TISSUE CULTURE STUDIES IN HODGKIN'S DISEASE*
Morphologic, Cytogenetic, Cell Surface, and Enzymatic Properties of Cultures Derived from Splenic Tumors

By JOHN C. LONG,‡ PAUL C. ZAMECNIK, ALAN C. AISENBERG, AND LEONARD ATKINS

(From the Immunopathology Laboratory, Department of Pathology and the Huntington Laboratories, Massachusetts General Hospital, Boston, Massachusetts 02114; the Departments of Pathology and Medicine, Harvard Medical School, Boston, Massachusetts 02115)

In earlier work long-term monolayer cell cultures were prepared from Hodgkin's disease tumors in the spleen (1). Cells from these monolayers contained an antigen on the surface and within the cytoplasm that could be isolated and purified from supernatant medium (2, 3). The antigen was not demonstrated in fresh (noncultured) Hodgkin's disease tumors suggesting that prolonged in vitro replication of cells derived from the tumor was necessary for its detection. Cells from Hodgkin's disease monolayers, but not fresh noncultured tumors, could be transplanted to nude mice with production of malignant tumors in recipient animals (4). Monolayer cultures prepared from normal adult spleen and human fetal spleen and thymus were found to contain only a very small amount of the Hodgkin's disease culture antigen, and could not be transplanted to nude mice.

The present report describes experiments undertaken to identify and characterize the cells of Hodgkin's disease monolayer and suspension cultures. Cytogenetic analyses were done, and the cultured cells were examined microscopically, assayed for lymphocyte and monocyte surface characteristics, and tested for a variety of intracellular enzymes by cytochemical staining and spectrophotometric techiques.

Materials and Methods

Hodgkin's disease tissue cultures were prepared from tumor nodules in the spleen. All patients had Hodgkin's disease of the nodular sclerosis or mixed cellularity types, and underwent splenectomy during staging laparotomy. Techniques of preparation of primary explants from splenic Hodgkin's disease tumors, and growth characteristics of the monolayer culture cells have been described (1, 4). The Hodgkin's disease cultures used in the present study were divided into four groups: (*a*); three long-term monolayer cultures carried more than 3 yr in vitro, and serially passaged more than 200 times; (*b*); one monolayer culture of a transplanted tumor in a nude mouse obtained by subcutaneous inoculation of cells from a Hodgkin's disease monolayer (4); (*c*); six short-term monolayers maintained less than 6 mo in vitro, and passaged less than 20 times, and (*d*); three suspension cultures derived from Hodgkin's disease monolayer cell lines. Cells from

* This work was supported by grant no. 1-RO-1 CA19514-01 awarded by the National Cancer Institute, Department of Health, Education, and Welfare, and NCI contract NIH-NCI-E-71-2174.

‡ Address correspondence to Dr. John C. Long, Immunopathology, Cox 5, Massachusetts General Hospital, Boston, Mass. 02114.

these three Hodgkin's disease monolayers (HR, FR, and KB cell lines), after 18–30 serial passages, became nonadherent, lifted off the plastic surface, and proliferated in the supernatant medium. All monolayer adherent cells from these three cultures spontaneously went into suspension, and the cell lines have been maintained thereafter in stationary suspension flasks for 54–68 serial weekly passages.

Normal monolayer cultures were derived from adult spleen and fetal thymus and spleen. Normal adult cultures were prepared from spleens removed for trauma or incidentally during another surgical procedure. Fetal cultures were obtained from the Naval Biomedical Research Laboratories, Oakland, Calif. Raji cell lymphoblastoid suspension cultures, derived from a patient with Burkitt's tumor (5), were provided by Dr. A. N. Theofilopoulos, Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif.

All monolayer culture lines were maintained in Falcon 75 cm^2, 250 ml, plastic flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and fed twice weekly with Dulbecco's modified Eagle's medium that contained 15% cobalt irradiated fetal calf serum (Microbiologic Associates, Bethesda, Md.) and 4.5 g/liter glucose. The Hodgkin's disease suspension cultures were fed weekly with RPMI medium (Grand Island Biological Co., Grand Island, N. Y.) containing 15% irradiated fetal calf seurm. Raji cell suspension cultures were grown in Eagle's minimum essential medium supplemented with 15% serum of the same type. All cultures were routinely and repeatedly checked for contamination with Mycoplasma before the cells were used in experiments.

Cytogenetics. Monolayer cells in exponential growth were trypsinized, washed with Earle's balanced salt solution, and suspended in 0.75 M hypotonic potassium chloride for 8–10 min. The cells were fixed with three changes of freshly prepared methanol and glacial acetic acid (3:1) solution for 30 min, and stained with Giemsa stain by the method of Moorehead et al. (6). Hodgkin's disease suspension culture cells were collected directly, without trypsinization, and prepared in the same way for chromosome analysis.

Cell Surface Studies. Cells from Hodgkin's disease and normal cultures were examined for the presence of lymphocyte and monocyte surface characteristics. Monolayer cells were trypsinized, washed with Earle's balanced salt solution, and grown in suspension with Dulbecco's medium for 15 h before tests were done. Viability of monolayer cells cultured overnight in suspension ranged between 85 and 94% by trypan blue dye exclusion. Cultured cells forming spontaneous rosettes with sheep erythrocytes were assessed in the presence of 9% type AB serum (7). The cells were incubated at 37°C for 10 min, centrifuged, and reincubated at 4°C for 6 h. The cell mixtures were resuspended and the number of rosettes counted in a hemocytometer. Reactivity of cultured cells with a 1:50 dilution of anti-thymocyte serum (ATS)[1] was done by indirect immunofluorescence. ATS diluted 1:5 in the presence of complement was added to a suspension of cultured cells and sheep erythrocytes to measure inhibition of E-rosette formation. Details of the preparation of ATS and its effect on E rosette formation have been previously published (8, 9). Cell surface immunoglobulin was identified with fluorescein-conjugated goat polyvalent antisera to human immunoglobulin heavy and light chains (10). Acetone and ethanol fixed tissue culture cells were examined for intracellular immunoglobulin by immunofluorescence and the immunoperoxidase technique of Garvin et al. (11).

Surface Receptors for IgGFc. Rabbit IgG and IgM anti-Forsmann antisera to sheep erythrocytes were obtained from Cordis Laboratories, Miami, Fla. Assays for the presence of cell surface receptors for cytophilic antibody were done by the method of Jaffe et al. (12). Sensitized sheep erythrocytes (EA) were prepared with a 1:200 dilution of IgG and IgM antibodies at 37°C. IgMEAC complexes were made with mouse serum as the complement source. 2×10^6 cells from the tissue cultures were incubated with 1×10^8 IgGEA and IgMEAC suspensions at 37°C for 30 min with gentle shaking of the tube. Rosette formation was also assayed by the pelleting technique described by Braylan et al. (13). Cultured cells and IgGEA or IgMEAC complexes were mixed, centrifuged at 200 *g* at room temperature for 10 min, and the pellet incubated at 37°C for 30 min. The pellet was resuspended, and rosettes counted in a hemocytometer. Rosettes done with sheep erythrocytes and sensitized with IgM antibody (IgMEA rosettes) served as a control (12). Aggre-

[1] *Abbreviations used in this paper:* AHG, aggregated human gammaglobulin; ATS, anti-thymocyte serum; BTEE, N-benzoyl-L-tyrosine ethyl ester; EA, erythrocyte antibody; EAC, erythrocyte antibody complement; PNPP, *p*-nitrophenyl phosphate; SRBC, sheep erythrocytes; TAME, *p*-tosyl-L-arginine methyl ester.

gated human gammaglobulin (AHG) was prepared by the method of Theofilopoulos et al. (14). Tissue culture cells were incubated with 50 μl of a 2-mg/ml dilution of AHG with and without 50 μl of human serum as a complement source. Indirect immunofluorescence was done to demonstrate binding of AHG to cultured cells by using 50 μl of a 1:10 dilution of fluorescein-conjugated goat antiserum to human IgG (Cappel Laboratories, Inc., Downingtown, Va.).

Enzyme Histochemistry. Monolayers were trypsinized, and 1–2 $\times$ 10^4 cells were incubated overnight at 37°C on four chamber glass tissue culture slides (Lab-Tek Products, Div. Miles Laboratories Inc., Naperville, Ill.). Unfixed monolayer cells, as well as acetone, formalin, and absolute-alcohol fixed cells were tested. Cells from Hodgkin's disease suspensions and Raji cell cultures were air dried on glass slides for histochemical stains. The cultured cells were assayed for esterase activity by using alpha-naphthol acetate, naphthol AS-D-acetate, and naphthol AS-D chloracetate as substrates by the methods of Li et al. and Yam et al. (15–17), and for peroxidase and acid phosphatase by the procedures of Kaplow and Barka and Anderson (18, 19).

Spectrophotometric Enzyme Assays. Cells from monolayer cultures were removed with a rubber policeman, washed twice with phosphate-buffered saline, and 6 $\times$ 10^6 cells were disrupted by freeze-thawing three times in an aqueous solution of 0.1% Triton-x-100 (Fisher Scientific Co., Pittsburgh, Pa.). The suspension of disrupted cells was centrifuged, and the supernatant fraction examined for lysozyme (muramidase), acid and alkaline phosphatase, and trypsin and chymotrypsin-like activities. All bioassays were done by using a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) fitted with an automatic recorder. Protein concentrations of the supernatant fractions of disrupted cells were determined by the method of Lowry et al. (20). Lysozyme (muramidase) activity was measured by the procedure of Litwack (21) as a decrease in OD_{410} of a suspension of lyophilized *Micrococcus lysodeikticus* by using the Worthington assay kit (RLK) (Worthington Biochemical Corp., Freehold, N. J.). 50 μl of sample was added to 1.0 ml of the bacterial suspension; egg white lysozyme was used as a standard. Alkaline phosphatase activity was measured with *p*-nitrophenyl phosphate (PNPP) as substrate (22, 23). The release of *p*-nitrophenol was followed by the change in absorbancy at 410 nm in a 1.0-ml mixture composed of 1 $\times$ 10^{-3} M PNPP in 1.0 M Tris-HCl buffer, pH 8.1, that contained 50 μl of sample. *Escherichia coli* alkaline phosphatase (Worthington Biochemical Corp.) was used as a standard. Acid phosphatase was measured by adding 25 μl of sample to 50 μl of 5 $\times$ 10^{-3} M PNPP in 0.15 M sodium acetate buffer, pH 5. The mixture was incubated for 30 min at 37°C, and the reaction stopped by the addition of 1.0 ml of 0.1 NaOH (23). Optical densities were read at 410 nm and compared with wheat germ acid phosphatase used as a standard (Worthington Biochemical Corp.). 1 U of enzyme activity was defined as an increase in OD_{410} of 1.0 above the blank. Assays for trypsin and chymotrypsin-like activity were done by the method of Hummel (24). The enzyme catalyzed hydrolysis of *p*-tosyl-L-arginine methyl ester (TAME) and N-benzoyl-L-tyrosine ethyl ester (BTEE) (Sigma Chemical Co., St. Louis, Mo.) was monitored spectrophotometrically at 247 and 256 nm, respectively. The BTEE assay was modified to use 10% methyl alcohol (wt/wt) and 2.7 $\times$ 10^{-4} M substrate by the method of Orenstein et al. (25).

Results

Morphologic Features (Table I). Primary explants derived from splenic Hodgkin's disease tumors were composed of a pleomorphic mixture of adherent reticular, round, and stellate cells admixed with variable numbers of multinuclear giant cells (Fig. 1). The presence of numerous clusters of giant cells with multiple small nuclei was a characteristic feature of Hodgkin's disease monolayers after 7–10 days in vitro, and was not observed in cultures derived from normal spleen. After the Hodgkin's disease cultures had been trypsinized and passaged 6–10 times, clusters of multinuclear cells disappeared, and the monolayers were overgrown by irregularly contoured, reticular, and polygonal cells with loss of the spindle (fibroblast-like) cells (Fig. 2). Long-term Hodgkin's disease monolayers (those carried for more than 200 passages) were composed of round and polygonal cells that propagated in a mosaic pattern (Fig. 3). Numerous binuclear and multinuclear cells were conspicuous and numerous in long-

TABLE I
Morphologic Features and Cytogenetics of Tissue Culture Cells

Cell culture lines	Number of passages in culture	Number of chromosomes*	Morphology
Hodgkin's disease monolayers (long-term)			
RB	292	78–80	Proliferative very rapidly, uniform population of round and polygonal (epithelioid) cells that replicate in a mosaic pattern; many binucleate and multinuclear cells
SpR	208	64–87	
FQ	376	64–83	
$FQNMI_2$‡	18‡	59–79	
Hodgkin's disease monolayers (short-term)			
RY	19	58–69	Replicate rapidly; pleomorphic mixture of reticular stellate, and round cells with occasional multinuclear cells.
ML	9	67–84	
HT	8	71–78	
GL	7	60–81	
CT	6	70–78	
GM	6	68–72	
Hodgkin's disease suspension§ cultures			
FR	54	71–80	Replicate in stationary suspensions as single round cell and aggregates of 8–10 cells; numerous multinuclear and binuclear cells.
KB	58	74–81	
Normal spleen monolayers			
LW	12	44–46	Replicate sluggishly; spindle and reticular cells with fibroblast-like features; occasional binuclear cells; no multinuclear or epitheloid cells.
MP	11	43–46	
GR	39	44–46	
NH	14	44–47	
FL	36	42–48	
FT	40	43–46	
FS	41	45–46	

* Results of cytogenetic studies are expressed as the range of the number of chromosomes per 20 mitoses. Cells from long-term Hodgkin's disease monolayers contained many chromosomal fragments.

‡ $FQNMI_2$ refers to a culture line derived from FQ cells transplanted into nude mice (4). Cells from the second xenograft were re-established in monolayer culture, and passaged 18 times. Chromosomes of cultured cells derived from xenografts were of human type similar to FQ cells before transplantation into nude mice (4).

§ Hodgkin's disease suspensions were derived from monolayer cultures with spontaneously altered growth characteristics.

term Hodgkin's disease monolayers (Fig. 4). The Hodgkin's disease suspension cultures that were derived from monolayers with altered growth properties, were composed of round cells, 8–9 μm in diameter, with abundant basophilic cytoplasm, and large nuclei with distinct nucleoli. Binuclear and multinuclear cells were numerous in these suspension cultures. The cells that propagated in suspension were similar microscopically to the polygonal and round cells in Hodgkin's disease monolayers. Monolayer cultures derived from normal adult spleen and human fetal spleen and thymus were composed predominantly of spindle cells without multinuclear giant cells.

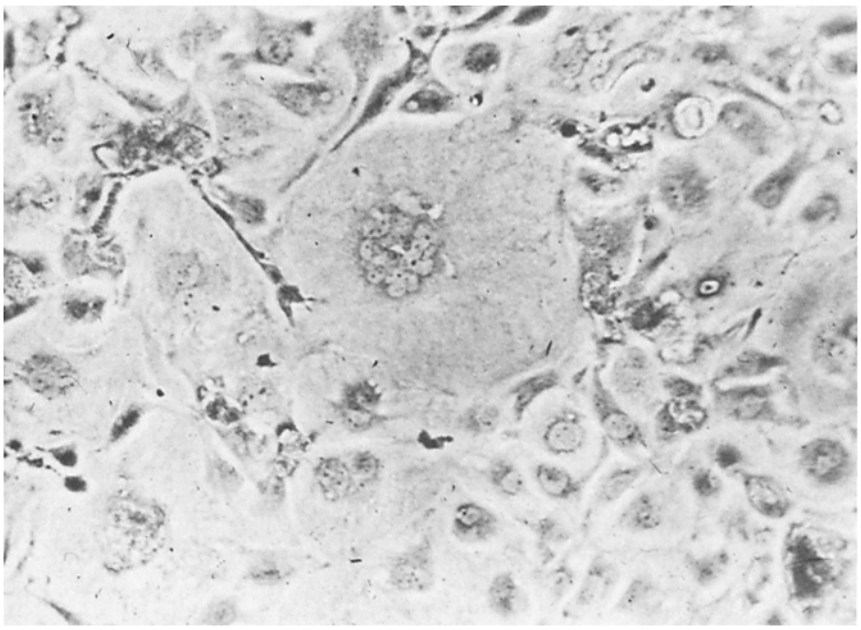

FIG. 1. Primary monolayer explant from a Hodgkin's disease tumor in the spleen showing a multinuclear giant cell surrounded by spindle and reticular cells (phase contrast × 120).

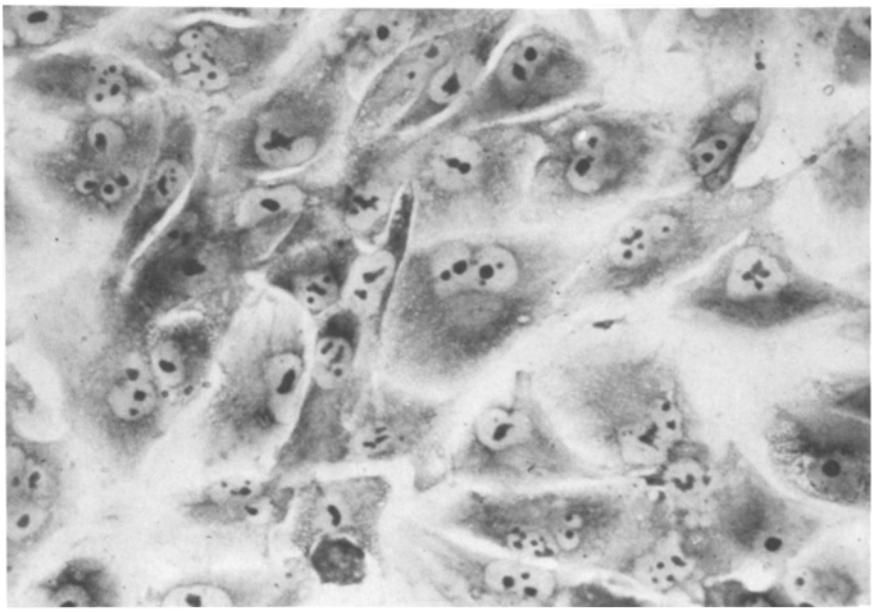

FIG. 2. A Hodgkin's disease monolayer, after 10 passages in culture, composed of pleomorphic, polygonal, reticular cells with scattered binuclear cells with large nucleoli. (Giemsa stain × 230).

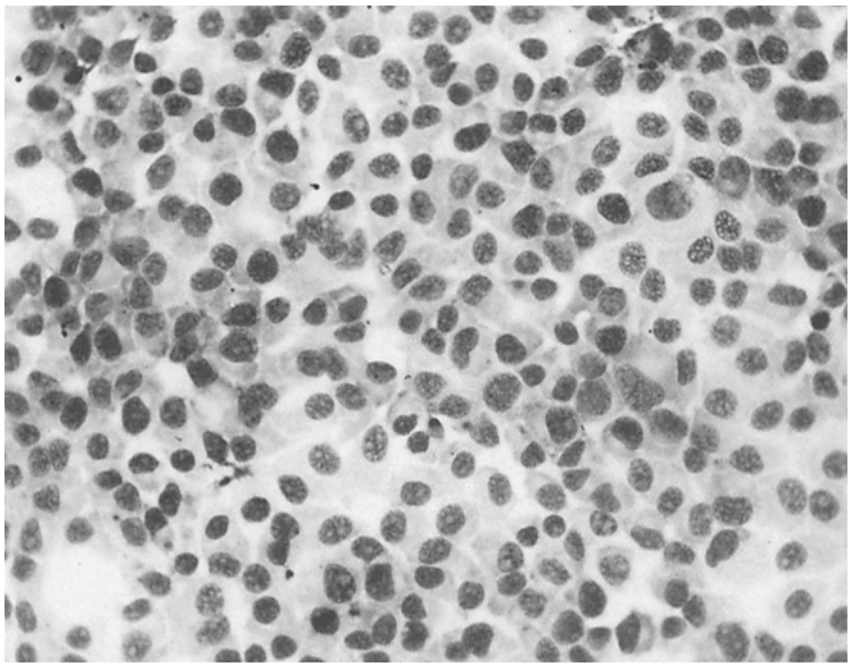

FIG. 3. A long-term Hodgkin's disease monolayer maintained in culture for more than 4 yr (370 passages) composed of round and polygonal cells that replicate in a mosaic pattern (Giemsa stain × 260).

Cytogenetics. Chromosome analyses were done on 19 tissue culture lines (Table 1). Cells from Hodgkin's disease monolayers contained aneuploid karyotypes with numerous abnormal chromosomes and chromosomal fragments (Fig. 5). Similar abnormal, aneuploid chromosomes were observed in short-term Hodgkin's disease monolayers passaged less than 20 times and in established cell lines except that chromosomal fragments were more conspicuous in long-term cultures. Suspension cultures derived from Hodgkin's disease monolayers were composed of cells with 71–81 chromosomes similar to those of the monolayer lines. In contrast, normal cultures derived from adult spleen and fetal spleen and thymus contained cells with normal diploid chromosomes.

Cell Surface Studies. Results of assays of cultured cells for lymphocyte and monocyte surface characteristics are summarized in Table II. Cells from Hodgkin's disease monolayers formed weakly adherent rosettes with sheep erythrocytes (SRBC), but did not react by immunofluorescence with an ATS (8). Moreover, a 1:5 dilution of ATS, which has been demonstrated to inhibit E-rosette formation by normal blood T lymphocytes (9), had no effect on the adherence of SRBC to Hodgkin's disease monolayer cells. Finally, spontaneous rosette formation was observed when monolayer cells were incubated with rabbit, human, and guinea pig erythrocytes. Cells from Hodgkin's disease monolayers did not contain definite surface receptors for either complement or IgGFc; the cells did not form IgMEAC or IgGEA rosettes, nor react with aggregated human gammaglobulin by immunofluorescence in the presence or

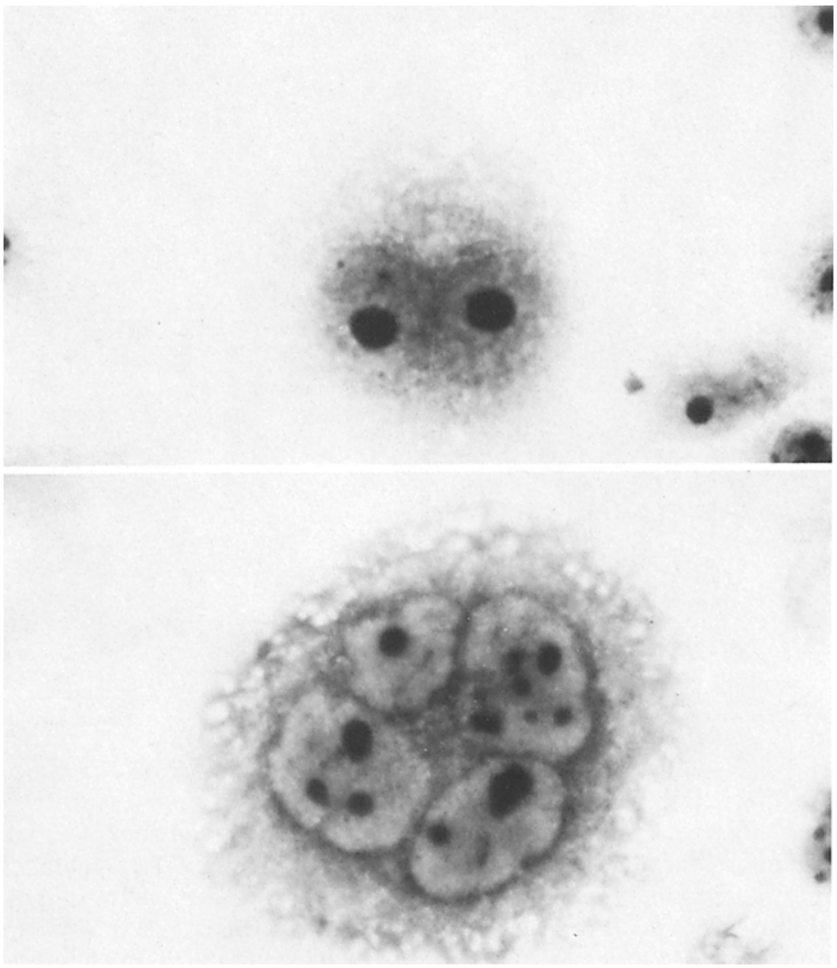

FIG. 4. Multinuclear and binuclear cells from a long-term Hodgkin's disease monolayer. (Giemsa × 680).

absence of complement. Finally Hodgkin's disease monolayer cells did not contain surface or cytoplasmic immunoglobulin, and did not exhibit phagocytosis in the presence of latex particles, or neutral red dye.

Hodgkin's disease suspension cultures (derived from monolayers with spontaneously altered growth characteristics) exhibited different surface characteristics than the monolayer cells. Cells from Hodgkin's disease suspensions formed avidly adherent IgMEAC and IgGEA rosettes, and bound aggregated human gammaglobulin in the presence or absence of complement by indirect immunofluorescence (Table II). The EA and EAC′ rosette forming suspension culture cells contained more than 12 erythrocytes that were firmly adherent to the cell surface (Fig. 6). The suspension culture cells did not form spontaneous rosettes with SRBC, react with ATS, contain surface or cytoplasmic immunoglobulin, nor exhibit phagocytosis.

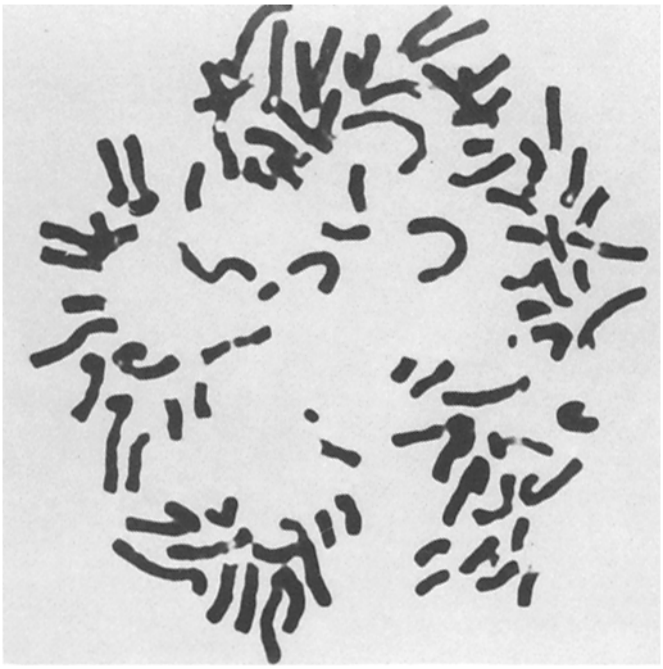

FIG. 5. Aneuploid chromosomes with many chromosomal fragments in cells from a long-term Hodgkin's disease monolayer passaged more than 300 times. Similar abnormal chromosomes were found in Hodgkin's disease suspensions and short-term monolayer cultures passaged 6–20 times in vitro.

Cells from normal cultures were negative in all the cell surface assays except that one cell line (GR) formed E-rosettes similar to those observed in the Hodgkin's disease monolayers. Cells from this normal monolayer failed to react with ATS, and E-rosette formation was not inhibited by prior addition of ATS. Raji suspension culture cells, used as a control, had surface receptors for IgGFc and complement, were devoid of surface and cytoplasmic immunoglobulin, and did not form E-rosettes or react with ATS. EA and EAC rosettes with Raji cells contained three to six erythrocytes adherent to the cell surface (many fewer than those illustrated in Fig. 6 for Hodgkin's disease suspension culture cells).

Enzyme Histochemistry. Cells from Hodgkin's disease monolayer and suspension cultures were found to contain similar enzymes (Table III). Cytochemical stains of the Hodgkin's disease cultures revealed abundant esterase activity when alpha-naphthol acetate and naphthol AS-D acetate were used as substrates. Alpha-naphthol acetate (nonspecific) esterase staining persisted in the presence of 4×10^{-2} M sodium fluoride (Fig. 7). Hodgkin's disease cultured cells displayed faint cytoplasmic staining for acid phosphatase and equivocal staining for alkaline phosphatase, and stains for chloracetate esterase and endogenous peroxidase were negative. Normal cultured spleen cells contained similar esterase and phosphatase activities. Nonspecific esterase staining of normal human fibroblasts (WI-38 monolayer cells) was sensitive to fluoride ion, and fibroblasts were devoid of naphthol AS-D acetate esterase and alkaline phosphatase (Table

TABLE II
Cell Surface Studies of Tissue Culture Cells

	IgMEAC	IgGEA*	AHG‡	IgMEA	E	ATS§	E+ATS‖	Ig¶	Phagocytosis**
Hodgkin's disease monolayers									
FQ	5	0	0	0	75	0	67	0	0
$FQNMI_2$	4	0	0	0	62	0	64	0	0
RB	0	0	0	0	61	0	42	0	0
SpR	0	0	0	0	52	0	50	0	0
RY	0	0	0	0	14	0	12	0	0
Hodgkin's disease suspensions									
HR	60‡‡	19‡‡	47	0	0	0	–	0	0
FR	74	16	55	0	0	0	–	0	0
KB	52	17	64	0	0	0	–	0	0
Normal spleen monolayers									
MP	0	0	0	0	0	0	0	0	0
LW	0	0	0	0	0	0	0	0	0
NH	0	0	0	0	0	0	0	0	0
GR	0	0	0	0	54	0	46	0	0
Raji Cells	95	5	87	0	0	0	0	0	0

The results are expressed as the percent positive cells per 200 cells counted.

* Assays for IgGFc receptors with cytophilic antibody (IgGEA) done in suspension and by a pelleting technique (13) gave similar results.

‡ Binding of AHG to the surface of cultured cells were done by indirect immunofluorescence by using human serum as a complement source and fluorescein conjugated goat antiserum to human IgG.

§ Reactivity of a 1:50 dilution of ATS with cultured cells was tested by indirect immunofluorescence (8).

‖ A 1:5 dilution of anti-thymocyte serum was added to a mixture of cultured cells and SRBC to test inhibition of E rosette formation (9).

¶ Cell surface immunoglobulin was tested by direct immunofluorescence (10) and cytoplasmic immunoglobulin by an immunoperoxidase method (11).

** Phagocytosis was tested using latex particles 1.1 μm in diameter (1×10^9 particles/ml) in minimal essential medium and with neutral red dye.

‡‡ IgMEAC and IgGEA rosettes with Hodgkin's disease suspension cells were avidly adherent and contained 12 SRBC/rosette; Raji cell rosettes were composed of 3–6 SRBC.

III). Raji lymphoblastoid suspension culture cells were negative for all enzymes tested by histochemical stains.

Spectrophotometric Assays. Hodgkin's disease monolayer and suspension culture cells contained abundant lysozyme (muramidase) activity as measured by solubilization of lyophilized *M. lysodeikticus* (Table III; Fig. 8 A). In contrast, normal spleen monolayers, WI-38 fibroblasts, and Raji cells were devoid of lysozyme activity. Acid phosphatase activity was demonstrated in all cultured cells tested. The level of acid phosphatase activity per mg protein was greatest for Hodgkin's disease and normal spleen monolayers (mean of 9.7 and 6.2 U, respectively), somewhat less for Hodgkin's disease suspension cells (mean of 5.1 U), and least for Raji cells and WI-38 fibroblasts (2.4 U). Alkaline phosphatase activity was abundant in Hodgkin's disease monolayers and suspension cultures, was demonstrable in normal spleen monolayers, and could not be detected in Raji cells (Fig. 8 B). Finally, chymotrypsin-like activity was present in Hodgkin's disease monolayers and suspension cultures, but absent in other cell lines (Fig. 8 C).

Discussion

Uncertainty regarding the identity and origin of the malignant cell of Hodgkin's disease, and the absence of a morphologically similar counterpart of the lymphoma in animals, have hampered experimental studies of the disorder.

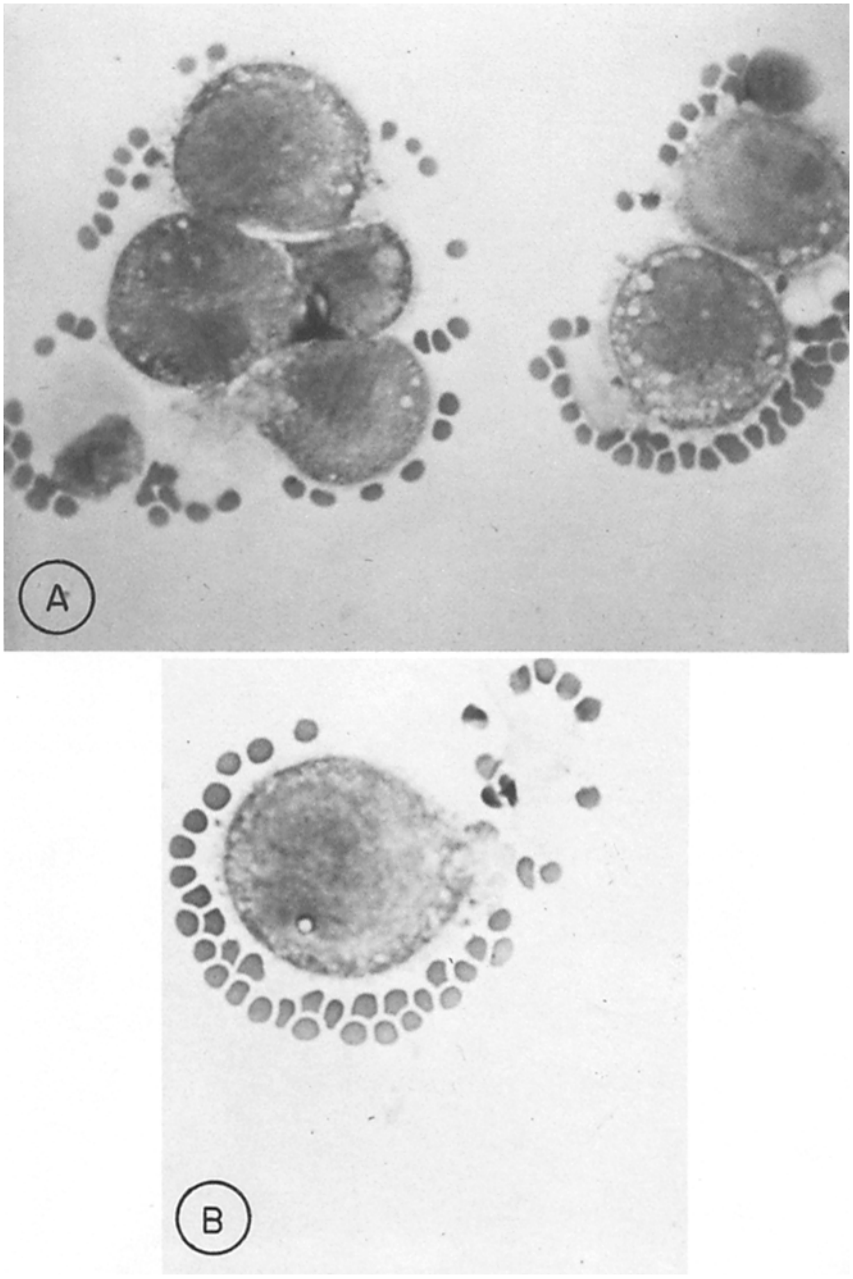

FIG. 6. IgMEAC (A) and IgGEA (B) rosette formation by cells from a Hodgkin's disease suspension culture (Giemsa stain × 1,450).

This limitation could be circumvented by the isolation and in vitro propagation of neoplastic cells from Hodgkin's disease. To this end we have prepared monolayer and suspension cell cultures from Hodgkin's disease splenic tumors, and detail their morphologic, cytochemical, cell surface, and enzymatic properties in the present report.

TABLE III

Enzyme Activities of Cultured Cells by Histochemical and Spectrophotometric Assays

Enzyme reaction	Hodgkin's disease monolayer cultures	Hodgkin's disease suspension cultures	Normal spleen monolayers	WI-38 Cells	Raji cells
Histochemistry					
Alpha-naphthol esterase	++	++	+	+	−
Alpha-naphthol acetate esterase + sodium fluoride	++	++	+	−	−
Naphthol AS-D acetate esterase	+	+	+	−	−
Naphthol AS-D chloracetate esterase	−	−	−	−	−
Peroxidase	−	−	−	−	−
Acid phosphatase	+	+	+	+	−
Alkaline phosphatase	±	±	−	−	−
Spectrophotometric assay					
Muramidase (lysozyme)	++	++	−	−	−
Acid phosphatase	+	+	+	+	+
Alkaline phosphatase	+	+	+	+	±
BTEE (chymotrypsin-like activity)	++	+	−	−	−
TAME (trypsin-like activity)	−	−	−	−	−

Four Hodgkin's disease monolayer, three suspension cell lines, and four normal adult spleen cultures were tested. Similar enzyme activities were demonstrated in short-term and established Hodgkin's disease monolayers. Fluoride-resistant alpha-naphthol acetate esterase staining is illustrated in Fig. 7. Results of spectrophotometric assays are plotted in Fig. 8.

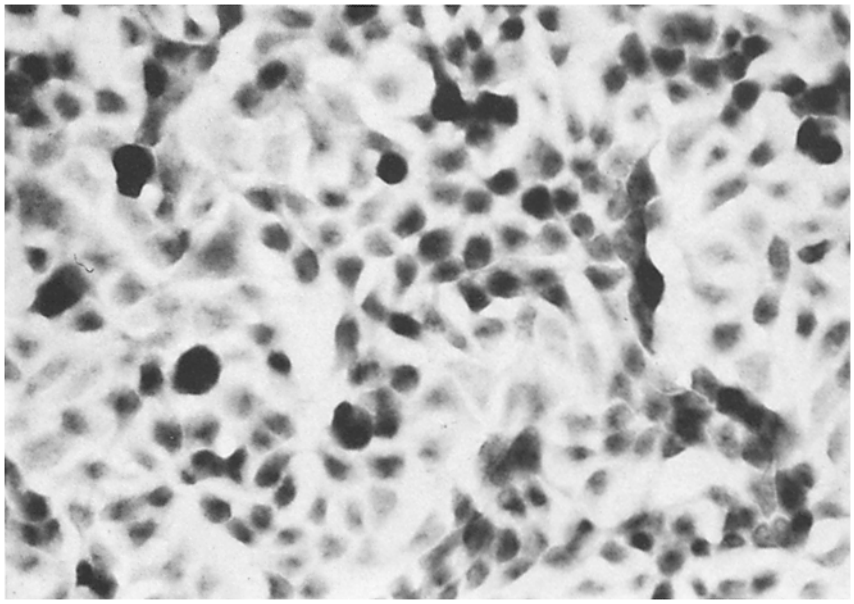

FIG. 7. Alpha-naphthol acetate esterase stain in the presence of sodium fluoride of a Hodgkin's disease monolayer. Dark staining of the cytoplasm indicates the presence of fluoride-resistant nonspecific esterase (× 260).

Hodgkin's disease monolayer cells were found to contain abundant lysozyme, fluoride-resistant acetate esterase, acid and alkaline phosphatases, and chymotrypsin-like activity (BTEE assay). Similar enzymes were found in suspension culture cells derived from Hodgkin's disease monolayers that spontaneously altered their growth characteristics and began to propagate in the supernatant medium of stationary flasks. Hodgkin's disease suspension culture cells were found to contain surface receptors for complement and the Fc portion of immunoglobulin. The suspension cells formed IgMEAC and IgGEA rosettes, and bound heat aggregated human gammaglobulin by immunofluorescence in the presence or absence of complement. Moreover, Hodgkin's disease monolayer and suspension cells were devoid of cytoplasmic or surface immunoglobulin, and did not form E rosettes nor react with an anti-thymocyte serum. However, Hodgkin's disease monolayer cells did not bear surface receptors for either complement or IgGFc. Although the monolayer cells formed E rosettes, the cells did not react with anti-thymocyte serum and E-rosette formation was not inhibited by the addition of ATS. The monolayer cells also formed spontaneous rosettes with rabbit and guinea pig erythrocytes. These findings are interpreted as representing a nonspecific adherence of SRBC to monolayer cells rather than indicating the presence of a T-cell surface marker. It is unclear why Hodgkin's disease suspensions, and not monolayers, had surface receptors for complement and IgGFc. It is possible that replication in suspension permits detection of Fc and complement receptors on Hodgkin's disease cultured cells which are not expressed on cells that replicate as monolayers. However, monolayers derived from normal blood monocytes have readily demonstrable Fc and C′ receptors and synthesize complement components (26, 27) (a property not detected in our Hodgkin's disease cultures). It should be emphasized that although our Hodgkin's disease suspension and monolayer cultures had dissimilar cell surface properties both were found to contain similar enzymes.

Cultures derived from normal spleen were composed predominately of spindle cells rather than the round, and polygonal epitheloid cells observed in Hodgkin's disease cultures. In contrast to Hodgkin's disease cultures, normal monolayers contained a diploid number of chromosomes and could not be transplanted to nude mice (4). Normal spleen cell lines did not have lysozyme, or chymotrypsin-like activity although similar esterases and phosphatases were demonstrable. Thus, normal cultured spleen cells have properties shared in common with normal fibroblasts, and appear to be quite different from Hodgkin's disease monolayer cells.

Three lines of evidence suggest that the Hodgkin's disease tissue culture cells under investigation are neoplastic: they are capable of continuous growth in vitro, have aneuploid karyotypes, and produce malignant tumors in nude athymic mice (4). Similar findings have been obtained from tissue cultures derived from other human lymphomas, including Burkitt's tumor and histiocytic lymphoma (28–30). It should be emphasized that while our Hodgkin's disease monolayer cultures readily produced invasive tumors in nude mice, cultured normal human cells and fresh (noncultured) Hodgkin's disease tumors could not be transplanted. Moreover, aneuploid karyotypes could be demonstrated in Hodgkin's disease monolayers in early passages, as well as in established culture lines, suggesting that the chromosome abnormalities were not acquired

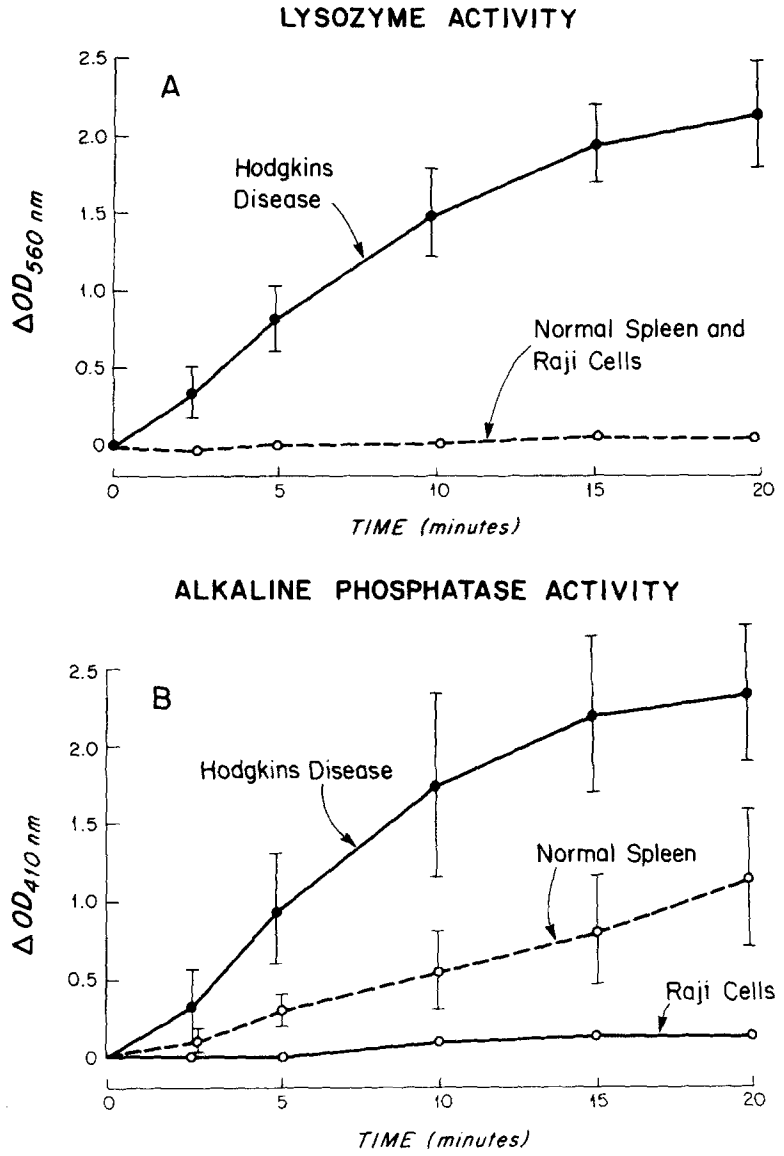

FIG. 8. Spectrophotometric assays for (A) lysozyme (muramidase); (B) alkaline phosphatase; and (C) chymotrypsin-like activity (BTEE assay). Four Hodgkin's disease monolayer cultures and three suspension cell lines were tested; four normal spleen monolayers and a Raji lymphoblastoid suspension culture were used as controls.

in vitro as a result of long-term serial propagation. Thus, normal cultured cells had diploid chromosomes after 40 passages in cultures, whereas Hodgkin's disease monolayers exhibited aneuploidy after only 6 passages.

Our evidence suggests that cells that propagate in monolayer and suspension cultures derived from Hodgkin's disease tumors have some features in common with monocytes rather than either lymphocytes or fibroblasts (15–17), (31–33).

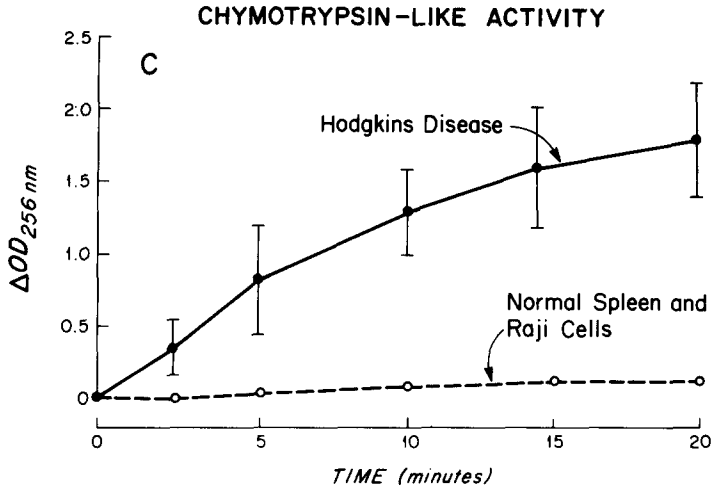

FIG. 8C.

However, interpretation of our findings is complicated by recent evidence suggesting that a lymphocyte rather than a monocyte is the neoplastic cell of Hodgkin's disease (34, 35). Garvin and associates demonstrated intracytoplasmic immunoglobulin in Reed-Sternberg and large lymphoid cells (immunocytes) in Hodgkin's disease lymph nodes with an immunoperoxidase bridge technique, and other investigators have observed that suspension cultures prepared from this tumor consist of a variety of large lymphocytes admixed with proliferating Reed-Sternberg-like cells (36, 37). These data suggest that one type of neoplastic cell of Hodgkin's disease is an abnormal lymphocyte, while the evidence available to date indicates that cells of our Hodgkin's disease monolayer and suspension cultures may be neoplastic monocytes rather than lymphocytes.

Summary

Monolayer and suspension cell cultures prepared from Hodgkin's disease tumors in the spleen were examined microscopically and by cytogenetics, tested for lymphocyte and monocyte cell surface properties, and assayed for enzymes by histochemical and spectrophotometric techniques. Hodgkin's disease monolayer cultures were composed of rapidly proliferating round and polygonal cells that were capable of propagation in vitro for an indefinite period of time. Abnormal aneuploid chromosomes were found in short-term Hodgkin's disease monolayers that had been passaged 16–20 times, and in established cell lines carried in culture longer than 3 yr and passaged more than 200 times. Cells from Hodgkin's disease monolayers contained lysozyme (muramidase), fluoride-resistant alpha naphthol acetate esterase, acid and alkaline phosphatase, and chymotrypsin-like activity. The monolayers did not exhibit specific cell surface markers or phagocytosis. Suspension cultures derived from Hodgkin's disease monolayers were composed of cells with aneuploid karyotypes and similar enzymes. The Hodgkin's disease suspension culture cells had surface receptors

for complement and IgGFc, lacked surface or cytoplasmic immunoglobulin, and did not form E-rosettes, react with an antithymocyte serum, nor exhibit phagocytosis. Normal monolayer culture cells, derived from adult spleen and human fetal spleen and thymus, were composed of spindle cells with a diploid number of chromosomes that could be carried for only a finite period of time in vitro. Normal cultured cells contained similar esterases and phosphatases, but were devoid of lysozyme and chymotrypsin-like activity.

The morphologic, cytogenetic, cell surface, and enzymatic findings indicate that our Hodgkin's disease monolayer and suspension cultures are composed of cells with many properties suggesting an origin from monocytes (macrophages) rather than lymphocytes or fibroblasts. The presence of aneuploid karyotypes is consistent with a neoplastic origin and derivation from a malignant cell of Hodgkin's disease.

We are indebted to Ms. Cathyrn Stamatos and Barbara Wilkes for excellent technical assistance and to Doctors Neil Orenstein, Harold Dvorak, and Robert Colvin for helpful advice.

Received for publication 28 January 1977.

References

1. Long, J. C., A. C. Aisenberg, and P. C. Zamecnik. 1974. An antigen in Hodgkin's disease tissue cultures: Fluorescent antibody studies. *Proc. Natl. Acad. Sci. U. S. A.* 71:2285.
2. Long, J. C., A. C. Aisenberg, and P. C. Zamecnik. 1974. An antigen in Hodgkin's disease tissue cultures: Radioiodine-labeled antibody studies. *Proc. Natl. Acad. Sci. U. S. A.* 71:2605.
3. Long, J. C., A. C. Aisenberg, and P. C. Zamecnik. 1977. Chromatographic and electrophoretic analysis of an antigen in Hodgkin's disease tissue cultures. *J. Natl. Cancer Inst.* 58:223.
4. Zamecnik, P. C., and J. C. Long. 1977. Growth of cultured cells from patients with Hodgkin's disease and transplantation into *nude* mice. *Proc. Natl. Acad. Sci. U. S. A.* 74:754.
5. Theofilopoulos, A. N., C. B. Wilson, V. A Bokisch, and F. J. Dixon. 1974. Binding of soluble immune complexes to human lymphoblastoid cells. II. Use of Raji cells to detect circulating immune complexes in animal and human sera. *J. Exp. Med.* 140:1230.
6. Moorhead, P. S., P. C. Nowell, W. J. Mellman, D. M. Battips, and D. A. Hungerford. 1960. Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp. Cell Res.* 20:613.
7. Bentwich, Z., S. D. Douglas, F. P. Siegal, and H. G. Kunkel. 1973. Human lymphocyte sheep erythrocyte rosette formation: Some characteristics of the interaction. *Clin. Immunol. Immunopathol.* 1:511.
8. Aisenberg, A. C., K. J. Bloch, J. C. Long, and R. B. Colvin. 1973. Reaction of normal human lymphocytes and chronic lymphocytic leukemia cells with an anti-thymocyte antiserum. *Blood.* 41:417.
9. Aisenberg, A. C., J. C. Long, and B. Wilkes. Chronic lymphocytic leukemia cells: Rosette formation and adherence to nylon fiber columns. 1974. *J. Natl. Cancer Inst.* 52:13.
10. Aisenberg, A. C., and K. J. Bloch. 1972. Immunoglobulins on the surface of neoplastic lymphocytes. *N. Engl. J. Med.* 287:272.

11. Garvin, A. J., S. S. Spicer, and P. E. McKeever. 1976. The cytochemical demonstration of intracellular immunoglobulin in neoplasms of lymphoreticular tissue. *Am. J. Pathol.* 82:457.
12. Jaffe, E. S., E. M. Shevach, E. H. Sussman, M. Frank, I. Green, and C. W. Berard. 1975. Membrane receptor sites for the identification of lymphoreticular cells in benign and malignant conditions. *Br. J. Cancer.* 31(Suppl. 2):121.
13. Braylan, R. C., E. S. Jaffe, J. W. Burbach, M. M. Frank, R. E. Johnson, and C. W. Berard. 1976. Similarities of surface characteristics of neoplastic well-differentiated lymphocytes from solid tissues and from peripheral blood. *Cancer Res.* 36:1619.
14. Theofilopoulos, A. N., F. J. Dixon, and V. A. Bokisch. 1974. Binding of soluble immune complexes to human lymphblastoid cells. I. Characterization of receptors for IgGFc and complement and description of the binding mechanism. *J. Exp. Med.* 140:877.
15. Li, C. Y., T. Yam, and W. H. Crosby. 1972. Histochemical characterization of cellular and structural elements of the human spleen. *J. Histochem. Cytochem.* 20:1049.
16. Yam, L. T., C. Y. Li, and W. H. Crosby. 1971. Cytochemical identification of monocytes and granulocytes. *Am. J. Clin. Pathol.* 52:283.
17. Yam, L. T., M. Tavassoli, and P. Jacobs. 1975. Differential characterization of the "reticulum cell" in lymphoreticular neoplasms. *Am. J. Clin. Pathol.* 64:171.
18. Kaplow, L. S. 1965. Simplified myeloperoxidase stain using benzidine dihydrochloride. *Blood.* 26:215.
19. Barka, T., and P. J. Anderson. 1962. Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler. *J. Histochem. Cytochem.* 101:90.
20. Lowry, O. H., N. H. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265.
21. Litwack, G. 1955. Photometric determination of lysozyme activity. *Proc. Soc. Exp. Biol. Med.* 89:401.
22. Garen, A., and C. Levinthal. 1960. A fine structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli* I. Purification and characterization of alkaline phosphatase. *Biochim. Biophys. Acta.* 38:470.
23. Dvorak, H. F. 1968. Metallo-enzymes released from *Escherichia coli* by osmotic shock. *J. Biol. Chem.* 243:2640.
24. Hummel, B. C. W. 1959. A modified spectrophotometric determination of chymotrypsin, trypsin and thrombin. *Can. J. Biochem. Physiol.* 37:1393.
25. Orenstein, N. S., M. E. Hammond, and H. F. Dvorak. 1976. Esterase and protease activity of purified guinea pig basophil granules. *Biochem. Biophys. Res. Commun.* 72:230.
26. Davies, P., and A. C. Allison. 1976. Secretion of macrophage enzymes in relation to the pathogenes of chronic inflammation. *In* Immunobiology of the Macrophage. David S. Nelson, editor. Academic Press Inc. New York. 428–463.
27. Einstein, L. P., E. E. Schneeberger, and H. R. Colten. 1976. Synthesis of the second component of complement by long-term primary cultures of human monocytes. *J. Exp. Med.* 143:114.
28. Epstein, A. L., and H. S. Kaplan. 1974. Biology of the human malignant lymphomas. I. Establishment in continuous cell culture and heterotransplantation of diffuse histiocytic lymphoma. *Cancer.* 34:1851.
29. Epstein, A. L., M. M. Herman, H. Kim, R. F. Dorfman, and H. S. Kaplan. 1976. Biology of the human malignant lymphomas. III. Intracranial heterotransplantation in the nude athymic mouse. *Cancer.* 37:2158.
30. Povlsen, C. O., P. J. Fialkow, E. Klein, G. Klein, J. Rygaard, and F. Wiener. 1973. Growth and antigenic properties of a biopsy derived Burkitt's lymphoma in thymusless (nude) mice. *Int. J. Cancer.* 11:30.

31. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes: Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* **121**:153.
32. Cohn, Z. A., and E. Wiener. 1963. The particulate hydrolases of macrophages. *J. Exp. Med.* **118**:991.
33. Gordon, S., J. Todd, and Z. A. Cohn. 1974. In vitro synthesis and secretion of lysozyme by mononuclear phagocytes. *J. Exp. Med.* **139**:1228.
34. Glick, A. D., J. H. Leech, J. M. Flexner, and R. D. Collins. 1976. Ultrastructural study of Reed-Sternberg cells. *Am. J. Pathol.* **85**:195.
35. Dorfman, R. F., D. F. Rice, A. D. Mitchell, R. L. Kempson, and G. Levine. 1973. Ultrastructural studies of Hodgkin's disease. *Natl. Cancer Inst. Monogr.* **36**:221.
36. Boecker, W. R., D. K. Hossfeld, W. M. Gallmeier, and C. G. Schmidt. 1975. Clonal growth of Hodgkin's cells. *Nature (Lond.).* **258**:235.
37. Kadin, M. E., and A. K. Asbury. 1973. Long-term cultures of Hodgkin's disease: A morphologic and radioautographic study. *Lab. Invest.* 28:181.