Brief Definitive Report

IDENTIFICATION OF A p69,71 COMPLEX EXPRESSED ON HUMAN T CELLS SHARING DETERMINANTS WITH B-TYPE CHRONIC LYMPHATIC LEUKEMIC CELLS*

By CHANG YI WANG, ROBERT A. GOOD, PATRIZIA AMMIRATI, GINY DYMBORT, AND ROBERT L. EVANS

From The Memorial Sloan-Kettering Institute for Cancer Research, New York 10021

A key limitation to the analysis of the immune response in man has been the difficulty of defining and characterizing the unique surface components of human lymphocyte subpopulations. Whereas specific lymphocyte differentiation antigens have been defined by heterologous antibodies from immune sera, many of the technical limitations of this approach can now be resolved by the generation of monoclonal antibodies with somatic cell hybridization techniques (1). We have therefore applied these techniques to study differentiation antigens on human T lymphocytes.

In this report we define and characterize by a monoclonal antibody a surface antigen complex of 69,000 and 71,000 daltons, p69,71 (Leu 1),[1] that has a thymus-dependent distribution on normal lymphocytes, but shares determinants with leukemic cells from patients with B-type chronic lymphatic leukemia. The distribution and molecular nature of p69,71 suggest that this complex may be related to the G_{IX} system in the mouse (2).

Materials and Methods

Production of Monoclonal Antibody

IMMUNIZATION AND SOMATIC CELL HYBRIDIZATION. BALB/c mice were immunized subcutaneously with 1×10^7 sheep erythrocyte (SRBC)-rosetting human peripheral blood lymphocytes and boosted three times at 2-wk intervals. 3 d after the last immunization, the splenocytes were fused with P3 $\times$ 63 Ag-8 myeloma cells, and cultured in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) that contained 15% fetal calf serum (FCS) and hypoxanthine-aminiopterine-thymidine following the procedure developed by Köhler and Milstein (1).

SELECTION AND CLONING OF HYBRIDIZED CELLS. Approximately 10–14 d later, supernates were collected from wells with hybrid cell growth and tested for antibody specific to T cells by indirect immunofluorescent analysis as described below. Hybrids from wells that contained antibodies specific to peripheral T lymphocytes were harvested and cloned by a limiting-dilution method in the presence of feeder cells (3) and plated into a Linbro 96-well flat-bottom tissue culture plate (Linbro Chemical Co., Hamden, Conn.) for subsequent screening and recloning.

NUDE MICE SERUM THAT CONTAINED MONOCLONAL ANTIBODY. After recloning positive hybrids 8–10 times, 1×10^7 hybrid cells were implanted subcutaneously into nu/nu mice on a Swiss strain background. Sera were obtained from these tumor-bearing mice ~3 wk later.

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[1] In developing a terminology for a series of leukocyte differentiation antigens, this antigen has been designated leukocyte determinant number 1 (Leu 1).

Specificity Analysis of the Monoclonal Antibody

ISOLATION OF T LYMPHOCYTE POPULATION.   Mononuclear cells were separated from fresh heparinized blood of normal individuals by Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) -Hypaque (Winthrop Laboratories, New York) density gradient centrifugation. T cells were isolated from other mononuclear cells by a two-step process that involved passage over nylon-wool columns followed by E-rosette formation and density gradient centrifugation (4).

ISOLATION OF MONOCYTES.   Monocytes were isolated by plating peripheral blood mononuclear cells at a density of 5×10^6/ml in RPMI-1640 that contained 10% FCS over glass Petri dishes for 2 h. The nonadherent cells were removed by vigorous lavage, and the adherent cells are recovered after incubation for 1 h at 37°C in medium that contained 0.1% EDTA. Greater than 90% of these cells had the morphological characteristics of monocytes or macrophages.

ISOLATION OF B CELLS.   Lymphocytes depleted of phagocytic cells by iron carbonyl treatment were brought to a concentration of $10-20 \times 10^6$/ml and mixed at a 1:1 vol with a 5% suspension of neuraminidase-treated SRBC (4). The non-E-rosette-forming cells (B cells) were separated from the E-rosette-forming cells on Ficoll-Hypaque gradients.

CYTOFLUOROGRAPHIC ANALYSIS.   Cytofluorographic analysis of all cell populations was performed by indirect immunofluorescence with fluorescent-conjugated F(ab')$_2$ fragments of affinity-purified goat anti-mouse IgG (G/M FITC) on a cytofluorograph (model FC200-4800 A50, Ortho Instruments, Westwood, Mass.) as previously described (5).

In brief, 5×10^5 cells were treated with 0.15 ml of serum from mice bearing the α-Leu 1-secreting tumors at a 1:10^4 dilution, incubated at 4°C for 1 h, and washed twice. The cells were then mixed with 0.15 ml of G/M FITC at a 1:20 dilution for 1 h, washed three times, and then analyzed on the cytofluorograph. Background staining was obtained by using a serum from a nude mouse that was tumor free.

Biochemical Analysis of the Target Antigen

IODINATION OF THE CELL SURFACE.   A modification of the method of Marchalonis et al. (6) was used to iodinate the surface as described previously (7).

IMMUNOPRECIPITATION.   For immunoprecipitation experiments, 100 μl of the tumor-bearing nude mouse serum at a 1:10^3 dilution was incubated for 30 min at room temperature with 1 $\times$ 10^7 ^{125}I-iodinated cells followed by two washes in phosphate-buffered saline. The antibody-bound radioiodinated cells were lysed with 0.5 ml of 0.5% nonidet P-40 (NP-40) (Shell Chemical Co., New York) in 10 mM Tris-HCl buffer (pH 8.0) that contained 10 mM iodoacetamide and then spun at 3,000 rpm for 10 min to remove the nuclear debris. F(ab')$_2$ fragments of affinity column purified goat antimouse IgG reagent were coupled to cyanogen bromine-activated Sepharose 4B gel (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) at 3 mg/ml gel and used to bring down the antibody-bound, radiolabeled surface antigens. The immunoprecipitates were then washed twice with buffer that contained 10 mM Tris HCl (pH 8.0), 0.5 M LiCl, and 0.1% NP-40 followed by a third wash with the same buffer that contained 0.1% sodium dodecyl sulfate (SDS). The bound ^{125}I-labeled surface antigens were eluted by boiling the beads with 4M urea and 2% SDS in 0.1 M Tris HCl (pH 8.0) buffer and electrophoresed on SDS-polyacrylamide gels as described by Laemmli (8) with 1.5-mm-thick slab gels with a 5-20% gradient of acrylamide.

Results and Discussion

Mouse splenocyte-myeloma cell hybrids that produced antibodies that reacted with normal T but not B cells were screened by indirect immunofluorescent analysis on a cytofluorograph. One such hybrid, which produced an antibody that reacted with the majority of SRBC-rosetting cells, was cloned 8-10 times in vitro and implanted subcutaneously into a nude mouse. The resulting high-titered antiserum was shown to recognize a p69,71 by immunoprecipitation experiments. This was performed by harvesting T cells that were stimulating in culture for 5 d with concanavalin A (Con A) and surface labeling the cells with ^{125}I by the lactoperoxidase-catalyzed reaction.
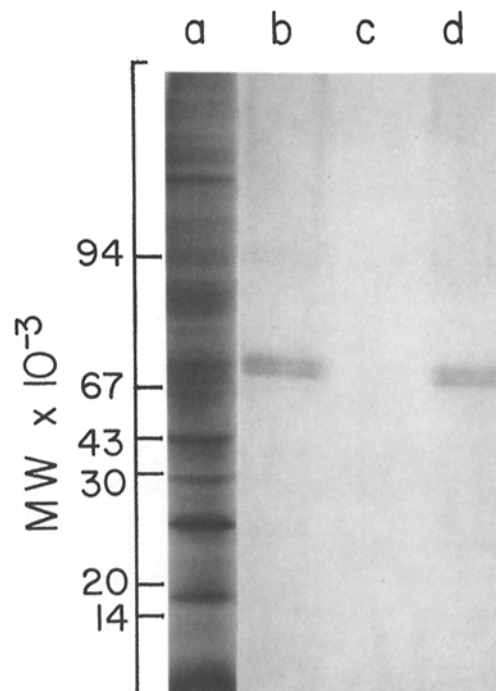
Fig. 1.   Autoradiographs of SDS gels (5–20%) of immunoprecipitates prepared from NP-40 lysates of surface-radioiodinated Con A-activated (15 μg/10⁶ cells per ml) T cell blasts. (a) Total cell lysate of the T cell blasts; (b) immunoprecipitate with a nude mouse serum with monoclonal antibody against human T cells; (c) immunoprecipitate with a control serum from a tumor-free nude mouse; (d) specific nude mouse serum from (b) was reacted with an excess of FCS before the incubation with the Con A-activated T cell blasts for immunoprecipitation. The position of the molecular weight (MW) markers is shown at the left: phosphorylase b 94,000; bovine serum albumin 67,000; ovalbumin 43,000; carbonic anhydrase 30,000; soybean trypsin inhibitor 20,100; and α-lactalbumin 14,400.

The cells were reacted with specific antibody, washed, and lysed with NP-40 detergent. The immune complexes in the lysate were absorbed onto Sepharose CL-4B beads that had been coupled with F(ab')₂ fragments of goat-antimouse IgG reagent, and analyzed by SDS-polyacrylamide gel electrophoresis.

Under nonreducing conditions, the total cell lysate of the Con A-activated blasts gave more than 40 discernible bands that ranged from 5,000 to 220,000 daltons (Fig. 1 a). The immunoprecipitate performed with the monoclonal antibody gave two distinct bands with molecular weights of 69,000 and 71,000 daltons (Fig. 1 b). As a control, a normal nude mouse serum did not precipitate any discernible component (Fig. 1 c). To exclude the possibility that the defined antigen was a component of FCS that had been passively absorbed onto the cells, the antibody was first incubated with an excess of FCS before reaction with the labeled cells as shown in Fig. 1 d. Similar results were obtained with other positive target cells.

The specificity of the α-Leu 1 nude mouse serum was studied by indirect immunofluorescence on a cytofluorograph with techniques that have been previously described (5). Background reactivity was assessed by using a serum at the same dilution (1:10⁴) from a tumor-free animal of the same strain. Fig. 2 A and B show representative binding curves of the α-Leu 1 serum and a normal nude mouse serum
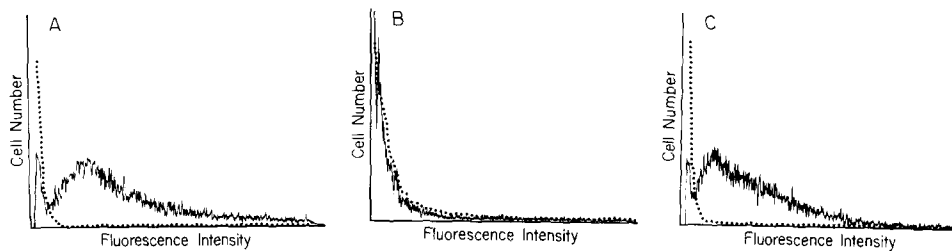
Fig. 2. Cytofluorograph analysis of indirect immunofluorescent staining of human lymphoid cells with specific nude mouse serum (Leu 1) at a 1:10,000 dilution (——) or with control nude mouse serum (. . .). 10,000 cells were analyzed per sample. (A) E-rosette-positive peripheral blood lymphocytes; (B) E-rosette-negative tonsillar lymphocytes; (C) cells from a patient with CLL that were positive with sIg and Ia antigen. The abscissa and ordinate record the number of cells and the fluorescence intensity per cell, respectively.

reacted with peripheral blood T cells, and with tonsillar B cells. As can be seen, the great majority of T cells exhibited binding to α-Leu 1 above background. In contrast, B cells did not fluoresce above a normal serum control.

As shown in Table I, 80–95% of SRBC-rosetting cells from the peripheral blood of 20 normal individuals and >95% of thymocytes from 5 children undergoing cardiac surgery were found to express the Leu 1 antigen. Ongoing studies in our laboratory (Memorial Sloan-Kettering Cancer Center, New York) indicate that the 5–20% E-rosette-positive, Leu 1-negative cells do not express other thymus-dependent surface antigens. This finding suggests that α-p69,71 defines the entire population of circulating thymus-derived lymphocytes, and that some cells of a non-thymic origin may carry the receptor for SRBC (9).

Data presented in Table I also indicate that α-Leu 1 does not react with normal lymphoid or hematopoietic cells that do not carry thymus-dependent markers. Thus, bone marrow cells, monocytes, and B cells from the peripheral blood, tonsils, and spleen were found to be unreactive with α-Leu 1. Splenocytes were obtained from patients with Hodgkin's disease that had been splenectomized for staging. Spleens that were pathologically positive for Hodgkin's disease were not included.

Because of the demonstration that α-Leu 1 was unreactive with normal B cells, it was surprising to find that this antibody reacted with Ig^+ cells from 11 of 14 patients examined who had the clinical diagnosis of chronic lymphatic leukemia (CLL) (Fig. 2 C; and Table I). It should be mentioned that the great majority of cells from patients with CLL have several characteristics that indicate that the malignant cells are of B cell origin. These include surface markers characteristic of B cells and the capacity to differentiate into plasma cells under certain conditions (10). Furthermore, none of the 14 cases examined in this report reacted with monoclonal antibodies to thymus-dependent antigens, including T_{H_2} (5) (data not shown). The possibility that the expression of Leu 1 by surface (s)Ig^+ CLL cells resulted from transformation events that can also be triggered in normal B cells seems unlikely in view of the demonstration that Epstein-Barr virus (EBV)-infected B cell lymphoblasts were Leu 1 negative (Table I).

An alternative explanation of our results lies in the possibility that CLL represents a malignancy of a small subpopulation of Leu 1-positive B lymphocytes. However, this does not seem likely in light of the demonstration that this subset was not

TABLE I

Reactivity of α-Leu 1 with Subpopulations of Lymphoid Cells by Immunofluorescent Analysis

Cell source	Purified cell population	Percentage of positive cells
		%
PBL (20/20)*	E-RFC‡ enriched	80–95
	E-RFC depleted	<5
Tonsil (5/5)	E-RFC depleted	<5
Spleen (4/4)§	E-RFC depleted	<5
Bone marrow (5/5)	E-RFC depleted	<5
Thymocytes (5/5)		>95
Chronic lymphocytic leukemic cells (11/14)		Positive with variable intensity
B lymphoblastoid cell lines (10/10)‖		Negative

* 20 of 20 samples analyzed.
‡ SRBC E-rosette-forming cells.
§ Spleens obtained from patients with Hodgkin's disease that were not pathologically involved.
‖ Established in culture from normal PBL by transformation with EBV.

detectable in the blood, tonsils, spleen, or bone marrow. Furthermore, these findings are consistent with the results of other studies that demonstrate that T cells and sIg⁺ CLL cells share surface markers not found on normal B cells. These include determinants defined by heterologous anti-human T cell sera (11), receptors for Helix pomatia agglutinin (12), and receptors for IgM (13). Our findings, therefore, raise questions regarding the classification of certain human lymphocyte surface antigens as markers of particular differentiation pathways, because induction of the determinant recognized by α-Leu 1 seems to take place under at least one set of pathological conditions outside the thymic environment.

Finally, it is relevant to our studies that malignant transformation of murine lymphocytes may be associated with the induction of membrane alloantigens that have a normal tissue distribution that is restricted by either strain phenotype or by cell lineage (14). The mouse G_{IX} system exemplifies such an antigen because its distribution on normal lymphoid cells is restricted to thymus-dependent lymphocytes of certain strains, but it is found on both thymic and nonthymic leukemias of $G_{IX}{}^{+}$ and $G_{IX}{}^{-}$ strains (2). It is also notable that G_{IX} is associated with a membrane complex of 69,000 and 71,000 daltons and that this complex shares biochemical and antigenic properties with the major glycoprotein component for the murine leukemia virus envelope (15–17). We are therefore investigating a possible relationship between the human p69,71 complex and the murine G_{IX} system.

Summary

In the course of generating monoclonal antibodies to human thymus-dependent differentiation antigens, we were able to define specificities shared by T cells and by cells from patients with chronic lymphatic leukemia that were not detectable on normal B cells. In particular, one of these antibodies was reactive by indirect immunofluorescence with >95% of the thymocytes and 80–95% of nonadherent sheep erythrocyte-rosetting peripheral blood lymphocytes (PBL), but was unreactive with normal B cells or cell lines derived from PBL by Epstein-Barr virus transformation. However, the leukemic cells from 11 of 14 patients with B-type chronic lymphatic leukemia were found to express detectable concentrations of this surface determinant.

The target antigen recognized by this monoclonal antibody was shown by immu-

noprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be a p69,71 complex. Our findings suggest a possible relationship between this antigen and the previously described G_{IX} system in the mouse.

References

1. Köhler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.)*. **256:**495.
2. Stockert, E., L. J. Old, and E. A. Boyse. 1971. The G_{IX} system. A cell surface allo-antigen associated with murine leukemia virus; implications regarding chromosomal integration of the viral genome. *J. Exp. Med.* **133:**1334.
3. Yelton, D. E., B. A. Diamond, S. P. Kwan, and M. D. Scharff. 1978. Lymphocyte hybridomas. *Curr. Top. Microbiol. Immunol.* **81:**1.
4. Hoffman, T., and H. G. Kunkel. 1976. The E rosette Test. *In* In Vitro Methods in Cell-Mediated and Tumor Immunity. B. R. Bloom and J. R. David, editors. Academic Press, Inc., New York. 71.
5. Evans, R. L., H. Lazarus, A. C. Penta, and S. F. Schlossman. 1978. Two functionally distinct subpopulations of human T cells that collaborate in the generation of cytotoxic cells responsible for cell-mediated lympholysis. *J. Immunol.* **120:**1423.
6. Marchalonis, J. J., R. E. Cone, and V. Santer. 1971. Enzymatic iodination. A probe for accessible surface proteins of normal and neoplastic lymphocytes. *Biochem. J.* **124:**921.
7. Wang, C. Y., S. M. Fu, and H. G. Kunkel. 1979. Isolation and immunological characterization of a major surface glycoprotein (gp54) preferentially expressed on certain human B cells. *J. Exp. Med.* **149:**1424.
8. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. **226:**680.
9. Evans, R. L., L. Chess, H. Levine, and S. F. Schlossman. 1978. Antibody-dependent cellular cytotoxicity by allosensitized human T cells. *J. Exp. Med.* **147:**605.
10. Fu, S. M., N. Chiorazzi, and H. G. Kunkel. 1979. Differentiation capacity and other properties of the leukemic cells of chronic lymphocytic leukemia. *Immunol. Rev.* **48:**23.
11. Boumsell, L., A. Bernard, V. Lepage, L. Dejos, J. Lemerle, and J. Dausset. 1978. Some chronic lymphocytic leukemic cells bearing surface immunoglobulins share determinants with T cells. *Eur. J. Immunol.* **8:**900.
12. Hellstrom, U., H. Mellsleat, P. Perlmann, G. Holm, and O. Petterson. 1976. Receptors for Helix pomatia A hemagglutinin on leukemic lymphocytes from patient with chronic lymphocytic leukemia (CLL). *Clin. Exp. Immunol.* **266:**196.
13. Ferrarini, M., T. Hoffman, S. M. Fu, R. Winchester, and H. G. Kunkel. 1977. Receptors for IgM on certain human B lymphocytes. *J. Immunol.* **199:**1525.
14. Old, L. J., and E. A. Boyse. 1973. Current enigmas in cancer research. *Harvey Lect.* **67:**273.
15. Obata, Y., H. Ikeda, E. Stockert, and E. A. Boyse. 1975. Relation of G_{IX} antigen of thymocytes to envelope glycoprotein of murine leukemia virus. *J. Exp. Med.* **141:**188.
16. Tung, J. S., E. Fleissner, E. S. Vitetta, and E. A. Boyse. 1975. Expression of murine leukemia virus envelope glycoprotein p69/71 on mouse thymocytes. Evidence for two structural variants distinguished by presence vs. absence of G_{IX} antigen. *J. Exp. Med.* **142:**518.
17. Del Villano, B. C., B. Nave, B. P. Croker, R. A. Lerner, and F. J. Dixon. 1975. The oncornavirus glycoprotein p69/71: a constituent of the surface of normal and malignant thymocytes. *J. Exp. Med.* **141:**172.