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Comparison of Normal and CLL Lymphocyte Surface Ig Determinants Using Peroxidase-labeled Antibodies. I. Detection and Quantitation of Light Chain Determinants

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Chronic lymphocytic leukemia (CLL) and normal human blood lymphocytes were investigated with regard to their membrane-associated light (L) chains. Peroxidase-labeled anti-kappa or anti-lambda antibodies were used to visualize by light microscopy the cells bearing L chain determinants. In addition, the activity of the enzyme coupled to the antibody was measured spectrophotometricly in order to determine the number of antigenic determinants on each positive cell. The results demonstrated that 16%–25% of lymphocytes from six normal control subjects were positive for kappa chains, and 4%–10% were positive for lambda chains. The average number of antigenic sites on the surface of each positive cell was calculated to be 90,000. Lymphocytes of each of the 14 CLL patients studied carried either kappa or lambda antigenic determinants on their surface, but not both; 50%–70% of the lymphocytes were stained. The average number of antigenic sites per positive CLL was calculated to be 9000. These results confirm previously reported studies indicating that CLL is a monoclonal proliferative disease. In addition, the quantitative results demonstrate that the CLL lymphocyte surface membrane bears, on the average, only 10% of the L chain determinants present on the normal lymphocyte.

SINCE MÖLLER DEMONSTRATED the presence of antigens on the surface of mouse lymphoid cells,[1] immunoglobulins (Ig) on the surface of lymphocytes of several species have been visualized by various methods, including fluorescent antibody techniques,[2,3] and immunoelectron microscopy with antibodies labeled by tracers such as ferritin,[4] peroxidase,[5] or radioisotopes.[6]

The quantitation of Ig antigenic sites on the lymphocyte cell surface has generally been carried out by combining two techniques: The fluorescent antibody technique to determine the proportion of cells bearing a particular Ig determinant, and either a hemaglutination inhibition[7] or radioimmunoassay technique[8] to determine the quantity of the Ig determinant present in the cell suspension studied. By contrast, both quantitation and distribution of membrane-bound Ig can be determined with peroxidase-labeled antibody[9] or radiolabeled antibody.[10]

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Membrane-associated immunoglobulins have been demonstrated on human peripheral blood lymphocytes of normal and diseased subjects, and significant differences have been found.[7,11,15]

The purpose of this study was threefold: (1) morphological comparison of chronic lymphocytic leukemia (CLL) and normal human blood lymphocytes with respect to surface L chain antigenic determinants, (2) quantitation of the number of lymphocytes bearing kappa or lambda chains, and (3) determination of the number of antigenic determinants on each positive cell.

MATERIALS AND METHODS

A total of 20 cases were studied; 14 had an established diagnosis of chronic lymphocytic leukemia, two of these were being treated with chlorambucil, while the rest were untreated, and six normal subjects were professional blood donors.

The source of chemicals were Boehringer for horseradish peroxidase, grade 1; Merck for 3-3′-diaminobenzidine tetrahydrochloride; Sigma for 3-amino-9-ethylcarbazole; NBC for *o*-dianisidine; Pharmacia for Ficoll; and Nyegaard and Co. for Isopaque 440.

Preparation of Anti-kappa and Anti-lambda Chain Antibodies

Antisera to kappa and lambda chains were raised in goats immunized with Bence Jones proteins obtained from the urine of patients with multiple myeloma. They were rendered monospecific for kappa or lambda chains by adsorption with an immunoadsorbent of the opposite type, prepared by coupling Bence Jones proteins to glutaraldehyde-treated polyacrylamide beads.[16] Pure anti-kappa or anti-lambda antibodies were isolated by adsorption and elution from homologous insoluble light chain adsorbents. They have been shown to react with ten different Bence Jones proteins of the same light chain type by a double diffusion technique.[17]

Peroxidase Labeled Antibodies

Purified antibodies were coupled to peroxidase by glutaraldehyde using a two-step procedure previously described.[18] Labeled antibodies were separated from free peroxidase by precipitation with an equal volume of saturated ammonium sulfate solution at neutral pH, then resuspended in and dialyzed against Hanks' solution.

Purification of Peripheral Blood Lymphocytes

Thirty to fifty milliliters of blood from patients with CLL was collected in tubes containing heparin and allowed to sediment for 1 hr at 37°C. The plasma was aspirated, and the cells were sedimented and washed three times by centrifugation in Hank's medium (7 min at 1200 *g* at 4°C). The cell preparation thus obtained contained an average of 90% lymphocytes and rare erythrocytes.

Lymphocytes from normal donors were isolated in the following way: 300–500 ml of heparinized blood was filtered on glass beads and diluted with 3 volumes of Hanks' solution. Sixty milliliters of diluted blood were layered over 30 ml of a freshly prepared mixture of Ficoll-Isopaque (24 parts of a 9% w/v Ficoll + 10 parts of a 34% w/v Isopaque) and centrifuged at 18°C for 20 min at 1000 *g*.[19] The lymphocytes were aspirated in the layer above the Ficoll-Isopaque, sedimented by centrifugation (8 min at 2500 *g* and 4°C), and then washed three times with Hanks' solution. With this method, pure lymphocytes were obtained with a yield of 70%–90% of the original count.

Both lymphocyte preparations were exposed to 0.87% ammonium chloride solution for 10 min at 4°C to lyse the remaining erythrocytes, and washed three times with Hanks' solution. This was necessary because the peroxidase activity of hemoglobin gave a high background level in the quantitative assay, if not removed. Cells were counted with a hemocytometer and their viability determined by trypan blue exclusion. Cell death never exceeded 10%.

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Reaction of Cells With Enzyme-Labeled Antibody

Samples of 5×10^7 cells were incubated either at 4°C or 37°C with 0.5 ml of peroxidase-labeled anti-kappa or anti-lambda antibodies, or with normal IgG, for 2 hr. The concentration of the reagents was determined by a preliminary experiment in which the same number of cells was incubated with increasing concentrations of labeled antibodies. A curve of bound antibodies was obtained, showing a plateau at a concentration of 200 μg/ml.[9] This concentration of labeled antibodies was employed in the present study.

Following incubation, the cells were washed three times and processed for microscopy or for the spectrophotometric assay.

Light and Electron Microscopy

Aliquots of cells treated at 37°C were fixed in 4% formaldehyde in 0.1 *M* phosphate buffer, pH 7.4, for 1 hr and washed three times by centrifugation. Two techniques were then used; the one allowing observations both in the light and the electron microscope has been previously described,[15] and can be summarized as follows. The peroxidase staining was performed with a solution of 3-3'-diaminobenzidine and hydrogen peroxide (5 mg of diaminobenzidine in 10 ml of 0.1 *M* Tris-HCl buffer, pH 7.5, containing 0.03% H_2O_2) for 10 min.[20] Cells were next washed three times with the phosphate buffer containing 0.2 *M* sucrose. They were then postfixed in a 2% osmium tetroxide solution in the phosphate buffer for 1 hr and embedded in Epon after dehydratation. Sections 0.5 μ thick were mounted on glass slides for light microscopy. The second procedure, allowing observations only by light microscopy, was carried out by staining the cells with a solution of 3-amino-9-ethylcarbazole and hydrogen peroxide (2 mg amino ethylcarbazole are dissolved in 0.5 ml of dimethylformamide + 9.5 ml of 0.05 *M* acetate buffer, pH 5, containing 0.03% H_2O_2) for 30 min.[21] One drop of the cell suspension was mounted under a coverslip, and positive cells were counted immediately. The percentage of labeled versus unlabeled cells was obtained in both procedures after counting 500 cells.

Measurement of Peroxidase Activity

The pellets of cells treated with labeled reagents at 4°C were suspended in 0.5 ml of Hanks' medium. Three 0.1 ml aliquots were taken from each tube. One was used to determine the exact concentration of cells and the number of dead cells (which never exceeded 10%). To the two other aliquots, 2.9 ml of the *o*-dianisidine reagent was added (0.83 ml of 1% *o*-dianisidine in methyl alcohol + 100 ml of 0.003% H_2O_2 in 0.01 *M* phosphate buffer, pH 6, all reagents freshly prepared). The reaction was stopped after 15 min with a drop of 6 *N* HCl. Tubes were centrifuged at 5000 *g* for 5 min, and the absorbance of the supernatant was read at 400 nm.[9] Nonspecific peroxidase activity was determined in the sample which was incubated with peroxidase-labeled normal IgG, and this value was subtracted from the activity obtained with labeled antibody. Taking into account the number of cells and the activity found, the quantity of peroxidase fixed per 10^6 cells was calculated by referring to a standard curve of absorbance versus reaction time for known quantities of peroxidase. Since the mean ratio of peroxidase coupled to antibodies is one molecule to one molecule,[18] the number of molecules of antibody bound per 10^6 cells could be calculated, and then the mean number of antigenic sites on the surface of each positive lymphocyte could be determined.

The reaction of cell-bound peroxidase with its substrate was generally allowed to proceed for 15 min for 10^6 cells. This reaction time can be reduced if excessively high peroxidase activity is obtained. However, if very weak activity is obtained, it is preferable to increase the number of cells in the reaction mixture, rather than increase the reaction time, to avoid a large increase of background peroxidase activity.

RESULTS

The circulating lymphocytes of 14 CLL patients and six normal donors were examined.

The spectrophotometric measurements demonstrated that all CLL patients had lymphocytes bearing a single light chain type, twelve of the kappa type

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Table 1.

Donors*		Blood Lymphocyte Count (per cu mm)	Lymphocytes (%)	Peroxidase Antibodies Bound Specifically: Anti-kappa Stained Cells (%)	Anti-kappa No. of Molecules per Cell × 10^3	Anti-lambda Stained Cells	Anti-lambda No. of Molecules per Cell × 10^3
Patients	1	52,500	92	68	6.6	0	
with	2	99,600	99	47.2	6	0	
chronic	3	27,000	88	0		75	6.5
lymphatic	4	218,000	99	22.6	7.9	0	
leukemia	5	26,200	90	35	3	0	
	6	42,100	84	71.6	6.3	0	
	7	52,400	94	61	7.4	0	
	8	20,400	81	76	3.5	0	
	9†	26,800	92	70	21.4	0	
	10	42,000	92	55	8.2	0	
	11	27,800	92	70	12.8	0	
	12	17,900	84	68	13.2	0	
	13†	110,000	94	52	15	0	
	14	52,200	96	50	7.3	0	
	15	700,000	99	0		96	75
Normal	1	5,300	39	16.4	185	4	97
subjects	2	5,900	40	20	86	10	190
	3	6,200	42	19.6	88	10	32.5
	4	5,300	45	18	112.5	6	106
	5	6,500	43	17.1	153	8	140
	6	7,000	47	25.2	111	9.5	70

*Patients were untreated except No. 4 and No. 5, who received chlorambucil.

†Patient No. 14 is the same patient as No. 10 examined 9 mo later.

and two of lambda type (Table 1). The optical density obtained with the antibody to the opposite chain never exceeded that obtained with the labeled normal IgG; this provided further evidence of the monospecificity of the anti-L chain preparations. The CLL lymphocytes were so faintly labeled that for light microscopy it was necessary to use semithick sections to count the positive cells. Since this procedure was not satisfactory for routine use, we modified the technique by incubating the aliquot of cells to be counted at 37°C to concentrate the surface L chain determinants at one pole of the cell.[22] The number of positive cells counted either on semithick sections or in cell suspension being approximately the same, the second procedure was subsequently used exclusively.

The results of light microscopic observations are in agreement with the spectrophotometric data. Twelve patients had positive cells with anti-kappa and two with anti-lambda antibodies. The number of stained cells varied from 50% to 70%, except for the two treated patients (Nos. 4 and 5) who had 22.6% and 35%, respectively, and case No. 15, who had 96% positive lymphocytes. Cells which reacted with the other anti-light chain antibody or the normal IgG never constituted more than 2% of the total number of stained cells. Stained polymorphonuclear cells or monocytes were occasionally seen, but they were easily distinguishable from lymphocytes by a very abundant and topographically different labeling.

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Lymphocytes from the normal controls reacted with the two classes of antibodies: 16%–25% of lymphocytes were stained with anti-kappa and 4%–10% with anti-lambda antibodies (Table 1). The average number of antibody molecules bound per positive CLL cell (if No. 15 was excluded) ranged from 3000 to 21,400 (mean value 9000), whereas there were 86,000–185,000 (mean value 93,700) kappa antigenic sites and 32,500–190,000 (mean value 106,000) lambda antigenic sites on the surface of a normal lymphocyte. The results obtained in one patient (experiment 10) were virtually the same 9 mo later, when the study was repeated (experiment 14). Case No. 15 possessed the highest number of positive cells, having 75×10^3 antigenic sites on the surface of each lymphocyte, and was distinctly atypical of the CLL patients as a group. In order to exclude the possibility that an apparent low density of L chain antigenic determinants on CLL lymphocytes resulted from the presence of a constituent on the leukemic cell capable of inactivating or reducing the activity of peroxidase, the following experiment was performed. Equal quantities of peroxidase were incubated with equal numbers of CLL or normal lymphocytes. After 2 hr, or 24 hr, the amount of peroxidase activity present in each mixture was determined spectrophotometricly. No difference was detected in the activity of peroxidase incubated with CLL or normal cells.

DISCUSSION

Our results confirm earlier findings. First, L chain determinants are present on the membrane of CLL lymphocytes; second, only one class of L chain is found, and third, the number of antigenic sites is reduced as compared to normal lymphocytes.

All the 14 leukemic patients possessed lymphocytes bearing either kappa or lambda determinants, but the two chains were never found together in the same patient. The majority of cells were positive in all cases, although some were faintly stained. All positive cells had a similar appearance in the light microscope; investigations are in progress to compare labeled lymphocytes in CLL and normal donors at the electron microscopic level.

The second and new finding is that the number of antigenic sites is about tenfold lower on the surface of CLL cells than on normal lymphocytes. The one case which possessed a number of lambda determinants similar to a normal control is atypical.[23] This patient is analogous to the case T.P. described by Klein et al., whose cell reactivity with fluorescent anti-mu and anti-kappa antibodies was very marked.[7,13]

The results do not show any correlation between the number of positive cells, the quantity of antigenic sites found on each stained cell, and the leukocyte count. Chlorambucil treatment decreased lymphocyte counts and the number of positive cells, but did not affect the monoclonal aspect of the disease, since the other chain determinant never appeared.

There is convincing evidence that this method is a useful tool for the diagnosis of CLL. The criteria must be (1) reactivity with labeled antibody directed against only one light chain type, (2) labeling of the majority of the lymphocyte population, and (3) number of antigenic determinants dramaticly lower than that of the normal lymphocyte.

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The notable diminution of membrane-associated light chain determinants of CLL lymphocytes is to be compared with the decreased number of binding sites for concanavalin A,[24] to the unresponsiveness to phytohemagglutinin and pokeweed mitogen, and the absence of activity in mixed lymphocytes culture.[25] The average number of antigenic sites can be calculated to be about 10% that of a normal lymphocyte. The findings cannot be solely attributed to a reduction of size of the CLL lymphocyte, since it can be calculated from published data[26] that its mean surface area is approximately 80% that of a normal lymphocyte. The difference may reflect, then, a structural difference of the membrane. It is possible that the CLL lymphocyte has been arrested at an immature stage in which the membrane possesses fewer of its components,[27,28] or that it is unable to continue its maturation due to the absence of factor(s) possibly essential or important for further differentiation.

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