IDENTIFICATION WITH A MONOCLONAL ANTIBODY OF A PREDOMINANTLY B LYMPHOCYTE-SPECIFIC DETERMINANT OF THE HUMAN LEUKOCYTE COMMON ANTIGEN

Evidence for Structural and Possible Functional Diversity of the Human Leukocyte Common Molecule*

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The leukocyte common (LC)[1] antigen, originally described in the rat using conventional absorbed rabbit anti-rat lymphocyte sera (1), and later in the rat (2) and mouse (where it was termed T200) (3) using monoclonal antibodies, is a well characterized and major glycoprotein of the rodent lymphocyte surface. It is widely distributed among leukocytes, being found on T and B lymphocytes, thymocytes, macrophages, and granulocytes, but is absent from other tissues (1–3). An interesting feature of this molecule is that its mobility on sodium dodecyl sulfate (SDS) gels varies, depending on the cell type being studied. Thus, the LC antigens of rat thymocytes, T lymphocytes, and B lymphocytes migrate on SDS gels with apparent mol wt of 150,000, 170,000, and 200,000, respectively (2); whereas in the mouse, LC antigens from T and B lymphocytes have mol wt of 190,000 and 220,000, respectively (3). These differences in mobility of the molecule on functionally different cell populations are extremely interesting, and the structural basis for the differences are at the moment completely unknown.

We have recently characterized the human homologue of the rodent LC antigen (4) with a monoclonal antibody. The human LC antigen is very similar to the rodent homologue, being found on T and B lymphocytes, thymocytes, monocytes, and granulocytes, but absent from other tissues; and it is a major, lentil-lectin-binding glycoprotein of the human lymphocyte. When purified from blood lymphocytes, two peaks are seen, one at 190,000 and the other at 215,000 mol wt, which are probably the LC antigen from T and B lymphocytes, respectively (4).

In this paper we demonstrate that in addition to the determinant common to all

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter; LC, leukocyte common; LN, lymph node(s); PAGE, polyacrylamide gel electrophoresis; PBL, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; RAH, immunoadsorbent-purified rabbit $F(ab')_2$ anti-human $F(ab')_2$; RAM, immunoadsorbent-purified rabbit $F(ab')_2$ anti-mouse $F(ab')_2$; RBC, erythrocyte(s); SDS, sodium dodecyl sulphate.

human LC molecules, a subset of LC molecules express a new determinant, and that this new determinant is selectively expressed principally on the LC molecules of B lymphocytes. This selective expression, on a functionally discrete population of lymphocytes, of a serologically identifiable conformational or other structural change has interesting implications for the function(s) of the LC molecules. The data also forcefully illustrate how the target of monoclonal antibodies must be considered at the determinant rather than the molecular level.

Materials and Methods

Cell Suspensions. Except for the target cells in the binding assay, all cell suspensions were prepared fresh immediately before use, and for most experiments were in 0.5% bovine serum albumin (BSA) (Miles Laboratories, Stoke Poges, Slough, England) in phosphate-buffered saline (PBS) (Dulbecco's A and B; Oxoid Ltd., London). Spleen was taken from splenectomy cases for trauma or from cadaver kidney donors, and cervical lymph nodes were taken at surgery from patients without systemic illness. Thymus was taken as 1-cm^3 biopsies from prepubertal patients at open heart surgery. Bone marrow came from ribs removed at thoracotomy. Granulocytes were prepared from EDTA anti-coagulated blood as described by Böyum (5), and peripheral blood mononuclear cells (PBL) were prepared from defibrinated blood by centrifuging over Lymphoprep (Nyegaard, Oslo, Norway). Rat lymph nodes were a mixture of cervical and mesenteric nodes obtained from freshly exsanguinated DA rats (Bantin and Kingman Ltd., Aldbrough, Hull, Yorkshire, England). Contaminating erythrocytes (RBC) were removed from all cell suspensions except bone marrow by incubating with Tris-buffered ammonium chloride (6). Viability was assessed by trypan blue exclusion and was almost always >80%. Relatively pure RBC suspensions were obtained from heparinized blood by washing three times and removing supernate, buffy coat, and upper RBC layer after each wash. Cell counts were performed by using a model DN Coulter Counter (Coulter Electronics, London). Pure suspensions of platelets were prepared by removing leukocytes and RBC from EDTA anti-coagulated blood by two sequential spins at 200 *g* for 20 min. Platelets were washed twice by centrifugation and counted under phase-contrast microscopy on a hemocytometer.

The targets in the binding assays were human spleen cells fixed for 5 min in 0.25% glutaraldehyde and stored at −40°C in 5% BSA/PBS (7).

Tissue Homogenates. Liver, heart, and kidney were obtained fresh from cadaver kidney donors and a piece of cerebrum was obtained fresh from the Neurosurgery Unit, John Radcliffe Hospital, Oxford, England. The capsule of the organ to be homogenized was removed and the tissue minced in PBS using mechanically driven blades, followed by mechanical homogenization with a Teflon pestle and then manual ground-glass homogenization. Large particles were removed by centrifuging at 40 *g* for 1 min. The homogenate was washed twice by centrifuging at 35,000 *g* for 20 min. The second pellet was resuspended by manual ground-glass homogenization, aliquoted, and stored at −40°C. All procedures were carried out at 4°C or on ice. The protein content of the homogenates was estimated by the method of Lowry et al. (8) using BSA as a standard.

Antibodies. Immunoadsorbent-purified rabbit $F(ab')_2$ anti-mouse $F(ab')_2$ (RAM) and rabbit $F(ab')_2$ anti-human $F(ab')_2$ (RAH) were prepared as previously described for other immunoadsorbent-purified anti-immunoglobulin sera (9). The pepsin degradation of the mouse and human immunoglobulins was carried out at pH of 4.1 and 4.5, respectively.

The antibodies were iodinated using the chloramine T method and fluorescein labeled using bicarbonate buffer, pH 9.5, for 3 h at room temperature, as previously described in detail (10).

With the fluorescence-activated cell sorter (B-D FACS Systems, Mountain View, Calif.), cross-reactivity between mouse and human immunoglobulins resulted in unacceptably high backgrounds where B lymphocytes were present in the cell population under study. This was overcome by passing the fluorescein-labeled RAM through a 10-ml Sepharose 4B (Pharmacia, Uppsalla, Sweden) column to which had been coupled 10 mg of human $F(ab')_2$/ml of gel.

Binding Assay. Binding assays were performed essentially as described by Morris and Williams (11). All procedures were at 4°C or on ice. Duplicate 25-μl samples of immune ascites or culture supernate to be assayed were transferred to LP3 tubes (Luckham Ltd., Burgess Hill,

Sussex, England). If the samples to be assayed had been used in absorption analysis, they were centrifuged at 6,000 *g* for 5 min immediately before assay. 25 μl of glutaraldehyde-fixed spleen cell targets at 10^8/ml were then added to each tube and this was incubated for 1 h on ice. The cells were then washed twice in 0.1% BSA/PBS and 100 μl of ^{125}I-labeled RAM in 0.5% BSA/PBS (~300,000 cpm/tube) was added to the pellet of the second wash. This was resuspended, incubated for a further 1 h on ice, and the cells washed twice as above. The pellet of the second wash was resuspended in 0.5 ml of PBS, transferred to fresh LP3 tubes, and the target cell-bound radioactivity measured in a Packard gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Absorption Analysis. Initial titrations of immune ascites or culture supernate were performed to choose a dilution that represented conditions of target antigen excess in the assay system. Absorptions were then performed at this dilution by incubating equal volumes (80 μl) of antibody sample at the chosen dilution with tripling dilutions of homogenates or cell suspensions in 0.5% BSA/PBS. Again, all procedures were at 4°C or on ice. Absorbing tissue was removed by centrifugation and absorbed samples stored at −40°C until all the absorptions for a particular analysis had been completed.

For the absorptions, homogenates were used at starting concentrations as prepared above: i.e., 50% solid tissue, which corresponded to 25–35 mg homogenate protein/ml. Alignment of absorption curves for different homogenates was done on the basis of protein content. Cell suspensions were used at starting concentrations of 10^9/ml.

Production of Monoclonal Antibodies. This was performed essentially as previously described by Köhler and Milstein (12), and the details of the technique used in this laboratory have been published previously (4).

Mice were immunized with fresh human lymph node lymphocytes depleted of surface-immunoglobulin-positive cells. The depletion was done by rosetting the lymph node lymphocytes with human RBC to which RAH had been coupled using the $CrCl_3$ method (13) and then centrifuging over Lymphoprep of specific gravity 1.077. BALB/c mice were then injected intravenously with 10^7 cells on three occasions, the third injection being 5 d before the fusion.

Fusions were carried out with the myeloma P3-NS1/1Ag 4.1 (NS-1), kindly provided by Dr. C. Milstein (MRC Laboratory of Molecular Biology, Cambridge, England). Immunized mice were killed by cervical dislocation and a single cell suspension prepared from their spleens. This was mixed with NS-1 cells in the ratio of 10 nucleated spleen cells:1 myeloma cell, and the cells fused using polyethylene glycol. Hybrids were selected in hypoxanthine-aminopterin-thymidine medium. The culture conditions, cloning procedures by dilution, and production of immune ascites were by standard techniques (4).

For partial purification of the monoclonal antibody, ascites was pooled, dialyzed against 0.15 M NaCl, 0.025 M Tris, 0.02%, sodium azide, pH 8.5, at 4°C, and passed through DEAE-CL 6B (Pharmacia) previously equilibriated with the above buffer. The monoclonal antibody activity passed unretarded through the column, and all antibody activity was recovered with ~15% of the ascites protein.

Monoclonal Antibody Affinity Chromatography. Pilot experiments titrating antigen activity of different detergent extracts of spleen homogenate established that Brij 96 (Sigma Chemical Co., London) gave the best results. Extracts with 2% Brij 96 contained the antigen in good yield (~50%) and antigen activity remained in the supernate after centrifugation at 85,000 *g* for 75 min. As previously described, 2% Brij 96 also solubilized the LC antigen (4). Bulk extraction of antigen from spleen homogenate was done by incubating the homogenate at a concentration of 10% solid tissue in 2% Brij 96 in 0.15 M NaCl, 0.025 M Tris, 0.02% sodium azide, pH 7.5, at 5°C for 1 h on ice, and centrifuging at 1,500 *g* for 15 min to remove nuclei.

In some experiments ^{3}H-labeled antigen was prepared from lymph node lymphocytes. For these studies, lymph node lymphocytes depleted of RBC were labeled with $NaB[^3H_4]$ (14). Briefly, 1×10^8–2×10^8 lymphocytes were incubated on ice for 5 min at 2.5×10^7/ml in 1 mM $NaIO_4$ in PBS. The reaction was stopped by adding 0.1 volume of 0.1 M glycerol, and the cells were washed three times in PBS at 4°C. They were resuspended to 1×10^8/ml in PBS, and 1 mCi (40 μl) of $NaB[^3H_4]$ (Radiochemical Centre, Amersham, England) in 0.01 M NaOH was added per 10^8 lymphocytes. This was incubated for 30 min at room temperature, and the cells washed three times at 4°C. After the third wash, the cells were resuspended at 10^8/ml in 2%

Brij 96 in PBS that contained 2.5 mM iodoacetamide and 2 mM phenylmethylsulphonyl fluoride for inhibition of proteolysis (all chemicals from Sigma Chemical Co.). After 30 min on ice, this was centrifuged at 1,500 *g* for 15 min to remove nuclei. More than 90% of the counts were recovered in the supernate, which was immediately frozen at −80°C. Approximately 1.5–2.0 × 10^6 cpm were recovered per 10^8 starting cells.

Monoclonal antibody affinity columns were prepared by coupling the partially purified monoclonal antibody to cyanogen bromide-activated Sepharose 4B (Pharmacia) essentially as recommended by the manufacturers. Coupling was done at a ratio of 5 mg protein:1 ml of swollen gel. The columns consisted of 0.7 ml of gel in 2-ml glass syringes. 10 ml Brij extracts of spleen homogenate or 1 ml Brij extracts of ^{3}H-lymph node lymphocytes (containing ~10^6 cpm) was passed through the columns at 5 and 2 ml/h, respectively. The columns were then washed with 10–20 ml of 0.5% Brij 96 in 0.15 M NaCl, 0.025 M Tris, 0.02% NaN_3, pH 7.5, at 5°C. Unlabeled antigen was eluted with 0.05 M diethylamine, 0.5% Brij 96, pH 11.5, at 5°C, and eluted fractions were immediately neutralized with solid glycine. With this system, all antigen activity was recovered in the elution volume of 0.3–1 ml. With the ^{3}H-labeled samples, better elution was obtained by removing the beads from the column, adding an equal volume of 4% SDS, and placing in a boiling water bath for 2 min.

Polyacrylamide Gel Electrophoresis (PAGE) in SDS. The technique for PAGE-SDS was essentially as described by Laemmli (15), using 3-mm thick 5% polyacrylamide analytical gels with 3% polyacrylamide stacking gels (Bio-Rad Laboratories, Richmond, Calif.). At the completion of electrophoresis, the portion of the gel containing the molecular weight standards (Bio-Rad Laboratories) was cut from the main part of the gel and stained with Coomassie blue. The remainder of the gel was cut into strips corresponding to the samples analyzed, and fixed overnight in methanol/acetic acid. 2-mm thick slices were then cut, placed into counting vials, and 0.6 ml of nuclear Chicago solubilizer (NCS; Hopkins and Williams, Romford, Essex, England) was added. This was incubated at 50°C for 2 h and then overnight at room temperature. 2 ml of scintillant was then added, and the samples counted in a Packard scintillation counter.

Fluorescence-activated Cell Sorter (FACS). Immune ascites was always used for FACS analysis and a dilution was chosen which represented conditions of antibody excess. This dilution was chosen by performing saturating binding assays, where the second incubation of the assay contained 25 μg/ml of unlabeled RAM in addition to the ^{125}I-RAM (11). Cells were prepared in binding assays as described above; the second incubation was with fluorescein-labeled RAM or RAH at the saturating concentration (11) of 25 μg/ml, and in some experiments, both RAM and RAH together. As a control monoclonal antibody, a monoclonal anti-dog Thy-1 (16) was used. The control for RAH was RAH blocked with 250 μg/ml of human $F(ab')_2$ for 1 h at 4°C.

Results

Tissue Distribution of the F8-11-13 Determinant

QUANTITATIVE ABSORPTION ANALYSIS. The results of absorptions with various tissue homogenates are given in Fig. 1, and it is clear that kidney, liver, heart, and brain give little or no absorption, so the determinant recognized by the F8-11-13 antibody is specific to leukocytes. Fig. 2 shows that single cell suspensions of spleen and lymph node lymphocytes have large and approximately equal amounts of F8-11-13, whereas bone marrow and thymus have ~30 and 10% as much, respectively, as spleen and lymph node. Granulocytes, as well as platelets, erythrocytes, and serum, do not absorb the antibody. The major difference with previous studies of the LC antigen is that granulocytes gave excellent absorption for the LC antigen (4).

FACS ANALYSIS. To better characterize the F8-11-13 determinant in leukocyte populations, thymocytes, bone marrow cells, granulocytes, PBL, and lymph node lymphocytes, each from several individuals, were studied on the FACS. The results of typical FACS analyses with each tissue are given in Fig. 3, and the percentage of F8-

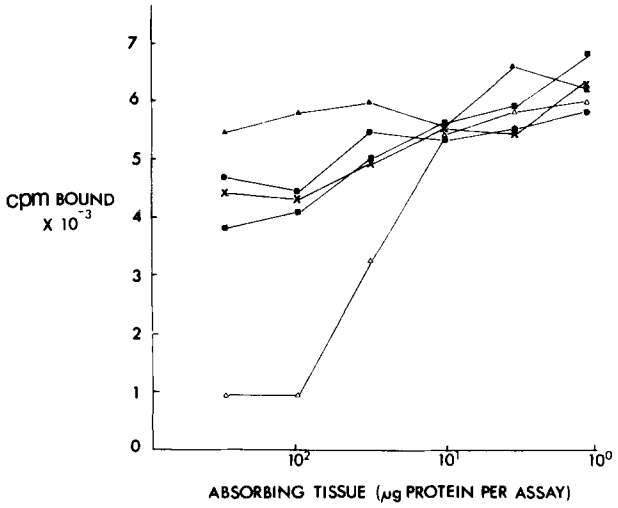

FIG. 1. Quantitative absorption analysis of F8-11-13-immune ascites. Immune ascites at a 1:5,000 dilution was absorbed with tripling dilutions of homogenates of spleen, △; liver, ×; heart, ▲; brain, ●; and kidney, ■. The residual antibody was assayed for binding to glutaraldehyde-fixed spleen cells using ^{125}I-labeled RAM.

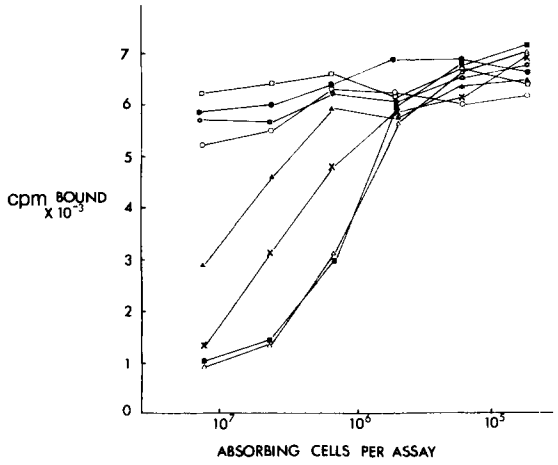

FIG. 2. Quantitative absorption analysis of F8-11-13-immune ascites. Immune ascites at a 1:5,000 dilution was absorbed with tripling dilutions of normal serum, □, and the following cell suspensions: spleen, △; lymph node, ■; bone marrow, ×; thymus, ▲; granulocytes, ○; erythrocytes, ☆; and platelets, ●. The starting concentration of cells was 10^9/ml except for platelets, which were used at an initial concentration of 6×10^9/ml and plotted such that six platelets were equivalent to one lymphocyte. Normal serum was arbitrarily plotted such that undiluted serum was equivalent to cells at 10^9/ml. Residual antibody was assayed as in Fig. 1.

11-13-positive cells in the tissues studied is given in Table I. It should be noted that with the F10-89-4 antibody, which is directed at the common determinant of the human LC antigen, 100% of thymocytes, granulocytes, PBL, and lymph node (LN) lymphocytes, and 83% of bone marrow cells are positive (4). With the F8-11-13 antibody, only a small proportion of thymocytes was positive, and even then only weakly so (Fig. 3 a), whereas granulocytes were completely negative. With PBL and

LN lymphocytes, only ~50% of the cells were F8-11-13 positive. PBL had a broad, positive peak and appeared to be the most strongly labeled cell population studied (Fig. 3 d), whereas LN lymphocytes had a strongly staining peak, with a group of more weakly staining cells to the left of this main peak (Fig. 3 e).

Given that subpopulations of PBL and LN lymphocytes were being labeled, an obvious question was to ask was whether or not the F8-11-13-positive cells could be designated as being in the B or T lymphocyte lineage. Double-labeling experiments were therefore performed on LN lymphocytes using the F8-11-13 antibody and fluorescein-labeled RAM in combination with fluorescein-labeled RAH to label the B lymphocytes. The results are given in Table II, and show that most, but not all, of the B lymphocytes (defined as immunoglobulin-positive cells) are F8-11-13 positive. Most of the surface-immunoglobulin-negative (presumably T) lymphocytes were F8-11-13 negative, and in two of the three persons studied, the number of F8-11-13-positive T lymphocytes was very small.

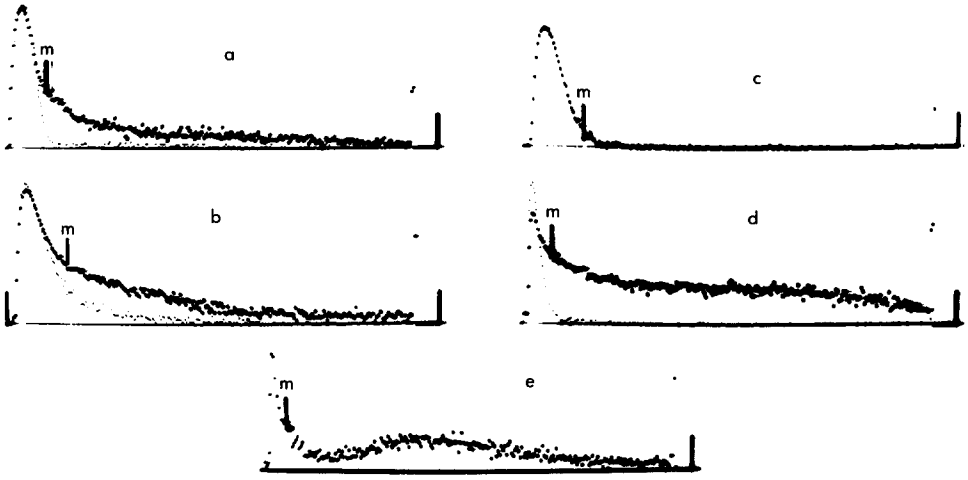

FIG. 3. FACS analysis of F8-11-13-immune ascites on various cell populations. Immune ascites was used at a 1:500 dilution and tested on (a) thymus; (b) bone marrow; (c) granulocytes; (d) PBL; and (e) LN lymphocytes. In (a), (b), and (d) the faint profile to the left represents the fluorescence profile with an irrelevant monoclonal antibody. The marker, m, represents the channel beyond which 0.5, 1.9, 0.7, 0.7, and 0.9% of cells in (a), (b), (c), (d), and (e), respectively, were labeled with an irrelevant monoclonal antibody.

TABLE I

FACS Analysis of Various Cell Populations with the F8-11-13 Monoclonal Antibody

Cell type	(Percentage) F8-11-13-positive cells*	Mean ± SD
Thymocytes	4.8, 5.9, 6.0, 7.6	6.1 ± 1.2
Bone marrow cells	9.9,‡ 19.4, 25.7, 32.9	22.0 ± 9.8
Granulocytes	0, 0	
PBL	37.0, 54.7, 58.4, 60.0	52.5 ± 10.6
LN lymphocytes	36.5, 48.9, 58.5	48.0 ± 11.0

* Each figure represents a different individual.

‡ This sample of bone marrow came from an adult, and the other three from children.

TABLE II

Double Labeling Experiments to Determine by FACS Analysis the Overlap between F8-11-13-positive and Surface-Immunoglobulin-positive LN Lymphocytes

Individual number	Percentage LN cells positive with F8-11-13 and fluorescein-labeled RAM*	Percentage LN cells positive with fluorescein-labeled RAH‡	Percentage LN cells positive using combination of F8-11-13, and fluorescein-labeled RAM and RAH§	Percentage of B lymphocytes‖ which are F8-11-13 positive	Percentage of T lymphocytes‖ which are F8-11-13 positive
1	36.5	28.7	37.3	97.2	21.1
2	48.9	45.1	52.6	91.8	13.7
3	58.5	37.2	63.5	86.8	41.9

* First incubation with F8-11-13 antibody, second incubation with fluorescein-labeled RAM.

‡ First incubation with medium, second incubation with fluorescein-labeled RAH.

§ First incubation with F8-11-13 antibody, second incubation with a mixture of fluorescein-labeled RAM and RAH.

‖ B and T lymphocytes of lymph node defined as surface-immunoglobulin-positive and -negative cells, respectively.

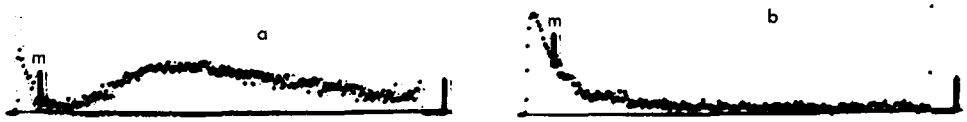

FIG. 4. FACS analysis with F8-11-13-immune ascites on separated T and B lymphocytes from LN. Immune ascites was used at a 1:500 dilution and tested on (a) purified B lymphocytes and (b) purified T lymphocytes from LN. LN lymphocytes from the same individual were divided into two aliquots, and B cells prepared from one aliquot by rosetting with papainized sheep RBC and T cells prepared from the other aliquot by rosetting with human RBC to which RAH had been coupled, as outlined in Materials and Methods. The purity of the B and T cells was checked by FACS analysis using fluorescein-labeled RAH, and the B and T cell populations were found to contain of 91 and 6% Ig-positive cells, respectively. The marker m, represents in (a) the point beyond which 93% of the cells were positive with the F8-11-13 antibody and 0.9% were positive with an irrelevant monoclonal antibody and in (b), the point beyond which 33% of the cells were positive with the F8-11-13 antibody and 0.6% were positive with the irrelevant monoclonal antibody.

Two lines of evidence suggest that B lymphocytes express quantitatively much more of F8-11-13 determinant than T lymphocytes. First, the T and B lymphocytes from the LN of individual 2 of Table II were separated and studied for the expression of the F8-11-13 determinant on FACS analysis, and the results are given in Fig. 4. Fig. 4b shows that the F8-11-13-positive T lymphocytes express the determinant only weakly, much like thymus cells (Fig. 3a). The B lymphocytes, however (Fig. 4a), express the determinant strongly. The second line of evidence is that fluorescence studies (performed as in [4]) on frozen sections of LN from three different persons show that the F8-11-13 antibody stains predominantly the lymphatic nodules and germinal centers. It is interesting that with the monoclonal antibody (F10-89-4) detecting the determinant common to all LC molecules, we have shown that T cells stain more strongly than B cells (4).

Thus the F8-11-13 determinant is not entirely restricted to the B lymphocyte lineage, but most B and only some T cells express it, and B cell express much more of the determinant than do the F8-11-13-positive T cells. The relatively strong staining of PBL with the F8-11-13 antibody (Fig. 3d) raises the possibility that monocytes

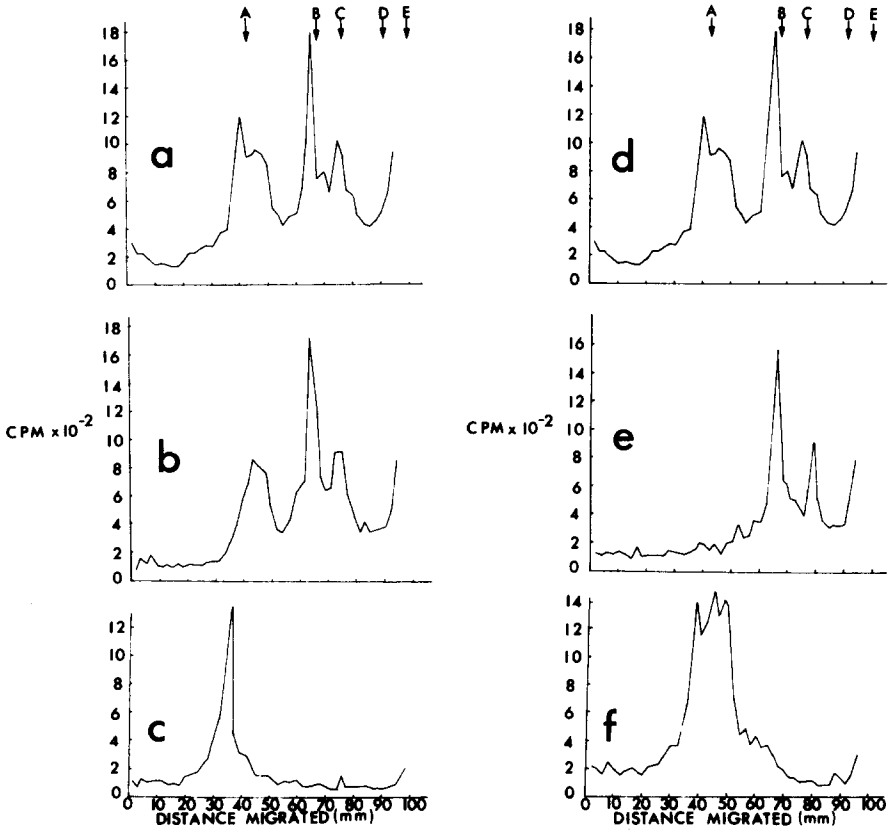

FIG. 5. SDS-PAGE analysis of ^{3}H-labeled membrane sialoglycoproteins. LN lymphocytes were treated with periodate, labeled with NaB[^{3}H$_4$], and solubilized with Brij 96 as outlined in Materials and Methods. Samples were run on 5% polyacrylamide gels in SDS, and the gels were sliced, and the radioactivity measured in each slice. (a–c) experiments with F8-11-13 monoclonal antibody columns; (d–f) experiments with F10-89-4 (anti-LC [4]) monoclonal antibody columns. (a) and (d) represent the solubilized membrane before passage through the affinity columns; (b) and (e) represent the solubilized membrane after passage through the affinity columns; and (c) and (f) represent the material that was eluted from the columns. The number of cpm added to the gels for (a), (b), and (c) were 1.8×10^5, 8×10^4, and 1.4×10^4, respectively, whereas for (d), (e), and (f) the cpm added were 1.8×10^5, 6.4×10^4, and 2.1×10^4, respectively. Marker proteins were (molecular weights in parentheses): A, myosin (200,000); B, β galactosidase (130,000); C, phosphorylase B (94,000); D, BSA (68,000); E, ovalbumin (43,000); and F, dye front.

and/or null cells might also express the determinant strongly, and this is currently under study.

Biochemical Characterization of F8-11-13-bearing Molecule. ^{3}H-labeled, detergent-solubilized LN lymphocyte membranes were passed through F8-11-13 monoclonal antibody affinity columns and the bound antigen eluted. The starting membrane, the membrane depleted of F8-11-13, and the eluted F8-11-13 antigen were analyzed on SDS-PAGE. For comparison, the same starting material was passed through a monoclonal antibody (F10-89-4) affinity column directed at the LC antigen, and the results are given in Fig. 5.

If one looks first at the results with the F8-11-13 column (Fig. 5 a–c), one can see that the starting membrane shows the three major peaks seen previously with ^{3}H-

labeled PBL (4). These are: (*a*) a broad band in the 170,000- to 220,000-mol wt region representing the LC antigen (4); (*b*) an unidentified large peak at 120,000 mol wt; and (*c*) the F10-44-2 antigen (possibly homologous to the W3/13 antigen of the rat [17]) at the 105,000-mol wt region (18). The first peak of the LC antigen at the 215,000-mol wt region is depleted by the F8-11-13 column (Fig. 5 b) and can be eluted from the column (Fig. 5 c). These data establish that the molecule bearing the F8-11-13 determinant is a glycoprotein of 215,000-mol wt, and strongly suggest that the F8-11-13 determinant is borne by the large molecular weight component of the LC antigen. This is reinforced by showing, in Fig. 5 d–f, the corresponding experiments with the monoclonal antibody F10-89-4 affinity column directed at the LC antigen (4). Strict specificity of the affinity columns was established by showing that another monoclonal antibody (F10-44-2) affinity column depletes the peak at 105,000 mol wt, leaving the LC peaks untouched (18).

Serological Proof That the F8-11-13 Determinant Is Borne by the LC Antigen. On the basis of the biochemical data presented in Fig. 5, we hypothesized that the LC antigen on all leukocytes bears a common determinant recognized by the F10-89-4 antibody (4) but that there is an additional determinant, restricted largely to B lymphocytes, which is recognized by the F8-11-13 antibody. If this hypothesis is correct, one would expect that the material purified from the F8-11-13 affinity column would inhibit both the F8-11-13 and F10-89-4 antibodies, but that solubilized spleen depleted of F8-11-13-bearing molecules should inhibit F10-89-4 and not F8-11-13 antibody. In addition, one would expect that the solubilized spleen depleted of F10-89-4 antigen would inhibit neither the F8-11-13 nor the F10-89-4 antibodies, and, conversely, that purified F10-89-4 antigen would inhibit both antibodies. As the F10-89-4 antigen could not be eluted in good yield without denaturation, this last possibility was not examined, but the other three were studied and the results are given in Fig. 6. Fig. 6 A shows that the F8-11-13 antibody was inhibited by the starting spleen extract and the purified F8-11-13 antigen, but not by the spleen extract depleted by passage through the F8-11-13 or F10-89-4 columns. Fig. 6 B shows that the F10-89-4 antibody was inhibited by the starting spleen extract, by the purified F8-11-13 antigen, and also by the extract depleted by passage through the F8-11-13 column, but not by the extract depleted by passage through the F10-89-4 column. Fig. 6 C shows control inhibitions of the F10-44-2 antibody (18) discussed above, and shows, as expected, that the starting spleen extract and the extracts depleted by passage through F10-89-4 and F8-11-13 columns did inhibit, and that the purified F8-11-13 antigen did not inhibit at all. It is clear, therefore, that the molecule bearing the F8-11-13 determinant also carried the F10-89-4 determinant, but that not all F10-89-4-bearing molecules carry F8-11-13.

Discussion

The data presented in this paper clearly indicate that the LC "antigen" or "molecule" should not be considered as a single, discrete entity, but as a family of structurally related molecules with possibly different functions. We show that, in addition to the antigenic determinant common to all LC molecules and previously defined by the monoclonal antibody F10-89-4 (4), the LC molecule of some—predominantly B—lymphocytes express an additional determinant defined by the monoclonal antibody F8-11-13. This selective expression of a serologically identifiable

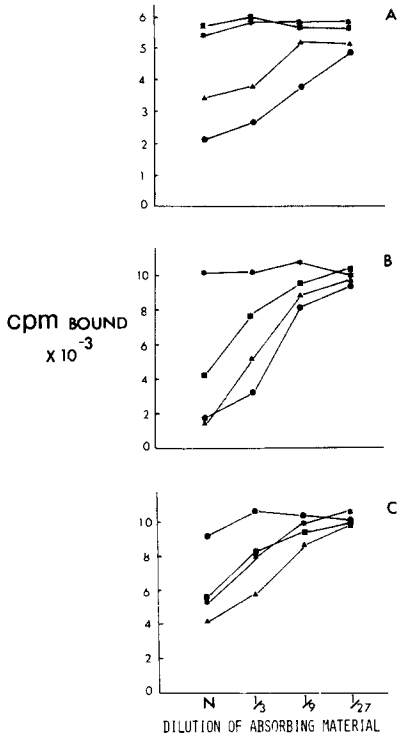

FIG. 6. Cross-inhibition studies using affinity-column-purified and -depleted spleen extracts. Inhibition studies were carried out with: A, F8-11-13 antibody; B, the monoclonal antibody F10-89-4 directed against the deteminant common to all LC molecules (4); and C, an irrelevant monoclonal antibody, F10-44-2 (18), reacting with another antigen in the extract of spleen. The inhibiting material consisted of Brij 96 extract of spleen, ▲; spleen extract after passage through the F8-11-13 affinity column, ■; purified F8-11-13 antigen, ●; and spleen extract after passage through the F10-89-4 column, ✱ . Purified F10-89-4 antigen was not used as it could not be eluted from the column without denaturation. The purified F8-11-13 antigen was concentrated threefold relative to the starting spleen extract, as it was eluted from the column in one-third the volume of spleen extract applied.

conformational or other structural change on the LC molecules of a functionally discrete lymphocyte population suggests that the conformational change detected might be of functional significance. For this reason, when considering possible functions for the LC molecule, one should not think exclusively of a single function likely to be relevant to all LC-bearing leukocytes, but should consider the possibility that the LC molecule of B lymphocytes, and, by inference, that of the other different LC-bearing leukocyte populations, might be different. Whether or not the antigenic determinant common to the LC molecules of all leukocyte populations (detected by the F10-89-4 antibody [4]) is of functional importance, either in its own right or in relation to the more restricted determinant described here, is a matter for speculation.

The elucidation of the structural basis for the conformational difference detected in the LC molecule from B lymphocytes will be very interesting. One possibility is that the polypeptide chains of all the LC molecules are identical and coded for by the same gene, and that the differences that arise are secondary to differences in the number, size, and/or composition of the carbohydrate side chains. These carbohydrate

differences could form new antigenic determinants themselves or lead to alterations in the folding of the polypeptide chain. It is interesting that differences have been noted in the carbohydrate composition of the rat Thy-1 molecule when brain and thymus Thy-1 were compared (19), but serologically identifiable differences between brain and thymic Thy-1 have not been found. In any case, if this explanation is correct, our data suggest that cellular differentiation and functional specialization can result not only as a result of the expression or not of a particular molecule, but in addition can be a result of secondary alterations in different cells of the same polypeptide chain.

However, other possibilities exist, such as gene duplication from an ancestral gene giving rise to a variety of different but related structural genes in a manner analogous to that seen with the K and D region genes of the H-2 complex, and each gene coding for the LC polypeptide chain in a different population of leukocytes. It is also possible that the LC molecule from B lymphocytes has a longer polypeptide chain and the new determinant could be located on this extra chain. Various genetic mechanisms could be invoked for this such as differences in splicing of essentially the same gene (20) or else separate but related genes with different numbers of domains, as occurs with the μ and γ chains of immunoglobulin (21).

It is unlikely that our data could be a result of masking of the F8-11-13 determinant on the F8-11-13-negative leukocyte populations. For example, the possibility that the F8-11-13 determinant is masked by other molecules on the membranes of F8-11-13-negative cells is excluded by the biochemical data. With the LC molecules free in solution, the F8-11-13 affinity columns interacted with only the high molecular weight (215,000) component (Fig. 5). In the rat (2) and mouse (3), it is the LC molecule from B lymphocytes that has the highest molecular weight, so the selective interaction of the F8-11-13 antibody on the one hand predominantly with B lymphocytes and on the other with the high molecular weight component of the LC molecules makes a coherent picture. Masking of the F8-11-13 determinant by carbohydrate side chains on the LC molecule itself is also unlikely. Of all the LC molecules, those from B lymphocytes have the highest apparent molecular weight on SDS-PAGE, and are therefore the ones that would be most heavily glycosylated. The absence of the F8-11-13 determinant on the less heavily glycosylated LC molecules is the reverse of what one would expect if glycosylation were a likely cause of masking.

The Ly-1 (22) and Ly-5 (23) lymphocyte alloantigen systems in the rat and mouse, respectively, have recently been shown to be allodeterminants on the LC molecule (24, 25). There has been uncertainty as to whether or not these allodeterminants are specific to T lymphocytes, or are only apparently so, because of the use of lymphocytotoxicity as the indicator system (24). Certainly, the Ly-5 alloantigen of the mouse has now clearly been shown to be present on both T and B lymphocytes (26), but our data raise the possibility of T or B lymphocyte specific allodeterminants on the LC molecule.

The finding that the F8-11-13 and F10-89-4 monoclonal antibodies, which interact with very different populations of leukocytes, are in fact detecting different determinants on the same molecule was unexpected. This result strongly demonstrates that it is essential, when comparing monoclonal antibodies, to think of the targets at the determinant rather than the molecular level. In the longer term, it will be interesting to see if this phenomenon of determinant differences (of possible functional signifi-

cance) on the same or similar molecules in different cell types is peculiar to the LC molecule or is also found with other membrane molecules.

Summary

Initial studies with the monoclonal antibody F8-11-13 described in this paper showed that it reacted strongly with B lymphocytes, did not react at all with granulocytes, and reacted only weakly with a small subpopulation of thymocytes and peripheral T lymphocytes. This picture was entirely different from that seen with monoclonal antibodies to the leukocyte common (LC) antigen, where 100% of all the above-mentioned leukocyte populations were positive. Biochemical studies using detergent solubilized membranes labeled with ^{3}H at the sialic acid residues showed that the molecule bearing the F8-11-13 determinant was a glycoprotein of 215,000 mol wt, and that the peak depleted by F8-11-13 monoclonal antibody affinity columns corresponded to the high molecular weight region of a broad peak previously shown to be completely depleted by monoclonal antibody (F10-89-4) affinity columns directed at the LC antigen. Proof that the F8-11-13 determinant was expressed on some LC molecules was established by cross-inhibition studies with affinity-column-purified and depleted material. This finding of a serologically identifiable conformational or other structural change selectively expressed on the LC molecule of a functionally discrete population of lymphocytes has interesting implications for the structure and function of the LC molecule, and might be relevant to functional considerations of other membrane molecules.

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