An Antigen in Hodgkin's Disease Tissue Cultures: Radioiodine-Labeled Antibody Studies

(cell culture/direct and indirect radioimmunolabeling)

JOHN C. LONG, ALAN C. AISENBERG, AND PAUL C. ZAMECNIK

The John Collins Warren Laboratories of the Huntington Memorial Hospital, and the James Homer Wright Pathology Laboratories,
Massachusetts General Hospital, Boston, Mass. 02114; and the Departments of Medicine and Pathology, Harvard Medical School,
Boston, Massachusetts 02115

ABSTRACT   An antiserum was prepared in rabbits against an antigen obtained by density gradient sedimentation of centrifuged medium from monolayer cultures of spleens involved by Hodgkin's disease. The antiserum was tested by isotopic antibody techniques with cells from each of eight cultures derived from spleens involved by Hodgkin's disease, four cultures derived from normal adult spleen, and one culture each of fetal spleen and thymus. By an indirect radioiodine-labeled antibody assay, anti-Hodgkin's disease globulin reacted with an antigen on the surface of cells from the Hodgkin's disease cultures, the quantity of which was related to the number of target cells and the amount of antibody used. This Hodgkin's disease tissue-culture antigen did not react with a rabbit antiserum against fractionated medium from a normal spleen culture, nor against noncultured Hodgkin's disease tumor tissue. The tumor specificity of the Hodgkin's disease tissue-culture antigen was assessed by a direct technique using ^{125}I-labeled anti-Hodgkin's disease globulin absorbed with either cultured Hodgkin's disease cells or with cultured normal cells. By this method the quantity of antigen on cells from Hodgkin's disease cultures was 15- to 30-fold greater than that on cells from normal cultures.

The Hodgkin's disease tissue-culture antigen is intimately associated with the propagation of the tumor in monolayer cultures, but its identity has not been established: it could be a viral component, a tumor or fetal antigen, or a normal tissue constituent.

An antigen has been demonstrated recently in serially passaged monolayer cultures derived from spleens involved by Hodgkin's disease (HD). The antigen was detected with a rabbit antiserum prepared against material recovered from the culture medium by sedimentation in an isopycnic sucrose gradient. Reaction of the antigen and antiserum was first demonstrated by agar gel diffusion and immunoelectrophoresis (1).

In the studies described in the preceding paper, the antigen was observed by fluorescence microscopy in the cell membrane and cytoplasm of cells from Hodgkin's disease cultures, but not in noncultured Hodgkin's disease tumor tissue (2). Although experiments with the fluorescent microscope have clarified the cellular localization of the Hodgkin's disease tissue-culture antigen, the evidence regarding the tumor specificity of the antigen was inconclusive; i.e., a quantitative difference in an antigen common to Hodgkin's disease and

normal spleen cultures, but not demonstrable in normal spleen cultures by gel diffusion could not be excluded. In the work to be described, the reaction of anti-HD and control antisera with tissue-culture target cells has been tested with the radioiodine-labeled antibody technique to assess the binding of anti-HD globulin to normal tissue culture cells and to quantitate more precisely the amount of antigen on cultured Hodgkin's disease cells.

MATERIALS AND METHODS

Monolayer Culture Cells. Ten culture lines derived from pathologically positive spleens of patients with Hodgkin's disease, eight cultures of normal adult spleen, and one culture line each of fetal thymus and spleen were used as target cells for the radioiodine-labeled antibody assays. The preparation, maintenance, and cytological features of these serially passaged, long-term monolayer cultures have been described (1, 2).

Antisera. An anti-HD antiserum was prepared by immunizing rabbits with material of specific gravity 1.15–1.21 obtained by density gradient sedimentation of centrifuged medium from Hodgkin's disease cultures. The globulin fraction of the antiserum was absorbed with noncultured normal spleen cells, fetal-calf serum, and type A positive human erythrocytes. Details of the preparation and absorption of anti-HD globulin have been published (1). Two antisera used as controls were prepared by immunizing rabbits with medium from a normal spleen culture line that had been fractionated by sedimentation gradient centrifugation, and with minced tissue from a *noncultured* Hodgkin's disease lymph node. The globulin fractions of these antisera had been used as controls in the gel diffusion (1) and fluorescent microscopy experiments (2).

Indirect Radioiodine-Labeled Antibody Experiments. ^{125}I-Conjugated antiserum against rabbit immunoglobulin was prepared as follows. Goat antiserum to rabbit immunoglobulin (Meloy Laboratories, Springfield, Va.) was precipitated three times with 35% (w/v) ammonium sulfate, and the globulin fraction thereby obtained was dialyzed against 50 mM phosphate buffer, pH 7.0. The immunoglobulin concentration was adjusted to 2 mg/ml by the addition of phosphate buffer. Immunoglobulin (200 μg) was then reacted with 10 mCi of carrier-free ^{125}I (New England Nuclear Corp.) by the chloramine-T method (3), and dialyzed against phosphate-buffered saline, pH 7.4, to remove unbound iodine. Seventy-six percent of the radioiodine was bound to the immunoglobulin,

99% of which was precipitable in 10% trichloroacetic acid. The specific activity of the radioiodine-labeled antibody to rabbit immunoglobulin was 38 μCi/μg of immunoglobulin. In the absence of chloramine-T, less than 1% of the ^{125}I was conjugated to the immunoglobulin.

For the indirect isotopic antibody assay, cells obtained by mechanical disruption of monolayer cultures with a rubber policeman were washed three times with phosphate-buffered saline. Counted aliquots of cells that were 96–99% viable by trypan blue dye exclusion were added to 12 × 75-mm siliconized glass tubes and incubated for 30 min at 20° in the presence of 50 μl of diluted rabbit antiserum (either anti-HD globulin, or control antisera). After four additional washes with phosphate-buffered saline, 50 μl of a 1:20 dilution of ^{125}I-conjugated antibody to rabbit immunoglobulin was added, and the cells were again incubated. The cells were then washed four times, suspended in 1 ml of phosphate-buffered saline, and counted in an automatic gamma scintillation counter (Nuclear Chicago). Three additional incubations were done to control each experiment: (*i*) tissue culture target cells + phosphate-buffered saline + 50 μl of ^{125}I-labeled antibody to rabbit immunoglobulin (background counts); (*ii*) cells + 50 μl of normal rabbit globulin at the same dilution as the rabbit antiserum used in the test + 50 μl of ^{125}I-labeled antibody to rabbit immunoglobulin; (*iii*) cells + 100 μl of undiluted rabbit antiserum to pooled cells from a Hodgkin's disease culture line + 50 μl of ^{125}I-labeled antibody to rabbit immunoglobulin. This unabsorbed antiserum reacted uniformly with 100% of target cells from both Hodgkin's disease and normal cultures by immunofluorescence, and was used as an indicator of cell count in the indirect isotopic antibody assay. Only experiments in which the number of target cells in aliquots from Hodgkin's disease and normal cultures varied by less than 5%, measured by the reaction of this nonspecifically reacting antiserum, were considered valid.

Direct Radioiodine-Labeled Antibody Experiments. ^{125}I-Conjugated anti-HD globulin was prepared as follows. Crude (nonabsorbed) anti-HD globulin, adjusted to an immunoglobulin concentration of 2 mg/ml, was dialyzed against 50 mM phosphate buffer, pH 7.0. One hundred micrograms of immunoglobulin were reacted with 5.0 mCi of carrier-free ^{125}I by the chloramine-T method (3), and were dialyzed against phosphate-buffered saline. Sixty-four percent of the radioiodine was bound to the antibody, 96% of which was precipitable with 10% trichloroacetic acid. The specific activity of the ^{125}I-labeled anti-HD globulin was 30 μCi/μg of immunoglobulin.

One milliliter of a 1:10 dilution of ^{125}-labeled anti-HD globulin was absorbed as described with noncultured normal human spleen cells, fetal-calf serum, and type A positive erythrocytes (1). The absorbed 1:10 dilution of radioiodine-labeled antibody was then divided into two 500-μl aliquots. One aliquot was absorbed three times with 2.0 to 2.5 × 10^6 washed cells, 98% viable by trypan blue dye exclusion, from two different Hodgkin's disease lines. The second aliquot was similarly absorbed with the same number of cells from two different normal adult spleen culture lines. Thus, for the direct radioiodine-labeled antibody experiments, anti-HD globulin was first absorbed as described for the fluorescent microscopy experiments (2) and for the indirect ^{125}-labeled antibody assay above, after which two additional absorptions were carried out with cells from either Hodgkin's disease or normal spleen cultures.

The direct radioiodine-labeled antibody experiments were done as described for the indirect assay except that 100 μl of ^{125}-labeled anti-HD globulin was added to tissue-culture target cells, and the number of washes was reduced from 11 to 7 since there was only one incubation of cells with antibody in the direct test.

RESULTS

The Indirect Assay. Results of the indirect isotopic antibody experiments were calculated by subtracting the background counts (target cells + phosphate-buffered saline + ^{125}I-labeled antibody to rabbit immunoglobulin) from the counts obtained when the target cells were incubated with rabbit antiserum plus ^{125}I-labeled antibody. Background counts, which indicated nonspecific binding of ^{125}I-labeled antibody against rabbit immunoglobulin to the target cells, were similar for Hodgkin's disease and normal cultures and ranged between 400 and 1250 cpm per 10^6 cells. Results of replicate experiments done with equal numbers of target cells and quantities of reagents varied from 4 to 11%. Target cells (10^6) from Hodgkin's disease cultures bound 1.5–1.8% of the radioiodine in 50 μl of ^{125}I-labeled antibody to rabbit immunoglobulin diluted 1:20 in the presence of similarly diluted anti-HD globulin.

The relationship between the number of target cells incubated with anti-HD globulin and the ^{125}I cpm is plotted in Fig. 1. By serially increasing the number of target cells from each of six Hodgkin's disease cultures from 0.5 to 5.0 × 10^6 per aliquot, there was an increase in the ^{125}I cpm from 200 to 11,250 which was not linear over the entire range of cell counts tested. This nonlinearity in ^{125}I cpm was possibly related to more nonspecific binding of the iodinated anti-rabbit immunoglobulin because of less effective washing in tests done with greater than 3.0 × 10^6 target cells.

The ^{125}I cpm for similar serial increments (from 0.5 to 5.0 × 10^6 target cells per aliquot) of cells from five different normal cultures ranged from 200 to 2150 when they were incubated with similar dilutions of anti-HD globulin and ^{125}I-labeled antibody against rabbit immunoglobulin (Fig. 1). The ^{125}I cpm for target cells from Hodgkin's disease and normal cultures incubated with a 1:20 dilution of normal rabbit globulin were similar and ranged from 75 to 980. The ^{125}I cpm for target cells from normal cultures incubated with anti-HD globulin were slightly, but consistently, greater than those observed when the cells from normal cultures were incubated with similarly diluted normal rabbit globulin (Fig. 1).

The effect of the concentration of anti-HD globulin on the ^{125}I cpm is plotted in Fig. 2. These experiments were done with 50 μl of serially diluted anti-HD globulin per 10^6 target cells and with ^{125}I-labeled antibody against rabbit immunoglobulin diluted 1:20. The ^{125}I cpm ranged from 4210 to 1124 per 10^6 target cells from each of six Hodgkin's disease cultures reacted with anti-HD globulin diluted from 1:20 to 1:2560. For five different normal cultures, the ^{125}I cpm ranged from 1250 to 254 per 10^6 target cells incubated with similar dilutions of anti-HD globulin. As in the data plotted in Fig. 1, the ^{125}I cpm for target cells from normal cultures incubated with anti-HD globulin were slightly greater than those observed when the normal cells were incubated with similarly diluted normal rabbit globulin (Fig. 2).

The reaction of anti-HD globulin with tissue-culture target cells was then compared with that of two control antisera (antiserum prepared against fractionated medium from a normal spleen culture, and another prepared against *non-*
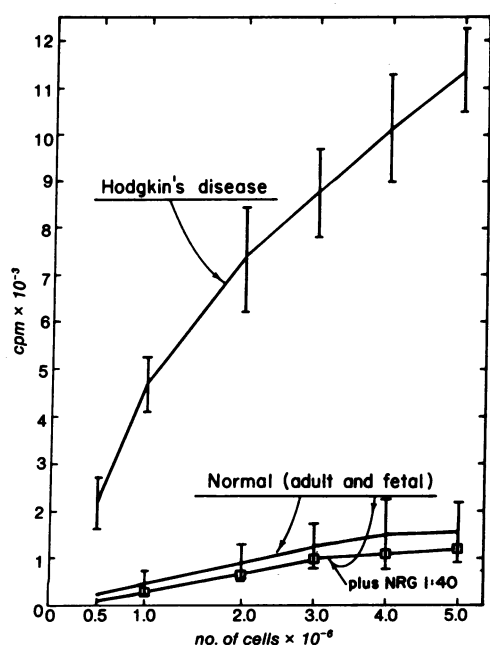
FIG. 1. Effect of increasing the number of target cells on the binding of anti-HD globulin·to cells from Hodgkin's disease and normal cultures by the indirect ^{125}I-labeled antibody technique. Results were calculated by subtracting background counts (cells + phosphate-buffered saline + ^{125}I-labeled antibody against rabbit immunoglobulin) from the counts obtained when target cells were incubated with 50 μl of a 1:40 dilution of anti-HD globulin or normal rabbit globulin (NRG) before addition of 50 μl of ^{125}I-labeled antibody diluted 1:20. Target cells were obtained from each of six different Hodgkin's disease culture lines, three cultures of normal adult spleen, and one culture each of fetal spleen and thymus. The binding of ^{125}I-labeled antibody to target cells from Hodgkin's disease cultures incubated with 50 μl of a 1:40 dilution of normal rabbit globulin (not shown) was similar to that plotted for target cells from normal cultures.

cultured Hodgkin's disease tumor tissue) (Table 1). Anti-HD globulin diluted 1:40 and 1:80 reacted strongly with cells from each of six Hodgkin's disease cultures (mean of 3560 and 3099 cpm per 10^6 target cells, respectively), and weakly with cells from each of five normal cultures (mean of 640 and 417 cpm per 10^6 target cells, respectively). In contrast, the reactivity of the two control antisera with tissue-culture target cells differed only slightly from that of similarly diluted normal rabbit globulin. Thus, the mean ^{125}I cpm ranged from 533 to 646 per 10^6 target cells incubated with either of the two control antisera diluted 1:40, with no difference in the
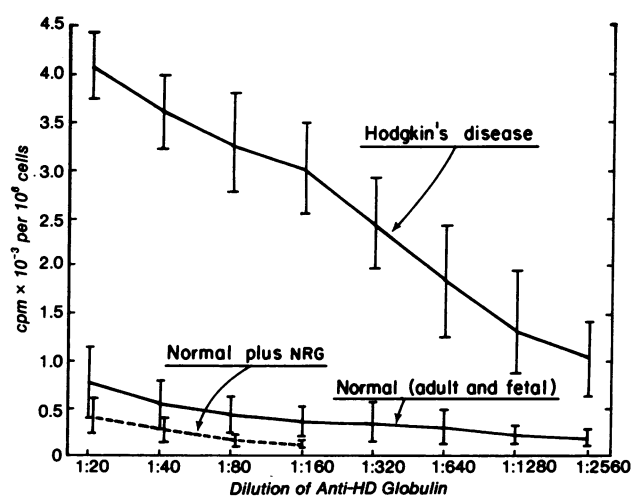

FIG. 2. Indirect ^{125}I-labeled antibody studies of the effect of the dilution of anti-HD globulin on the reaction with target cells from Hodgkin's disease and normal cultures. Each aliquot of 10^6 target cells was incubated with 50 μl of diluted anti-HD globulin followed by the addition of 50 μl of a 1:20 dilution of ^{125}I-labeled antibody against rabbit immunoglobulin. Results are expressed as ^{125}I cpm per 10^6 target cells calculated by subtracting the background counts from the test counts, as indicated in legend to Fig. 1. Target cells were from the same six Hodgkin's disease and five normal culture lines used in the experiments in Fig. 1. NRG: normal rabbit globulin.

reactivity of cells from Hodgkin's disease cultures compared with cells from normal cultures.

Brief incubation of washed target cells with various dilutions of trypsin prior to incubation with anti-HD antiserum globulin had no effect on the subsequent binding of ^{125}I-labeled antibody to rabbit immunoglobulin (4). Cell suspensions from each of four normal cultures (two adult spleen, one each of fetal spleen and thymus), treated with trypsin-EDTA (Grand Island Biological Co.) diluted 0.25, 0.1, and 0.01% for 30, 60, or 90 sec before incubation with a 1:40 dilution of anti-HD globulin, did not bind greater amounts of ^{125}I-labeled antibody than controls that had not been treated with trypsin. Similarly, treatment of target cells from each of three Hodgkin's disease cultures with trypsin in the same manner did not alter the reactivity of the cells with anti-HD globulin.

The Direct Assay. Cells from each of eight different Hodgkin's disease and six normal cultures were tested with ^{125}I-labeled anti-HD globulin by the direct isotopic antibody assay

TABLE 1. *Indirect ^{125}I-labeled antibody studies of the reaction of anti-HD globulin and control antisera with target cells from Hodgkin's disease and normal spleen cultures**

Culture lines	No. of culture lines tested	Anti-HD globulin		Normal rabbit globulin	Antiserum to normal cultured spleen	Antiserum to noncultured tumor tissue
		1:40	1:80	1:40	1:40	1:40
Hodgkin's disease	6	3650 ± 590	3099 ± 431	360 ± 126	533 ± 149	646 ± 245
Normal adult spleen and fetal spleen	5	640 ± 87	417 ± 80	370 ± 170	575 ± 273	573 ± 231

* The results are expressed as the arithmetic mean and the standard deviation of the cpm of ^{125}I bound to 10^6 target cells. The numbers were calculated by subtracting the background cpm (cells + ^{125}I-labeled anti-rabbit immunoglobulin from goat) from the cpm in the presence of the appropriately diluted rabbit antiserum (cells + anti-HD globulin or globulin fraction of control anti-serum + ^{125}I-labeled anti-rabbit immunoglobulin from goat).

TABLE 2. *Reaction of* [125]*I-labeled anti-HD globulin with tissue-culture target cells after absorption of the antibody with cells from Hodgkin's disease and normal spleen cultures*

Dilution of globulin	Tissue culture line used for absorption	Target Cells*		Ratio of antigen on Hodgkin's disease culture cells to antigen on normal culture cells‡
		Hodgkin's disease	Normal	
1:40	1. Normal	5436 ± 654	1421 ± 166	
	2. Hodgkin's disease	1489 ± 317	1290 ± 184	
		3897 ± 980 (P < 0.01)†	174 ± 48 (P > 0.1)†	23:1
1:80	1. Normal	4822 ± 621	1006 ± 118	
	2. Hodgkin's disease	908 ± 220	887 ± 162	
		3304 ± 885 (P < 0.01)	134 ± 53 (P > 0.1)	24:1
1:100	1. Normal	3402 ± 751	625 ± 128	
	2. Hodgkin's disease	578 ± 167	522 ± 135	
		3023 ± 869 (P < 0.01)	102 ± 40 (P > 0.1)	30:1
1:200	1. Normal	1811 ± 393	365 ± 85	
	2. Hodgkin's disease	371 ± 87	270 ± 74	
		1422 ± 401 (P < 0.01)	75 ± 39 (P < 0.05)	19:1
1:300	1. Normal	1229 ± 220	312 ± 58	
	2. Hodgkin's disease	339 ± 54	245 ± 36	
		906 ± 81 (P < 0.01)	67 ± 29 (P < 0.02)	14:1

* Eight Hodgkin's disease culture lines and six normal adult spleen culture lines were used. The results are expressed as the mean and one standard deviation of the ^{125}I cpm per 10^6 target cells.

† Antigen-specific counts for target cells from Hodgkin's disease and normal cultures were calculated by subtracting line 2 from line 1 for each dilution of ^{125}I-labeled anti-HD globulin (expressed as mean of the difference, and the standard deviation of the difference).

‡ Ratio of the mean antigen-specific counts for Hodgkin's disease culture cells to the mean antigen-specific counts for normal culture cells.

(Table 2). The quantity of antigen per 10^6 target cells was calculated as follows: antigen-specific ^{125}I cpm/10^6 cells from Hodgkin's disease for normal cultures = (cpm/10^6 cells incubated with ^{125}I-labeled anti-HD globulin absorbed with cells from *normal* spleen cultures) minus (cpm/10^6 cells incubated with ^{125}I-labeled anti-HD globulin absorbed with cells from Hodgkin's disease cultures). The antigen-specific counts for target cells from both Hodgkin's disease and normal cultures, therefore, were calculated in terms of the difference in the reactivity of ^{125}I-labeled anti-HD globulin absorbed with cells from two different tissue culture sources. The ratio of the quantity of antigen on cells from Hodgkin's disease cultures compared with that on cells from normal cultures varied from 14:1 to 30:1, depending upon the dilution of ^{125}I-labeled anti-HD globulin used. For example, at an antibody dilution of 1:40, the mean of the antigen-specific ^{125}I cpm per 10^6 target cells from the Hodgkin's disease cultures was 3897 ± 980. At the same antibody dilution, the mean antigen-specific ^{125}I cpm per 10^6 normal target cells was 174 ± 48. The ratio of these two mean values was 23:1, indicating that the quantity of antigen on cells from Hodgkin's disease cultures was 23-fold greater than the quantity of antigen on cells from normal cultures. At an antibody dilution of 1:100, the ratio was 30:1, whereas at antibody dilutions of 1:200 and 1:300, the ratios were 19:1 and 14:1, respectively (Table 2).

DISCUSSION

In the present investigation the indirect and direct isotopic antibody techniques were used to study the reaction of an antiserum with target cells from Hodgkin's disease and normal spleen and thymus cultures. The antiserum was prepared in rabbits against an antigen isolated by density gradient sedi-

mentation of the medium from Hodgkin's disease monolayer cultures. In other systems (5–8), the isotopic antibody method has proven to be a sensitive and quantitative assay of cell surface antigens.

The anti-HD globulin reacted with an antigen on the surface of Hodgkin's disease tissue-culture cells as measured with the indirect technique; radioiodinated goat antibody to rabbit immunoglobulin was bound to the cell surface in the presence of anti-HD globulin. The quantity of bound antibody varied with the number of target cells from Hodgkin's disease cultures (Fig. 1) and with the dilution of anti-HD globulin (Fig. 2), although the relationship was not completely linear in either instance. The globulin fraction of an antiserum prepared against medium from normal spleen cultures that had been fractionated by density gradient sedimentation was used as a control, and did not react significantly with target cells from either Hodgkin's disease or normal cultures. A second control antiserum, prepared against *noncultured* Hodgkin's disease tumor tissue, in earlier work, did not react by gel diffusion (1) and immunofluorescence (2) with the Hodgkin's disease tissue-culture antigen. The globulin fraction of this antiserum also failed to react with target cells from Hodgkin's disease cultures when investigated with the indirect isotopic antibody method.

The antiserum to the Hodgkin's disease tissue–culture antigen was unreactive with *noncultured* Hodgkin's disease tumor tissue by gel diffusion (1) and immunofluorescence (2), nor could reaction be detected with the very sensitive indirect isotope technique. Unreactivity of noncultured Hodgkin's disease tissue as measured with the latter method is of some concern, but may be attributed to the presence of many dead cells in suspensions of freshly minced tumor, which cause nonspecific absorp-

tion of antibody and high background counts. We have found that reliable determinations of cell-surface antigen with the isotopic antibody method require greater than 96% cell viability by trypan blue dye exclusion, while viability of fresh Hodgkin's disease tumor tissue is usually less than 80%.

Since antiserum to the Hodgkin's disease tissue-culture antigen consistently displayed a low level of reactivity with cultured *normal* spleen cells, the specificity of the Hodgkin's disease antigen, on the basis of the indirect radioiodinated antibody experiments, remains in doubt. This reactivity could not be attributed to nonspecific adherence of rabbit globulin to the surface of normal cells because the binding of anti-HD globulin to normal target cells was slightly greater than that of normal rabbit globulin (Figs. 1 and 2, Table 1). Moreover, "unmasking" of the antigen (4) was not observed after gentle trypsinization of target cells from normal cultures.

The direct isotopic antibody assay was designed to permit more precise measurement of the quantity of "tumor" antigen on target cells from Hodgkin's disease and normal cultures, and to take into account any residual reactivity of anti-HD globulin with normal cellular antigens which might result from incomplete absorption of the antibody. By this assay, the ratio of the quantity of antigen on the surface of cells from Hodgkin's disease cultures to the quantity of antigen on cells from normal monolayers ranged from 14:1 to 30:1, depending on the dilution of iodinated anti-HD globulin used (Table 2). The small amount of reactivity with target cells from normal cultures suggests the presence of the antigen on normal cells. This could be explained by differences in the cell populations comprising the normal and the Hodgkin's disease monolayers, a possibility suggested by the different morphologic appearances of the respective cultures (2). Alternatively, the small amount of reactivity of normal spleen cultures may reflect only background "noise" inherent in the highly sensitive direct isotopic method. Finally, despite absorption of anti-HD globulin with both noncultured *and* cultured spleen cells, it remains possible that reactivity with a normal component has not been completely removed and still contaminates the antibody.

The Hodgkin's disease tissue-culture antigen could be a virus component. The antigen banded in the isopycnic density sedimentation gradient at specific gravity 1.15–1.21 (1) may be present in small amounts or in a cryptic state in cultured normal adult and fetal cells, and was demonstrable in cells carried in culture but not in noncultured tumor tissue; all data consistent with a hypothetical virogene (9–14). However, we have not found the Hodgkin's disease tissue-culture antigen to be immunologically related to several known oncogenic viruses, including the Rauscher murine leukemia, the Epstein-Barr, the herpes simplex, the RD-114, and the avian myeloblastosis viruses (1). Moreover, cell-free extracts from several Hodgkin's disease culture lines have not induced the formation of the antigen in normal cells carried in culture, and addition of 5-iododeoxyuridine and dimethylsulfoxide, or the administration of ultraviolet light [procedures to increase the amount of a viral antigen (15, 16)] did not increase the titer of antigen in Hodgkin's disease cultures as measured with the iodinated antibody technique (unpublished). Therefore, the

evidence linking the Hodgkin's disease tissue culture with an oncogenic virus remains inconclusive: the demonstration of such a virus will require other means.

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