The Surface Antigenicity of Serially Transplantable Malignant Human Lymphoid Cells Derived from Subjects with Infectious Mononucleosis, Hodgkin's Disease, Chronic Lymphatic Leukemia, or Acute Lymphoblastic Leukemia

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SUMMARY

Human lymphoid cells can be maintained as serially transplantable malignant tumors in rabbit anti-hamster thymocyte serum-immunosuppressed neonatal Syrian hamsters, and these tumors can be assigned to either of two categories on the basis of their biological behavior in serial transplantation. Some (Category A) have exhibited the manifestations of acute leukemia but cannot be demonstrated to synthesize immunoglobulins; these have been isolated solely from children with active acute lymphoblastic leukemia secondary to lymphosarcoma. The remainder (Category B) have been isolated from a wide diversity of malignant and nonmalignant states and, in the hamster, these secrete immunoglobulin and provide no convincing evidence of their capacity to progress to acute leukemia.

It can be demonstrated, by the techniques of quantitative absorption and indirect immunofluorescence, that the Category B cell surface is characterized by the presence of common antigen(s) which is probably not surface immunoglobulin and which is restricted at or absent from the cell surface of the Category A acute lymphoblastic leukemia cells and from that of normal peripheral blood leukocytes as well. Some of the implications of these findings for the study of cell populations in the lymphoproliferative disorders are discussed.

INTRODUCTION

The xenogeneic implantation of human lymphoid cells or cell lines into normal or rabbit anti-hamster thymocyte serum-immunosuppressed neonatal Syrian hamsters has in a number of instances resulted in malignant lymphoid tumors that can be propagated by conventional methods of serial transplantation (1,3-6,8,10). The serially transplantable malignant tumors thus derived have originated not only from subjects with overt lymphoid neoplasia (3,5,8) or Hodgkin's disease (4,9) but also—with many interesting implications—from subjects with infectious mononucleosis (4,6,9,10) and from normal subjects as well (4). All the tumors so produced and maintained have retained karyotypic evidence (25,26) and/or one or another immunological evidence of their human ancestry and origins (4,9), and these tumors have been progressively growing, disseminating, and lethal to the alien host.

Each of the tumors falls into either Category A or B, defined on the basis of an apparently inverse relationship between 2 characteristics exhibited in serial transplantation, i.e., the ability of the tumor to progress to the manifestations of acute lymphoblastic leukemia in the hamster (1,5), or the ability of the tumor to secrete human immunoglobulins detectable in the sera of the tumor-bearing hamsters (4,6,9). The larger of the 2 categories thus defined is Category B, which consists of tumors derived from all of the diagnostic categories and clinical states thus far examined. In the hamster, these secrete immunoglobulins but have not been observed to progress to acute leukemia. These tumors have been referred to as the "malignant immunoblastomas" (9), to emphasize their experimental derivation, their malignant behavior, their resemblance to each other rather than to the disease or clinical state from which derived, and their probable relationship to lymphoid cells of B lineage. By contrast, Category A, thus far derived solely from children with active or terminal acute lymphoblastic leukemia secondary to lymphosarcoma, consists of 3 tumors which in the hamster progress either occasionally or routinely to acute leukemia but cannot be demonstrated to synthesize immunoglobulin.

This report focuses upon the malignant immunoblastoma Category B, principally because of the occurrence therein of the tumors derived by the direct transplantation of peripheral blood buffy coat cells from subjects with infectious mononucleosis. The subject who during the acute phase of infectious mononucleosis served as the original donor for tumor therefore represents, after convalescence, a consistent source of peripheral blood leukocytes (that reasonably can be considered normal) for autochthonous absorption of a heterologous antiserum to the hamster-grown tumor cells. The purpose of these experiments was to explore, by immunofluorescence and quantitative absorption, antigenic distinctions between populations of A and B experimentally...
isolated and maintained tumor cells, relative to normal peripheral blood leukocytes. The results suggest that the behavioral differences in heterotransplantation that distinguish the Category A from the Category B neoplastic lymphoid cells may be associated with differences in surface antigenicity of the cells, the surface of the Category B malignant immunoblastoma cells possessing a common antigen or antigens restricted at, perhaps entirely absent from, the surfaces of both the Category A acute leukemic cells and the surfaces of autochthonous and allogeneic normal peripheral blood leukocytes.

MATERIALS AND METHODS

Experimental Plan. The reference tumor in this study, i.e., that used to raise a heterologous antiserum in rabbits, was H-CO-1, isolated by direct transplantation from a subject (Patient C. O.) during the acute phase of infectious mononucleosis (9, 10). The 1st objective was to examine the ability of varied numbers of H-CO-1 tumor cells to quantitatively absorb the surface immunofluorescence reaction between anti-H-CO-1 antiserum and the H-CO-1 cells and to compare this with the absorptive ability of cells from other Category B tumors and from Category A tumors and with that of normal leukocytes both of the same subject and of unrelated normal blood donors. The 2nd objective was to compare the ability of anti-H-CO-1, after quantitative absorption with the autologous normal leukocytes, to react with the H-CO-1 tumor cells and with the autologous leukocytes. The 3rd objective was to examine the ability of an anti-H-CO-1 antiserum to react with these cell populations after it had been absorbed with autologous leukocytes and then concentrated by lyophilization.

Tumor Cells. All of the tumor lines used in this study (Table 1) were taken from a panel of about 30 which have been described in several previous reports (1, 3, 4, 6, 9, 10). These reports detail the production of tumor lines, either from cells taken directly from patient material or from cells first isolated in culture, and describe methods of serial propagation. All tumor cells, whether used for immunization of rabbits, for quantitative absorption with the autologous normal leukocytes, to react with the H-CO-1 tumor cells and with the autologous leukocytes. The 3rd objective was to examine the ability of an anti-H-CO-1 antiserum to react with these cell populations after it had been absorbed with autologous leukocytes and then concentrated by lyophilization.

Heterologous Antiserum to the H-CO-1 Tumor Cells. The anti-H-CO-1 anti-tumor antiserum was raised by the immunization of New Zealand White rabbits, weighing 3 to 4 kg, with 3 doses of $1.0 \times 10^6$ H-CO-1 tumor cells administered at weekly intervals. One-half of the cells were administered i.p. and the remainder were injected s.c. in multiple sites as a 1:1 suspension in complete Freund’s adjuvant (Difco Laboratories, Detroit, Mich.). One week after the final sensitizing dose, the rabbits were boostered with 3 consecutive daily i.v. injections of $1.0 \times 10^6$ H-CO-1 cells. The rabbits were exanguinated 10 days later, and the pooled serum was heat inactivated at 56° for 0.5 hr, filtered through a 0.45 Millipore filter, divided into aliquots, and stored at -20° for periods of 3 to 24 months without perceptible diminution in activity. Although anti-H-CO-1 antisera have not been tested for their ability to inhibit the growth of H-CO-1 tumor in hamster neonates, antisera prepared in this manner against other human lymphoblastoid cells previously have been shown to possess such neutralizing anti-tumor activity in vivo (2).

Absorptions. Absorptions preliminary to quantitative absorption with autologous peripheral blood leukocytes were initially with BLP and HRBC, theoretically to remove nonspecific and hamster species-specific antibodies. Absorption with BLP (Sylvana Co., Millburn, N. J.) was carried out at room temperature for 90 min in a proportion of 100 mg BLP to 1 ml antiserum. Neither the unabsorbed nor the absorbed anti-H-CO-1 could be demonstrated to react with normal human serum in immunodiffusion. Because the main purpose of this study was to implicate broad differences among the various cell populations, the specific antigens or antigens restricted at, perhaps entirely absent from, the surfaces of both the Category A acute leukemic cells and the surfaces of autochthonous and allogeneic normal peripheral blood leukocytes.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Human lymphoid cells maintained as serially transplantable malignant tumors in neonatal Syrian hamsters</th>
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<tr>
<td>Diagnosis</td>
<td>Source</td>
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<tr>
<td>Category A, leukemic, non-immunoglobulin synthesizing</td>
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<tr>
<td>ALLa</td>
<td>H-SB-2 Blood</td>
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<td></td>
<td>H-HM-1 Bone marrow</td>
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<td>Category B, nonleukemic, immunoglobulin synthetizingb</td>
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<tr>
<td>BT</td>
<td>H-EB-10 Mandibular</td>
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<tr>
<td></td>
<td>CLL</td>
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<td>Normal</td>
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<td>Melanoma</td>
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a All tumors except H-SB-2 require treatment of the neonatal hamster with ATS for growth and serial propagation.

b Category A: acute lymphoblastic leukemia; BT, Burkitt’s tumor; CLL, chronic lymphocytic leukemia; HD, Hodgkin’s disease; IM, infectious mononucleosis.

- The abbreviations used are: PBS, phosphate-buffered saline; BLP, bovine liver powder; HRBC, hamster red blood cells; NRS, normal rabbit serum; COPL, peripheral leukocytes of Patient C. O. after his recovery from infectious mononucleosis.
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exclusion of other antigens that can be anticipated to be present at the lymphoidal cell surface (cf. "Discussion") has been left for detailed study. Absorption with HRBC was at 4° for 30 min in a proportion of 0.5 ml packed, threewashed erythrocytes per ml antiserum.

Quantitative Absorption with Autologous or Allogeneic Peripheral Blood Leukocytes and Allogeneic Tumor Cells. Autologous or allogeneic normal peripheral blood leukocytes (buffy coat cells) were obtained from postconvalescent Patient C. O. or from unrelated normal donors, respectively, by the sedimentation of 5 parts heparinized fresh whole blood with 1 part 6% Gentran 75 (dextran 75) in 0.9% NaCl solution at room temperature for 2 hr. The leukocyte-rich supernatant was mixed 3:1 with 0.87% aqueous ammonium chloride, a modification of Boyle's method of red cell lysis (12), and then centrifuged at 1500 rpm for 10 min at 20° in an International PR-2 refrigerated centrifuge; the pellet was resuspended and washed in PBS 3 times. For quantitative absorption of the heterologous antiserum, the washed autologous or allogeneic normal leukocytes or the hamster-grown allogeneic tumor cells were prepared as described above, counted in a hemocytometer, and 0.2- to 0.4-ml aliquots of serial doubling dilutions of the cell suspensions were added to equivalent volumes of antiserum diluted 1:100 with PBS. The absorptions were carried out first at 37° for 1 hr and then overnight at 4° (32). The absorbed antisera were examined for immunofluorescence reactivity either immediately or after storage for 1 to 3 months at -20°.

Immunofluorescence. Indirect immunofluorescence ("sandwich") methods with living cell suspensions were as described previously (1) and are essentially those of Möller (28) and others (13, 24) with little modification. Target cells were washed 3 times in PBS, brought to a concentration of 1.5 x 10⁷ cells/ml, incubated for 0.5 hr with appropriately diluted and absorbed rabbit antiserum, washed 3 times in PBS, incubated for an additional 0.5 hr with fluorescein-conjugated goat anti-rabbit globulin globulin (Behring Diagnostics, Inc., Somerville, N. J.) in a 1:10 dilution in the presence of rhodamine bovine albumin (Hyland Laboratories, Costa Mesa, Calif.), 1:20 as counterstain, washed again 3 times in PBS, and mounted under sealed coverslips in 50% glycerine in PBS. The slides were examined with a Reichert Zetopan microscope equipped with an Osram mercury light source and OG-1 and BG-1 pass and barrier filters. Negative control was provided by substituting PBS, or NRS at dilutions of 1:50 or 1:100, for antiserum in the first incubation. NRS controls (Chart 3) were done with NRS subjected to the same procedures of BLP and HRBC absorption, dilution in PBS, then concentration by lyophilization as used in the production of the concentrated anti-H-CO-1 autologous leukocyte-absorbed antiserum. Control staining of all tumor and all normal peripheral leukocyte targets was in no instance greater than 6 and 20%, respectively. Control staining of COPL was never greater than 5%. All slides were examined by 1 person (S. J. G). Initially, the degree of brightness was estimated subjectively as intense (++++), moderate (+ + ), or faint (+ ), and the cells were differentiated as to completeness of peripheral staining: complete ("halos"), partial ("necklaces"), segmental ("tags"). Ultimately, all reactions were scored less subjectively, however, on the basis of the percentage of 200 counted cells exhibiting any degree of pattern of surface reactivity.

RESULTS

Common Surface Antigenicity of the Category B Tumor Cells. Representative results of quantitative absorption of the anti-H-CO-1 antiserum with the H-CO-1 tumor cells themselves, or with Category A or B allogeneic tumor cells at final dilutions of 1:200 are given in Chart 1. Cells selected from the Category B group of tumors seemed to have a capacity equivalent to that of H-CO-1 cells themselves to absorb the reaction between anti-H-CO-1 and H-CO-1 cells. By contrast, Category A cells had little or no capacity to absorb the reaction in the range examined. These findings constitute presumptive evidence for the presence of antigen common to surfaces of the Category B tumor cells that is either restricted at or entirely absent from the surfaces of the Category A cells. The inability of autochthonous normal peripheral blooduffy coat cells to absorb the antitumor anti-H-CO-1 antiserum (Chart 1) leads to the further inference that antigen common to the Category B tumor cell surfaces is also restricted among mixed populations of autochthonous or allogeneic mature

![Image](cancerres.aacrjournals.org) Chart 1. Membrane immunofluorescence reactivity of rabbit anti-H-CO-1 antiserum with the hamster-grown H-CO-1 cells after quantitative absorption with various hamster-grown human tumor cells or autologous normal peripheral blood leukocytes. Identity of tumor lines as in Table 1. •, H-CO-1 cells; •¿, Category A-absorbing cells; V, Category B-absorbing cells; O, COPL-absorbing cells. Vertical bars connect points representing results of 2 tests of each absorbed antiserum performed on different days with the use of different batches of H-CO-1 target cells. Curves are drawn through points representing arithmetic mean of these 2 values.
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leukocytes of the peripheral blood. H-PR-1 melanoma cells had a similar inability to absorb the reaction (data not included in Chart 1). Although absorption with these normal leukocytes or with Category A tumor cells slightly diminished the subjectively estimated intensity of membrane fluorescence of H-CO-1 target cells, the staining pattern (halos, necklaces) of the target cells as well as the percentage of positively staining cells suggested that appreciable amounts of antibody were not absorbed by these Category A or normal cells.

Difference in Surface Antigenicity between Autochthonous Normal Leukocytes and Hamster-grown Tumor Cells. The inferred difference in surface antigenicity between the autochthonous (COPL) normal leukocytes and the experimentally derived tumor (H-CO-1) cells from this patient can be more directly demonstrated by comparing the ability of the antitumor anti-H-CO-1 antiserum to react with H-CO-1 tumor cells and with autochthonous normal leukocytes after quantitative absorption with the autochthonous leukocytes; representative data are shown in Chart 2. Absorption in the range 2.0 x 10^7 COPL per ml antiserum resulted in fewer than 10% COPL and more than 95% H-CO-1 cells reacting.

Reactivity of Concentrated Autologously Absorbed Antiserum. An attempt was made to concentrate an antiserum absorbed with autologous normal leukocytes for testing against Category A and B tumor cells and autologous and allogeneic normal peripheral blood buffy coat cells (Chart 3). Concentration of the absorbed antiserum by lyophilization resulted in the return of its ability to react with autologous normal cells at antiserum dilution equivalents of 1:50. Under these conditions, 8 to 42% autologous normal peripheral blood leukocytes also reacted (extremely weakly), fewer than 35% Category A acute lymphoblastic leukemia cells reacted, and more than 80% Category B cells reacted (intensely). H-EB-10 cells reacted in an intermediate fashion. These relationships, which are the inverse of those obtained in the quantitative cross-absorption studies (cf. Chart 1), suggest that autologous and allogeneic normal cells and Category A acute lymphoblastic leukemia cells may be antigenically distinct from Category B tumor cells by virtue of restrictions in the concentration rather than complete absence of surface antigen common to both populations.

Such quantitative distinctions could reflect differences either in amounts of antigen per cell or in the relative proportions of cells of equivalent antigenicity, or both.

DISCUSSION

Several recent studies of tumor antigens in man have variously exploited the techniques of immunofluorescence, immunodiffusion, cytotoxicity, the mixed leukocyte reaction, and colony inhibition in vitro, for example, in the characterization of antigens associated with and in some instances perhaps specific for leukemia (17, 36), Burkitt's tumor (22, 23), Hodgkin's disease (30), melanoma (19, 21, 29) osteogenic sarcoma (14), neuroblastoma (18, 19, 21) cancers of the alimentary tract (15, 16) and hepatoma (35), to mention several. This study, in its reliance upon absorption of heteroantiserum with autochthonous normal cells, resembles the initial studies of Gold and Freedman in
defining carcinoembryonic antigen (16); with its reliance upon immunofluorescence techniques and its principal attention to antigens of lymphoreticular neoplasms, it further resembles several recent studies with Burkitt’s tumor (23) and Hodgkin’s disease (30). This investigation, however, differs substantially in its exclusive use of human lymphoid cells experimentally isolated and propagated as malignant tumor in the hamster both for the production of heteroantiserum and as the source of the target cell however, differs substantially in its exclusive use of human lymphoid cells experimentally isolated and maintained by these experimental methods but may not be entirely without bearing on the question of human tumor antigens.

As regards their antigenicity, individual lymphocytic cells may differ from each other in having or not having particular antigen, in having differing concentrations of the same antigen, or in not expressing an antigen under certain conditions or at specific times during the cell cycle, and specific examples of these conditions among mouse and human cells appear in many recent reports. Furthermore, many antigens are either known to exist or may be anticipated to exist at the lymphoidal cell surface, for example, nonspecific, Forssman, and heterophil antigens, species-specific antigens, immunoglobulins, HL-A, Epstein-Barr virus-associated antigen, lymphoid-specific or “differentiation” antigen (31, 33, 34), and possibly (in the case of experimentally isolated and maintained cells), antigens acquired from the milieu. Thus, populations of lymphocytic cells may be quite heterogeneous antigenically and may differ from each other in several different ways. Although absorption of antisera to H-CO-1 with differing populations of lymphoid cells might be expected in large measure to remove a great many specificities common to neoplastic or normal lymphoid cells, distinctions in net antigenicity between 2 populations of such cells determined by the quantitative absorption procedures used in this study may nevertheless reflect quantitative differences in antigen or antigens common to both cell populations. Hence it is conceivable, for example, that although populations of Category B tumor cells can be shown at appropriate dilutions of antibody to differ markedly from unfractinated populations of peripheral blood buffy coat cells, peripheral blood may nevertheless contain small numbers of “stem cells” of antigenic constitution equivalent to that of the majority of cells in a Category B tumor. Similar considerations may also apply not only to Category A experimentally isolated tumor cell populations, but also to cells isolated and maintained in culture from normal and malignant sources and to cell populations present in the peripheral blood of patients with acute leukemia. Clearly, additional investigations along these lines are required before far-reaching conclusions can be drawn.

Nevertheless, antigenic differences among experimentally isolated lymphocytic tumor cells that might relate to their differing behavior in transplantation, irrespective of the precise nature of the antigen(s) involved, would seem in some measure to have been implicated by the present studies, and some comment concerning the nature of the antigens, although in no way conclusive, may be appropriate. For example, although heteroantiserum are notoriously rich in species-specific antibody (11), it would seem unlikely that the antigenic distinctions demonstrated are referable to hamster species specificities, since this would require that the acquisition of antigen from the hamster by the human cells is a phenomenon uniformly specific to the group of neoplasms comprising Category B; in any event, other data make it fairly certain that anti-hamster species-specific antibody has been absorbed out of the antiserum by the procedures employed. Similarly, it is a fairly safe assumption that the antigenic distinctions cannot refer solely to the known differences in immunoglobulin associated with the cells, since the native or BLP- and HRBC-absorbed anti-H-CO-1 antisera, for reasons still not clear, cannot be shown to react with human serum in immunodiffusion, hence presumably they contain no antibody to human immunoglobulins (cf. “Materials and Methods”), an observation not entirely unlike that of Takahasi et al. (33) in their definition of the PC-1 antigen of murine immunoglobulin-secreting plasmacytoma cells with the appropriate alloantiserum. Although more inferential evidence exists that would tend to exclude them from interpretation of these results, the formal exclusion of human species-specific antigen, Forssman, heterophil, immunoglobulin HL-A, and Epstein-Barr virus-associated antigens, or proper assessment of their participation on a quantitative basis, requires specific absorption of the antiserum to H-CO-1 prior to the quantitative absorption with lymphoid cells and much additional investigation; these matters are at present under consideration as separate studies.

Thus, although it is to be acknowledged that the distinctions in antigenicity among the experimentally isolated and maintained lymphoid cells of this study may be attributable to quantitative differences in a number of well-known antigens characterizing the surfaces of lymphocytic cells, other explanations are at this time of equal validity and are equally worthy of pursuit. For instance, these antigenic distinctions may refer to quantitative, possibly qualitative, differences in “differentiation antigen” (31, 33, 34) peculiar to the surfaces of primitive B lymphocytes, which by their regular association with the surfaces of these cells capable of immunoglobulin synthesis may constitute not only a marker for primitive B lymphocytes, but a tumor-associated antigen as well for those neoplastic states involving proliferation of cells of the B lymphocyte cell lineage. Subsequent communications will describe the biochemical isolation and characterization of Category B tumor cell surface antigens defined by these methods, studies of their immunogenicity in rabbits, and their distribution in clinical material.

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REFERENCES

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