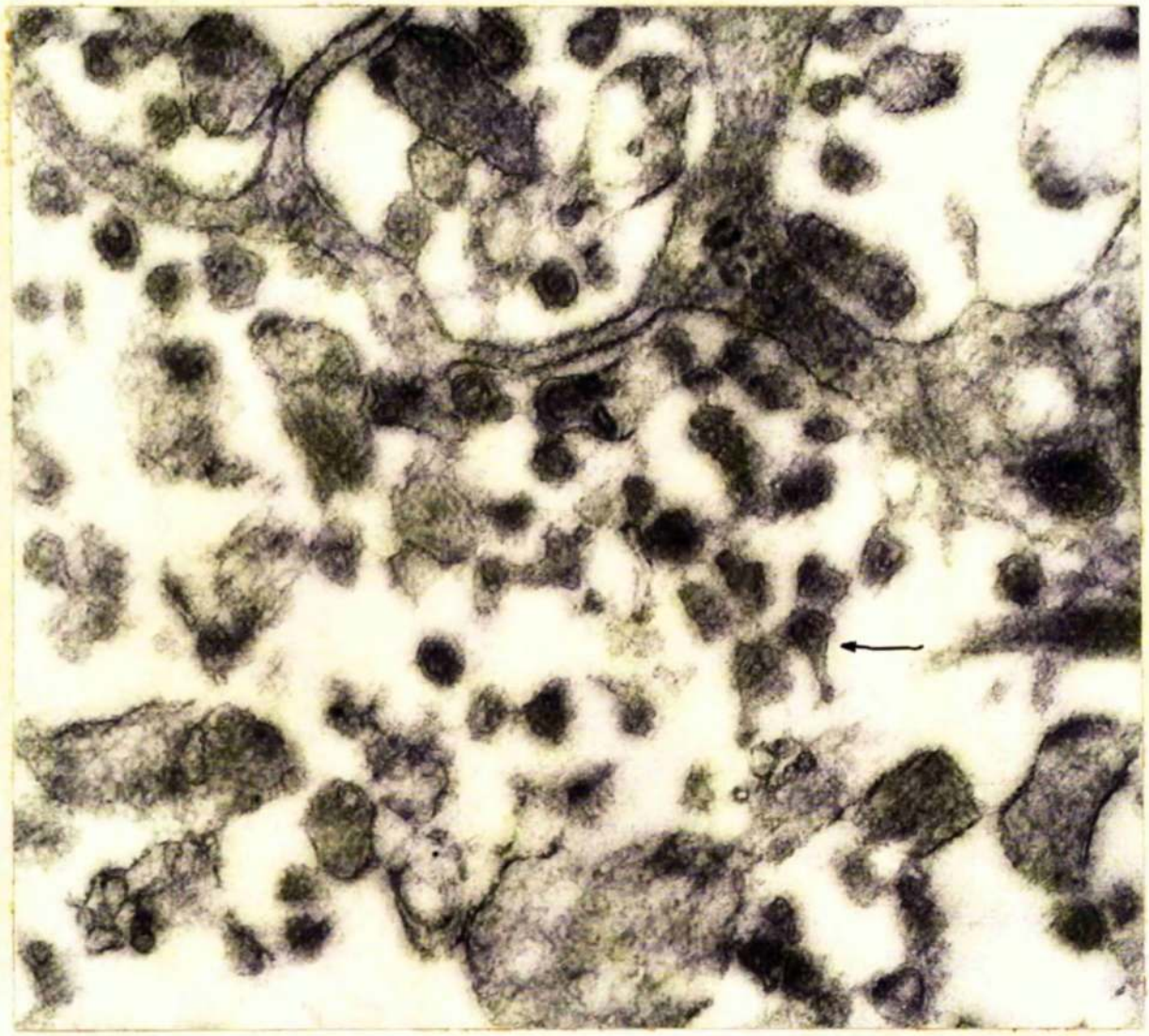


Fig. 20 Inoculated kitten, 15 months after infection. Mesenteric lymph node. Part of a megakaryocyte showing many complete and forming particles within intracytoplasmic vacuoles. A 'tailed' particle is shown (⟶). x 100,000.

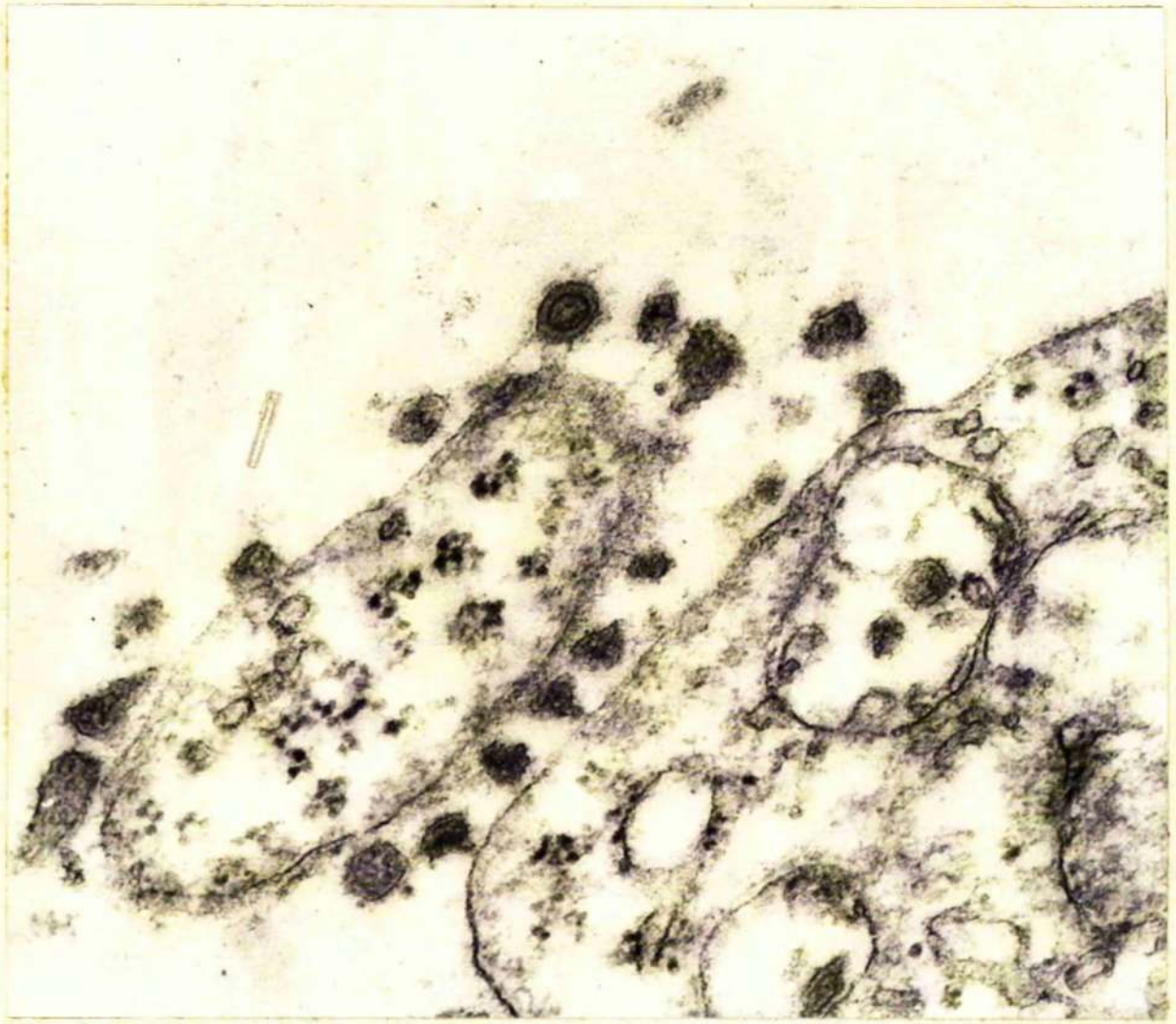


Fig. 21 Inoculated kitten, 15 months after infection. Mesenteric lymph node. Group of particles near a cell surface; one complete Type 1 particle demonstrates the triple-membraned structure. Within a vacuole 2 particles are shown. x 100,000.

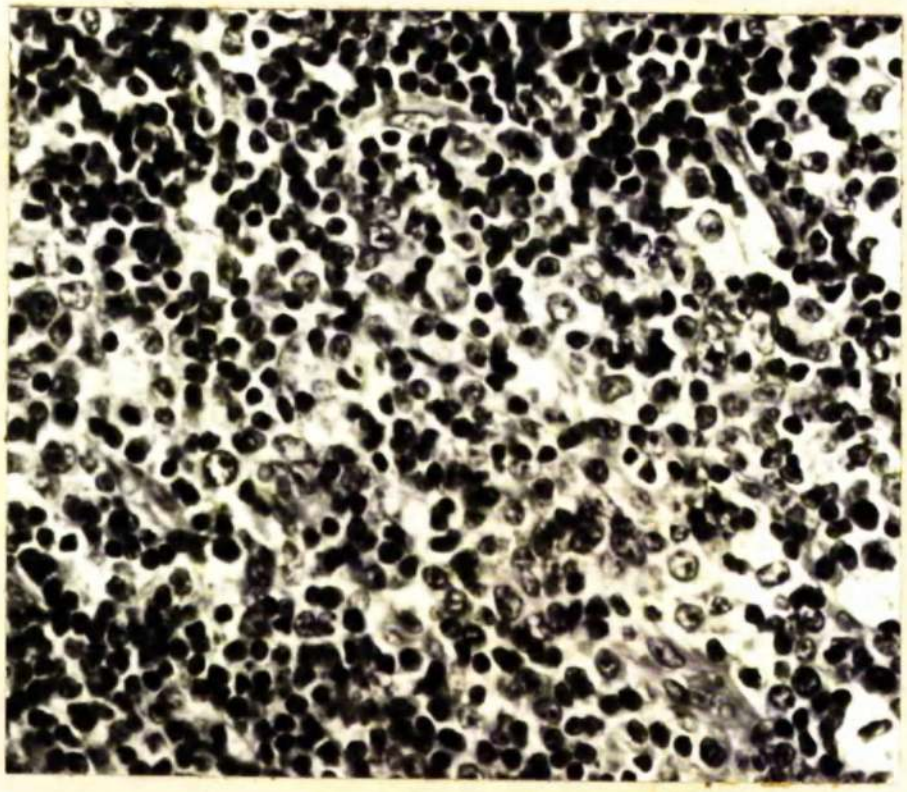


Fig. 22 Spontaneous feline lymphosarcoma. CL1. Lymph node sections showing a mixed population of lymphoid tumour cells. H & E x 420.

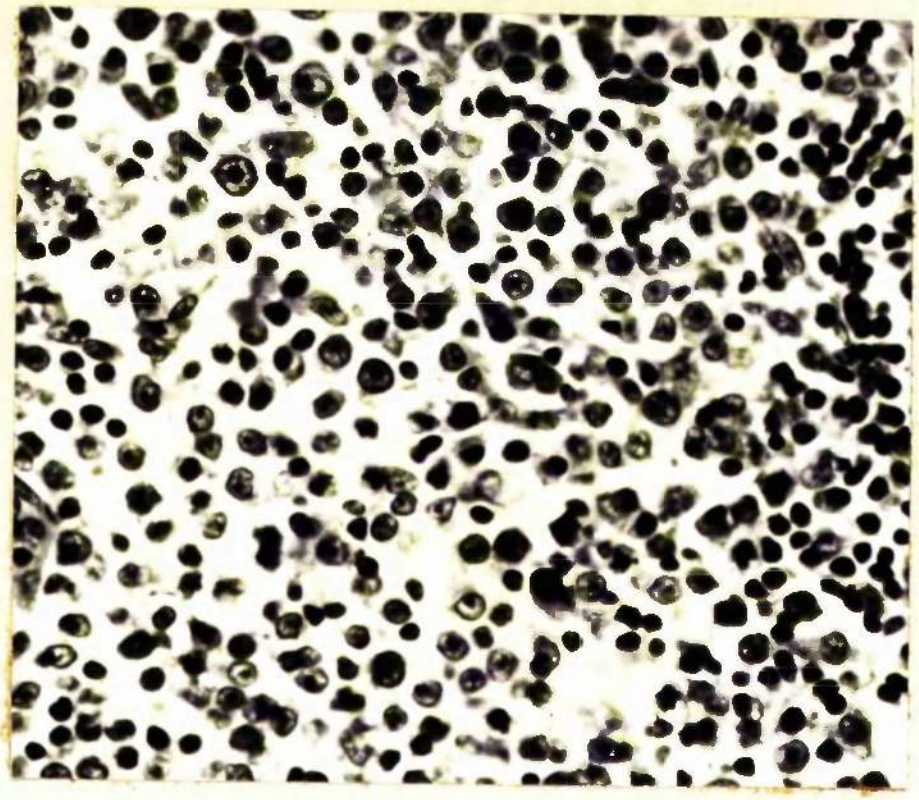


Fig. 23 Spontaneous feline lymphosarcoma. CL6. Lymph node section showing an immature cell population of malignant lymphoblasts and stem cells. H & E x 420.

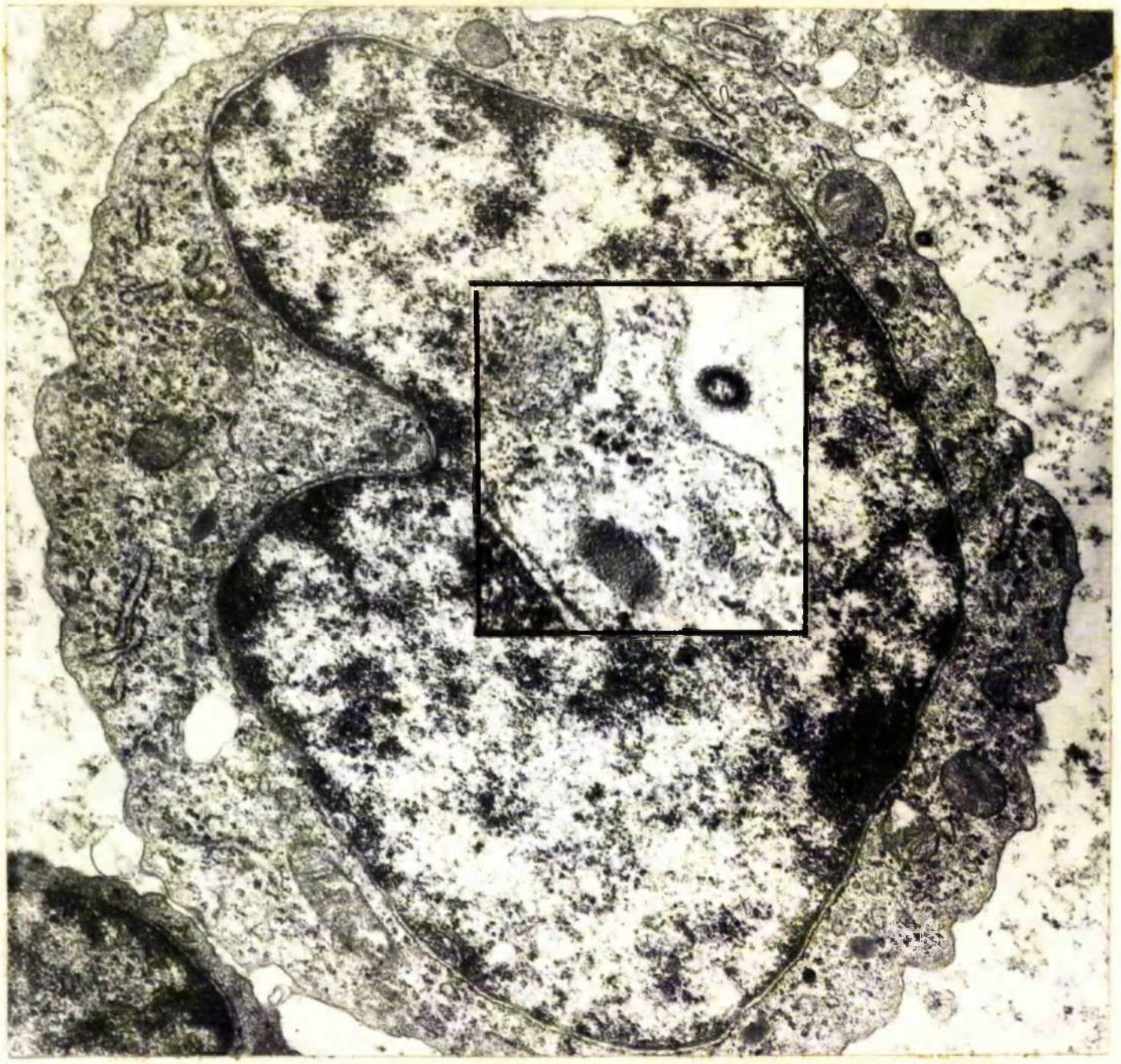


Fig. 24 Field case. Bone marrow. Type 1 particle near the surface of a lymphoid cell. x 25,000.

Inset shows detailed structure of the particle and residual connection of amorphous material between the particle and the region of the cell surface from which it detached. x 75,000.

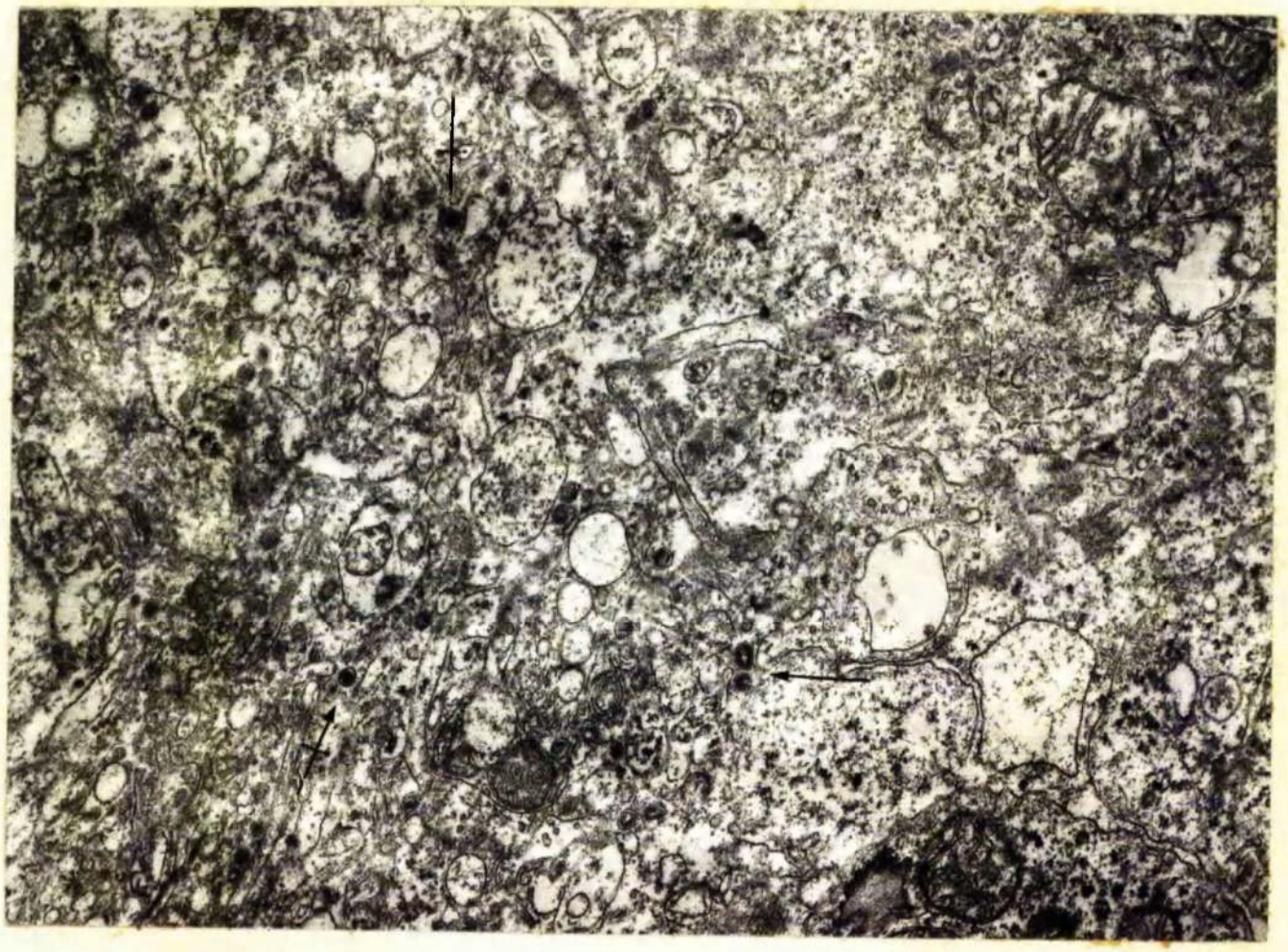


Fig. 25 Field case. Iliac lymph node. Part of a megakaryocyte with many particles in vacuoles and intracytoplasmic channels. (⟶ Type 1 particle ⟶ Type 2 particles). x 25,000.

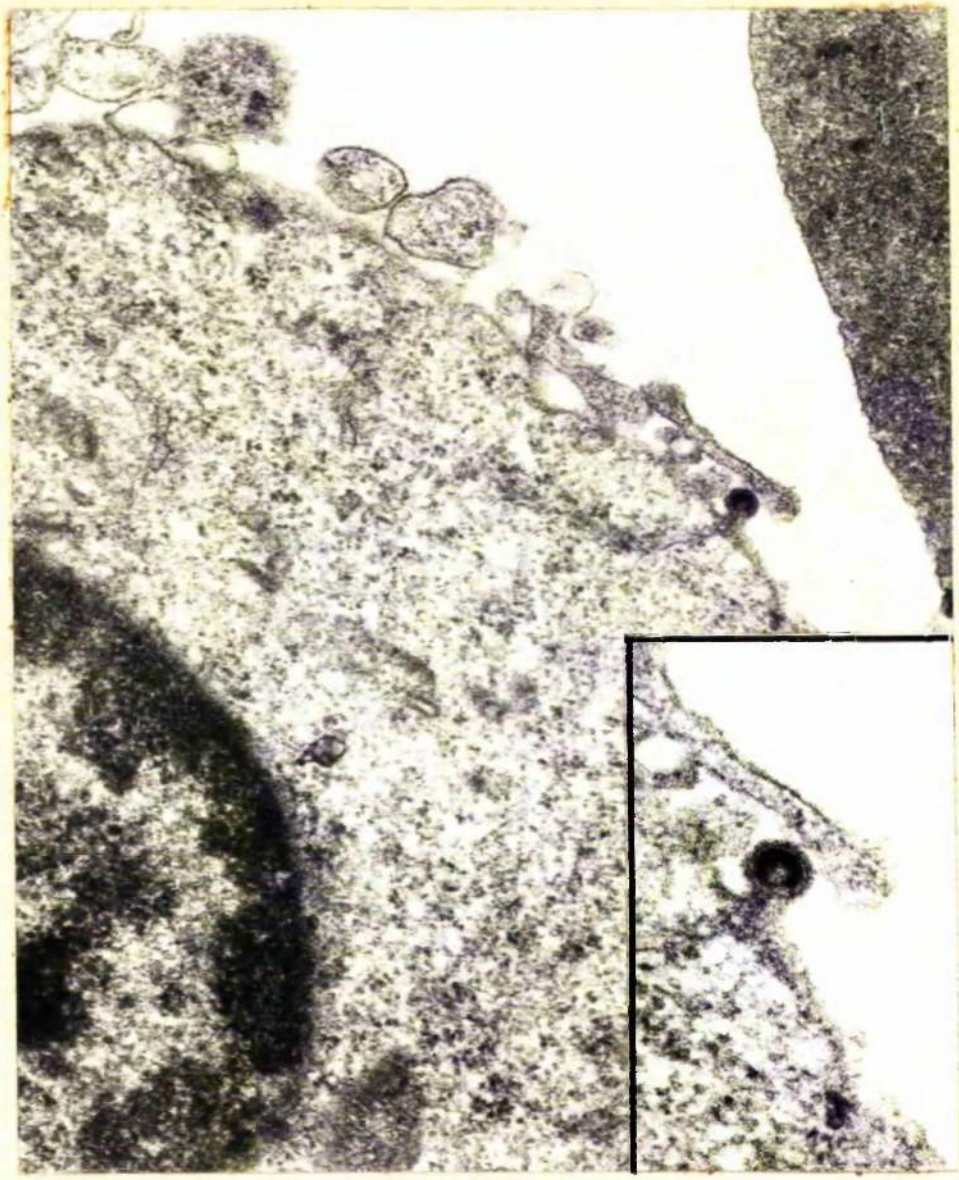


Fig. 26 Field case. Bone marrow. Particle budding from surface of lymphoid cell. x 37,500.

Inset shows detailed structure of particle with 'coating layer' and some condensation of electron-dense material in the core. x 75,000.

Fig. 27 Field case. Mesenteric lymph node. 3 particles budding into intracytoplasmic vacuole. x 75,000.

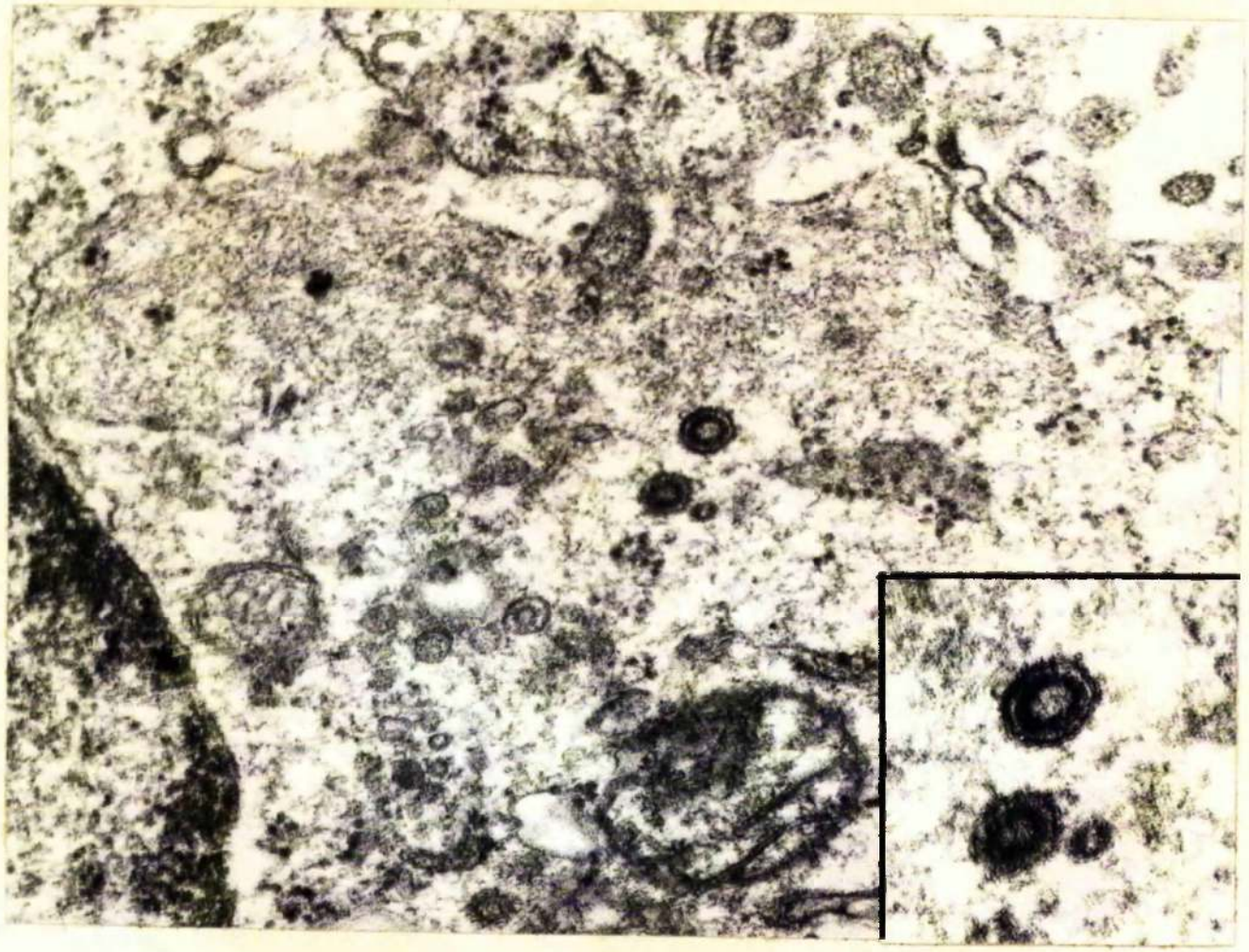


Fig. 28 Field case. Mesenteric lymph node. 2 particles in cell debris of disintegrating cell. x 62,500.

Inset shows detail of particle structure. x 125,000.

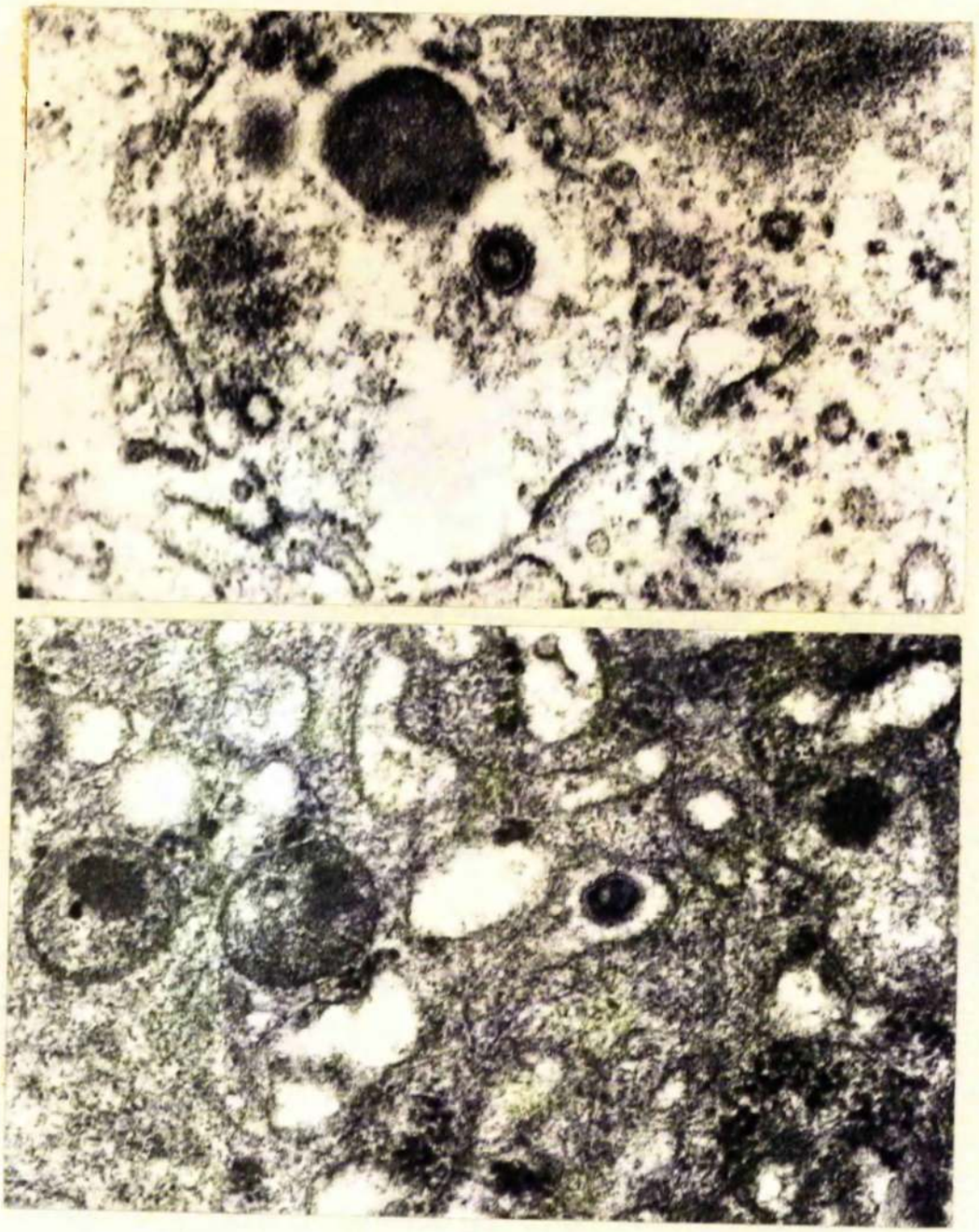


Fig. 29 (Top) Field case. Spleen. Type 1 particle in intracellular vacuole. The 'coating layer' is clearly defined. x 100,000.

Fig. 30 (Bottom) Field case. Bone marrow. Type 2 particle in megakaryocyte vacuole. x 75,000.

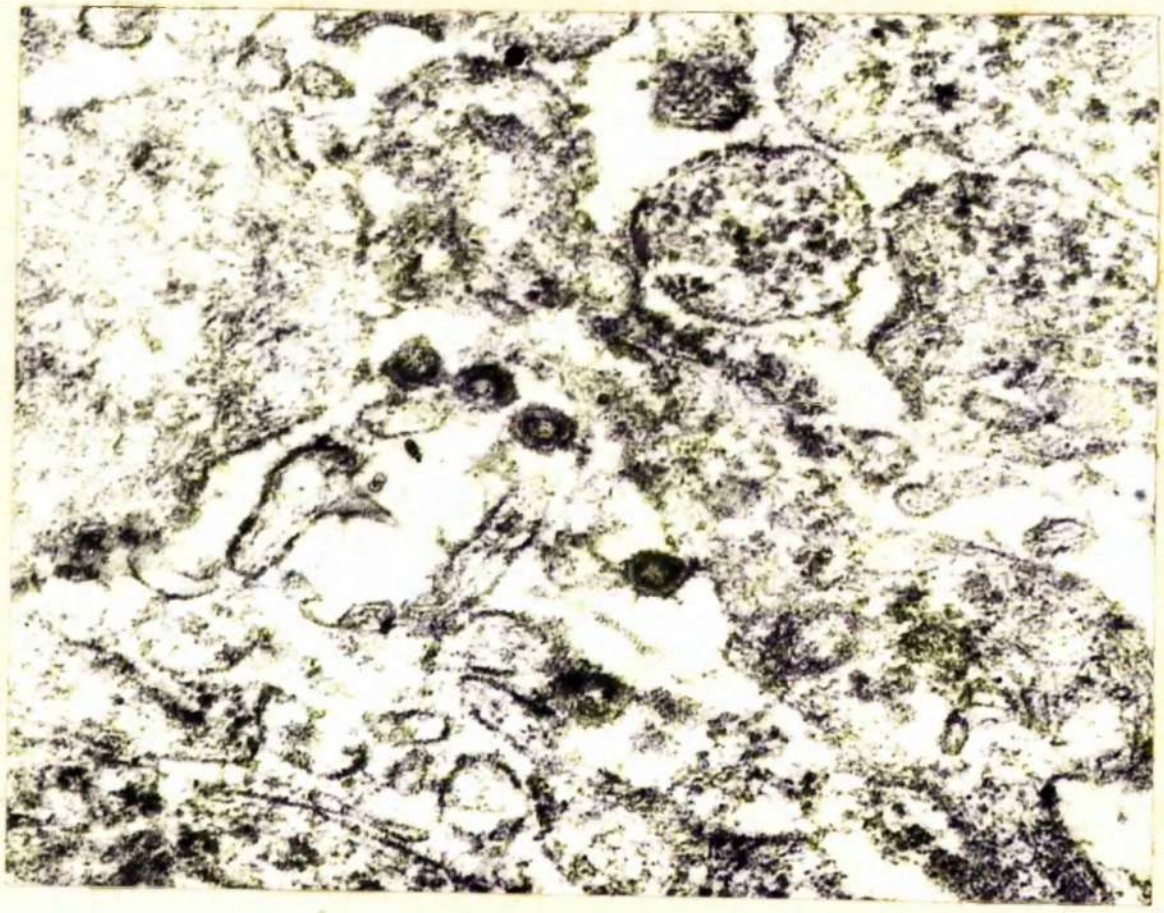
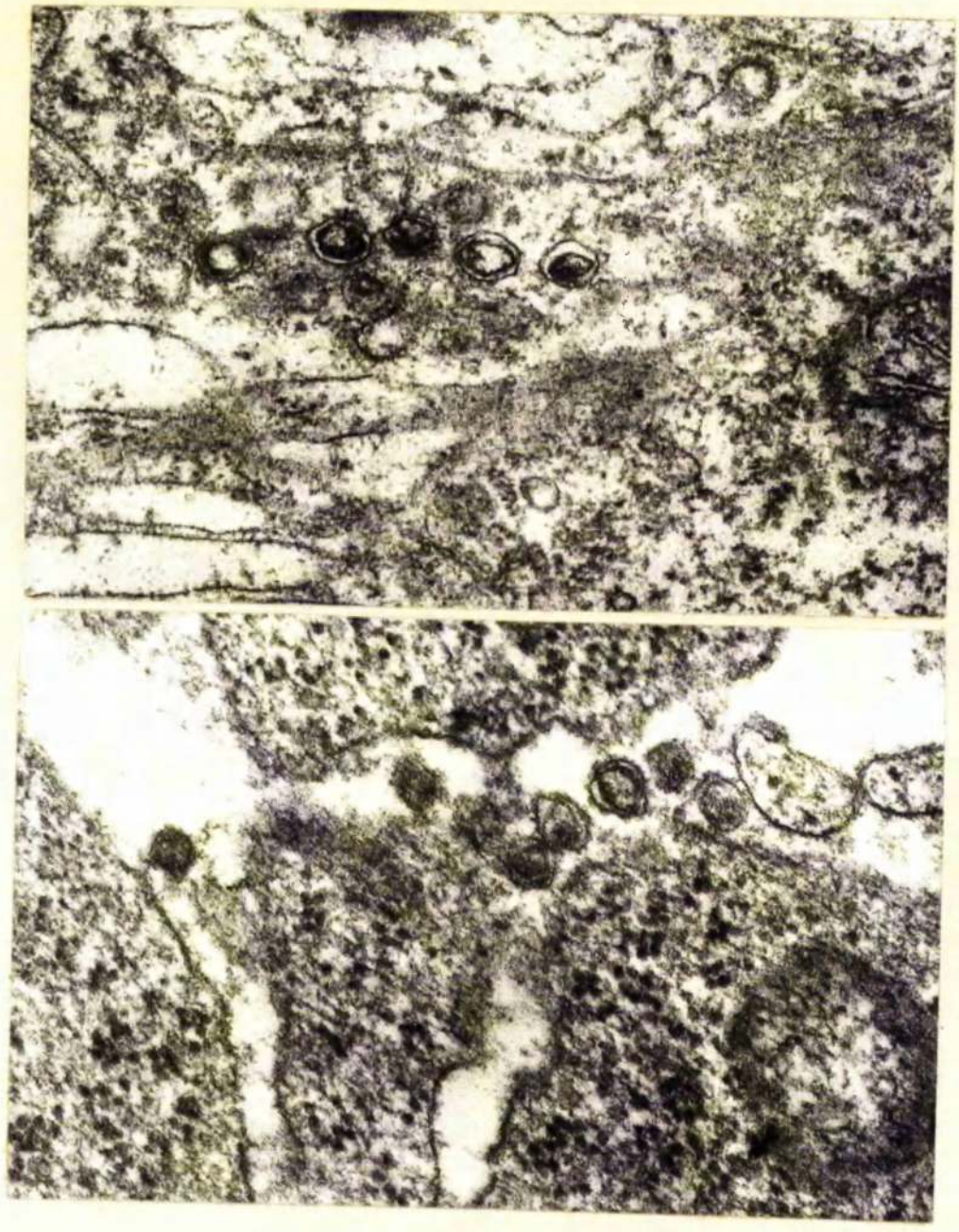


Fig. 31 Field case. Mesenteric lymph node. Group of 5 Type 1 particles. x 62,500.



Field cases with groups of particles with electron-dense nucleoids.

Fig. 32 (Top) Iliac lymph node. One Type 1 particle to the left; the other particles show varying degrees of condensation of electron-dense material in the core; the outer membrane is irregular and the nucleoid eccentric in the particle to the right. x 75,000.

Fig. 33 (Bottom) Thymus. Extracellular Type 2 particles with wrinkled outer membranes. x 75,000.

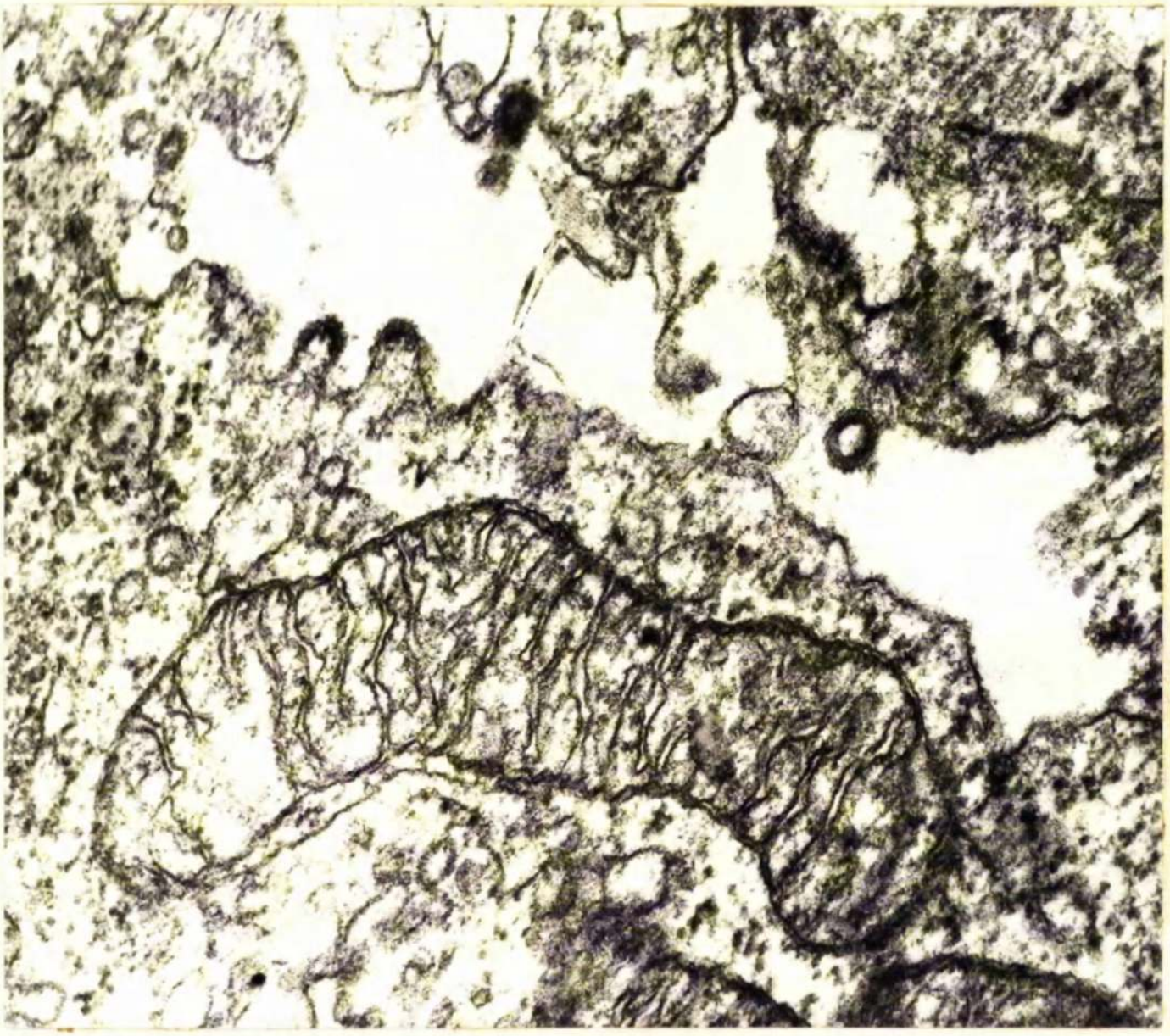


Fig. 34 Field case. Mesenteric lymph node. 2 particles budding from a short length of plasma membrane: the inner electron-dense membranes and intermediate membranes are partially formed. 2 fully formed particles are also seen. x 75,000.

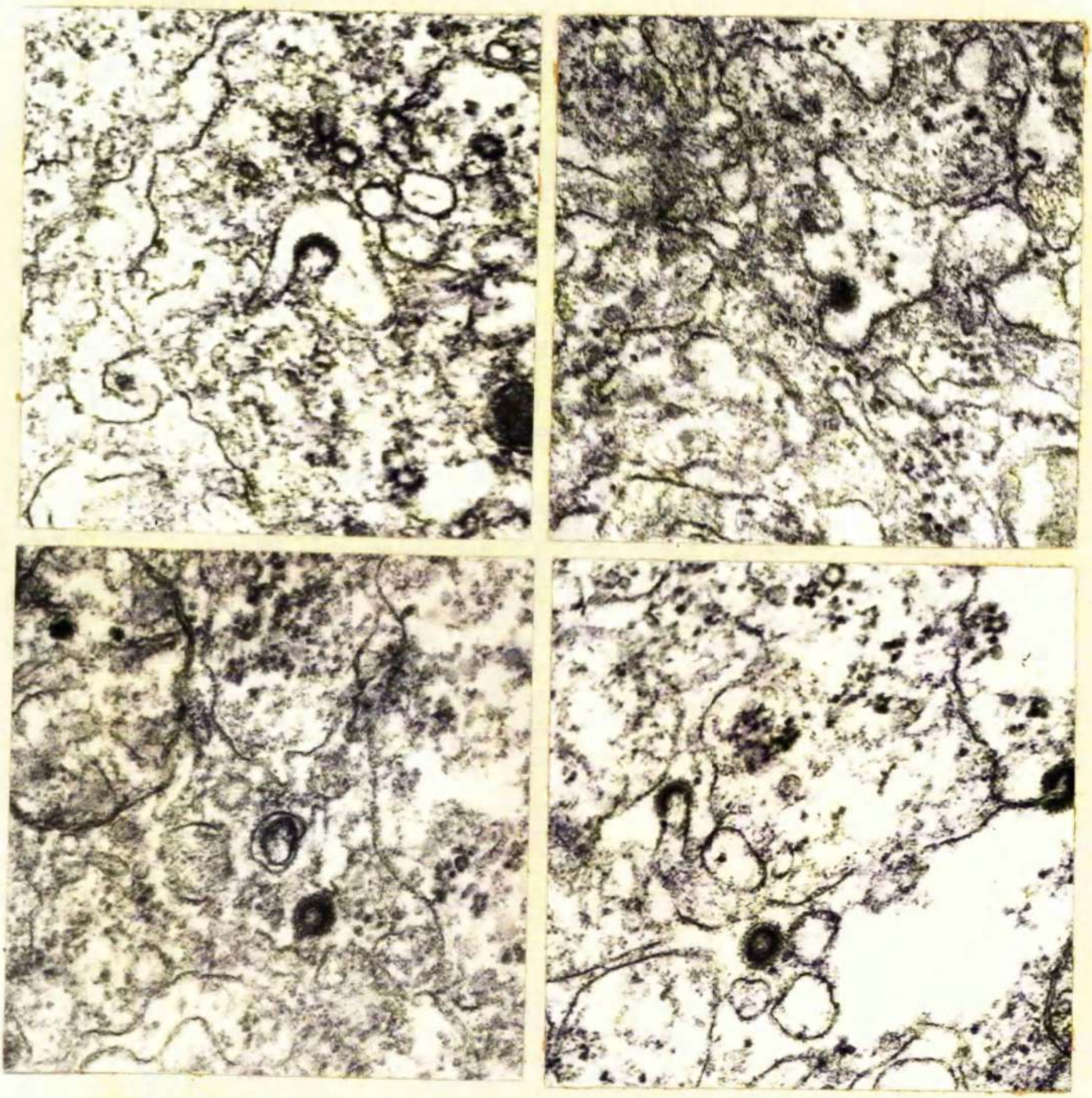


Fig. 35 (Top left) Field case. Triple-membrane layered particle budding into vacuole. The outer membrane shows the 'coating layer'. x 75,000

Fig. 36 (Top right) Field case. Particle with dense nucleoid forming at a vacuole surface. x 75,000

Fig. 37 (Lower left) Field case. Complete particle with 'tail'. x 75,000

Fig. 38 (Lower right) Field case. One complete and two forming particles; all have a surface 'coating layer'. x 75,000.

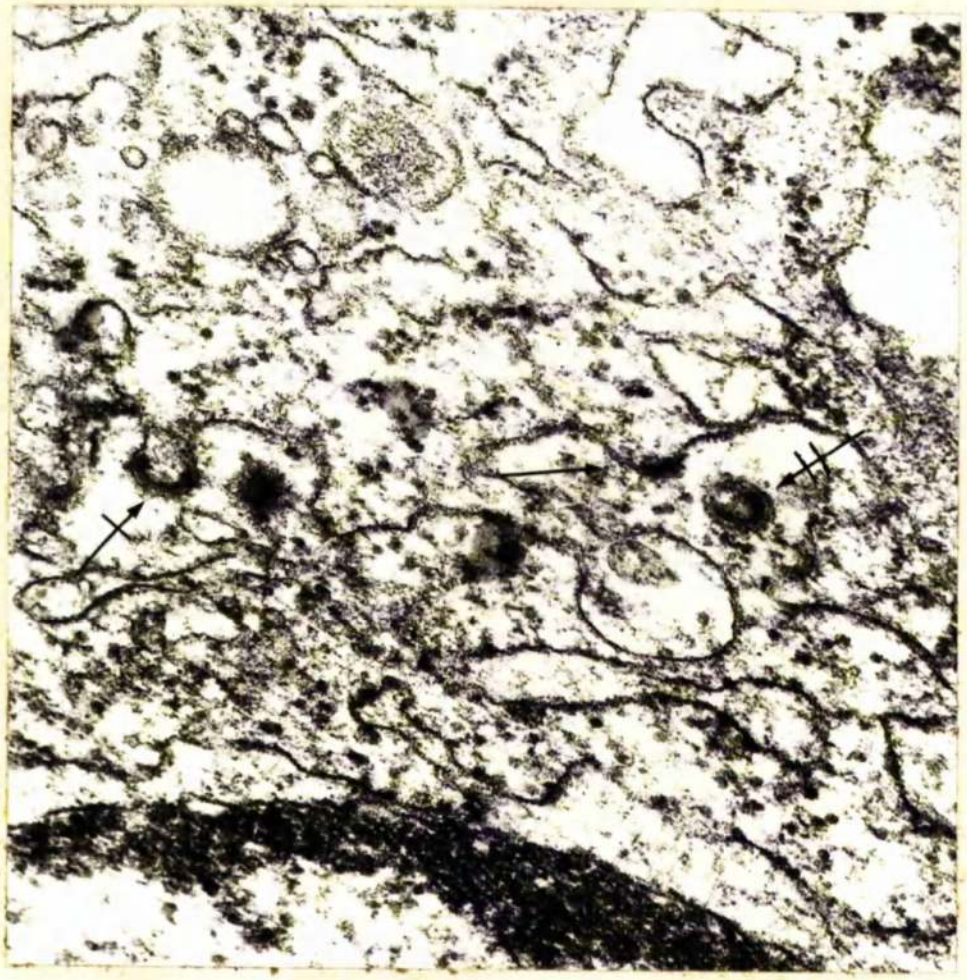
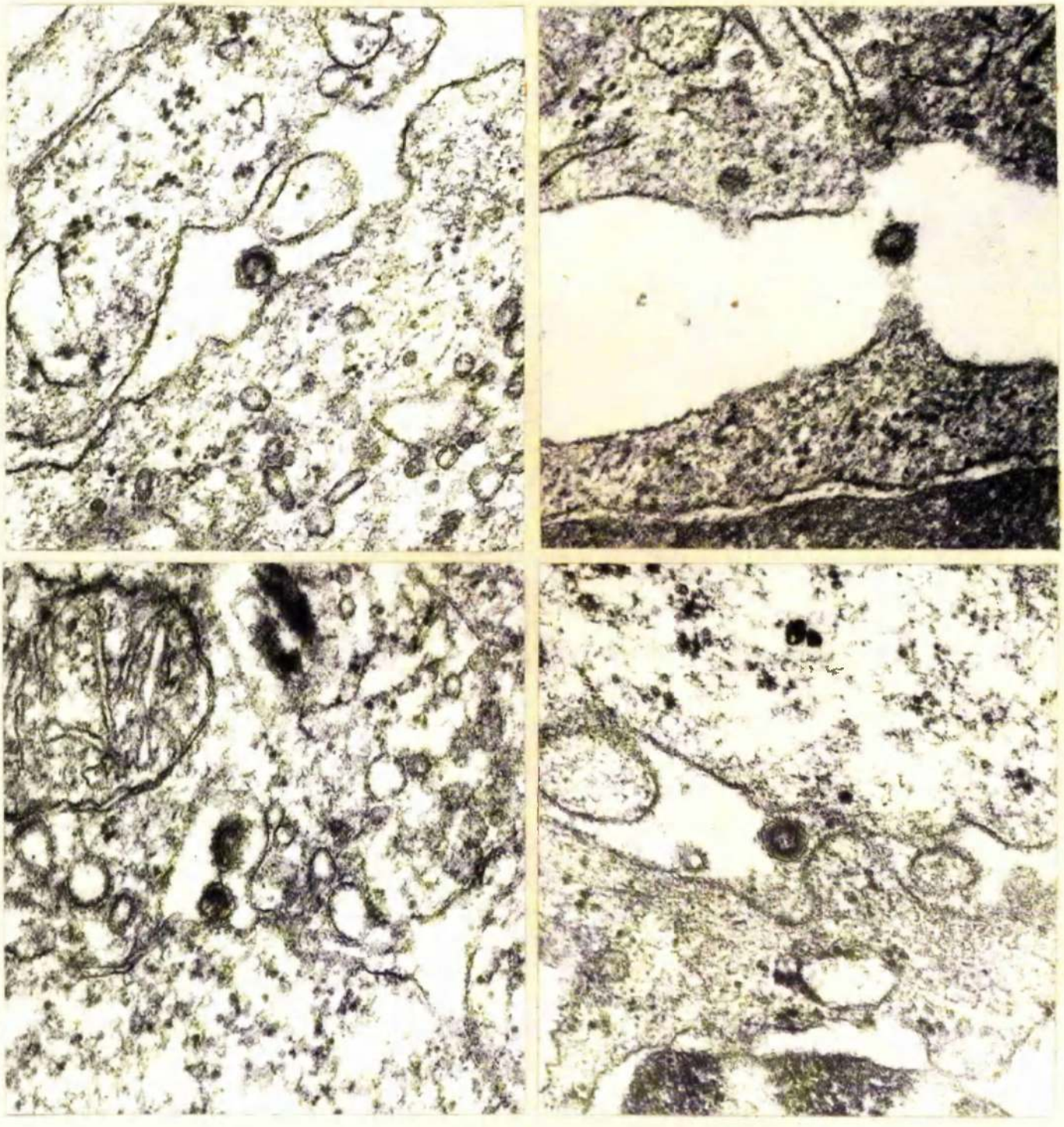


Fig. 39 Field case. Mesenteric lymph node. An early stage of budding (⟶) and a more fully formed bud (⟶+) are shown. The triple-membraned structure is clearly defined in the bud (⟶+) and in a complete particle (⟶‖). x 75,000.



Field cases. Particles at plasma membranes

Fig. 40 (Top left) Outer membrane of particle attached to cell membrane. x 75,000

Fig. 41 (Top right) Particle free from cell surface with amorphous material between particle and membrane. x 75,000

Fig. 42 (Lower left) Completely formed particle in intercellular space. x 75,000

Fig. 43 (Lower right) 2 particles joined by thread of membrane. x 75,000.

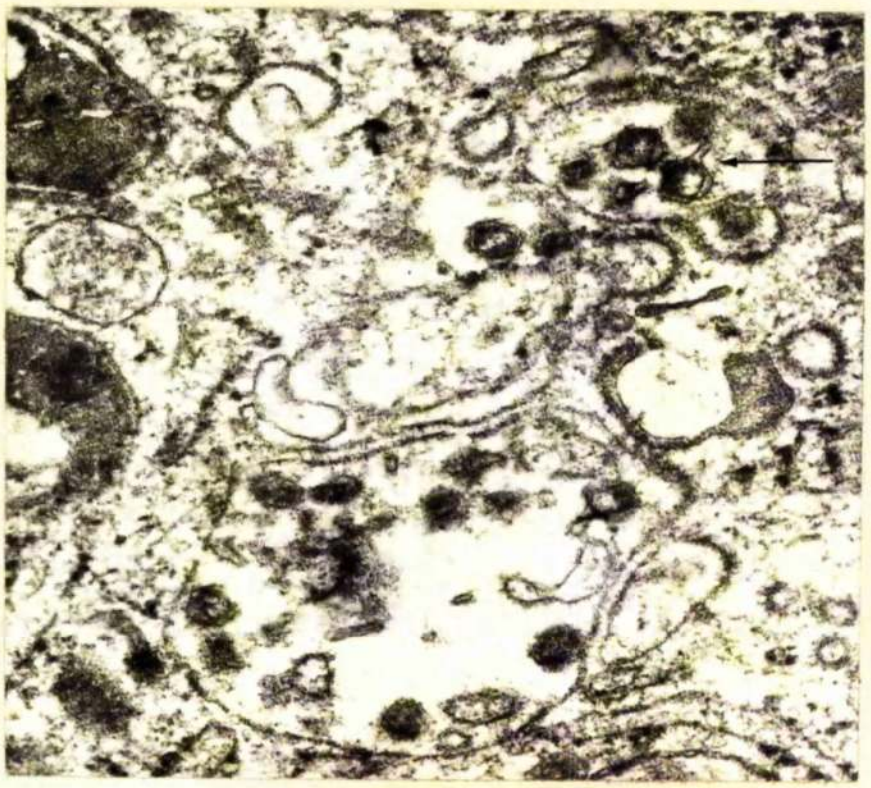
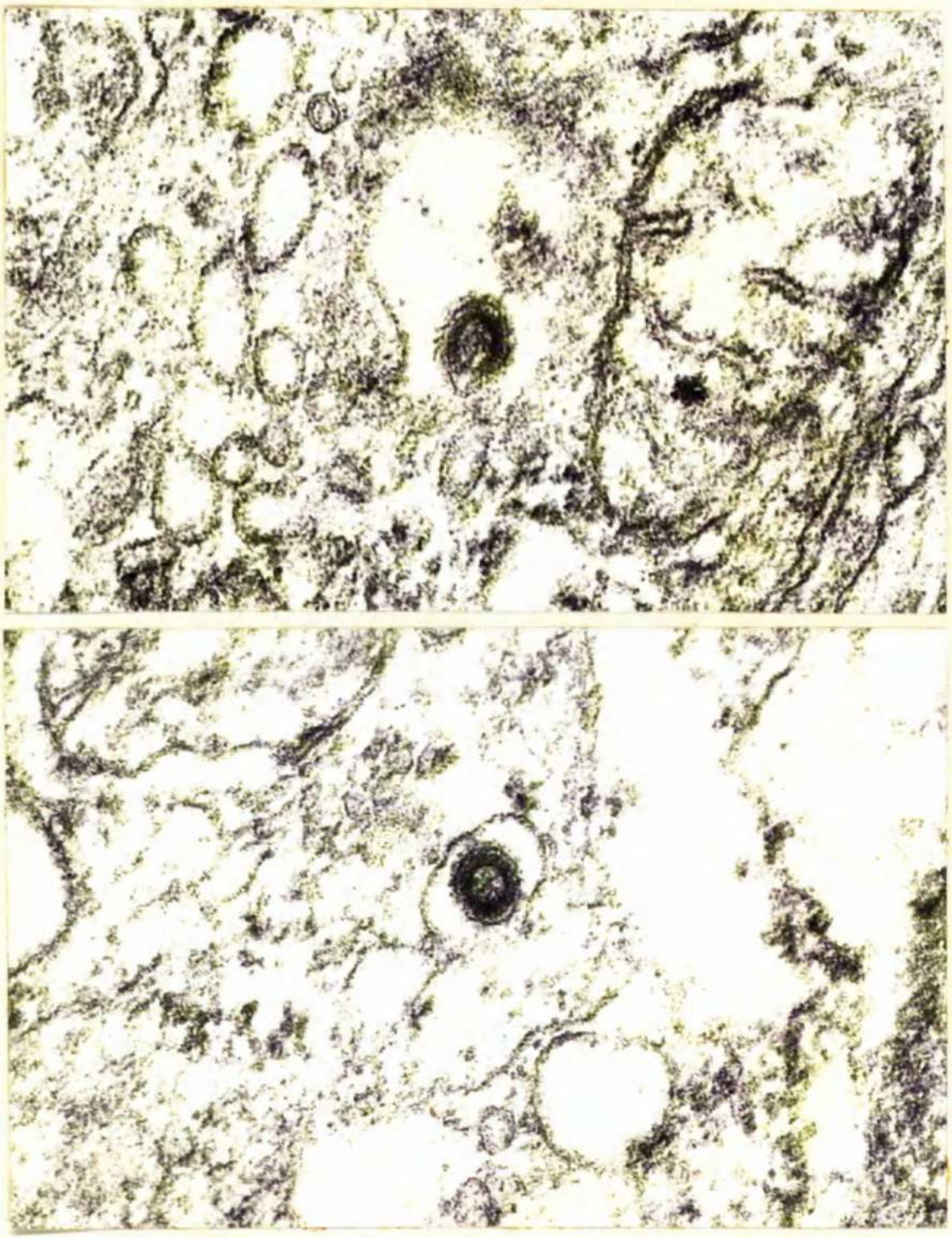


Fig. 44 Field case. Thymus. Groups of particles in vacuoles. A particle with a "tail" is shown (⟶). x 62,500.



Field case. Mesenteric lymph node

Fig. 45 (Top) Detailed structure of budding particle showing 3 membranes; the inner and intermediate membranes are almost complete and the outer membrane 'pinching off' from the vacuole surface. A diffuse 'coating layer' is on the outer surface. x 120,000.

Fig. 46 (Bottom) Complete Type 1 particle, in a vacuole. The 3 membranes and 'coating layer' are seen. x 120,000.

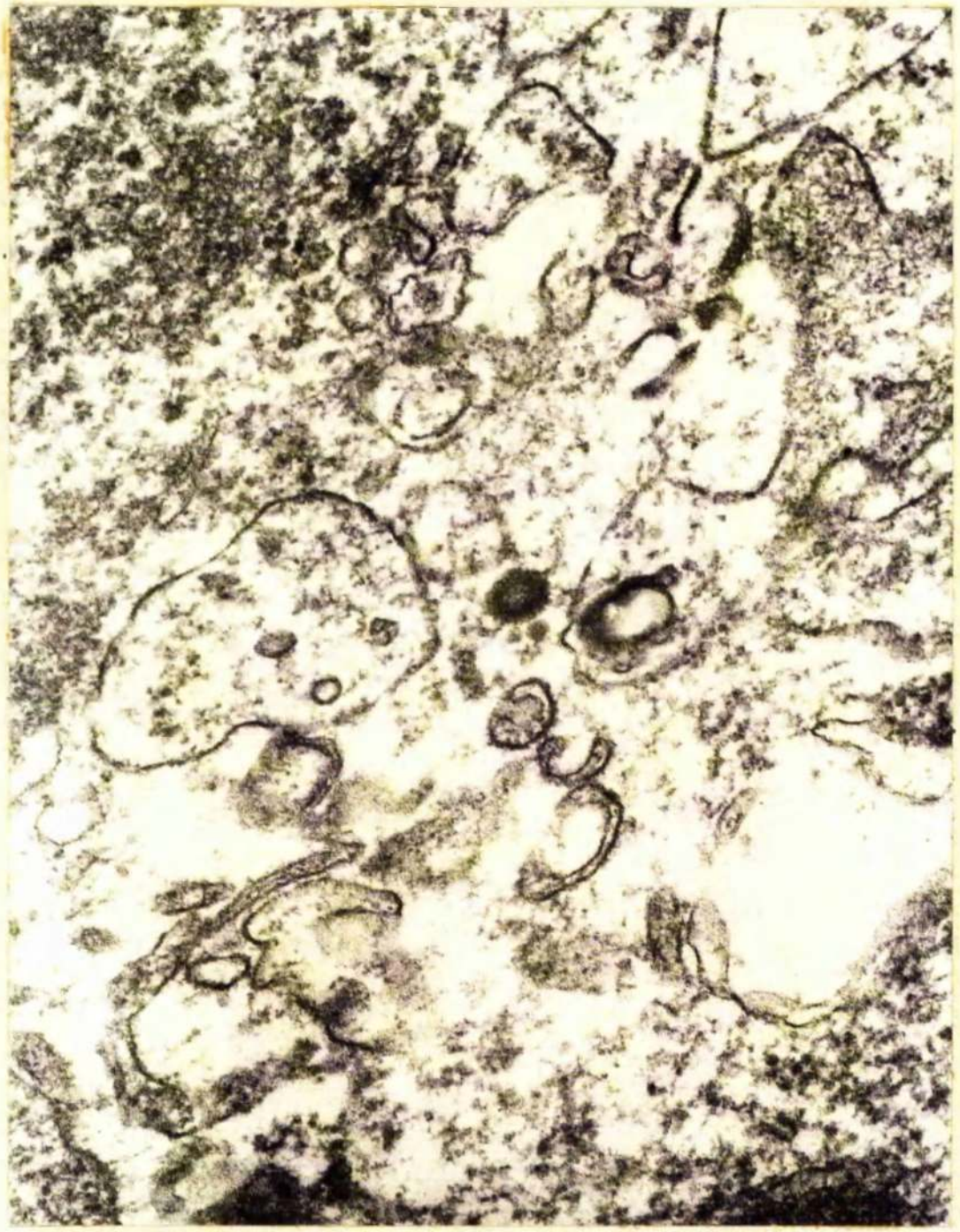


Fig. 47 Field case CL2. Prescapular lymph node. The single particle found in this case. The particle, approximately 100 mμ in diameter has an electron-lucent core and is seen extracellularly amongst cellular debris. x 75,000.

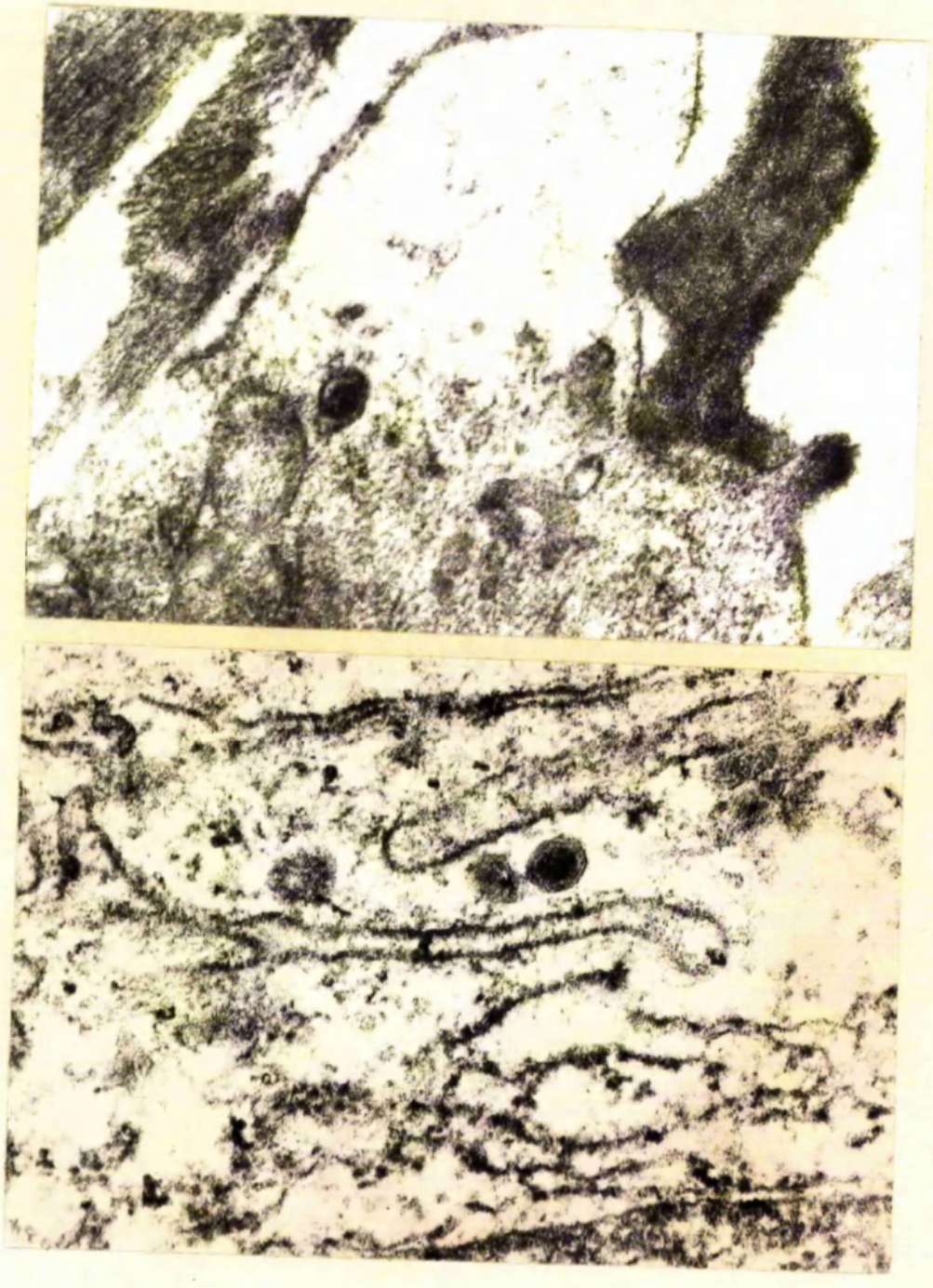


Fig. 48 (Top) Field case. Virus-like structure and possible bud in platelet pellet. x 75,000.

Fig. 49 (Bottom) Field case. CL7. Mesenteric lymph node. Virus-like structures with electron-dense nucleoids. x 75,000.

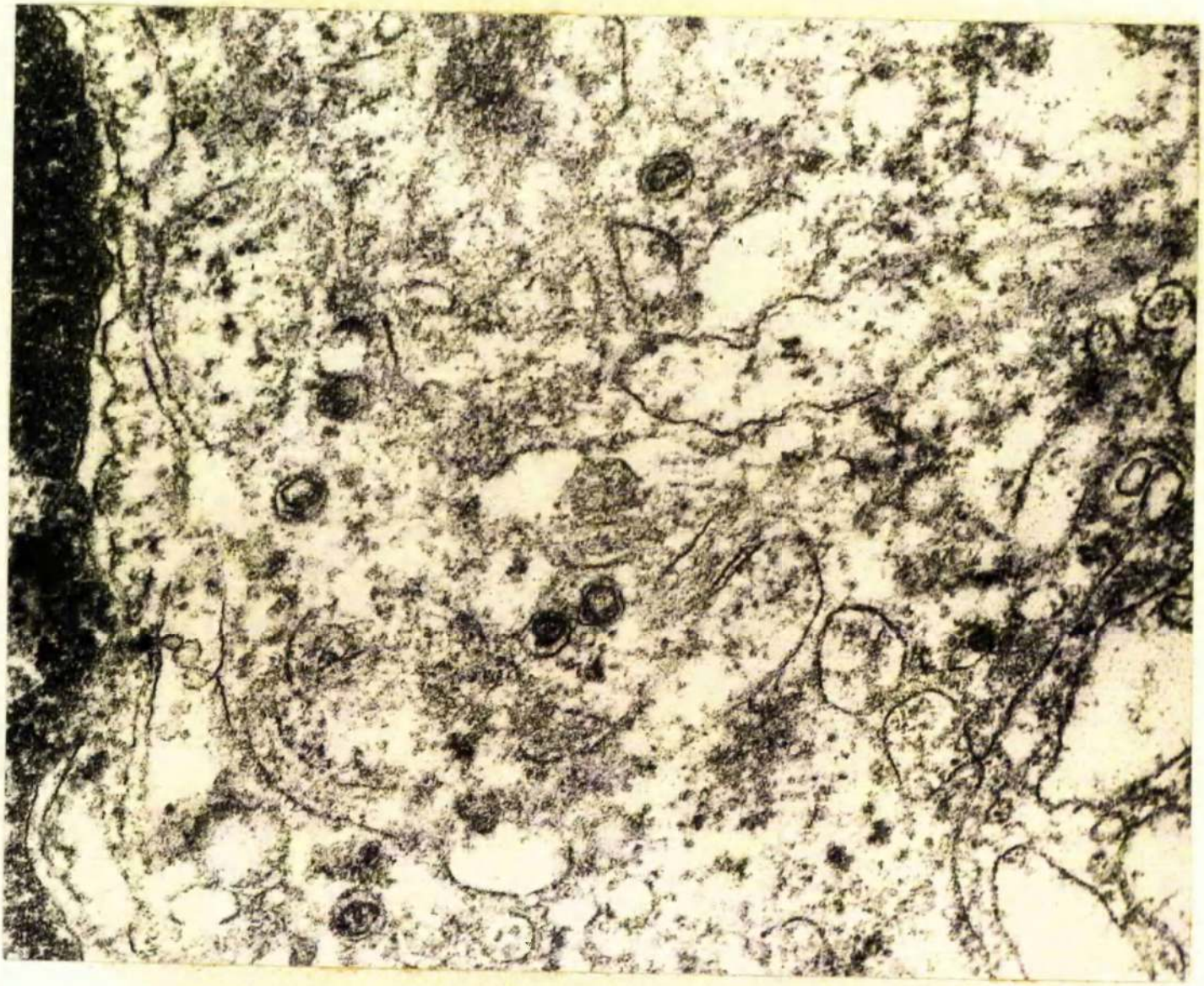


Fig. 50 Field case CL8. Iliac lymph node. The only particles found in the examination of this case. The particles, approximately 100 mμ in diameter, show electron-dense, eccentric nucleoids. x 75,000.

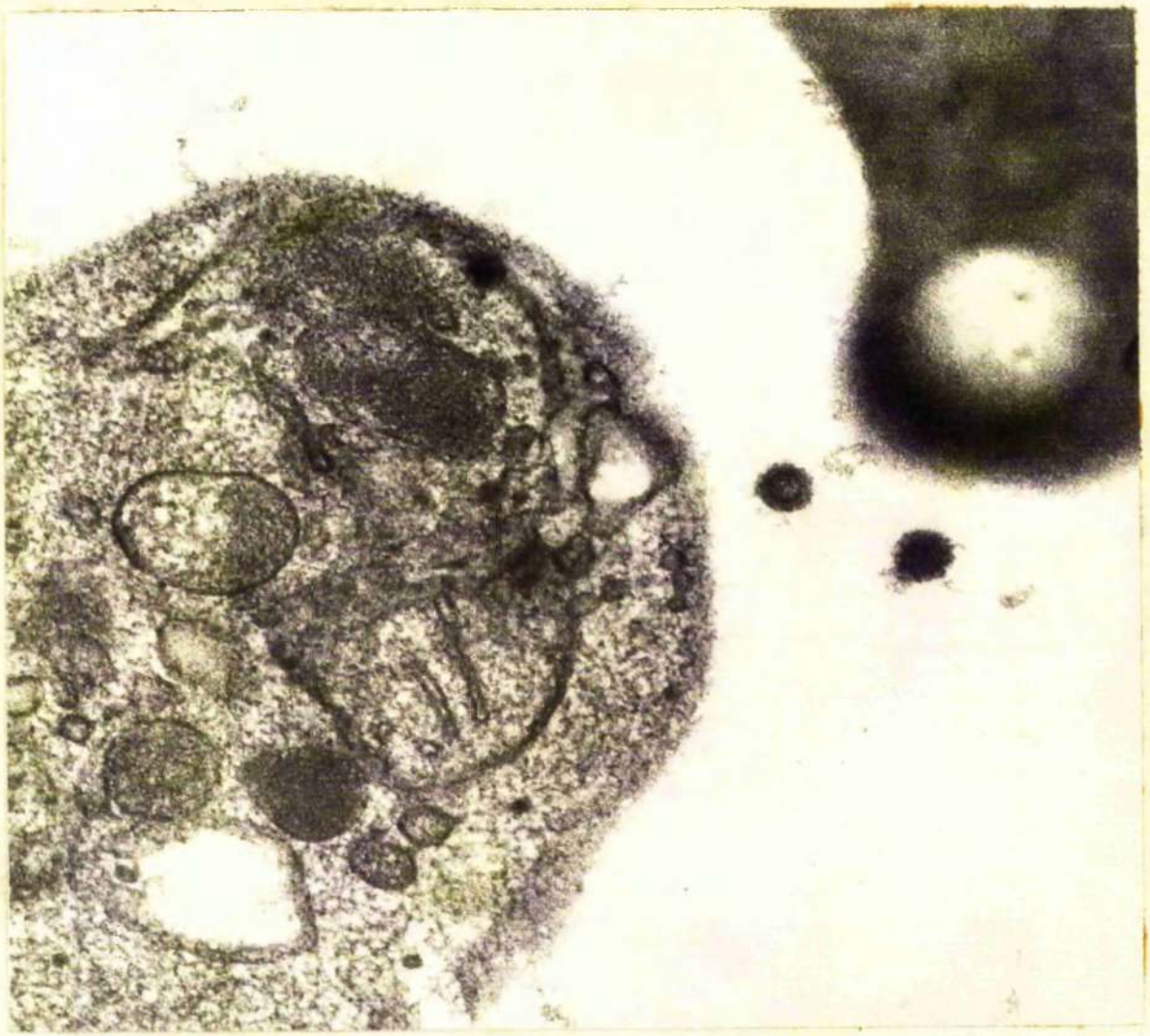


Fig. 51 Field case CL9. Blood platelet pellet. 2 extracellular particles; one particle has a relatively electron-lucent core and electron-dense inner membrane; the other particle has an electron-dense core and shows a 'coating layer' over part of the outer surface. x 62,500.

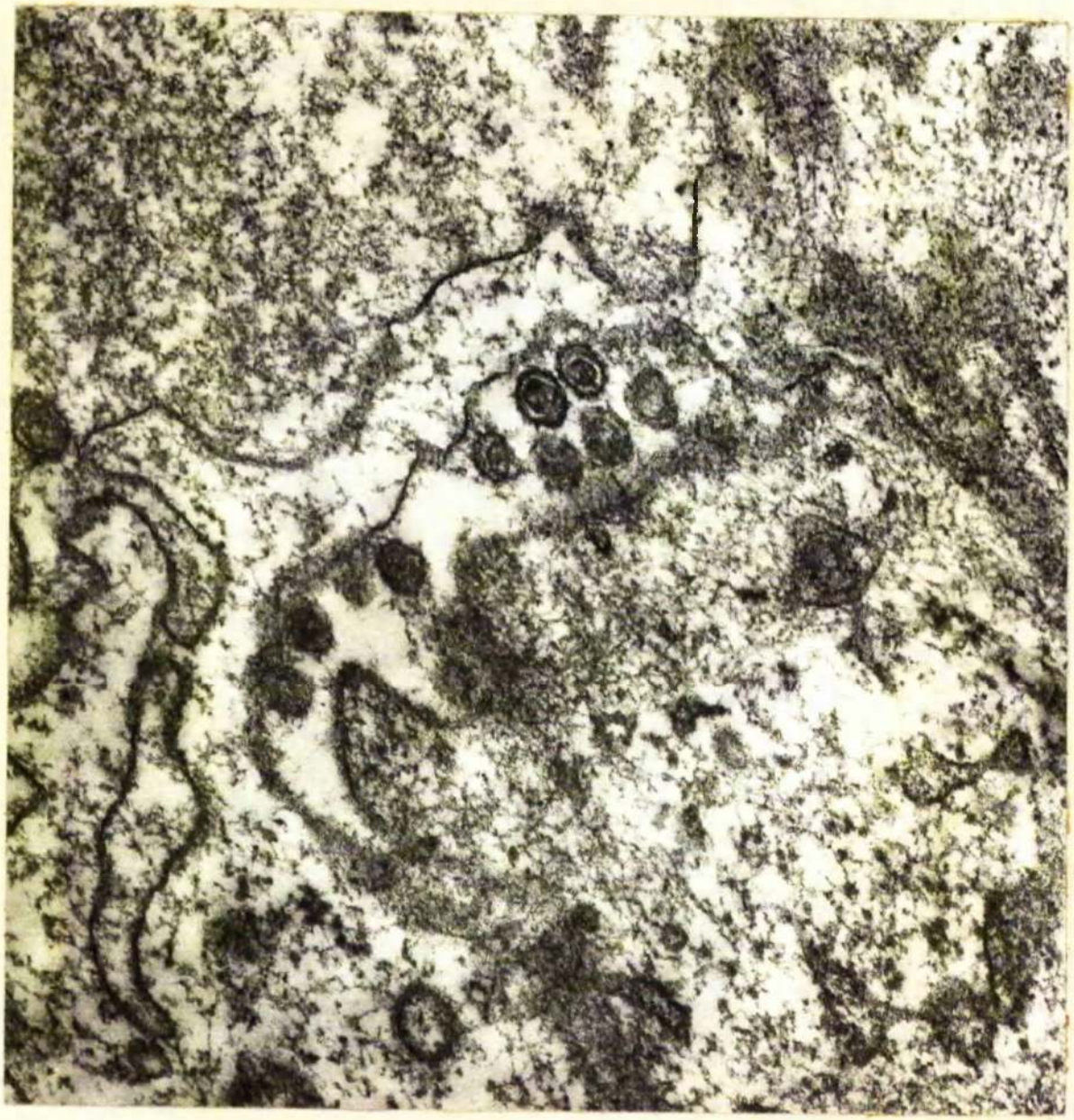
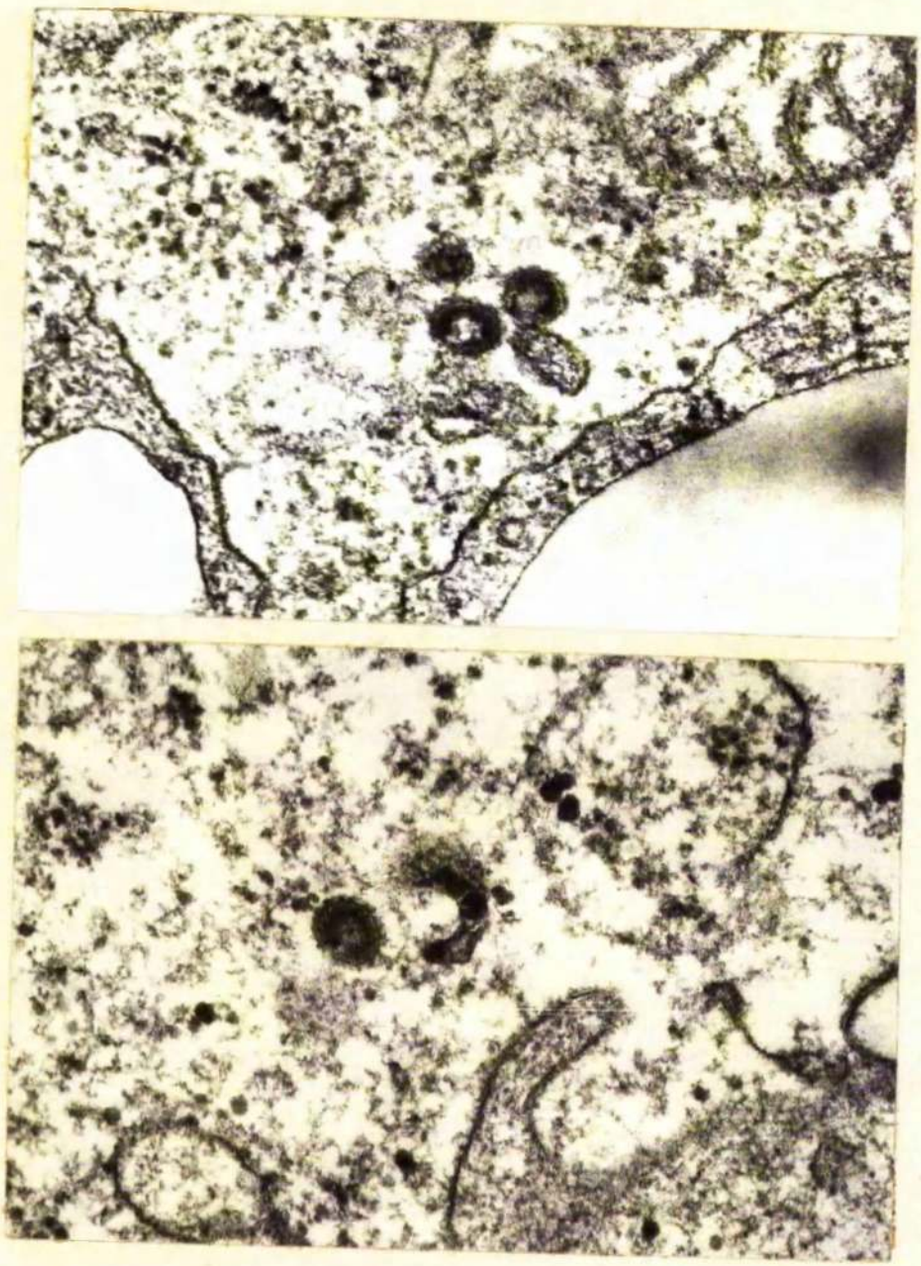


Fig. 52 Field case CL9. Bone marrow. Group of particles with electron-dense nucleoids. x 75,000.



Field case CL9

Fig. 53 (Top) Thymus. 2 triple-membraned particles near the surface of an epithelial cell. x 75,000.

Fig. 54 (Bottom) Lymph node. Particle with distinct 'coating layer'. x 75,000.

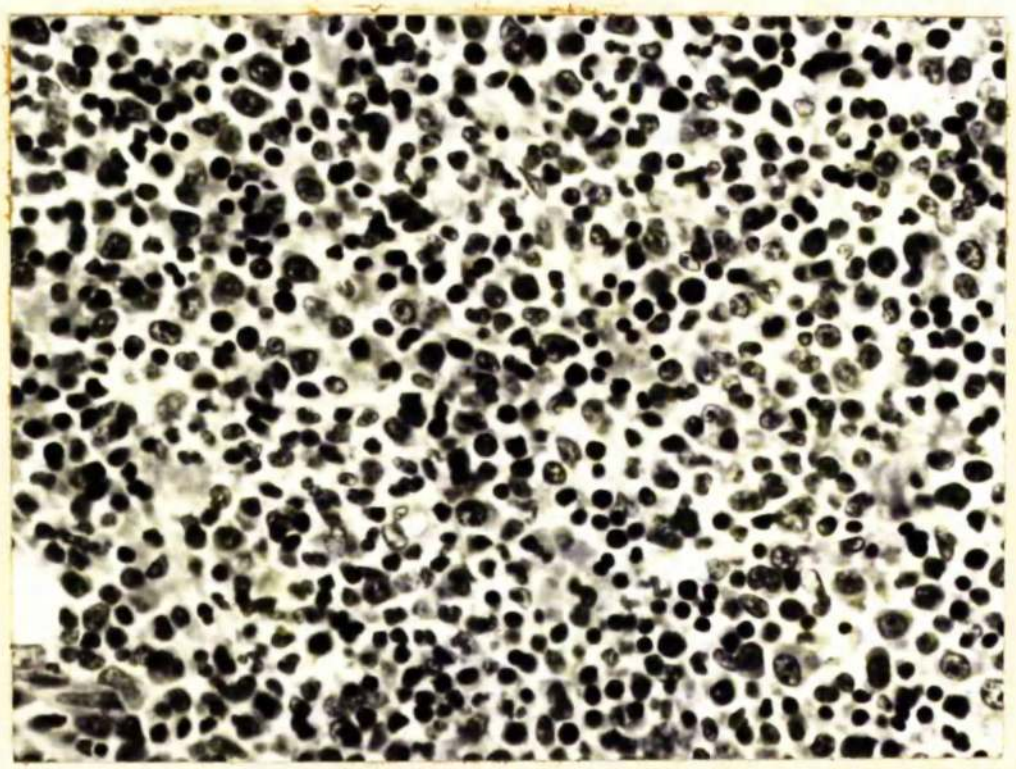


Fig. 55 Inoculated kitten CL5/4. Inoculum CL5/CFS. Day 56. Bone marrow section showing a predominance of blast cells. H & E x 420.

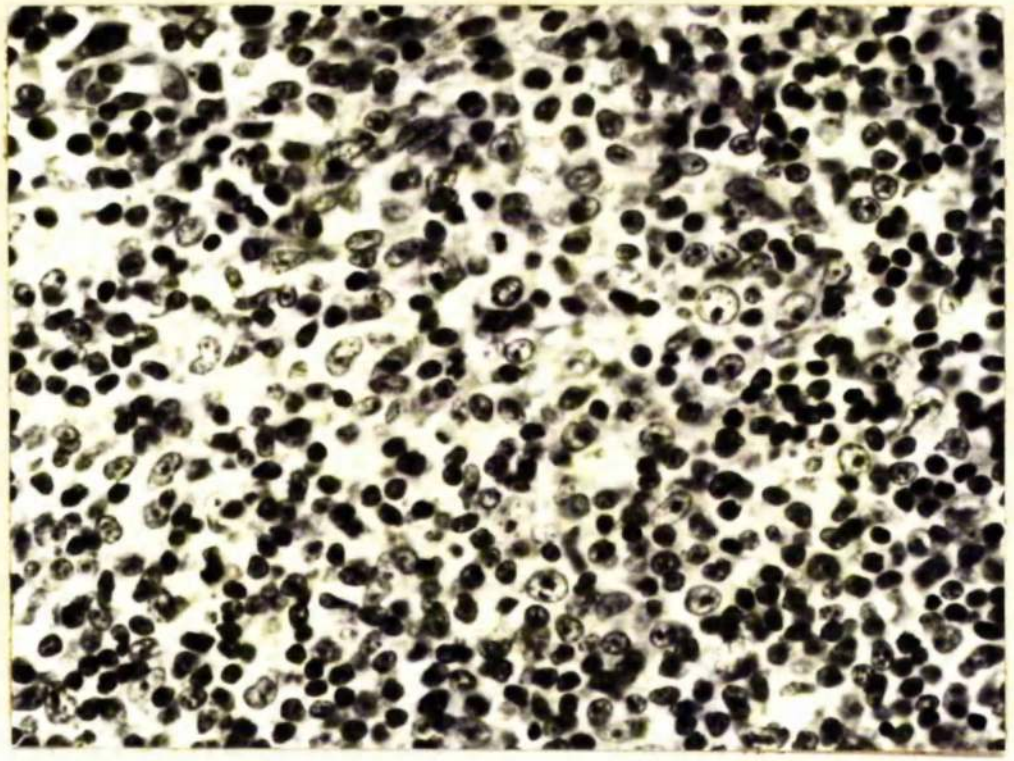


Fig. 56 Inoculated kitten, CL5/7. Inoculum CL5/CFS. Day 85. Lymph node section. The tumour cell population ranges from medium lymphocytes to bizarre stem cells. H & E x 420.

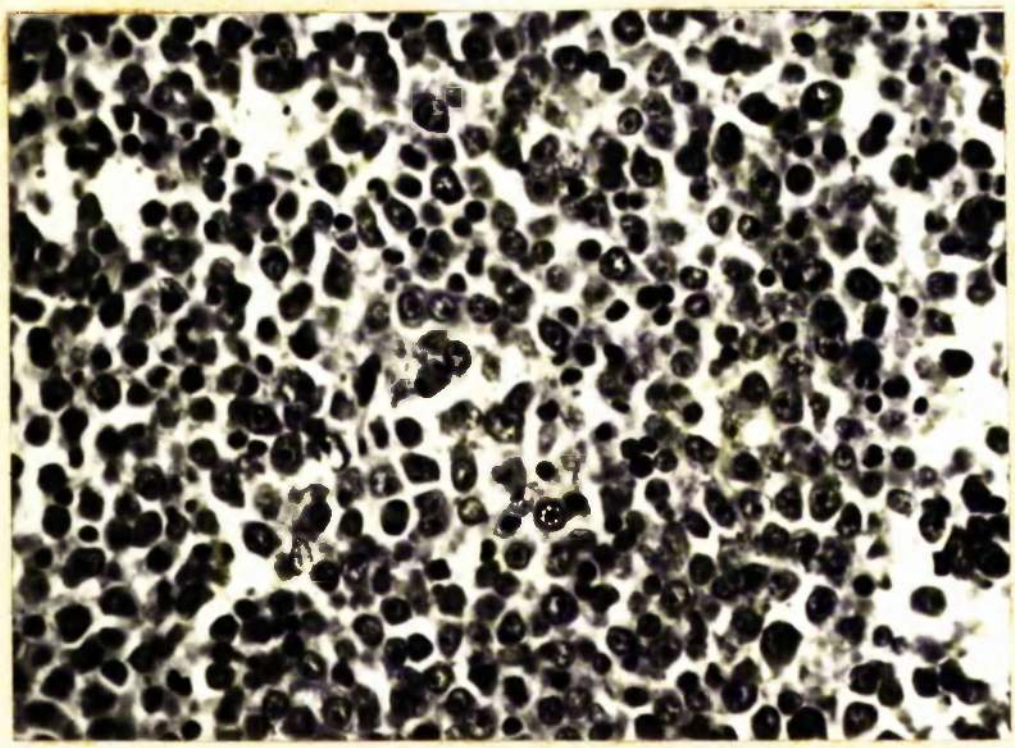


Fig. 57 Inoculated kitten CL5/7. Inoculum CL5/CFS. Day 85.
Bone marrow section showing massive infiltration of the marrow by lymphoblasts. H & E x 420.

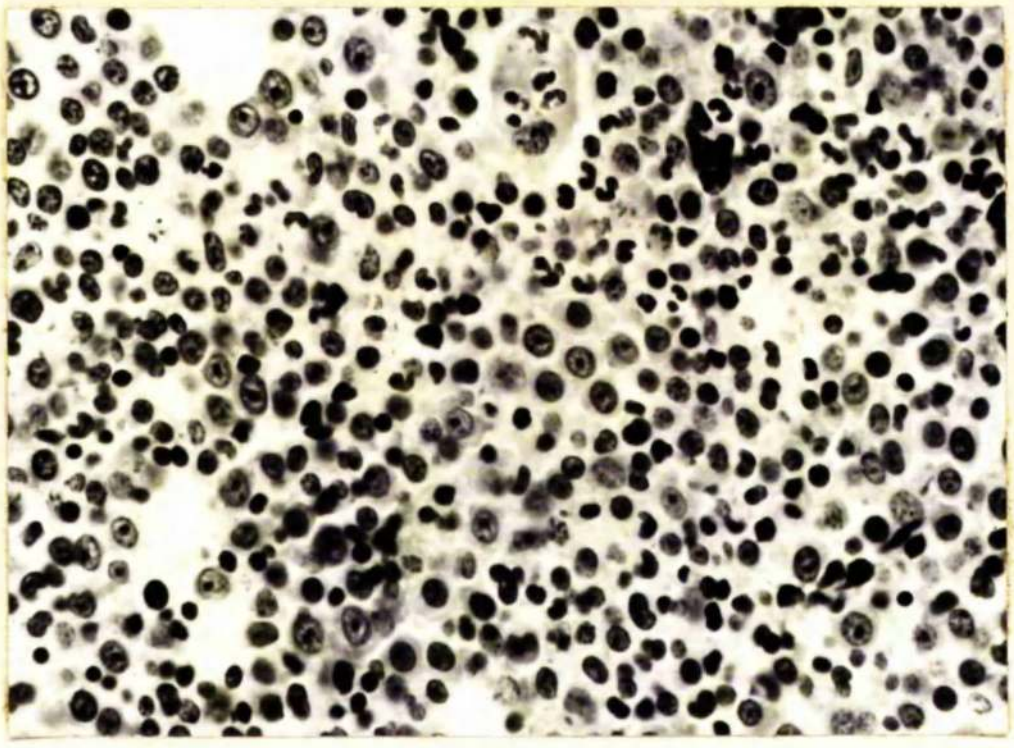


Fig. 58 Inoculated kitten CL5/10. Inoculum CL5/CFS.
At 15 weeks. Bone marrow section showing large numbers of immature cells, groups of which are focally arranged. H & E x 420.

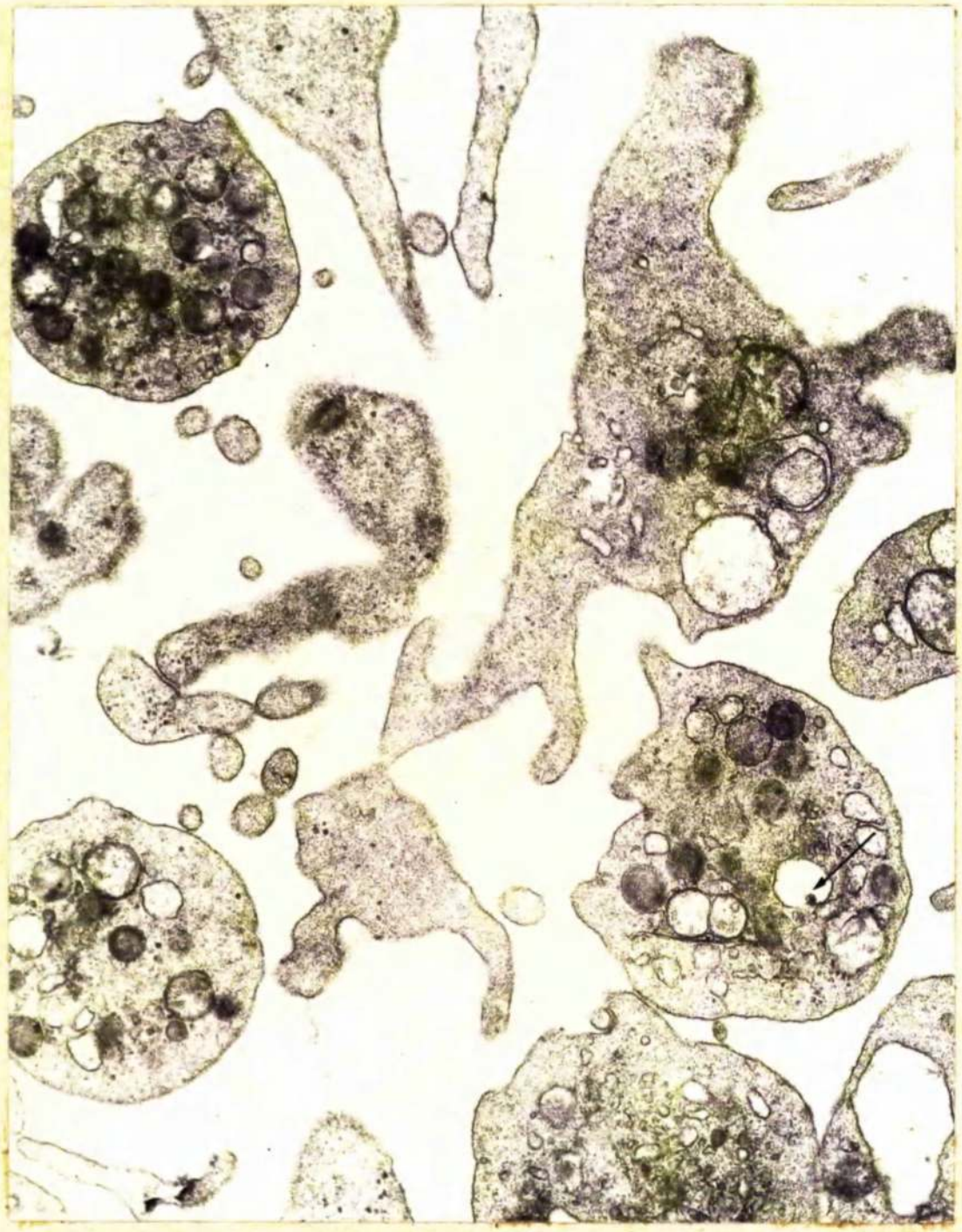


Fig. 59 Inoculated kittens, 28 days after inoculation with CL5/CFS. Pooled blood platelet pellet. One particle is seen in a vacuole in the platelet at the lower right (⟶). x 25,000.

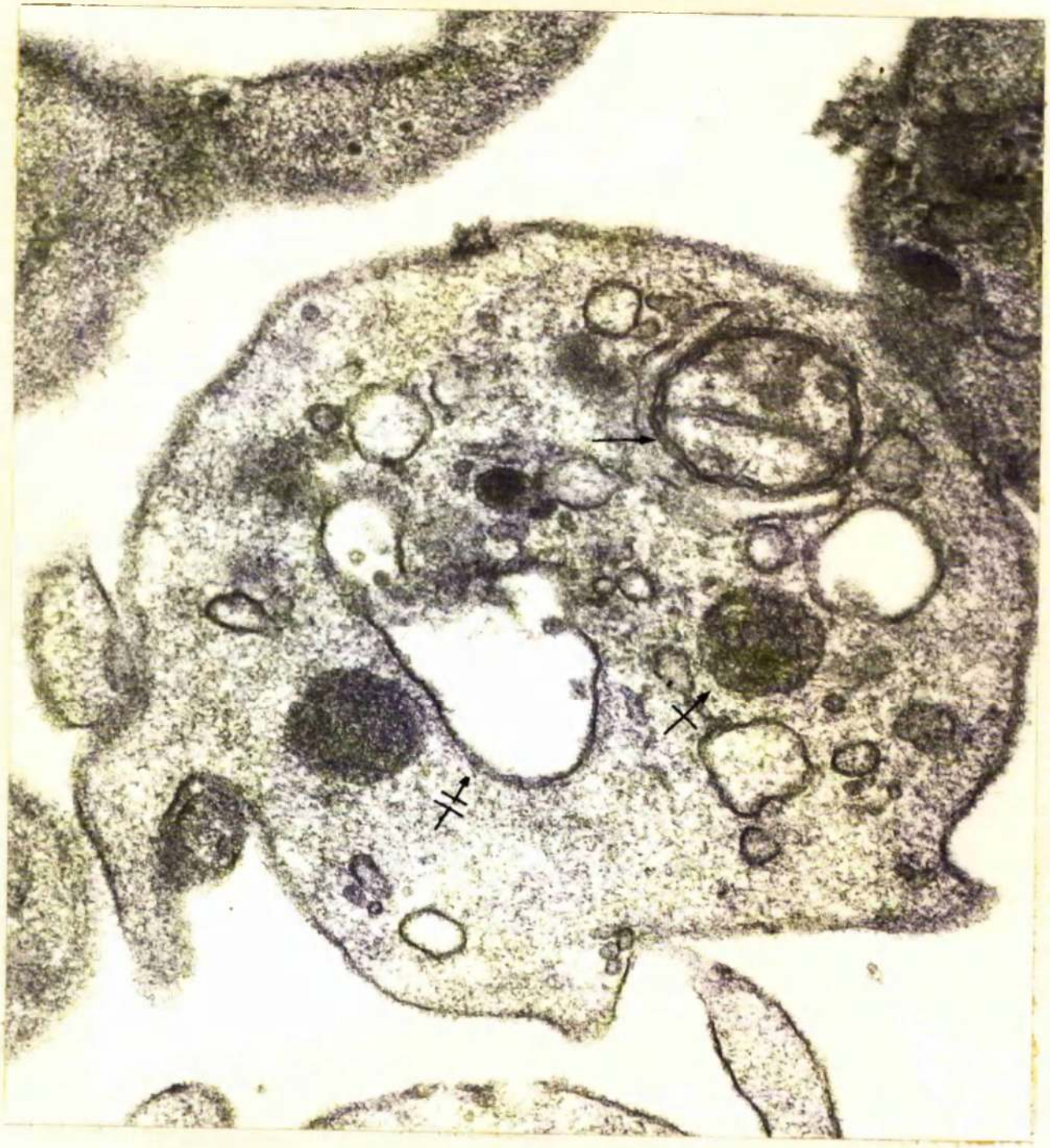


Fig. 60 Normal kitten platelet, showing mitochondria (⟶), granules (⟶+) and vacuoles (⟶‖). x 75,000.

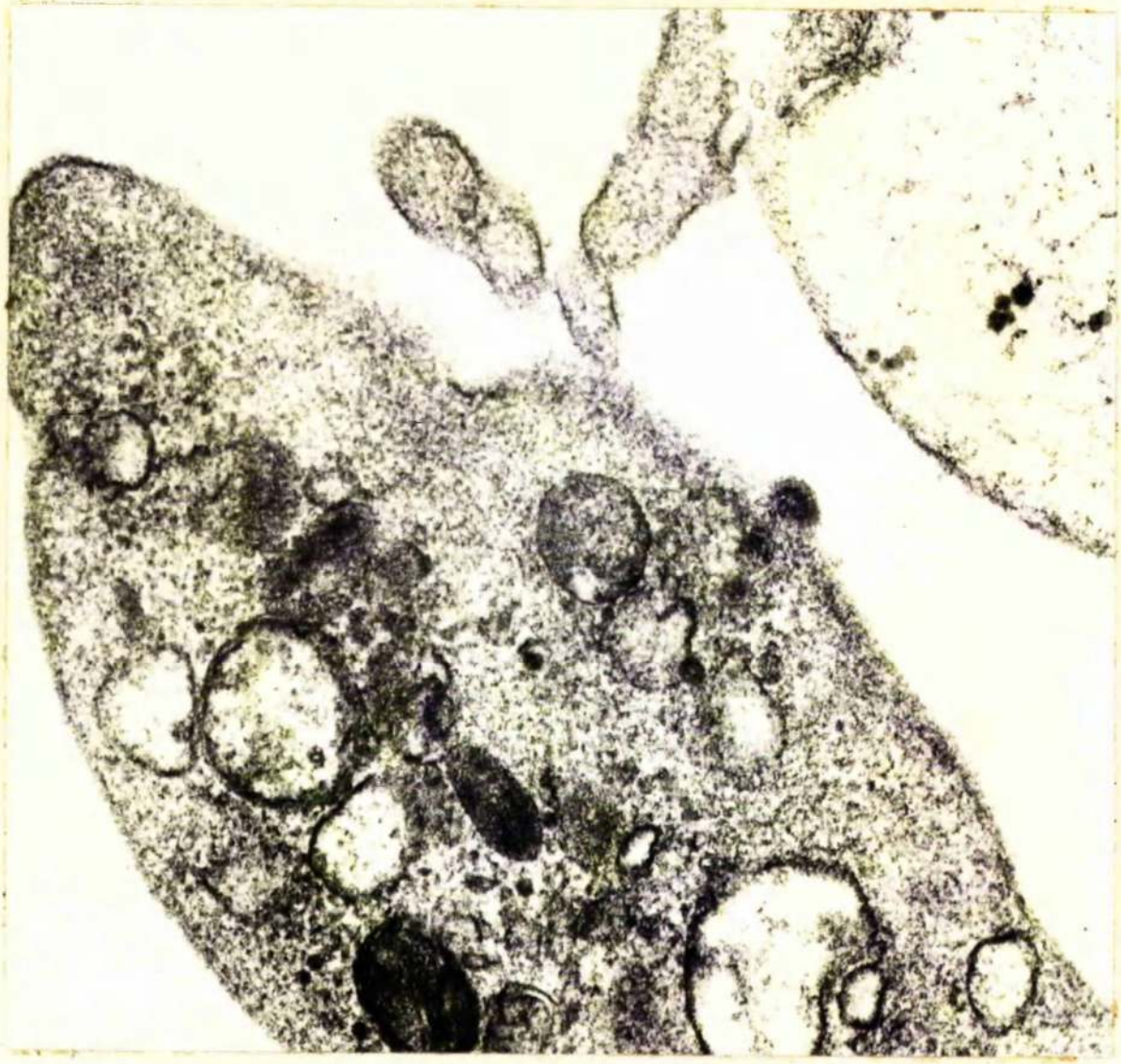


Fig. 61 Pooled blood from kittens 28 days after infection with CL5/CFS. Particle in close association with platelet surface. x 75,000.

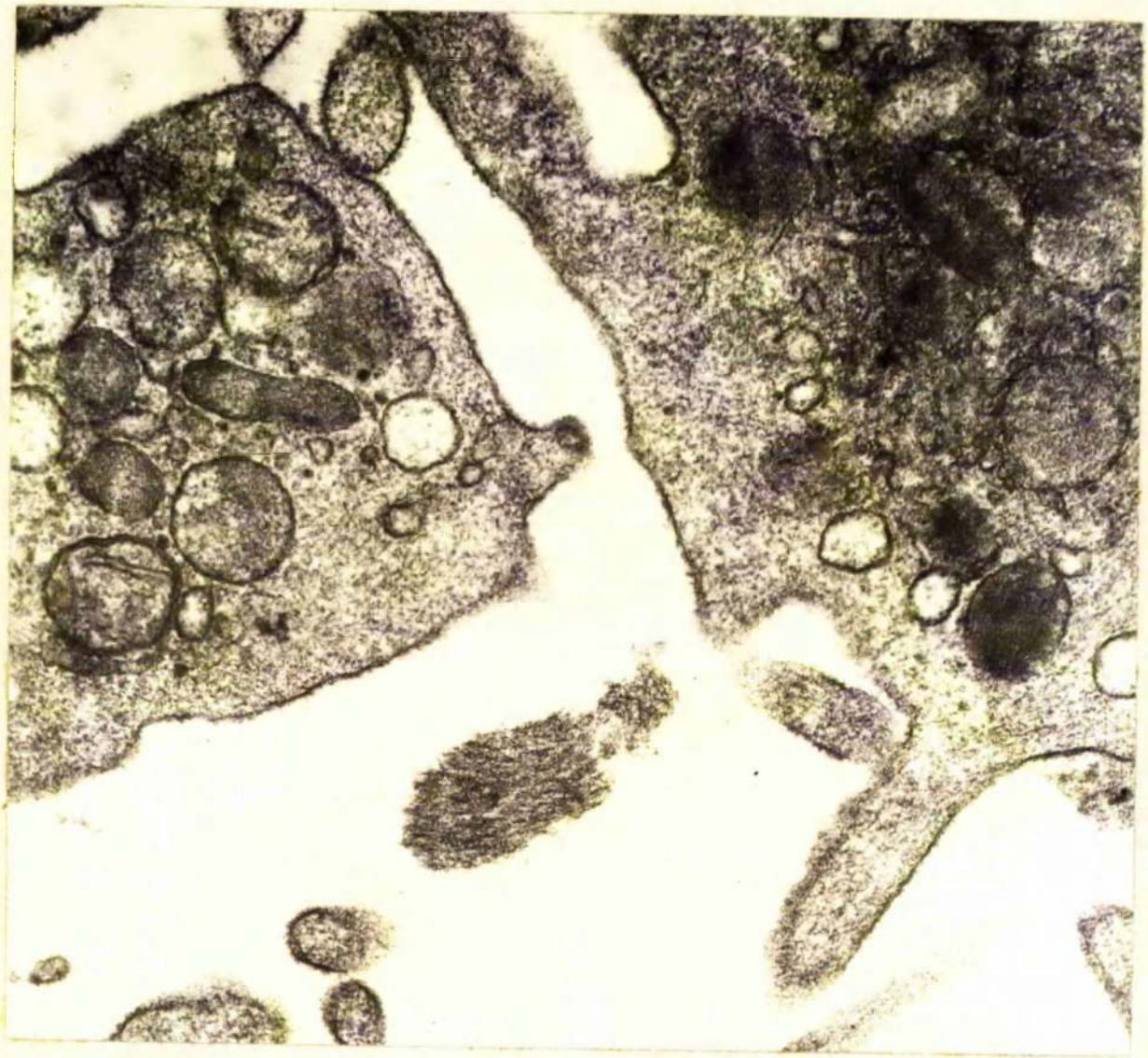


Fig. 62 Pooled blood from kittens 28 days after infection with CL5/CFS. Early stage of particle budding showing continuity between the platelet membrane and outer particle membrane. x 60,000.

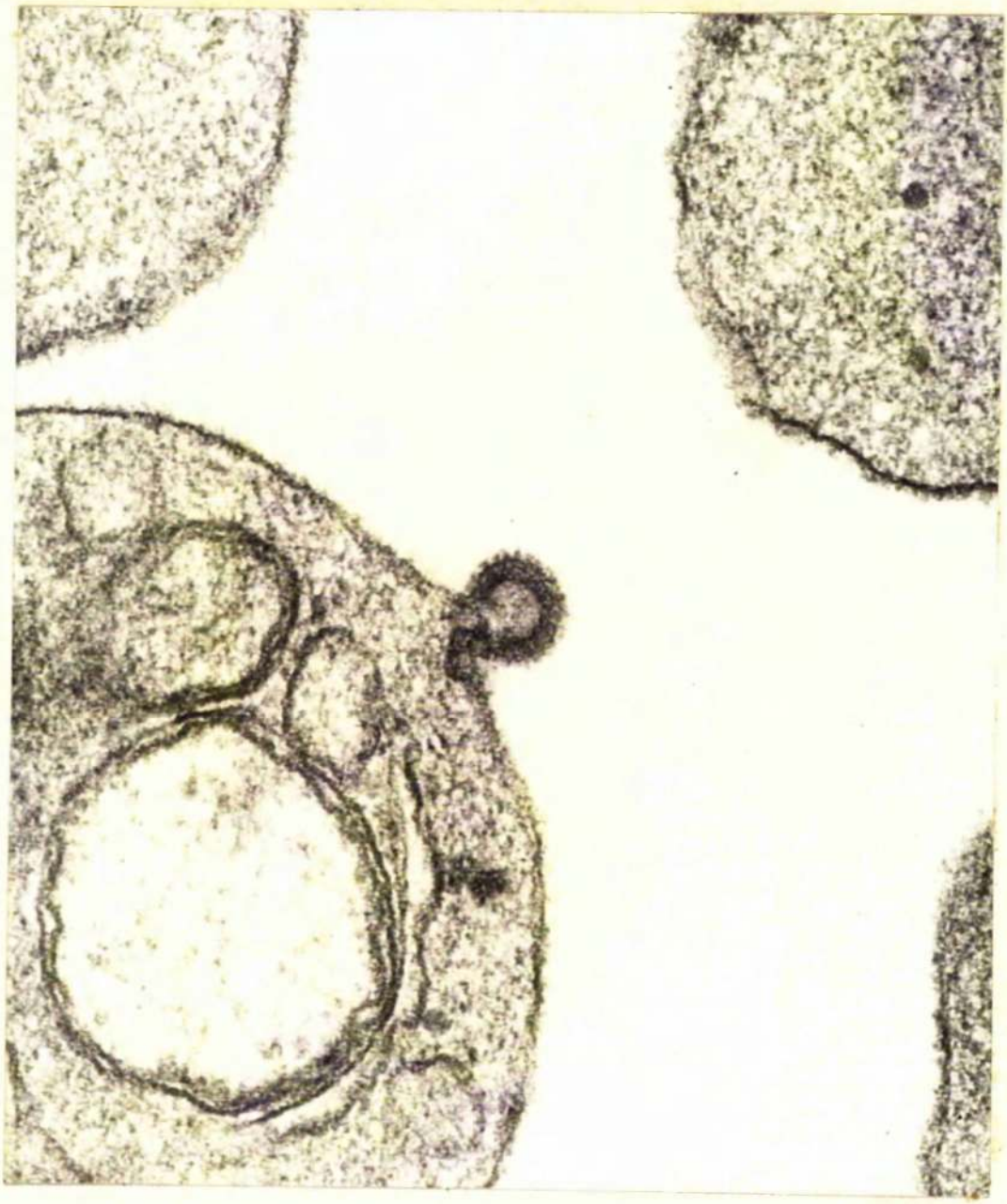


Fig. 63 Detail of budding particle. The triple-membraned structure and 'coating layer' are well defined. No surface structure is seen on the outer platelet membrane. x 120,000.

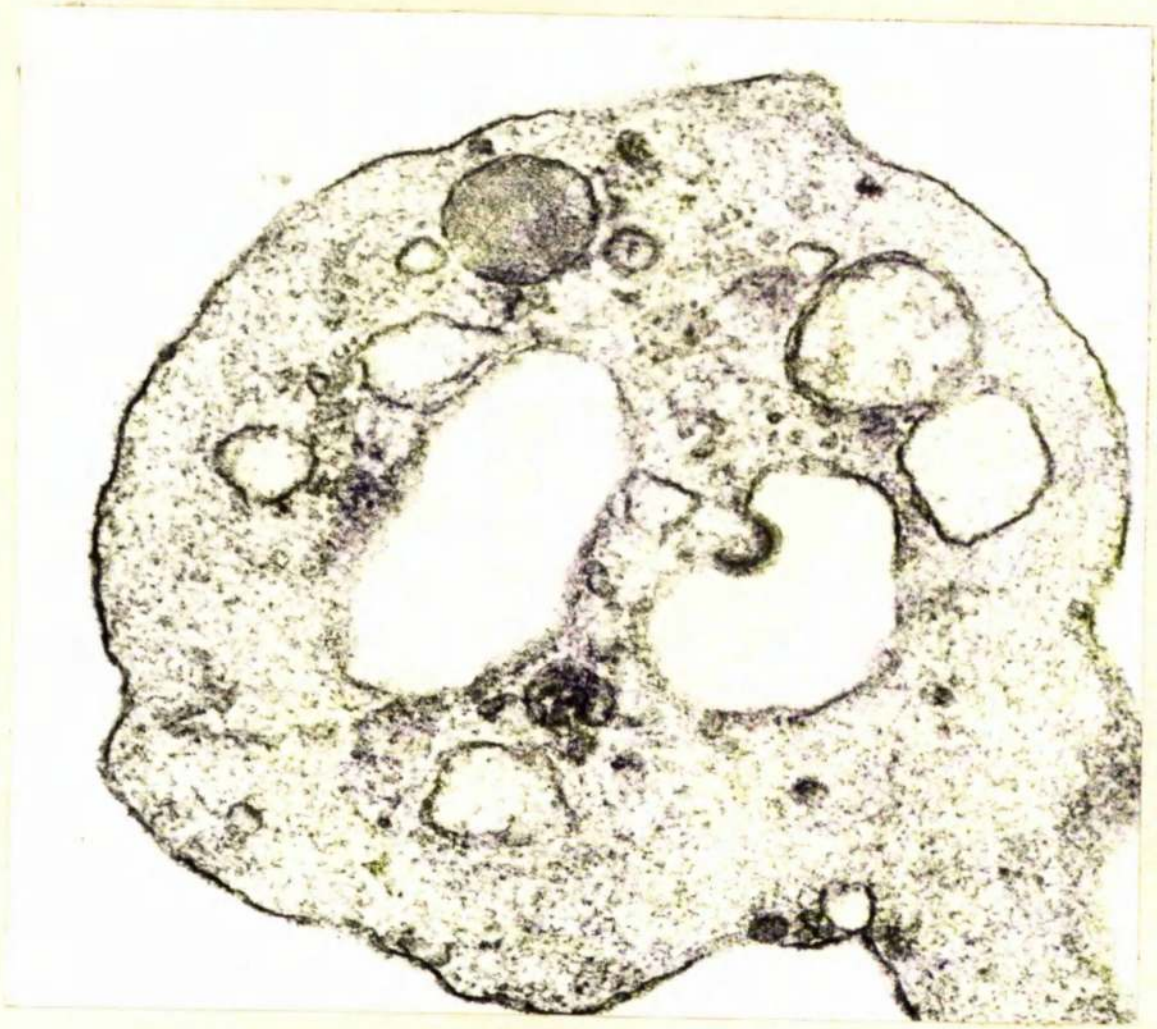


Fig. 64 Pooled blood from kittens, 28 days after infection with CL5/CFS. Particle budding into platelet vacuole. The inner electron-dense membrane is well developed. x 75,000.

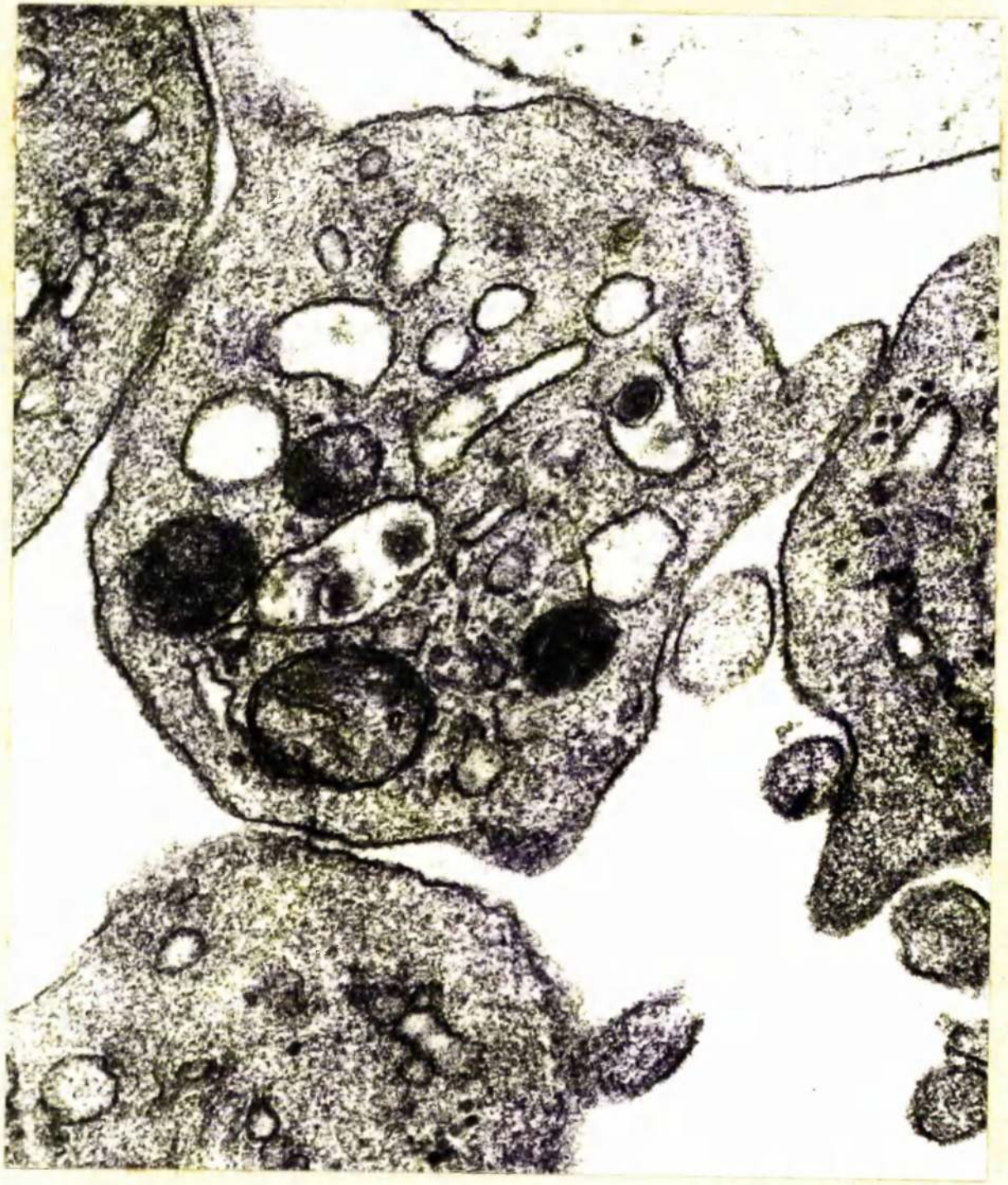


Fig. 65 Platelets from pooled blood from kittens 28 days after infection with CL5/CFS. In one vacuole a complete Type 2 particle is seen; in another vacuole parts of 2 particles are shown. x 75,000.

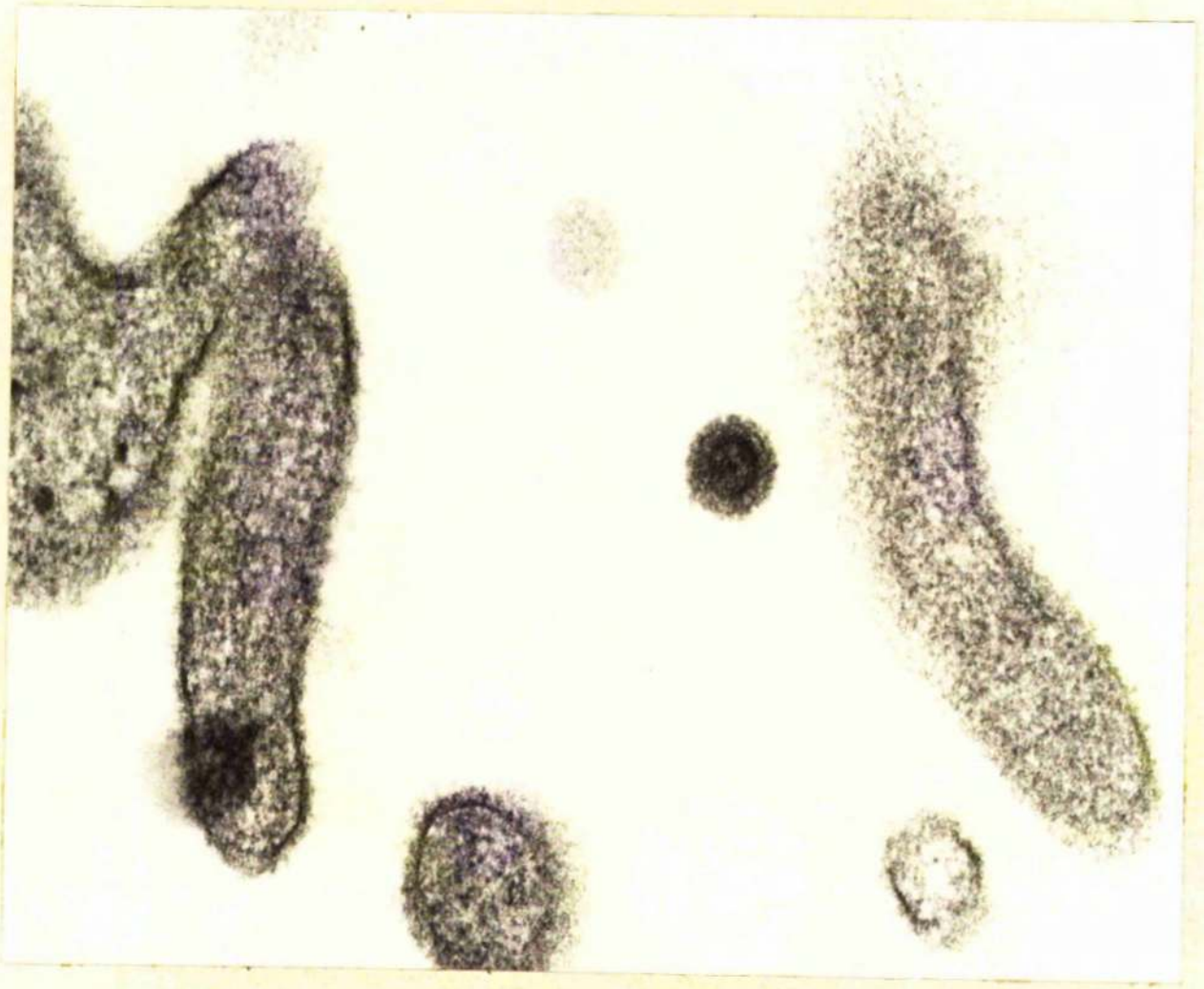
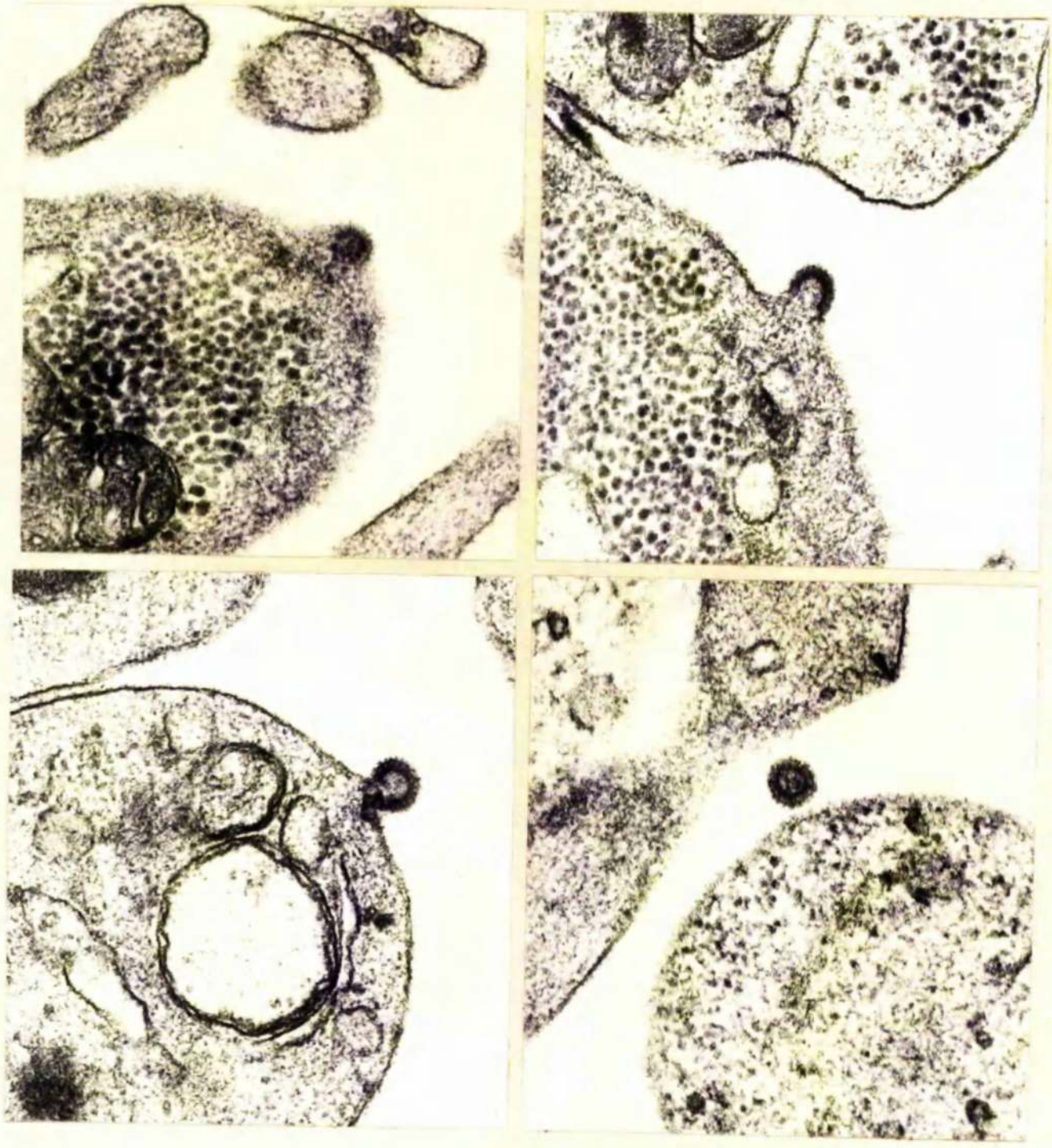


Fig. 66 Complete particle in pellet of pooled blood from kittens 28 days after infection with CL5/CFS. The membrane structure and 'coating layer' are clearly defined. x 120,000.



Blood platelets from pooled blood from kittens 28 days after infection with CL5/CFS

Fig. 67 (Top left) Tangential section showing particle in close association with platelet. x 75,000

Fig. 68 (Top right) Partially formed particle with distinct inner membrane and developing intermediate membrane. x 75,000

Fig. 69 (Lower left) Almost complete particle with "coating layer". x 75,000

Fig. 70 (Lower right) Complete particle in interplatelet space. x 75,000.

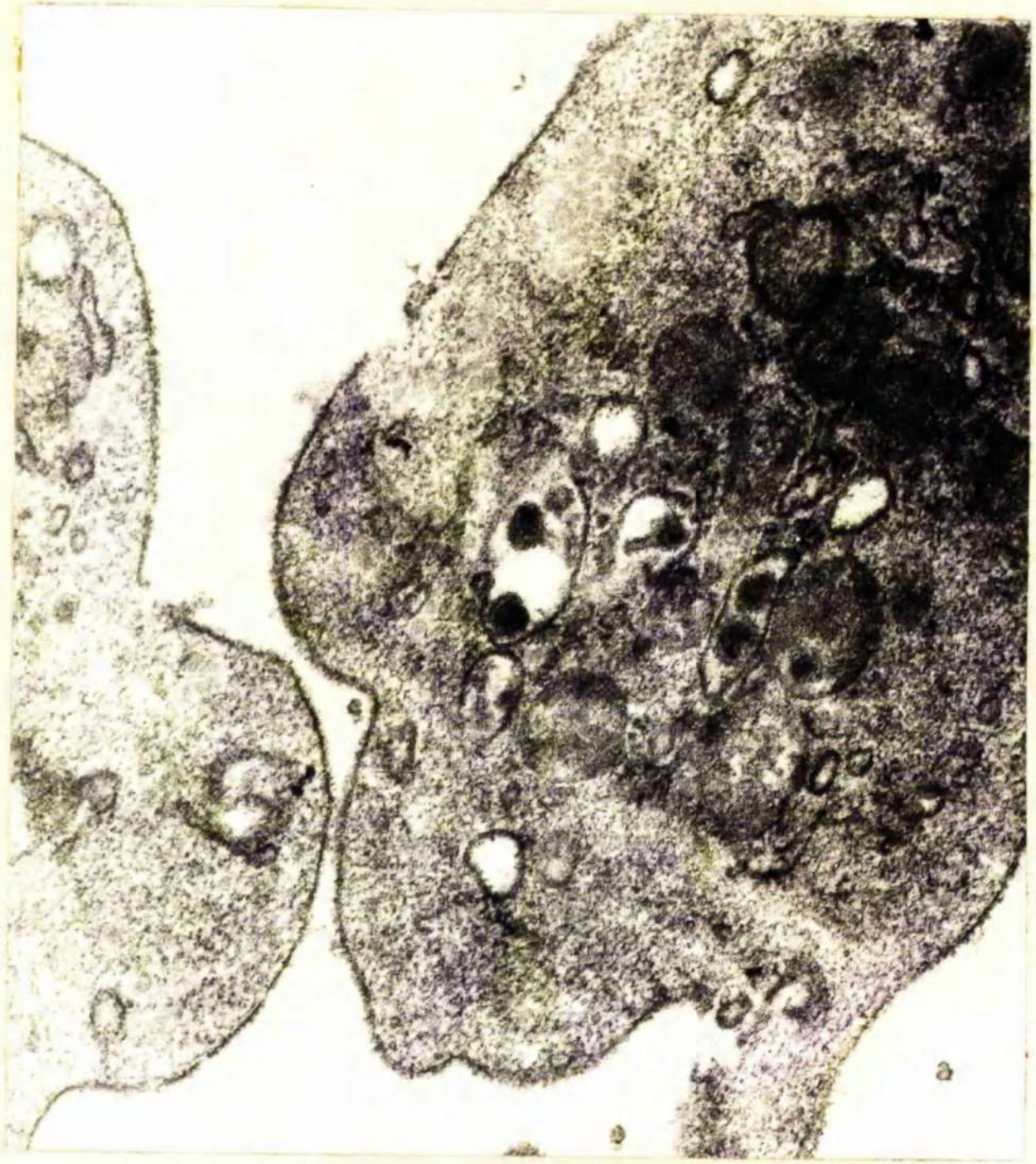
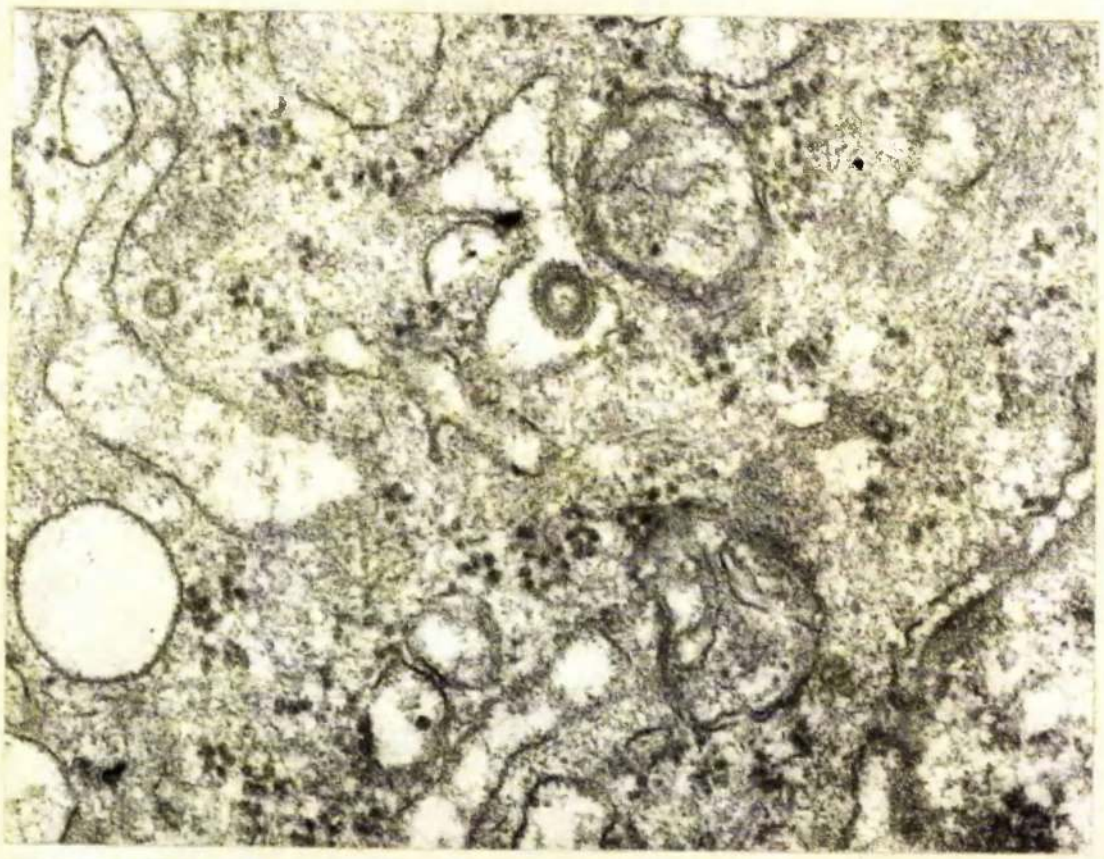
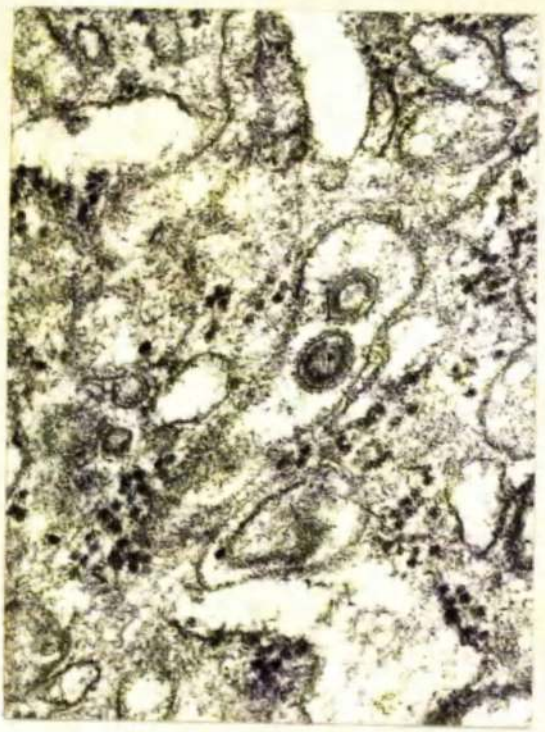
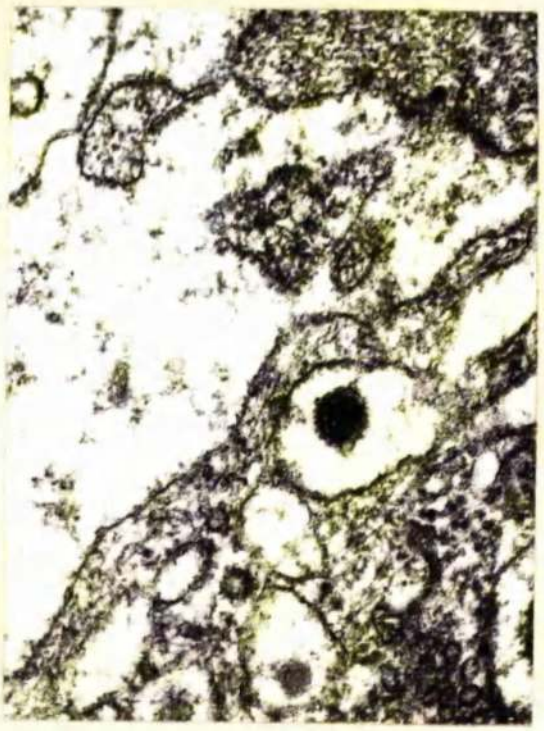


Fig. 71 Inoculated kitten. Blood platelets. 42 days after infection with CL5/CFS. 3 vacuoles containing Type 2 particles. One particle in the centre vacuole and one in the vacuole to the right have 'tails'. x 75,000.







Inoculated kitten 28 days after infection with CL5/CFS. Spleen megakaryocyte.

Fig. 72 (Top) Type 1 particle, with 'coating layer', in vacuole. x 75,000.

Fig. 73 (Lower left) In a vacuole, 2 Type 1 particles with 'coating layer'. x 75,000.

Fig. 74 (Lower right) In a vacuole, a Type 2 particle with a 'coating layer'. x 75,000.

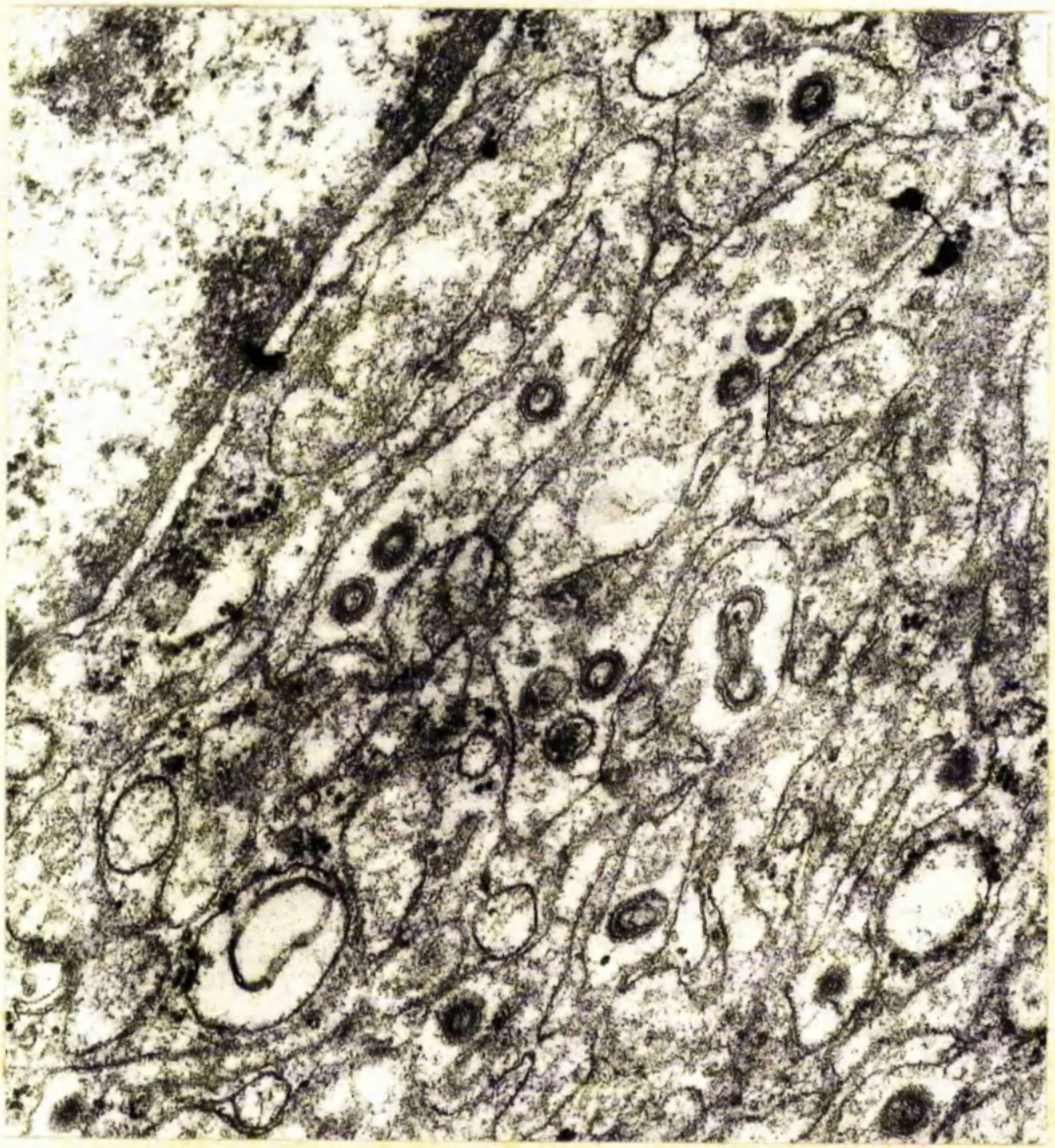


Fig. 75 Inoculated kitten 28 days after infection with CL5/CFS. Spleen megakaryocyte. Many particles, mostly Type 1, in intracytoplasmic vacuoles and channels. One Type 2 particle is seen in the central vacuole. (See Fig. 77 for detailed structure of particles). x 75,000.

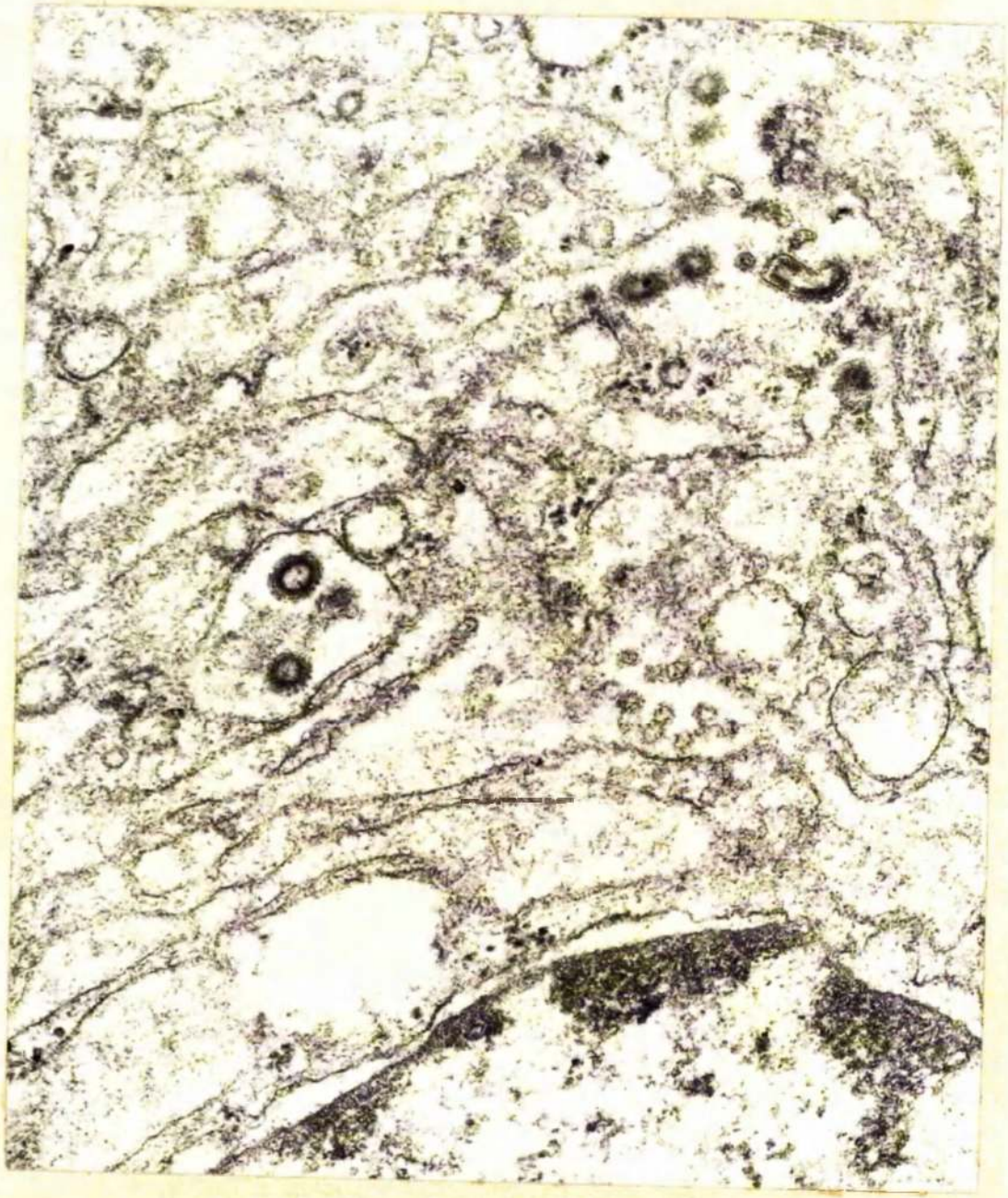


Fig. 6 Inoculated kitten 28 days after infection with CL5/CFS. Spleen megakaryocyte. To the left, 2 particles with clearly defined membrane and 'coating layer' structure; to the upper right, bizarre cylindrical particle, triple-membraned and with a 'coating layer'. x 75,000.

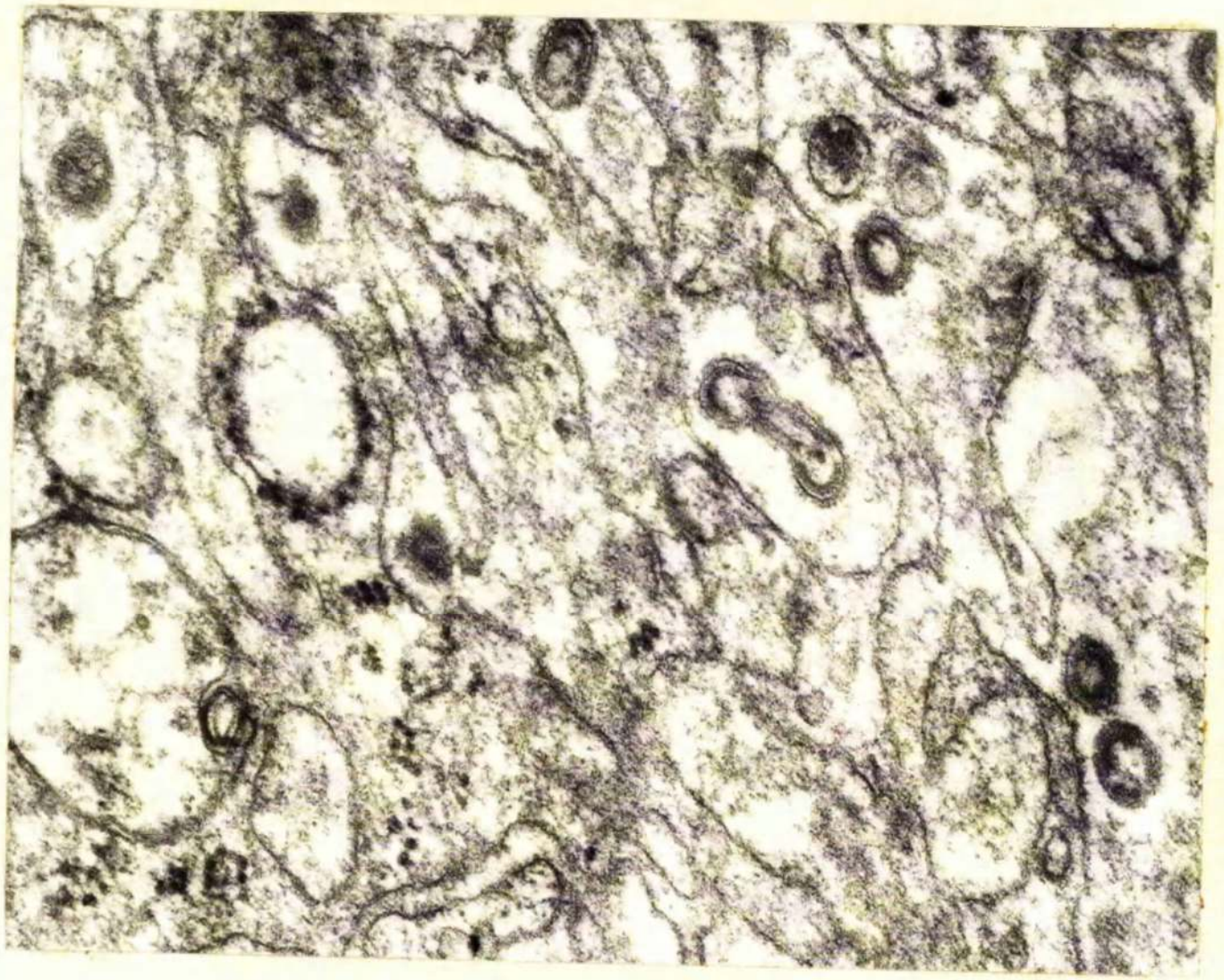


Fig. 77 Inoculated kitten 28 days after infection with CL5/CFS. Part of a spleen megakaryocyte. High magnification of area of Fig. 75. Type 1 particles, some with clearly defined 'coating layer'; a Type 2 particle (upper right) and 2 particles joined by a cylindrical section. x 100,000.

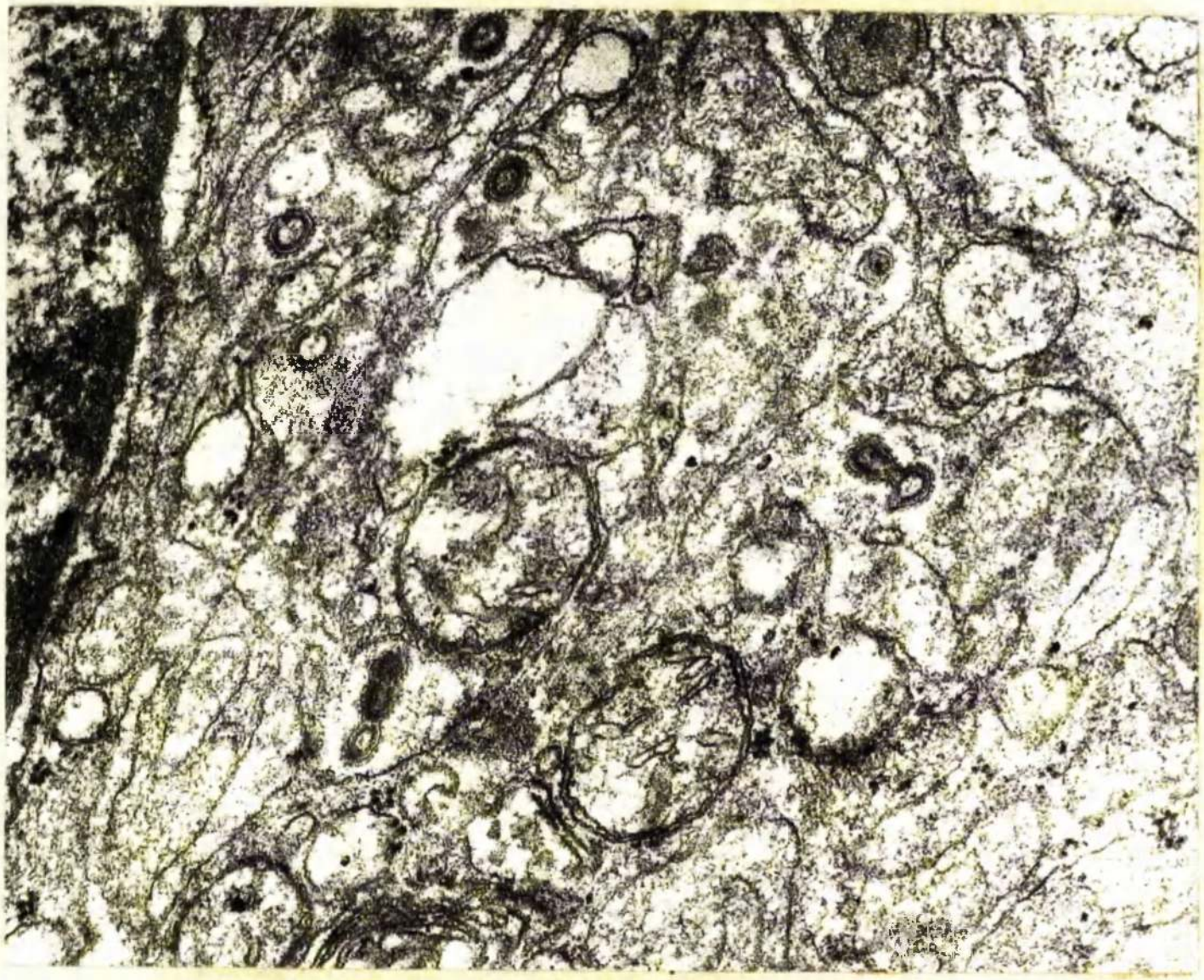


Fig. 78 Inoculated kitten, 28 days after infection with CL5/CPS. Spleen megakaryocyte containing many triple-membraned particles each with distinct 'coating layer'. At lower left, 3 particles are attached linearly and in a vacuole to the right, 2 particles are attached at right angles. x 75,000.

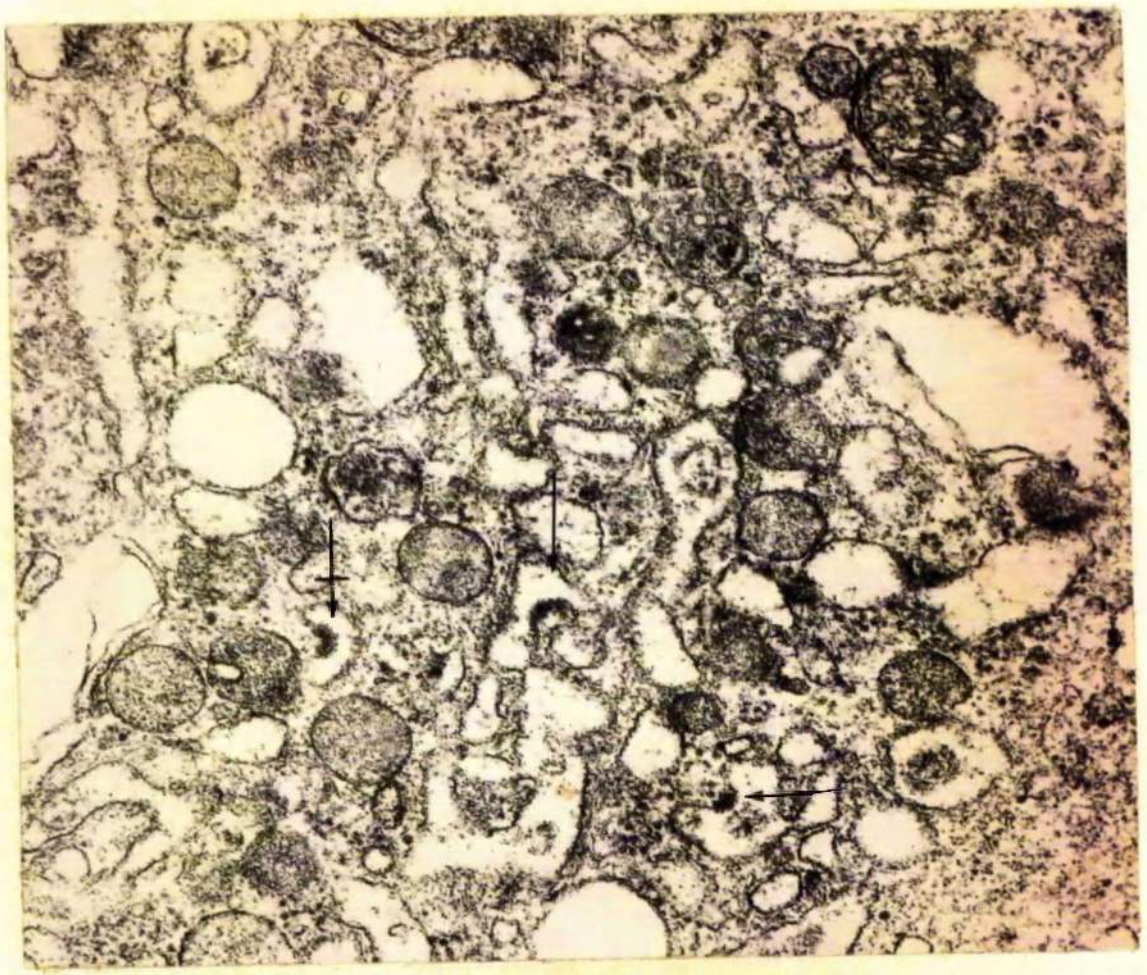


Fig. 79 Inoculated kitten 28 days after infection with CL5/CFS. Spleen megakaryocyte. Early stages of budding into vacuoles (⟶) and a budding particle with a clearly defined "coating layer" (⟷). x 75,000.

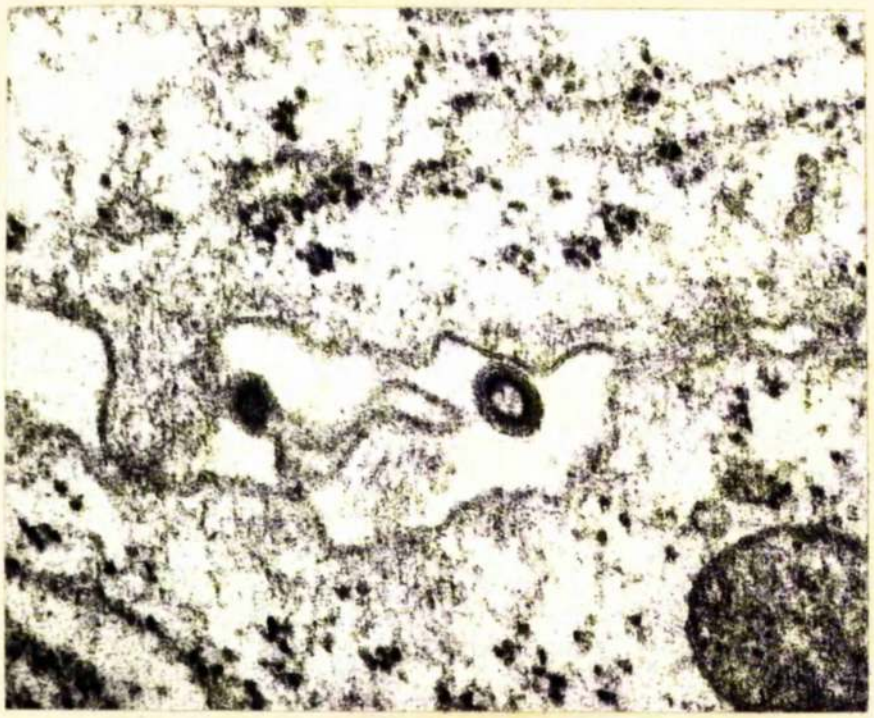
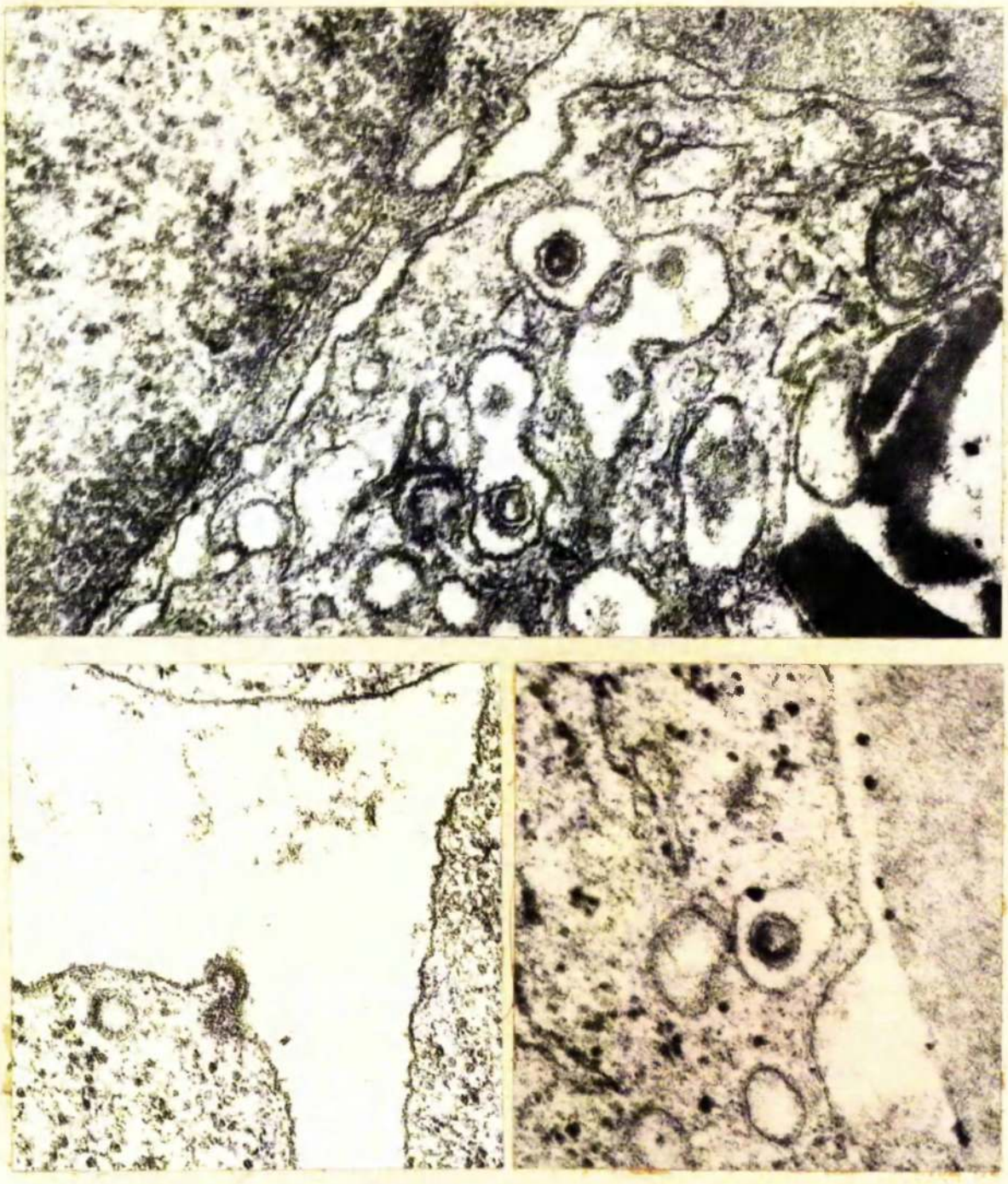


Fig. 80 Inoculated kitten, 15 weeks after infection with CL5/CFS. Spleen. In an intercellular space a complete Type 1 particle and a budding particle are seen. x 75,000.



Inoculated kitten 28 days after infection with CL5/CFS. Bone Marrow.

Fig. 81 (Top) 2 fully formed particles in vacuoles. x 75,000.

Fig. 82 (Lower left) Particle budding from a lymphoid cell. x 75,000.

Fig. 83 (Lower right) Type 2 particle in a vacuole near the surface of a lymphoid cell. x 75,000.

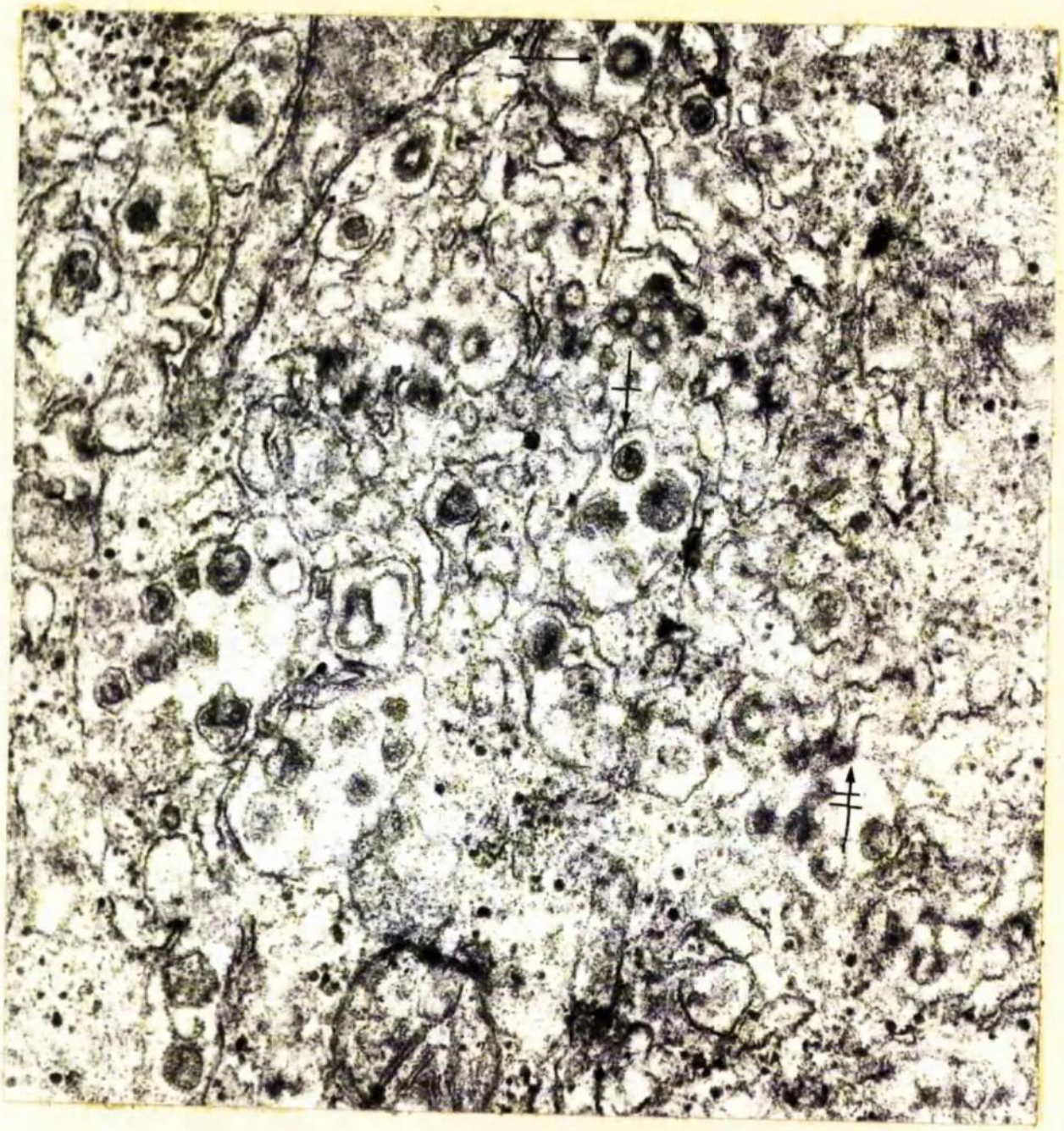


Fig. 84 Inoculated kitten 70 days after infection with CL5/CFS. Bone marrow megakaryocyte. Type 1 particles (⟶), Type 2 particles (⟶+) and budding particles (⟶‖). x 50,000.

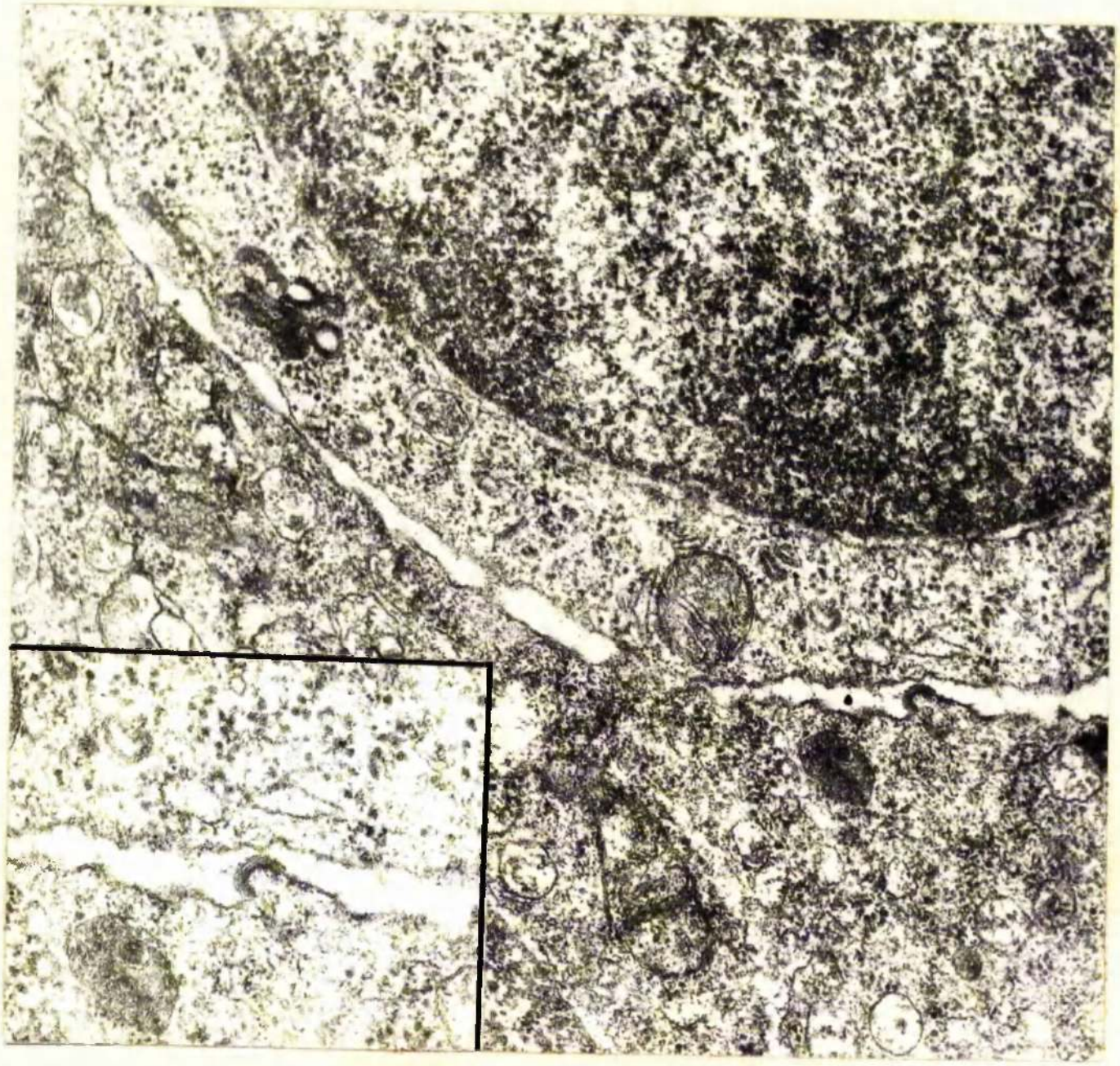
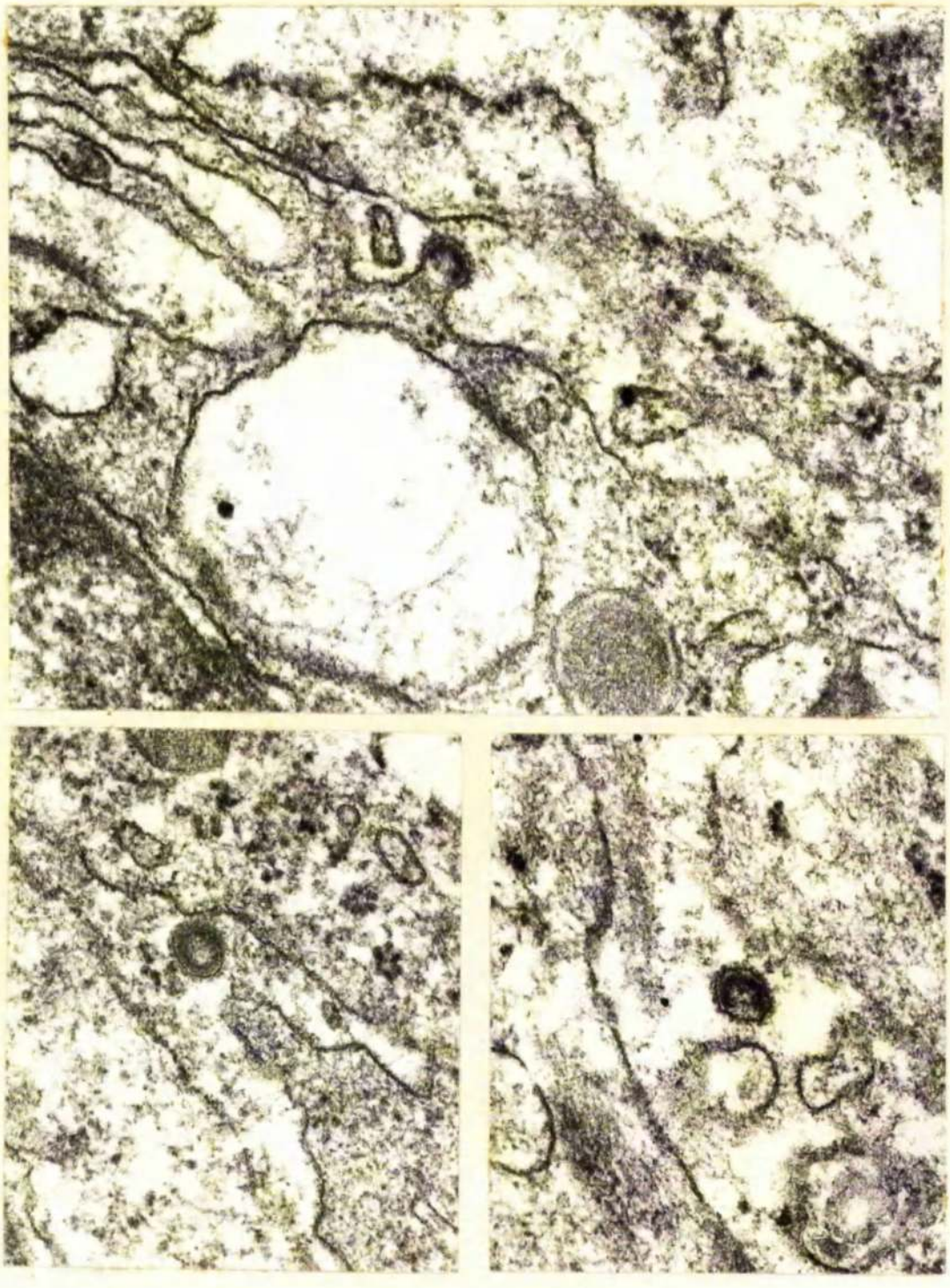


Fig. 85 Inoculated kitten, 28 days after infection with CL5/CFS. Bone marrow. Particle budding from lymphoid cell into intercellular space. x 40,000.

Inset shows detail of particle structure. x 75,000.

Fig. 86 Inoculated kitten, 28 days after infection with CL5/CFS. Thymus. 2 particles at an early stage of budding from a lymphoid cell surface. x 60,000.



Inoculated kitten, 28 days after infection with CL5/CFS. Thymus.

Fig. 87 (Top) Particle budding from epithelial cell surface. x 75,000.

Fig. 88 (Lower left) Complete Type 1 particle in intercellular space. "Coating layer" is seen on the particle surface. x 75,000.

Fig. 89 (Lower right) Particle with complete outer membrane with "coating layer". The inner and intermediate membranes are not continuous. x 75,000.

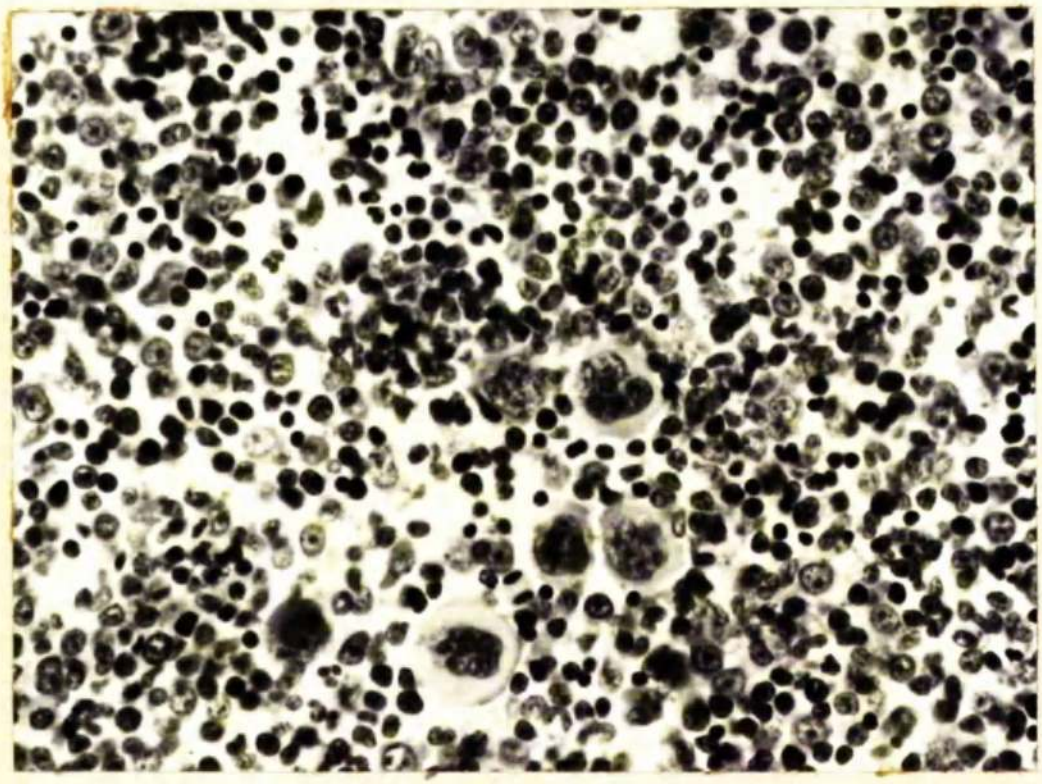


Fig. 90 Inoculated kitten CL4/3. Inoculum CL4/CFS. Day 56. Bone marrow. Numerous large immature cells are distributed throughout the section. A group of megakaryocytes is present in the lower central area. H & E x 420.

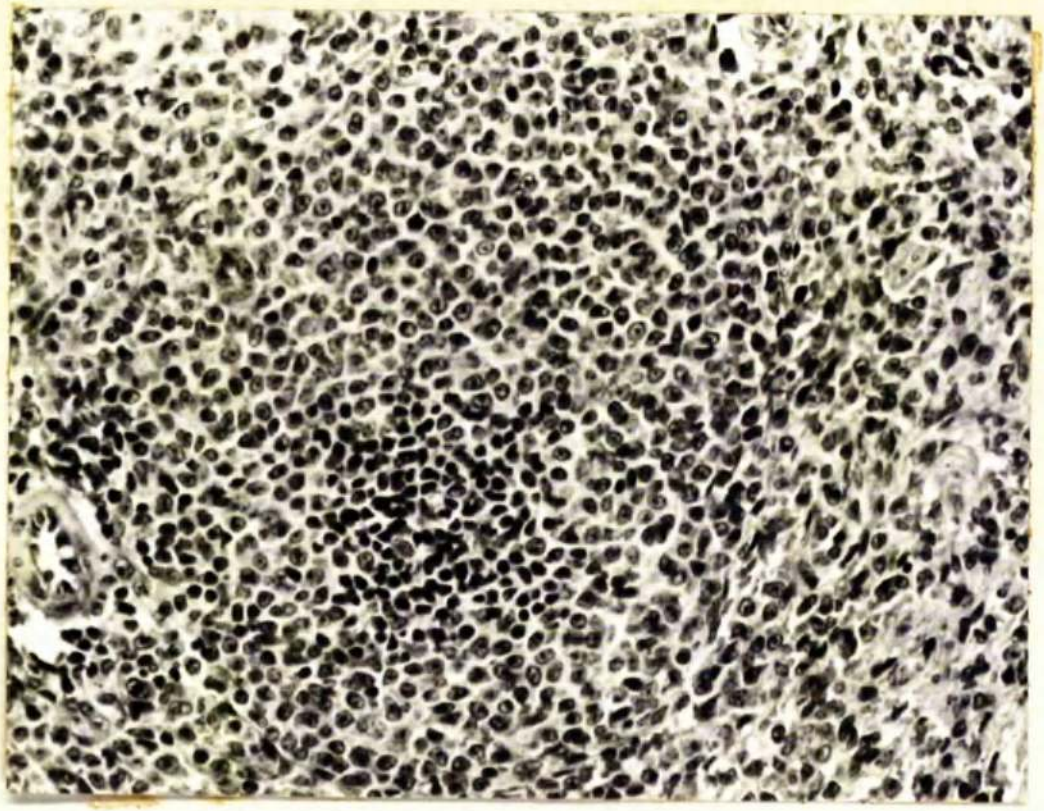
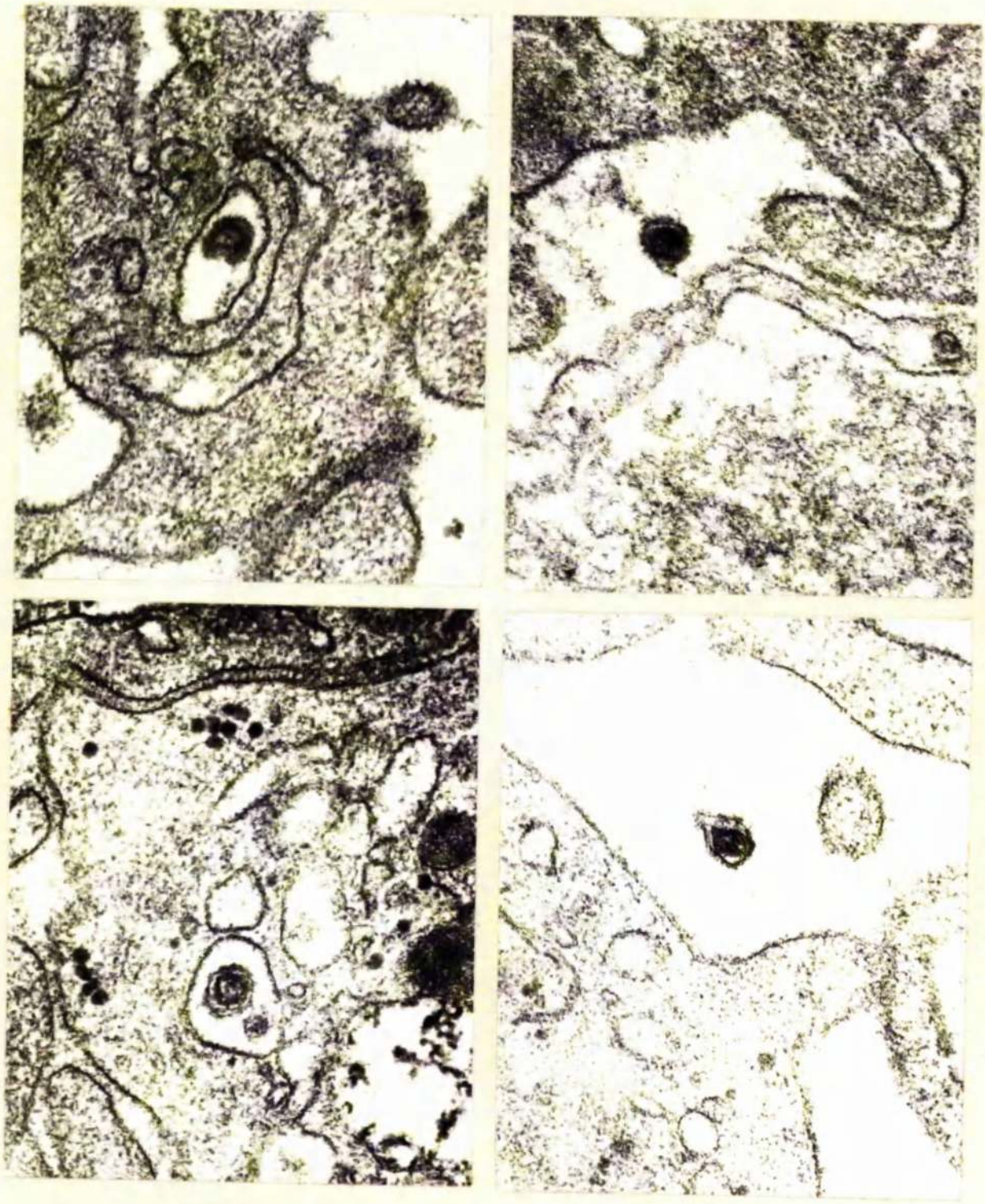


Fig. 91 Inoculated kitten, CL4/7. Inoculum CL4/CFS. At 13 weeks. Spleen. A markedly enlarged Malpighian corpuscle occupies most of the field. There is a central area of mature lymphocytes surrounded by lymphoblasts. The red pulp is shown at the right. H & E x 260.



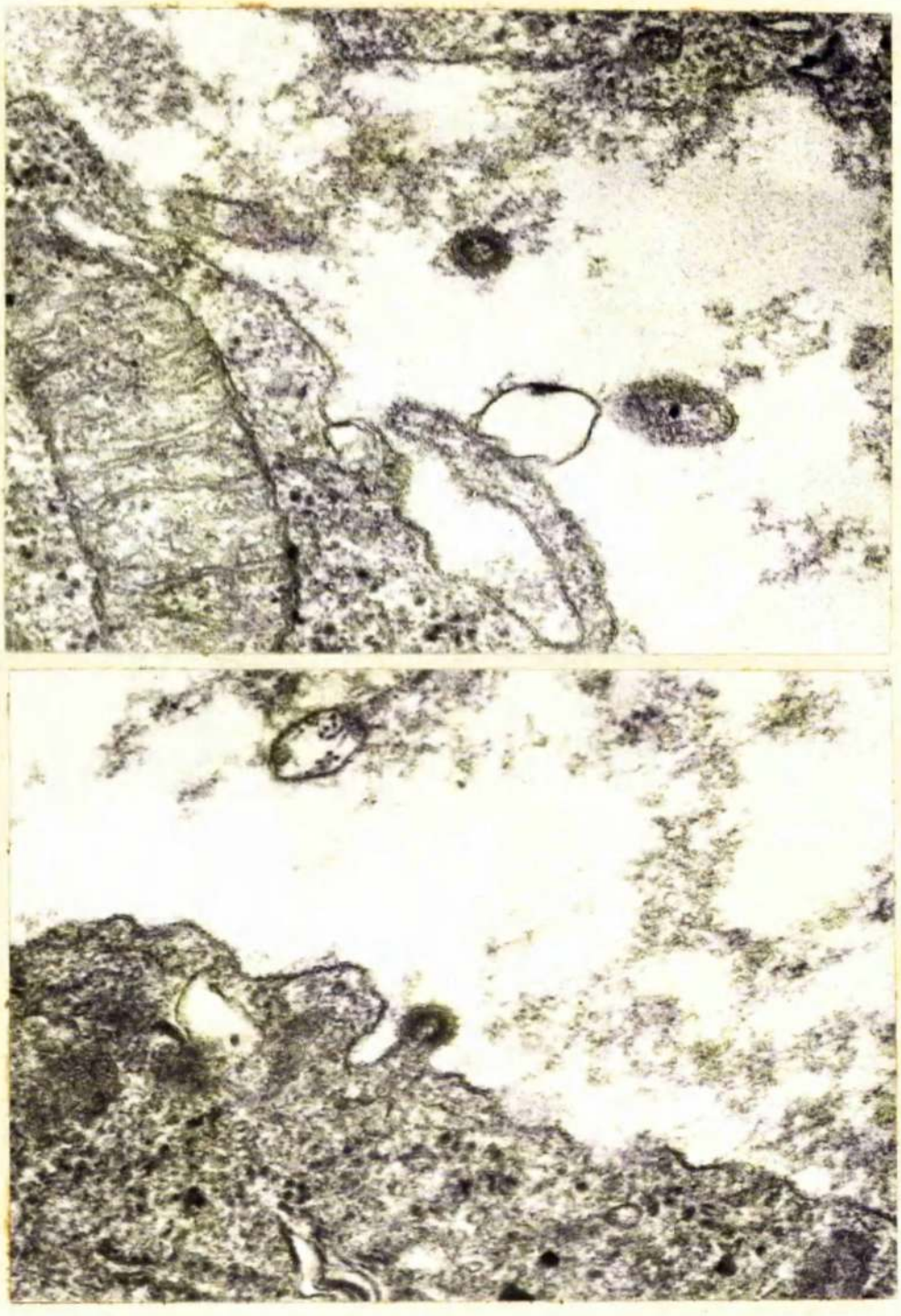
Blood platelets from kittens inoculated with CL4/CFS.

Fig. 92 (Top left) Type 1 particle in platelet, 42 days after infection. x 75,000

Fig. 93 (Lower left) Type 2 particle in a vacuole, 42 days after infection. x 75,000.

Fig. 94 (Top right) Type 2 particles in interplatelet space 56 days after infection. x 75,000.

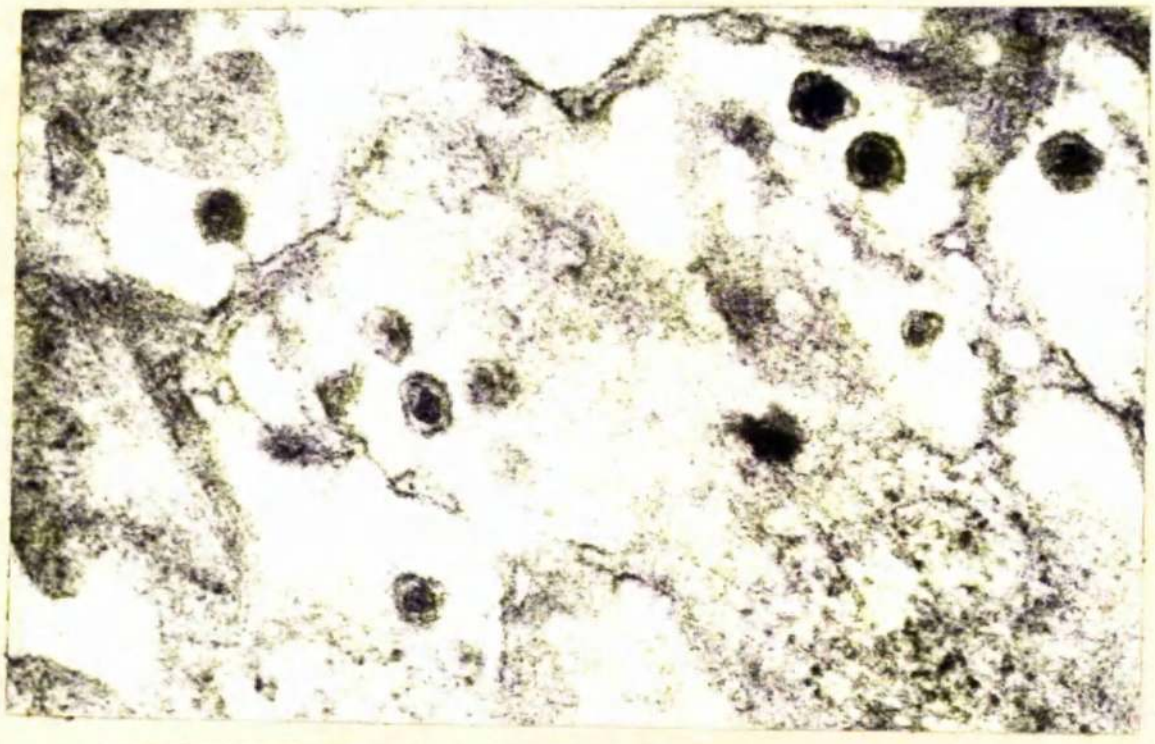
Fig. 95 (Lower right) 9 months after infection. Type 2 particle. The outer membrane is irregular, forming a slight tail.

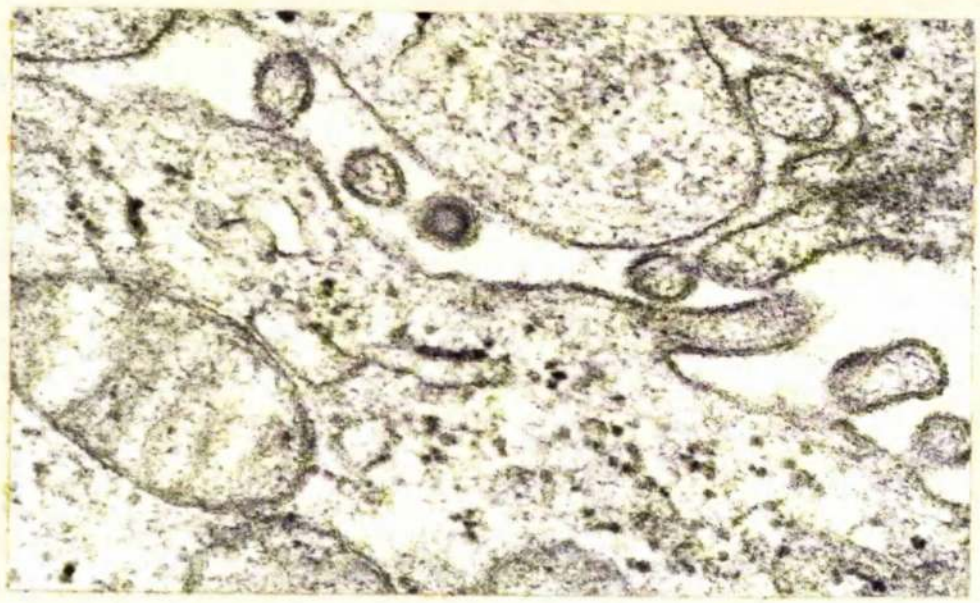


Inoculated kitten, 42 days after infection with CL4/CFS. Bone marrow.

Fig. 96 (Top) Complete triple-membraned particle, with"coating layer", in extracellular space. x 75,000.

Fig. 97 (Bottom) Particle budding from the surface of a lymphoid cell. x 75,000.

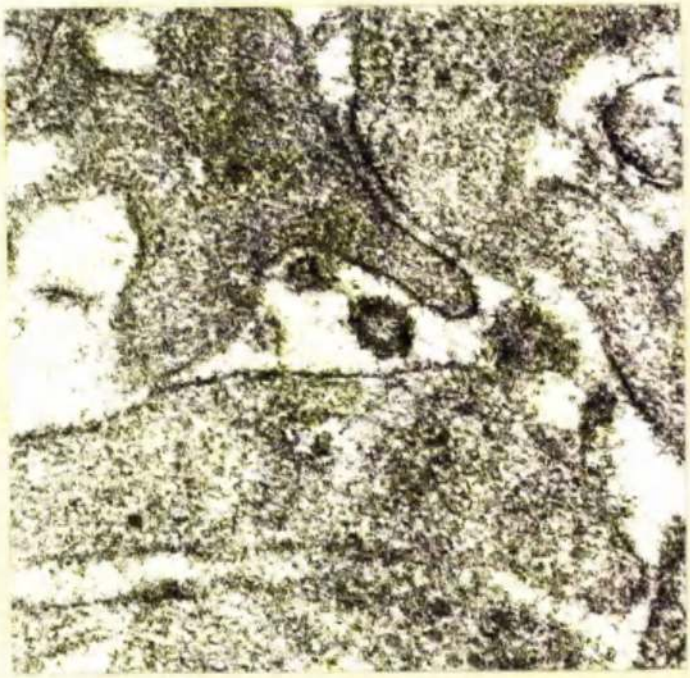


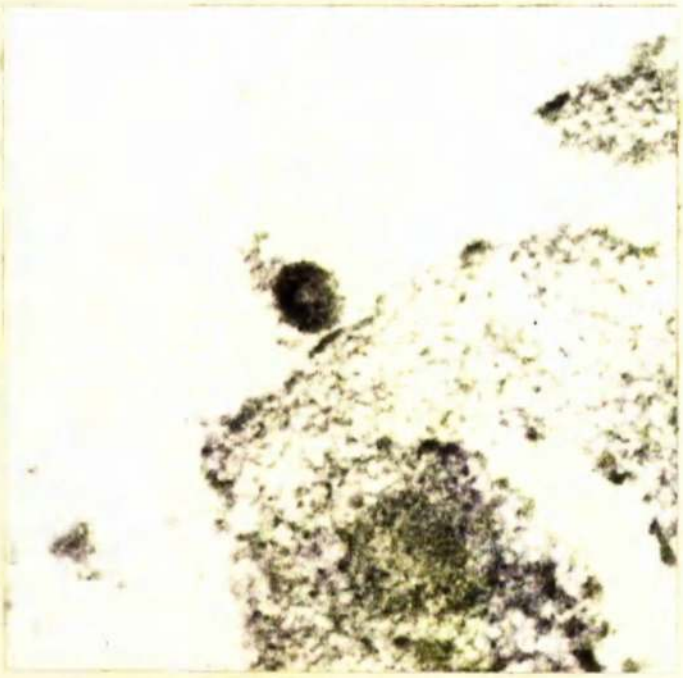


Inoculated kitten 13 weeks after infection with CL4/CFS. Bone Marrow.

Fig. 98 (Top) Type 2 particles in the vacuoles of a megakaryocyte. x 75,000.

Fig. 99 (Bottom) Type 1 particle in an intercellular space. x 75,000.





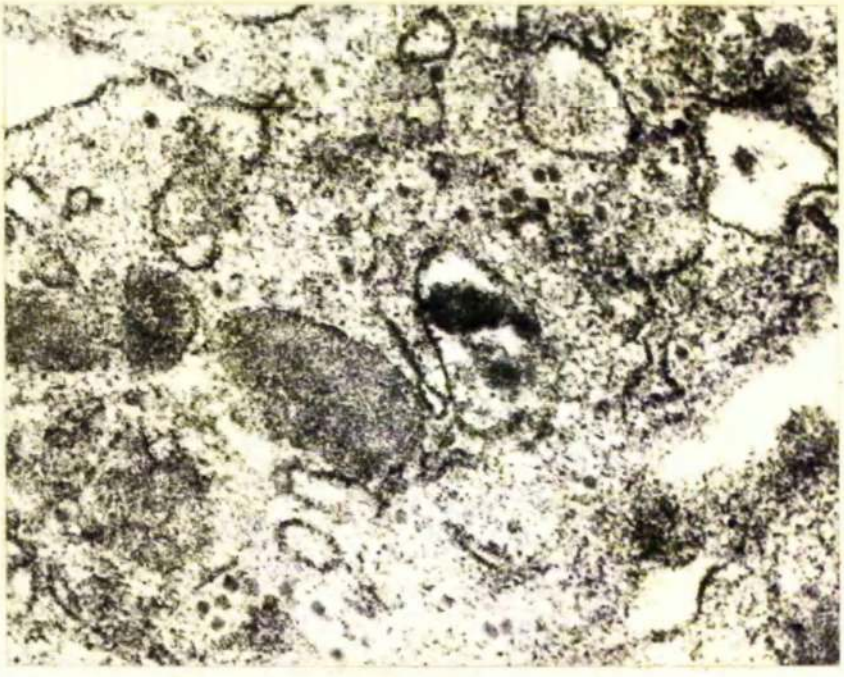


Fig. 100 (Top right) Inoculated kitten 42 days after infection with CL3/CFS. Particle near platelet surface. x 75,000.

Fig. 101 (Top left) Inoculated kitten, 56 days after infection with CL3/CFS. Platelet pellet: virus-like structure in inter-platelet space. x 75,000.

Fig. 102 (Bottom) Inoculated kitten 72 days after infection with CL2/CFS. Virus-like structure in platelet vacuole. x 75,000.

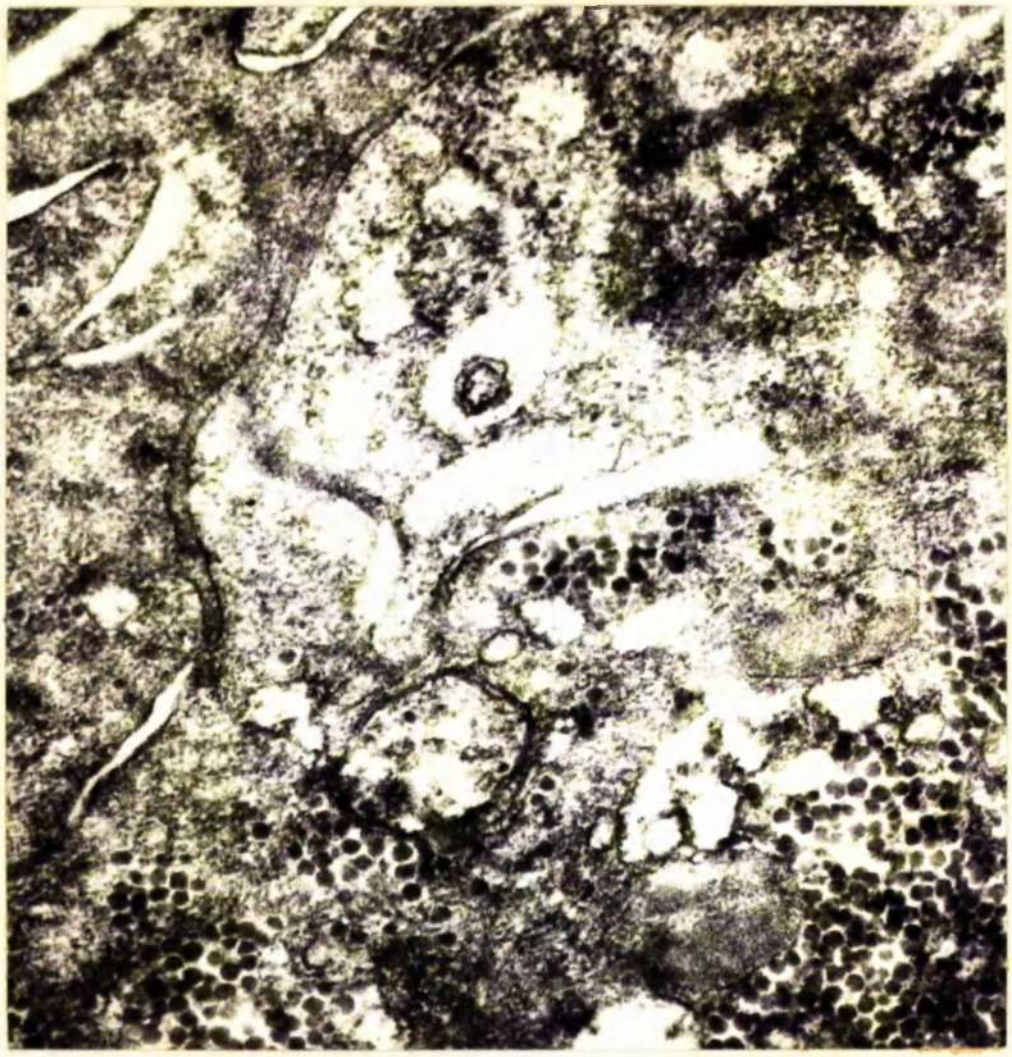
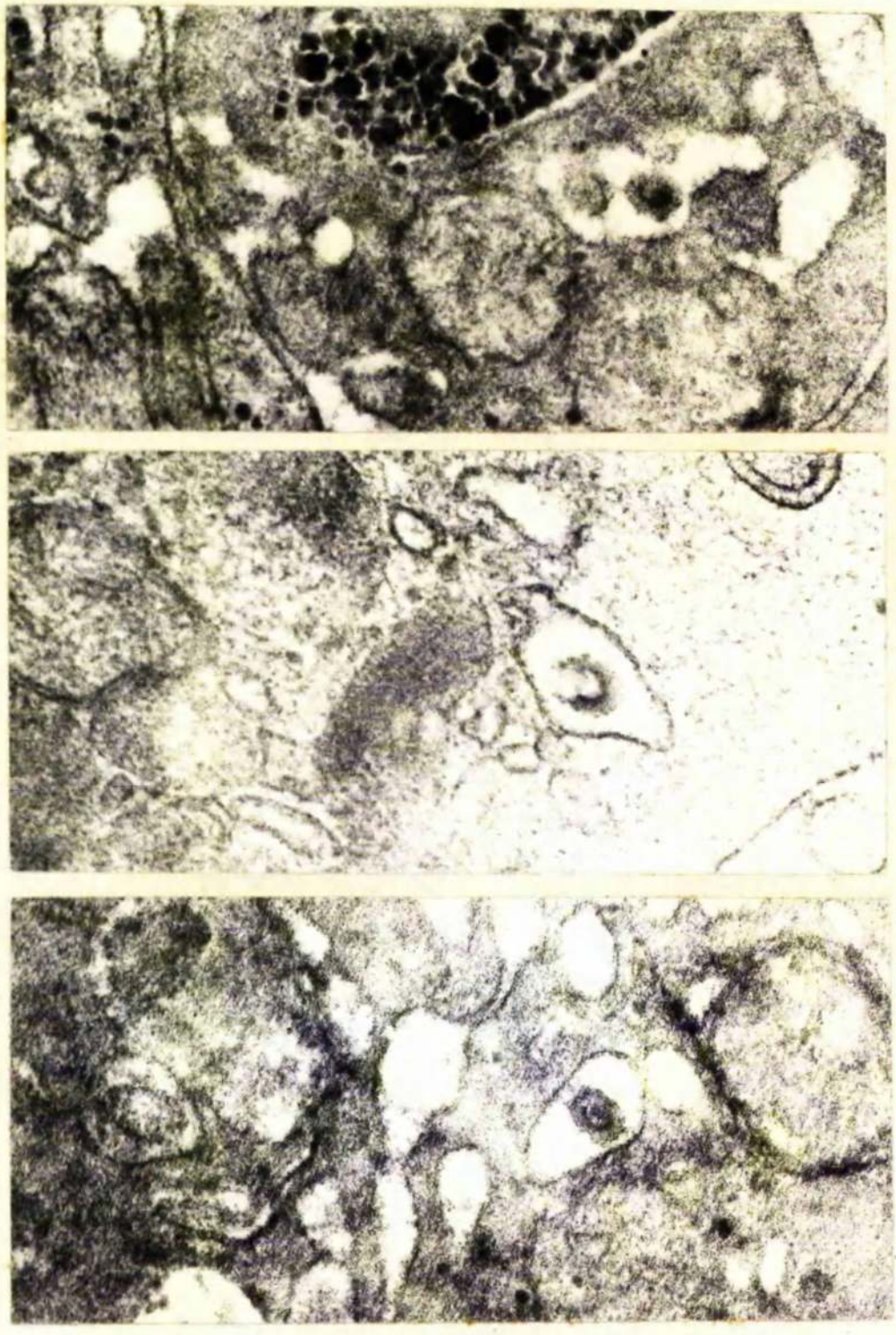


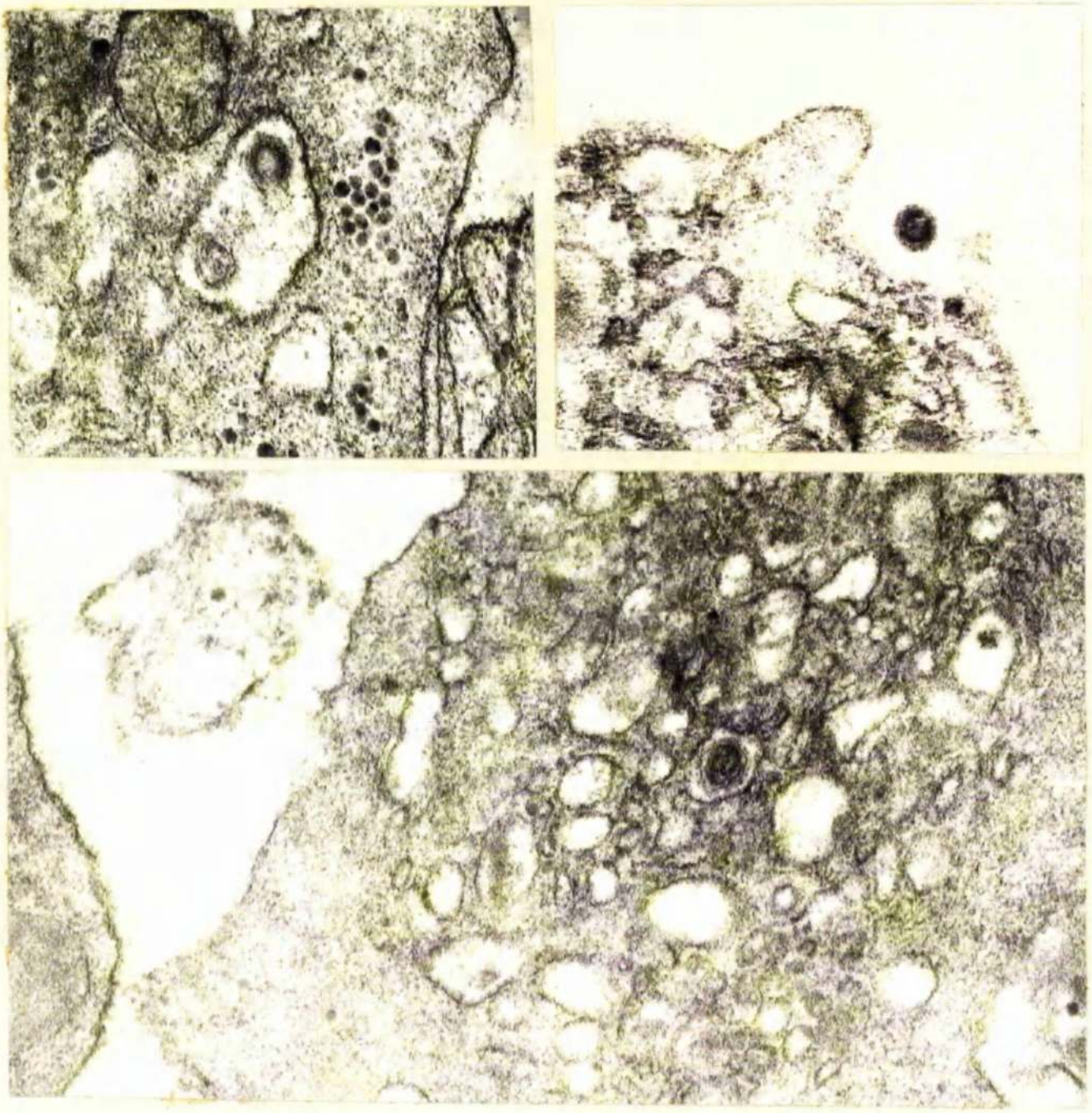
Fig. 103 Kitten inoculated with normal lymph node extract. Single particle in blood platelet vacuole. The particle is approximately 100 mμ in diameter; a distinct electron-dense inner membrane is seen. x 75,000.



Figs. 104 - 106 Particles, approximately 100 mμ in diameter, in vacuoles in platelets from normal kittens. x 75,000.

Top and bottom: platelets from 70 day old kitten.

Middle: platelet from 28 day old kitten.



Inoculated kittens. Filtered inoculum CL5/CFSF

Fig. 107 (Top left) 2 particles in a platelet vacuole 26 days after infection. x 75,000.

Fig. 108 (Bottom) Type 2 particle with electron-dense nucleoid and "coating layer" in platelet 28 days after infection. x 75,000.

Fig. 109 (Top right) Particle in interplatelet space, 42 days after infection. x 75,000.

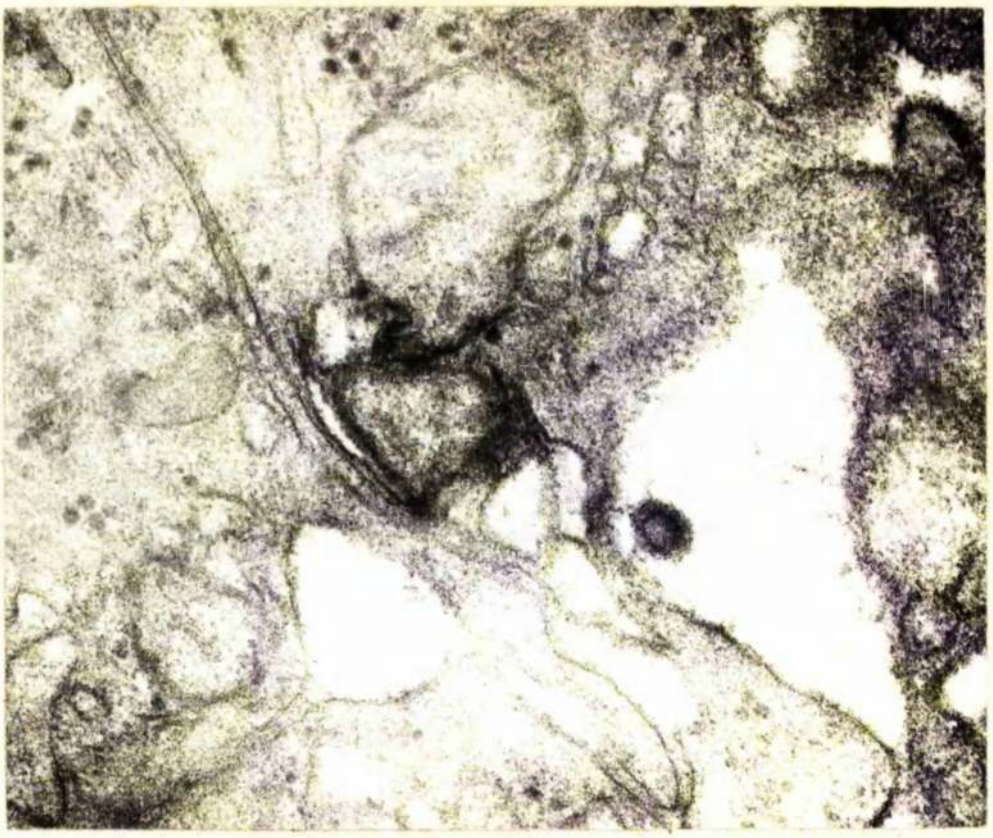


Fig. 110 Inoculated adult cat 26 days after infection with CL5/CFS. Platelet pellet showing particle in platelet vacuole. x 75,000.

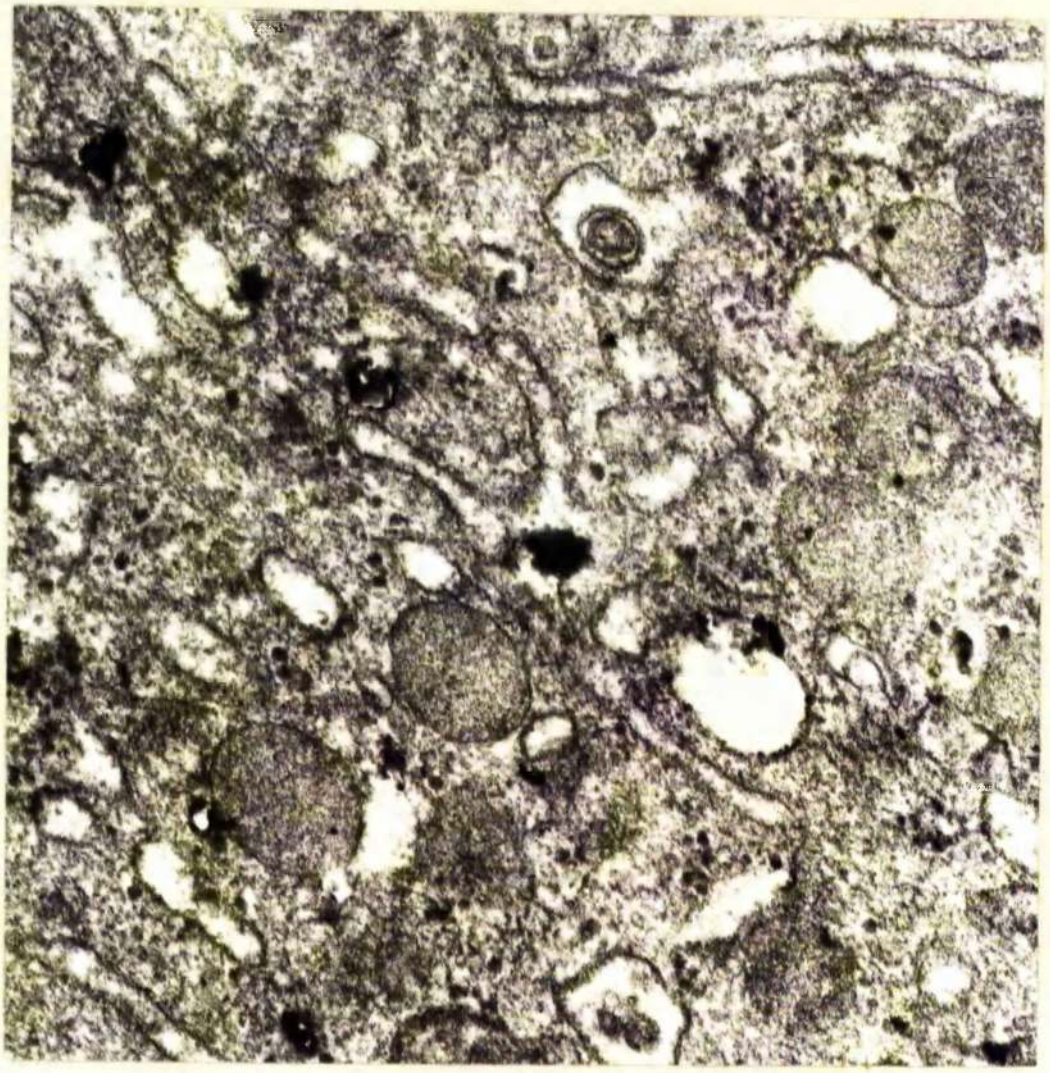
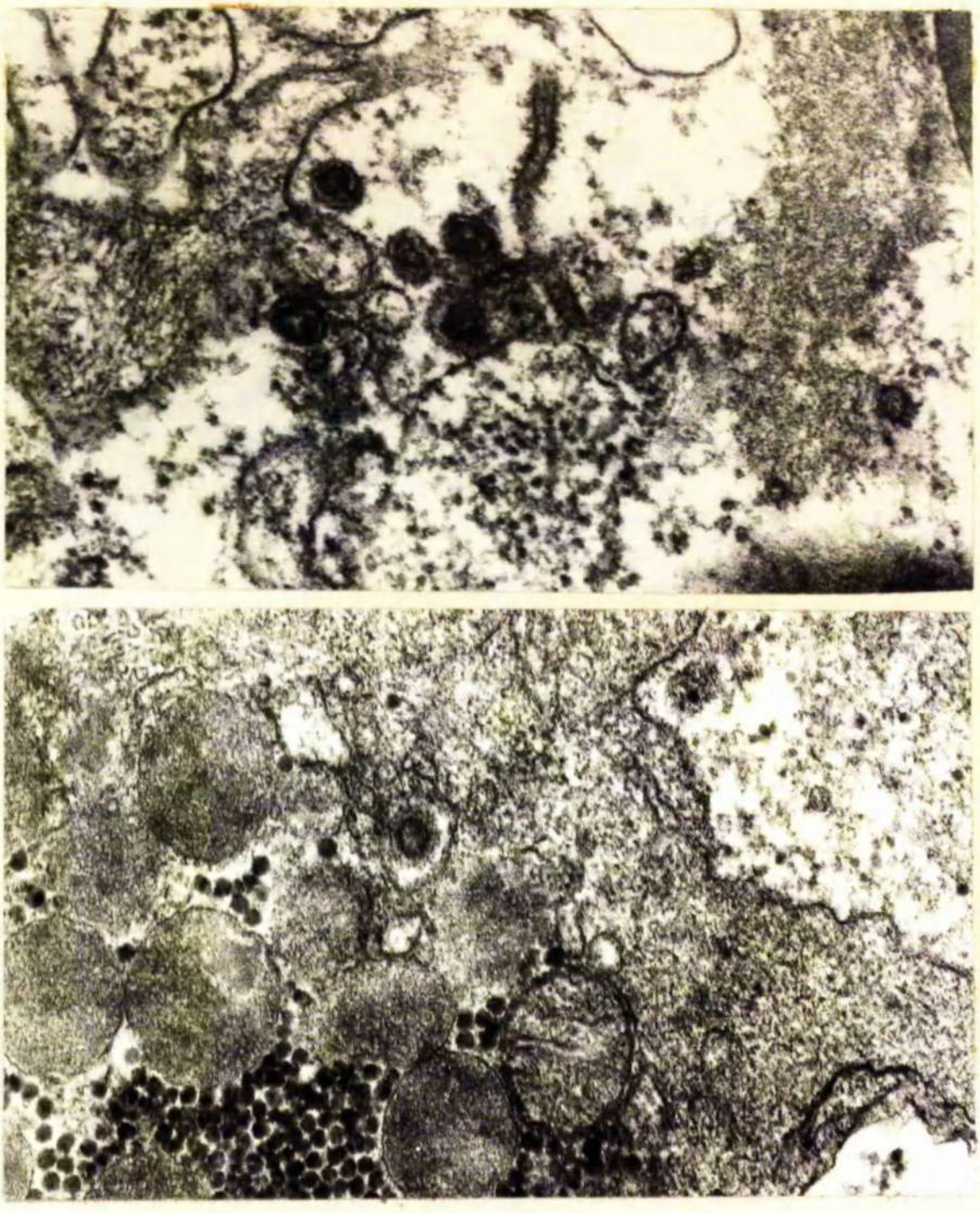


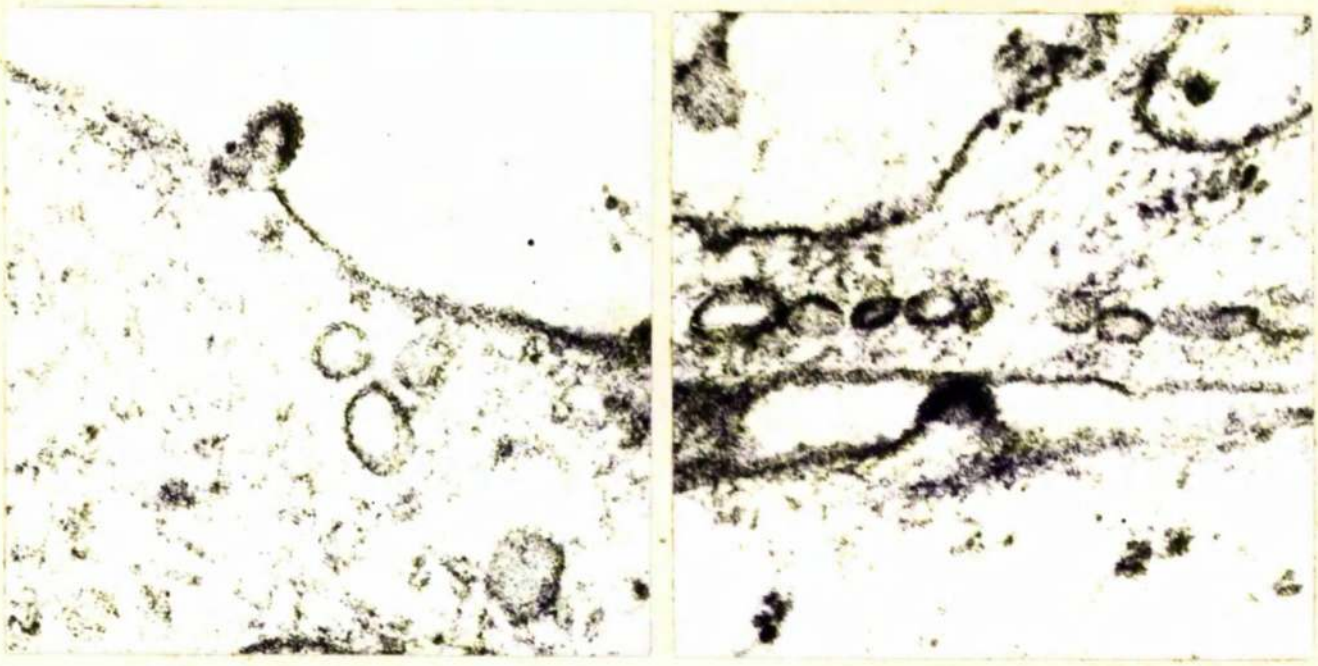
Fig. 111 Inoculated kitten 14 weeks after infection with CL5/P2. Particle, in platelet vacuole, with electron-dense nucleoid and well-defined "coating layer". x 75,000.



Inoculated kitten, 14 weeks after infection with CL5/P2

Fig. 112 (Top) Bone marrow. Extracellular group of Type 2 particles. x 75,000.

Fig. 113 (Bottom) Spleen. Single particle, with distinct inner membrane, in a platelet vacuole. x 75,000.



NK tissue culture infected with CL5/CFS

Fig. 114 (Left) Budding particle in culture 15 days after infection. x 75,000.

Fig. 115 (Right) Particle budding from cell membrane 25 days after infection. x 75,000.

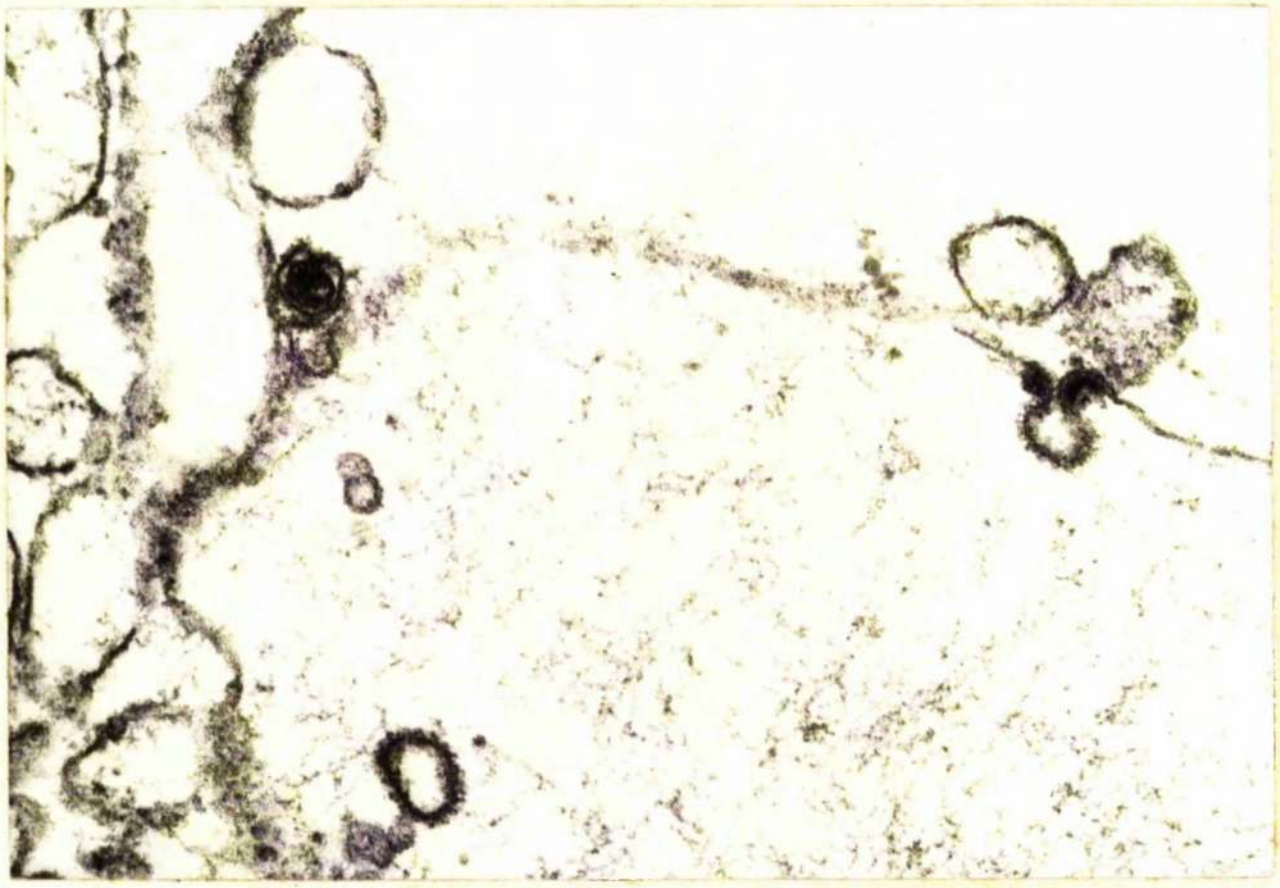


Fig. 116 NK tissue culture 15 days after infection with CL5/CFS. Two budding particles are seen in association with a cellular invagination. The inner electron-dense membrane is clearly seen and continuity between the cell and particle outer membranes is shown. A Type 2 particle is seen extracellularly. x 75,000.

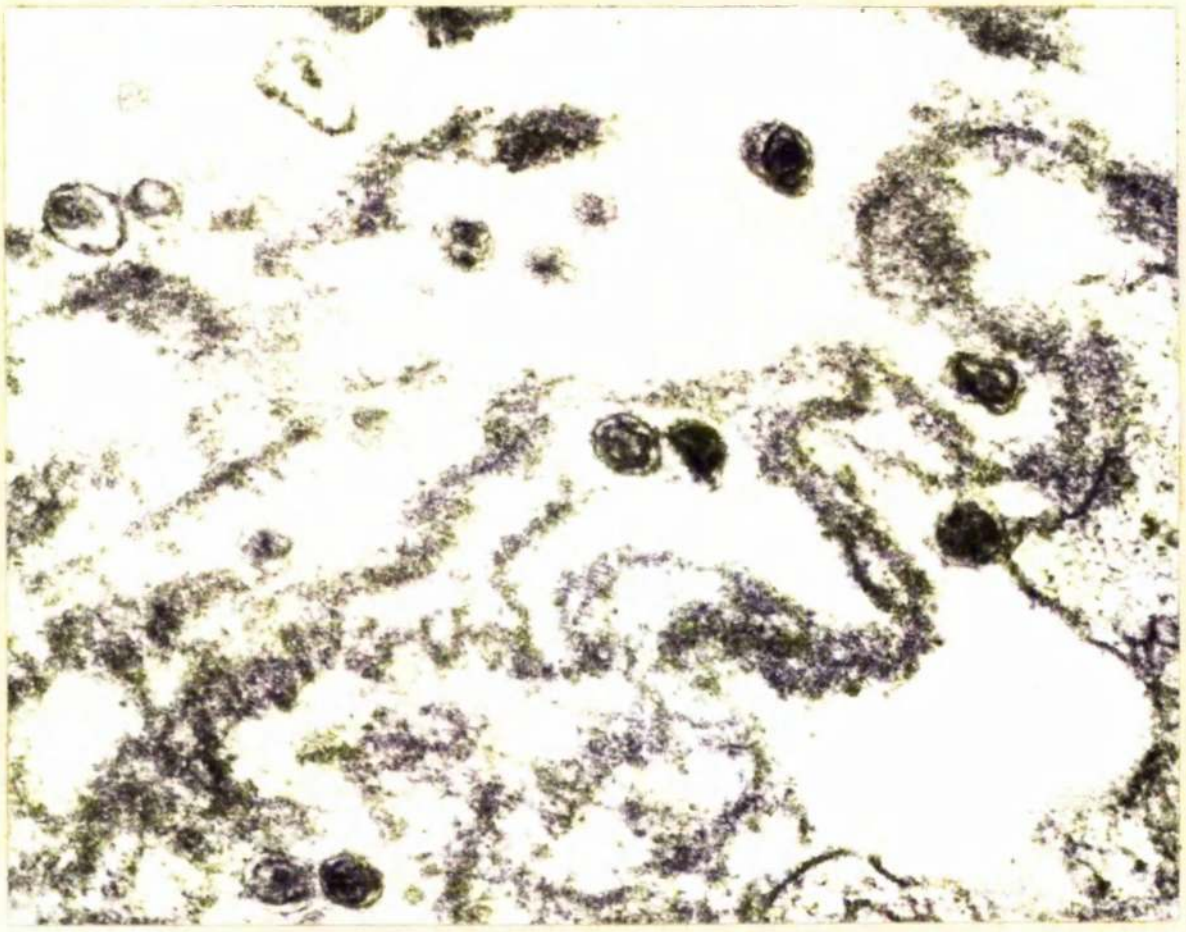
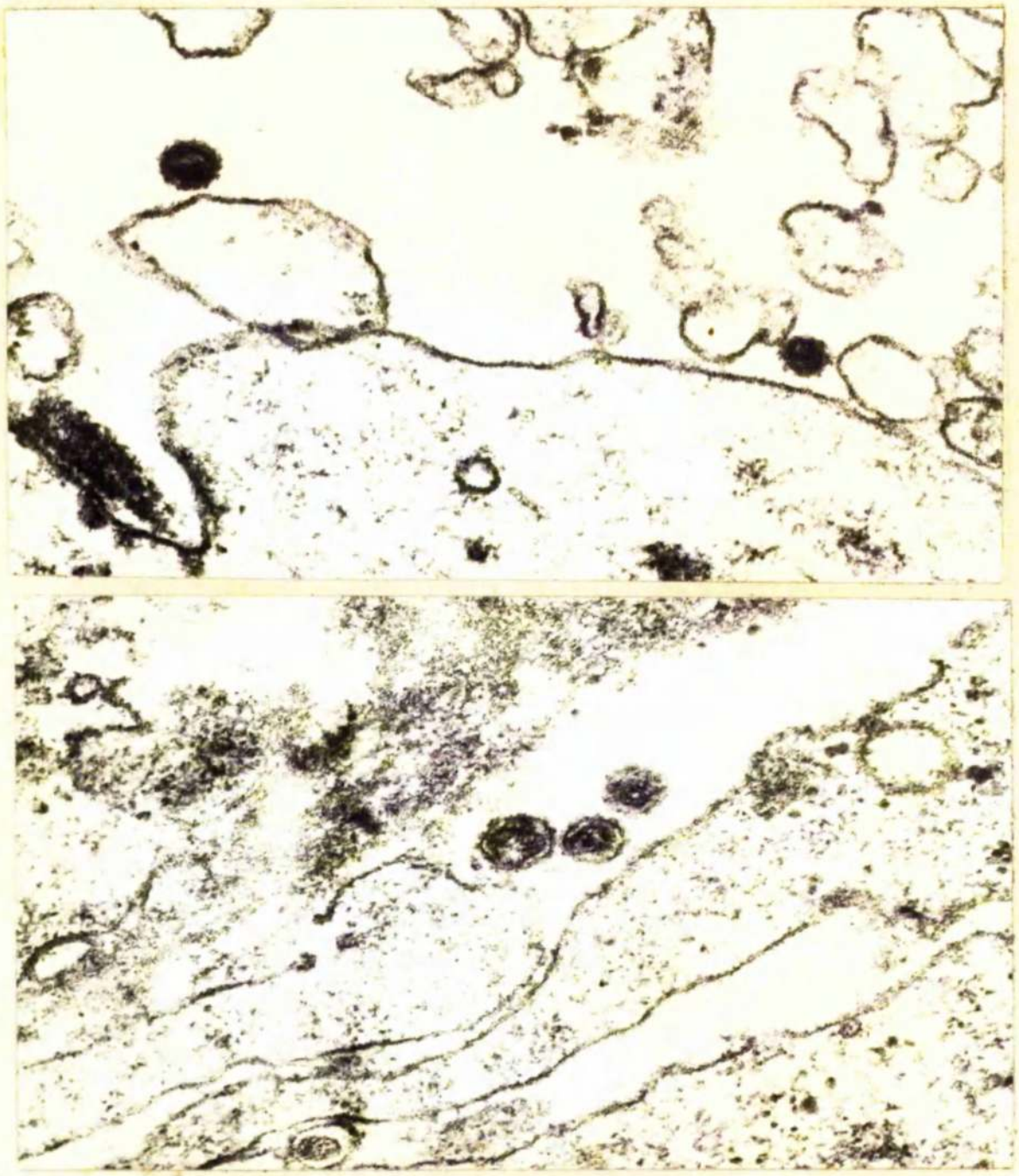


Fig. 117 NK tissue culture 25 days after infection with CL5/CFS. Fully formed particles with electron-dense nucleoids and wrinkled external membranes. x 75,000.



NK tissue culture infected with CL4/CFS

Fig. 118 (Top) 20 days after infection. Two complete particles in extracellular space. The "coating layer" is seen on the particle to the left. x 50,000.

Fig. 119 (Bottom) 25 days after infection. Three Type 2 particles in an extracellular space. x 75,000.

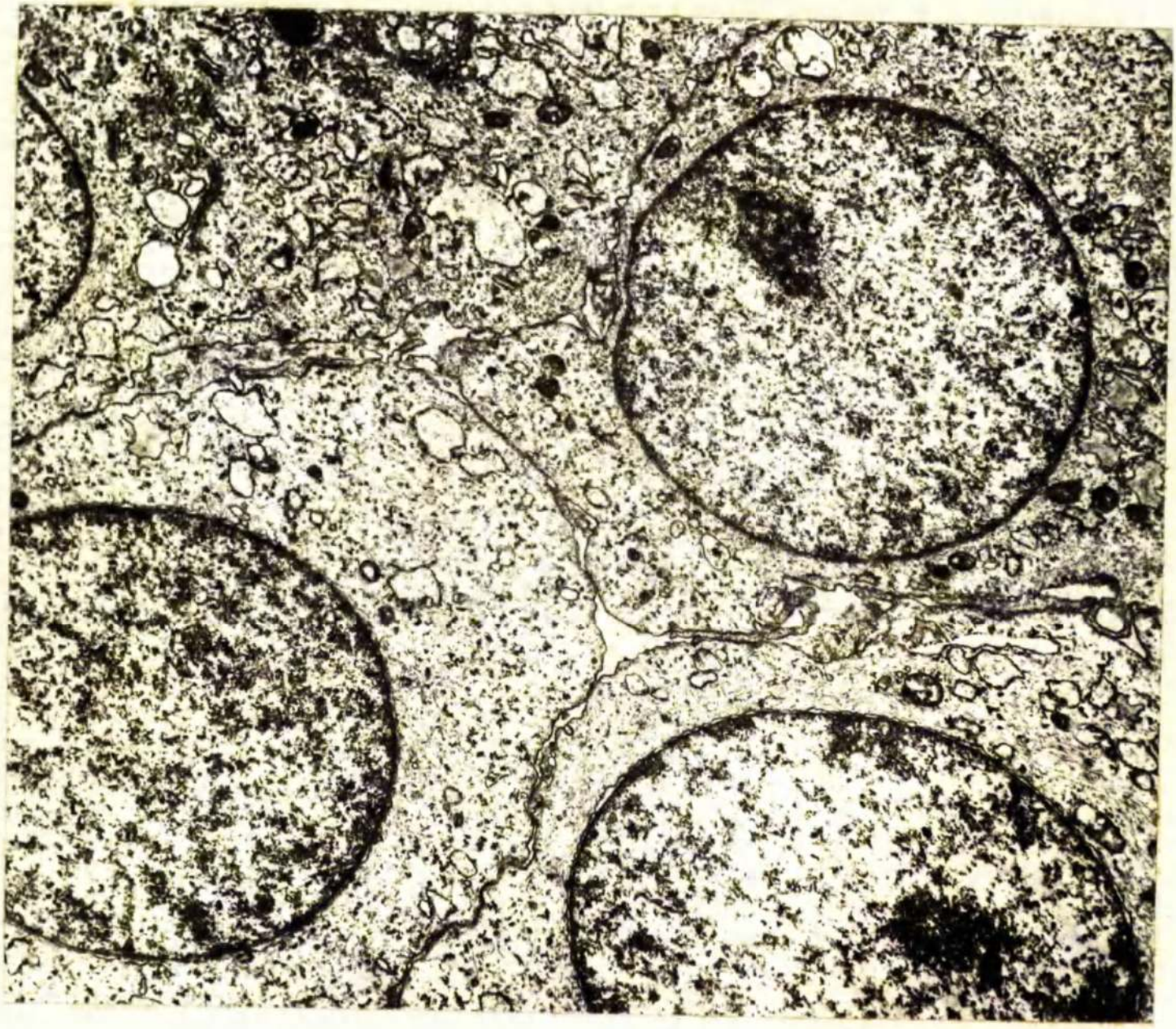


Fig. 120 FL control tissue culture at 7 days. x 12,500.

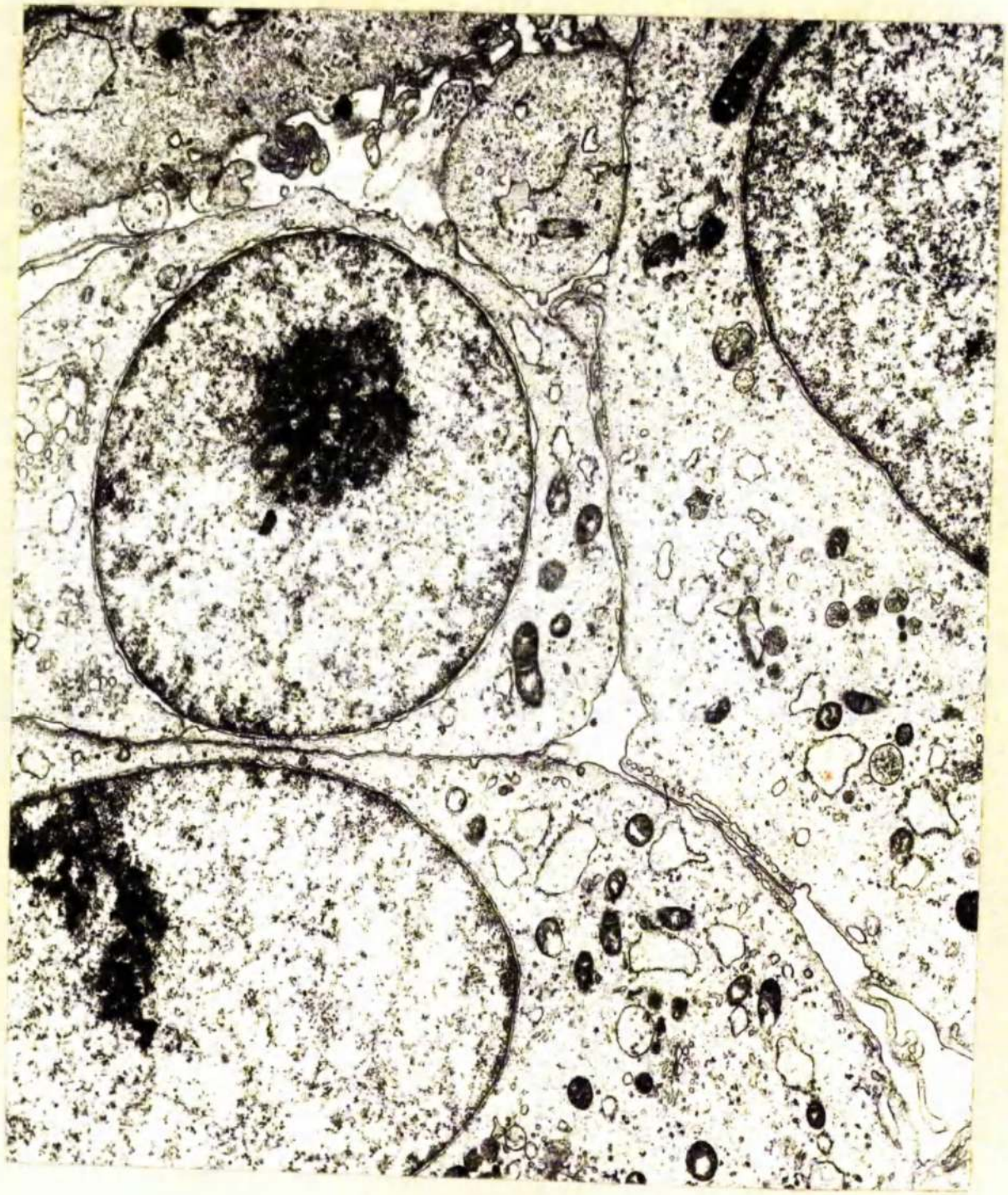
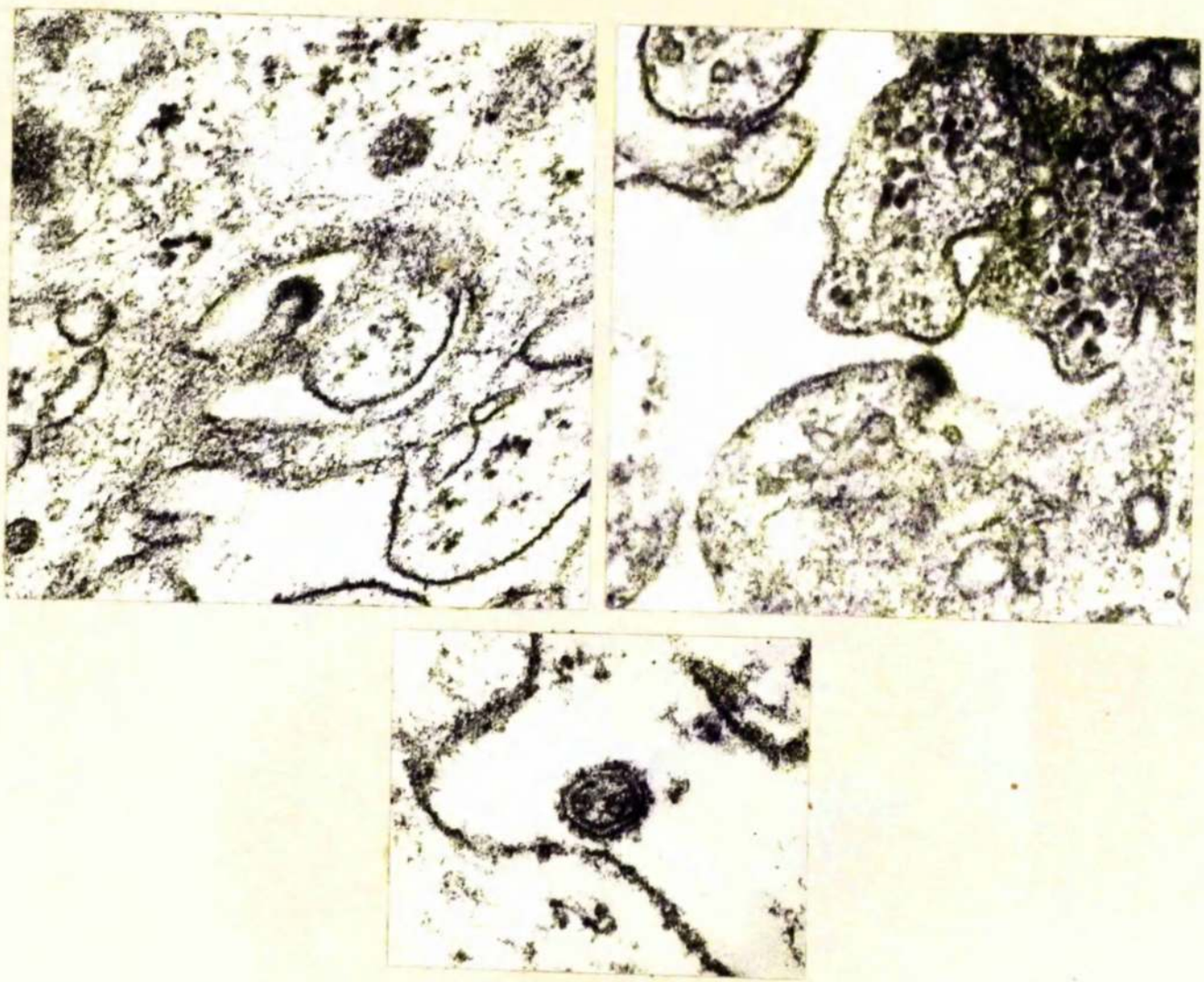


Fig. 121 FL tissue culture 14 days after infection with CL5/CLV5. One particle is in the central intercellular space near the cell surface. x 18,250.



FL tissue culture infected with CL5/CLV5. Particle budding 14 days after infection

Fig. 122 (Left) Early bud from cell surface. x 75,000.

Fig. 123 (Right) Bud showing triple membraned structure. x 75,000.

Fig. 124 (Bottom) Detailed structure of Type 2 particle with "coating layer". x 120,000.

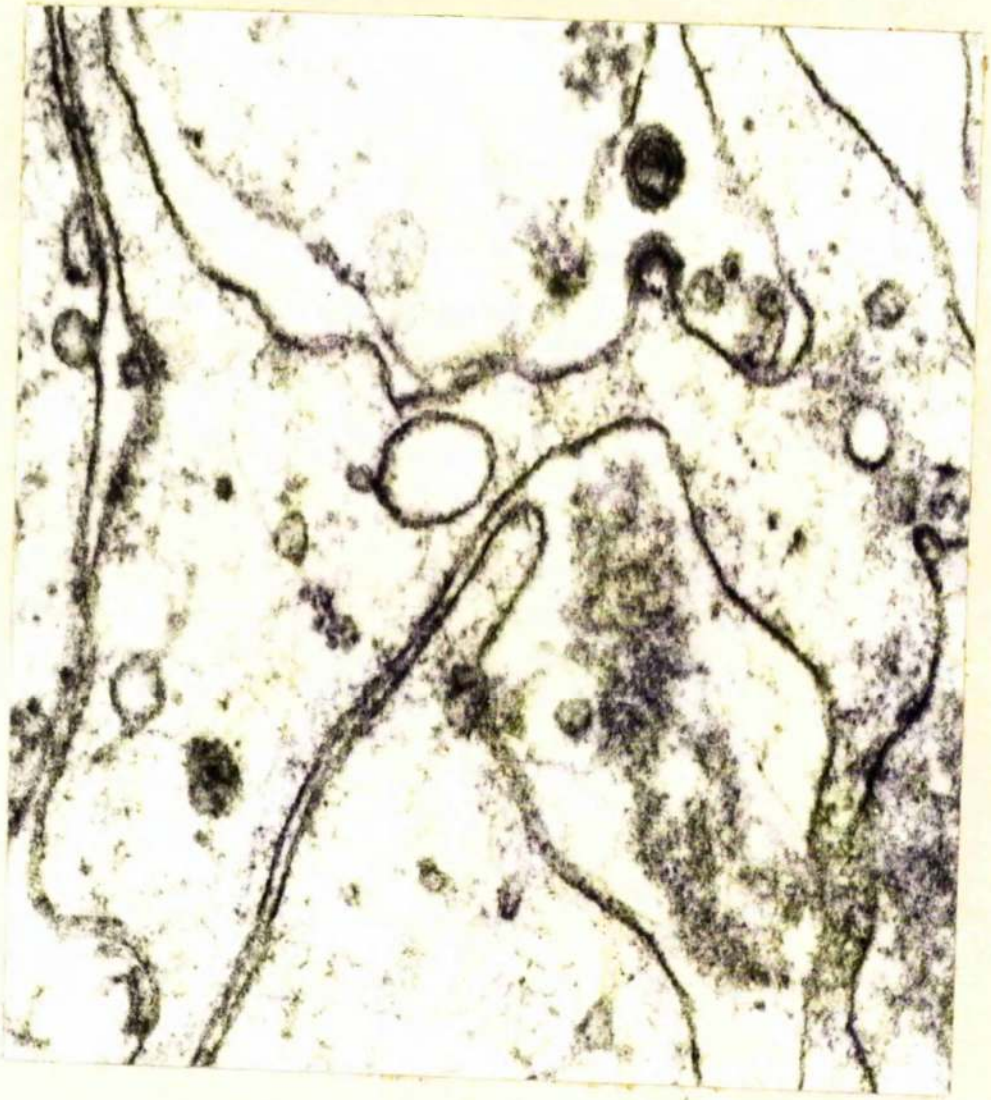
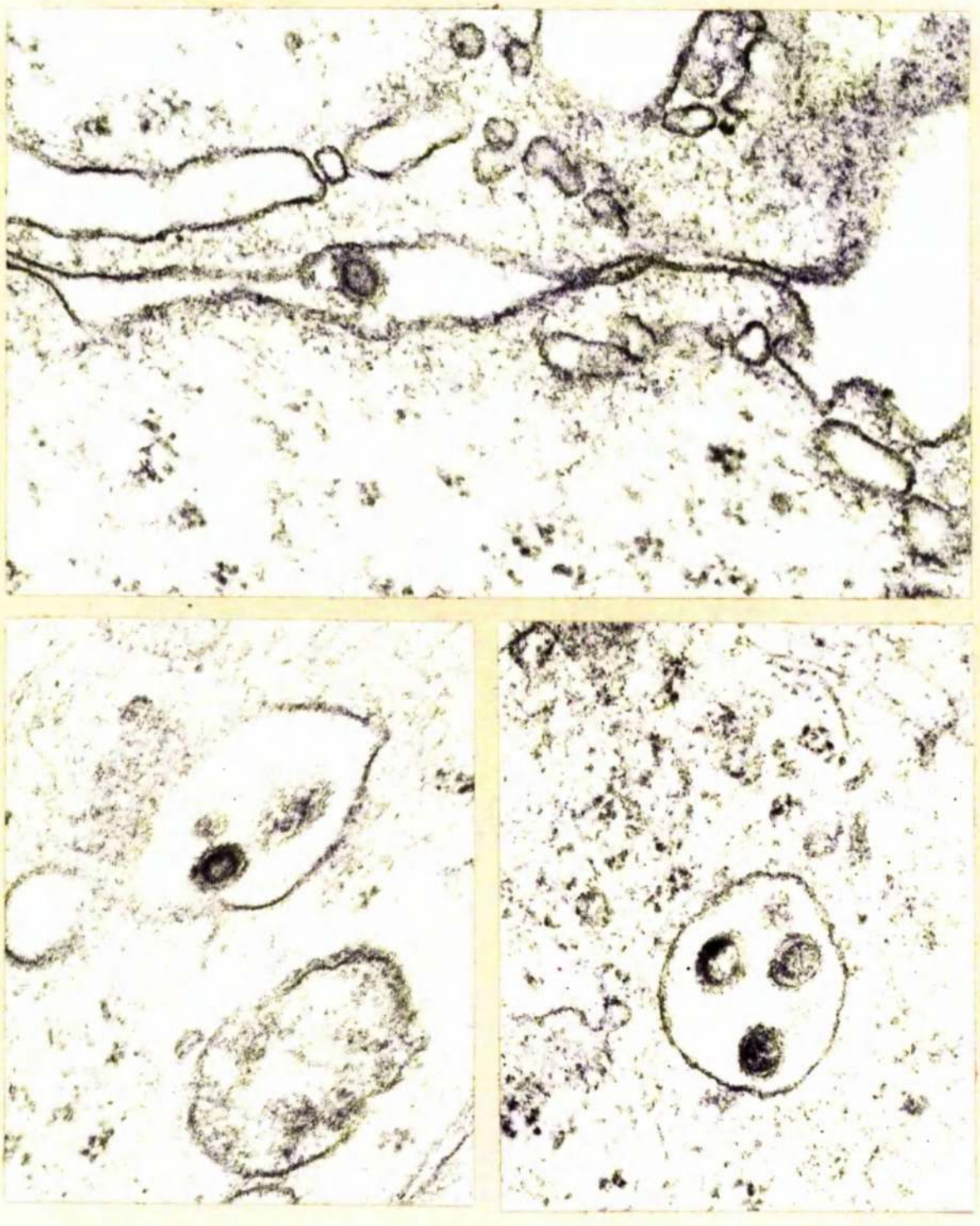


Fig. 125 FL tissue culture infected with CL5/CLV5 14 days after infection. One budding and one complete particle are shown. x 75,000.

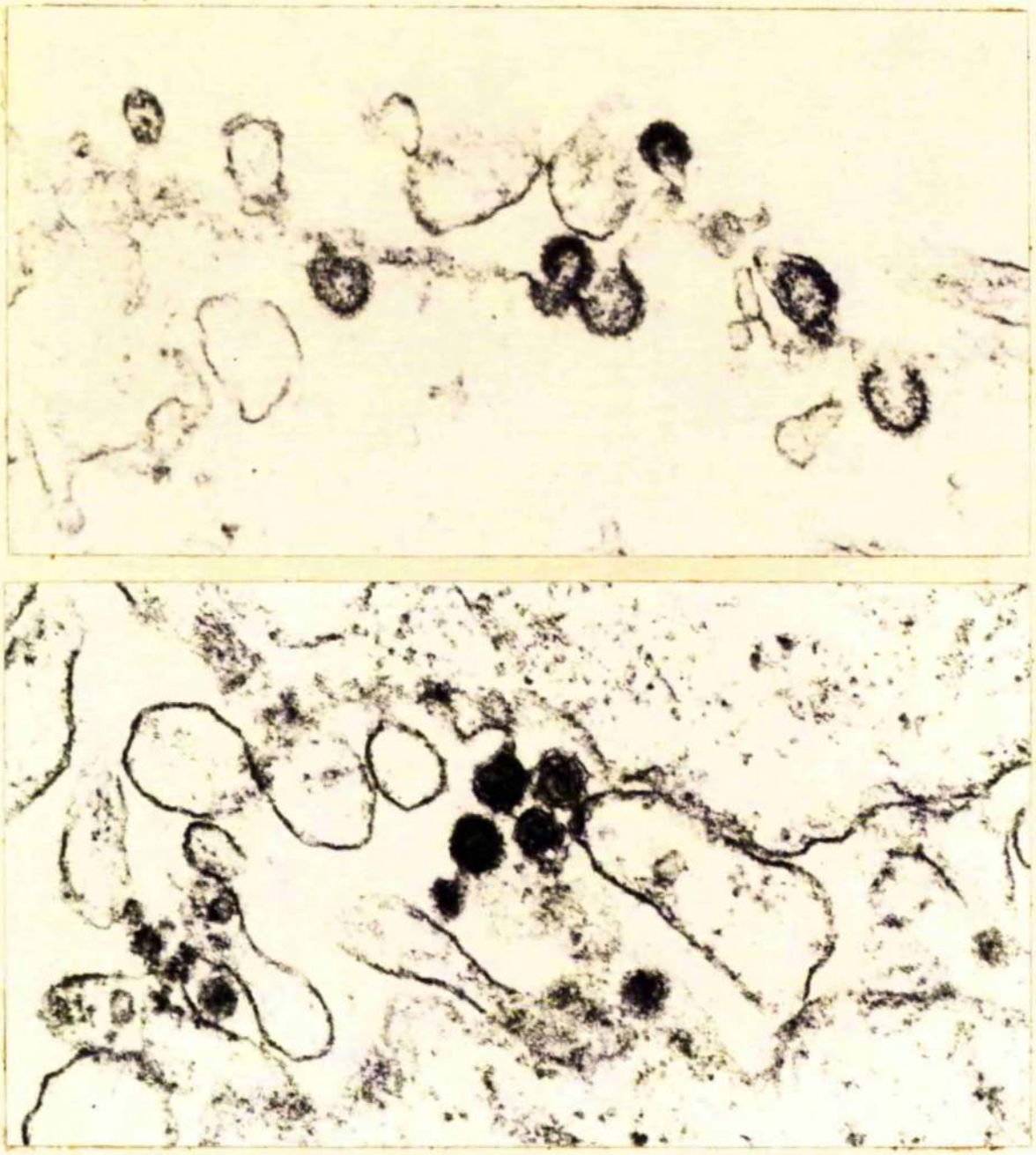


Fully formed particles in FL tissue culture 14 days after infection.

Fig. 126 (Top) CL5/CLV5 infected culture. Single Type 1 particle in an intercellular space. x 75,000.

Fig. 127 (Lower left) CL5/CFSF infected culture. Single particle in a vacuole. x 75,000.

Fig. 128 (Lower right) CL5/CFSF infected culture. Group of particles in vacuole; Two Type 2 particles and one Type 1 particle. "Coating layer" is seen on the particles to right and bottom. x 75,000.



FL tissue culture infected with CL5/CFSF. 8 days after infection.

Fig. 129 (Top) Particles budding from cell membran. x 75,000.

Fig. 130 (Bottom) Group of extracellular particles. One particle shows a relatively electron-lucent core; the other particles are Type 2. x 75,000.

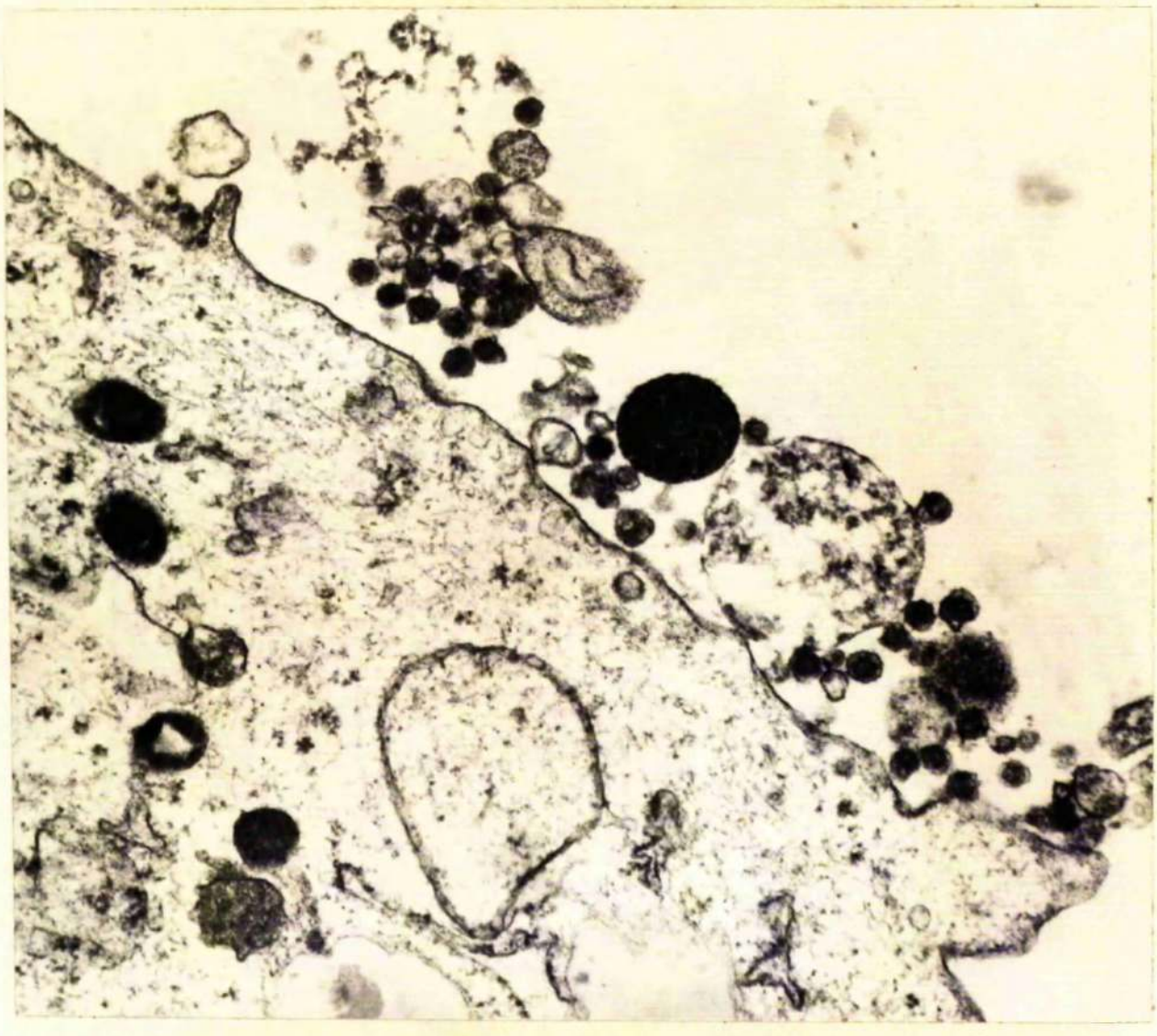


Fig. 131 FL tissue culture 29 days after infection with CL5/CFSF. Many particles are seen extracellularly near plasma membrane. Most particles are Type 2 and have irregular outer membranes. x 37,500.

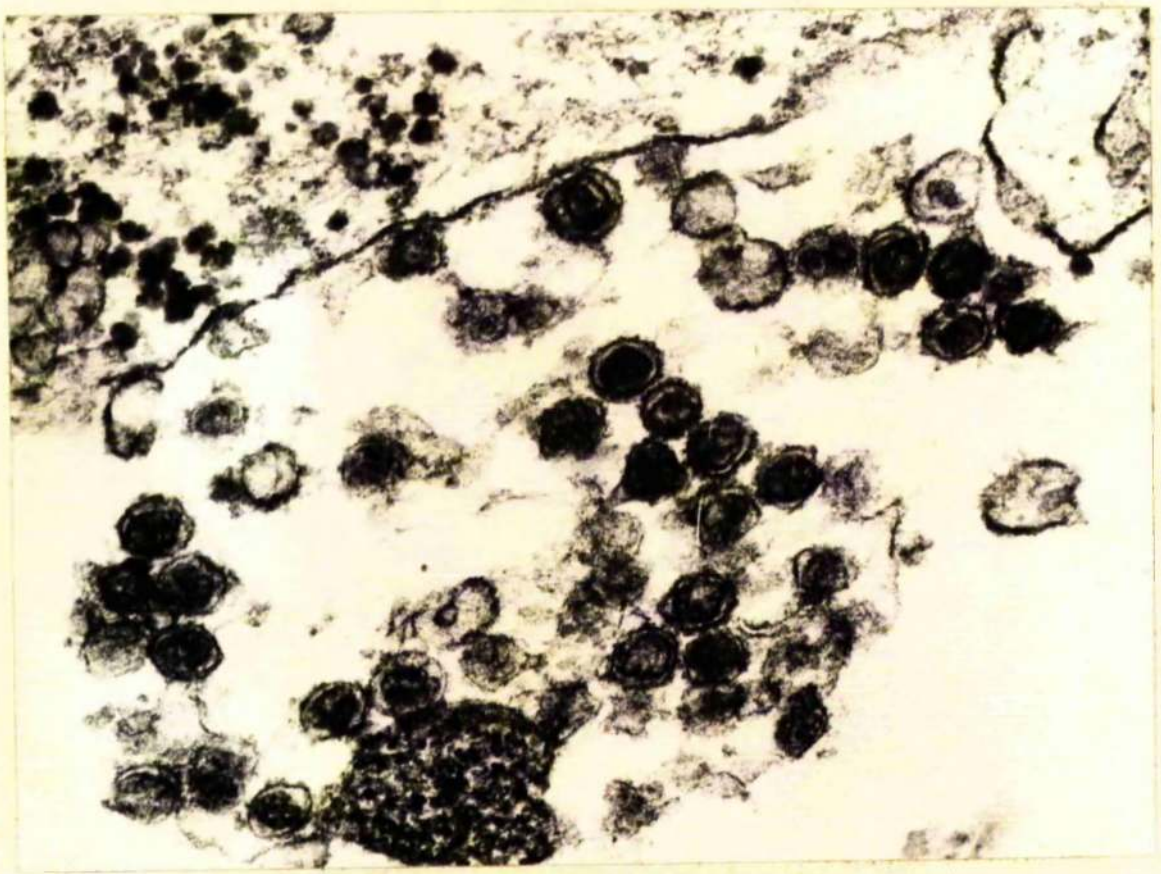


Fig. 132 FL tissue culture 29 days after infection with CL5/CFSF. Large extracellular group of Type 2 particles. The irregularity of the outer membranes and the eccentricity of the nucleoids is shown. x 75,000.

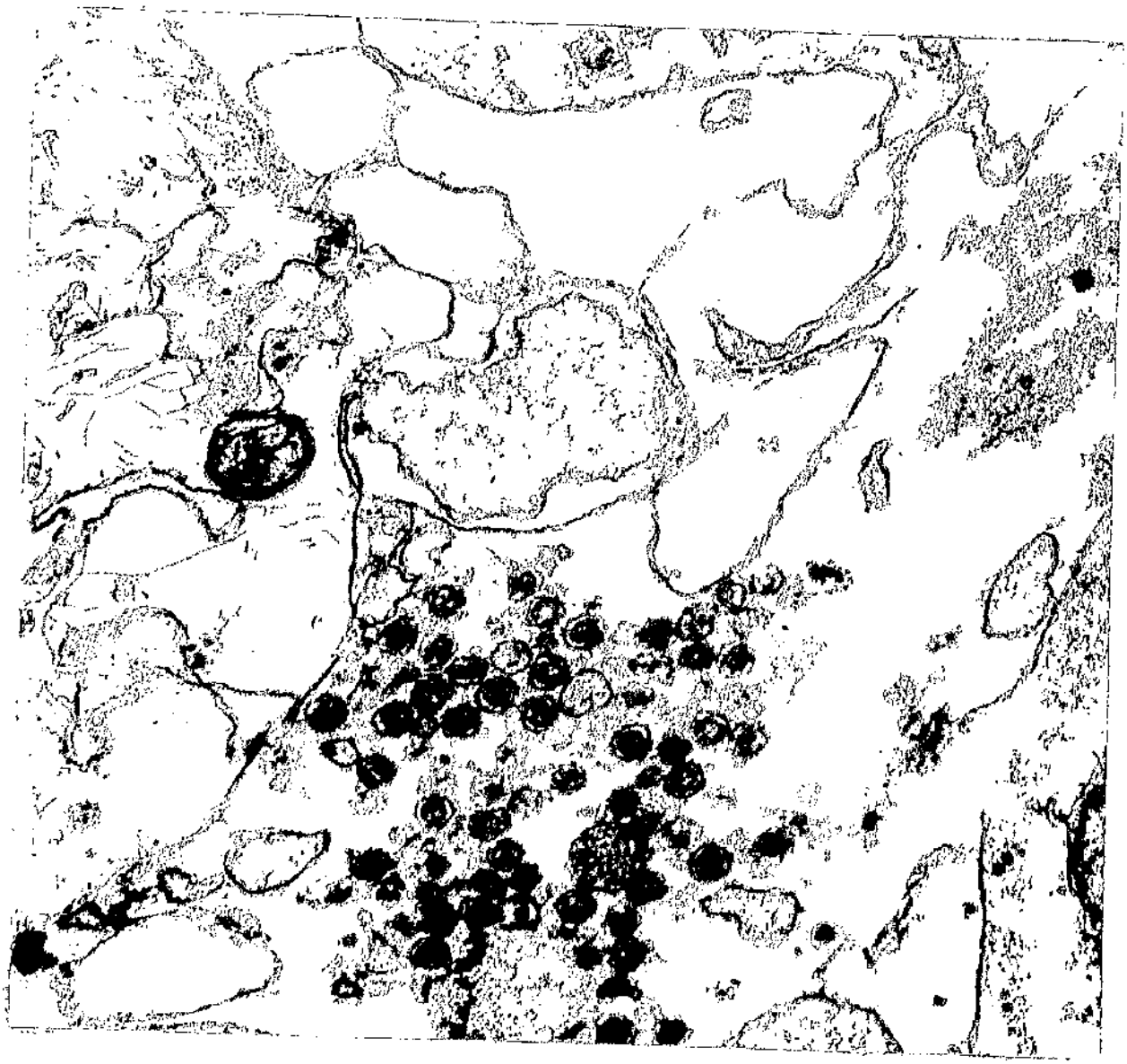


Fig. 133 Feline embryo tissue culture infected with CL5/CFSF. Large group of Type 2 particles in extracellular space. x 50,000.

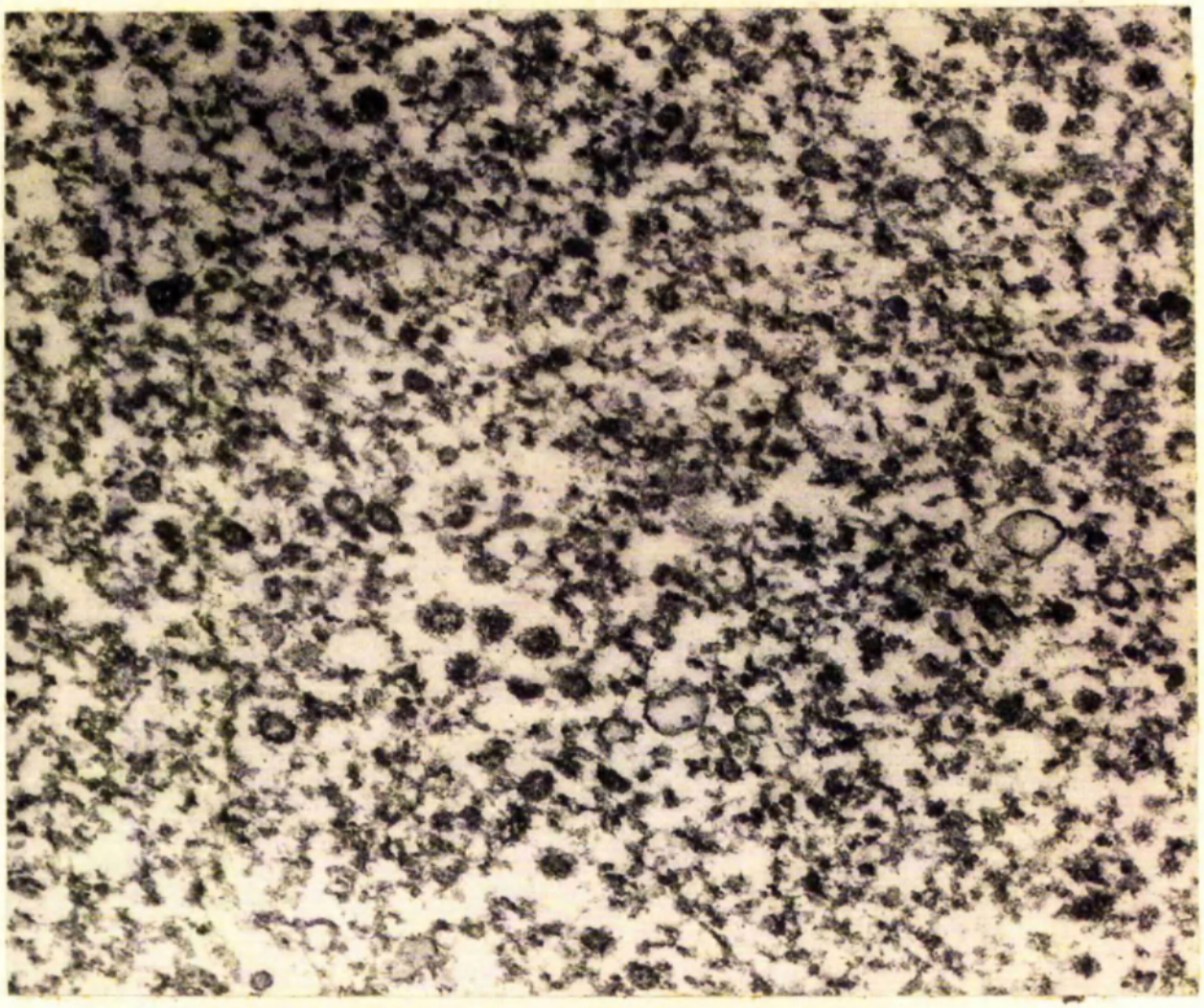


Fig. 134 Section of pellet from fraction 4 of leukaemic tissue extract from spontaneous feline lymphosarcoma CL3. Particles approximately 65 mμ are seen in a network of smaller particles and amorphous material. x 75,000.

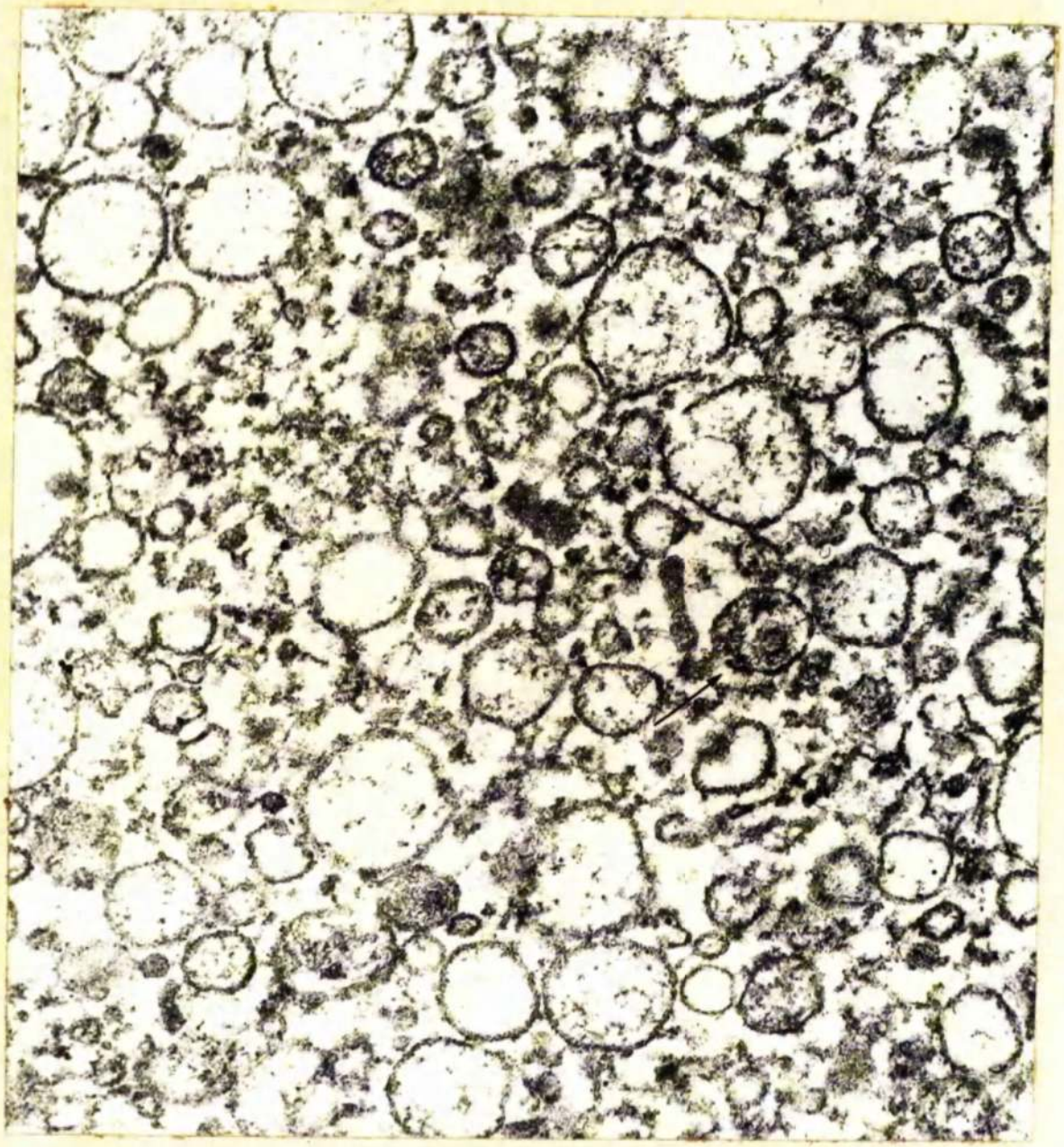


Fig. 135 Section of pellet from fraction 6 of leukaemic tissue extract from spontaneous lymphosarcoma CL3. One virus-like structure, approximately 100 mu is seen in a vacuole (⟶). x 75,000.

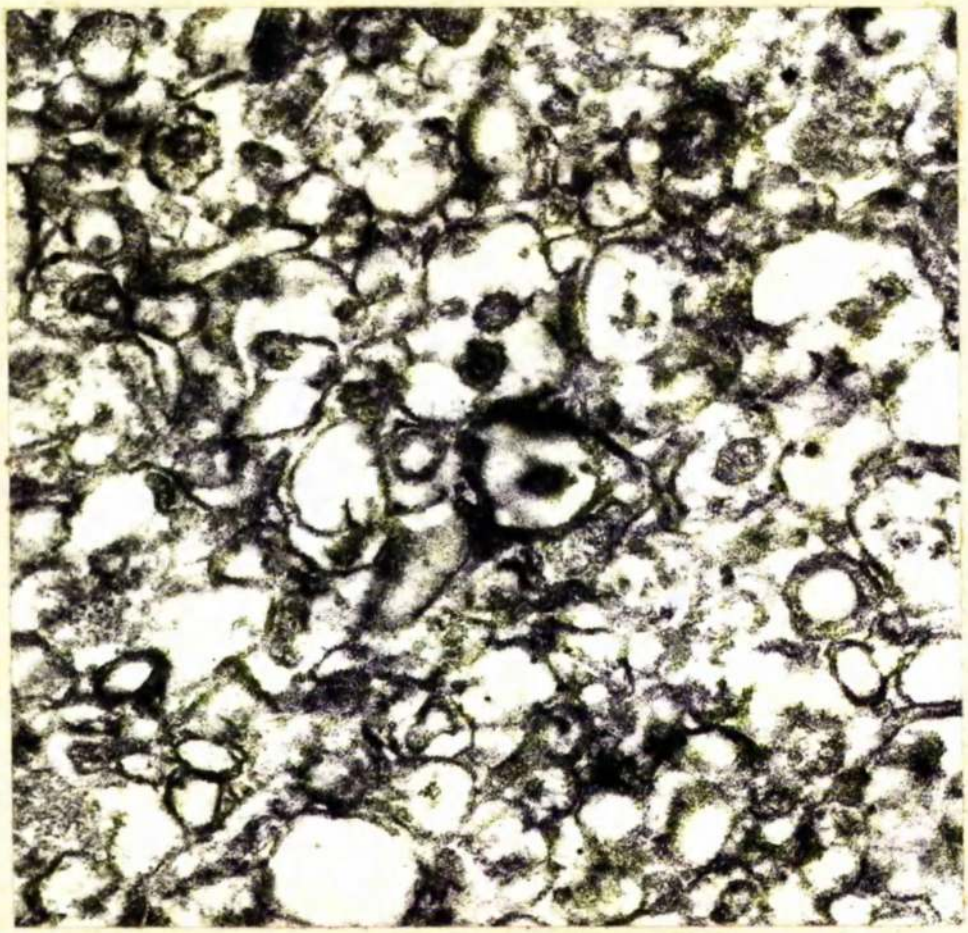


Fig. 136 Section of pellet from fraction 11 of leukaemic tissue extract from spontaneous feline lymphosarcoma CL3. Virus-like structure with electron-dense inner membrane. x 75,000.

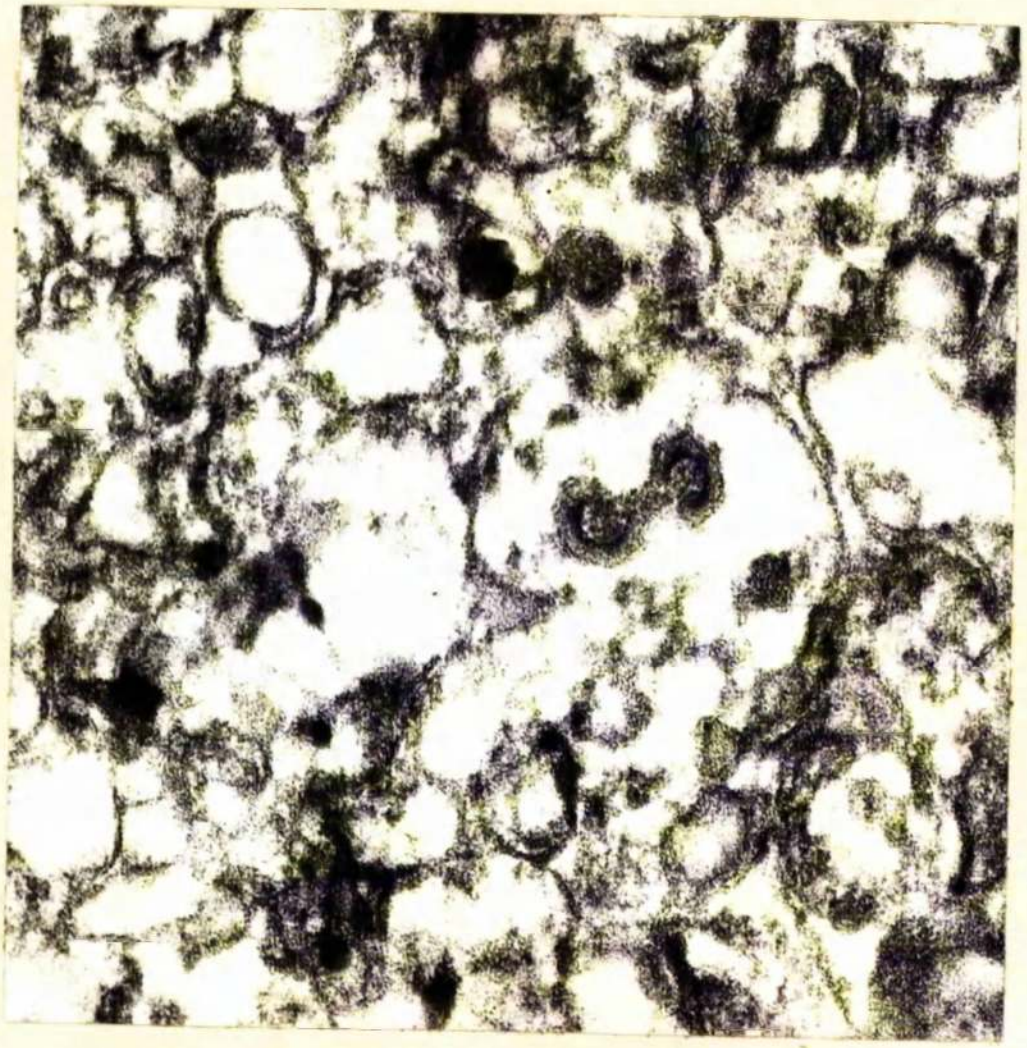


Fig. 137 Section of pellet from fraction 11 of leukaemic tissue extract from spontaneous lymphosarcoma CL3. x 125,000.

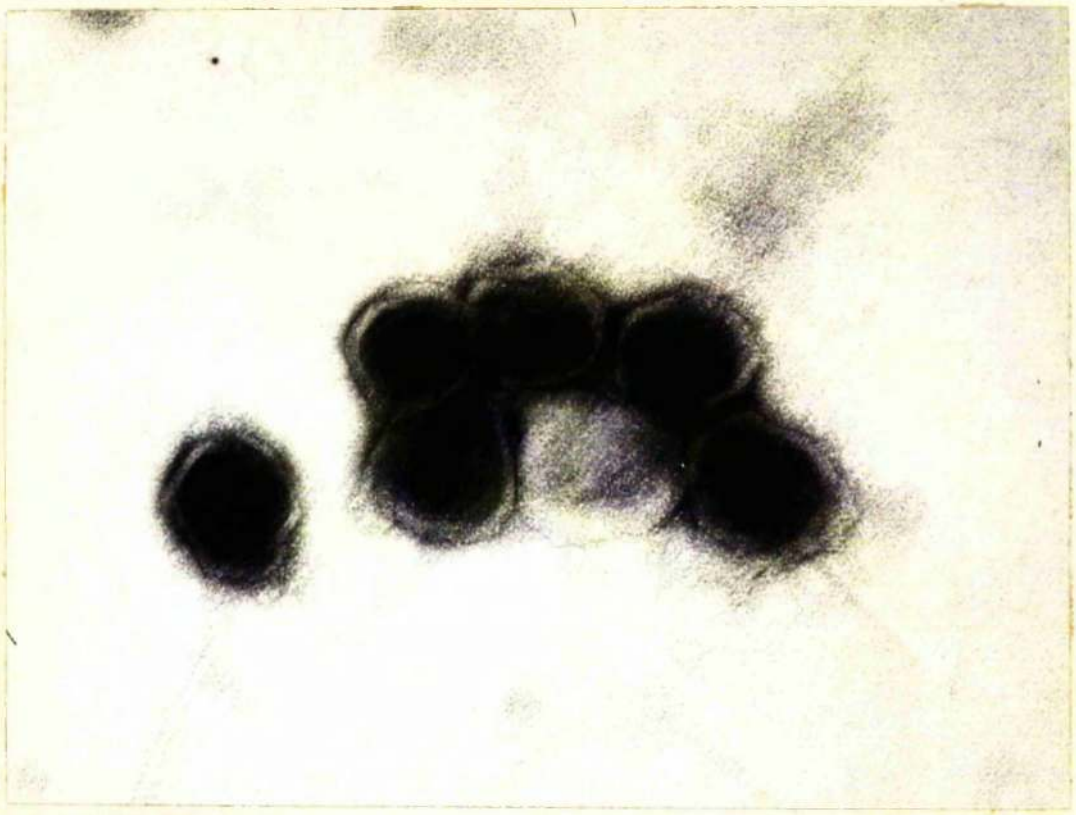


Fig. 138 Particles extracted from tissue culture fluid. Negative stained preparation;uranyl acetate. x 150,000.

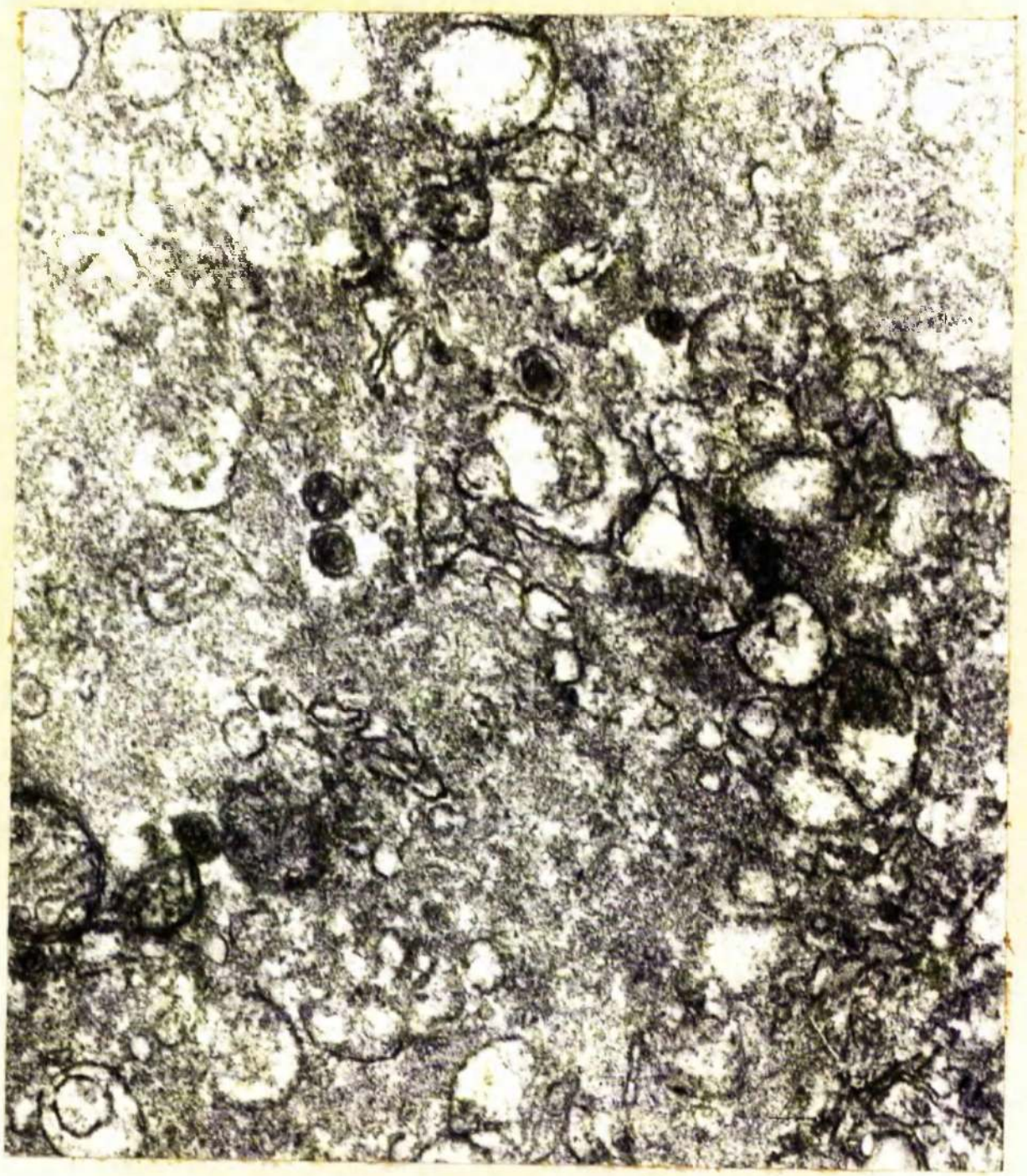


Fig. 139 Section of pellet of inoculum CL5/CLV1. Five Type 2 particles are seen in a matrix of amorphous and membranous material. x 75,000.

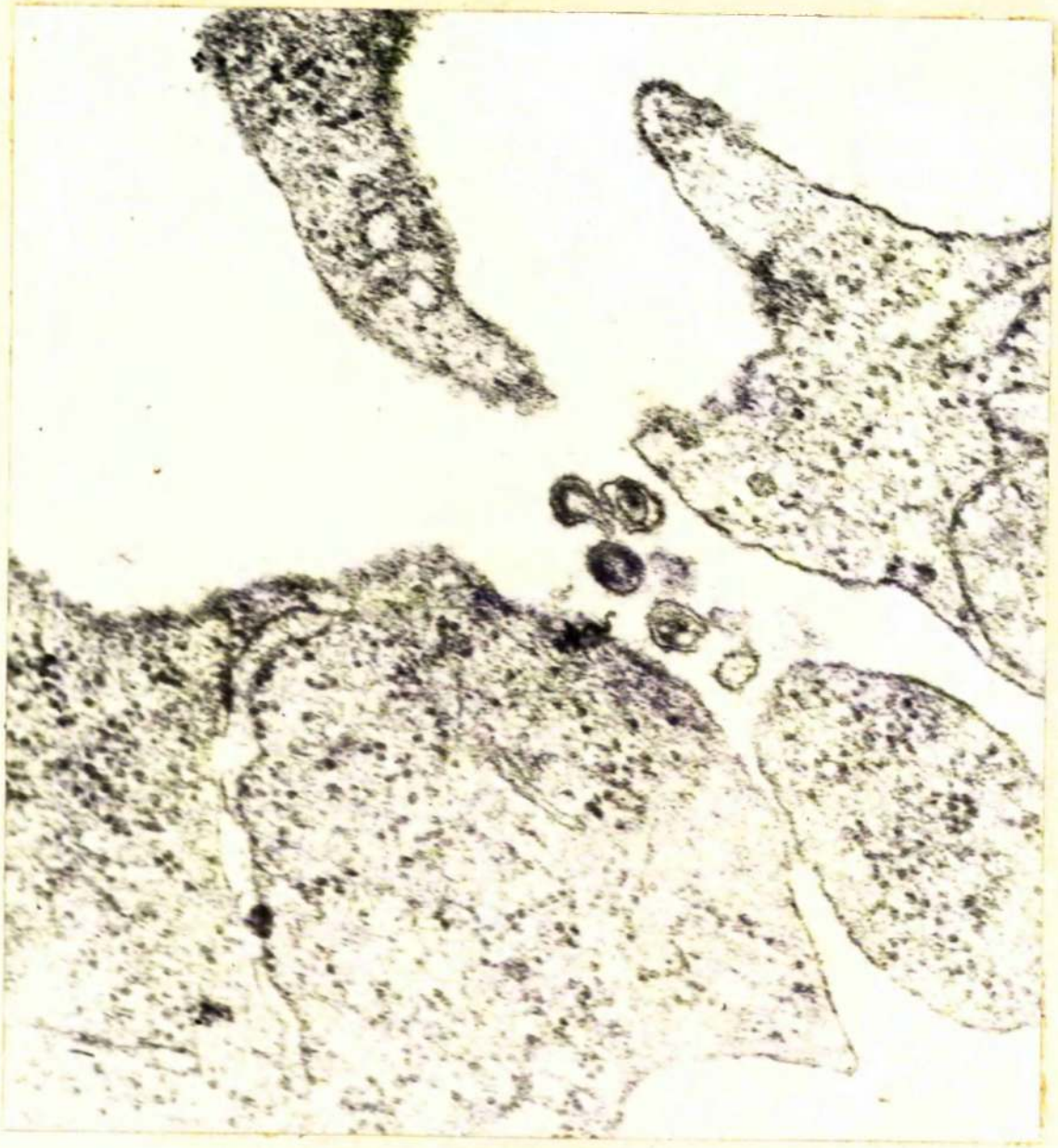


Fig. 140 Canine lymphosarcoma. Extracellular group of virus-like particles in section of lymph node. x 75,000.