Brief Definitive Report

IDENTIFICATION OF A HUMAN T LYMPHOCYTE SURFACE PROTEIN ASSOCIATED WITH THE E-ROSETTE RECEPTOR

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Human thymus-dependent lymphocytes (T cells) are defined by their ability to form spontaneous rosettes (E-rosettes) with sheep erythrocytes (SRBC) (1-3). Recently, several murine monoclonal antibodies that recognize distinct differentiation antigens on human T cells have been described (4-11). We report here a monoclonal antibody, designated 9.6, that identifies a 50,000-dalton surface protein that appears to be present on all E-rosette-forming cells. Blocking and lysostripping experiments indicate that this monoclonal antibody reacts either with the E receptor itself or with a closely associated structure.

Materials and Methods

Cells. Mononuclear cells from peripheral blood (PBL) or bone marrow were obtained from normal volunteers and isolated by centrifugation over Ficoll-Hypaque (LSM; Litton Bionetics Inc., Kensington, Md.). T cells were enumerated by rosetting with 2-aminoethylisothiouronium bromide hydrobromide (AET)-treated SRBC ($SRBC_{AET}$) in medium (RPMI-1640) with 12% fetal calf serum (12). For the isolation of purified T cells, PBL were passed over nylon wool (11) and then incubated with $SRBC_{AET}$. Rosette-forming T cells (E^+) were separated from non-T cells (E^-) by centrifugation over Ficoll-Hypaque. T cells were recovered from the rosettes by lysis of SRBC in Tris-buffered 0.83% ammonium chloride. Normal human thymocytes were prepared from thymus specimens obtained in the course of corrective open heart surgery from children <14 yr old. Long-term-cultured T cells, stimulated by alloantigen, were maintained in medium supplemented with T cell growth factor (TCGF) (13).

A selected panel of cultured lymphoid cell lines was used for serological analysis. Included were leukemic T cell lines (CEM, HSB2, 8402, Jurkat, Molt-4F, and KE37), the leukemic pre-B cell line NALM-6, Epstein-Barr virus-transformed B-lymphoid cell lines (PA-3, SB, 8392, HA, Swei), and the Burkitt's lymphoma cell line Daudi.

Leukemic blasts from peripheral blood of patients with acute lymphocytic leukemia (ALL), were separated over Ficoll-Hypaque, and cryopreserved. Cells were obtained either at presentation or relapse when the leukocyte count was >20,000/mm^3 and >90% of the cells were blasts. Subdivision of ALL into T and null cell types was based on the clinical criterion of a thymic mass, and on determination of whether the leukemic blasts formed E-rosettes or expressed Ia-like antigen.

Immunizations, Fusion, and Screening. The clone of hybrid cells producing the antibody described here was isolated from a previously described fusion experiment (9). Briefly, spleen cells from BALB/c mice immunized with human peripheral blood lymphocytes were fused with BALB/c MOPC21 NSI/1 myeloma cells. Antibody production by hybrid cells was assayed by testing tissue culture supernates in a complement-dependent microcytotoxicity assay against normal peripheral blood T cells, a B-lymphoid cell line, and continuously cultured T cells, all obtained from the same donor. One culture fluid was cytotoxic for both the normal and cultured T cells but nonreactive with the autologous B-lymphoid cell line. Cells from this

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culture, designated 9.6, were serially cloned four times by limiting-dilution and then inoculated intraperitoneally into Pristane-primed (Aldrich Chemical Co., Inc., Milwaukee, Wis.) BALB/c mice for the production of antibody-containing ascites fluid. The immunoglobulin produced by the 9.6 hybrid was identified as IgG_{2b} by immunodiffusion.

Serological Analysis. Methods for complement-dependent microcytotoxicity, quantitative cytotoxicity (using trypan blue as indicator), and indirect immunofluorescence assays have been previously described (9, 11). A fluorescein-conjugated affinity-purified goat anti-mouse IgG serum (Litton Bionetics Inc.) was used for indirect immunofluorescence. In some experiments rhodamine-conjugated goat $F(ab')_2$ fragments specific for mouse IgG were used (this reagent was generously provided by Dr. Shu Man Fu, The Rockefeller University, New York). Comparative studies were carried out using three additional monoclonal antibodies recognizing distinct markers for human lymphocytes. Antibody 9.3 reacts with a 45,000-dalton surface protein expressed by 70–80% of peripheral E^+ cells (9); antibody 10.2 reacts with a 65,000- to 67,000-dalton surface protein expressed by 85–95% of peripheral blood E^+ cells (11); and antibody 7.2 reacts with a framework determinant of the human Ia bimolecular complex (9).

Blocking of E Rosette Formation and Lysostripping. Cells were incubated in medium that contained monoclonal antibody for 30 min at 22°C and then washed three times. In blocking experiments, these cells were subsequently assayed for E rosette formation. In lysostripping experiments, treated cells were incubated with affinity-purified goat anti-mouse IgG serum (fluorescein conjugated) for 45 min at 37°C, washed three times, and then tested for rosette formation.

Immune Precipitation of Cell Membranes. Cells of the leukemic T cell line Jurkat were washed twice in phosphate-buffered saline, pH 7.2, and surface labeled by the ^{125}I-lactoperoxidase method (14) with minor modification (15). Labeled cells were disrupted in cell lysis buffer that contained 0.5% Nonidet P-40 (15) and 2% of the protease inhibitor aprotinin (Sigma Chemical Co., St. Louis, Mo.). The lysate was cleared of nonsolubilized cellular structures and free ^{125}I by ultracentrifugation and chromatography on a Sephadex G-25 column as described elsewhere (16).

Before use in immune precipitation analysis, the cell lysate was treated by sequential incubation (1 h each at 4°C) with AKR normal mouse serum (1:40 dilution) and *Staphylococcus aureus*, Cowan I strain (30 mg) (17). The *S. aureus* was removed from the reaction mixture by centrifugation at 1,500 *g* for 10 min. The concentration of sodium dodecyl sulfate (SDS) in the lysate was adjusted to 0.1% and the immune precipitation reactions initiated by the addition of 5 μl of appropriately diluted antibody to 95-μl (10^6 cpm) aliquots of radiolabeled lysate. The mixture was incubated for 1 h on ice and the reaction then terminated by the addition of 3.5 mg *S. aureus* for 30 min on ice. The *S. aureus* pellet was washed five times in buffer that contained 0.5% NP-40 and 0.1% SDS (15). Radiolabeled proteins bound to the pellet were extracted by incubation with 50 μl of sample electrophoresis buffer (0.062 M Tris-HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.02% bromphenol blue, pH 6.8) for 5 min at 100°C and then analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS (SDS-PAGE) in 10% slab gels (18). Radiolabeled bands in the dried gel were identified by radioautography (Kodak NS-2T film [Eastman Kodak Co., Rochester, N. Y.] with GAFMED Rarex B Mid Speed intensifying screen [GAF Corp., New York] with exposure at −70°C) (19).

Results and Discussion

The 9.6 antigen was present on peripheral blood T cells and thymocytes, but was not detected on mononuclear cells of peripheral blood or bone marrow after removal of T cells by E-rosetting (Table I). T cells purified by passage over nylon wool followed by centrifugation of E^+ cells over Ficoll-Hypaque were >99% positive with 9.6 antibody. Thymocytes were also >99% positive for the 9.6 antigen. Cells from B lymphoid lines were uniformly negative.

A high degree of concordance between E-rosette formation and expression of 9.6 antigen was observed in testing cells from a variety of sources (Table I). Similar numbers of 9.6-positive and E^+ cells were found in PBL, thymus, bone marrow, and long-term-cultured T cells. Expression of 9.6 antigen also correlated with E-rosetting in cells of leukemic T cell lines. Only the E^+ leukemic T cell lines 8402, Jurkat, and Molt-4F were 9.6 positive, each demonstrating equal numbers of E^+ and 9.6-positive

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TABLE I

*Comparison of the Number of E-Rosetting and Monoclonal Antibody 9.6-Positive Cells in Normal and Cultured Lymphoid Cells**

	Percent E^+	Percent 9.6 positive
Peripheral blood lymphocytes	72 (9)	68 (9)
Purified T cells	99 (5)	99 (5)
Non-T cells	<1 (3)	<1 (3)
Thymocytes	>99 (3)	>99 (3)
Bone marrow cells	9-17 (3)	10-15 (4)
T cell depleted	<1 (2)	<1 (2)
B-lymphoid cell lines‡§	<1 (7)	<1 (7)
Cultured T cells‖	>99 (3)	>99 (3)
Leukemic T cell lines§		
CEM	<1	<1
8402	23	20
Jurkat	90	98
HSB-2	<1	<1
KE37	<1	<1
Molt-4F	17	20
Molt-4F, E^+ enriched	85	93
Molt-4F, E^+ depleted	3	4

* Assays for E-rosette forming cells E^+ were performed with $SRBC_{AET}$. Testing with 9.6 antibody was performed by indirect immunofluorescence. The number of donors tested is indicated in parentheses. Where appropriate, results are expressed as the mean (or range) of multiple samples.

‡ NALM-6, Daudi, PA3, SB, 8392, HA and Swei.

§ Cell lines CEM and PA3 were obtained from Dr. D. Mann, National Institutes of Health, Bethesda, Md.; HSB-2, SB, and Jurkat from Dr. W. D. Peterson, Wayne State University, Detroit, Mich.; 8402, 8392, Molt-4F, and NALM-6 from Dr. J. Minowada, Roswell Park Memorial Institute, Buffalo, N. Y.; KE37 from Dr. S. M. Fu; and Daudi from Dr. W. Newman, Fred Hutchinson Cancer Research Center, Seattle, Wash. Derivation of B-lymphoid cell lines HA and Swei has been previously described (20).

‖ Normal T cells stimulated with alloantigen and cultured >3 mo in TCG Factor.

TABLE II

Effect of Antibody 9.6 on E-Rosette Formation

Cells treated	E-Rosette formation after incubation with antibody*		
	Control	Antibody 10.2	Antibody 9.6
Peripheral T cells‡	86	92	<1
Thymocytes	99	98	<1
Cultured T cells	99	99	<1
Leukemic T cell line, Jurkat	92	96	<1
PHA blasts§	ND	94	<1

* Results expressed as the percent rosette-forming cells. Ascites fluids that contained antibody 10.2 or 9.6 were tested at a dilution of 1:1,000. Rosette inhibition with antibody 9.6 persisted to a dilution of 1:64,000. ND, not determined.

‡ Nylon wool-nonadherent peripheral blood lymphocytes.

§ Nylon wool-nonadherent peripheral blood lymphocytes were cultured for 72 h in complete medium with 10% pooled human serum and 12 μg/ml phytohemagglutinin (PHA-P; Burroughs Wellcome & Co., Research Triangle Park, N. C.).

cells. Molt-4F cells, enriched for E^+ cells were 9.6 positive, whereas Molt-4F cells depleted of E^+ cells were 9.6 negative. Cells from patients with T cell ALL whose leukemic blasts formed E-rosettes were 9.6 positive. Leukemic blasts from patients with null cell ALL were 9.6 negative.

The association between the E receptor and the 9.6 antigen was further investigated by blocking experiments. Cells preincubated with antibody 9.6 did not form E-rosettes (Table II). Incubation with either of the two other monoclonal T cell antibodies, 9.3 (data not shown) or 10.2, did not inhibit E-rosetting. Rosette inhibition by antibody 9.6 paralleled its activity in a complement-mediated cytotoxicity assay.

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TABLE III
Lysostripping

Lysostripping antibody*	E^+	Treated cells tested by indirect immunofluorescence‡	
		9.6	10.2
None	94	94	91
9.6	4	0	92
10.2	95	77	0

* Cells were incubated with monoclonal antibody diluted in medium for 0.5 h at 22°C and washed three times. Cells were then incubated with fluorescein-conjugated goat anti-mouse IgG serum for 45 min at 37°C and the washing was repeated. Fluorescein staining was detected only in polar caps. Rosetting was assayed with $SRBC_{AET}$. Numbers in the Table represent the percentage of rosetting cells.

‡ To determine whether any uncapped mouse immunoglobulin remained on the cells after lysostripping, cells were stained with rhodamine-conjugated goat $F(ab')_2$ fragments specific for mouse immunoglobulin. No rhodamine staining could be detected outside the polar caps. Treated cells were also tested with 9.6 antibody and 10.2 antibody by indirect immunofluorescence to determine whether the respective antigens had been completely capped. The rhodamine-conjugated antiserum facilitated distinction between staining of previously formed polar caps and circumferential staining of antigens that remained distributed on the cells. Numbers in the Table represent percent positive cells.

Lysostripping of cells incubated with monoclonal antibody was performed to determine whether the E receptor and the 9.6 antigen could be distinguished by co-capping. Cells lysostripped of 9.6 antigen did not form E-rosettes, whereas cells lysostripped of the 10.2 antigen were still capable of rosette formation (Table III). Capping of antigen-antibody complexes appeared complete, as assessed by indirect immunofluorescence (Table III) and quantitative cytotoxicity (data not shown). It appeared, therefore, that the failure of cells stripped of 9.6 antigen to form E rosettes was not a result of residual blocking antibody. When cells were lysostripped of 9.6 antigen and then incubated in fresh medium at 37°C for 18 h, they again expressed 9.6 antigen and formed E-rosettes.

Results of precipitation assays with antibody 9.6 and an ^{125}I-labeled lysate of the leukemic T cell line Jurkat are presented in Fig. 1. The antibody precipitated a single polypeptide of ~50,000 mol wt. In this same assay are shown the results with antibodies 9.3 and 10.2, which precipitated proteins of 45,000 and 67,000 daltons, respectively. The 45,000-dalton protein precipitated by 9.3 antibody was not associated with β_2-microglobulin. This was in contrast to that observed with precipitates formed by antibodies against HLA heavy chain (W6/32) and β_2-microglobulin. Control reactions performed with normal mouse serum and monoclonal antibody 7.2 against human Ia antigen failed to show precipitation with the cell lysate. In additional precipitation assays with ^{125}I-labeled lysates of normal T cells (data not shown), the 9.6 antibody demonstrated essentially similar results to those found with the Jurkat cell line, although the antigen precipitated from normal T cells showed a slightly more disperse pattern on the gel. These results suggested that the antigen precipitated by 9.6 may have variable glycosylation patterns on T cells from different sources. Alternatively, the different pattern on gels might reflect mild proteolytic degradation of the antigen that varied from one T cell type to another.

Summary

We describe a new monoclonal murine antibody that reacts with a 50,000-mol wt polypeptide that appears to be present on all E-rosetting cells. We conclude that this

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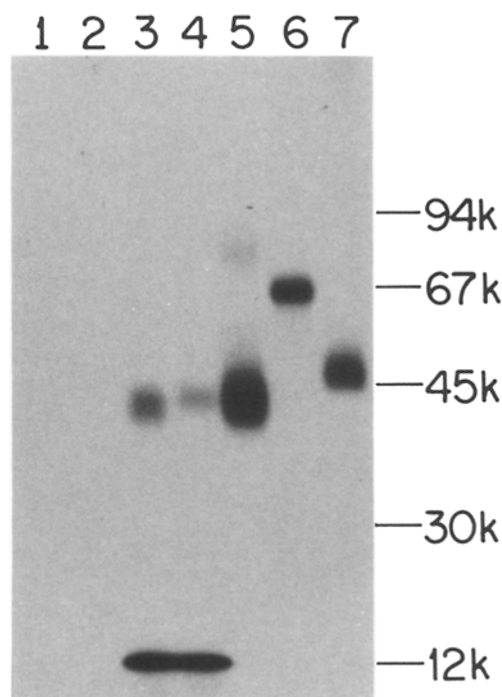

FIG. 1. Radioimmune precipitation assays with monoclonal antibodies and a ^{125}I-surface-labeled lysate of the leukemic T cell line Jurkat. Aliquots of radiolabeled cell lysate were tested in immune precipitation assays with: (1) normal mouse serum (1:50 dilution); (2) monoclonal 7.2 antibody from ascites fluid (1:500) which recognizes a monomorphic determinant of human Ia antigens (9); (3) rabbit anti-human β_2-microglobulin serum (Dakopatts, Copenhagen, Denmark; 1:50 dilution); (4) monoclonal antibody W6/32 (Allied Chemical Corp., Morristown, N. J.; 1:100 dilution) which recognizes a monomorphic determinant of the HLA-A,B,C heavy chain (21); (5) monoclonal 9.3 antibody from ascites fluid (1:500 dilution) which recognizes a 45,000-dalton protein specific for T cells (9); (6) monoclonal 10.2 antibody from ascites fluid (1:500 dilution) which recognizes a 65,000–67,000 dalton protein specific for T cells (11); and (7) monoclonal 9.6 antibody from ascites fluid (1:500 dilution). Immune precipitates were collected on *S. aureus*, eluted in electrophoresis buffer that contained 2-mercaptoethanol, and analyzed by SDS-PAGE. Radioautography of the dried gel was enhanced for 2 d on x-ray intensifying screens.

antigen is either identical to or closely associated with the E receptor because of (*a*) the high degree of concordance between E-rosette formation and 9.6 antigen expression, (*b*) the inhibition of rosette formation by preincubation of cells with 9.6 antibody, and (*c*) the observed failure of cells lysostripped of 9.6 antigen to form E-rosettes. This last finding suggests cocapping of 9.6 antigen and the E receptor.

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