Comparative Density of the Human T-Cell Antigen T65 on Normal Peripheral Blood T Cells and Chronic Lymphocytic Leukemia Cells

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A 65,000 dalton T-cell specific antigen previously demonstrated to be present on the surface of normal and malignant T cells, but not normal B cells, has been detected on the surface of leukemic cells from patients with nonsecretory, surface immunoglobulin-positive chronic lymphocytic leukemia (CLL). By means of immunofluorescence and flow cytometry, the relative surface density of the T65 antigen on CLL cells was compared to that on normal peripheral blood T cells and human thymocytes, as well as cell lines of T-cell lineage. In all cases, the CLL cells had a more homogeneous and a lower median fluorescence intensity than that of normal circulating T cells. Thymocytes were composed of three populations, two with low surface density of T65 resembling the CLL cells and the other with higher density similar to normal T cells. The staining of cell lines varied from bright, heterogeneous staining (8402) to uniform, low-density staining (Molt-4). The implications of these findings with regard to lymphocyte differentiation are discussed.

CHRONIC lymphocytic leukemia usually is a proliferation of B lymphocytes bearing monoclonal surface immunoglobulins (slg), with the light chain in each case restricted to either κ or λ type.1,3 CLL cells were shown by immunofluorescence (IF) and cytofluorographic analysis4 to differ from normal B lymphocytes by the presence of significantly lower densities of slg. Recently, another antigen, T65, was demonstrated on nonsecretory CLL cells with the use of a previously described monoclonal antibody (MoAb) termed T101.3 In contrast, T65 was not found on normal B cells, B cell lines, or secretory CLL associated with a circulating M-protein, but was present on all normal circulating T lymphocytes, as well as greater than 95% of thymocytes. By IF microscopy it appeared that the density of the T65 surface antigen was lower on CLL cells than on normal peripheral blood T cells. In this study, we have confirmed and extended this finding with flow cytometry.

Histogram analyses of peripheral blood lymphocytes (PBL) from patients with CLL were performed and compared to those of normal donors. Utilizing dual parameter analysis, we also detected a relative size difference between CLL and normal T lymphocytes. For purposes of comparison, hematopoietic cell lines of T-cell lineage as well as human thymocytes were analyzed.

MATERIALS AND METHODS

Cells and Cell Lines

Heparinized peripheral blood samples were obtained from 10 patients with clinically diagnosed CLL and from 7 healthy adult donors. The four leukemic T-cell lines, CEM,6 6 Molt-4,6 6 HPB-ALL,6 and 8402,8 8 were used in this study, were propagated at 37°C in RPMI-1640 media supplemented with 10% fetal calf serum (FCS) without antibiotics. The cell lines have the characteristics of the original malignant cells.6 8 Two samples of human thymus were used in this study. Fetal thymus (a kind gift of Dr. S. Sarkar, UCSD, La Jolla, Calif.) was obtained from a fetus of 14–16 wk gestation following saline abortion. Child thymus tissue was obtained during corrective cardiac surgery performed on a 9-yr-old patient (a kind gift of Dr. R. Fox, Scripps Clinic and Research Foundation, La Jolla, Calif.).

Antisera

T101, a monoclonal mouse antibody belonging to the IgG2A subclass, specific for human T cells,11 was used to determine the presence of T65 antigen (65,000 dalton) on the cell surface (concentration 20μg/ml). A purified mouse myeloma protein of the same isotype (RPC-5, Litton Bionetics Laboratories, Kensington, Md.) was used as a negative control (concentration 20μg/ml). Fluorescein-conjugated rabbit anti-mouse IgG (FL-RAMIG, Miles Laboratories, Elkhart, Ind.) was used as a secondary antibody for indirect immunofluorescent staining.

Cell Preparation

Fresh heparinized venous blood (10 ml) was mixed with 3 ml of Plasmagel (Roger Bellon Laboratories, Neuilly, France) and incubated for 20 min at 37°C. The leukocyte layer was collected, centrifuged at 400 g for 7 min, resuspended, and then incubated with 3 ml of an iron filing solution (Lymphocyte Separator Reagent, Technicon Instruments Corp., Tarrytown, N.Y.) for 30 min at 37°C to remove phagocytic cells. This mixture was then layered over 5 ml of Ficoll-Hypaque (Pharmacia Inc., Piscataway, N.J.) and centrifuged for 40 min at 400 g. The lymphocyte layer was collected and washed. Cytophilic proteins were shed by incubation overnight in RPMI-1640 media supplemented with 10% fetal calf serum (10% FCS), 1% l-glutamine, and 1% penicillin-streptomycin (Grand Island Biological Co., Grand Island, N.Y.) at 37°C and 5% CO₂ humid atmosphere. Thymus tissue was placed in 10% FCS, finely minced into a single cell suspension, and viable mononuclear cells were recovered.

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Fig. 1. Fluorescence profiles (control versus positive) of peripheral blood lymphocytes from a normal donor (A), a patient with CLL (B), and normal thymocytes (C) stained with T101. The arrows designate the median intensity fluorescence values.

Fig. 2. Summary of median intensity fluorescence values for normal PBL, CLL cells, thymocytes, and cell lines.
separated by Ficoll-Hypaque centrifugation. All cell preparations, including cell lines, were washed 3 times prior to staining, and in all cases viability was greater than 95%.

**Immunofluorescent Staining**

Cells were resuspended in 1640-10% FCS + 0.02% sodium azide (1640-10% FCS-AZ) to a concentration of 5.0 x 10⁷ cells/ml. Two 25-μl aliquots of each sample were removed and stained with 50 μl each of TIOI (positive) and RPC-5 (control). After a 30-min incubation at 0°C, the samples were layered over 1 ml heat-inactivated FCS and centrifuged, washed with 1640-10% FCS-AZ, and resuspended in 25 μl 1640-10% FCS-AZ. Fifty microliters of secondary antibody, FL-RAMIG diluted 1:30, was added to each sample and incubated at 0° for 30 min. The cells were then passed through FCS and washed as above and finally resuspended in 2 ml of 1% formalin. Cells were analyzed on the same day as staining.

**Cyttofluorographic Analysis**

Analysis was carried out using an Ortho Cyttofluorograf 50H (Ortho Instruments, Westwood, Mass.). A detailed description of the system has been published. A 5 W argon laser source (488 nm) was used for fluorescence activation as well as generation of scatter signal as a measure of cell size. In each case 20,000 cells were analyzed. Cytograms were obtained by using dual parameter analysis, with fluorescence intensity on the horizontal axis and scatter on the vertical axis. Normal PBL scatter signals were located centrally for convenience. In addition, fluorescence profiles were presented as histograms of relative intensity over 510 channels (x-axis) versus number of cells in each channel (y-axis). The channel representing the median fluorescence intensity of each positive sample was determined. The percent of cells reactive with TIOI antibody was determined by subtracting the percent positive cells stained with the control from the percent positive cells stained with TIOI.

**RESULTS**

The immunofluorescence profiles of cells stained with TIOI are shown in Fig. 1 (the arrows point to the median fluorescence channels). Normal peripheral blood T cells (Fig. 1A) show a broad heterogeneous distribution. In this study, the range of TIOI cells in normal PBL was 60%-77%. In contrast, the distribution of TIOI cells in patients with CLL (Fig. 1B) shows a more homogeneous and decreased fluorescence intensity than do normal PBL. The range of TIOI cells in patients with nonsecretory CLL was 70%-90%. Thymocytes show two peaks of fluorescence intensity (Fig. 1C). The first peak was of low, uniform intensity (median channel 84), and the other was of heterogeneous higher intensity (median channel 332) staining. Greater than 95% of the thymocytes were positive for TIOI.

The median fluorescence intensity of each case studied is shown in Fig. 2. All CLL cases had a lower value than did normal PBL, while the values for cell lines varied widely. The median value for the less intense thymocyte subpopulation was comparable to that for CLL, whereas the more intense thymocyte subpopulation was comparable to the normal T cells in PBL. Seventy-five percent of the TIOI thymocytes fall in the first peak.

Cytograms displaying scatter (vertical) versus fluorescence intensity (horizontal) are presented in Fig. 3. Although the degree of light scatter does not directly coincide with the cell size, a positive correlation has been found between relative size measured by light scatter and sedimentation at unit gravity. In all cases tested, the CLL cells had a greater degree of light scatter than normal PBL and tended to be more broadly distributed. The cytogram for thymocytes revealed that the low fluorescence intensity peak consisted of two distinct size populations. The higher fluorescence intensity peak consisted of a single population of cells midway between the other two in relative size.

We also examined two cases of CLL shown to be TIOI (Fig. 4). Analysis of the PBL from the first case (Fig. 4 A and B), considered to be of non-T, non-B origin (TIOI, slg), revealed no TIOI cells above background control. Analysis of the second case (Fig. 4 C and D), which was associated with a circulating M-protein of IgMs, revealed a small population (4.8%) of TIOI PBL with a fluorescence distribution expected for normal T cells.

**DISCUSSION**

A 65,000 dalton T-cell-specific antigen termed T65 has been shown by Royston et al. to be present on slg cells of patients with nonsecretory (absence of circulating M-protein) CLL. We have compared by cyttofluorographic analysis using a monoclonal antibody, termed T101, the relative density of T65 on the surface of normal peripheral blood T lymphocytes and cells from patients with CLL. For further comparison, four cell lines of T-cell lineage and normal human thymocytes were examined. The CLL cells, in all cases studied, had lower surface density of T65 and were of a more homogeneous nature than normal circulating T lymphocytes. The cell lines varied from high-density heterogeneous staining of 8402 to the low-density uniform staining of Molt-4.

Investigators have previously suggested that CLL cells represent immature cells arrested early in B-cell differentiation, manifested in part by the faint immunofluorescent staining of slg on the CLL cells compared to normal B cells. The presence of T65 on nonsecretory CLL cells, also in decreased density, provides further evidence in support of this hypothesis and may indicate arrest at an even earlier stage of lymphocyte differentiation.

The absence of T101 reactivity with abnormal cells from other B-cell proliferative diseases is consistent
with the hypothesis that these other B-cell malignancies probably represent proliferations of more differentiated B cells.\textsuperscript{15,16} Based on this reasoning, Royston et al.\textsuperscript{5} postulate that any CLL that shows evidence of further B-cell differentiation, such as an association with a serum M-protein, will not carry the T65 antigen. Consistent with this hypothesis was patient LK (Fig. 4 C and D), who has "CLL" associated with serum monoclonal IgM (Waldenstrom's macroglobulinemia). His leukemic cells stained brightly for IgM (not shown) and had less than 5% cells reacting with T101, which resembled normal T cells in their fluorescence profile.

Other investigators have confirmed the binding of monoclonal anti-human T-cell antibodies to CLL cells. Wang et al.\textsuperscript{17} have described a p69,71 antigen (Leu 1), detected by immunofluorescence, on 80%--95% of normal human T cells, which form rosettes with sheep erythrocytes (E), and on slg' CLL cells from 11 of 14 patients. Boumsell et al.\textsuperscript{18} have described a monoclonal antibody, termed A50, which reacts by complement-mediated cytotoxicity with 70%--90% of E PBL and with greater than 70% of slg' CLL cells from 17 of 39 patients. The relationship of monoclonal antibodies T101, A50, and anti-Leu 1 await further study.

Recently, Reinherz et al.\textsuperscript{19} have postulated an intrathymic differentiation scheme based on immunofluorescent staining with a series of monoclonal anti-human T-cell subset antibodies. Their studies suggest that
null cell CLL

three major compartments of thymic differentiation exist in humans. By virtue of the fact that T101 reacts with greater than 95% of thymocytes, we feel that T65 is present on all thymus cells in varying amounts regardless of the stage of differentiation. Furthermore, based on our own hypothesis that cells with less surface T65 are arrested at an earlier stage of differentiation, Molt-4 may be considered to be less differentiated than the other T-cell lines examined (Fig. 2). This notion is consistent with the findings of Reinherz et al. that Molt-4 expresses fewer stage II differentiation antigens than CEM.

Our studies showed three size populations of thymocytes, the majority being small and large cells with uniform, low-density T65 and a third population of medium sized cells with higher, more heterogeneous staining, resembling circulating T cells. Similar subgroups of mouse thymocytes have been demonstrated by Fathman et al. using light scatter and fluorescence analysis with anti-Thy-1.2. The majority of mouse thymocytes are small (90%) and large (5%) cells with high and intermediate levels of Thy-1.2, respectively. The third population of medium sized cells (5%) has low Thy-1.2 surface density. Weissman et al. have further characterized the mouse thymocytes and shown that the small and large cells with high Thy-1.2 are derived from the cortex and have low surface H-2, while the medium sized cells are medullary thymic lymphocytes and have the same phenotype (low Thy-1.2, high H-2) as peripheral T cells. We propose that the weaker staining populations in the human thymus may also represent cortical thymocytes and are in early stages of differentiation, while the more mature thymocytes, perhaps medullary thymocytes, make up the brighter population. Studies are now underway to both localize these subpopulations on frozen sections of thymus and sort them for functional studies.

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