Chronic Lymphatic Leukemia (CLL): Cell Surface Changes Detected by Lectin Binding and Their Relation to Altered Glycosyltransferase Activity

By Stephen F. Speckart, David H. Boldt, and Richard P. MacDermott

Binding studies with five purified plant lectins were used to investigate membrane alterations in lymphocytes from patients with CLL. Compared to normal human B lymphocytes, CLL lymphocytes had fewer receptors for E-phytohemagglutinin, wheat germ agglutinin, and concanavalin A, and more receptors for L-phytohemagglutinin. Receptors for Ricinus communis agglutinin were the same on both normal and CLL cells. Since the lectins bind to complex carbohydrates on the cell surface, these data suggested that the carbohydrate composition of CLL membranes differed from that of normal lymphocytes. To investigate this point, exposed membrane sialoglycoproteins on intact cells were radiolabeled by a combination of mild periodate oxidation followed by reduction with NaB₄H₄. Analysis by SDS polyacrylamide gel electrophoresis of extracts from the radiolabeled cells indicated that the CLL cell membranes contained the same protein components as normal lymphocyte membranes but that these components were generally less heavily glycosylated in CLL. To investigate mechanisms responsible for the altered glycosylation of CLL cell membrane components, we examined glycosyltransferase activities in cells from CLL patients and normal donors. Compared to normal cells, CLL cells showed decreased ability to transfer N-acetylglucosamine from UDP-N-acetylglucosamine to appropriate exogenous or endogenous glycoprotein acceptors. We conclude that CLL lymphocytes are characterized by a specific pattern of altered cell surface glycoproteins that can be detected by lectin-binding studies. These alterations may be related to decreased glycosyltransferase activity in the CLL lymphocyte.

Peripheral Blood Lymphocytes (PBL) from most patients with chronic lymphatic leukemia (CLL) carry B cell surface markers. Recent evidence indicates that CLL cells in a given patient all bear the same Ig idiotypic determinants and thus presumably represent the progeny of a single specific clone of B lymphocytes. Such homogeneity provides an excellent cell system for the further study of surface changes that might be unique to the CLL lymphocyte. Previous studies have shown that compared to normal lymphocytes CLL cell membranes possess alterations in the expression of certain antigenic determinants, changes in lipid content and in cell membrane...
microviscosity,20 and a decrease in sialic acid.21 Reported abnormalities of CLL membrane function include reduced cap formation in response to both anti-Ig antiserum22 and con A,23 increased agglutinability with con A,23 and increased adhesiveness to plastic Petri dishes23 or nylon wool columns.24,25 In addition, others have shown that relative to normal PBL, CLL lymphocytes carry reduced receptor sites for the plant lectins E-PHA,21 con A,26 and Agaricus bisporus hemagglutinin,27 and it has been suggested that this observation may explain in part the decreased responsiveness of the CLL lymphocyte to stimulation by the plant mitogens.21

In the present study we employed a panel of plant lectins as probes to investigate in a systematic fashion the surface properties of CLL lymphocytes. Our results indicate that the CLL cell is characterized by a diffuse abnormality of membrane glycosylation detectable as a specific alteration in the lectin binding pattern relative to normal human B lymphocytes. To examine the mechanism of the membrane alteration in CLL, we studied the activity of the glycosyltransferase enzymes responsible for completing the synthesis of nascent cell membrane saccharide components.

MATERIALS AND METHODS

Dextran T250 (mol wt 250,000 daltons) and Ficoll were purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Sigma Chemical, St. Louis, Mo. supplied jackbean meal, p-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitrophenyl-β-D-galactose, bovine serum albumin, α-methyl-D-mannose, and ovalbumin. Fetuin (B grade) was from Calbiochem, San Diego, California; Triton X-100 from Packard Instrument, Downers Grove, Ill.; and sodium diatrizoate (Hypaque sodium, 50% w/v) from Winthrop Laboratories, New York, N.Y. Con A and wheat germ agglutinin (WGA) were obtained from Miles-Yeda, Kankakee, Ill. and PHA-P came from Difco Laboratories, Detroit, Mich. NaB₃H₄ (320 mCi/mM) was purchased from New England Nuclear, Boston, Mass. All other radiochemicals came from Amersham/Searle, Arlington Heights, Ill. who also supplied NCS solubilizer. Hydromix scintillation counting cocktail was purchased from Yorktown Research, S. Hackensack, N.J. Tissue culture medium 199 came from Microbiological Associates, Bethesda, Md.

Patient population. Thirteen untreated patients with CLL were studied. The diagnosis was based on characteristic clinical, peripheral blood, and bone marrow findings. Profiles of these subjects are given in Table 1. At time of study, their ages were 55-84 yr and the duration of disease had been 1-240 mo. All patients had a significant absolute lymphocytosis of 7.2 × 10⁹ cells/liter.

Preparation of lymphocytes. CLL lymphocytes were isolated from defibrinated peripheral venous blood by dextran sedimentation followed by Ficoll-Hypaque centrifugation. More than 99% of the cells isolated were lymphocytes as seen with both Wright and peroxidase stains. Viability was greater than 97% of trypan blue exclusion. Normal human PBL from healthy donors were prepared in the same manner; 85%-98% of the mononuclear cells were lymphocytes, and trypan blue exclusion was greater than 95%.

Purified populations of B cells were prepared as previously reported.28 Briefly, mononuclear cells obtained from heparinized blood by Ficoll-Hypaque centrifugation were passed over an immunoabsorbent column consisting of rabbit anti-human Fab convanently linked to Sephadex G-200. The nonadherent cells included the T and null cell populations. The column-adherent B cells were eluted with media containing 1% human Ig. Greater than 97% of the eluted B cells bore surface Ig, 95% of EAC rosettes, and less than 2% formed E rosettes. Latex particle ingestion was less than 10%, and viability assessed by trypan blue exclusion was greater than 95%.

Lectins. E-PHA and L-PHA were purified by fractionation of phytohemagglutinin P according to the method of Weber et al.29 RCA-I was prepared from crude ricin by affinity chromatography on ovomucoid sepharose.30 The ricin and ovomucoid Sepharose were gifts of Dr. L. Shapiro.

Lymphocyte-binding studies. The five purified plant lectins were radioiodinated with ¹²⁵I (100 mCi/ml) using a 10-sec exposure to chloramine-T.31 Con A was iodinated in the presence of
Table 1. Clinical Profile of Patients With CLL

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Clinical Duration of Disease (mo)</th>
<th>Leukocyte Count ($\times 10^9$/liter)</th>
<th>Lymphocytes (%)</th>
<th>Hemoglobin (g/dl)</th>
<th>Platelet Count ($\times 10^9$/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F.C.</td>
<td>67</td>
<td>4</td>
<td>36.0</td>
<td>62</td>
<td>14.5</td>
<td>254</td>
</tr>
<tr>
<td>J.R.</td>
<td>82</td>
<td>13</td>
<td>18.8</td>
<td>80</td>
<td>13.7</td>
<td>275</td>
</tr>
<tr>
<td>L.D.</td>
<td>84</td>
<td>16</td>
<td>15.3</td>
<td>62</td>
<td>14.7</td>
<td>270</td>
</tr>
<tr>
<td>F.L.</td>
<td>70</td>
<td>6</td>
<td>23.5</td>
<td>70</td>
<td>14.4</td>
<td>234</td>
</tr>
<tr>
<td>C.V.</td>
<td>72</td>
<td>8</td>
<td>16.7</td>
<td>78</td>
<td>10.1</td>
<td>295</td>
</tr>
<tr>
<td>W.D.</td>
<td>55</td>
<td>9</td>
<td>23.4</td>
<td>68</td>
<td>14.2</td>
<td>331</td>
</tr>
<tr>
<td>J.D.</td>
<td>47</td>
<td>2</td>
<td>12.0</td>
<td>60</td>
<td>14.6</td>
<td>245</td>
</tr>
<tr>
<td>J.T.</td>
<td>55</td>
<td>10</td>
<td>51.8</td>
<td>90</td>
<td>13.4</td>
<td>252</td>
</tr>
<tr>
<td>R.B.</td>
<td>55</td>
<td>60</td>
<td>44.0</td>
<td>95</td>
<td>13.0</td>
<td>310</td>
</tr>
<tr>
<td>L.V.</td>
<td>55</td>
<td>5</td>
<td>43.5</td>
<td>77</td>
<td>14.7</td>
<td>391</td>
</tr>
<tr>
<td>J.H.</td>
<td>77</td>
<td>240</td>
<td>25.0</td>
<td>69</td>
<td>12.9</td>
<td>170</td>
</tr>
<tr>
<td>C.K.</td>
<td>59</td>
<td>1</td>
<td>30.0</td>
<td>92</td>
<td>10.6</td>
<td>114</td>
</tr>
<tr>
<td>G.K.</td>
<td>60</td>
<td>5</td>
<td>29.0</td>
<td>85</td>
<td>10.6</td>
<td>136</td>
</tr>
</tbody>
</table>

0.2 M $\alpha$-methyl-$\alpha$-mannose, which was removed by dialysis after completion of the iodination procedure. Lectin-binding studies were performed in 13 x 100 mm glass culture tubes (Kimble, Toledo, Ohio) that had been presoaked with 5 mg/ml BSA. Lymphocytes, 1-2 x $10^6$, were incubated for 45 min at 25°C in 0.4 ml medium 199 containing 1 mg BSA and 0.55-36 $\mu$g labeled lectin. Control studies were run in parallel with all reactants except lymphocytes to correct for nonspecific binding of lectin to the tubes. In certain experiments control studies containing appropriate monosaccharide haptenic inhibitors of lectin binding also were included. Residual binding in the presence of these haptenes $^{28}$ (0.1 M N-acetylglucosamine for WGA, 0.1 M mannose for lentil-PHA, 0.1 M lactose for RCA-I) was consistently less than 10% of the total. Since con A binds nonspecifically to glass tubes, parallel studies with this lectin routinely were carried out in the presence of 0.1 M $\alpha$-methyl-$\alpha$-mannoside. Residual nonspecific binding in the presence of this sugar (generally 10%-25% of the total) was subtracted from total binding to obtain specifc con A binding. Following incubation all tubes were washed twice with 5 ml BBS. To further reduce nonspecific binding, all con A studies were transferred to a second set of tubes following the first wash. The cell pellets were then counted in a LKB-Wallac 80000 automatic gamma sample counter (LKB Instruments, Rockville, Md.).

Radiolabeling of sialoglycoproteins on intact cells. $^{33}$ Cells (10$^8$/ml) were suspended in PBS pH 7.4 containing 2 mM sodium metaperiodate and incubated at room temperature for 10 min. Cells were then washed three times and resuspended in PBS, 10$^6$ cells/ml, followed by addition of 50 $\mu$Ci/ml NaB$_3$H$_4$ in 0.05 N NaOH. After a 45-min incubation at room temperature under a hood, the cells were washed four times in PBS and transferred to 15-ml thick-wall glass tubes (Corex) for the extraction procedure. Parallel control studies were processed in the same manner but without the addition of sodium metaperiodate to the first incubation.

Extraction of glycoproteins. Extraction of the labeled glycoproteins was performed by addition of 0.5 ml of cold 0.5%, Triton X-100 (Packard Instrument, Downers Grove, Ill.) in 0.056 M borate buffer pH 8.0 to 10$^6$ cells followed by incubation for 30 min at 4°C. The tubes were then spun at 20,000 g for 30 min, and the supernatant containing the solubilized radiolabeled sialoglycoproteins was collected. Aliquots of the extract were measured for radioactivity (Beckman LS-345 Liquid Scintillation System, Beckman Instruments, Fullerton, Calif.) and protein by the method of Lowry et al. $^{34}$ Lowry standards were prepared with BSA dissolved in Triton X-100 borate buffer.

SDS-PAGE. Samples of the cell membrane extracts containing approximately 100 $\mu$g protein were analyzed by SDS-PAGE according to the method of Neville and Glossman. $^{35}$ Molecular weights were estimated by calibration with the following series of standards: cytochrome C (12,500 daltons), ovalbumin (45,000), BSA (68,000), glucose oxidase (150,000) and catalase (240,000). For visualization of proteins, gels were stained with Coomassie blue and traced in a Gilford gel scanner, Model 2520 (Gilford Instrument, Oberlin, Ohio). For radioactivity determinations gels were sliced into 1-mm segments and solubilized by incubation with 100 $\mu$l 30% H$_2$O$_2$ at 60°C for
14 hr and then mixed with 1 ml NCS solubilizer. This mixture was dissolved in 10 ml Hydromix scintillation cocktail and counted in a Beckman Liquid Scintillation System.

Glycosyltransferase Assays

Glycoprotein acceptors. To prepare asialofetuin, 1 g fetuin was dissolved in 100 ml 0.5 N H$_2$SO$_4$ and incubated for 1 hr at 80°C. The mixture was neutralized with NaOH to a pH of 7.2 and extensively dialyzed. Analysis of the desialized fetuin for residual sialic acid by the method of Warren showed that 90%--95% of the sialic acid was removed from native fetuin by this procedure. Asialoagalactofetuin was prepared from asialofetuin by periodate oxidation followed by sodium borohydride reduction and mild acid hydrolysis. As previously described, this procedure resulted in release of approximately 80%--90% of galactose residues from asialofetuin. N-acetylglucosamine was removed from ovalbumin by incubation with $\beta$-N-acetylhexosaminidase prepared from jackbean meal by the method of Li and Li. Ovalbumin 1 g, was dissolved in 100 ml 0.05 $M$ sodium citrate buffer pH 5.0 containing 12.5 U/ml $\beta$-N-acetylhexosaminidase. Incubation was carried out at 37°C under toluene for 72 hr. The enzyme was inactivated by heating, the preparation centrifuged to remove precipitated material, and the supernatant retained and dialyzed. Gas chromatographic analysis (kindly performed by Dr. G. Pier) indicated that the derivative compound retained only 12% of the N-acetylglucosamine but all of the mannose of the parent compound.

Enzyme activity. Assays for glycosyltransferase activities in whole cells were based upon a modification of the method of Lamont et al. Reaction mixtures for determination of glycosyltransferase activity contained the following components in 335 $\mu$l 0.1 $M$ sodium cacodylate pH 7.4–0.15 $M$ NaCl: 4.5 x 10$^6$ lymphocytes, 1.5 $\mu$moles MgCl$_2$, 1.5 $\mu$moles MnSO$_4$, 0.4 $\mu$ mole of either CMP$^{14}$C-sialic acid (specific activity 196 mCi/$mM$), UDP-$^{14}$C-D-galactose (196 mCi/$mM$), or UDP-$^{14}$C-N-acetyl-d-glucosamine (300 mCi/$mM$). Certain tubes also contained 250 $\mu$g of either asialofetuin, asialoaagalactofetuin, or $\beta$-N-acetylhexosaminidase-treated ovalbumin. Incubations were carried out in 15-ml Corex tubes for 45 min at 37°C in a shaking water bath. Incubations were terminated by the addition of 1 ml cold cacodylate buffer, and the tubes were centrifuged at 15,000 $g$ for 10 min. Supernatants from tubes containing added glycoprotein acceptors were saved for further processing to determine cell-associated glycosyltransferase activity towards exogenous acceptors, while the cell pellets from these tubes were discarded. To process these supernatants, 5 ml ice-cold 1% PTA in 0.5 N HCl were added and the tubes were centrifuged at 15,000 $g$ for 10 min to collect the precipitate. The precipitate was washed a second time in PTA, dissolved in 0.5 ml NCS, and added to 10 ml Hydromix for liquid scintillation counting.

To determine cell-associated glycosyltransferase activity toward endogenous cell acceptors, the cell pellets from assays without added glycoproteins were saved. The pellets were resuspended in 5 ml 1% PTA in 0.5 N HCl and disrupted by sonication at a probe intensity of 40 for 20 sec (Biononix; Bronwill Scientific, Rochester, N.Y.). The resultant precipitate was collected by centrifugation, washed a second time with cold PTA, dissolved in NCS, and mixed with Hydromix for scintillation counting. All assays were carried out in triplicate. Parallel control studies contained all reactants except lymphocytes. Cells remained viable during the assay procedure as assessed by trypan blue exclusion exceeding 90%, following a 45-min incubation under these conditions.

To measure glycosyltransferase activities in whole cell extracts, preparations were made as described by Gottlieb et al., except that Triton X-100 was used in place of Emulphogene. Briefly, 5 x 10$^6$ cells suspended in BBS were disrupted by sonication. The sonicate was taken to 0.5%, in Triton X-100, incubated 1 hr at 4°C, and then centrifuged at 100,000 $g$ for 60 min to obtain a soluble extract containing glycosyltransferase activities. This material was used to measure galactosyl and $\beta$-N-acetylglucosaminyl transferase activities as described. Assays were carried out in 0.01 $M$ Tris maleate buffer pH 6.8 containing 0.0125 $M$ MnSO$_4$. Radiolabeled nucleotide sugars were added as described. Some reaction mixtures contained appropriate exogenous acceptors prepared as described above. Reactions, carried out at 37°C for 45 min were terminated by adding 5 ml cold 1% PTA in 0.5 N HCl, and the mixtures were centrifuged to collect the precipitate, which was washed a second time with PTA, dissolved in NCS, mixed with scintillation cocktail, and counted in a liquid scintillation counter.

Glycosidase activities. A modification of the method of Li and Li was used. Lymphocytes,
4.5 x 10^6, were incubated for 45 min at 37°C in a shaking water bath in 0.5 ml of either 2 mM p-nitrophenyl-β-N-acetylglucosaminide or 2 mM p-nitrophenyl-β-D-galactose in 0.1 M sodium cacodylate pH 7.4-0.15 M NaCl containing 5 mM MgCl₂ and 5 mM MnSO₄. After incubation, the reaction mixtures were centrifuged to pellet the cells. To the supernatant was added 3 ml 0.2 M sodium borate buffer pH 9.8, and the optical density at 400 nm was then determined.

RESULTS

Lectin-binding studies. Representative binding curves depicting the binding of five different plant lectins to normal PBL, purified normal B lymphocytes, and CLL lymphocytes are shown in Fig. 1. The results are plotted according to the method of Steck and Wallach. By this technique the intercept on the ordinate is used to calculate the number of lectin binding sites per cell (see legend to Fig. 1) and the intercept on the abscissa is a measure of the affinity of the lymphocyte receptor for the lectin. As illustrated in Fig. 1, the affinities of the lymphocyte receptors for a given lectin did not change significantly among the three lymphocyte groups studied. By contrast, the number of receptor sites for four of the five lectins (E-PHA, WGA, con A, and L-PHA) differed greatly between normal PBL and purified B cells on one hand and CLL lymphocytes on the other.

Fig. 1. Binding of 125I-labeled lectins to lymphocytes from normal individuals and patients with CLL in glass tubes, 1-2 x 10⁶ lymphocytes were incubated at 25°C for 45 min in 0.4 ml medium 199 containing 1.0 mg BSA and (A) 0.5-36.0 μg 125I-E-PHA, (B) 0.7-22.0 μg 125I-WGA, (C) 1.0-36.0 μg 125I-con A, (D) 1.3-26.0 μg 125I-L-PHA, or (E) 0.6-30 μg 125I-RCA-I. Cells were harvested and bound radioactivity determined as described in Materials and Methods. Data were analyzed by the method of Steck and Wallach according to the equation

\[
\frac{1}{(L - \text{bound})} = \frac{1}{kn} \frac{1}{L} + \frac{1}{nc},
\]

where \(L\) is the concentration of free lectin, \(n\) the number of lectin binding sites per cell, \(c\) the number of cells, and \(k\) the affinity constant of the lectin. Molecular weights of both E- and L-PHA were taken as 128,000 daltons; WGA 25,000; con A 110,000, and RCA-I 120,000. Normal PBL or B lymphocytes; a, CLL lymphocytes.
Although percentages of monocytes in the PBL preparations from the normal donors ranged from 2\% to 15\%, no relationship was observed between the number of bound lectin molecules and the number of monocytes in a given preparation. In addition, no difference in lectin binding was observed between studies carried out at 25°C and 4°C, indicating that phagocytosis of radiolabeled lectin by monocytes present in the normal PBL preparations did not contribute significantly to the calculated lectin binding in these experiments. Nonetheless, to exclude the possibility that differences in the monocyte content of normal and CLL lymphocyte preparations might explain the observed differences in lectin binding, normal PBL were processed by nylon fiber filtration followed by incubation in glass Petri dishes as previously described.\(^4\) Resultant cell preparations contained fewer than 0.3 peroxidase-positive cells,\(^4\) and when tested for binding of E-PHA, L-PHA, and lentil-PHA did not differ from the nondepleted PBL. Although we previously showed that nylon fiber filtration does deplete normal PBL of a subclass of cells specifically enriched for con A receptors,\(^2\) the fact that no differences were observed for the other lectins tested indicates that the relative percentages of monocytes in the normal and CLL lymphocyte preparations cannot account for the observed differences in lectin binding.

The data from the binding studies on all 13 CLL patients are summarized in Table 2, which compares the mean (±SE) number of lectin-binding sites on CLL lymphocytes to the number of sites on normal PBL and B cells. As we previously reported,\(^4\) using these five lectins we found no difference between normal PBL and purified B cells. However, Table 2 shows that CLL lymphocytes differ dramatically from the normal pattern. Compared to normal B cells, CLL cells bind significantly fewer molecules of E-PHA, WGA, and con A. This reduction in lectin binding varies for different lectins, representing a 60\% reduction in binding capacity for E-PHA and con A and a 40\% reduction in WGA-binding capacity. Although the data in Table 2 represent the group means for all 13 patients, the same pattern was given by the lymphocytes of each patient when analyzed individually. In contrast to the results for E-PHA, con A, and WGA, CLL cells bind more than twice as many L-PHA molecules as the normal controls. Again this pattern was seen in each individual patient.

**Table 2. Lectin Receptor Sites on Peripheral Blood Lymphocytes From Normal Individuals and Patients With CLL**

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Molecules Bound per Cell (x 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal*</td>
</tr>
<tr>
<td>E-PHA</td>
<td>2.43 ± 0.36</td>
</tr>
<tr>
<td>WGA</td>
<td>35.59 ± 1.96</td>
</tr>
<tr>
<td>Con A</td>
<td>1.12 ± 0.04</td>
</tr>
<tr>
<td>L-PHA</td>
<td>0.63 ± 0.18</td>
</tr>
<tr>
<td>RCA-I</td>
<td>4.50 ± 1.12</td>
</tr>
</tbody>
</table>

Binding studies were carried out as described in Materials and Methods and the data were analyzed by the method of Steck and Wallach\(^4\) to determine the number of lectin molecules bound per cell at saturation.

*Virtually identical results were obtained utilizing either unfractionated normal PBL or purified normal B lymphocytes.\(^4\)

†Difference between results for CLL and normal statistically significant (p < 0.001).
Fig. 2. PAGE of extracts from radiolabeled normal and CLL lymphocytes. Lymphocytes were radiolabeled and extracts prepared as described in Materials and Methods: 100 μg protein was applied to each gel and electrophoresis was carried out as described. Following electrophoresis, gels were sliced into 1-mm segments, solubilized, and counted in a liquid scintillation spectrometer. Each tracing, composite of four studies; all peaks were identified in each individual experiment; cpm corrected for both nonspecific radiolabeling as assessed in parallel experiments described in Results and for background counts determined from gel slices samples 5–6 mm ahead of tracking dye.

analyzed. Finally, the fifth lectin used in this study, RCA-I, did not discriminate between normal and CLL lymphocytes. These data establish a specific lectin-binding profile for CLL lymphocytes, which are characterized by decreased receptor sites for E-PHA, con A, and WGA, increased receptor sites for L-PHA, and equivalent numbers of RCA-I receptor sites relative to normal PBL and B cells.

Analysis of radiolabeled sialoglycoproteins. Exposed cell surface sialic acid groups on intact normal PBL and CLL cells were radiolabeled as described in Materials and Methods. Nonspecific labeling, monitored by parallel control experiments in which cells not preincubated in sodium metaperiodate were exposed to NaB₃H₄ under otherwise identical conditions, generally represented about 15% of the total radioactivity incorporated into both normal PBL and CLL cells and was subtracted from all results. Equivalent amounts of protein were extracted from both normal and CLL lymphocytes, approximately 9 mg/10⁶ CLL cells versus 11.6 mg/10⁶ normal PBL, but the specific activity of the extracted radiolabeled PBL proteins averaged 430 cpm/mg protein, compared with only 141 cpm/mg protein in the CLL extracts, a difference of approximately threefold. These data indicate that normal and CLL lymphocytes contain equivalent amounts of detergent extractable proteins but that these proteins are significantly less sialylated in CLL.

When the CLL and normal extracts were electrophoresed on 11% polyacrylamide gels in SDS, stained with Coomassie blue, and scanned densitometrically, no differences could be regularly identified between them. However, as shown in Fig. 2, when the gels were sliced and analyzed for the distribution of radiolabel, striking differences emerged. As expected, most CLL components showed decreased glycosylation relative to the normal, but at least three components (Rⱡ 081, mol wt ≈ 165,000 daltons; Rⱡ 148, mol wt ≈ 140,000; Rⱡ 721, mol wt ≈ 32,000) were more heavily glycosylated in the CLL.
extracts than in the normals. Each tracing in Fig. 2 is a composite tracing of four separate gels. All peaks were identifiable on each gel. These data establish that the CLL cell surface is characterized by a diffuse alteration of membrane protein glycosylation.

Glycosyltransferase activities. The altered pattern of lectin binding and the abnormalities of membrane protein glycosylation in the CLL lymphocytes suggested that these cells might have a defect in the synthesis of complex oligosaccharides. Current evidence suggests that the synthesis of terminal trisaccharide units of certain glycoproteins having the sequence sialic acid → galactose → N-acetylglucosamine occurs by the stepwise addition of monosaccharide units catalyzed by specific glycosyltransferases that utilize nucleotide sugars as the carbohydrate donors.48 To test for a possible role of these enzymes in the altered CLL cell membrane carbohydrate pattern we compared the activities of three glycosyltransferases in CLL and normal lymphocytes toward both their appropriate exogenous glycoprotein acceptors and their endogenous intracellular acceptors. Mean results from a group of six CLL patients and six controls are summarized in Table 3. For endogenous acceptors, the activities of each of the three glycosyltransferases assayed were reduced significantly in CLL lymphocytes relative to their activities in normal PBL. The data for glycosyltransferase activity toward exogenous glycoprotein acceptors presents a more varied picture; transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to its appropriate ovalbumin acceptor was clearly reduced, while by contrast transfer of both sialic acid from CMP-sialic acid and galactose from UDP-galactose to their appropriate fetuin acceptors was increased in CLL cells compared to normal PBL.

It was thought possible that the glycosyltransferase assay system employing whole cells (Table 3) might measure only ectoglycosyltransferase activity located on the cell surface and not accurately reflect the true intracellular activity of these enzymes.49 Therefore we examined the ability of Triton extracts prepared from sonicated lymphocytes to carry out galactosyl and N-acetylglucosaminyl transferase reactions. Results are summarized in Table 4. N-acetylglucosaminyl transferase activities in the CLL cell extracts averaged only one-third to one-fourth the level of comparable activities in extracts from normal

| Table 3. Glycosyltransferase Activities in Intact Lymphocytes From Normal Individuals and Patients With CLL |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Enzyme Activity | Acceptor        | pmoles Sugar Incorporated/10^7 | Normal | CLL |
|                 |                 | Lymphocytes/45 min (±SE) | Normal | CLL |
| Sialyltransferase | Endogenous      | 0.18 ± 0.05            | 0.07 ± 0.05* |     |
|                 | Asialofetuin    | 0.50 ± 0.17            | 0.97 ± 0.29* |     |
| Galactosyltransferase | Endogenous | 0.44 ± 0.07            | 0.14 ± 0.10* |     |
|                 | Asialoagalactofetuin | 1.08 ± 0.10      | 1.32 ± 0.10   |     |
| N-acetylglucosaminyl transferase | Endogenous | 0.49 ± 0.24            | 0.25 ± 0.07* |     |
|                 | α-N-acetylglucosaminovalbumin | 0.50 ± 0.34     | 0.19 ± 0.08*   |     |

Enzyme assays were carried out using intact cells as described in Materials and Methods with or without addition of appropriate exogenous glycoprotein acceptors.

*Difference between results for CLL and normal statistically significant (p < 0.01).
Table 4. Glycosyltransferase Activities in Triton X-100 Extracts of Lymphocytes From Normal Individuals and Patients With CLL

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Acceptor</th>
<th>pmoles Sugar Incorporated/mg Protein/45 min&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>CLL</td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>Endogenous</td>
<td>1.36 1.11</td>
</tr>
<tr>
<td></td>
<td>Asialoagalactofetuin</td>
<td>69.5 69.9</td>
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<tr>
<td>N-acetylgalcosaminyl transferase</td>
<td>Endogenous</td>
<td>0.915 0.314</td>
</tr>
<tr>
<td></td>
<td>α-N-acetylgalcosaminovalbumin</td>
<td>78.3 20.3</td>
</tr>
</tbody>
</table>

Enzyme assays were carried out using cell extracts in Triton X-100 prepared as described in Materials and Methods.

*Results represent mean of at least four determinations.

cells. When an exogenous acceptor was used, galactosyltransferase activity was identical in CLL and normal extracts, but it was slightly decreased relative to normal in the CLL extracts when endogenous activity was assessed. Because of the differences between the assay systems used to measure activities in intact cells and cell extracts, the data in Tables 3 and 4 are not directly comparable. However, the relative values are consistent, and both sets of data point to a striking deficit in N-acetylgalcosaminyl transferase activity in CLL lymphocytes.

To examine the possibility that the apparently decreased glycosyltransferase activity in the CLL lymphocytes might actually represent enhanced glycosidase activity not detected by our assay system, lymphocytes from CLL patients and normal donors were incubated with the p-nitrophenyl derivatives of either galactose or N-acetylgalcosamine under conditions identical to those used for the glycosyltransferase assays. Both normal and CLL lymphocytes hydrolyzed the synthetic p-nitrophenyl galactoside at a rate of 0.02 μmoles/min/5 × 10⁶ cells and the p-nitrophenyl-β-N-acetylgalcosaminide at a similar rate of 0.02–0.03 μmoles/min/5 × 10⁶ cells. Therefore under the assay conditions employed there was no enhancement of glycosidase activity in CLL lymphocytes relative to normal PBL.

**DISCUSSION**

These experiments indicate that the CLL lymphocyte is characterized by specific defects in cell surface structure that can be detected by lectin-binding studies. Compared to normal human PBL or purified B lymphocytes, the CLL lymphocytes from all 13 patients studied had fewer receptors for E-PHA, WGA, and con A and more receptors for L-PHA. Receptors for RCA-1 were the same on both normal and CLL cells. These studies both confirm and extend earlier studies that showed decreased receptor sites for certain individual lectins on the CLL lymphocyte. By using a group of lectins we identified a shared, reproducible pattern to the surface structure of CLL lymphocytes from

*In contrast to the results in this report and in ref. 26, a recent study reports finding no difference in con A binding between normal and CLL lymphocytes. However, insufficient data is presented in the latter report to allow meaningful comparison with our results.
13 different individuals. These data show that at the molecular level all patients with CLL may share certain basic structural abnormalities. The data therefore complement other evidence based on idiotypic determinants of CLL cell surface Ig indicating that the lymphoproliferation in CLL is clonal in nature.7,9

Plant lectins interact with cells by binding to specific cell surface saccharide structures.32 The pattern of lectin binding to the CLL cells becomes more understandable when available information concerning the structure of lectin receptor glycoproteins on human erythrocyte membranes is reviewed. In the red cell, E-PHA binds to an oligosaccharide structure termed the type I glycopeptide, which is present on the major sialoglycoprotein of the erythrocyte membrane.51 It contains sialic acid, galactose, N-acetylglucosamine, and mannose.51 In this structure the core mannose and peripheral galactose residues are critical determinants for E-PHA binding.51 It has been suggested that con A may bind to the same structure as well as others.53 Data indicate that important determinants for con A binding are the presence of α-linked mannose54,55 and terminal β-N-acetylglucosamine groups.54 WGA is also known to bind to major sialoglycoprotein of human erythrocytes,30 where the important binding determinants are N-acetylglucosamine and sialic acid groups.30 By contrast, RCA-I is known to bind to a different oligosaccharide unit that contains predominantly galactose and N-acetylglucosamine.30 The structural nature of the specific receptor site for L-PHA is not known. Therefore in the CLL cells a block in the synthesis of type I-like oligosaccharide units could account for the decreased binding of E-PHA, con A, and WGA without affecting RCA-I binding. The observed increase in L-PHA receptor sites on the CLL cells could result from exposure of previously "hidden" sites and/or the synthesis of additional membrane components. In addition the lectin-binding data suggest that the CLL membrane may be deficient in sialic acid, galactose, N-acetylglucosamine, and mannose. In fact, Kornfeld reported that the cell surface sialic acid content of CLL lymphocytes approximated only 50% of normal.21

Since certain plant lectins are known to bind to glycolipids as well as glycoproteins,57 it is possible that an alteration in glycolipid composition may also contribute to the observed abnormalities in lectin binding to the CLL cells. Both normal lymphocytes and granulocytes contain substantial quantities of glycolipid (about 16% of total lipid weight),58 present mainly as lactosylceramide.18 Hildebrand et al. reported that CLL cells possess only small amounts of glycolipid, present mainly as glucosylceramide.19 These data provide the possibility that alterations in membrane glycolipids as well as glycoproteins may play a role in the altered lectin binding given by CLL cells.

We conclude that the altered lectin binding given by the CLL cells provides strong evidence that these cells are characterized by significant changes in membrane carbohydrates relative to normal PBL. Based on the observation that the cell surface sialic acid content of CLL lymphocytes was considerably reduced, Kornfeld also suggested that the CLL lymphocyte might be characterized by a grossly abnormal cell surface.21 Other evidence in support of this hypothesis includes reports from several groups describing abnormal antigenic features of CLL lymphocytes10,17 including altered expression of A, B, H,10 and
HLA\textsuperscript{11,12} antigens. To visualize these proposed differences, we radiolabeled intact lymphocytes from normal donors and patients with CLL by a process of mild periodate oxidation followed by reduction with sodium borotritiide to introduce a tritium label into exposed sialic acid groups at the cell surface.\textsuperscript{33} Protein extracts of the radiolabeled cells were prepared using the nonionic detergent Triton X-100 and then analyzed by PAGE in SDS. As shown in Fig. 2, the pattern of sialylation of the proteins extracted from CLL cells differed markedly from that of normal PBL. These differences were not likely due to the presence of different protein components in the CLL extracts, since we and others\textsuperscript{59} have found no difference between Coomassie blue-stained gel patterns from normal and CLL membrane extracts. Although certain individual radiolabeled CLL components in Fig. 2 bore equal or even increased amounts of sialic acid groups relative to normal, the overall pattern implied generally decreased glycosylation in the CLL extracts. This is borne out by the very low average specific activity of the CLL extracts, 140 cpm/mg protein for CLL versus 430 cpm/mg protein for normal PBL. These data indicate that the CLL lymphocyte is characterized by a diminished exposure of sialic acid groups on many different glycoproteins at the cell surface.

Anderson et al. labeled exposed glycoproteins on purified normal human T and B lymphocytes and CLL cells by the galactose oxidase-sodium borotritiide method.\textsuperscript{60} By this technique, T, B, and CLL lymphocytes were characterized by specific labeling patterns when analyzed by SDS-PAGE.\textsuperscript{60} Since the studies of Anderson et al.\textsuperscript{60} were carried out under conditions different from those used in the present study, detailed comparisons with our data are not possible. However, their data provide additional evidence that the CLL lymphocyte is characterized by significant alterations in cell surface glycoproteins relative to normal T and B cells.

The data in Tables 3 and 4 compare several glycosyltransferase activities between normal and CLL lymphocytes. We measured the activities of these enzymes both in viable, intact lymphocytes (Table 3) and in whole cell extracts prepared in Triton X-100 (Table 4). Both methods indicate that compared to normal PBL the CLL lymphocytes are deficient in N-acetylglucosaminyl transferase activity towards both endogenous and exogenous acceptors. The apparent diminished enzyme activities in CLL cells cannot be explained on the basis of enhanced glycosidase activity, since CLL cells did not show an increased tendency to cleave synthetic p-nitrophenylglycosides under the conditions employed.

These data strongly imply that the CLL lymphocyte may be deficient in its capacity to complete the synthesis of nascent oligosaccharide chains on certain cell membrane glycoproteins. Since the oligosaccharide chains synthesized by these enzymes have the sequence sialic acid $\rightarrow$ galactose $\rightarrow$ N-acetylgalcosamine, with the sialic acid in the peripheral, nonreducing terminus,\textsuperscript{48} and since the multiglycosyltransferase system can function only in strict sequence with the product of one enzyme serving as substrate for the next,\textsuperscript{49} impaired activity of N-acetylglucosaminy transferase would represent a critical deficiency. Inability to add N-acetylgalcosamine to a growing oligosaccharide chain would automatically abrogate addition of penultimate galactose and terminal sialic acid
residues. Thus despite apparent increased galactosyl- and sialyltransferase activities in the CLL lymphocyte towards exogenous fetuin acceptors (Table 3), these activities are likely to be of little or no practical consequence in vivo because the cells are clearly limited in their capacity to produce appropriate substrates for these two enzymes. Similarly the apparent decrease in galactosyl- and sialyltransferase activities towards endogenous acceptors (Tables 3 and 4) may very likely reflect decreased acceptor availability rather than actual differences in enzyme activities.

The enzymatic activities listed in Table 3 reflect functions of viable, metabolically active cells as evidenced by the fact that greater than 90% of the cells excluded trypan blue and also by the fact that the various activities could be abolished by incubation at 4°C or if the cells were boiled prior to the assay. We considered that changes in the rate of transport of radiolabeled substrates or their breakdown products might explain our results for endogenous acceptors. This is unlikely because kinetic analysis of the glycosyltransferase-mediated incorporation of radioactivity into endogenous acceptors in our system indicated that the reaction is initiated immediately, without the 10-15-min lag phase characteristic of systems in which nucleotide sugars are degraded prior to uptake into the cells. Furthermore, the rates of increase in glycosyltransferase activities toward endogenous acceptors in our system actually decreased and plateaued with increasing time of incubation, a kinetic pattern quite different from that given by systems that measure only uptake of sugars derived from nucleotide sugar breakdown. With reference to experiments using exogenous glycoprotein acceptors, the specificities of the assays were monitored by “cross-over” experiments in which “inappropriate” acceptors and nucleotide sugars were interchanged in various combinations. Results of these experiments showed less than 20% nonspecific incorporation.

Although the data in Table 3 using intact cells may imply that the glycosyltransferase activity measured in this system was located at the cell surface, our studies do not rigorously exclude an intracellular location. Although a small percentage of glycosyltransferase activity may be located at the cell surface, the majority of activity is located intracellularly. Therefore it was important to measure enzyme activity in cell extracts to be certain that the activity detected in intact cells was representative of the total enzyme activity. Although the data in Tables 3 and 4 are not directly comparable because differences in the assay systems may influence the levels of activities observed (e.g., certain transferase activities may be enhanced by the presence of detergents), results in the two sets of experiments were consistent, indicating that measurements of glycosyltransferase activities in intact human lymphocytes may be a valid reflection of the total cellular activities of these enzymes.

The altered glycosyltransferase activity of the CLL cell appears to be but one of several alterations in enzyme activity in CLL. It seems reasonable to suggest that the markedly altered cell surface of the CLL lymphocyte as the chief manifestation of defective glycosyltransferase activity may be important in the abnormal functional properties of these cells.
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Chronic lymphatic leukemia (CLL): cell surface changes detected by lectin binding and their relation to altered glycosyltransferase activity

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