HUMAN HEMATOPOIETIC NEOPLASIA:
A STUDY DIRECTED TOWARD
ONCOGENIC VIRUS ISOLATION

by

Mary Jo Florey, B. A.

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....

(Professor in Charge of Thesis)

[redacted]

.....

(Chairman, Graduate Council)

CONTENTS

Introduction 1

Statement of the Problem 3

Historical Review of the Literature 3

Early Morphological Work, in Culture 4

Recent Morphological Work, in Culture 5

Peripolesis, Emperipolesis 7

Oncogenic Virus in Tissue Culture 8

Review of Tumor Immunology 8

Animal Tumor Work 9

Human Tumor Work 10

Review of Lymphoid Tumor Histochemistry 10

Lactic Dehydrogenase 10

Beta-Hydroxybutyrate Dehydrogenase 11

Acid Phosphatase 11

Methods and Materials 13

Culture Techniques 13

Photographic Processing 14

Immunofluorescent Methods 17

Histochemical Procedures 18

Electron Microscopy Methods 20

Results 22

Morphological Study 22

General Behavior of Lymphoid Tissue 22

Reaction to Vital Dyes 24

Cell Destruction 27

Four Morphological Changes 31
Type I, Spreading Lymphocyte 35
Type II, Transitional Lymphocyte 35
Type III, Transitional Reticulum Cell 35
Type IV, Foreign-Body Giant Cell 36
Lymphocytic Lymphoma 44
Hodgkin's Granuloma and Paragranuloma 48
Reticulum Cell Sarcoma 51
Reactive Hyperplasia 54
Lymphadenitis 54
DeGuglielmo syndrome 55
Metastatic Adenocarcinoma 56
Multiple Myeloma Metastasis 61
Boeck's Sarcoid 61
Monolayer Morphology 64
Mycoplasma Cultures 72
Summary of Morphological Study 73
Electron Microscopic Observations 85
Immunofluorescent Findings 108
Autologous Sera 109
Cross-Reactions 111
Murine Leukemia Anti-Sera 111
Rauscher Virus (RLV) 112
Moloney Virus (MLV) 114
Histochemical Findings 122
Lactic Dehydrogenase (LDH) 122
Beta-Hydroxybutyerate Dehydrogenase (BHBDH) ... 122

Acid Phosphatase 123

Discussion 126

Morphology 126

Media Factors and General Behavior in Culture 126

Mycoplasma 128

Transitional Forms 130

Virus Inclusion 132

Emperipolesis, Peripolesis 133

Electron Microscopic Significance 135

Immunofluorescence Work 136

Immplication of Autologous Sera 136

Immplications of Murine Leukemia Antisera 137

Histochemical Findings 140

Immunofluorescent Correlation 141

Acid Phosphatase 142

Conclusion 143

References 144

Appendix 173

ILLUSTRATIONS

Figure 1.	Diagram Sykes-Moore Chamber Cultures	16
Figure 2.	Cells from Non-Malignant Cultures	26
Figure 3.	Cell Degeneration	30
Figure 4.	Sheath and Cell Islands	33
Figure 5.	Lymphocyte Spreading, Type I	37
Figure 6.	Transitional Lymphocyte, Type II	39
Figure 7.	Transitional Reticulum Cell, Type III	41
Figure 8.	Foreign-Body Giant Cell, Type IV	43
Figure 9.	Lymphocytic Lymphoma	46
Figure 10.	Hodgkin's Granuloma-Paragranuloma	50
Figure 11.	Reticulum Cell Sarcoma	53
Figure 12.	Metastatic Adenocarcinoma	58
Figure 13.	Transitional Cells from Metastatic Adenocarcinoma	60
Figure 14.	Emperipolesis	63
Figure 15.	Transitional Cells	66
Figure 16.	Stained Monolayer Cultures	69
Figure 17.	Stained Monolayer Cultures	71
Figure 18.	Diagrammatic Representation of Chamber Cultures over a 30-Day Incubation Period	75
Figure 19.	Diagrammatic Representation of Chamber Cultures from Lymphocytic Lymphoma	77
Figure 20.	Diagrammatic Representation of Chamber Cultures from Hodgkin's Granuloma and Paragranuloma Involved Human Lymph Nodes .	79
Figure 21.	Diagrammatic Representation of Reticulum Cell Sarcoma Cultures	81

ILLUSTRATIONS

Figure 22. Diagrammatic Representation of Chamber Cultures from Lymph Nodes with Metastatic Cells 83

Figure 23. Normal Lymphocytes 88

Figure 24. Abnormal Lymphocytes 90

Figure 25. Abnormal Lymphocytes 92

Figure 26. Abnormal Reticulum Cell 94

Figure 27. Reed-Sternberg Cells 96

Figure 28. Reed-Sternberg Cells 98

Figure 29. A Reticulum Cell 101

Figure 30. A Portion of a Reticulum Cell 102

Figure 31. A Portion of a Reticulum Cell 104

Figure 32. A Portion of a Reticulum Cell Nucleus 106

Figure 33. Immunofluorescent Reactions 116

Figure 34. Specific Fluorescence Reactions and BHBDH Histochemical Reactions 120

Figure 35. Acid Phosphatase Histochemical Reaction 125

TABLES

Table 1. Number and Diagnostic Distribution of Lymph Nodes Cultured 23

Table 2. Correlation of Cell Types with the Number of Cases and Diagnosis 34

Table 3. Immunofluorescent Tests of Autologous Serum 109

Table 4. Immunofluorescent Tests: Cross-Reactions of Lymphocytic Lymphoma Tissue with Lymphoma and Normal Sera 112

Table 5. Immunofluorescent Tests: Cross-Reactions of Lymphoma and Non-Lymphoma Tissue with Lymphoma and Normal Sera 113

Table 6. Immunofluorescent Tests with Anti-Rauscher Sera 117

Table 7. Correlation of Immunofluorescent Response with Beta-Hydroxybutyrate Dehydrogenase and Lactic Dehydrogenase 118

Dedicated To

My Very Patient Husband

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INTRODUCTION

Increasing evidence that human hematopoietic neoplasms are of viral origins has gradually accumulated (1,2,3,4,5, 6,7,8,9,10). From time to time viral agents have been isolated (3,9,11,12) from human lymphoma and leukemia but often these have been known agents not considered to be oncogenic (11,12). Other agents isolated are still in question (9,10). Attention is currently centered on the Reo-3 virus and the herpes-like agent repeatedly isolated from Burkitt's lymphoma (13,14,15). However, until determination is made whether this agent is a deoxyribose nucleic acid (DNA) or ribonucleic acid (RNA) virus and more detailed morphological studies are done, the relationship to other agents of similar structure cannot be overlooked (15,16,17). Except for the recent work on Burkitt's lymphoma and on lymphocytic leukemia (18,19,20), conventional virus-isolation techniques employing many types of continuous lines and cytopathic effect (CPE) as criteria of virus presence were used (4,9,10,11). Cell lines have been found to differ greatly in susceptibility to known and unknown human agents isolated (9,10,21,22). In confirmation of the oncogenic nature of these agents, animal inoculations (usually mice or hamsters) of both tumor tissue and culture media from inoculated cultures have produced a variety of tumors in a variable percentage of the animals used. Both processes have left uncertainties as to the

source and nature of the agents demonstrated (4,5,9,10,11). These questions arise from the possibility of: (1) The presence of latent virus in the cell lines (22,23,24,25), (2) the presence of virus in serum and embryo extract (26), (3) the presence of helper virus as occurs with the Bryan strain of Rous sarcoma (27,28), (4) the ability of some viruses to hybridize as is seen in tumor enhancement with some adenoviruses and SV-40 infections (29,30). Some of the uncertainty can be reduced by using tumor tissue as the vehicle of isolation, as has occurred with Burkitt's lymphoma (13,14,15) and recently reported isolations from other lymphocytic leukemias (19,20,21). The morphological changes and cell behavior in cultures where CPE is not observed may be an aid to the recognition of the presence of virus in lymphoid tissue. A limited knowledge of the behavior of lymphoma under conditions of culture that restrict the presentation of new antigen was available when this project began. Only a few reports of the finding of virus-like particles in human lymphoma were known (7,10, 11,12).

The purpose of this present work is to determine what known clues to the presence of latent virus in tissue may exist in the lymphomas and often hematopoietic neoplasias by the application of a number of tests on the same sample of tissue. To this orentation the following literature review is directed in outlining those tests.

Early morphological studies (31,32,33) of rabbit and chick lymphoid tissues indicated within the limited time of observation (5 days) that early plasma clot techniques allowed: (1) transformation of lymphocytes into blasts, (2) transformation of large lymphocytes into histiocytes, (3) development of sinusoidal reticulum cells into new lymphocytes, (4) formation of polykaryocytes, (5) the identification of two types of reticulum cells both with hematopoietic potential. These functions of lymphoid tissue have recently gained more wide acceptance as normal responses (31). In pathological human node tissue Lewis and Webster (34,35) were able to follow the formation of polykaryocytes. In Hodgkin's-derived cultures these were considered distinct from Reed-Sternberg cells, however, differentiation of normal and pathological cultures was not possible. In 1921 Sabin (36) used vital dyes to follow the differentiation of cell types in human lymphoid tissue, describing in detail the nature of cell movement. Later in a series of articles Osgood (37,38,39) reported the culture of lymphocytes in gradiant and monolayer culture, which transformed to attached spindle cells in long-term culture. When rabbit serum was used to culture human peripheral blood, chiefly "macrophages" remained that later assumed characteristics of embryonic mesenchyme (33). Lambert (40) in a study of chick tissue concluded that polykaryocytes were foreign-body giant cells resulting from the fusion of

three types of outgrowth cells in the cultures. Additional studies have confirmed this (41,42). Lewis and Webster (43) later, in a larger study, were able to differentiate between normal and pathological node tissue, particularly in the formation of foreign-body giant cells and in the appearance of wandering cells. The wandering cell had the size of a large lymphocyte or lymphoblast but with histiocytic movement, a crescent-shaped nucleus, and hairlike processes, and was most numerous in Hodgkin's cultures. Grand (44,45) reported virus inclusions in Hodgkin's cultures, which were transmissable to normal human lymphocytic tissue by cell-free filtrate. However, secondary agents have been shown to occur in a percentage of Hodgkin's cases (46,47) and in other lymphomas (48,49). No identification of the agent was made. Liquefaction of the plasma clot (50) used in most of the early research was attributed to enzymatic action by Rettino and Hollender in 1949 (51). Later inhibition of liquefaction by trypsin inhibiters (52) supports this contention as does some chemical data (53). In addition to Hodgkin's tissue, Lündback and Löfgren (11) cultured a large series of nodes from cases of Boeck's sarcoid. A unique lysis of portions of the outgrowth and their regrowth with the presence of a vague greenish pigment typified these cultures.

For almost a decade there was an absence of reported studies of lymphoma in tissue culture. In 1961 Siegel and

Smith (52) confirmed the formation of foreign-body giant cells in Hodgkin's and lymphocytic lymphoma cultures. Again no clear distinction could be drawn between normal and pathological involved tissue. Plasma-clot techniques and trypsin inhibiter were used in these cultures, which may have contributed to this situation. Hoster and Reiman (54) indicated that two distinct types of Hodgkin's disease could be identified in cultures based on the degree of reticular cell outgrowth. Histologically, three types are identifiable (55): paragranuloma, granuloma, and a sarcoma form that is chiefly reticular in nature.

In summary through 1961, polykaryocytes are distinct from Reed-Sternberg cells; morphology and function indicate that these are foreign-body giant cells. The appearance of these cells in normal tissue may be stimulated by foreign fibrin used in the making of the plasma clot (51) or other antigens including small numbers of virus (51,54). Chicken lymphoma virus may possibly be introduced into the cultures with plasma or embryo extract (51,52,56). Transformations of various cell types had been observed by early researchers.

By dispersing node tissue with trypsin and not utilizing embryo extract, only a few multinucleated cells were found by Sykes et al (57) in a series of 72 cultures over a 4-year period. From this procedure, in most cases, a giant-cell outgrowth resulted that demonstrated a series of events involving enlargement of the Golgi zone, vacuolization,

and area degeneration with eventual loss of the culture. Electron micrographic studies indicated the presence of virus-like particles in these cells.

From areas of research not involving human lymphoma additional information was drawn pertinent to the problem. Morphological classification of the cells involved can be accomplished under either bright-field or phase-contrast microscopy by attention to the type of motility and general configuration of the cell (58). The use of vital dyes (31,36,59) is sometimes of assistance in this identification. Inflammatory responses of guinea pig lymphoid tissue to culture conditions similar to those used in this study was not remarkably different than some of the previously mentioned changes except for apparent amitotic division of certain large reticular cells (60,61). In the skin-window technique of Rebuck (62,63) it is not clear whether the secondary mononuclear cell is a transformed lymphocyte or a histocytic cell derived from circulating blood monocytes. The formation of the foreign-body giant cell in inflammatory reactions and certain pathological situations in vivo is well known (64).

In tissue-graft rejection lymphoid tissue is involved in two ways; first, that of circulating-antibody production; and second, the fixed-tissue, delayed-hypersensitivity mechanism, requiring contact with lymphocytes (65,66). Peripolesis, the attraction of lymphocytes to other cell

types, and emperipolesis, the inside wandering about of lymphocytes within other cell types, are involved in tissue-graft rejection (66,67,68) in an as-yet-unknown mechanism. Emperipolesis is seen normally in the function of lymphocytes crossing the gut epithelium (69), in the thymus (70) reticulum cells, and in the crossing of lymphocytes from the circulating blood to sinusoids in lymph nodes (71). Time-lapse studies have confirmed the motility of lymphocytes within the host cell for many hours and often their subsequent release (72,73,74). The peripoletic association of lymphocytes with macrophages seems to be chemotactically stimulated, and an attraction to late anaphase cells was observed when lymphocytes were within 100μm (68,69). Involvement of lymphocytes in graft rejection, hence tumor distruction by contact involving cell death, has been shown in vitro studies (75,76,77). Electron-micrographic studies have confirmed the presence of mouse lymphoma cells within membrane-enclosed vacuoles of giant reticulum cells (78). Cultured lymphoma cells were observed to undergo mitosis within reticulum cells in both mouse and human tissue (73).

Briefly, the action of known oncogenic virus on cells in culture indicates the slow release of virus without the disruption of the cell (79,80,81) by membrane budding. Recent reports confirm this observation for murine leukemia agents also (82,83) and a persistent low

percentage of cells demonstrating the virus. Fragmentation of nucleoli and an increase in nucleolar volume have been reported in response to some oncogenic viruses (84,85,86), though neither is exclusively a finding of virus infection. Loss of contact inhibition described initially by Abercrombie and Ambrose (87) and others (88,89,90) is a more consistent attribute of the malignant cell. Studies with SV-40 indicate transformation of human cells in culture (91). These indicate little difference in behavior from corresponding rodent cells more thoroughly studied. Immunofluorscent techniques were used in following these infections that result in transformations, suggesting application of this technique in this study.

Specific antibodies in the case of human neoplasia must initially come from autologous serum, involving dealing with two or more unknown systems complicating the interpretation of the results. Rapport and Graf in 1961 (92) theorized that antibodies should exist against oncogenic virus or altered metabolic products and altered-cell structural protein in human neoplasia. Though tumor-distinctive antigens were demonstrated in human cancer by Witebsky, Rose, and Shulman (93) and others (94,95,96,97) little information concerning the presence of antibody in autologous serum against tumors has been reported (92,96). Antibodies against erythrocytes (Coombs' positive) are known to develope often in lymphoma and leukemia late in the course of the disease. The existence of many types of

antigens in human tumors has been gradually established (98,99,100). There is indication that some new antigens may result from a reversion to the production of embryonic protein, mucopolysaccharide, or lipid electrophoretically distinct from the adult counterpart (101,102). In mice, viral-induced antibodies are known to be protective against tumor growth as is antitumor antibody (103,104,105).

Tumor antibodies have been shown to be protective against tumor growth but immunological enhancement is also reported (106,107) in studies using murine leukemia as a model system. Attia, Deome, and Weiss (108) in 1966 confirmed in the mammary tumor virus system that, though variable, tumor antigens do induce the production of a cytotoxic immune response. A few studies (109,110,111,112) indicate that a percentage of human patients do have antibodies against tumor, or antigenic agents within their tumor. Gluck in 1962 (113) showed the effectiveness of immunofluorescent techniques, indicating their usefulness in tumor investigation, particularly the two-stage technique of Coons (114). The questions to be examined by this method are: What is the frequency of the appearance of autologous serum antibody in lymphoma patients? Is the antibody individual-specific? Can it be found in normal individuals?

Enzymatic changes with virus infection have been studied extensively in bacterial genetics but only recently

has application of this knowledge to virus-infected mammalian cells been made (115,116,117). Lactic dehydrogenase (LDH) induction by Riley agent and other virus (118,119,120,121), and elevation of nucleoside kinases were investigated in reference to oncogenic virus in recent studies (115,116). The general shift in metabolism is not sufficiently specific to serve as an indicator of virus mechanism or presence within the cell, but specific enzymes involved with the glycolytic pathways may prove of interest. Histochemical techniques are more applicable to this study where not all cells are necessarily involved than are biochemically more quantitiative procedures. Enzymatic alteration of human lymphoma tissue was studied extensively by Braunstein (122,123,124). Hodgkin's tissue demonstrated elevated aminopeptidase, acid phosphatase, and alkaline phosphatase in the abnormal reticulum cells. One lymphocytic lymphoma case had a marked elevation of beta-hydroxybutyrate dehydrogenase (BHBDH). Both LDH and acid phosphatase are elevated in inflammatory response generated by the skin-window technique used by Rebuck (62) and Wulff (125,126).

Acid phosphatase also has been shown to be elevated in the presence of Rauscher virus infection (129). In this study it has been possible to work with only a few enzymes in a few cases; a much more extensive study is of course desirable, as has been done in bone marrow and peripheral

blood by Hayhoe, Quaglino, and Doll (130).

Before oncogenic virus isolation could be considered it was necessary to examine the morphological characteristics of the human lymphoid tissue by methods not involving known additional antigenic stimulation, and to determine if there are clues recognizable that may suggest the presence of such agents. At the inception of this project, information available suggested that virus-like particles were present in human tissues from blood dyscrasias. Some methods and clues are needed to follow these particles in culture with greater economy of time than is allowed by electron micrographic methods. Morphological changes other than CPE were observed for such clues. As these were found insufficient, immunofluoresence using autologous serum on original node tissue was considered in an attempt to find a usable system of antigen detection. This too was not consistent. Anti-specific murine leukemia virus sera were also tried in an effort to find antigen antibody systems. Histochemical alterations were considered also as clues to the presence of oncogenic virus or other foreign agents. The problem is not only one of the presence of virus but one of certainty of the source of the agents isolated, which can best be gained by immunological methods utilizing the patients' serum or tissue in initial testing.

METHODS AND MATERIALS

Through the cooperation of the surgical staffs and pathology departments of the University of Oregon Medical School and local U. S. Veterans' Hospitals, human lymph nodes from pathological conditions and normal nodes from surgery unrelated to neoplastic disease or known infectious process were secured. The larger portion of each pathological node, bisected at the hilus, was reserved for histological examination by the surgical pathologist whose reports constitute the diagnostic information used in this paper. Imprints of the nodes were made for subsequent immunofluorescent tests and May-Grünwald Giemsa staining. Explants were cultured in Sykes-Moore chambers and in milk-dilution bottles. Mycoplasma cultures were done on many but not all specimens. Tissues for electron microscopy were fixed in osium tetroxide (131). From the larger specimens portions were frozen in liquid nitrogen for later histochemical and additional antibody studies.

Culture Techniques

In Sykes-Moore chambers (132), small explants approximately 1 mm were trapped under a complete dialysis membrane diaphragm, thus isolating the tissue from the medium portion of the chamber (133,60,61) as shown in Figure 1. In milk--dilution bottles tissue fragments were trapped under or between small cover slips, and about ten fragments were placed in each bottle.

Medium 199 (134) with 10% calf serum, modified by the addition of 1mM each of glutamine, glycine, cysteine, serine, and pyruvate in addition to 100 mg/liter of ascorbic acid, was used initially in this study for the chamber cultures. The bottle cultures were fed twice weekly with medium containing 20% calf serum. Later, to obviate the use of nucleotides and to secure better survival of the monolayer cultures, a modification of Minimum Essential Medium of Eagle (135) was used. A detailed description of these media appears in the Appendix.

Vital staining was done after the technique of Rose (136) using 0.1% of Neutral Red and Methylene Blue. Iodonitrotetrozolium (INT) was used as a mitochondrial stain in place of the more toxic Janus Green B. This dye reaction under these conditions is felt to demonstrate succinic dehydrogenase (137).

Photographic Processing. Chambers were observed and photographed at frequent intervals by bright-field microscopy employing Köhler illumination. High-constrast copy film (Eastman Kodak), developed for maximum contrast and resolution, was used. Best results in developing were obtained with Dectol (Eastman Kodak) 1:1 for 7 minutes at 70° F. Film exposure was adjusted to a minimum time consistent with this developing using a green filter.

For fluorescent microscopy, Tri-X pan film was used. FR-22 (FRco) was the developing process used enhancing

Figure 1. Diagram of Sykes-Moore Chamber Cultures Approximately 4X actual size; Ex = explant, D = dialysis membrane extending under R, R = silicone rubber ring seal, CS = No. 1 cover slips 25 mm round, M = media space, FCA = free cell area around the explant. Medium was changed through holes in the metal casing with #25 hyponeedles. Medium space was completely filled with 1 cc of medium. The silicone rubber ring allows for gaseous exchange requiring for incubation a moisture-saturated 3-5% CO_2 atmosphere.

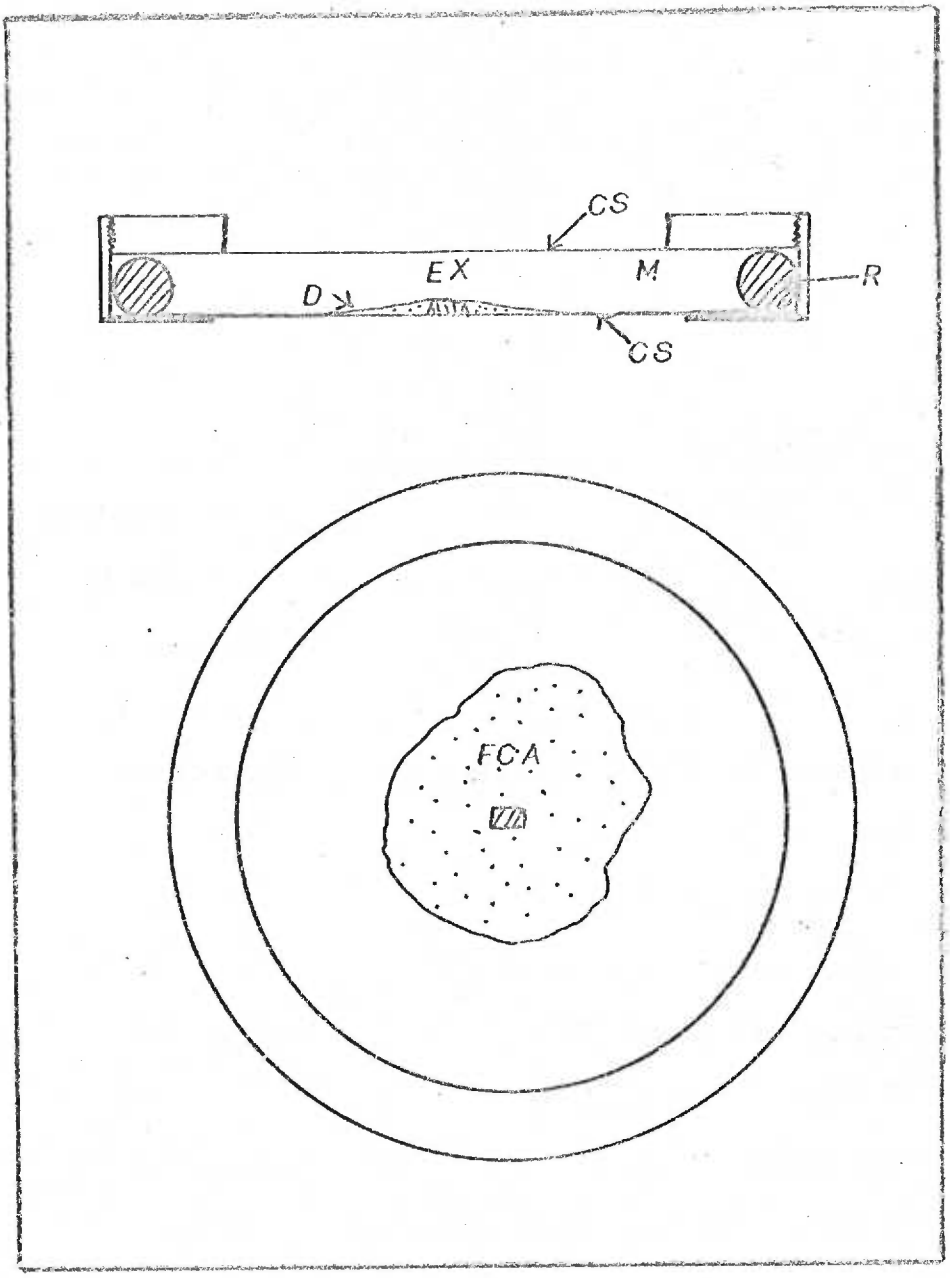


FIG. 1 Diagram Sykes-Moore Chamber Cultures

slightly the effective film speed, and offering a finer grained negative than other processes. Exposures under oil immersion were for 30 seconds, 10 seconds under lower magnification.

Immunofluorescent Methods.

Imprints and liquid nitrogen-frozen cryostat sections of mouse and human tissue (stored at -70 C) were fixed in acetone for 10 minutes at room temperature. Following fixation, storage with a desiccant for up to 3 months was found to be possible, but in general the fluorescent antibody (FA) tests (except for cross reactions) were completed within two weeks. With human sera an indirect test (138) utilizing a second reaction with labeled rabbit antihuman globulin was used. A rhodamine albumin counter-stain was used to help control some of the autofluorescence of the tissue. Rabbit antihuman globulin was labeled by Rinderkrechect's method (139), passed through a Sephadex 50 column to remove excess fluorescence and nonspecific protein, and then frozen in 2-ml aliquots. Absorption with mouse liver powder and dilution to the working concentration followed by storage in single-use containers completed preparation of the antihuman globulin for testing. All sera were stored frozen in small aliquots and thawed only once. Two negative control procedures were employed to evaluate extraneous binding of the antihuman globulin serum either to cells containing antibody or to damaged cells that might become globulin coated. For the second control, only

rhodamine counterstain was used; here specific yellow-green fluorescence was observed in the granules of eosinophils and mast cells. This may be attributed to the natural fluorescence of some of the monamines contained by these cells (140,141).

In the absence of a supply of globulin-containing tissue for a positive control, a drop of serum was air dried on a slide and fixed in acetone. Positive, specific fluorescence was easily observed. Immunofluorescent complement fixation was done in a few cases (138) using labeled anti-complement sera. Test sera were mixed with equal parts of guinea pig complement diluted 1:10. Following this reaction the labeled anticomplement sera were allowed to react for 30 minutes at 37 C. Saprophytic Mycobacterium were used as a positive control test of the complement-anticomplement reacting system. The antiserum from rabbits immunized with Freund's adjuvant contain complement-fixing antibodies against Mycobacterium, making it a convenient test system. The direct fluorescent test (138) was used with anti-Rauscher (RLV) serum produced in rabbits in this laboratory and with the labeled anti-RLV produced in monkeys supplied by Doctors Rauscher and Fink of the National Institutes of Health (142), and the indirect fluorescent test was used with the unlabeled anti-Moloney virus serum supplied by Doctors Moloney and Pinkel (143) after their recommendations. The anti-RLV serum produced in our laboratory was labeled using the techniques of Rinderkerchect (139).

Capillary precipitation indicated a liter of 1:100 or above after absorption with normal mouse tissue. The antirabbit globulin serum was secured from commercial sources and labled by the same method. Immunization schedules used in producing the sera are in the Appendix.

Histochemical Procedures.

With diphosphopyridine nucleotide (DPN)-dependent LDH and BHBDH, histochemical analyses were done without prior fixation of cryostat sections of liquid nitrogen--frozen tissue stored at -70 C. The LDH substrate concentrations were those of Pearse (144). Substrate for BHBDH, concentrations were 0.2 mg/ml of beta-hydroxybutyrate in the dehydrogenase buffer of Pearse (144). Higher concentrations of specific substrate were found to be inhibitory to the reaction. Nitro blue tetrazolium (Nitro BT), 4 mg/10 ml of substrate, was used as hydrogen ion acceptor in both substrates. Incubation time was 15 minutes for LDH and 45 minutes for BHBDH.

Acid phosphatase reactions using the lead techniques of Gomori as modified by Novikoff et al (145) were done following 10 minutes cold glutaraldehyde fixation (146). (See Appendix.) Incubation time was 2 hours at 37 C. Controls were incubated in complete substrate without the specific reaction compound. The diazo-dye technique of Burstone (147) was also used in some studies without prior fixation other than air drying. The specific substrate

used was naphthol-ASMX phosphate. Incubation time was 30 minutes to 2 hours.

Phospholipids were stained using the techniques of Ackerman (148): Briefly, formalin fixation for 30 seconds, periodic acid treatment, and staining for 24 hours in Sudan Black B in propylene glycol. This was followed by a rapid rinse in 70% alcohol, counterstaining with safranine, and mounting in polyvinyl pyrolidine (PVP).

Schiff's periodic acid stains (PAS) were done after the method of Pearce (144). Glutaraldehyde (2% v/v) in o.1 caccodolate buffer, pH 7.2, was used for fixation. Light green (o.2%) was used as counterstain followed by dehydration in alcohol and mounting in Permount.

For imprints and cultured cells, air-dried slides were fixed in methanol and stained first with May-Grünwald stain, then washed and stained with stock giemsa diluted 2:25 with tap water. The May-Grünwald stain is used in the same manner as the more familar Wright's stain. In comparison with blood smears, lymph node or organ imprints required longer staining times.

Electron Microscopy Methods.

Small fragments of node tissue taken from different areas of the node were fixed in 1% osmium tetroxide at 10 C for one hour. Following rapid alcohol dehydration and embedding in Epon after Luft's technique (149), the

tissue was sectioned on either a Sorval-Porter-Blum ultramicrotome or an LKB Ultratome. The ultrathin sections were picked up on a Formvar-coated grid and strained with uranyl acetate and lead citrate (150). Specimens were examined on an RCA EMU electron microscope.

RESULTS

Morphological Study.

Listed in Table 1 are the number and types of cases studied. When the type of medium was changed, six parallel cultures were followed. The only differences noted were improved survival, more consistent outgrowth, and a consistent though not marked higher level of motility in the chamber cultures. Fifteen normal nodes were followed for between 30 and 60 days. Those cultures in which free lymphocytes were not found proved to be chiefly fibrous tissue and were excluded from this table.

Under the conditions of culture diagrammed in Figure 1, in the Sykes-Moore chamber (132) following 4 to 6 hours' incubation, many free cells were found in the area surrounding most explants. These demonstrated motility typical of their particular cell type, or remained nonmotile. In normal node tissue lymphocytic cells (representing 95-98%) demonstrated slow motility, often recognized by the hand-mirror shape and haloplasmic membrane consistent with earlier descriptions (31,32,35). Granulocytes displayed a rapid apparently random pseudopodial motion frequently bipolar in nature. All granulocytes lost motility after 72 hours and rapidly disappeared from the cultures. Eosinophils persisted for a slightly longer period. Mast cells were occasionally observed. These had a slow progression with an extended haloplasmic membrane, which

TABLE 1
NUMBER AND DIAGNOSTIC DISTRIBUTION
OF LYMPH NODES CULTURED

Normal	14
Hodgkin's granuloma	4
Hodgkin's paragranuloma	2
Lymphocytic lymphoma	3
Lymphocytic leukemia	1
Chronic lymphocytic leukemia	1
Lymphoblastic lymphoma	3
Lymphosarcoma	2
Reticulum cell sarcoma	2
Dermatopathic lymphadenitis	5
Lymphadenitis (bacterial)	2
Reactive hyperplasia (Follicular) (Reticulum cell) (Lymphocytic)	10
Fatty replacement	2
Sarcoidosis	1
Metastatic carcinoma	8
Multiple myeloma	1
Erythremic myelosis (DiGuglielmo syndrome, bone marrow)	2
Total cases	63

* The only patient of the series that had been extensively treated with cytotoxic drugs.

tended to be ruffled and more changable in direction than the lymphocytic cells, as described for histiocytic cells (35). Room temperature and lower temperatures reduced the general level of cellular motility as did loss of CO_2, causing pH ranges above 7-7.1.

Within the first 18 hours in both pathological and normal node cultures reaggregation of the lymphoid cells was observed. Figure 2 shows these aggregates. Central to the smaller units were found nonmotile granulocytes, lymphoblasts, or reticulum cells; in larger units the center cell was obscured. All stages of lymphoid development and reticulum-cell elements were represented in these units as were, occasionally, mast cells. Though Robineaux (60,61) described, in guinea pig spleen, an unequal amitotic division of the reticulum cells found in the centers of these aggregations, such a mitote division was not observed in cultures of human lymphoid tissue. Plasma cells were never numerous and only in one culture, where erythrocytes from a recent transfusion were present, was formation of plasma cells observed.

In normal node cultures vital dyes at 6 to 18 hours demonstrated the same general pattern of response described by Ackerman in bone marrow (59). In motility in lymphocytic and reticulum cells, the mitochondria and other organelles trailed the nucleus (Fig. 2). Blasts in general displayed a greater number of granules with Methylene Blue and

Figure 2. Cells from Nonmalignant Tissue Cultures in Sykes-Moore Chambers.

A&B. These pictures show cell aggregates as formed in most cultures after 6 to 8 hours of incubation. These cells were at times actively motile and all cell types were represented. The central cell in A may be a plasma cell. The difference in size and appearance is due to mechanical flattening by the dialysis membrane in A.

C. This picture shows an actively motile lymphoblast with the cell organelles trailing; above it is a slowly moving mature lymphocyte, moving away from the blast, partially out of focus.

D. Plasma cells, when present, were consistently non-motile in culture.

E. The large cell is a hemocytoblast; its appearance suggests motility more closely allied to the reticulum cell type than to the lymphocytic series at this stage of development. Note the extremely large nucleoli.

F. The large central cell with the finger-like projections was actively phagocytizing. Lymphocytes were not observed to enter this cell, though they moved extensively on its surface. From a reactive hyperplasia in a 9-month-old child.

G. Macrophages from another culture showing no evidence of active phagocytosis. This is the more common situation in most of the cultures.

Bright field; 2100X inclusive of photographic magnification.

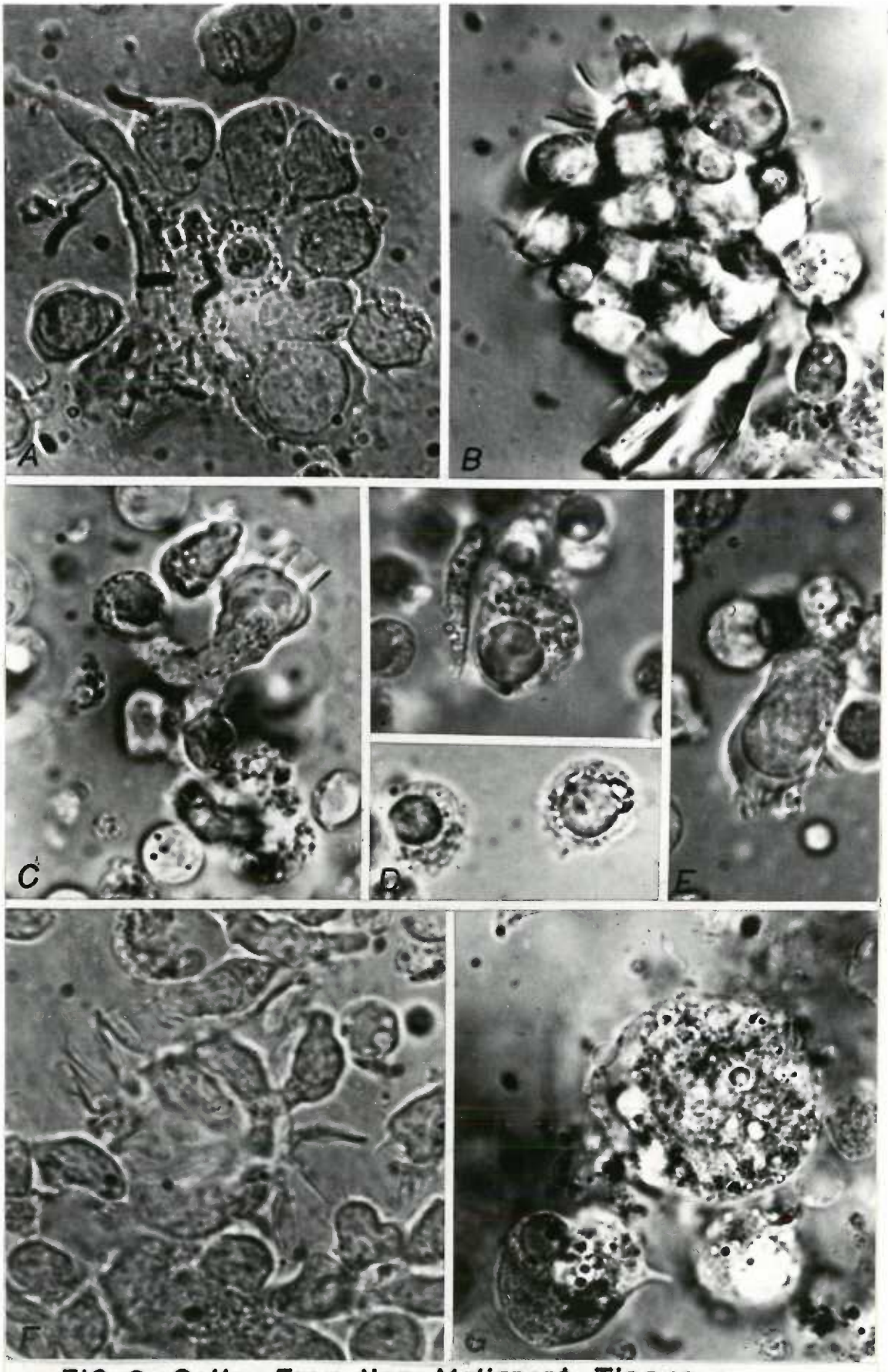


FIG. 2 Cells From Non-Malignant Tissue

Neutral Red than did mature cells. Staining of the mitochondria with INT demonstrated from two to five reddish granules in mature lymphocytes, and Methylene Blue demonstrated one or two larger vacuoles. As any one cell lost its metabolic integrity a faint blue spread to the entire cell. Observation over a 4- to 6-hour period was possible though both Neutral Red and Methylene Blue are more toxic than the INT. Cultures stained with INT seemed to clear the formazan or the mitochondria from the cells over a 5-day period with some increased distruction not seen in unstained cultures. Blasts and reticulum cells contained a larger number of mitochondria than did the mature lymphocytes. Three pathological cultures had greatly elongated mitochondria in cells of the lymphocytic series. These cases were a lymphadenitis, a reactive follicular hyperplasia, and a lymphoblastic lymphoma culture. Motility in the reactive hyperplasia case was more frequent and rapid than other cultures studied. Macrophages (Fig. 2) were seen in new cultures but were more numerous at about 10 days except in the culture containing many erythrocytes.

In the first twenty cultures studied an increase in both size and number of Neutral Red-stained granules in lymphocytes occurred between 18 and 72 hours; a lysis of many cells was subsequently noted. Following this lysis only cells containing small granules were observed.

Staining with Sudan Black B confirmed the phospholipid nature of the granules, which were also found to contain acid phosphatase. These reactions confirmed the lysosomal nature of the enlarged granules (150,151,152).

Degeneration of the lymphoid elements in both normal and pathological cultures occurred slowly. Area differences within a chamber were recognizable. Different forms of degeneration (Fig. 3) including changes described by Hirsch (191) as attributable to lysosomal leakage, loss of membrane competence, or rupture were present. The formation of a large central, deep Neutral Red staining body was the predominant type of degeneration observed in normal cultures. This may represent derangement of the lipid metabolism of the cell. Both karyorrhexis and karyolysis occurred (Fig. 3), in addition to a karyoclastic process associated with loss of cytoplasmic osmolarity, recognizable by Brownian movement. In pathological tissue cell lysis and zeosis predominated.

By the end of the first week most normal cultures had a partial or complete organized sheath derived from sinusoidal reticulum cells around the outer edges of the explant (Fig. 4). This occurred in the absence of node capsule but not in tissue where heavy fibrosis existed in the explant, or where lymphocytes were the only cell type present, as in some lymphomas. The cells forming this sheath were consistently granular and lymphocytes exhibited some peripoletic activity with them. Emperipoelesis was not

Figure 3. Cell Degeneration

A. The first and fourth cells seem normal but are at a different depth of focus. The second and third cells are examples of karyorrhexis. The last of this group exhibits zeosis (cell boiling or blebing). Three to four days in culture.

B. It is not uncommon to find dividing cells exhibiting a marked zeosis that does not necessarily result in degeneration of the cell as shown here.

C. In this picture only one cell shows signs of degeneration. In the central cell karyorrhexis seems to be occurring.

D. The swelling of the nuclei and cytoplasm indicates hydropic degeneration.

E. The lower cell contains a large liped ball and no nuclear structure. The other intact cell has lost its nuclei, possibly through karyolysis and retains its normal size.

F. This cell corresponds more directly with the descriptions of cloudy swelling described in most texts of pathology.

G. On the right are two normal nonmotile lymphocytes. The body on the left is the remains of a cell undergoing a degenerative process involving lysosomal enzyme release as described by Hirsch (153).

Bright field; 2100X inclusive of the photographic magnification.

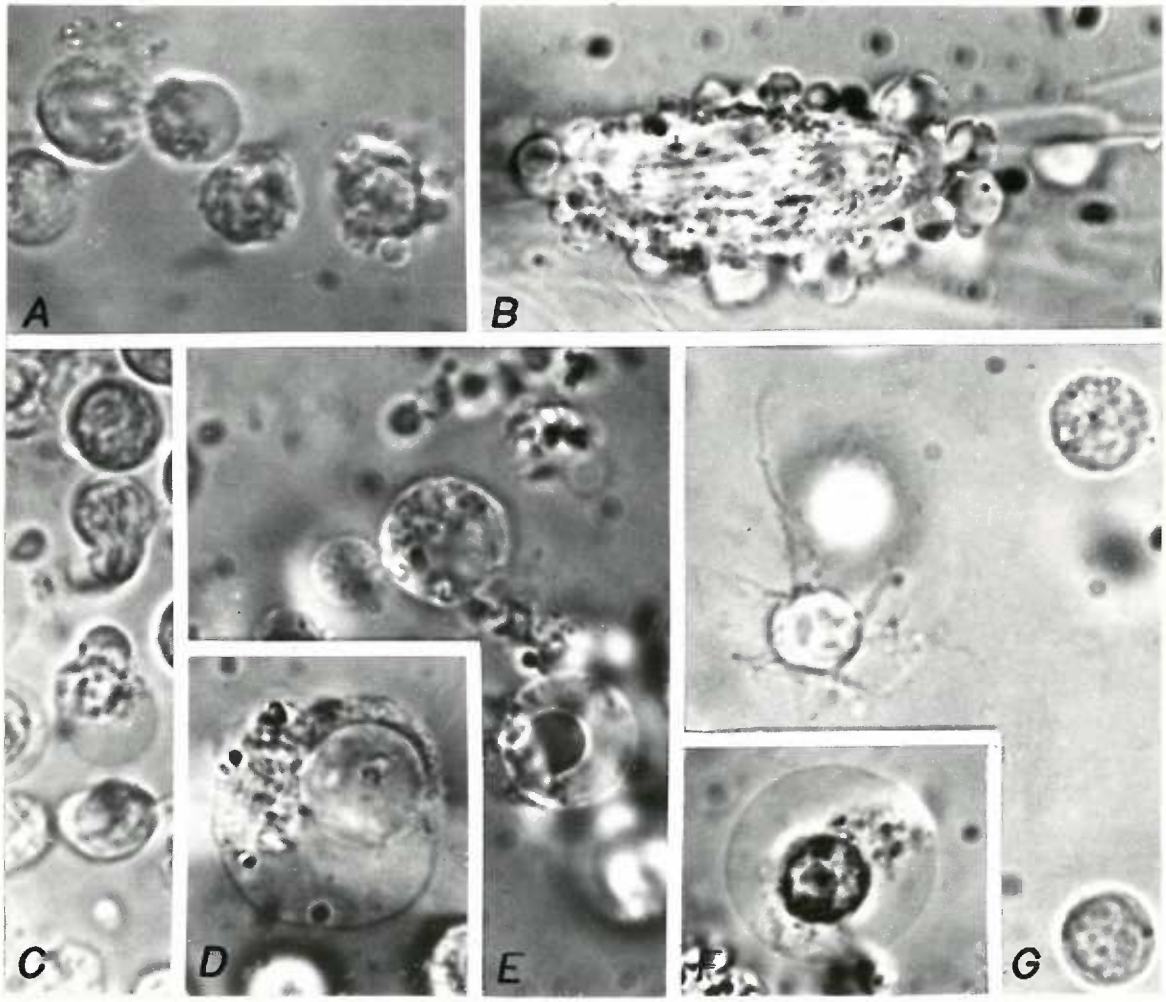


FIG. 3 Cell Degeneration

observed in the normal cultures. Eventually between 30 and 90 days' slow proliferation of these cells occurred, forming a monolayer of giant spindle cells, epithelial cells, and smaller fibroblasts from the capillary endothelium. Changes in the first two cell types suggested hemocytoblastic potential, more readily observed in some of the pathological conditions. In pathological cultures the extent of malignant involvement seems to be reflected by failure of this sheath to form or its abortive formation. In these cultures, islands or balls of new cell activity (Fig. 4) formed. Individual cells were elongated, smooth, and granular. When free of the island and motile, similarities to the wandering cell of Lewis and Webster (35) were observed (Fig. 4), including the crescent-shaped nucleus, light granulation, and hairlike projections.

As the result of an article published by Shereck and Donnely (154) about hairlike projections on certain peripheral blood cells in certain histiocytic leukemias, a careful review of the cultures being dealt with here produced some significant findings.

Table 2 shows the correlation of four cell types, three having projections from the cell membrane with the diagnosis of the patient. The cultures in table 2 were selected from the total cultures handled because of more consistent media factors and survival for 4 weeks or longer, as well as the fact that they were scanned for

Figure 4. Sheath and Cell Islands.

A & B. The denser tissue on the left is the explant now bounded by one of the overlapping granular spindle cells forming the sheath. The clear area immediately around the explant was a consistent finding with the formation of the sheath. 800X.

C. A motile transitional cell such as this is though to be the wandering cell described by Lewis and Webster (35). 2100X.

D. Cell islands composed of the transitional cells were found in lymphoma cultures and some reactive hyperplastic cultures. These were always free of the explant, having the appearance of balls or nodules of over-lapping slightly elongated cells with fine granulation, large nuclei, and prominent nucleoli. Examples are from a Hodgkin's disease case. 400X.

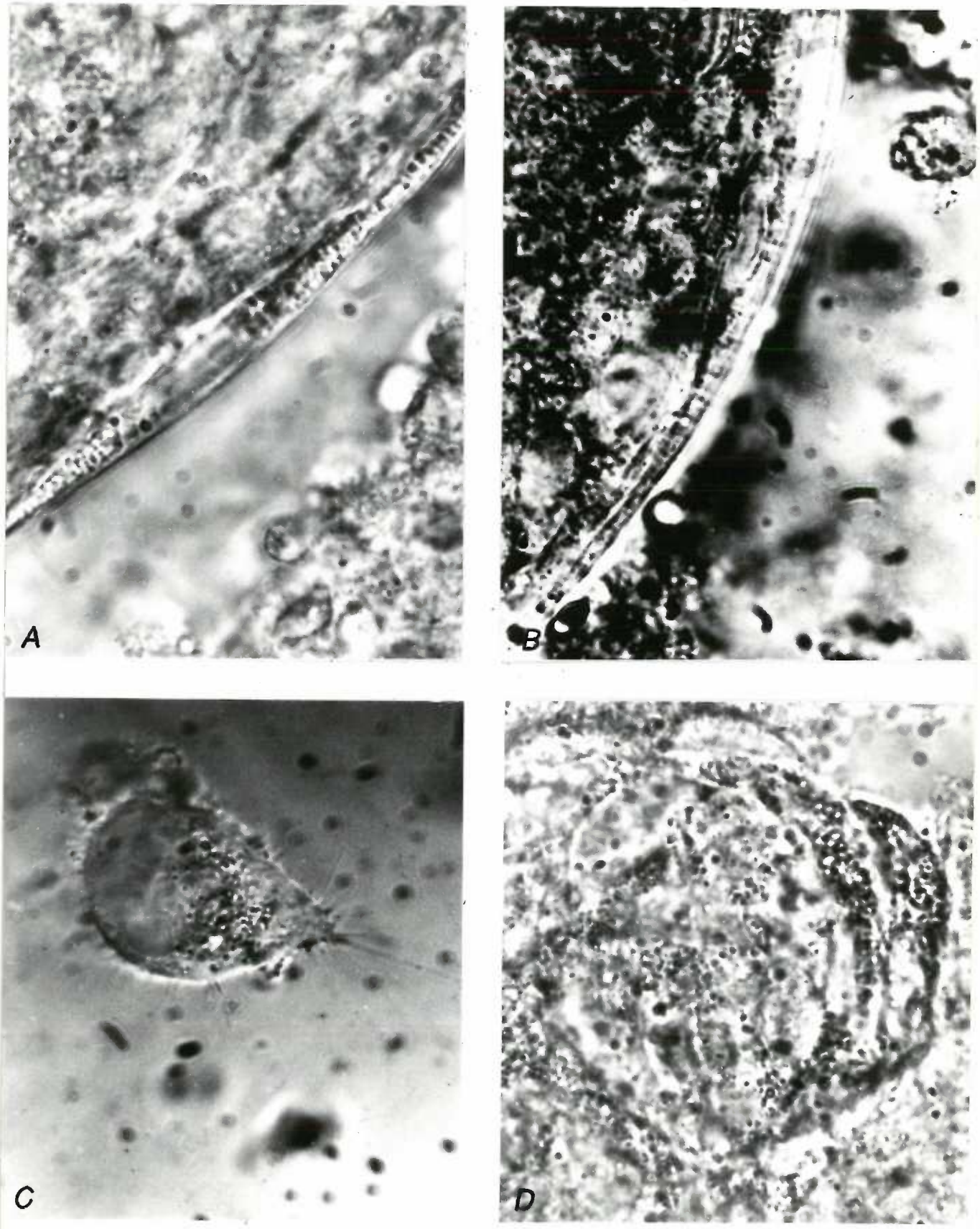


FIG. 4 Sheath And Cell Island

TABLE 2
CORRELATION OF CELL TYPES WITH THE NUMBER OF CASES AND DIAGNOSIS

No. of cases	Diagnosis	I	II	III	IV
6	Normal	0	0	0	0
2	Adenocarcinoma metastases	+	+	0	+
2	Reactive hyperplasia (lymphocytic)	+	+	0	+
1	Granuloma (mycological)	+	+	0	+
1	Fatty replacement	+	+	0	+
2	Lymphadenitis (bacterial)	+	+	0	+
2	Hodgkin's granuloma	+	+	+	+
1	Lymphoblastic Lymphoma	+	+	+	+
1	Lymphosarcoma	+	+	+	+
2	Reactive hyperplasia (reticulum cell)	+	+	+	+
2	Dermatopathic lymphadenitis	+	+	+	+
3	Reticulum cell sarcoma	+	+	+	+
2	Hodgkin's sarcoma	+	+	+	+

0 - not demonstrated
+ - cell type demonstrated

these cell types.

Type I was a spreading and attachment of a few lymphocytes on the cover slip (Fig. 5). These spread cells ruptured and remained as fragments after the first 24 hours. In pathological cultures 1-2% of the lymphocytes in each chamber were involved. In normal cultures a rare cell was found in some chambers.

Type II had the morphology of a large lymphocyte with long projections extending from the cell membrane. Transformation of this cell over the next few days gradually occurred (Fig. 6). The enlarged cell, became more granular, and often attached to the glass. The protrusions of cell membrane increased and became points of attachment to the cover glass. In the mycological granuloma from which Nocardia was cultured, 60% of the lymphocytes were Type II; in Hodgkin's granulomas about 10% of the cells were of this type. Other cultures demonstrated smaller numbers of these cells.

Type III was found after 2 weeks of culture. This cell may represent further transformation of the Type II cell but it was not possible to determine sequence of changes (Fig. 7). These cells were distinctly spiny in appearance and of histocytic type, with granular cytoplasm, a crescent shaped nucleus, and a prominent nucleolus. Moderate hypertrophy and attachment usually occurred. In most cases this cell type was not numerous but was striking in appearance, not easily overlooked and sometimes remained

Figure 5. Type I Morphological Alteration, Lymphocyte Spreading.

This example is from a culture containing metastatic adenocarcinoma of the lung. The small nonmotile lymphocyte in the drawing is a size reference for the next four drawings. The photographic magnification was unavoidably altered.

Approximately 2100X.

Type I

FIG. 5 Lymphocyte Spreading

Figure 6. Type II, Lymphocyte Transformation.

In the drawing the short arrows indicate progression of transformation over 2-5 days in culture. The long arrow indicates the direction of motility in the transformed cell. The normal round lymphocyte is a size key relative to the next four figures.

A & B. These cells are from the same culture photographed several days apart.

C. This modified cell was taken of a normal culture showing a lymphocyte modified in response to cell-free Hodgkin's material.

D. This cell demonstrates the reticulum cell-like motility apparent after further hypertrophy of these cells (only infrequently followable).

E. Transformed lymphocytes from a culture containing Nocardia; note the extremely long filaments extending from the cell membrane (arrow).

Approximately 2100X.

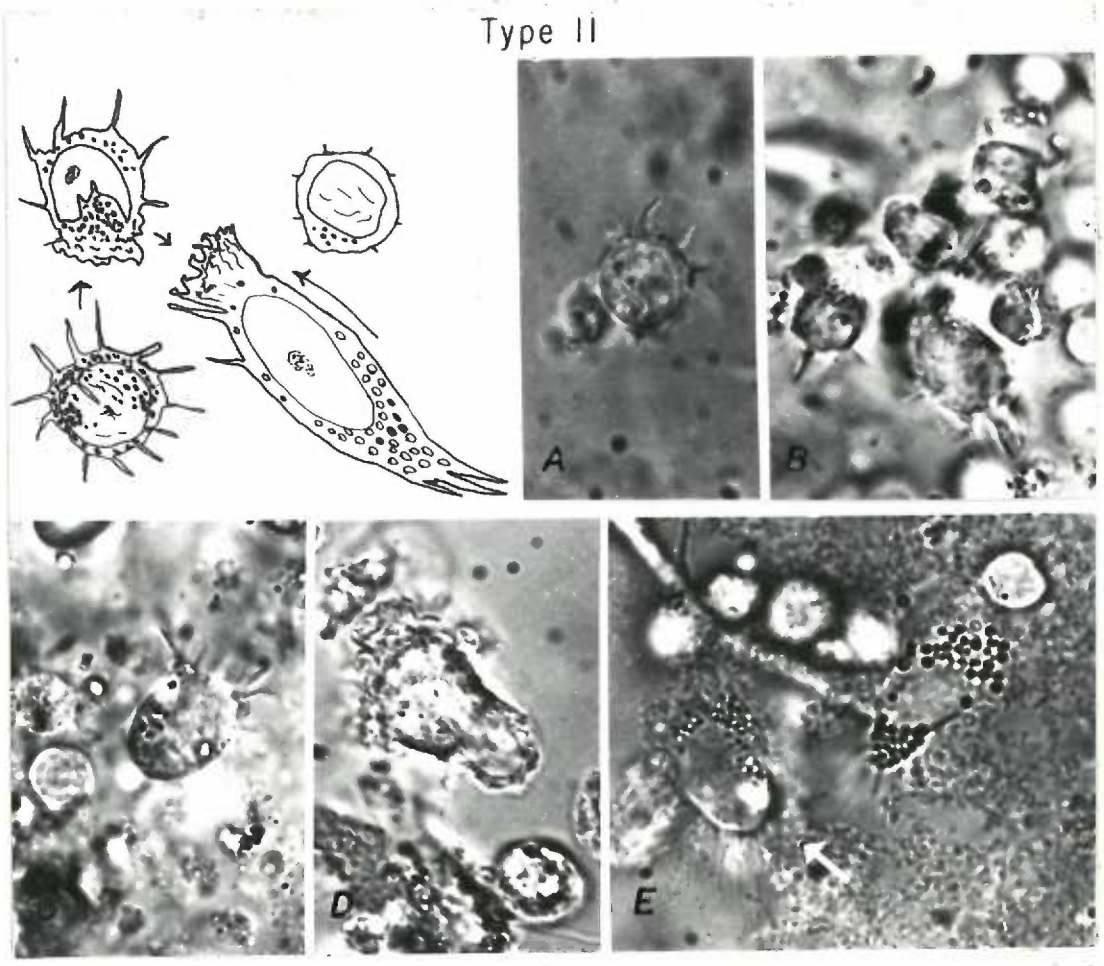


FIG. 6 Lymphocyte Transformation

Figure 7. Type III. Transitional Reticulum Cell.

These cells, found after 10 to 14 days of culture, were nonmotile in this form. With further hypertrophy they may become slowly motile or attach to the glass by the extended membrane processes.

A & B. These pictures are different depths of focus of the same cell. The arrow in B indicates the large crescent-shaped nucleus. Approximately 1500X.

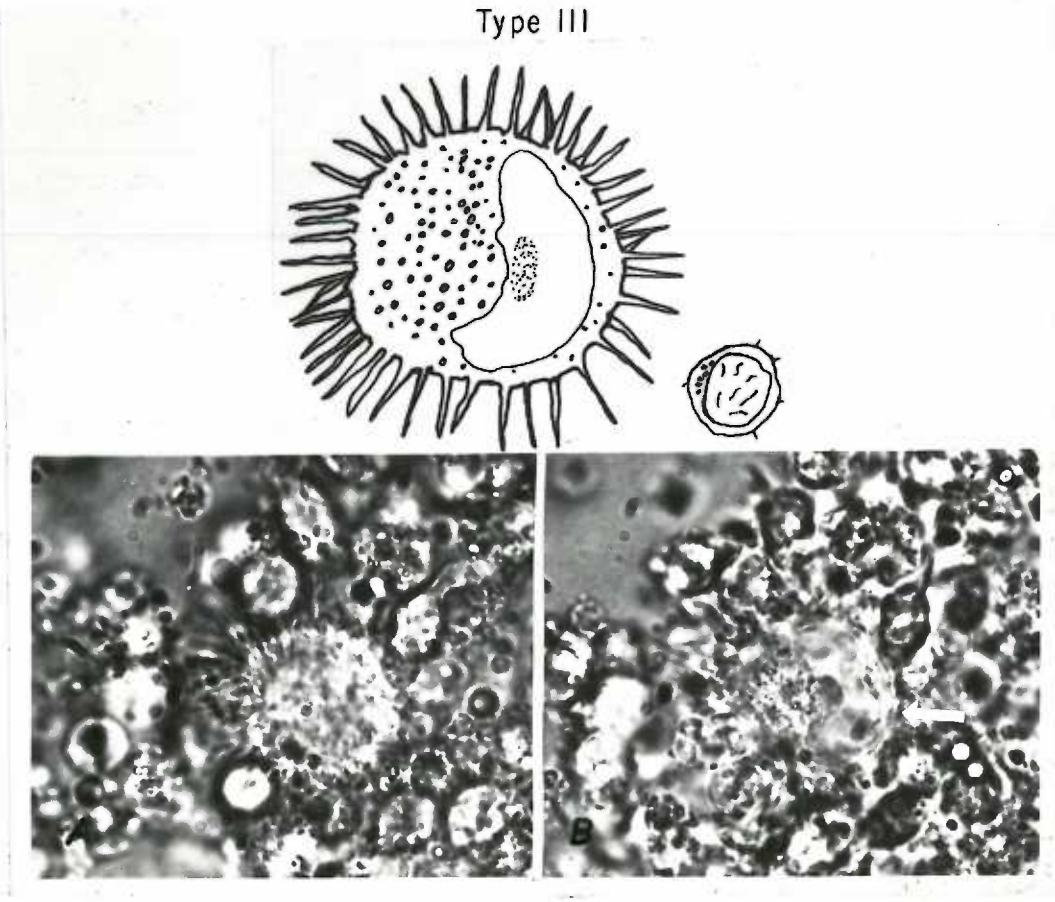
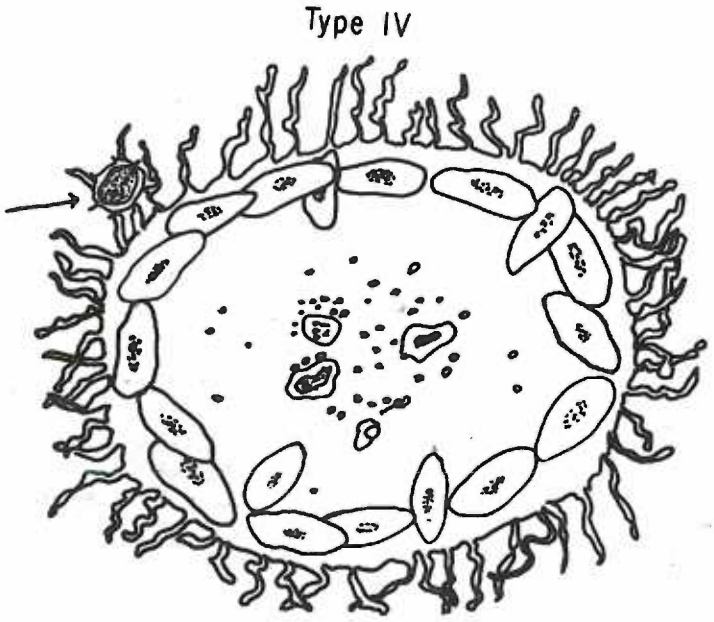


FIG. 7 Transitionol Reticulum Cell

Figure 8. Type IV. Foreign-body giant cell.

The arrow in the drawing indicates a small lymphocyte included as a size reference. This dramatic example of what is identified as a Langhan's-type foreign--body giant cell was from a reactive hyperplastic node culture.

Approximate magnification 1500X.



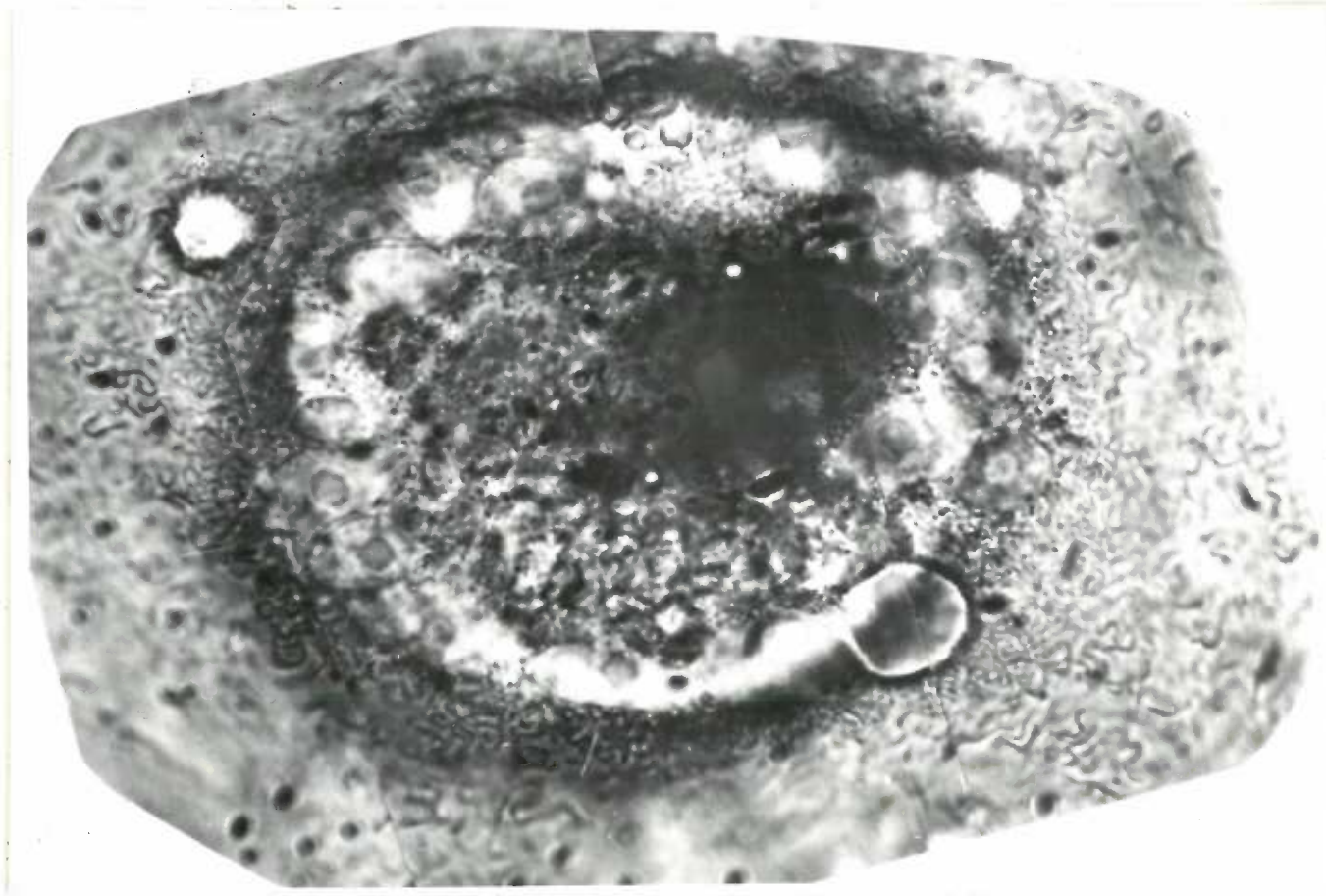


FIG. 8 Foreign-Body Giant Cell

unchanged in one place for over a week.

The foreign-body giant cell is Type IV, as shown in Figure 8. Frequently these cells are smaller than the illustration and occasionally motile. Lymphocytes are attracted to these cells but were not observed within them. In any one culture or chamber their number was varible, usually appearing between 10 and 14 days, and often persisted in the same general area for a week or more. This cell type did not appear in normal nonreactive cultures.

Lymphocytic Lymphoma. As seen in Table 1, seven cases under this general heading were cultured. Most cultures had less frequent motile lymphocytes, and fewer aggregates than were found in other pathological cultures. An aggregate of such nonmotile cells at the edge of the explant is shown in Figure 9. With hypertrophy, over a period of weeks motility was regained, but it was of a more histiocytic type. Extremely lobulated nuclei, as seen in some Burkitt's lymphoma cultures (13), were present for a few hours in one culture. Later observation revealed the typical large nonmotile lymphoblast with a slightly exaggerated nuclear hof. Over the first 3 days the cells enlarge slightly and the nucleoli become more apparent. Individual differences were present in each culture that seemed to correlate with the degree of immaturity reflected by lymphocytes in the May-Grünwald giemsa-stained imprints.

Plasma cells were not found. Reticular cells and macrophages were few. Cell destruction between 5 and 72 hours was more extensive than in other cultures. Hypertrophy of the remaining cells and formation of new cell islands continued slowly. Some mitosis was observed, but only in three cases did it increase and a semiestablished culture result in the milk-dilution bottles. One culture has become an established culture. In this culture from periotoneal effusion the persistance of small motile lymphocytes moving among the initially nonmotile blasts was most striking and could still be detected at 2 months when the culture was stored in liquid nitrogen. In these cultures slow hypertrophy and attachment with the formation of new cells continued. Lymphocyte transformation as described for Type II morphology was similar to the changes observed in these abnormal cells. Type III morphology, the transitional reticulum cells, and Type IV foreign-body giant cells were few but present in each culture. Division of lymphoma cells within reticulum cells (73) was not found though a few examples of emperipolesis were present in each culture. (Fig. 9) studied.

Dermatotrophic lymphadenitis cases presented a similar picture initially except for greater lymphocyte motility and the more frequent appearance of macrophages and reticulum cells. Plasma cells were rare. Sheath formation, though abortive, did begin and new cell islands

Figure 9. Lymphocytic lymphoma cultures.

A. Part of a foreign-body giant cell formed after 15 days of culture, showing three nuclei but probably containing more.

B. A rare motile large lymph among giant hypertrophic giant cells.

A & B. These cells are from a lymphosarcoma case found to have antigen common to moloney leukemic virus (MLV) infected mice and histochemical similarities.

C. An aggregate of nonmotile malignant lymphoblasts from another lymphocytic lymphoma.

D. An example of sequestered lymphocytes in a reticulum cell with an unusually large nucleolus. These cells were rare in any one culture but present in each. Magnification 2100X.

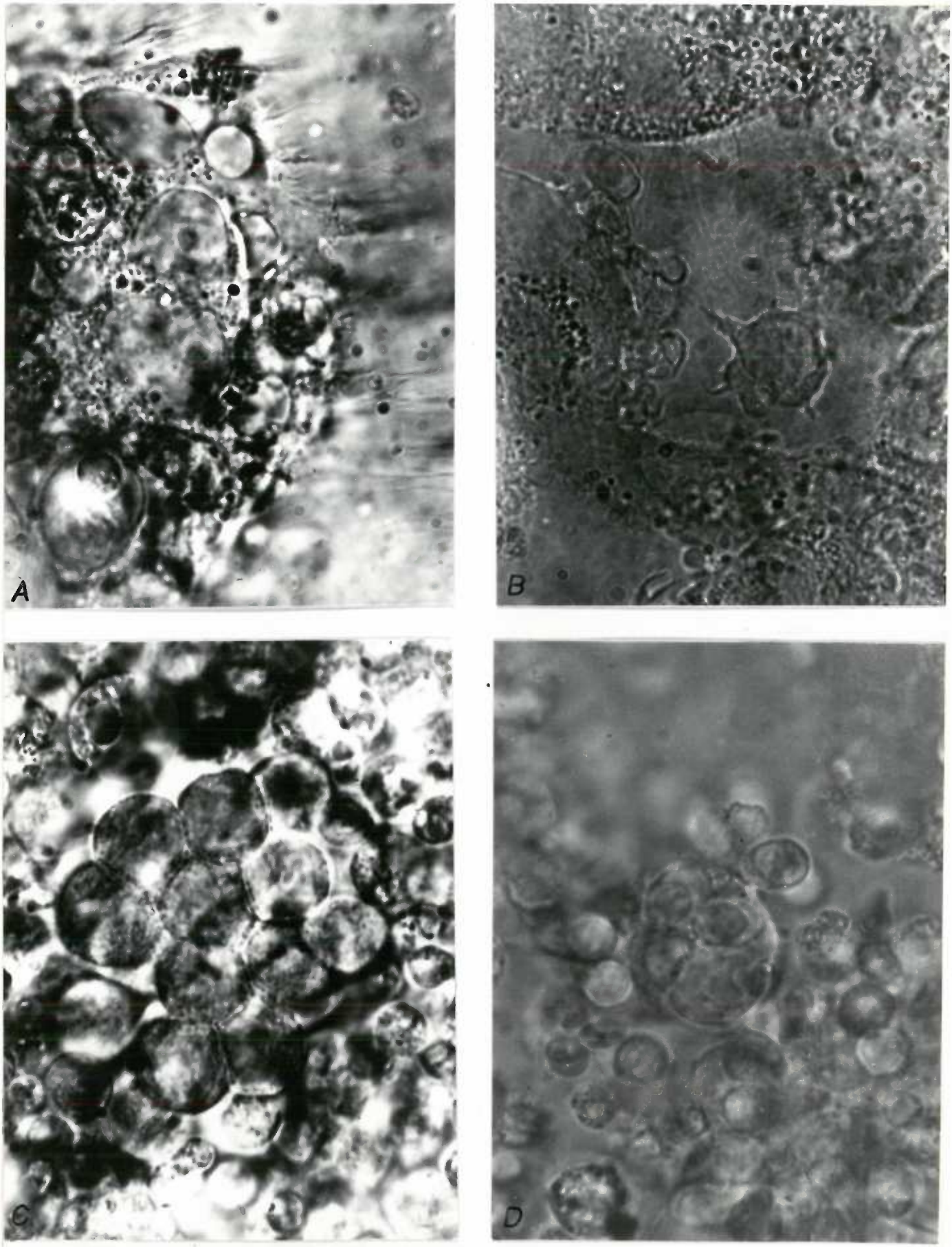


FIG. 9 Lymphocytic Lymphoma

were few in number. Peripolesis and emperipolesis involved a larger number of cells than in most lymphocytic lymphomas.

Hodgkin's Granuloma and Paragranuloma. Reed-Sternberg cells were occasionally found in the cultures. Though at times motile, more often these cells remained as large round cells showing some peripoletic attraction to the lymphocytes (Fig. 10). The fate of these cells was not apparent but large abnormal reticulum cells, hypertrophied and attached, were morphologically recognizable as epithelioid giant cells and large spindle cells. In monolayer cultures these comprised the major cell types, forming starlike hyperplastic colonies (see Fig. 16). Lymphocytes displayed a particular deep nuclear hof and heavy granulation (Fig. 10). Emperipolesis and peripolesis were noticeable than in the lymphocytic lymphoma cultures. New cell islands were frequently formed (Fig. 10). On one occasion an attempt to pass infected material from a Hodgkin's culture to a normal node culture was possible. A cell-free filtrate of thawed Millipore-filtered material was injected under the dialysis membrane of four Sykes-Moore chambers containing normal node tissue. Specific changes were observed including an increase in lymphoblast formation and islands free of the explant (Fig. 10A). Plasma cell formation was not observed over a 30-day period. Reed-Sternberg cells were not identified but typical giant cells with some nucleolar fragmentation were present in great

Figure 10. Hodgkins' granuloma and paragranuloma.

A. Blastic lymphocytes typical of Hodgkins cultures after 10 days of incubation.

B. A hemocytoblast from a similar culture.

C. A large abnormal reticulum cell surrounded by motile lymphocytes, 48 hours in culture.

D. A large reticulum cell with what seem to be non-motile lymphocytes within the cytoplasm, 5 days in culture.

E. New cell island after 10 days of culture. 400X.

F. A Reed-Sternberg cell with peripoletic lymphocytes after 48 hours of culture. 2100X except where indicated.

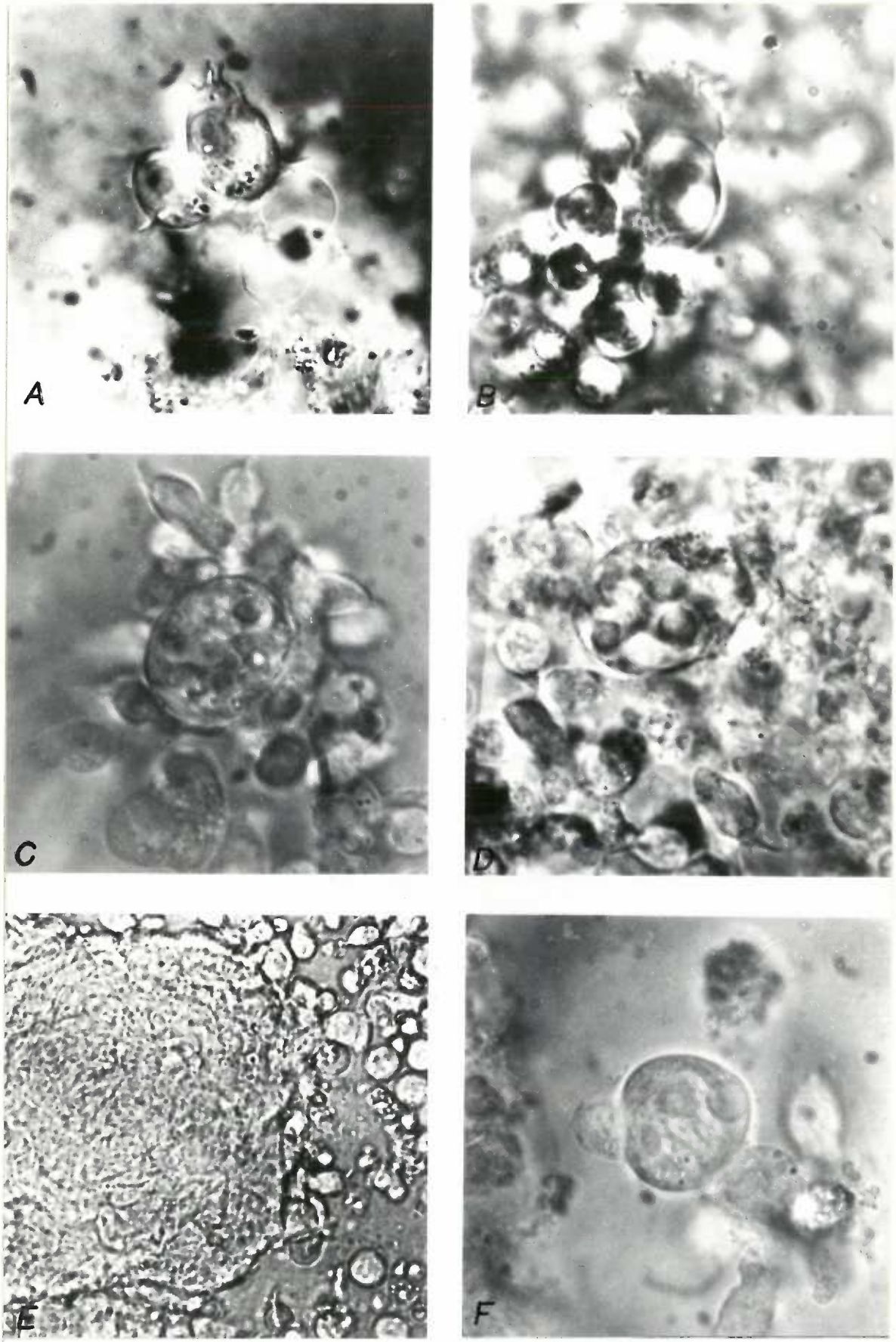


FIG. 10 Hodgkins Granuloma - Paragranuloma

numbers. Hypertrophic cells and Type IV foreign-body giant cells were found in the Hodgkins-injected normal culture chambers but not in uninoculated chambers of the same culture. A total of five Hodgkins cultures were studied (Table 1). One culture, the most recent, resulted in a continuous culture of rapidly dividing cells currently stored in liquid nitrogen.

Reticulum Cell Sarcoma. Two such cultures were studied (Table 1). One of these cultures rapidly degenerated and was followed for only 7 days. Slight exposure to formalin vapor in the original handling of tissues was found to be conducive to such degeneration. Such exposure might result from the handling of fresh tissue in the tissue pathology laboratory of the hospital, where a residual vapor exists when no containers of the fixative are open. The same caution must be exercised in the use of osmium tetroxide or of storage refrigerators. In both cultures in general the predominant cell was an initially nonmotile abnormal reticulum cell (Fig. 11). Lymphocytes were present and actively motile. Figure 11 shows the peripoletic attraction of a dividing sarcoma cell to motile small lymphocytes. No sheath formation occurred but new cell islands formed free of the explant. Among the new cells formed were large blasts of the reticular cell type. Cell destruction was again heavy but hypertrophy of the remaining sarcoma cells to giant fibroblastic cell occurred within 14 days.

Figure 11. Cells from Reticulum Cell Sarcoma Cultures.

A. Typical of one of the two cultures studied are these large nonmotile sarcoma cells.

B. A hypertrophied nonmotile sarcoma cell 10-15 days in culture.

C. Smaller motile sarcoma cells just beginning to develop motility.

D. A dividing sarcoma cell in late telophase, demonstrating the peripoletic attraction of lymphocytes to the mitotic process.

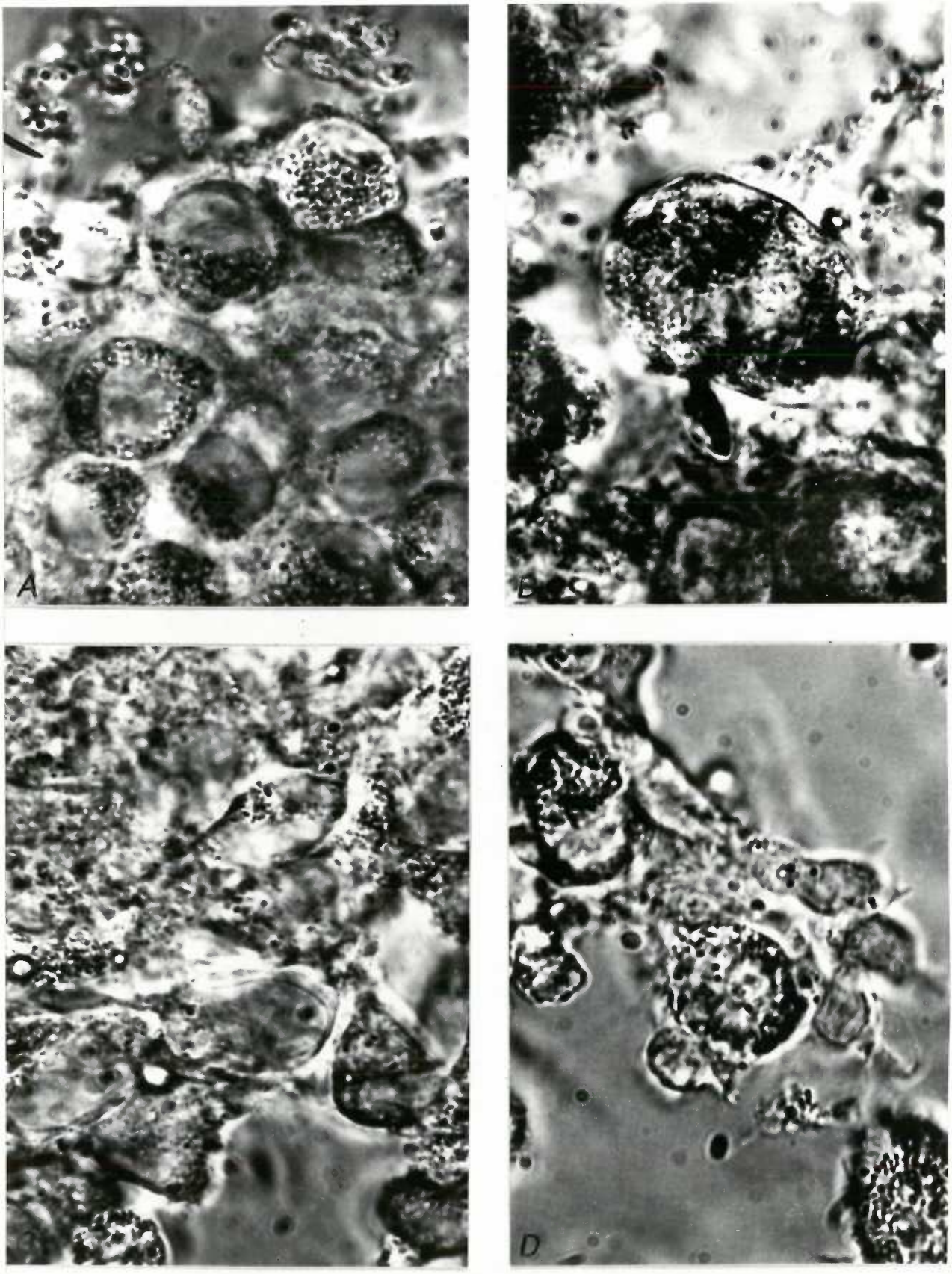


FIG. 11 Reticulum Cell Sarcoma

Figure 16 shows the stages of such alterations. Macrophages were few as were foreign-body giant cells. The Type III cell was present in greater numbers than in other cultures.

Reactive Hyperplasia. These cultures tend to follow the pattern of the lymphomas, with individual variations that reflect the histopathology of the particular case. In general lymphocytes were actively motile in larger numbers than they were in lymphocytic lymphoma. Macrophages were numerous and emperipolesis was observed in a few reticulum cells. As shown in Table 2, cell Types I, II, and IV were found. In reticulum-cell hyperplasia, Type III morphology was more frequently found. In some cases extremely active phagocytic activity, easily observable without cinematography, occurred. Finger-like projections closed over debris and a large vacuole was pinched off from the cell membrane, followed by the formation of new projections (Fig. 2). Lymphocytes moving on the surface of the cell were not phagocitized though they remained in contact for 5 minutes moving 2/3 of the circumference of the macrophage. Elongated mitochondria were observed in the lymphocytic series of this particular culture, which was observed in only three widely differing cases. In reactive hyperplastic cultures sheath like cell formation occurred to a variable degree, and reticulum cell outgrowth was rapid and heavy.

Lymphadenitis. The nonspecific lymphadenitis

developed in chamber culture much as do moderately involved lymphocytic lymphomas, but sheath formation was more complete, and new cell islands were rare after the first two weeks. Two node cultures were found to have specific agents present. Macrophage activity was initially heavy, a few bizarre cells could be found, and lymphocyte transformations Type I and particularly Type II was heavy (Fig. 4), though the survival of the culture was short owing to the agents involved. A high overall percentage of motile cells were present, both neutrophils and lymphocytes. Plasma cells were not formed though lymphoblasts became more numerous on the fifth day of culture. Small foreign-body giant cells were formed. One culture contained Nocardia asteroids, and the second a Gram-negative rod with biochemical characteristics consistent with the Brucella. In the later culture a few plasma cells were present initially and eosinophils were more numerous. After 10 days bacterial forms were recognized in the culture; antibiotics suppressed but did not inhibit this growth. Cell destruction and possibility of laboratory contamination made termination of these cultures necessary.

DiGuglielmo's syndrome. In the first of two cases, necroposy tissue, spleen, and nodes were the only tissue available. An overgrowth of E. coli destroyed the culture. This was the only autopsy tissue included in this study. Several attempts were made to utilize autopsy material but

it consistently was autolyzed to a degree that lymphocyte motility did not retunr with incubation nor was outgrowth consistent. The second DiGuglielmo's syndrome case was cultured from bone marrow of an acute "blastic" crisis. These hemocytoblasts underwent transformation to giant epithelioid morphology with highly fragmented nucleoli within 2 weeks. A modification of medium was necessary to secure continued cell division. High levels of lysine were necessary (390 mg/L). Unfortunately this culture was lost in a laboratory accident before transfer or investigation could be made. Both will be discussed later.

Metastatic Adenocarcinoma. Though not lymphomas, eight metastatic nodes were cultured (Table 1) and presented findings meriting inclusion. Five cultures involved the presence of adenocarcinoma cells; the other three cultures failed to show identifiable malignant cells, though the pathology reports indicated their presence in representative sections. Four of the nodes containing adenoma cells were scalene nodes; the fifth was an inguinal node. The morphology of the cells reflects the difference in origin. Figures 12 and 13 are from two such cultures exhibiting this difference of morphology from the two cases. In one culture metastatic cells were found only after 5 days of incubation. Histological sections were negative except on resectioning. Initially these cells were non-motile, developing motility as hypertrophy and attachment

Figure 12. Metastatic adenocarcinoma in lymph node cultures.

A & B. Photographs are from an inquinal node culture.

A. This cell shows the enlarged lysosomes that lead to cell destruction; Neutral Red had been used to stain these bodies.

B. The large signet-ring cell was a form of degeneration found in this culture and in one of the lymphosarcoma cultures. Many cells assumed this morphology. On the viable cell, the nuclear cap of granules may represent an active Golgi zone, and was typical of this culture as proliferation of the adenoma cells continued.

C. Small aggregates of metastatic cells of this type appeared in this culture of a scalene node after 5 days of culture. These cells gradually hypertrophied, attaching to the glass as giant cells.

D. Metastatic cells were more abundant in this culture from another scalene node. These cells have an appearance similar to one culture of reticulum-cell sarcoma; however on closer observation differences are apparent.

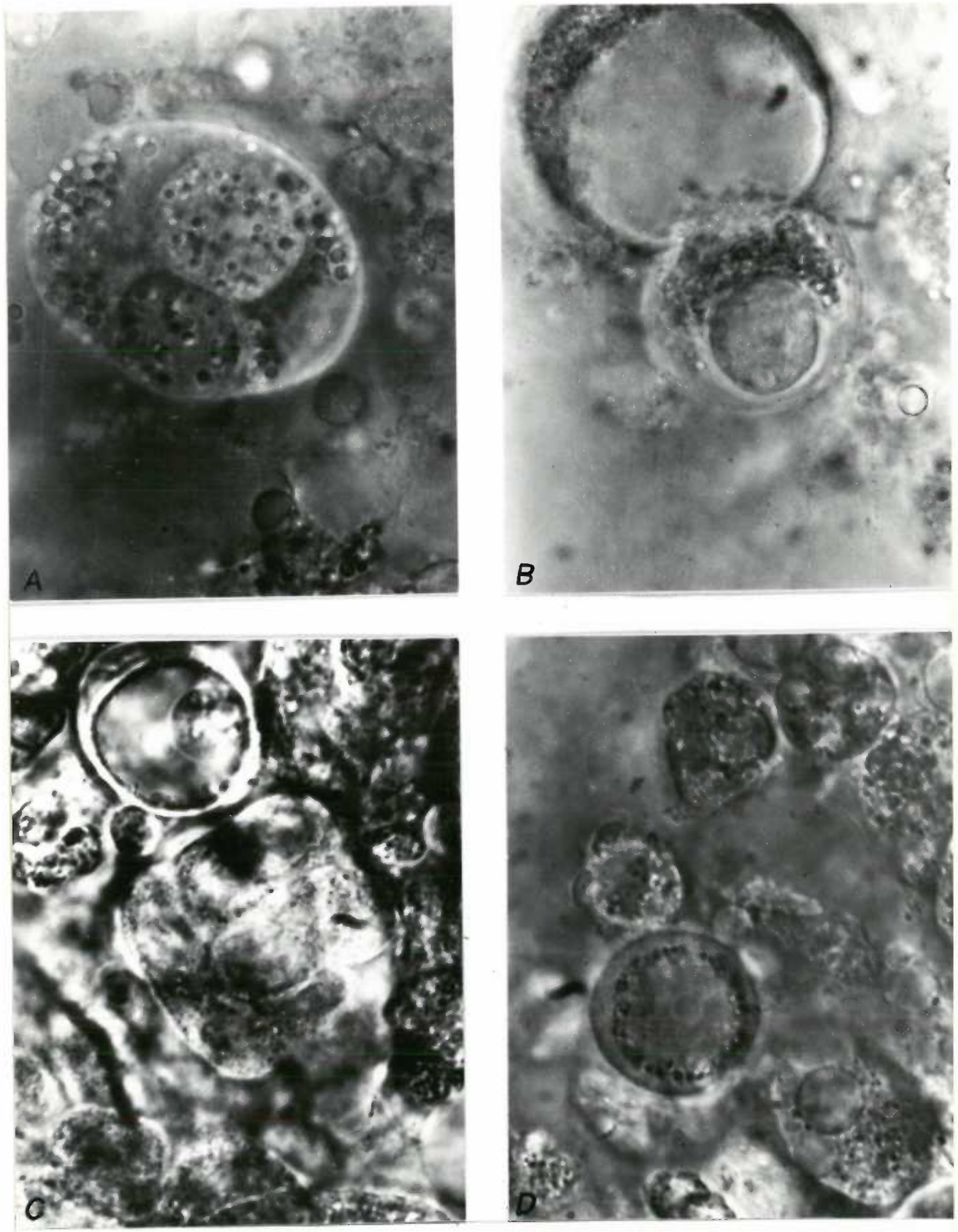


FIG. 12 Metastatic Adenocarcinoma In A Lymph Node

Figure 13. Transitional cells from metastatic adenocarcinoma.

A. A hypertrophic adenoma cell, heavily granular, attached to the glass by the many fine membrane protrusions. The nucleus and nucleoli are partially obscured by the granulation.

B. Motile adenocarcinoma cells exhibiting peripoletic attraction to a few lymphocytes (note the arrow).

C. A motile partially hypertrophied adenocarcinoma cell showing an extremely ruffled haloplasmic membrane.

D. An aggregate of cells lymphocytes, and an adenocarcinoma cell with many heavy protrusions from the cell membrane.

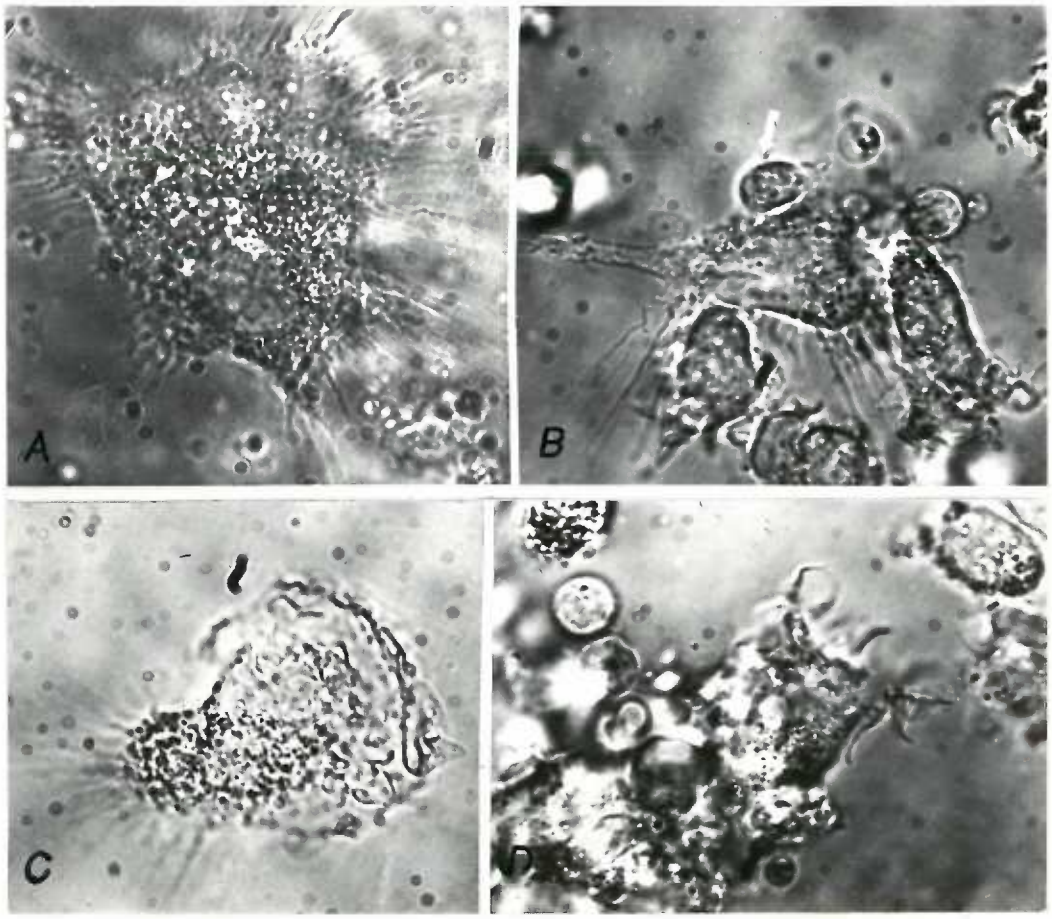


FIG. 13 Transitional Cells From Metastatic Adenocarcinoma

occurred (over 14 to 21 days). In one culture it was difficult to distinguish between small adenocarcinoma motile forms and large transforming Type II lymphocytes (Fig. 13). In nonmotile cells organelles and vacuoles were distributed around the nucleus similarly to those in reticulum cell sarcoma. Lymphocyte motility was consistently active, peripolesis and occasionally emperipolesis were observed. With INT staining, Mitochondria were in excess of six and were consistently small round granules. Neutral Red staining indicated a lipid nature of the vacuolization shown in Figure 12. Cell destruction was heavy in some cultures. Plasma cells were occasionally present and granulocytes were rare. Monolayer cultures were not followed.

Multiple Myeloma. One culture was studied from a relatively well-differentiated case in which abnormal plasma cells and plasma cell blasts were predominant. Little motility was observed in lymphocytes and metamyelocytes. Degeneration was heavy but a rapid heavy hypertrophy of the blastic cells to giant cell followed. Outgrowth persisted for 95 days, gradually slowing in division; appearance of RNA barring and loss of the culture followed.

Boeck's Sarcoid. One culture was studied (Table I) in which reticulum cells hypertrophied within 24 hours to giant-cell morphology (Fig. 14). Figure 14 shows the emperipoletic phenomenon that resulted occasionally in

Figure 14. Emperipolesis in a giant reticular cell from a case of Boeck's sarcoid. This is the same cell at two depths of focus about 30 seconds apart; note changes in the lymphocytes within the cell. Though lymphocytes were occasionally found in other cultures within reticulum cells, they were rarely motile. In this culture cell destruction by the lymphocytes was observed. 2100X.

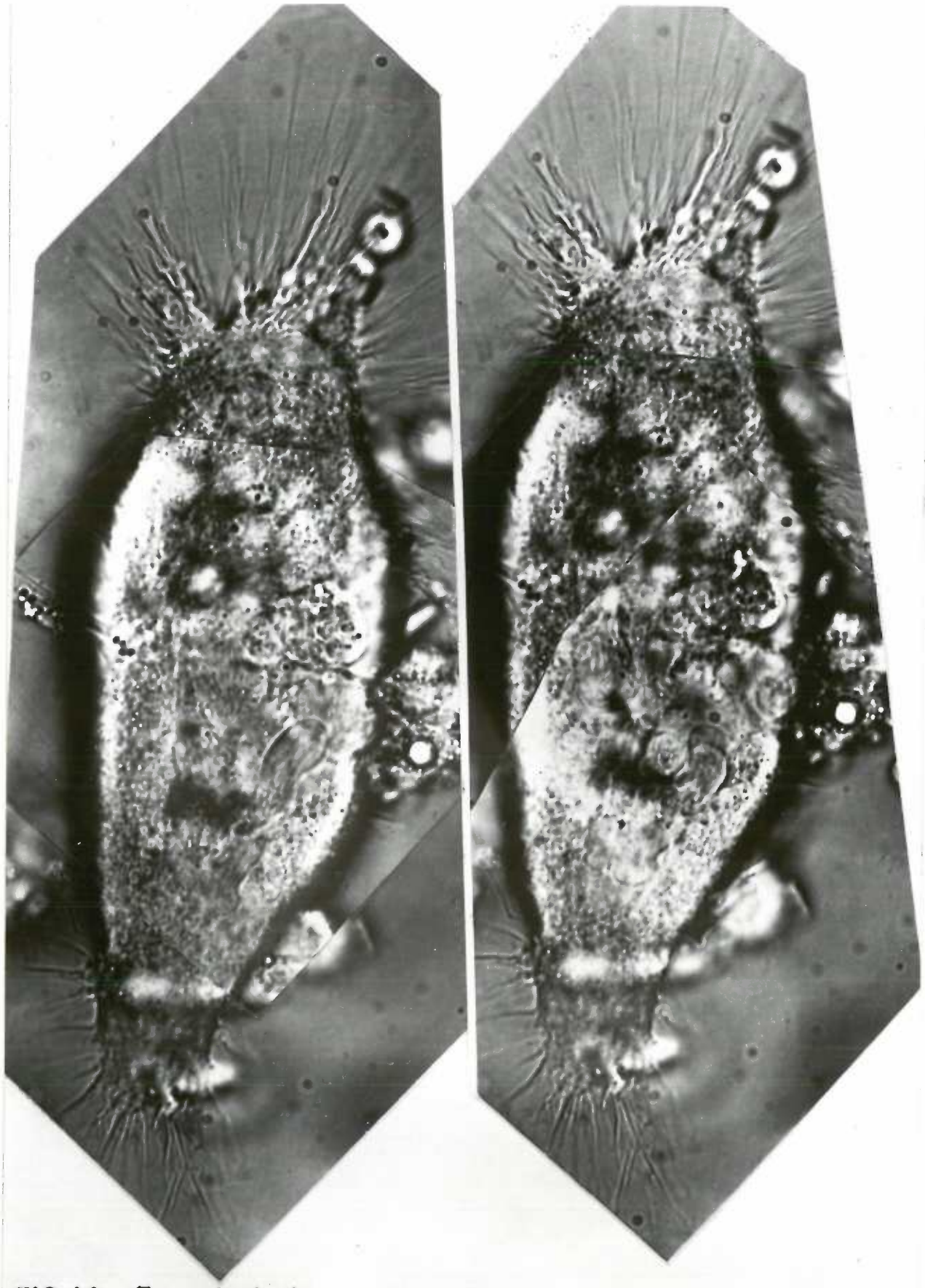


FIG. 14 Emperipolesis

death of the giant cell. Though foreign-body giant cells were formed in small number, none seemed to contain Schanmam bodies typical of this disease (64). In the monolayer outgrowth a vague greenish pigmentation was observed under low-power magnification, not found in other cultures. Area destruction described by Lündbeck and Löfgren (11) was present, followed by subsequent regrowth. Medium-sized bipolar cells dominated the outgrowth though epitheloid giant cells and smaller fibroblasts were present. Lymphocytes persisted for over 30 days, and sheath formation did occur in an abortive manner.

In most of the cultures just described transition of reticulum cells to giant cell morphology occurred. In some instances these changes were dramatic as shown in Figure 15. A chance juxtapositioning of cells showed the stages of development that were observed to occur over a period of 5 to 10 days. Whether lymphocytes in a few cases progress to cells of extreme giant morphology remains questionable, but alteration and attachment as small forms was followed.

In the monolayer outgrowth from normal lymph nodes and pathologically derived cultures, four basic cell types were recognized (Fig. 16,17). Three were from normal cultures: the small structural fibroblasts, few in number; medium bipolar granular cell derived from capillary endothelium; and larger bipolar and tripolar, more epitheloid cell from sinusoidal reticulum (Fig. 16).

Figure 15. Transitional Cells From a Reactive Hyperplasia. The large motile cell on the right seems to be binucleated with an active haloplasmic membrane. On the left is a series of three cells showing various degrees of hypertrophy. The smallest cell is the size of a large lymphocyte. 2100X.

FIG. 15 Transitional Cells

In lymphocytic lymphomas and leukemias a modified lymphocyte frequently predominated (Fig. 17). In Hodgkin's disease cultures, among the previously mentioned cells is a large abnormal reticulum cell with epitheloid characteristics as shown in Figure 17. Their persistence in cultures was seemingly influenced by the medium and the initial number was related to the degree of pathological involvement of that particular tissue. Stellar hyperplastic foci formed and in some cases lymphocyte formation was noted in conjunction with this formation. Long-term or continued maintenance of these cells was initially poor. A requirement for vitamin B_{12} and higher levels of amino acids was shown by paired cultures. After 6 weeks of incubation, medium D (appendix) improved the division, doubling time of the culture, when the last Hodgkin's case cultured was converted to the new formula. Some of the reactive hyperplastic cultures also show a variable number of giant epitheloid cells with nucleolar fragmentation. The foci formation of the Hodgkin's disease tissue was not as pronounced in other types of cultures, but organization of the monolayers was irregular. Trypsin (0.25%) was ineffective for releasing human lymphoid outgrowth from the glass. A mixture of ethylenediamine tetrocetate (EDTA) and trypsin proved satisfactory.

In some cultures, when time permitted, outgrowth cells were transfered to observation chambers. A reticulum cell

Figure 16. May-Grunwald Giemsa stained Monolayer Cultures.

A. Three cell types can be identified in the outgrowth of this normal culture. 250X.

B. The giant epitheloid cells were more predominant in the outgrowth from this dermatotrophic lymphadenitis case. 250X.

C. A hyperplastic focus from a monolayer of a Hodgkin's paragranuloma culture. 186X.

D. Another area from the same culture showing the giant epitheloid cell in contrast with the smaller spindle cells and fibroblasts. 250X.

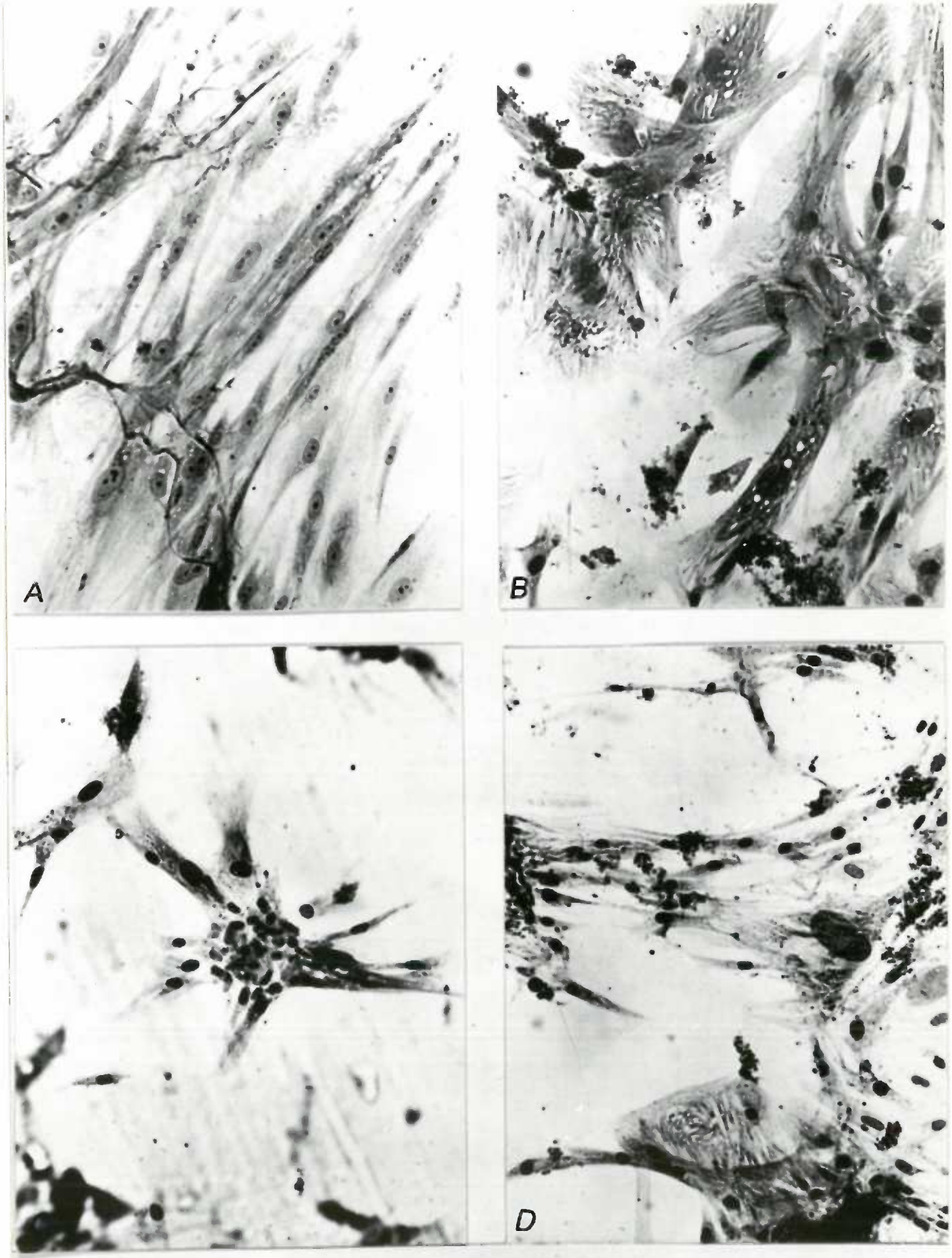


FIG. 16 Stained Monolayer Cultures

Figure 17. May-Grünwald Giemsa Stained Outgrowth Cells.

A. Giant cells taken from a reactive hyperplastic node culture.

B. Giant cells from multiple myeloma metastatic node culture showing a pronounced degree of RNA barring and evidence of a degenerating lymphocyte within the cell.

C. Cells from a lymphocytic lymphoma culture showing the giant epitheloid cells. The smaller spindle cell and a modified attached lymphocyte are more numerous in these cultures than in cultures from other pathology.

D. Lymphocytic leukemia node cultures show a predominance of transformed and dividing lymphoblasts. Note the nucleolar fragmentation. 1000X.

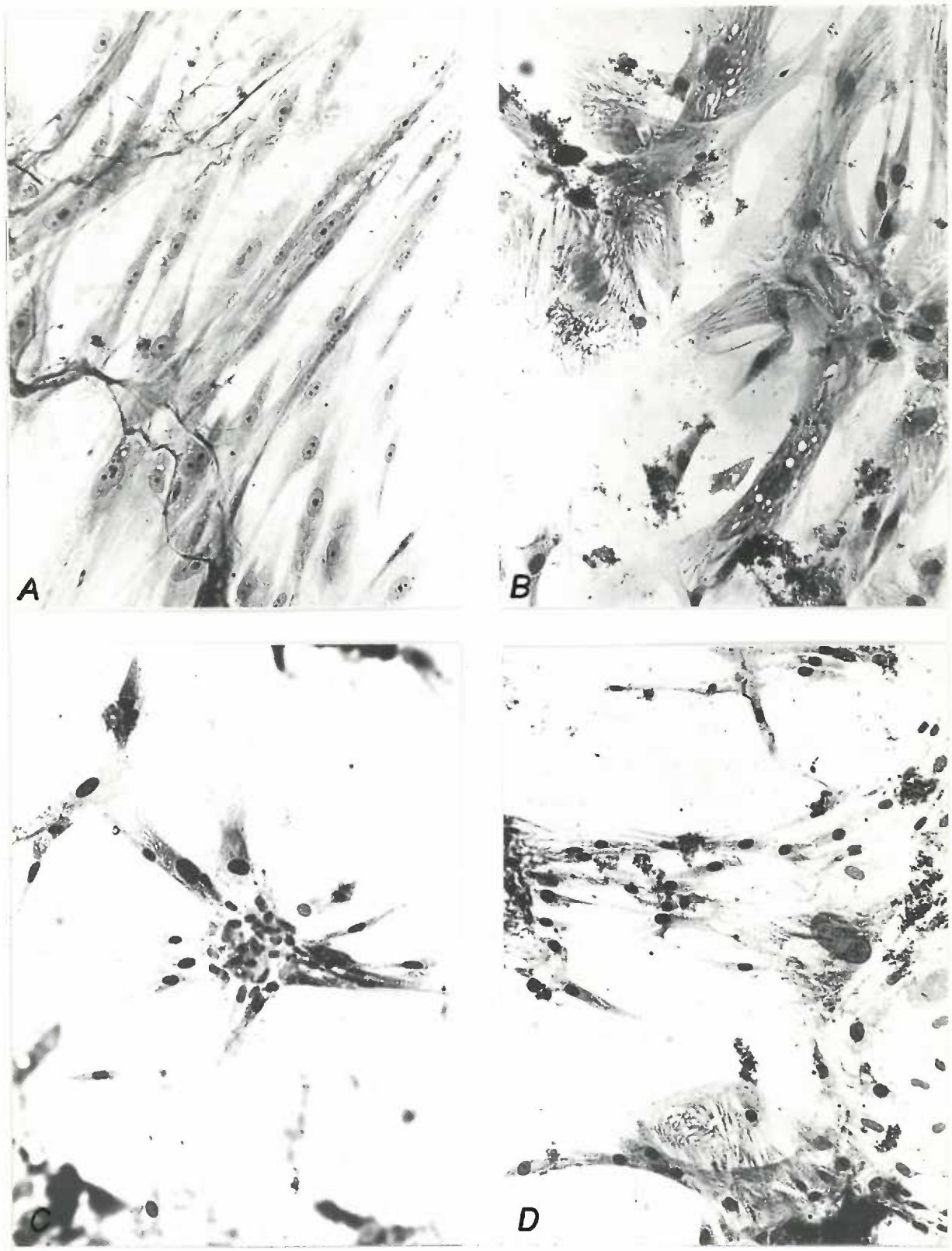


FIG. 16 Stained Monolayer Cultures

sarcoma, several Hodgkin's cultures, and two reactive hyperplastic cultures were so studied. Nucleolar fragmentation was observed in which some of the smaller fragments of nuceoli moved toward the nucleolar membrane and were released into the cytoplasm, losing their distinctive density and intergrity shortly thereafter. Zeosis, cell boiling, was heavy in all of the cultures. Mitochondria and lysosomes were found in some of the membrane-enclosed cytoplasmic balls that were pinched off. Vital dyes and acid phosphatase reactions confirmed the presence of lysosomes also. Rapid release of many vacuoles formed in the Golgi zone, that lead to cell destruction, were found in several cultures, particularly where the serine level was 1.5 mm. Some RNA barring was observed in these chambers as well as in the stained slides of many of the older cultures (Fig. 17).

Normal node outgrowth required 4 weeks to double; lymphoma cultures and many of the reactive hyperplasias required 2 weeks. Following change of the two continuous cultures to medium D, their doubling time decreased to 7 days. Unfortunately both carry indigenous bacteria with them making further study difficult.

Mycoplasma cultures were set up on many of the nodes received; but only the last three cultures, using a modified medium (see Appendix), demonstrated typical colonies of Mycoplasma. No attempt was made to subculture

these agents.

Particularly in some of the lymphomas cell blebs, associated with zeosis (Gr. boiling) were observed as one of the principal modes of cell destruction. In reviewing the imprints made from the node tissue, on the basis of thirteen cases of lymphoma this process could be detected in 5% to 80% of the nucleated cells. The nonpathological nodes demonstrated less than 0.5%. A great deal of debris in some imprints may be from zeosis.

In summary of the morphological changes reported here, diagrammatic drawings were prepared (Fig. 18-22). Reference to the legends with each drawing will show the differences presented in relation to clinical diagnosis. In general there was a positive relation between the number of motile lymphocytes and the number of aggregates formed, regardless of lymphocytes free of the explant. Sheath formation occurs more completely in normal node tissue cultures and abortively or not at all in lymphoma. Emperipolesis, though observed in the lymphomas, reflects the lack of motility of the lymphocytes within the reticulum cells. Motility of the lymphocytes was most noticeable in Boeck's sarcoid, where the cytotoxic effect of the lymphocytes was seen. Peripolesis was heavy in cultures where lymphocyte motility was high. A high incidence of cell destruction occurred in between 3 and 5 days, partially controllable by high levels of kanamycin and penicillin and increased

Figure 18. Diagrammatic Representation of Chamber Cultures over a 30-Day Period.

PMN - neutrophil
P - plasma cell
EO - eosinophil
L - lymphocyte
M - macrophage
PR - primitive reticulum cell
E - reticuloendothelial cell
H - degeneration "Hirsch" type
G - karyorrhexis
K - karyolysis
Z - zeosis
LB - lymphoblast
FR - free reticulum cell
MC - mast cell
EX - explant
SR - sinusoidal reticulum cell
ML - modified lymphocyte
C - lipid unmasking
T - hydrophilic necrosis
PB - plasmablast
FBC - foreign-body giant cell
A - aggregate of free cells

Type II, and Type I refer to cell morphologies previously discussed in the text. With antigenic stimulation, transformation of cells and the formation of foreign-body giant cells were observed. The small nucleated macrophage is thought to be a modified lymphocyte. Aggregates of cells are common to all cultures, being most infrequent where lymphocytes are nonmotile as observed in some cases of lymphocytic lymphoma. The left-hand side of the diagram presents the changes in a normal non-antigen-stimulated node culture. The right-hand changes are those of non-malignant antigen stimulation of many varied situations.

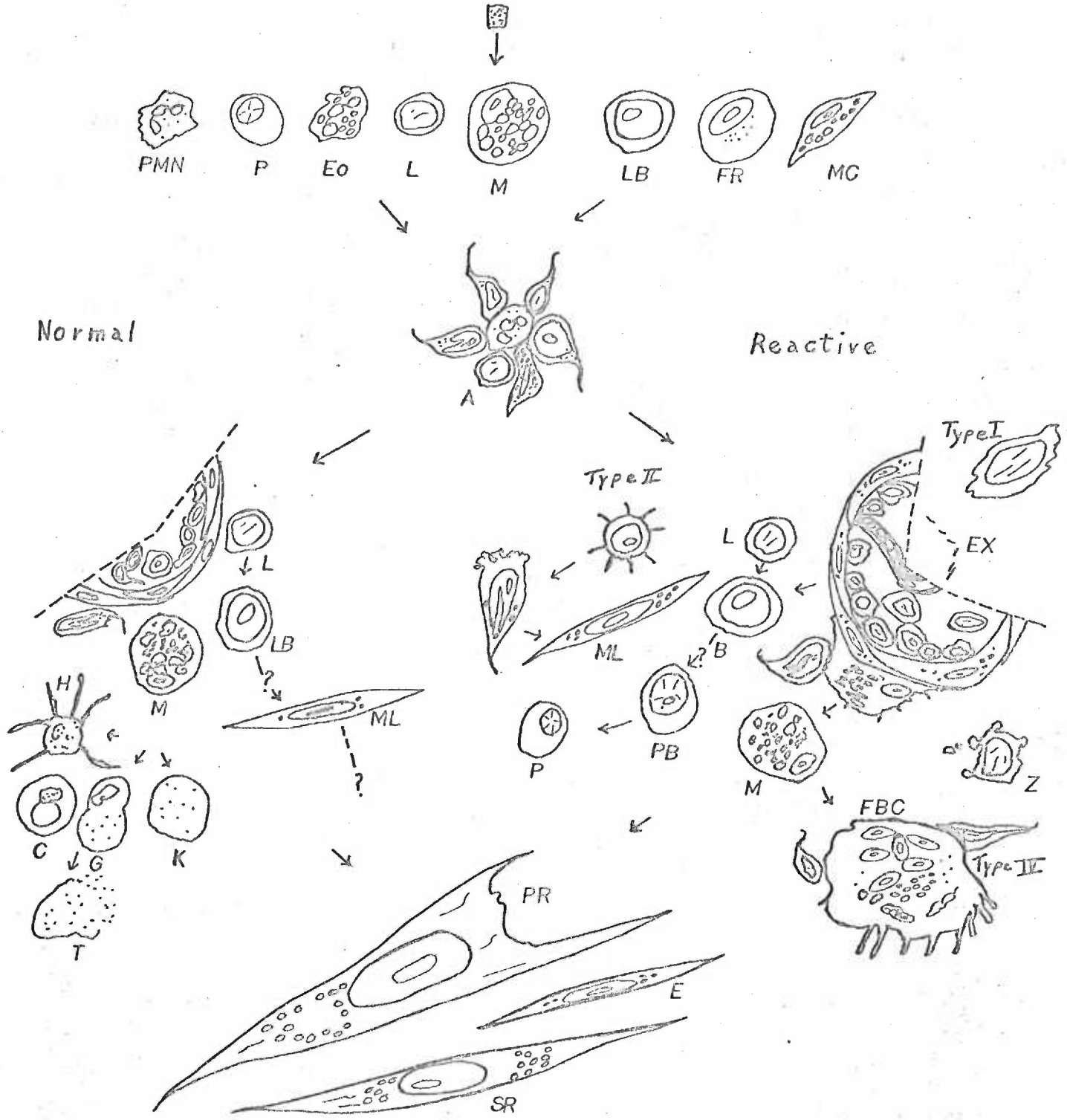


FIG. 18 Diagrammatic Representation of Chamber Cultures over a 30 Day Incubation Period

Figure 19. Diagrammatic Representation of Chamber Cultures from Lymphocytic Lymphoma and Leukemia.

PMN - neutrophil
L - lymphocyte
LB - lymphoblast
ML - modified lymph node
I - cell island
Tr - transitional cell
C - lipid unmasking
Em - emperipoletic situation

AL - atypical lymphocyte
M - macrophage
PR - abnormal primitive reticulum cell
SR - sinusoidal reticulum cell
FBC - foreign-body giant cell
Z - zeosis
K - karyolysis
A - aggregates of cells

In lymphocytic leukemia the modified lymphoblasts, lower left, predominate. In lymphocytic lymphoma the number of reticular cells found differs considerably from one culture to another reflecting the degree of malignant involvement of that particlar tissue. The number of examples of emperpolesis in any one culture was low but present. The abnormal changes observed in the reticulum cells behavior in organization of a sheath varied from one culture to another, seemingly dependent on the degree of malignant involvement of the particular tissue sample.

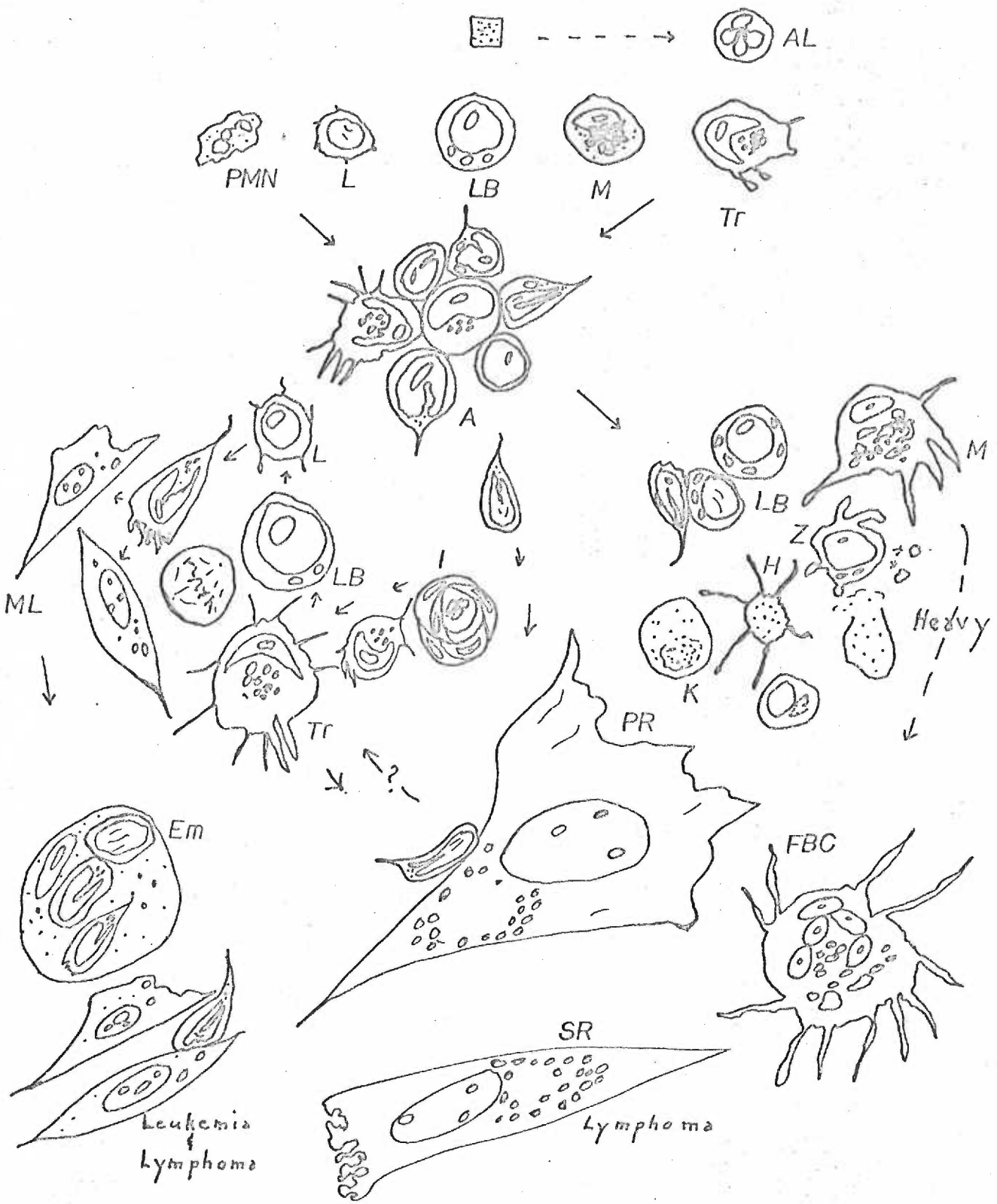


FIG. 19 Diagrammatic Representation of Chamber Cultures from Lymphocytic Lymphoma

Figure 20. Diagrammatic Representation of Chamber Cultures from Hodgkin's Granuloma and Paragranuloma-Involved Human Lymph Nodes.

PMN - neutrophil
P - plasma cell
L - lymphocyte
Eo - eosinophil
RS - Reed-Sternberg cell
R - abnormal reticulum cell
Tr - transitional reticulum cell
PR - primitive reticulum cell
MSR - medium abnormal reticulum cell
SR - sinusoidal reticulum cell
MC - mast cell
M - macrophage
H - Hirsch type of degeneration
Z - zeosis
K - karyolysis
ML - modified lymphocytes
NI - new cell islands
FBC - foreign-body giant cell
E - endoplasmic reticulum cell
MPR - abnormal primitive reticulum cell

Type II and type III refer to previously described morphologies. Here lymphocytes persisted and new cells were formed. Frequently a few large, modified, abnormal reticulum cells appeared attached to the glass around the explant within 24 hours but consistent outgrowth in chambers did not occur until later. New free-cell-islands appeared at about 7-10 days in culture. Emperipolesis was never heavy but was present in each culture. Peripolesis consistently was demonstrated in a selective manner to certain reticulum cells, but not to others of like morphology.

FIG. 20 *Diagrammatic Representation of Chamber Cultures from Hodgkins Granuloma and Paragranuloma Involved Human Lymph Nodes*

Figure 21. Diagrammatic representation of reticulum cell sarcoma cultures over the first 30 days in culture.

L - lymphocytes
S - sarcoma cell
M - macrophage
A - aggregates of cells
TR - transitional reticulum cell
ML - modified lymphocytes and blasts
E - endoplasmic reticulum
FBC - foreign-body giant cell
H - Hirschs form of degeneration
Z - zeosis
K - karyolysis, karyorrhexis
PR - primitive reticulum, abnormal
SR - sinusoidal reticulum, abnormal

In these cultures the appearance of large spindle and epithelial cells with abnormal characteristics occurred rapidly and heavily. Lymphocytes were actively motile but the number of such cells was often few. Some reticulum cells transformed rapidly to attached giant cell morphology, particularly prevelent were those of epitbelioed nature.

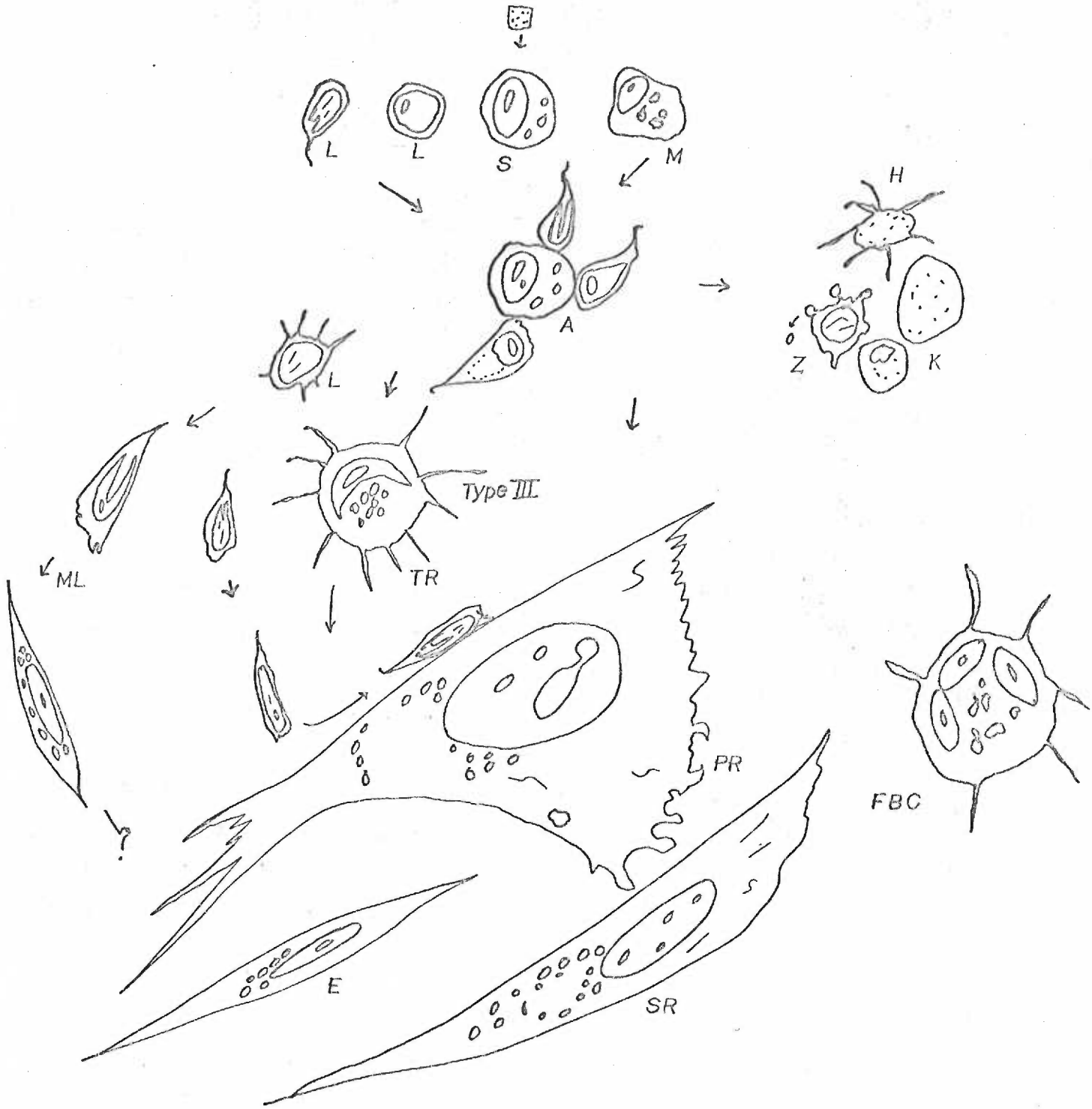


FIG. 21 Diagrammatic Representation of Reticulum Cell Sarcoma Cultures

Figure 22. Diagrammatic Representation of Chamber Cultures from lymph nodes containing metastatic cells.

PMN - neutrophil	Mn - metamyelocyte
L - lymphocyte	P - plasma cell
LB - lymphoblast	SR - sinusoidal reticulum
M - macrophage	MMR- modified myeloma cell
ADC - adenocarcinoma cells	A - aggregates of cells

Normal reticulum cells appear in variable numbers dependent upon the degree of metastatic invasion of the tissue cultured. Only the abnormal cells are shown here. In multiple myeloma a few lymphocytes were found in metastatic cells. Destruction of many cells occurred, as has been shown consistently to occur to some degree in all of the cultures. In the adenoma metastatic cultures lymphocytes were actively motile; peripolesis was frequent with certain cells and not with others. Transformation from round free cells to giant fibroblastic form in chambers was slow, requiring 15 to 30 days. The degree of involvement in the metastatic process is reflected in the number of abnormal cells observed in new cultures. Different types of adenomas, of course, exhibit differences in growth and morphology.

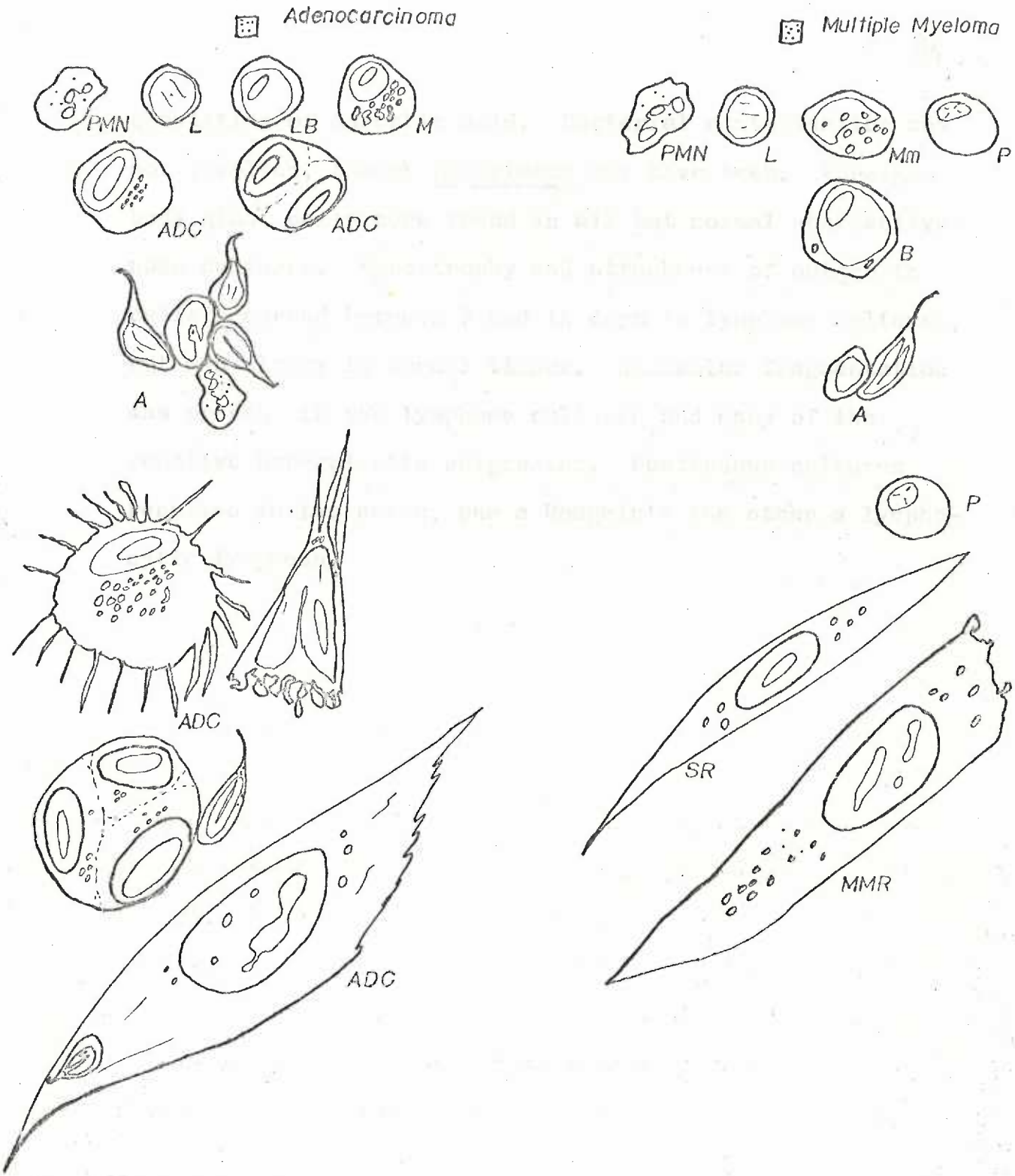


FIG. 22 Diagrammatic Representation of Chamber Cultures from Lymph Nodes with Metastatic Cells

quantities of ascorbic acid. Bacterial contamination was not involved, though Mycoplasma may have been. Foreign-body giant cells were found in all but normal nonreactive node cultures. Hypertrophy and attachment of outgrowth cells occurred between 7 and 14 days in lymphoma cultures, but was slower in normal tissue. Nucleolar fragmentation was present in the lymphoma cultures and many of the reactive hyperplastic outgrowths. Continuous cultures resulted in two cases, one a Hodgkin's the other a lymphocytic lymphoma.

Electron Microscopic Observations.

This study was not primarily of structural alterations but a search for virus-like particles, fibrils and Mycoplasma. Though other cases were examined, only those of lymphocytic lymphoma or leukemia, Hodgkin's disease and several normal nodes presented enough cases to be considered here. Figure 23 is of normal well-differentiated lymphocytes from normal tissue. Little endoplasmic reticulum can be detected and the Golgi zone is difficult to identify. In contrast, Figure 24 is from a relatively undifferentiated lymphocytic lymphoma. The nuclear chromatin is that of a less mature cell with sparse ribosomes attached to the erratic convolutions of a greatly dilated endoplasmic reticulum. This disorganization and the distinctness of the endoplasmic reticulum suggests the phenomena of asychrony often described by hematologist at the light level of microscopy. Asyhchrony of cytoplasm and nuclear chromatin was not displayed in all cells or in all cases. The dilatation of endoplasmic reticulum may also be related to abnormal secretion of some protein or other product of abnormal metabolism. Figure 25 is from a lymphocytic leukemia where asynchrony was not present, but a marked atypia demonstrated by the deep convolutions of the nucleus was common. Chromatin distribution of the nucleus was that of a mature lymphocyte but sparsity of ribosomes, and vacuolization are a departure from the normal cell;

as is the extremely deep nuclear convolutions. Often little evidence of cellular abnormality was found which is in keeping with recent reports of Bernhard and Leplus (155), but the predominance of immature cells all at one stage of maturity is significant. Three cases were studied. In these three cases no distinctive virus-like particles were found either of herpes-like morphology or of other nature.

Three Hodgkin's disease cases were also studied. Figure 26 depicts an abnormal reticulum cell showing the hypertrophic nucleoli typical of Hodgkin's disease. There is a sparsity of ribosomes and a tendency to rosette formation. Endoplasmic reticulum in this cell is almost nonexistent but the Golgi zone remains well defined. The mitochondria seem shrunken, which may be an embedding artifact. The relative ratio of nuclei to cytoplasma is typical of the more mature abnormal cell in this disease. The multi-nucleated or lobulated-nuclei with huge nucleoli are classical of this disease. Figure 27 shows a group of such cells, several having characteristics of Reed-Sternberg cells. The limiting membrane here is difficult to follow. At higher magnification, another Reed-Sternberg cell (Fig. 28), less mature, appears with more remnants of endoplasmic reticulum and inclusions, suggesting phagocytic activity. The sparsity of ribosomes and the rosette formation is again noticeable.

Figure 23. Lymphocytes from a Normal Lymph Node. 1720X stained with lead-uranyl acetate.

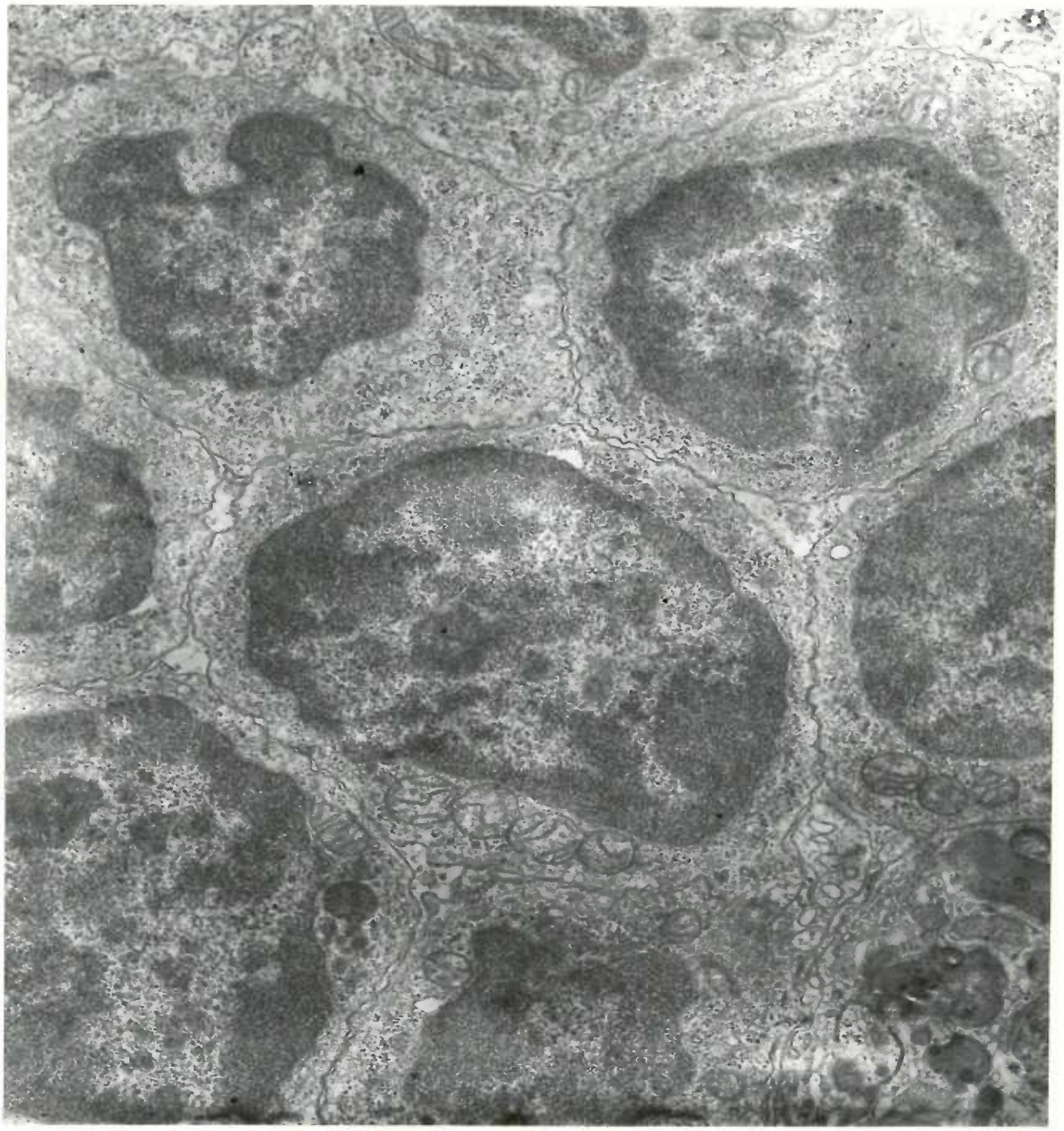


FIG. 23 Normal Lymphocyts

Figure 24. An Abnormal Small Lymphoblast.

This cell has a dilated (ER) endoplasmic reticulum and a prominent Golgi zone, showing some asynchrony of maturation. The nuclear chromatin retains the pattern of an extremely immature cell while the cytoplasm is that of a more mature blast. 1720X, lead-uranyl acetate staining.

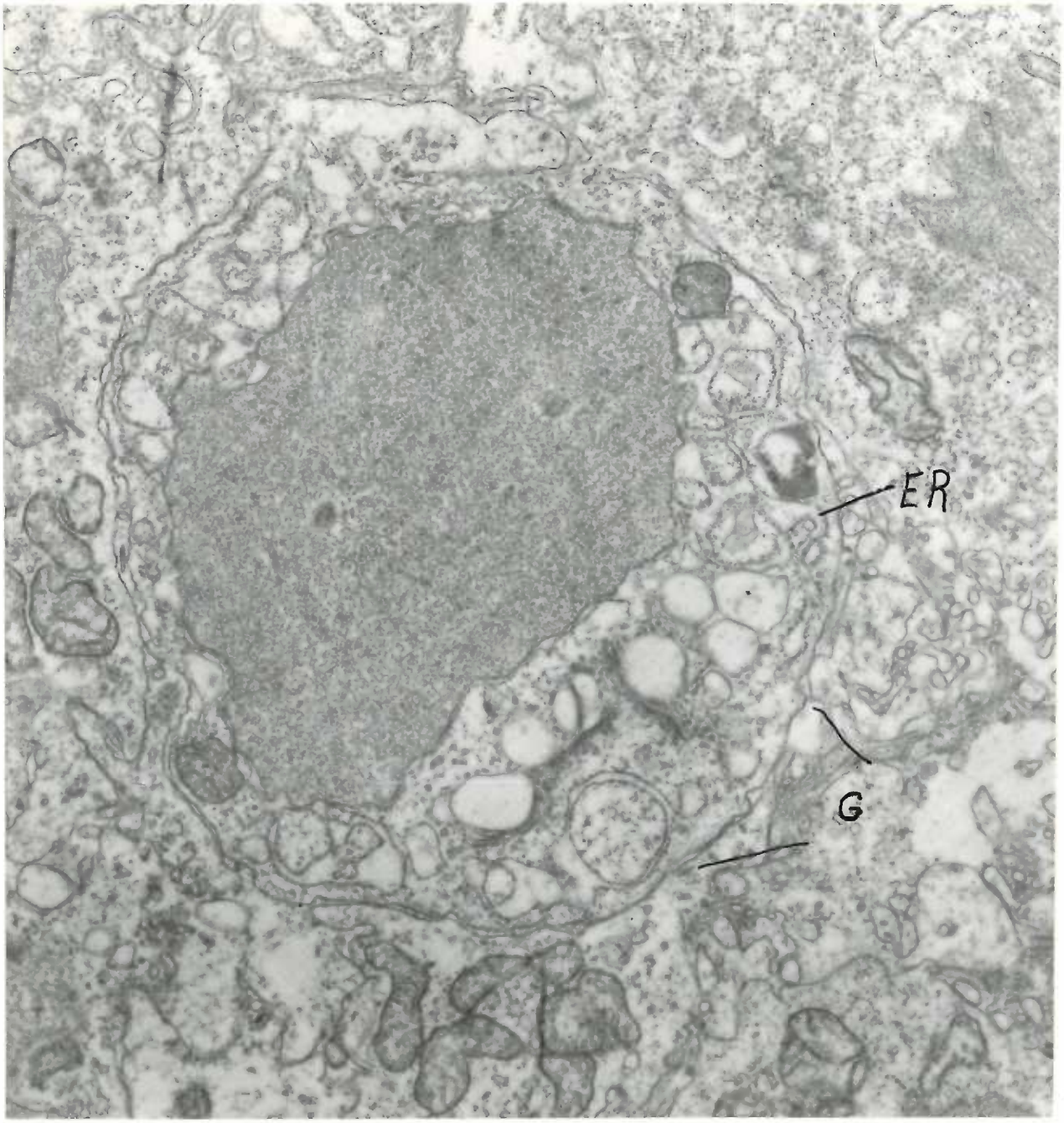


FIG. 24 Abnormal Lymphoblast

Figure 25. Mature (sl) small lymphocytes from another lymphocytic lymphoma, a leukemia. Note the deep nuclear invaginations and vacuolization. The nuclear chromatin here has a more mature arrangement though ribosomes are sparse and the rosette formation often observed in the Hodgkin's disease cells is in evidence. 1720X, lead-uranyl acetate staining.

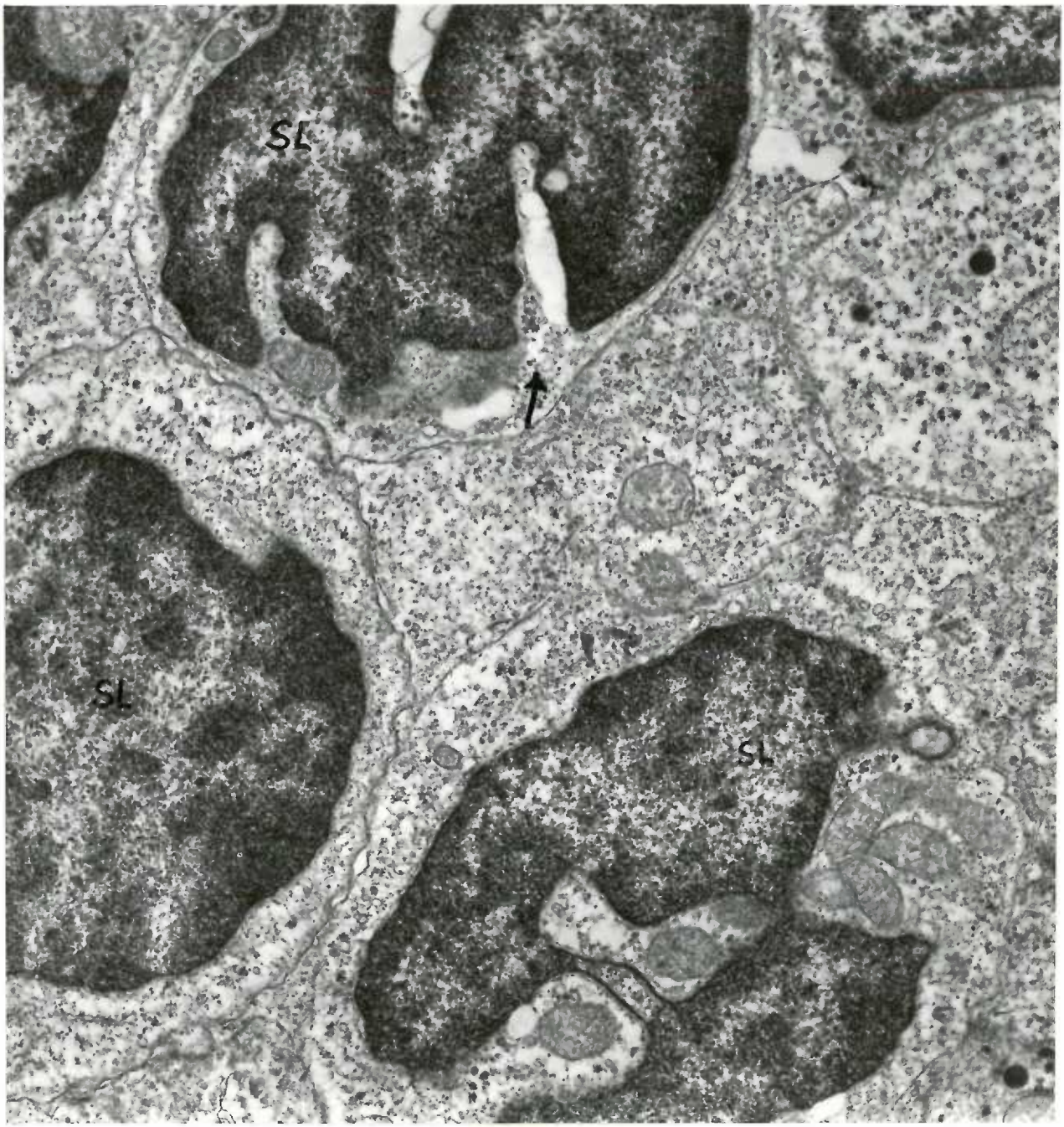


FIG. 25 Abnormal Lymphocytes

Figure 26. Abnormal Reticulum Cell from Hodgkin's disease Tissue. A typical abnormal reticulum cell from a Hodgkin's granuloma case. Some of the tubules of the (G) Golgi apparatus are in evidence. Note the nuclear cytoplasma ratio and large (nu) nucleoli. 17,200X, lead-uranyl acetate staining.

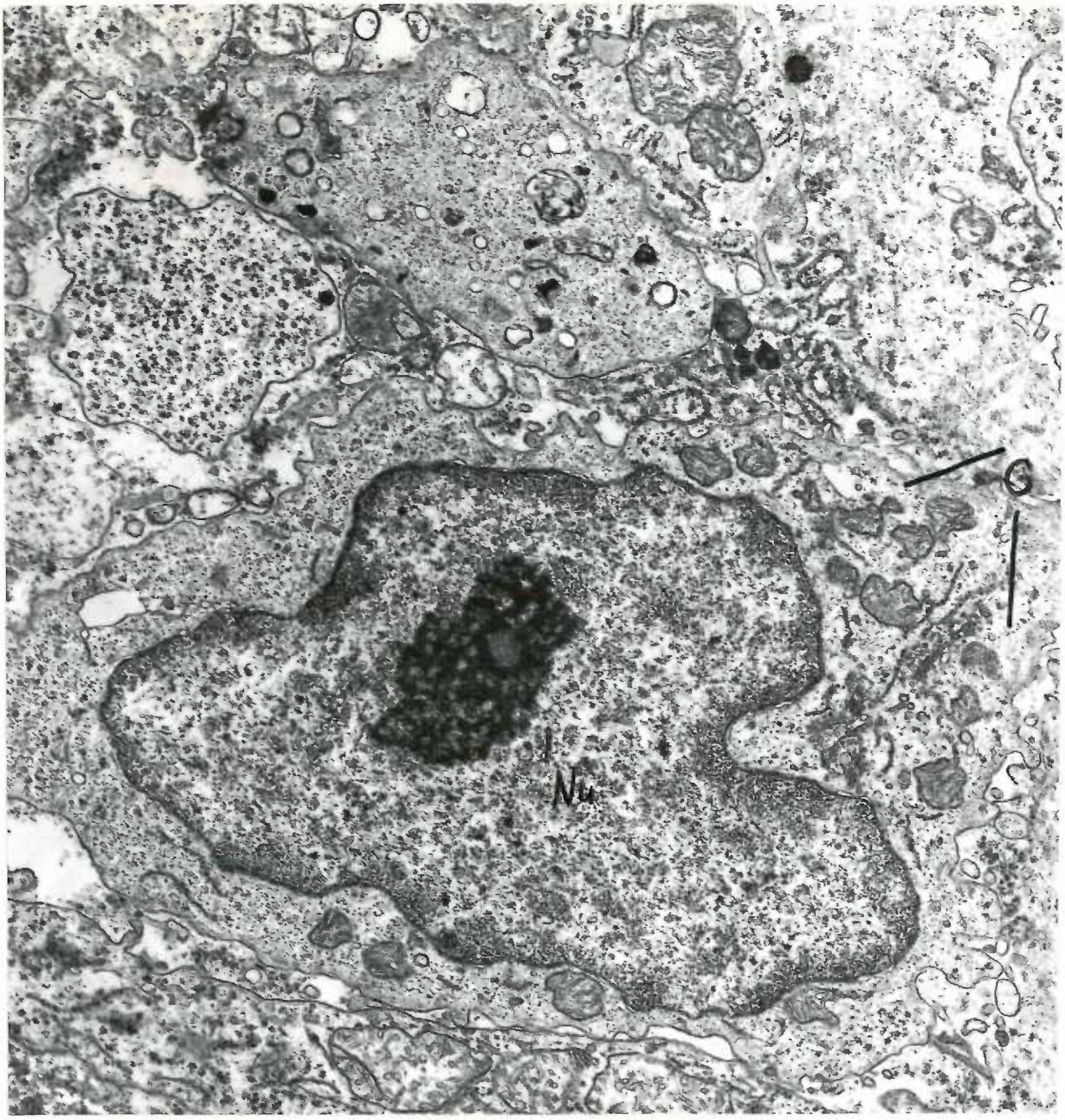


FIG. 26 Abnormal Reticulum Cell

Figure 27. A group of Reed-Sternberg cells from the same Hodgkin's case as figure 26. One cell contains nuclear bodies as described by Brooks and Siegel (156). Note the large nucleoli typical of this disease. Limiting membranes are difficult to follow in this picture. 6800X, lead-uranyl acetate staining.

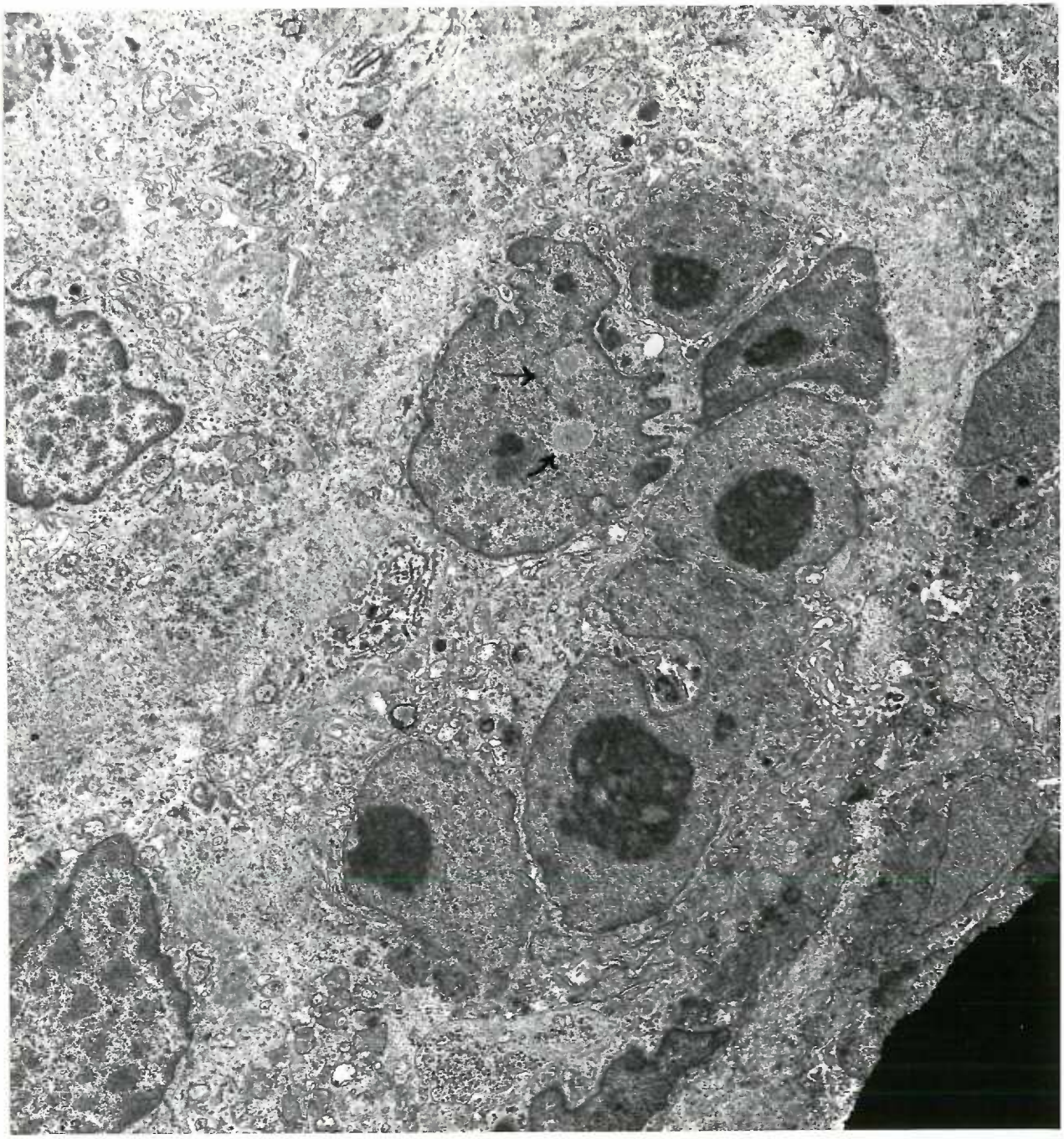


FIG. 27 Reed-Sternberg Cells

Figure 28. A Reed-Sternberg Cell from Hodgkin's disease tissue. Another Reed-Sternberg cell from the same tissue, as Fig. 27, showing the lobulated large nuclei and phagocytic inclusion material. The nuclei are just out of section. Several questionable bodies that may be virus-like containing are present (→). 17,200X, lead-uranyl acetate stained.

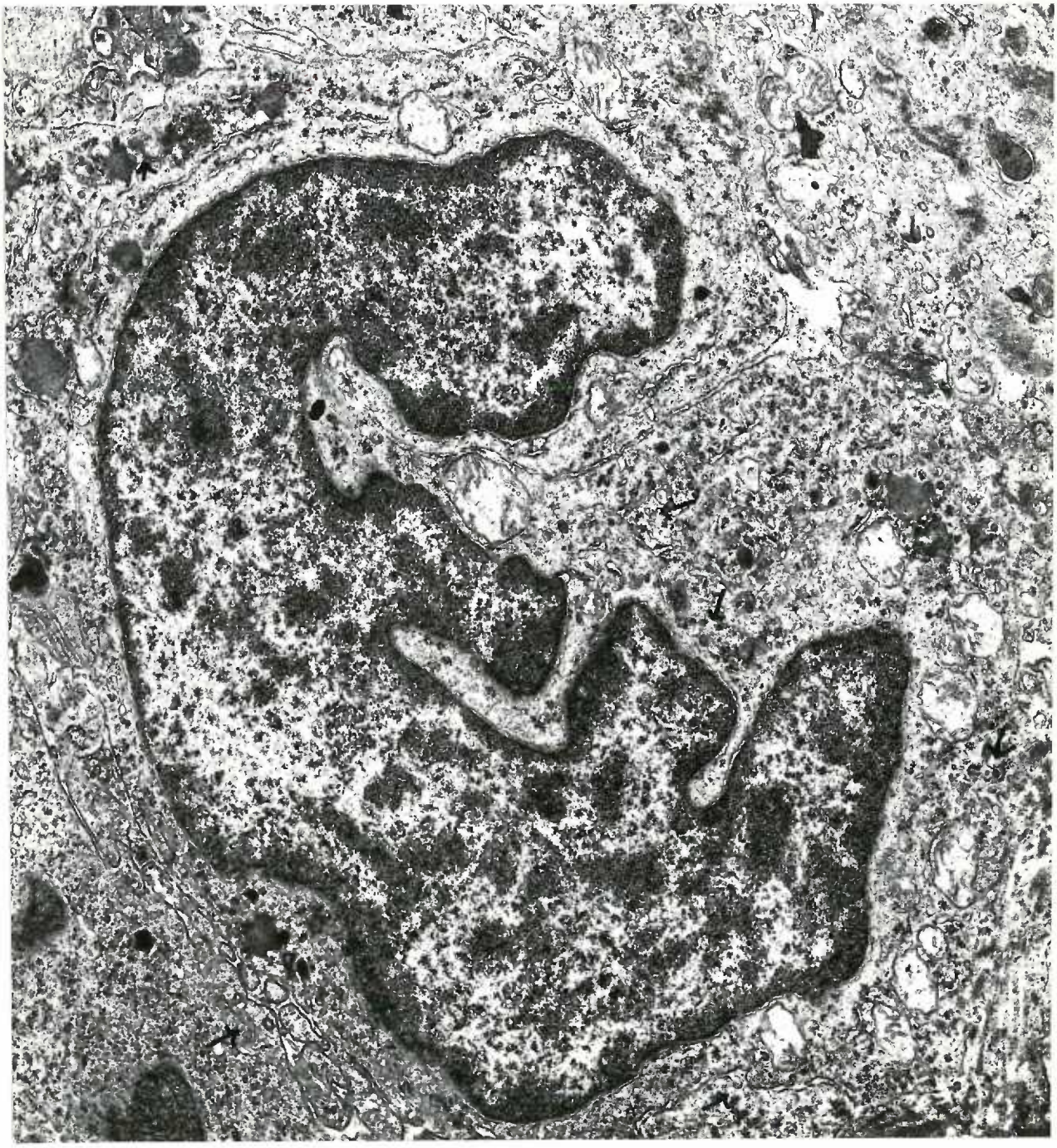


FIG. 28 A Reed-Sternberg Cell

Figure 29. A large reticulum cell. A large reticulum cell showing marked pinocytosis (→) and some rough (er) endoplasmic reticulum. Ribosomes are sparse. 17,200X, lead-uranyl acetate staining.

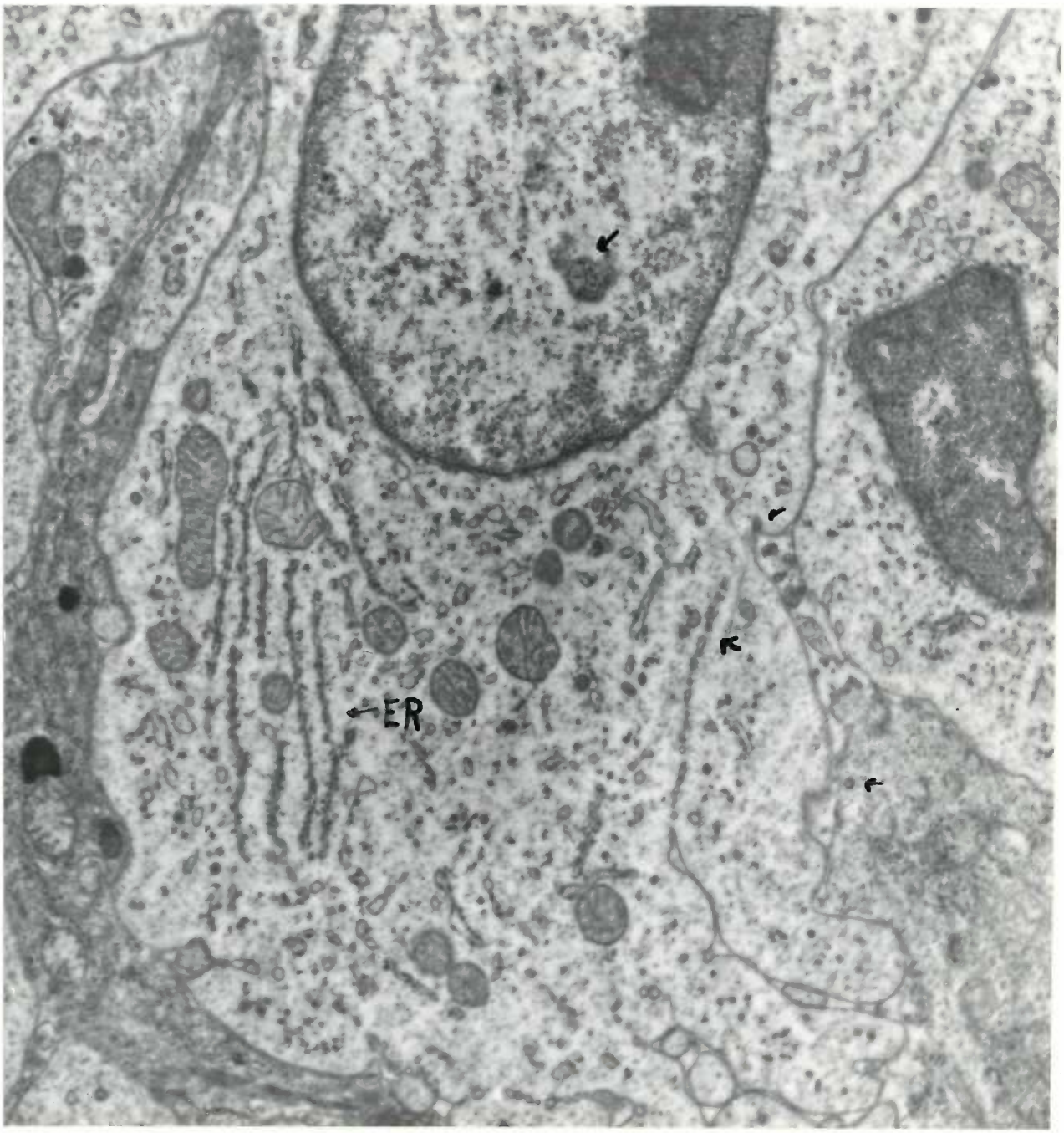


FIG.29 A Reticulum Cell

Figure 30. A Reticulum Cell Showing a Portion of The Nucleus. This is from another Hodgkin's disease case showing the fibrillar structures (⟶) and some of the small saccules (⟶) frequently observed possibly from an out of section Golgi zone. Several possible Mycoplasmal structures are also present. The nucleus (N) is that of a relatively mature reticulum cell. 33,200X.

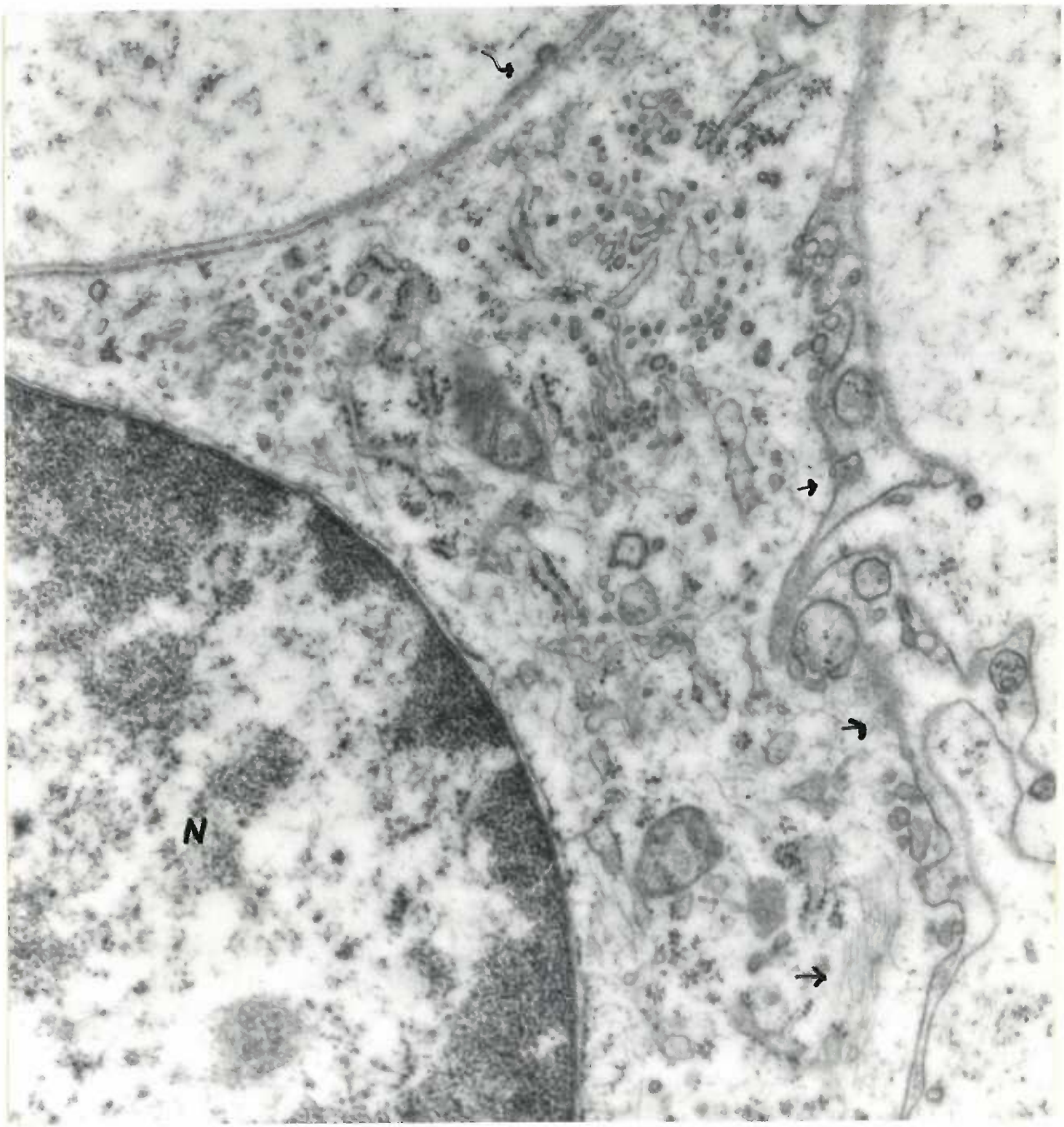


FIG.30 A Portion Of A Reticulum Cell

Figure 31. A portion of a mature reticulum cell. From the same Hodgkin's disease patient, as shown in Figure 30 was taken this reticulum cell demonstrating; a nuclear body (Nb), mycoplasma-like inclusions (M), fibrillar structures (F), degenerate mitochondria (dM), and a greatly dilated rough endoplasmic reticulum (→) suggesting an active secretory function for the cell. This cell also shows an alteration of nuclear membrane that seems related to the formation of some of the small vesicles (→). Note the mycoplasma-like structures (M). In the two dense bodies is slight evidence of structures of an unknown nature. 33,200X, lead-uranyl acetate staining.

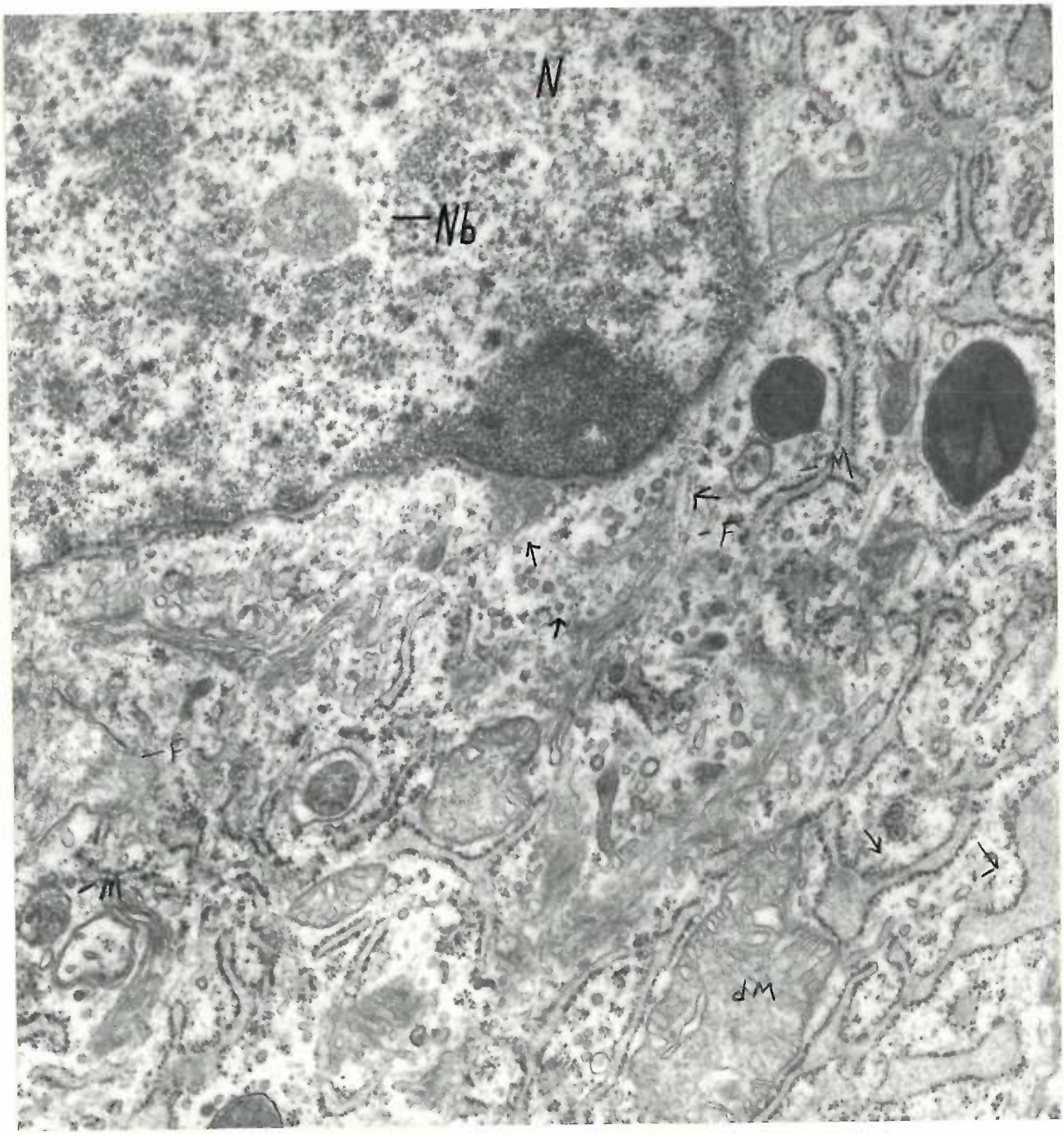


FIG. 31 A Portion Of A Reticulum Cell

Figure 32. A portion of the nucleus of an abnormal reticulum cell from Hodgkin's disease showing nucleolar fragmentation (Nu) and another unidentified structure of microtubules (T). What endoplasmic reticulum (→), appears to be rough and active as in Figure 31. 17,200X, lead-uranyl acetate staining.

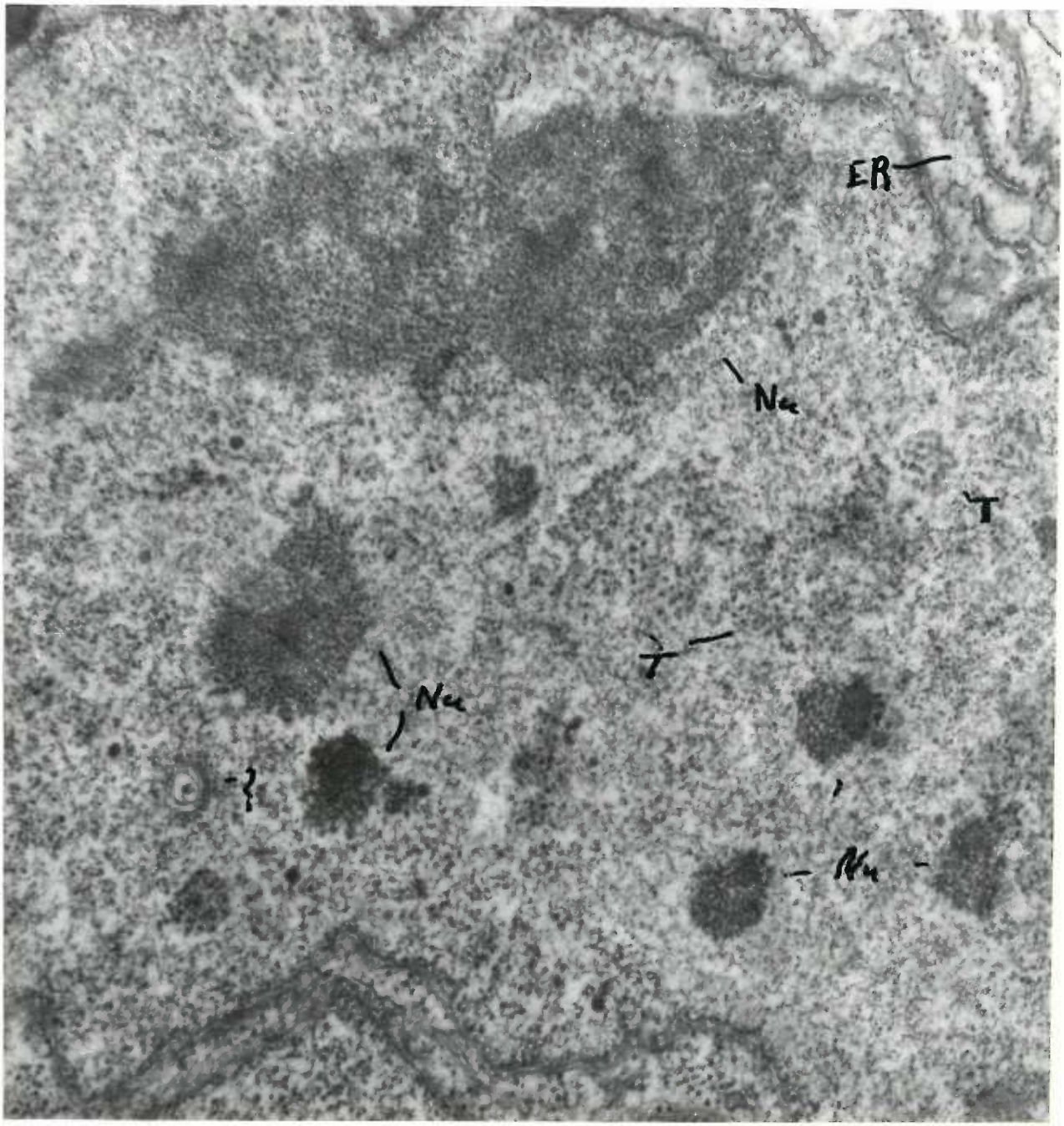


FIG. 32 Reticulum Cell Nucleus

Figure 29 depicts a more normal reticulum cell from another case; note the intense pinocytotic activity, but again the sparsity of ribosome is noticeable. The nucleus contains a nuclear body as described by Brooks and Siegel (156). No specific significance can be attached to this body at present. Many of the more immature reticulum cells have a greatly dilated endoplasmic reticulum (Fig. 31) and evidence of some phagocytic activity. Nucleolar fragmentation and inclusions, (Fig. 32) that may be virus-related, in addition to the nuclear body previously mentioned, were present. Fibrils present in Figures 30-31 may also be virus-related rather than unreleased reticulins; the small inclusions appear to be from an out-of-section Golgi area suggesting their lysosome nature. Small bodies of various types (Fig. 30-31) that can be called Mycoplasma-like and a few virus-like particles were found, but the rarity of occurrence made this an extremely unreqarding, time-consuming search. Higher magnification than that achieved here is necessary for more certain confirmation of the nature of such particles.

Immunofluorescent Results.

In Table 3 the individual cases tested with autologous serum are listed. Of 18 lymphomas tested 13 had circulating antibody against antigen in their tumor tissue, as determined by fluorescent indirect test (FA). Of the tissues, other than neoplastic tested, normal node tissue, and tissue from a non-specific lymphadenitis cases demonstrated antibody in autologous serum. The reaction patterns in these cases were not essentially different from those found in some cases that are considered neoplastic. The FA reaction in the non-neoplastic cases consisted of bright dots in a small number of macrophagic cells. Membrane coating was also demonstrated. In other cases predominately those of DiGugleilmo disease and two lymphoma cases a more general cytoplasmic reaction was found. In some cases both FA Bright spots and membrane coating was demonstrated on the same case. In sections examined as controls, on each tissue studied, only infrequently were plasma cells found by reacting the tissue with fluorescent labled anti-human globulin. In checking for cross-reaction, an imprint or section was used as positive control that reacted with the serum being tested. Stability of the antigen in the tissue has limited the number of cross-reactions tested. In a few of all cases tested there was a strong cytoplasmic reaction of many cells but more often the FA reaction involved only a few cells

TABLE 3
IMMUNOFLUORESCENT TESTS OF AUTOLOGOUS SERUM

		Lab no.	Fluor. anti - G	Autologous serum
1	Reactive hyperplasia	6	-	-
2	Lymphocytic lymphoma	7	-	+
3	Lymphocytic lymphoma	43	-	-
4	Lymphocytic lymphoma	63	-	+
5	Lymphocytic lymphoma	64	-	+
6	Lymphocytic leukemia	44	-	+
7	Lymphosarcoma	40	-	+
8	Lymphosarcoma	19	+	+ *
9	Lymphosarcoma	43	-	-
10	Lymphosarcoma	48	+	+ *
11	Lymphosarcoma	54	-	+
12	Normal	21	+	+ *
13	Normal	32	-	-
14	Normal	26	-	+
15	Lymphadenitis	14	-	-
16	Lymphadenitis	12	-	-
17	Lymphadenitis	17	-	+
18	Dermatopathic lymphadenitis	47	-	-
19	Hodgkin's	31	+	+ *
20	Hodgkin's	28	+	+ *
21	Hodgkin's	42	-	+
22	Hodgkin's	52	+	+ *
23	Hodgkin's	53	+	+ *
24	Reticulum cell sarcoma	24	-	-
25	DeGugleilmo syndrome	61	-	+
26	DeGugleilmo syndrome	62	-	-
27	Boeck's sarcoid	34	-	-
28	Adenocarcinoma	45	-	-
29	Fatty replacement	46	-	-

* These cases had fluorescent reactions that were differentially identifiable as positive in reference to the control slide treated with fluorescent labeled antihuman globulin serum.

\- Negative
\+ Positive, demonstration of specific fluorescence

in small clusters or single cells scattered across the imprints. In some cases small bright areas within cells predominated. At this time no specific pattern exists in relation to clinical diagnosis. The cell type was generally that charateristic of the disease process involved. In lymphocytic lymphoma a few lymphoblasts were involved, but the larger reticulum cells and macrophages were also occasionally fluorescent. In some cases a comparison of material, imprint, or frozen section treated with labled antihuman globulin was made. In one Hodgkin's disease case a moderate number of plasma cells were found on the stained imprint, and these were observed to react with the labled antihuman globulin serum used as control testing. Eosinophils and mast cells demonstrated autofluorescence of specific granules attributable to monamines that they contain (140). At no time did this specific fluorescence extend to mature rubricytic cells except when the Coomb's test was also known to be positive. Fixed imprints were stable for 3 months, blocks of frozen tissue up to a year at -70°C. This was demonstrated in Rauscher virus--infected mouse spleen with specific antiserum. However other antigen may not be this stable. Some suggestion of this was found in retesting as in cross-reacting sera on other node tissues.

When possible, serum that was known to contain antibody was tested on other tissue.

Tables 4 and 5 show the tests made, in addition to some normal sera checked against these tissues. It is interesting to note that normal serum MF (Table 4) reacted with four lymphocytic cases, one Hodgkin's, and one reactive hyperplastic tissue. Two of the lymphocytic lymphoma cases cross-reacted. These cases were brothers aged approximately 4 and 5 years, constituting a unique situation. Parents' serum, mother and father, were negative, as was normal MF at the time of diagnosis. Nine months later a recheck of the patients demonstrated the same cross-reaction and a now Coombs' positive antibody. Reaction with normal serum MF and gamma globulin from a pooled source were positive, while another normal serum checked at this time was negative. One Hodgkin's serum reacted on another tissue while another Hodgkin's serum was negative on the same tissue. Serum from another Hodgkin's case reacted strongly with two chronic lymphadenitis cases and one normal node that demonstrated autologous antibody. Another lymphadenitis case reacted with serum from a two lymphocytic lymphoma patients; autologous serum was also positive. Of the normal sera tested, which came from eight different persons tested against one or more tissues, only normal MF and the pooled gamma globulin were observed to react in FA tests.

In an effort to gain an additional antigen antibody system MLV and RLV antisera were used. With anti-RLV sera (Table 6), four cases demonstrated

TABLE 4

IMMUNOFLUORESCENT TESTS: CROSS-REACTIONS OF LYMPHOCYTIC LUMPHOMA TISSUE WITH OTHER LYMPHOMA AND NORMAL SERA

TISSUE	Lab. no.	SERUM	Reactions
Lymphosarcoma	19-19	Autologous	+
	- 0	Normal MF *	+
	- 0	Normal	-
Lymphosarcoma	43-43	Autologous	-
	-42	Hodgkin's	-
	-24	Ret. cell sarcoma	-
	-32	Normal	-
Lymphocytic leukemia	44-44	Autologous	+
	-32	Normal	-
Lymphocytic lymphoma	7- 7	Autologous	+
	- 0	Normal MF	+
Lymphocytic lymphoma	63-63	Autologous	+
	-64	Lymphocytic lymphoma	+
	- 0	Normal, mother	-
	- 0	Normal, father	-
	- 0	Normal MF	++
	- 0	Gamma globulin, pooled	+
Lymphocytic lymphoma	64-64	Autologous	+
	-63	Lymphocytic lymphoma	+
	- 0	Normal, mother	-
	- 0	Normal, father	-
	- 0	Normal MF	++
	- 0	Gamma globulin	+

* Normal MF denotes the researchers serum.

\- No immunofluorescence

\+ Specific fluorescence

TABLE 5

IMMUNOFLUORESCENT TESTS: CROSS-REACTIONS OF LYMPHOMA AND NONLYMPHOMA TISSUE WITH LYMPHOMA AND NORMAL SERA

TISSUE	Lab. no.	SERUM	Reactions
Hodgkin's disease	31-31	Autologous	+
	- 5	Hodgkin's	+
	-42	Hodgkin's	-
	-32	Normal	-
Hodgkin's disease	28-28	Autologous	+
	-29	Normal	+
Reticulum cell sarcoma	24-24	Autologous	-
	-19	Lymphosarcoma	+
	-32	Normal	-
Chronic lymphadenitis	12-12	Autologous	-
	-24	Ret. cell sarcoma	-
	-28	Hodgkin's	+
	-26	Normal	-
Chronic lymphadenitis	14-14	Autologous	-
	-24	Ret. cell sarcoma	-
	-28	Hodgkin's	+
	-26	Normal	-
Lymphadenitis	17-17	Autologous	+
	-19	Lymphosarcoma	+
	- 7	Lymphocytic lymphoma	+
	-32	Normal	-
Normal	26-26	Autologous	+
	- 0	Normal MF *	-
	-19	Lymphosarcoma	-
	-28	Hodgkin's	+
Normal	32-32	Autologous	-
	- 0	Normal MF *	-
	-31	Hodgkin's	-
	-19	Lymphosarcoma	-
Reactive hyperplasia	18-18	Autologous	0
	- 0	Normal MF *	+
	-28	Hodgkin's	-

* Normal MF denotes the researchers serum.

\- No immunofluorescence
\+ Specific fluorescence

a weak reaction in some cells. The fluorescence was either cytoplasmic or membranous. These cases were unrelated clinically. Rubricytes and certain granulocytes in peripheral blood smears of the first DiGuglielmo's syndrome patient had brillant specific FA reaction in the cytoplasm with anti-RLV serum. Autologous serum gave a similar response (Fig. 33). Cortisone therapy was in use at the time smears and serum were secured. Later tissue from necropsy, spleen, and aortic nodes failed to react with either serum. A small series of autopsy tissues was tested for use in this study. High levels of autofluorescence and erratic staining resulted in exclusion of such tissues from further study. A second erythemic myelosis case presented the typical erythrocytic involvement of the bone marrow. This case rapidly progressed to a terminal blastic crisis. Abnormal rubricytes and terminally present hemocytoblasts failed to demonstrate fluorescence with the anti-RLV serum; autologous serum was also negative. Table 6 shows the cases tested with this antiserum. Mouse tissue from animals infected with RLV virus served as positive controls. Figure 33 shows a group of megakaryocytes exhibiting brilliant fluorescence with anti-RLV serum. In mouse tissue this is the typical response. In rat tissue the FA reaction is more general in other cells.

With the application of anti-MLV serum to human node tissue in an indirect FA test, one case, a lymphosarcoma

Figure 33. Immunofluorescent Reactions.

A. The bright spot on the nucleus of this reticulum cell is considered to be in the cytoplasm or on the cell membrane rather than within the nucleus. 1800X.

B. At lower magnification, this picture allows some orientation to other cells in the immediate vicinity. 800X.

C. Membrane-binding reactions are illustrated in this example from another lymphocytic lymphoma. 800X.

D. The bright cells are from a mouse infected with RLV, tested with anti-RLV serum. 400X.

E. This group of prorubricytes and several metarubricytes were from a DiGuglielmo syndrome patient in an acute leukemic phase. The anti-serum used was anti-RLV. 1000X.

F. A slide tested with autologous serum showing neutrophils. 1000X.

G. These bone marrow smears were taken 9 months after diagnosis from a Coombs' positive patient and tested with autologous serum. 1000X.

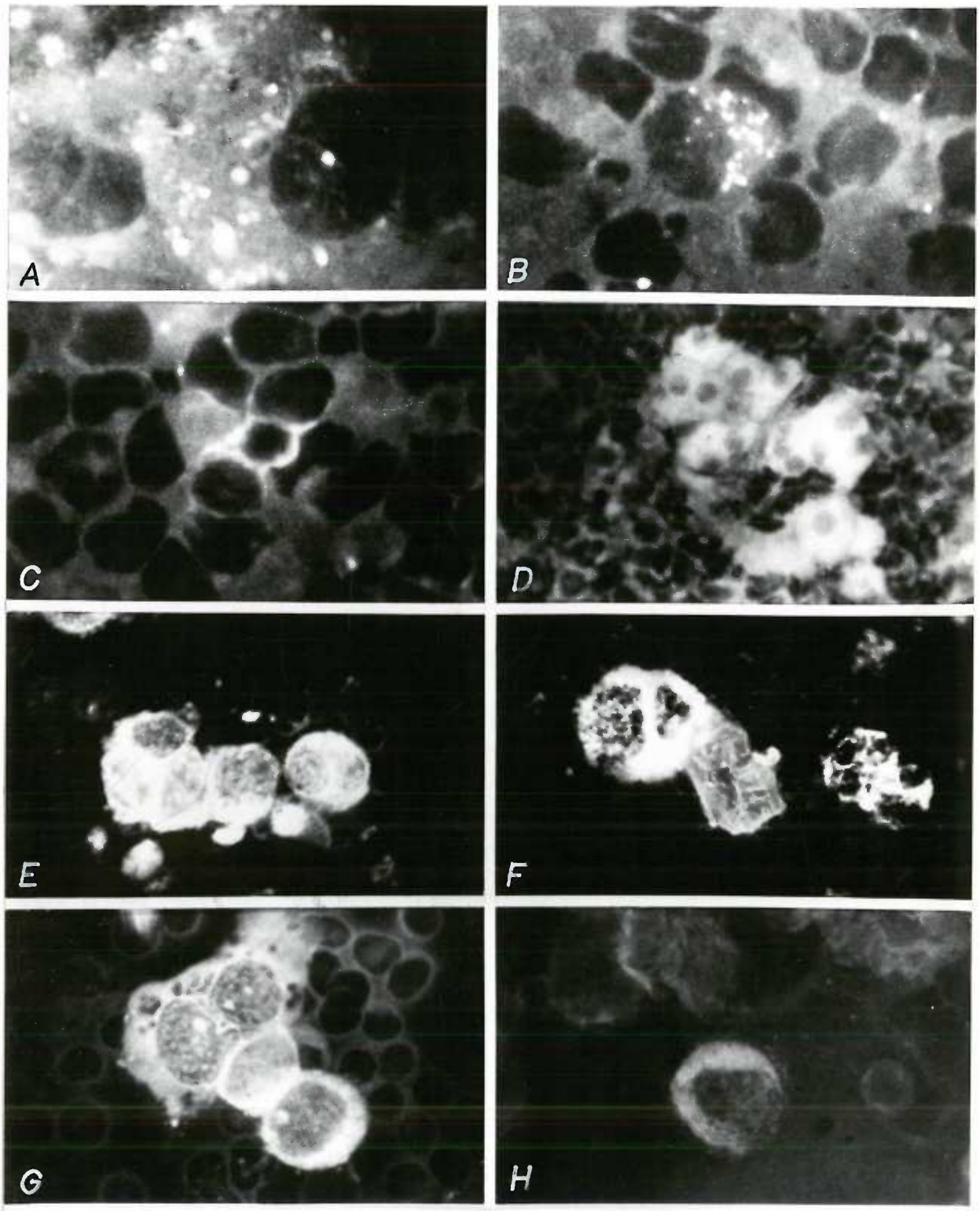


FIG. 33 Immunofluorescent Reactions

TABLE 6
IMMUNOFLUORESCENT TESTS WITH ANTI-RAUSCHER SERA

	Diagnosis	Lab. no.	Anti-RLV	Pos. (1) control
1	Mylogenous leukemia	A	-	+
2	Lymphocytic leukemia	44	-	+
3	Lymphosarcoma	40	-	+
4	Lymphocytic lymphoma	43	-	+
5	Hodgkin's disease	42	+*	+
6	Lymphosarcoma	48	+*	+
7	Lymphocytic lymphoma	63	-	+
8	Hodgkin's disease	53	-	+
9	Fatty displacement	46	+*	+
10	Adenocarcinoma	45	-	+
11	Dermatopathic lymphadenitis	47	+*	+
12	DeGugleilmo syndrome	61	+++*	+
13	DeGugleilmo syndrome	63	-	+

1 Positive controls were Rauscher virus-infected BALB/c mouse spleen imprints.

* These reactions were rechecked independently by a second test.

TABLE 7
CORRELATION OF IMMUNOFLUORESCENT RESPONSE WITH BETA-HYDROXYBUTYRATE DEHYDROGENASE AND LACTIC DEHYDROGENASE

Anti-Sera			Enzyme Reactions	
RLV	MLV	Source of Tissue	LDH	BHBDH
-	+	Lymphosarcoma	++	+++
-	-	Lymphosarcoma	++	-
-	-	Lymphosarcoma	++	-
-	-	Hodgkin's disease	++	-
-	+ a	Reactive hyperplasia	++	-
+ b	-	Dermatotrophic lympadenitis	++	-
+++	-	DiGuglielmo snydrome	0	0
-	-	DiGuglielmo syndrome	0	0
-	-	Normal human node	++	-
0	++	MLV-infected BALB/c spleen	++	+++
-	++	MLV-infected BALB/c spleen	++	+++
-	++	MLV-infected BALB/c spleen	++	+++
-	++	MLV-infected BALB/c spleen	++	+++
-	++	MLV-infected BALB/c spleen	0	0
-	++	MLV-infected BALB/c spleen	0	0
+++	-	RLV-infected BALB/c spleen	++	-
+++	-	RLV-infected BALB/c spleen	+++	-
++	-	RLV-infected BALB/c spleen	+++	-
+++	-	RLV-infected BALB/c spleen	++	-
++++	-	RLV-infected BALB/c spleen	0	0
+++	-	RLV-infected BALB/c spleen	0	0
0	0	RLV-infected BALB/c spleen	++	-
0	0	RLV-infected BALB/c spleen	++	-
-	-	Normal mouse spleen	+	-
-	-	Normal mouse spleen	+	-

0 - not done
- to ++++ extent of positive reaction neg. to 4.
Immunofluorescent response:
a. reaction in capillary endothelium
b. a very weak reaction

Figure 34. Specific Fluorescence and BHBDH Histochemical Reaction.

A. Anti-MLV antiserum showing specific fluorescence on Moloney-infected mouse spleen tissue. Frozen section, 1000X.

B. Anti-MLV serum showing specific fluorescence on a frozen section from a dermatotrophic lymphadenitis case. 186X.

C. No specific fluorescence, MLV antivirus serum on normal mouse tissue, frozen section.

D. Anti-MLV serum showing specific fluorescence on a human lymphosarcoma case, frozen section. 1000X.

E. BHBDH reaction on normal mouse tissue, frozen section.

F. BHBDH reaction on MLV-infected mouse spleen tissue, frozen section, graded 3 plus.

G, & H. The BHBDH reaction on the node tissue as shown in D, a case of lymphosarcoma. Normal human node tissue remained as in E, as did all other of the human lymphomas tested.

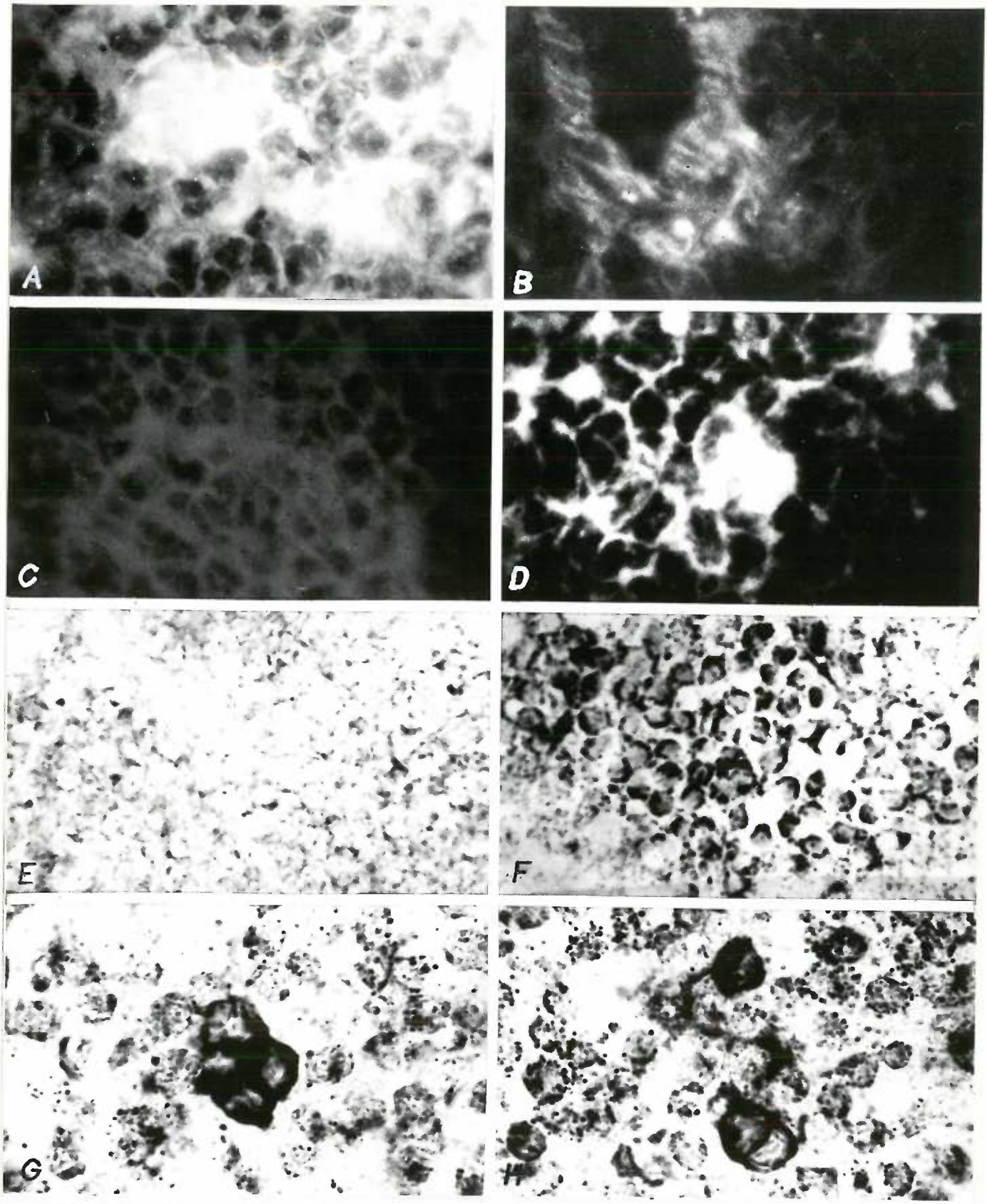


FIG. 34 Specific Fluorescence And BHBDH Histochemical Reaction

case, gave brilliant reaction as shown in Figure 34. A dermatotrophic lymphadenitis case also reacted with anti-MLV, showing weak specific reaction in sinusoidal reticulum cells (Fig. 34). Lymphoid tissue from MLV-infected BALB/cJax mice and the human lymphosarcoma case demonstrated small irregular areas of fluorescence with adjacent cells often weak or inactive. The areas of activity seemed to correspond to the degree of abnormal cell invasion into more normal tissue. The areas were more variable in mouse tissue where different degrees of involvement existed in different animals. Both May-Grünwald Giemsa-stained imprints and hematoxylin and eosinstained sections had corresponding irregular areas of larger immature mononuclear cells surrounded by mature cells of the lymphoid series. Mice infected with RLV, normal mice, and other human tissues tested did not react with this antiserum. Table 7 shows these FA reactions.

Patients' serum from the cases that reacted with the antimurine virus leukemia sera did not contain antibody against the virus even though there was antibody present against an antigen in their own tissue. Other human sera from this series were tested on mouse tissue, normal, and RLV- or MLV-infected. No positive reactions were found in the 15 tested.

Histochemical Results.

In Table 7 is listed the histochemical observations of LDH on a series of human and mouse tissues. Intensity was graded on a 0 to 4 plus scale. In this series of tissues no change could be detected between normal human and lymphoma tissue. A correlation was not shown by the human tissue and RLV-infected mouse spleen. In general the more immature cells and abnormal necrotic tissue displayed a higher level of enzyme, hence in the mouse spleen an overall increase from plus 2, to plus 3 was observed. Human node tissue remained plus 2, and had little evidence of necrosis.

With BHBDH substrate, MLV-infected mouse spleen and one human lymphosarcoma case demonstrated a plus 3 activity, while mouse and human normal tissue, RLV-infected mouse tissue, and other human lymphoma tissue failed to demonstrate the presence of the enzyme. In both the human lymphoma case and in the MLV-infected mouse spleen the same follicular pattern previously described as coincident with the immunofluorescent reaction to anti-MLV sera existed. The human lymphosarcoma case was the same patient in both cases. The BHBDH in the node tissue and in the MLV-infected mouse was partially resistant to iodoacetate whereas the normal enzyme in other mouse tissue was completely inhibited, suggesting the existence of an isozyme. Heated slides were negative with both enzymes tested. The BHBDH was found to be labile under -70 C storage after 4 months, as determined with MLV-infected mouse tissue.

Acid phosphatase testing was done both on imprints and on frozen section, using the di-azo technique of Burstone as described by Pearse (144). Three Hodgkin's cases, one lymphoblastic lymphoma, and a reactive hyperplasia were examined. Figure 35 is from the Hodgkin's cases. In general, abnormal reticulum cells and Reed--Sternberg cells were strongly positive, while lymphocytes also reacted more strongly than normal node tissue. A lymphoblastic lymphoma, a peritoneal node, also demonstrated a high level of acid phosphatase activity in the large histiocytic cells and a lower level though still above normal in the immature cells of the lymphocytic series. The reactive hyperplasia similarly demonstrated activation of this enzyme to a 3 plus level in histiocytic cells and to a lesser degree in the lymphocytes. Mouse liver slides were used as positive control and heated slides as negative controls. Counterstaining in general was not used nor found necessary for the identification of the cells. Imprints offer advantages of cell-type differentiation but frozen sections are often more easily stored and offer better area relationships. Reticular cells tend not to adhere to the slides in the making of imprints, hence frozen reactions should also be done.

Figure 35. Acid Phosphatase Histochemical Reactions.

A & B. This picture shows strongly reactive abnormal histiocytes and Reed-Sternberg cells from two Hodgkin's granuloma cases. 250X.

C & D. May-Grunwald Giemsa-stained imprints of the same two cases showing the abnormal cells in detail.

E. & F. Similar imprints at the same magnification from another Hodgkin's case showing the strong acid phosphatase reaction in a Reed-Sternberg cell and in the two small lymphocytes close to it. Incubation time, 2 hours at 37 C. Heated slides failed to react with the substrate. 2100X.

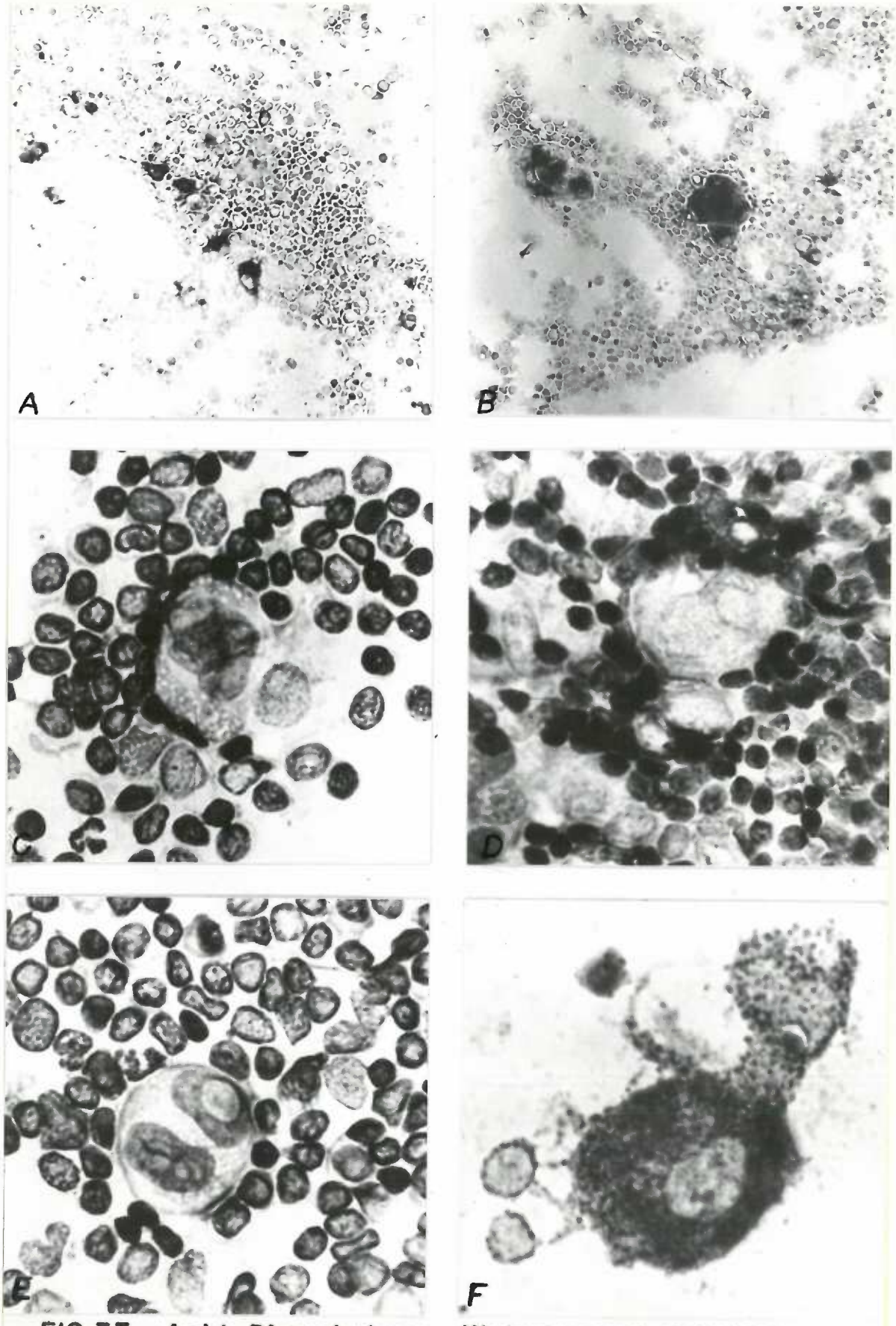


FIG.35 Acid Phosphatase Histochemical Reaction

DISCUSSION

Numerous reports of culturing human lymphoid tissue and of virus isolation have appeared with increasing frequency since the inception of this project. Some of these studies present significant differences in findings. Some of these differences may be related to differences in semidefined media used or other conditions of culture, which are at times subtle until fully recognized. Brooke and Osgood (37,38,39) found that oxygen tension, as reflected by the depth of medium, was significant in establishment of hematopoietic cultures. This may be a factor in repeated difficulties of establishing continued cultures from other than lymphoblastic lymphomas and myelogenous leukemias. Similarly this is reflected by the necessity of controlling the depth of medium with free fluid cultures, as results in Burkitt's lymphoma (13,14) and with other recently isolated cell lines (19,157). Though not extremely critical, medium depth reflecting oxygen tension is one factor. The persistence of round free blastic cells or the establishment of free hemocytoblasts as opposed to attached more fibroblastic cultures seems related to the source of tissue (bone marrow, peripheral blood, or biopsy), the pathology involved, and factors in the media and serum. Biopsy tissue and bone marrow (159,160,161) are more inclined to form attached cultures: peripheral blood, though immature cells are present that show loss of mature

differentiation in long-term culture, appears to favor the establishment of free cultures. In relation to the pathology involved, lymphoblastic lymphocytic lymphoma or leukemia, and myelogenous leukemia again favor free, fluid cultures (19, 157). Benysh-Melnick et al (159), Trujillo et al (160), Sinkovics et al (161), and this report have predominantly found attachment of all cell types, resulting in some cases in continued production of free blastic cells. Both types of cultures, one possible extract a clone, and one existing necessarily as a mixed population, offer two different avenues of approach to the study of this form of human neoplasia and virus isolation. The second duplicates more closely the cell relationships found in bone marrow and solid tissue. Fluid, free-cell cultures, even if a clone is made, are more analogous to circulating blood. Factors of metabolism and control involving interactions of hematopoietic cells are not yet fully understood nor has the maintenance of these cells in serum-free medium been accomplished. Metabolic studies of hematopoietic cells in culture have not yet been done though some observations were reported by Moore et al (158).

In the cultures reported in this thesis, monolayers of mixed cell types were formed. Production of free blasts occurred in some cultures, particularly the later group where media C and D, without hormones, were used. Many of these cultures initiated prompt fibroblastic and

epitheloid outgrowth, formed confluent monolayers and under went four to five subdivisions. After these subdivisions a slowing in doubling time was observed and development of RNA barring occurred (162), ending with the loss of the culture. Inclusion of vitamin B_{12} and trace metals reduced this situation, increasing the number of subdivisions.

Medium formula D with 10% calf serum was used with the second DiGugleilmo syndrome case cultured from bone marrow. Inclusion of the unusually high level of lysine (290 mg/liter) was necessary to maintain mitosis of the hemocytoblasts that were present in large numbers. Interestingly, these cells attached within 14 days as epitheloid giant cells. The calcium concentration of the media did not seem to be significant in relation to the attachment of these cells. In all cultures of this type, serum offers variables difficult to control and interpret (163,164,165). The current media of Moore et al (158), Ambrose et al (166,167) and medium D in the Appendix of this paper, approach complete chemical definition. Medium D has been used without serum for 7-day cultures of mouse lymphoid tissue in another project. * Use of this medium also resulted in reduction of the percentage of serum used and an increase in doubling time of the two continuous cultures now in storage.

Kanamycin (400 µg/ml) and penicillin (1000 ug/ml) were used in new cultures for 48-72 hours in an attempt

* Unpublished data.

to inhibit Mycoplasma that may be indigenous to this tissue (168,169). Pollock et al (170) reported effectiveness of this level of kanamycin in clearing Mycoplasma-infected tissue cultures of these agents. Isolation of Mycoplasma from leukemia and lymphoma cases, though erratic, suggests its presence in this type of tissue (19,20,157,168,169). Early attempts in this study to identify Mycoplasma as present were negative. In the last three lymph nodes handled, Mycoplasma were found in the original material cultured. A change in culture medium and technique of culture is considered responsible. Reduction in early cell lysis by the high level of antibiotics, determined by following six parallel cultures, is thought to result from suppression of growth of these agents. Lower concentrations of either antibiotic were not effective, reducing the possibility that metabolic inhibition of the cells by the action of the kanamycin was the responsible factor. Stabilization of the lysosomal membrane could be occurring. No bacterial contamination was found except in two cases so described. Maintenance of the cultures in an acid pH (6.5 - 6.8) also facilitated the suppression of this lysis and seems to encourage growth of the lymphocytic population. This suppression offers further evidence of the involvement of Mycoplasma in lysosomal swelling of the lymphoid cells. Viral origin of this lysis seems unlikely but cannot be ruled out.

Type II, the "Hairy" lymphocytes reported by Shereck and Donnely (154), were found in large numbers in response to Nocardia, indicating that this cell alteration is not restricted to malignant changes. Type II also seems involved in inflammatory reactions of a more chronic nature. Correlation of finding Type II cells free in peripheral blood in large numbers as did Shereck and Donnely (154) with a specific type of histiocytic leukemia chiefly involving spleen, did not occur in this study. The finding of this cell type in smaller numbers or in tissue in large numbers in other than this clinical condition should be a word of caution to diagnosticians, and suggests another mode of lymphocyte transformation as described in early research (31,32,33), in addition to the blastoid transformation in responce to antigen and phytohemagglutinine (PHA). This transformation, seen in Type II, paralleled changes in abnormal lymphocytes that hypertrophied and attached in lymphocyte-involved dyscrasias.

Type III, the transitional reticulum cell, was seen more frequently in diseases where reticulum cells were involved in the disease process. Shereck and Donnely (154) also reported this cell. The extremely spiny appearance is also seen in adenocarcinoma cells during transition to attached giant-cell morphology. When the cell was motile, its membrane became smooth and an extremely ruffled haloplasmic membrane was formed. This spiny appearance was not

a mode of degeneration or a phenomenon in response to physical factors of cold or light. The apparent function is implied by the name, transitional reticulum cell. It is interesting to note that not all attaching cells show this change; many remain relatively smooth in outline, but the tips of the protrusions do become points of attachment, suggesting a specific membrane function.

Type IV morphology has been described consistently (26,33,34,35,36,42,43,44,45) in cultured abnormal lymphoid tissue. Correlation of these cells with malignancy was not consistent. Foreign-body cells including the Langhan's type are found in many diseases where certain types of antigen exist (64,172). Among other antigens stimulating their production are a low level presence of many types of viruses (171). Polykaryocytes were found in all cultures in variable numbers except normal nonreactive node tissue. Where no bacterial, mycotic, or red-cell stimulation could be attributable, virus can be considered a possible cause.

Aside from the specific sequential morphologies just noted, other changes in these cultures are of significance and require mention. The formation of a new sheath in normal tissue and its abortive formation or lack of formation in malignant tissue reflects the loss of organization via loss of contact inhibition, and in some cases the physical absence of the requisite reticulum cells, as seen in highly involved lymphocytic lymphoma explants.

Changes in nucleolar volume in some of the epitheloid cells and fragmentation of the nucleoli were observed in variable numbers in the neoplastic cultures and in some of the hyperplastic tissue. These changes were shown by Bernhard (86) to be consistent with virus presence.

In many spindle cells from lymphoma and some reactive hyperplastic cultures, active Golgi zones were found. However, the balance of changes leading to cell death and presence of virus described by Sykes et al (57) were observed only in response to elevated serine levels, followed in four paired cultures. These Golgi areas stain a pinkish color with May-Grünwald Giemsa in both cultures and imprints of the node tissue.

Grand (44,45) observed virus-inclusion bodies in Hodgkin's disease and thought he had successfully passed these to normal tissues. Though no inclusions were observed a few possible virus-like particles were seen in electron micrographs of some tissues. In one instance normal tissue became available and cell-free filtrate was passed to representative chambers. The formation of Reed-Sternberg cells did not result but other changes including the formation of abnormal reticulum cells, occurred. A detailed study using a diploid human culture of lymphocytic tissue that should include the epitheloid giant cell would be of value and would allow evaluation in terms of transformation and agents present.

Emperipolesis in the form of massive development of lymphocytes within reticulum cells as described by Dryer et al (73) and Ioachim et al (172) was not observed in these cultures. When lymphocytes were found within some reticulum cells, it was only a transitory situation in the first week of culture. Peripolesis and emperipolesis were more common in other than lymphocytic lymphoma cultures. The abnormal lymphocytes were less often motile, forming fewer aggregates, and were hence less often in contact with reticulum cells in the chamber cultures. When lymphocytes were observed in reticulum cells they were in many cases nonmotile. In some cultures two populations of lymphocytes could be clearly followed. Normal small motile lymphocytes could be found moving on the surface nonmotile lymphoblastic cells. In other lymphomas and in adenocarcinoma cultures, though peripolesis was common to certain cells, emperipolesis was neither continual nor heavy. Only in Boeck's sarcoid was the phenomenon outstanding. Here motile lymphocytes within reticulum cells were observed to result in death of some of the reticulum cells. With current knowledge of involvement of sensitized lymphocytes in tumor destruction (65,173), emperipolesis and peripolesis became more meaningful. The inducing mechanisms are unknown as yet, though a chemotactic attraction to dividing cells is suspected (74), and antigenic sensitization has been shown necessary for

the cytotoxic effect (173).

The use of standard virological techniques for determining the presence of latent virus, interference (174), with a second agent (frequently vesicular stomatitis virus (VSV) is used), was not considered in this study due to an absence of information in this area. Currently Benyesh-Melnick (175) has shown equiviocal results with VSV interference; normal peripheral blood lymphocytes gave similar results. Low level production of interferon by some of the oncogenic virus has recently been demonstrated (176).

Some of the morphological factors presented concur in the thesis that oncogenic viruses are present in hematopoietic neoplasia. No isolation of virus was attempted in this study but the preparative information gained allows satisfactory culture of the tissues involved, and recognition of morphological clues to the possible presence of virus. Recent reports are divided upon the finding of virus-like particles in electron micrographic studies of the cultured cells (19,58,155,172,175,177,178,179,180). Variation of specific amino acid concentrations in media may enhance the production of virus in other lymphoma cultures, as was shown by Henle and Henle (191) working with Burkitt's lymphoma.

In the electron micrographic studies of the node tissue it was soon obvious that finding virus-like particles in node tissue was an extremely unrewarding effort, corroborated currently by Bernhard and Leplus (155), though

others have reported their presence (6,7). Concentration of serum from leukemia patients has more frequently shown virus-like particles, particularly from acute cases (182, 183). From these cases virus of a herpes-like morphology was found if sections were made rather than negative staining, which causes distortion of many of the delicate membrane structures (184). Recent studies at Roswell Park Memorial Hospital (158,179,185) and by Henle et al (180) have shown the herpes-like structures in cultured cells, occasionally in large numbers, including leukemic cells other than Burkitt's tumor from Africa.

Leukemic and lymphomatous lymphocytes tend to present a relatively normal ultrastructural picture. Asynchrony of maturation in nuclei or cytoplasm was found in one case, as long noted by hematologists at the light microscopy level. The abnormality of a given tissue was detectable only by the number of relatively similar stages of development of the majority of cells, as noted by Bernhard and Leplus (155).

In Hodgkin's disease a dilated endoplasmic reticulum cells was noted. Bernhard and Leplus (155) observed this also, concluding the presence of globulin in the dilated structures. Fluorescent antibody studies done concurrently on this tissue did not demonstrate the presence of antibody in this type of cell. The existence of a protein in Hodgkin's disease patients, responsible for febrile

episodes and isolation of this protein (186,187), suggests these cells as a possible source of the protein in question. Higher magnification in several later cases resulted in the finding of rare particles that could be virus or virus-like structures and Mycoplasma. Increasing the magnification above that used in this study would be necessary to confirm the morphology of such bodies as well as application of ferritin-labled specific antibody, when such is available. Added knowledge concerning the structure of mycoplasma of different types has facilitated recognition of these structures at this time.

It was evident that no one clue particularly in the area of morphology was adequate to determine the presence of virus of the non-CPE-producing types. It was evident from this study and that of others (12,30,57,158,159,179, 185,160) that the use of abnormal tissue, either giant cells or the free blastic cells, is desirable for virus isolation in these diseases. Recent work with Burkitt's lymphoma and with mouse leukemia agents has shown the slow release of mature virus from a percentage (1-10%) of the cells. These cells were identifiable as containing antigen (142,143) using FA techniques with specific antivirus or with serum as in Burkitt's lymphoma (181).

In order to further apply FA techniques to human oncogenic virus isolation, autologous serum, serum from other cases, and normal sera were investigated on the node

imprints and sections. Of the lymphomas tested 13 of 18 had antibody against antigen in some cells of their tumors. Three types of response can be characterized in abnormal lymphoid tissues; one reaction is chiefly in macrophages in the form of small FA-reactive dots, distributed in the cytoplasm of the cell; the second is that of membrane FA reaction involving large numbers of cells; the third is that of a general cytoplasmic, often brilliant, FA reaction of some but not all cells seen in peripheral blood and bone marrow, also present frequently in solid tissue. Complement fixing antibody, FA test, was demonstrated in a few cases but without additional cases it cannot be intelligently discussed.

In animal and tissue culture, working with Rous sarcoma virus (RSV), certain adenovirus, papova-virus SV-40, and mouse leukemia agents, the existence of five types of immunological reactions, some overlapping and not clearly distinct, are known (142,143,188,189): (1) a transplantation-rejection type of antigenic system in virus-immunized, tumor challenged animals (190,191), occasionally demonstrating enhancement of the tumor challenge; (2) a complement-fixing soluble antigen measurable in vitro, in animals developing tumors (192,193,194,195); (3) a nuclear antigen possibly the same as the complement-fixing system just mentioned and the same as the T-antigen (196,197); (4) a surface antigen

that reacts with serum of tumor-resistant animals (142,143, 193,198,199); (5) antigens at times in groups 1 and 4, foreign agents, viral (protein coat), mycoplasmal or L-phase bacterial of the Mycobacterium tumerofaciens group (199,200,201,202), some of which may not be directly related to the malignant process but are demonstrable.

The small FA-reactive bodies may represent the fourth and fifth group possibly not directly related to the malignant process, or may be a matter of relative quantity of antigen contained. The membrane coating seen in the tumor, in response to autologous serum, is related to the fourth group in location of antigen; however, the nature of the antigen, owing to the limited number of cells reacting in any one area, may be that of the fifth group. The general cytoplasmic response found in both autologous serum and normal serum suggest the fourth and fifth classifications. Recent studies have shown antibody in 40 % of the normal adult population against the herpes--like agent of Burkitt's lymphoma (180), and against soluble antigens, some complement fixing, from larger numbers of myelogenous and lymphocytic leukemias containing a herpes-like agent (203). An antigenic relationship of the agent from Burkitt's cultures to antibodies formed by convalescent infectious mononucleosis patients has been shown by Henle et al (180,181). The reactions of normal MF serum, the cross-reactions obtained in the lymphomas,

and chronic lymphadenitis cases may be related to this factor. It is interesting from a clinical standpoint that reactive hyperplasias and chronic lymphadenitis cases, known clinically to progress frequently to lymphomas, were reactive to known positive lymphoma serum. Additional information is needed to evaluate the significance of autologous antibody, particularly to clinical implications in respect to enhancement and possible therapy.

The antisera to RLV and MLV were also checked against a small number of lymphoma cases forming a second source of antibody against tumor agents. Siegel et al (204) described the morphological similarity of RLV-leukemia in BALB/c mice to human erythemic myelosis, DiGuglielmo syndrome. Fink et al (142) showed that for reasons yet unknown other types of cases also occasionally reacted. The anti-RLV serum used was from two sources. That prepared in this laboratory in rabbit and that generously supplied by Doctors Fink and Rauscher, made in monkey. Both reacted similarly with due attention to differences in titer and absorption. Partially purified virus was the antigen source for both sera. None of the sera produced nuclear fluoresence or cross-reaction, RLV with MLV.

In the first DiGugleilmo syndrome case a strong FA reaction was observed in response to anti-RLV sera; the second case was not antigenically related to RLV. Rapid transition, (4 weeks) of this latter adult case to a

terminal blastic crisis suggests a possibility of a distinctive clinical variant; though bone marrow initially demonstrated erythrocytic involvement and PAS-positive material in the abnormal erythrocytic cells, factors were felt to be consistent with DiGugleilmo syndrome by Dameshek and Gunz (205). This nonreactive case just described, the sporadic cross-reactions observed with human sera, and the lymphosarcoma cases immunologically negative to MLV encourage three lines of reasoning: (1) No mature, or too few virus were being produced in cells of the negative cases. (2) There may be more than one agent involved in what we consider to be a clinically distinct group by descriptive pathology. (3) The antigenic relationship demonstrated has no direct association with the etiology of the disease in question. A significant situation exists with respect to the third point mentioned in the MLV-induced leukemia and the human lymphosarcoma case.

Enzymatic induction of BHBDH was demonstrated in tissues of both mouse and man, when normally it is not present in either. Enzymatic induction by a virus is not a unique situation, though it is far better investigated at present in bacteria-phage systems used in bacterial genetics. Recently many viruses have been shown to activate lysosomal acid phosphatase (127,128,129), and more specific activation exists in the case of Riley agent

and LDH, though this may be a common characteristic shared with a number of the murine tumor agents (119,120,121). Tissue culture studies have shown the elevation of thymidine kinase in response to SV-40 (115,116). The function of BHBDH is well established in lipid synthesis, but it is an enzymatic reaction not normally found in lymphoid tissue. Therefore activation of this enzyme in lymphoid tissue appears to be associated with the MLV-virus infection.

No clear picture of the frequency of such enzymatic findings in man is available, on the basis of this series or in the literature. However, Braunstein (124) reported one case of lymphosarcoma of eight tested as having a similar BHBDH elevation. One case does not offer conclusive evidence in this area but the possibility that enzymatic activation and antigenic relationship are unrelated is relatively remote. Isolation of the agent from human tissue is necessary for confirmation. Unfortunately a continuous culture did not result from this case and the electron micrographic blocks, owing to technical problems in polymerization of the Epon, could not be thin-sectioned.

The discussion just presented and an increasing number of reports of lymphomas and blood neoplasias in domestic animals of viral etiology (206,207) hopefully will encourage additional investigation using these known virus antibody systems to examine human tumor tissue. At

present the species specificity of the murine leukemia group, RSV, and other tumor-producing agents in primates, particularly man, is unknown. Mycoplasma, particularly those isolated from human leukemias, were initially thought to be highly species specific. Recent studies cast doubt on this, showing relationships through DNA-recombination studies to strains of Mycoplasma from rat and porcupine (208). Domestic animals and particularly genetically inbred strains of laboratory mice, with the exception of axenic animals have been subjected to human contagion for many generations. It is conceivable that some of the agents carried by man have been transmitted to susceptible murine individuals allowing adaptation and continued transmission. Even axenic animals are not tumor or virus free (207), and were derived obviously from existing strains of inbred animals.

The histochemical work reported here was cursory and represents a few exploratory procedures. Acid phosphatase has shown merit in following the possible presence of virus, as has the BHBDH of the MLV-infected mice and the one human lymphosarcoma case. The high level of LDH normally present in lymph node tissue did not show a difference in enzyme level, normal versus lymphoma, as did normal mouse versus RLV-infected tissue. The use of node tissue in man rather than spleen as in the mouse may be one of the factors involved in the lack of difference

shown. In general the use of fixatives, autopsy tissue, and widely differing methods in early reports are hard to correlate with current findings.

This study has confirmed the relation of foreign-body giant cells to pathological tissue and their absence in non-reactive normal tissue. A transmission of some characteristics of Hodgkin's disease by cell-free filtrate to normal tissue occurred in one case. Contrasts in morphology and behavior of cultures from hematopoietic neoplasia were discussed. A medium was developed that will support good rapid sustained growth of hematopoietic tissue. Immunofluorescent antibody studies have shown that many patients do have antibody against antigen in some of their tumor cells. Cross-reactions and antibody present in some normal serum indicate that this antibody may be foreign--agent system in some cases or tumor related antigen in others, but not an individual-specific system. The relationship of RLV to human erythemic myelosis, and the relationship of certain lymphosarcomas to MLV is suggested. Autologous antibody in cases that reacted with anti-MLV and -RLV was not antimurine viral specific. Use of cultured tumor tissue in conjunction with autologous and specific antibody sera, when available; as well as histochemical tests of possible viral induced enzymatic changes; and electron microscopy offer good methods of working toward oncogenic virus isolation, (208,209,210,211,212).

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APPENDIX

APPENDIX

Immunization Schedule Used For Rabbit Anti-guinea Pig Complement Serum

1. Fresh whole guinea pig serum was used, mixed equal parts with complete Freund's adjuvant, and injected into an adult rabbit, 1 ml IV, 1 ml in two sites IM.

2. Ten days later ½ ml of fresh serum was given IV at 4-day intervals for five doses. Test bleeding had an anti-guinea pig serum titer of 1:50,000.

3. The antiserum was labled as whole serum by method of Rinderkrechet (139). No absorption with mouse tissue was necessary and no antimycobacterium antibody was detected in the complement test after a 1:5 dilution of the labled serum.

APPENDIX

Method for Anti-RLV Serum Production

1. Newborn rabbits were made tolerant to mouse tissue with 0.2 ml of a 100 mg/ml homogenate of BALB/c spleen, kidney and liver, given on the day of birth and then daily for 20 days. Then once a week 0.2 ml was given until the rabbits were 9 weeks of age; 0.1 ml was then given until immunization was started at 12 weeks. A test bleeding indicated that anti-mouse was not present in the serum.

2. Spleens from RLV infected mice were concentrated using Moloney's technique (213).

   A. Spleen tissue was mixed with equal amounts w/v of potassium citrate (0.306M) containing 3 mg/100 ml of hyluronidase and allowed to stand 1 hr at room temperature.

   B. Mixture was centrifuged 1,200G for 15 min. Sediment: Repeated steps A and B. Supernatant: Step C.

   C. Supernatant from B was centrifuged at 2,300G for 20 min. discarding sediment.

   D. Supernatant from C was centrifuged 10,000G for 2 min.

   E. Supernatant from D was centrifuged 30,000G for 1 hr.

   F. Sediment was suspended in 1 ml of sodium citrate per centrifuge tube.

3. Complete Freund's adjuvant was mixed 1:1 with sediment suspension and given to the rabbit; 1 ml IP, ½ ml IM in two sites.

APPENDIX

4. After 30 days, ½ ml of virus suspension was given IV and IP.
5. Step 4 was repeated twice weekly for 4 weeks.
6. Animals were bleed by cardiac puncture, 60 ml; 25 ml of serum titered 1:200 with RLV was recovered.

Lactic Dehydrogenase (LDH) Substrate - Pearce (144)

Stock buffer	
Substrate 1.0M	0.1 ml
DPN or TPN 0.1M	0.1
Sodium cyanide 0.1M	0.1
Magnesium chloride 0.05M	0.1
Tris buffer 0.2M, pH 7.0	0.25
Distilled water	1.25
Polyvinylpyrrolidone 11,000 MW	75 mg

Beta-hydroxybutyric acid, 2.9 mg per ml of complete buffer. Nitro BT 3-4 mg per substrate volume of 10 ml was added at the time of use; pH 7.0.

Frozen DPN and TPN are stable at slightly acid pH should be stored separately from the stock buffer and added before use. Working substrate may be frozen and thawed without DPN - TPN.

DL-Lactic acid was neutralized to sodium salt with NaOH 1M. Incubation time was 15 min LDH.

Beta-Hydroxybutyrate Dehydrogenase - (BHDH) Substrate Beta-hydroxybutyric acid, 2.9 mg per ml of complete buffer. Nitro BT 3-4 mg per substrate volume of 10 ml was added at the time of use; pH 7.0. Incubation time; 1 hr BHBDH. Wash, counterstain if desired in nuclear fast read, fix in buffered formalin dehydrate, and mount in Permount.

APPENDIX

Acid Phosphatase

Naphthol AS-BI Phosphate Method (after Burstone) Pearce (144)

Dissolve 4 mg of naphthol AS-BI phosphate in 0.25 ml dimethylformamide and add 25 ml 0.2M acetate buffer pH 5.2. Add 35 mg of Red-Violet LB salt and 2 drops of 10% $MnCl_2$. Shake and filter. 1.5 g of polyvinylpyrrolidone after filtration.

Method.

1. Incubate sections for 2 hrs. at 37°C. Check at 30 minutes. If reaction is heavy, shorten the time, and record, as one perameter of the quantity present.
2. Wash in buffer, three changes.
3. Counterstain with Mayer's Haemalum, 1½ min.
4. Wash in buffer, three changes.
5. Drain and mount in glycerine jelly or Burstone's PVP mounting medium.

Note. Naphthol ASTR, ASMS, and ASMX all react but not with equal intensity.

APPENDIX

Modification A

Medium 199 - Parker (50,214)

NaCl	8.0	g/liter
KCL	0.4	
MgSO	0.2	
Na_2HPO_4	0.09	
KH_2PO_4	0.06	
Dextrose anhyd.	1.00	
Phenol Red	0.02	
$CaCl_2$ anhyd.	0.14	
$NaHCO_3$	0.35	
L-Arginine	70	mg/liter
L-Histidine	20	
L-Lysine monohydrochloride	70	
DL-Tryptophan	20	
DL-Phenylalanine	50	
DL-Methionine	30	
DL-Serine	50	+ 105 mg
DL-Threonine	60	
DL-Leucine	120	
DL-Isoleucine	40	
DL-Valine	50	
DL-Glutamic acid monohydrate	150	
DL-Aspartic acid	60	
DL-alpha-Alanine	50	
L- Proline	40	
L-Hydroxyproline	10	
Glycine	50	+ 75 mg
L-Glutamine	100	+ 396 mg
Sodium acetate trihydrate	50	
L-Cystine	20	
L-Tyrosine	40	
L-Cysteine hydrochloride	0.1	+ 121 mg
Adenine	10	
Guanine	0.3	
Xanthine	0.3	
Hypoxanthine	0.3	
Uracil	0.3	
Thymine	0.3	
Disodium alpha-tocopherol phosphate	0.01	
Thiamine	0.01	
Pyridoxine hydrochloride	0.025	
Riboflavin	0.01	
Pyridoxal hydrochloride	0.025	
Niacin	0.025	
Niacinamide	0.025	

APPENDIX

Modification A

Calcium pantothenate	0.01	
I-Inositol	0.05	
Ascorbic acid	0.05	100 increased to 200 mg
Folic acid	0.05	
P-Aminobenzoic acid	0.05	
Ferric nitrate	0.1	
Biotin	0.01	
Menadione	0.01	
Glutathione	0.05	
Vitamin A	0.1	
Calciferol	0.1	
Cholesterol	0.2	
Tween 80	5.0	
Adenosinetriphosphate Na Salt	1.0	
Adenylic acid	0.2	
Desoxyribose	0.5	
D-Ribose	0.5	
Choline chloride	0.5	

Kanamycin	400 µg/ml
Pencillin	1000 µg/ml

for 48 hrs. then reduced.

Kanamycin	50 µg
Penicillin	500 µg

APPENDIX

Modification B

Nutrient Mixture F 10 - Ham (215,216)

NaCl	7.4 g/liter
KCl	0.285
$Na_2HPO_4 \cdot 7H_2O$	0.290
KH_2PO_4	0.083
$FeSO_4 \cdot 7H_2O$	0.002
$CuSO_4 \cdot 5H_2O$	0.004 mg/liter
$ZnSO_4 \cdot 7H_2O$	0.051
$CaCl_2 \cdot 2H_2O$	0.044
$MgSO_4 \cdot 7H_2O$	0.153
Glucose	1.1 g + 1 g
Na pyruvate	0.110
L-Arginine . HCl	0.211
L-Histidine . HCl	0.021
L-Lysine . HCl	0.029
L-Tryptophan	0.0006
L-Methionine	0.004
L-Phenylalanine	0.005
L-Tyrosine	0.002
L-Alanine	0.009
Glycine	0.008 + .075
L-Serine	0.001 + .055
L-Threonine	0.004
L-Isoleucine	0.003
L-Leucine	0.013
L-Proline	0.012
L-Valine	0.004
L-Aspartic acid	0.013
L-Glutamic acid	0.015 + .278
L-Asparagine	0.013 + .060
L-Glutamine	0.146
L-Cysteine	0.025
Hyoxanthine	0.004
Biotin	0.024 mg/liter
Folic acid	0.001
Riboflavin	0.376 mg/liter
D-Ca Pantothenate	0.715 mg/liter
Choline Cl	0.698 mg/liter
I-Inositol	0.541 mg/liter
Niacinamide	0.615 mg/liter
Pyridoxine . HCl	0.206 mg
Thiamine . HCl	0.001

APPENDIX

Modification B

Thymidine	0.001
Vitamin B_{12}	0.001
Lipoic acid	0.0002
Phenol Red	0.0012
$NaHCO_3$	1.200

Compounds Added

Ascorbic acid	0.100 g
Cholesteral acetate	2.0 mg
Sialic acid	0.243 mg
Linoleic acid	2.5 mg

Concentrations were determined by titer on mouse lymphoid tissue. Cell motility and survival was the end point at 72 hrs; when used as serum free media. This media will not support heavy cell population for more than 24 hrs.

APPENDIX

Modification C

Minimum Essential Medium (MEM) - Eagle (135)

NaCl	6.5 g/liter
KCl	0.45
$CaCl_2$	0.25
$MgSO_4$	0.10
$MgCl_4 \cdot 6H_2O$	0.10
$Na_2HPO_4 \cdot H_2O$	0.175
KH_2PO_4	0.03
Glucose	2.00
Na acetate anh.	0.5
Phenol Red	0.01
$NaHCO_3$	0.80
L-Arginine HCl	0.126
L-Cystine	0.024
L-Cysteine HCl	+ 0.105
L-Histidine HCl $\cdot H_2O$	0.042
L-Glutamine	0.292 + 0.292
L-Isoleucine	0.0525
L-Leucine	0.0524
L-Lysine HCl	0.073
L-Methionine	0.015
L-Phenylalanine	0.033
L-Threonine	0.048
L-Tryptophan	0.010
L-Valine	0.045
L-Serine	0.015 + 0.105
Glycine	+ 0.112
Choline Cl	0.001
Folic acid	0.001
I-Inositol	0.002
Nicotinamide	0.001
Ca-D-pantothenate	0.001
Pyridoxal HCl	0.001
Riboflavin	0.0001
Thiamine HCl	0.001
Pyruvate	0.110
L-Alanine	0.0089
L-Asparagine $\cdot H_2O$	0.015
L-Aspartic acid	0.0133
L-Glutamic acid	0.0147
Ascorbic acid	0.0147
P-Aminobenzoic acid	0.001

APPENDIX

Medium D

L-Alanine	13.5 mg/liter
L-Arginine	200.0
L-Aspartic acid	20
L-Asparagine	38.5
L-Cysteine HCl	95.0
L-Cystine	18.5
L-Glutamic acid	22.5
Glycine	180.0
L-Histidine . HCl	12.5
L-Isoleucine	60.0
L-Leucine	60.0
L-Lysine HCl	290.0
L-Methionine	11.5
L-Phenylalanine	25.0
L-Proline	20.0
L-Serine	100.0
L-Threonine	90.0
L-Tyrosine	43.0
L-Valine	50.0
L-Glutamine	390 mg
Vitamin E	0.192
Vitamin A	0.192
Tween 80	9.2
P-Aminobenzoic acid	11.5
Biotin	22.3
Folic acid	2.3
Choline Cl	14.5
Nicotinamide	2.3
D-Ca pantothenate	2.3
Pyridoxal HCl	2.3
Thiamine HCl	2.3
Riboflavin	0.23
I-Inositol	2.8
Myoinositol	23.0
Vitamin B	1.0
$FeCl \cdot 3H_2O$	0.615
$AlCl_3 \cdot 6H_2O$	0.69
$MnCl_2 \cdot 4H_2O$	0.077
$MnH_4Mo_7O_{32} \cdot 4H_2O$	0.154
$CdCl \cdot 2\frac{1}{2}H_2O$	0.077
$CuSO_4 \cdot H_2O$	0.0154
$ZnSO_4 \cdot H_2O$	0.31
Sialic acid	0.158
Ascorbic acid	150
Tri-iodothyronine	5 µg
Insulin	23 U
Linolenic acid	2.5

APPENDIX

Medium D

NaCl	6.0 g
KCl	0.45
$CaCl_2$	0.195
$Ca(NO_3) \cdot 4H_2O$ (spinner)	0.100 gm/liter
$MgCl \cdot 6H_2O$	0.035
Glucose	2.0
Na acetate	0.5
$Na_2HPO_4 \cdot 7H_2O$ (spinner)	0.15 X 100
$NaHCO_3$	0.8 to 2.0 gm
Hydroxycortisone	50 ug/liter

This medium was made from concentrates that are reasonably stable. The completed medium was stored frozen for not more than 4 months. The tri-iodothyronine, insulin, and hydroxycortisone were added before use. No serum should be necessary to support lymphoid tissue. This medium is not suitable for the support of Hela cells due to the high levels of sialic acid and linolenic acid adjusted here for lymphoid tissue.

Trace metals were added to an amino acid concentrate, which included arginine and asparagine, at a 50X concentration. This was stored under refrigeration, but not frozen. Commercial concentrates of amino acids and vitamins were used when possible. Minimum essential medium of Eagle (MEM) amino acid concentrate at 2X normal concentration. The vitamin concentrate used in the basic medium of Eagle (BME) at 3X normal concentration was used as the basic vitamin source. Basic salt solution was made up as a 10X

sterile concentrate. The 0.5N HCl used in the amino acid concentrate was neutralized with 1N NaOH raising the effective NaCl concentration.

APPENDIX

Modification of the Hayflick's Medium - (168)

PPLO agar Difco	3.4 g
Brain-Heart infusion agar	3.7 g
Difco yeast extract	0.2 g
Glass-distilled water	70.0 ml

dispensed in tubes, 10 ml each and autoclaved, may be stored this way.

Before use melt in a water bath and add

Fresh yeast extract	1.3 cc
AGVD Solution	0.2 cc
BME Vitamin concentrate	0.1 ml
Serum, human (preferably the patient's)	1.3 ml

AGVD solution is made up of the following

Glutamine	4.380 g
Glucose	6.200 g
Arginine	3.150 g

Store frozen in 10-cc lots

Incubate 5% CO_2 - aerobicly and anerobicly.