Reaction of Normal Human Lymphocytes and Chronic Lymphocytic Leukemia Cells With an Antithymocyte Antiserum

By Alan C. Aisenberg, Kurt J. Bloch, John C. Long, and Robert B. Colvin

An antiserum against human fetal thymus was prepared in rabbits and absorbed with liver cells from the thymus donor and lymphocytes from a patient with chronic lymphocytic leukemia (CLL). The antiserum reacted with 80%–95% of the peripheral small lymphocytes from 12 normal controls but with only 1%–11% of cells from 12 patients with CLL. The antithymocyte antiserum demonstrates a cell surface antigen present on the majority of normal human lymphocytes, but absent from the neoplastic CLL cell. This finding is consistent with the view that the CLL lymphocyte is devoid of T-cell antigen and with other evidence that this lymphocyte closely resembles the B-cell of experimental animals. It appears likely that leukemic cells will prove useful in preparing an antihuman thymocyte antiserum that reacts exclusively with T cells by removing B-cell reactivity.

THERE IS AMPLE EVIDENCE for a functional separation of morphologically indistinguishable lymphocytes of lower animals into two main populations termed T- (for thymus-derived or dependent) and B- (for bone marrow or bursal equivalent-derived or dependent) cells.[1,2] B-cells, are characterized by the presence in high density of surface immunoglobulins,[3–5] and there is some evidence for the existence of receptors for antigen-antibody complexes and complement on their surface;[6] these cells or their derivatives are responsible for antibody production. T-cells are characterized by very low concentrations of surface immunoglobulins and, in certain mouse strains, by surface isoantigens, Θ[7,8] and TL;[9,10] these cells are largely responsible for certain cell-mediated immune reactions. The recognition that normal B-cells bear surface immunoglobulins suggests that immunoglobulin-bearing neoplastic lymphocytes, for example those of chronic lymphocytic leukemia (CLL), belong to the B-cell line and leads to a new approach in the classification of human lymphoid neoplasms.[11–16] Further progress requires the specific identification of human T-cells; this report describes the method of production and characterization of an antihuman thymocyte serum.

From the Massachusetts General Hospital, Boston, Mass.

Submitted June 19, 1972; revised August 3, 1972; accepted September 11, 1972.

Supported by research grants from the Massachusetts Division of the American Cancer Society, the Damon Runyon Memorial Fund for Cancer Research, and the Massachusetts Chapter of the Arthritis Foundation, and by USPHS Grant GM 02212.

This is publication 1430 of the Cancer Commission of Harvard University.

Alan C. Aisenberg, M.D.: *Associate Professor of Medicine, Harvard Medical School at the Massachusetts General Hospital, Boston, Mass. 02114.* Kurt J. Bloch, M.D.: *Associate Professor of Medicine, Harvard Medical School at the Massachusetts General Hospital, Boston, Mass. 02114.* John C. Long, M.D.: *Clinical and Research Fellow, Department of Pathology, Massachusetts General Hospital, Boston, Mass. 02114.* Robert B. Colvin, M.D.: *Clinical and Research Fellow, Department of Pathology, Massachusetts General Hospital, Boston, Mass. 02114.*

MATERIALS AND METHODS

Lymphocyte Preparations

The diagnosis of chronic lymphocytic leukemia was based on accepted clinical and laboratory criteria, including examination of a Wright-stained peripheral blood smear. Blood from leukemic patients with white cell counts below 20,000/cu mm was not used in order to minimize contamination of lymphocyte preparations with nonneoplastic cells. Normal controls were laboratory personnel.

Twenty milliliters of defibrinated blood were centrifuged for 5 min at 610 *g*, and the serum, buffy coat, and upper red cell layer were removed. The cells were obtained by centrifuging for 7 min at 1650 *g* and were then suspended in 4 volumes of Veronal-buffered saline containing 0.1% gelatin (VBS). Two-milliliter aliquots of the cell suspension were layered over 4 ml of a solution composed of 24 parts of 9% Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.) and 10 parts of 34% Hypaque-M, 90% (sodium and meglumine diatrizoates obtained from Winthrop Lab., New York, N.Y.)[17,18] and were centrifuged at room temperature (800 *g* for 40 min). The leukocytes at the interface were aspirated with a Pasteur pipette and washed twice with VBS, interspersed with centrifugation at 610 *g* for 5 min. To remove the erythrocytes, the pellet from the second wash was suspended in 1 ml of a solution made from 9 parts of 0.83% NH_4Cl and 1 part Tris-HCl (20.6 g/liter adjusted to pH 7.65 with 2 *N* HCl) for 10 min at 37°C, centrifuged, and resuspended in Medium 199 (Microbiological Associates, Bethesda, Md.) containing 0.1% gelatin. Lymphocyte preparations were employed only if their purity was 98% or greater.

Antithymocyte Antiserum

Human thymus was obtained from a 19-wk-old fetus 1 hr after hysterotomy, and a cell suspension was prepared by careful mincing in Hank's solution. Rabbits were immunized by the footpad injection of 1×10^8 thymocytes in 0.5 ml of complete Freund's adjuvant,[19] followed 2 wk later by the intravenous injection of 1.9×10^8 cells obtained from the same source and stored at −80°C in the frozen state in 10% ethylene glycol. Viability of both fresh and frozen cells was greater than 95% as determined by trypan blue dye exclusion. Two weeks after the second injection, animals were bled, and the globulin fraction of the serum was obtained by salting out twice with equal volumes of saturated ammonium sulfate.[20] This fraction was restored to the original serum volume with saline and was dialyzed extensively to remove residual ammonium sulfate. After dialysis the protein concentration of the solution was 6.8 mg/ml. On paper electrophoresis it was found to contain γ-globulin, as well as albumin, α_1-, α_2-, and β-globulins. Based on densitometry, the concentration of γ-globulin was 2.0 mg/ml. On immunoelectrophoresis developed with goat antirabbit γG and γM immunoglobulin antiserums, only γG was detected. The presence of γA was not tested. No precipitating antibodies to human serum proteins were detected by gel diffusion against human serum. The antithymocyte globulin was then absorbed at room temperature for successive 1-hr periods in the following manner: once with an equal volume of washed packed type A human erythrocytes, twice with 0.2 volumes (1.1×10^8 cells/ml globulin) of disrupted fetal liver (greater than 90% large mononuclear cells and lymphocytes, presumably hematopoietic cells) obtained from the thymus donor and stored as described above, and three times with 0.2 volumes (2.8×10^8 cells/ml globulin) of purified lymphocytes (100% small lymphocytes of which 85% bore IgM, type lambda on their surface) from a patient with chronic lymphocytic leukemia. The absorbed antithymocyte globulin was diluted 1/25, 1/50, and 1/100 with Medium 199 before use. It was stored in the frozen state without added preservative in aliquots of the 1/25 dilution. The absorbed antithymocyte globulin was not tested for its ability to agglutinate or to lyse leukocytes.

Immunofluorescent Staining

The indirect fluorescent antibody technique was employed. Lymphocytes ($1.5–2.0 \times 10^6$ in 0.05 ml) were incubated with 0.05 ml of diluted antithymocyte globulin for ½ hr at

37°C. Thereafter, the cells were washed twice with phosphate-buffered saline (PBS) and were incubated for an additional hour with 0.05 ml of a 1:5 solution of fluorescein-conjugated goat antirabbit globulin antiserum (Meloy Laboratories) Springfield, Va.). Following three additional washes with PBS, the cells were suspended in phosphate-buffered glycerine, placed on a slide, overlayed with a cover slip, and examined with a Zeiss ultraviolet microscope equipped with an Osram HBD 200 mercury arc lamp and an interference primary filter FITC-495X. A minimum of 200 lymphocytes were examined.

RESULTS

Normal human peripheral blood lymphocytes were incubated with a 1:50 dilution of rabbit antihuman thymocyte globulin absorbed with lymphocytes from a single patient with chronic lymphocytic leukemia (CLL). After the initial incubation, the normal lymphocytes were reacted with a fluorescein-conjugated goat antirabbit globulin antiserum; approximately 80%–95% of

Table 1. Staining of Normal Human Lymphocytes and Thymocytes and Chronic Lymphocytic Leukemia Cells With Antithymocyte Globulin

	Per Cent Cells Stained Following Initial Exposure to Antithymocyte Globulin Diluted:		
	1:25	1:50	1:100
Normal lymphocytes			
1 (JS)	—	86	—
2 (ON)	—	87	87
3 (AA)	90	89	64
4 (BW)	95	95	83
5 (PC)	—	85	51
6 (DC)	—	80	45
7 (IH)	—	80	47
8 (PA)	—	82	63
9 (VG)	—	93	88
10 (JL)	—	81	82
11 (JB)	—	86	40
12 (SF)	—	88	79
Chronic lymphocytic leukemia lymphocytes			
1 (NE)	—	3	4
2 (FI)	3	1	0
3 (BL)	6	3	0
4 (RI)	—	7	7
5 (RO)	—	5	6
6 (DE)		5	3
7 (AR)		3	2
8 (BU)		3	3
9 (RE)		8	4
10 (HA)		1	1
11 (GO)		11	10
12 (CA)		10	8
Normal fetal thymocytes			
1 (M)		93	92
2 (B)		99	93

Table 2. Summary of Staining of Normal Human Lymphocytes and Chronic Lymphocytic Leukemia Cells With Antithymocyte Globulin and Antiimmunoglobulin Antiserum

	Lymphocytes Used for Absorption	Cells tested*	
		Normal	CLL
Antithymocyte	CLL	88 (80–95)	5 (0–11)
	Normal	1 (0– 1)	0 (0)
	None	Not done	99 (98–99)
Antiimmunoglobulin	None	14 (8–17)	84 (57–96)

*Results are expressed as arithmetic mean (%) of cells stained with the range indicated in parentheses. Twelve each of normals and CLL patients were studied with a 1:50 dilution of antithymocyte globulin absorbed with CLL cells, and four subjects in each group with this fraction absorbed with normal lymphocytes or unabsorbed. The antiimmunoglobulin antiserum was a polyvalent serum directed against both light and heavy chains of human immunoglobulin and was used in ten normals and 28 CLL patients.[16]

cells (Tables 1 and 2) showed discrete surface fluorescence (Fig. 1). A similar percentage of cells was stained following initial exposure to the antithymocyte globulin diluted 1:25, while fewer cells were stained after initial exposure to a dilution of 1:100. Lymphocytes from ten patients with CLL were similarly tested; only 1%–11% of cells reacted; the percentage of stained cells increased slightly following exposure to antithymocyte globulin diluted 1:25 and decreased slightly after an initial exposure to a dilution of 1:100. Incubation of CLL cells with unabsorbed rabbit antihuman thymocyte globulin, followed by the fluorescein conjugate, led to surface staining of 98%–99% of the cells, while antihuman thymocyte globulin absorbed with normal human lymphocytes (in the same quantity and under the same conditions used for absorption with CLL cells) failed to react with either normal or CLL cells (Table 2). Antithymocyte globulin absorbed with CLL cells reacted with 93%–99% of thymocytes obtained from two different fetal donors (Table 1).

Table 2 also summarizes previous experiments in which normal and CLL lymphocytes were tested with fluorescein-conjugated antihuman immunoglobulin antiserum. The results suggest that a reciprocal relationship may exist between staining of the lymphocyte population by antithymocyte and antiimmunoglobulin antiserums.

Fig. 1. Normal human lymphocytes stained with 1:50 dilution of antithymocyte globulin that has been absorbed with cells from a patient with chronic lymphatic leukemia. × 500.

DISCUSSION

The neoplastic cell in chronic lymphocytic leukemia closely resembles the B-lymphocyte of current terminology. In common with the B-cell and in contrast with the thymus-processed T-lymphocyte, cells from the majority of CLL patients bear surface immunoglobulin of restricted specificity (demonstrable by fluorescence microscopy); in most cases IgM type kappa or IgM type lambda is detected.[11-16] In addition, some CLL lymphocytes resemble B-cells, in possessing complement receptors[21] and in reacting sluggishly in vitro to phytohemagglutinin.[22] Finally, the immune deficiency of CLL patients involves antibody-mediated defences predominantly, resembling the B-cell defect of bursectomized animals rather than the T-cell deficit observed after thymectomy.[23,24] Thus, the available evidence favors the B-cell lineage of the CLL lymphocyte, although a derepressed T-lymphocyte could exhibit the B-cell properties observed in cells from patients with chronic lymphocytic leukemia.

The present investigation was based on the assumption that the CLL cell represents a relatively pure population of B-lymphocytes, or at least resembles B-cells by lacking thymus-specific antigens. Thus, a rabbit antihuman thymocyte serum was absorbed with lymphocytes from a patient with CLL in order to obtain a T-cell reagent. The resulting reagent did not react with cells from other patients with CLL but did react with 80%–95% of peripheral blood lymphocytes from normal donors. The percentage of stained cells corresponds to the proportion of normal lymphocytes that lack readily detectable surface immunoglobulin (Table 2)[11-16] and, hence, are held to represent, at least in part, circulating T-cells. The minor fraction of normal cells, which do not stain with the antihuman thymocyte serum but do stain with the antiimmunoglobulin serum, are the putative B-cells. However, this interpretation remains unproved until it is possible to demonstrate that antihuman thymocyte and antiimmunoglobulin antisera react with different peripheral blood lymphocytes of the same individual.

There is precedence in other species for the preparation of specific anti-T-cell antisera by absorption of antithymocyte antisera with leukemic B-cells. Shevach et al.[25] have recently characterized the L_2C leukemic cell, derived from an acute lymphocytic leukemia of strain 2 guinea pigs, as a "B"-cell. Their rabbit antiguinea pig thymocyte antiserum absorbed with L_2C leukemia cells lost all specificity for B-cells but continued to react with thymocytes and thymus-derived lymphocytes.

In the experiments described here, it seems unlikely that the antithymocyte antiserum is directed against conventional histocompatibility antigens in view of the uniform reactions with lymphocytes from all normal humans tested and the known presence of histocompatability antigens on CLL cells.[26] Furthermore, it is unlikely that the reactivity of this antiserum is attributable to the presence of antigen-antibody complexes; such complexes might be expected to adhere to CLL cells rather than to the majority of normal lymphocytes, since the available evidence suggests that the former bear immunoglobulin and complement receptors. The results of the present experiments indicate that the absorbed antithymocyte serum detects a lymphocyte surface

antigen that is not present on CLL cells; indirect evidence suggests that this antigen may be a thymus-specific determinant possibly similar to theta[7,8] or the TL[9,10] antigens of mice.

ACKNOWLEDGMENT

We are indebted to Mrs. Caroline Murray and Mrs. Barbara Wilkes for excellent technical assistance.

REFERENCES

1. Roitt, I. M., Greaves, M. F., Torrigiani, G., Brostoff, J., and Playfair, J. H. L.: The cellular basis of immunological responses. Lancet 2:367, 1969.

2. Davies, A. J. S., and Carter, R. L.: Systems of lymphocytes in mouse and man: An interim appraisal. *In* Hanna, H. G. (Ed.): Contemporary Topics in Immunology, Vol. I. New York, Plenum, 1972, p. 1.

3. Raff, M. C.: Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. Immunology 19:637, 1970.

4. Pernis, B., Forni, L., and Amante, L.: Immunoglobulin spots on the surface of rabbit lymphocytes. J. Exp. Med. 132:1001, 1970.

5. Unanue, E. R., Grey, H. M., Rabellino, E., Campbell, P., and Schmidtke, J.: Immunoglobulins on the surface of lymphocytes. II. The bone marrow as the main source of lymphocytes with detectable surface-bound immunoglobulin. J. Exp. Med. 133:156, 1971.

6. Bianco, C., and Nussenzweig, V.: Theta-bearing and complement-receptor lymphocytes are distinct populations of cells. Science 173:154, 1971.

7. Reif, A. E., and Allen, J. M. V.: The AKR thymic antigen and its distribution in leukemias and nervous tissues. J. Exp. Med. 120:413, 1964.

8. Owen, J. J. T., and Raff, M. C.: Studies on the differentiation of thymus-derived lymphocytes. Cell. Immunol. 1:536, 1971.

9. Boyse, E. A., and Old, L. J.: Some aspects of normal and abnormal cell surface genetics. Annu. Rev. Med. 3:269, 1969.

10. Lance, E. M., Cooper, S., and Boyse, E. A.: Antigenic change and cell maturation in murine thymocytes. Cell. Immunol. 1:536, 1971.

11. Klein, E., Eskeland, T., Inoue, M., Strom, R., and Johansson, B.: Surface immunoglobulin-moieties on lymphoid cells. Exp. Cell Res. 62:133, 1970.

12. Pernis, B., Ferrarini, M., and Forni, L.: Immunoglobulin on lymphocyte membranes. *In* Amos, B. (Ed.): Progress in Immunology, New York, Academic, 1971, p. 95.

13. Grey, H. M., Rabellino, E., and Pirofsky, B.: Immunoglobulins on the surface of lymphocytes. J. Clin. Invest. 50: 2368, 1971.

14. Papamichail, M., Brown, J. C., and Holborrow, E. J.: Immunoglobulins on the surface of human lymphocytes. Lancet 2:850, 1971.

15. Wilson, J. D., and Nossal, G. J. V.: Identification of human T and B lymphocytes in normal peripheral blood and chronic lymphocytic leukemia. Lancet 2:788, 1971.

16. Aisenberg, A. C., and Bloch, K. J.: Immunoglobulins on the surface of neoplastic lymphocytes. N. Engl. J. Med. In press.

17. Noble, P. B., Cutts, J. H., and Carroll, K. K.: Ficoll flotation for the separation of blood leukocyte types. Blood 31:66, 1968.

18. Harris, R., and Ukaejiofo, E. O.: Rapid preparation of lymphocytes for tissue typing. Lancet 2:327, 1969.

19. Shevach, E., Green, I., and Ellman, L.: Heterologous antiserum to thymus-derived cells in the guinea-pig. Nature [New Biol.] 235:19, 1972.

20. Goldman, M.: Fluorescent Antibody Methods. New York, Academic, 1968, p. 94.

21. Shevach, E., Herberman, R., and Frank, M.: Receptors for complement and immunoglobulin on human leukemias and human cell lines. Blood 38:787, 1971.

22. Rubin, A. D.: In vitro evaluation of lymphocyte proliferation in lymphoproliferative disorders. *In* Harris, J. E. (Ed.): Proceedings of the Leukocyte Culture Conference. New York, Academic, 1970, p. 239.

23. Block, J. B., Haynes, J.. and Thompson, W. L.: Delayed hypersensitivity in chronic lymphocytic leukemia. J. Natl. Cancer Inst. 42:973, 1969.

24. Cone, L., and Uhr, J. W.: Immuno-

logical deficiency disorders associated with chronic lymphocytic leukemia and multiple myeloma. J. Clin. Invest. 43:2241, 1964.

25. Shevach, E. M., Ellman, L., Davie, J. M., and Green, I.: L_2C guinea pig lymphatic leukemia: A "B" cell leukemia. Blood 39:1, 1972.

26. Pegrum, G. D., Balfour, I. C., Evans, C. A., and Middleton, V. L.: HL-A antigens on leukemic cells. Br. J. Haematol. 19:493, 1970.