

An Antigen in Hodgkin's Disease Tissue Cultures: Fluorescent Antibody Studies

(cell culture/immunofluorescence/density gradient sedimentation)

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ABSTRACT Rabbits were immunized with an antigen of specific gravity 1.15-1.21 isolated by density gradient sedimentation of the centrifuged medium of long-term monolayer cultures derived from spleens involved by Hodgkin's disease. The globulin fraction of the antiserum was absorbed to reduce reactivity with normal cellular antigens and tissue culture components, and was tested by the indirect fluorescent antibody technique with cells from 18 different Hodgkin's disease cultures, and 16 normal cultures derived from adult spleen and fetal spleen and thymus. With anti-Hodgkin's disease globulin diluted 1:40 and 1:80, positive surface staining was observed in 48% and 41%, respectively, of viable cells from Hodgkin's disease cultures, and in less than 5% of cells from normal cultures. Fluorescent staining of the cytoplasm without nuclear staining was observed in 51% of acetone-fixed cells from the Hodgkin's disease cultures and in 4-8% of cells from normal cultures. Reactivity of the antiserum with Hodgkin's disease target cells could be removed by absorption of the antibody with additional antigen of density 1.15-1.21 obtained from other Hodgkin's disease cultures. Antisera to fractionated medium from a normal spleen culture and to noncultured Hodgkin's disease tumor tissue were used as controls: 2-10% of viable and acetone-fixed target cells reacted and no difference was observed between Hodgkin's disease and normal cell cultures. *In vitro* propagation of tumor cells from patients with Hodgkin's disease is needed for detection of the Hodgkin's disease tissue culture antigen; the antigen could not be demonstrated in noncultured Hodgkin's disease tissue.

Recent experiments have demonstrated an antigen in serially passaged, long-term monolayer cultures derived from the spleens of patients with Hodgkin's disease (HD) (1). The antigen was prepared from Hodgkin's disease tissue culture media, purified by ultracentrifugation and isopycnic density gradient sedimentation, and used to immunize rabbits. After appropriate absorption, the globulin fraction of the antiserum reacted by gel diffusion and immunoelectrophoresis with antigen prepared from nine of ten Hodgkin's disease cultures, but not with material derived from 12 normal spleen and thymus monolayers. This Hodgkin's disease tissue culture antigen was not detected by these techniques in noncultured tumor tissue, and antibody against noncultured tumor did not react with the Hodgkin's disease cultures. Thus, propagation in tissue culture appeared to be essential for expression of this antigen, a critical difference from the Hodgkin's disease antigen described by Order and associates (2, 3).

Abbreviations: HD: Hodgkin's disease; EBV: Epstein-Barr virus.

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In the present experiments, the Hodgkin's disease tissue culture antigen was investigated with fluorescence microscopy. Viable and acetone-fixed cells from normal and Hodgkin's disease cultures were incubated with either anti-HD globulin or control rabbit globulin, after which fluorescein-conjugated antiserum to rabbit immunoglobulin was applied. These studies confirm the earlier work, and permit the cellular localization of the Hodgkin's disease tissue culture antigen.

METHODS

Monolayer Cell Cultures. The 34 different human monolayer cultures used for fluorescence microscopy in the present experiments were divided into three groups: (1) 18 cultures derived from tumor nodules of Hodgkin's disease from spleens removed at staging laparotomy (4); (2) 10 cultures derived from normal adult spleen removed for trauma or incidentally during another surgical procedure; (3) three cultures each of normal fetal thymus and spleen obtained from the Naval Biomedical Research Laboratories, Oakland, Calif. Monolayers were maintained in 75-cm², 250-ml, Falcon plastic flasks and fed twice weekly with RPMI medium containing 30% cobalt-irradiated fetal-calf serum (Microbiological Assoc., Bethesda, Md.). All cultures were passed 10-20 times, at weekly intervals, with repeated checks for mycoplasma contamination, before the cells were used for experiments. A suspension culture of Burkitt's tumor cells (HR-1 line) which produced and released Epstein-Barr virus (EBV) was provided by Dr. Werner Henle.

Antisera. The preparation, in rabbits, of an anti-HD globulin has been described in detail (1). Briefly, 1200 ml of medium harvested from Hodgkin's disease monolayer cultures was centrifuged for 20 min at 7500 × *g* to remove particulate cell fragments. The supernatant fraction was then centrifuged for 2 hr at 78,000 × *g*, and the pellet thus obtained was sedimented in a 15-60% (w/w) continuous sucrose gradient for 18 hr at 190,000 × *g*. An *A*₂₆₀ peak with specific gravity 1.15-1.21 was recovered from the gradient, filtered with a Millipore membrane filter (0.45-μm pore size), and used as immunizing and boosting antigen. The globulin fraction of the immune rabbit serum was absorbed successively with noncultured normal human spleen cells, fetal-calf serum, and type-A-positive human erythrocytes. Two anti-HD globulin preparations produced against antigen from two different Hodgkin's disease cultures gave identical results and were used interchangeably. Control experiments were performed with anti-HD globulin further absorbed with material from the Hodgkin's disease high density peak (the sedimentation gradient peak of specific gravity 1.15-1.21 from centrifuged

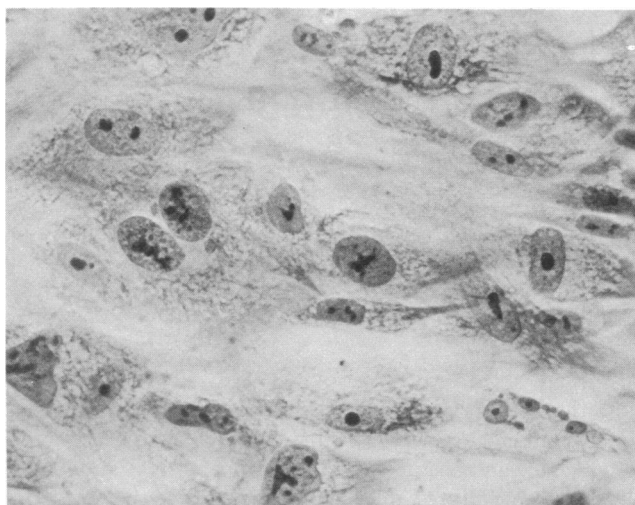


FIG. 1. Culture derived from a tumor nodule from the spleen of a patient with Hodgkin's disease. After 10 serial passages the culture was composed of pleomorphic, stellate reticular cells (Giesma stain, $\times 392$).

medium) of a different culture than that used to prepare the antiserum.

Antiserum to normal spleen high density peak was prepared by the immunization of rabbits with a density gradient isolate of specific gravity 1.13–1.16 obtained by sedimentation of centrifuged medium from a normal adult spleen monolayer culture. The globulin fraction of immune serum was absorbed as described for anti-HD globulin (1). An antiserum to non-cultured tumor tissue was prepared against cells from a freshly minced Hodgkin's disease lymph node, and the globulin fraction of the immune serum was absorbed with normal spleen cells and type-A-positive human erythrocytes (1).

Immunofluorescence of Monolayer Culture Cells. Cells (1 to 2×10^4) obtained by trypsin dispersion of monolayer flasks were planted on each chamber of 4-chamber tissue culture slides (Lab Tek Products, no. 4804, Division of Miles Laboratories, Westmount, Ill.). Use of the chamber slides permitted the side-by-side comparison of a single cell line with different antisera, or different cell lines with a single antiserum. After 12- to 15-hr incubation at 37° , slides were examined microscopically for adequate replication and adherence of cells. The plastic chamber dividers were then removed and the cells washed for 30 min with phosphate-buffered saline, pH 7.4. For membrane immunofluorescence, unfixed washed cells which were 96–98% viable by trypan blue dye exclusion were used. For immunofluorescence of fixed cells, washed monolayers were treated with acetone at 4° for 10 min and washed again with the buffered saline for 30 min. Each culture was tested at least three times.

Unfixed cell monolayers were tested for membrane staining, and acetone-treated cell monolayers were examined for cytoplasmic and nuclear staining: the indirect fluorescent antibody technique was employed. Fifty microliters of anti-HD or control globulin, diluted 1:20, 1:40, 1:60, and 1:80 were added to each of the four culture chambers and incubated at 20° for 20 min. The cells were washed with phosphate-buffered saline for 30 min and reincubated with $50 \mu\text{l}$ of a 1:8 dilution of fluorescein-conjugated goat antiserum to rabbit immuno-

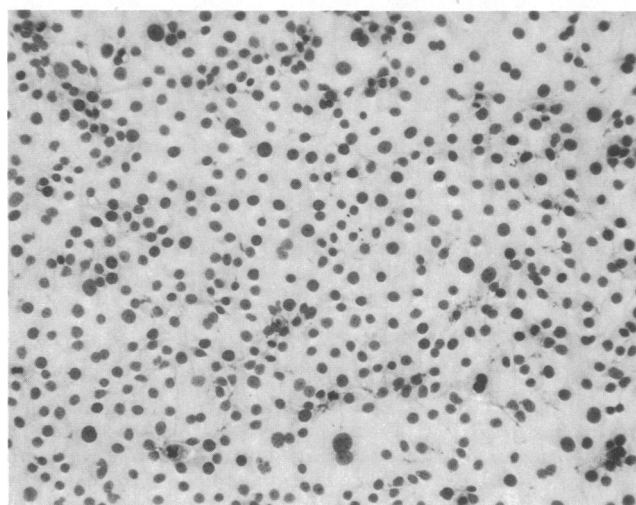


FIG. 2. Culture line derived from a nodule of Hodgkin's disease from the spleen shown in Fig. 1. After 24 serial passages the culture was composed of a uniform population of round cells with interspersed binucleate cells (Giesma stain, $\times 104$).

globulin (Meloy Laboratories, Springfield, Va.). In tests conducted with antisera against EBV, $50 \mu\text{l}$ of fluorescein-conjugated goat antiserum to human immunoglobulin (Meloy) was added to monolayers previously treated with $50 \mu\text{l}$ of serum from a patient with Burkitt's tumor (provided by Dr. Werner Henle). Following an additional 1-hr wash with phosphate-buffered saline, rubber bonding strips were removed, phosphate-buffered glycerin added, and the slides overlaid with a cover slip. Examination was carried out with a Zeiss ultraviolet microscope equipped with an Osram HBS 200 mercury arc lamp and an interference primary filter FITC-495X. A minimum of 200 cells from each individual chamber were examined.

RESULTS

Morphology of Monolayer Cultures. Primary explants derived from spleens of patients with Hodgkin's disease were composed of a heterogeneous population of adherent, pleomorphic spindle-shaped and round cells with numerous multinucleate giant cells. After 8 or 10 serial weekly passages in culture, irregularly contoured reticular cells predominated. Following 15 to 20 passages a population of stellate cells persisted in culture interspersed with occasional multinucleate cells and disappearance of the spindle cells (Fig. 1).

Two different Hodgkin's disease cell lines have undergone a striking morphological alteration in culture, with the appearance of rapidly proliferating round cells with numerous binucleate and trinucleate cells (Fig. 2). These two cell lines have continued to display this remarkable cytological appearance for more than 40 passages. Cultures derived from normal adult spleen were composed predominantly of spindle and reticular cells without multinucleate forms (Fig. 3).

Fluorescent Microscopy. The arithmetic mean and the standard deviation of the percent of positive cells from each of 34 different monolayer culture lines examined for surface fluorescent staining in the presence of anti-HD globulin are presented in Table 1. Positive cells showed discrete punctate, speckled staining of the cell surface without fluorescent cap-

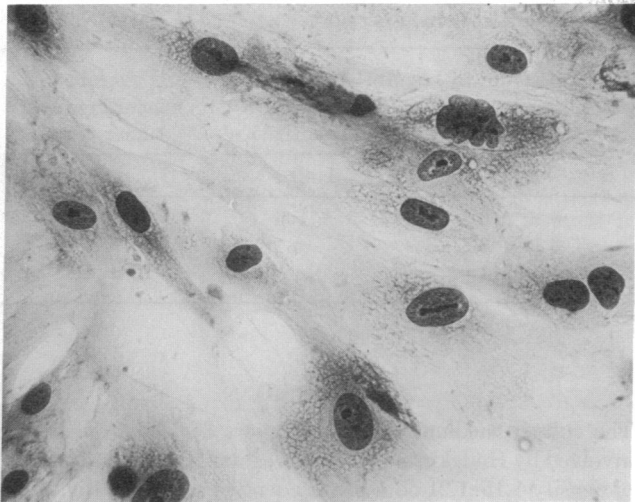


FIG. 3. Culture derived from a normal adult spleen after 16 serial passages. The cells are reticular and spindle-shaped (Giesma stain, $\times 237$).

ping (Fig. 4). Negative cells displayed either absent or faint, nonpunctate staining of the complete cell periphery (5). Anti-HD globulin diluted 1:20, 1:40, and 1:80 reacted respectively with an average of 54, 48, and 41% of target cells from the 18 different Hodgkin's disease cultures. A 1:40 dilution of anti-HD globulin absorbed with the Hodgkin's disease antigen (sedimentation gradient fractionated medium from a Hodgkin's disease culture) reacted with only 3% of cells from these Hodgkin's disease cultures. Target cells from each of 16 normal adult spleen and fetal spleen and thymus cultures were examined for membrane fluorescent staining in the presence of anti-HD globulin. An average of 5 and 2% of cells from these normal cultures reacted at antibody dilutions of 1:40 and 1:80, respectively. A 1:20 dilution of anti-HD globulin stained an average of 8% of cells from the normal cultures. Target cells from the 34 culture lines were also examined for surface fluorescent staining in the presence of two control antisera (Table 1). A 1:20 dilution of an antiserum to fractionated medium from a normal spleen culture and the same dilution of an antiserum to *noncultured* Hodgkin's

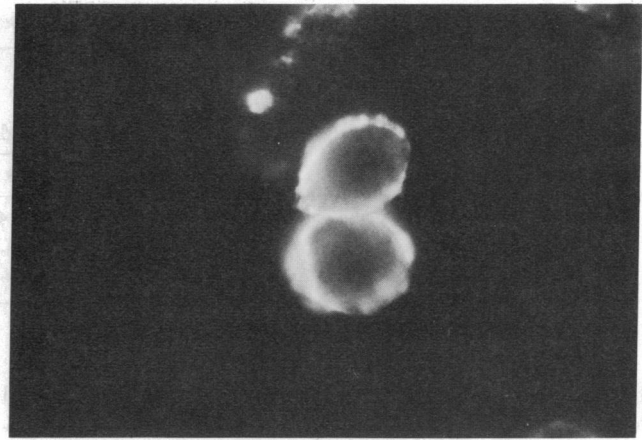


FIG. 4. Appearance of viable cells from a Hodgkin's disease culture incubated with anti-HD globulin and stained with fluorescein-conjugated goat antibody to rabbit immunoglobulin. Positive cells show discrete, punctate staining of the cell membrane ($\times 200$).

disease tumor tissue reacted with an average of less than 4% of the tissue target cells. No difference was observed in the staining of cells from Hodgkin's disease cultures compared with cells from normal cultures.

Acetone-fixed cells from each of 18 different monolayer culture lines were then reacted with anti-HD globulin (Table 2). Positively reacting cells showed brilliant granular fluorescent staining of the cytoplasm without staining of the nucleus (Fig. 5). Negative cells showed either absent or faint, nonpunctate, peri-nuclear staining. A 1:60 dilution of anti-HD globulin stained the cytoplasm of an average of 52% of target cells from each of eight Hodgkin's disease cultures, in contrast to 7% staining of cells from each of 10 normal spleen and fetal spleen and thymus cultures (the arithmetic mean and the standard deviation of the percent positive cells are indicated in Table 2). A 1:40 dilution of anti-HD globulin absorbed with the Hodgkin's disease antigen (sedimentation gradient fractionated medium from a Hodgkin's disease culture) stained an average of 6% of cells from the eight Hodgkin's disease cultures. Acetone-fixed tissue culture

TABLE 1. Membrane fluorescent staining of monolayer culture cells*

Culture lines	No. of culture lines tested	Anti-HD globulin			Antiserum to normal spleen culture		Anti-HD globulin absorbed with HD antigen†	Antiserum to noncultured HD tumor tissue	
		1:20	1:40	1:80	1:10	1:20	1:40	1:10	1:20
Hodgkin's disease (HD)	18	54 \pm 17‡	48 \pm 12‡	41 \pm 9‡	5 \pm 1.5	2 \pm 0.7	3 \pm 1.6	10 \pm 2.4	4 \pm 1.4
Normal adult spleen	10	8 \pm 2.5	4 \pm 1.3	2 \pm 1.3	7 \pm 1.1	3 \pm 2.0	1 \pm 0.3	4 \pm 1.7	2 \pm 0.8
Normal fetal spleen and thymus	6	8 \pm 2.4	5 \pm 2.0	2 \pm 1.6	6 \pm 2.3	4 \pm 2.2		5 \pm 0.4	2 \pm 0.6

* Results are expressed as the arithmetic mean and one standard deviation of the percent of fluorescent positive cells from all cultures tested in each of the three groups. Each culture line was tested three times. The dilution is that of the rabbit anti-globulin applied to the cells prior to the addition of 50 μ l of fluorescein-conjugated goat antiserum to rabbit immunoglobulin.

† Anti-HD globulin absorbed with material of buoyant density 1.15-1.21 from sedimentation-gradient-fractionated medium from a Hodgkin's disease culture different from that used to prepare the antiserum.

‡ Significant differences ($P < 0.01$ by paired *t*-tests) compared with staining of normal adult and fetal cells.

TABLE 2. *Cytoplasmic fluorescent staining of monolayer culture cells**

Culture line	No. of culture lines tested	Anti-HD globulin	Antiserum to normal spleen culture	Anti-HD globulin absorbed with HD antigen	Antiserum to noncultured HD tumor tissue
		1:60	1:20	1:40	1:2;
Hodgkin's disease (HD)	8	52 ± 14‡	6 ± 2.3	5 ± 1.6	9 ± 3.2
Normal adult spleen	6	7 ± 3.6	6 ± 1.8		10 ± 1.9
Normal fetal spleen and thymus	4	6 ± 4.2	7 ± 2.1		10 ± 4.6

* See footnote for Table 1. Acetone-fixed cells.

target cells were tested with the two rabbit antibodies used as controls (Table 2). A 1:20 dilution of an antiserum to fractionated medium from a normal spleen culture stained the cytoplasm of less than 7% of cells from each of the eight Hodgkin's disease and the 10 normal cultures. A similar dilution of the antiserum against *noncultured* Hodgkin's disease tumor tissue reacted with less than 10% of acetone-fixed cells from each of the 18 cultures: again no difference was observed between the Hodgkin's disease and the normal cell lines.

Anti-HD globulin was tested by indirect immunofluorescence with both cryostat-frozen sections and cell suspensions prepared from eight spleens involved with Hodgkin's disease. Preferential staining of tumor nodules was not observed in the frozen sections, and less than 2% of viable cells in suspension showed positive membrane staining.

An antiserum to the viral capsid antigen of EBV was tested by fluorescent microscopy with target cells from each of 7 Hodgkin's disease and four normal adult spleen monolayer cultures. A 1:20 dilution of this antibody incubated with fluorescein-conjugated antiserum to human immunoglobulin stained less than 3% of either viable or acetone-fixed cells from the 11 monolayer culture lines. In contrast, a similar dilution of the antibody to EBV stained the membrane of 34% of cells from the HR-1 Burkitt tumor suspension culture line. Anti-HD globulin diluted 1:20 reacted with less than 2% of cells from this Burkitt tumor suspension culture by immunofluorescence.



Fig. 5. Appearance of acetone-fixed cells from a Hodgkin's disease culture incubated with anti-HD globulin and stained with fluorescein-conjugated goat antibody to rabbit immunoglobulin. Positive cells show granular staining of the cytoplasm without staining of the nucleus ($\times 200$).

DISCUSSION

The culture medium from monolayers derived from spleens involved by Hodgkin's disease was centrifuged, and an antigen of density 1.15–1.21 obtained by density sedimentation of the pellet was used to immunize rabbits. Reaction of the heterologous antiserum with target cells in long-term, serially passaged, monolayer cultures was investigated by fluorescence microscopy after nonspecific reactivity had been reduced by absorption of the antibody with normal cellular antigens and tissue culture components. In 40–50% of cells in Hodgkin's disease cultures, the antiserum detected antigens located both on the cell surface and within the cytoplasm, but not within the nucleus. Reactivity of the antiserum with the Hodgkin's disease target cells could be removed by absorption of the antibody with additional antigen of density 1.15–1.21 obtained from other Hodgkin's disease cultures.

The antigen in Hodgkin's disease tissue culture cells did not react significantly with two control antisera. An antiserum prepared against a peak of similar density from sedimentation gradient fractionated *normal* spleen culture medium, and an antiserum against *noncultured* Hodgkin's disease tumor tissue reacted with less than 6% of target cells. With both control sera there was no difference between the staining of normal and Hodgkin's disease cells.

From fluorescence microscopy data it is not possible to exclude the presence of Hodgkin's disease tissue culture antigen in cells from normal cultures. Despite repeated absorption steps to reduce reactivity with normal cellular antigens, anti-HD globulin reacted with the surface of 2–4% of cells, and with the cytoplasm of 4–8% of cells, from cultures of normal adult spleen and fetal spleen and thymus. This reactivity with normal cells could be nonspecific fluorescent staining of cells in monolayers, since similar staining of cultured target cells was also observed with the antiserum against fractionated medium from normal spleen cultures. The control antiserum was absorbed with culture medium components and normal cellular antigens, and the residual fluorescent staining may represent incomplete removal of unbound antibody from the closely aggregated, glass-adherent cells. However, fluorescence microscopy has not established the specificity of the Hodgkin's disease tissue culture antigen with sufficient certainty. This specificity bears on the central question of the nature of the antigen: is it a viral component, a tumor or fetal antigen, or merely a normal tissue constituent? The tumor specificity of the Hodgkin's disease tissue culture antigen is explored in a subsequent paper with a radioiodine-labeled antibody technique, a more sensitive and quantitative assay than fluorescence microscopy (12).

Morphologically, the cells from our long-term, monolayer

cultures resemble stellate and round reticulum cells rather than lymphocytes or fibroblasts. It seems unlikely that these glass-adherent cells were derived from lymphocytes. Since suspension cultures of most normal and neoplastic lymphocytes contain Epstein-Barr virus (EBV), we examined our cell cultures for EBV antigen. A human antiserum to EBV did not react with cells from either Hodgkin's disease or normal cultures, and anti-HD globulin was unreactive by gel diffusion (1) and immunofluorescence with cells from an EBV-positive Burkitt's tumor suspension culture, additional evidence that the Hodgkin's disease cells in culture are not lymphocytes. Finally, the monolayer culture cells lack lymphocyte surface markers (surface immunoglobulin, spontaneous rosette formation with sheep erythrocytes, and thymus-specific antigens) (6-8) and do not secrete immunoglobulin into the medium (unpublished). Thus, it is unlikely that the Hodgkin's disease monolayer cells are lymphocytes, though their exact lineage and their relationship to the *in vivo* malignant cells (9) and to "Hodgkin's cells" which propagate in suspension culture (10, 11) are unknown.

Prolonged *in vitro* propagation is needed to detect the Hodgkin's disease tissue culture antigen. Thus, anti-HD globulin directed against a tissue culture antigen did not react with noncultured Hodgkin's disease tumor tissue by gel diffusion (1) and immunofluorescence, and an antiserum against noncultured tumor (2, 3) did not react with Hodgkin's disease tissue culture cells. It should also be noted that the Hodgkin's disease tissue culture antigen reacted with an antiserum directed against an isolate from fractionated medium rather than against whole cells. Isopycnic density sedimentation of the medium from long-term cultures may concentrate Hodgkin's disease tumor antigens. Although important questions about the specificity and biological significance of the Hodgkin's disease tissue culture antigen remain unanswered, it appears that tissue culture techniques will facilitate the study of Hodgkin's disease.

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