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Detection of Human Hematopoietic Differentiation-related Glycoproteins: The Western Enzyme-linked Lectin Analysis

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A technique is introduced (Western enzyme-linked lectin analysis, WELLA) for detecting lectin-reactive cellular glycoproteins after separation on the basis of molecular weight in sodium dodecyl sulfate (SDS) polyacrylamide gels. Lectin-reactive glycoproteins are detected on Western transfers by reaction with lectin-peroxidase conjugates followed by development with hydrogen peroxide and 4-chloro-1-naphthol which forms a purple-gray precipitate. WELLA is more rapid, more sensitive, and the bands are highly reproducible and better resolved than those obtained by autoradiography or fluorography.

Using this technique, we have detected human differentiation-related glycoproteins on cells of different hematological lineages. Both wheat germ agglutinin-peroxidase (WGA-P) and concanavalin A-peroxidase (ConA-P) detected distinct glycoprotein patterns on isolated peripheral blood platelets, lymphocytes, monocytes, erythrocytes and granulocytes. WGA-P detected numerous similarities between immature myeloid cells isolated from bone marrow and acute myelogenous leukemia cells, including major glycoproteins at 20 and 25 kDa. ConA-P detected a similar pattern of glycoproteins between isolated peripheral blood lymphocytes and T-cell acute lymphoblastic leukemia (T-ALL) cells. The T-ALL cells, however, had a major 200 kDa glycoprotein not present on lymphocytes. WGA-P also showed nearly identical patterns between the lymphocytes and the T-ALL cells, but detected prominent 200 and 250 kDa glycoproteins on the T-ALL cells which were absent from the lymphocytes. We have also detected polymorphic differences in the glycoproteins on lymphocytes from normal donors in the range of 95-110 kDa using ConA-P.

Abbreviations: WELLA, Western enzyme-linked lectin analysis; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PVP, polyvinylpyrrolidone; PBS, phosphate-buffered saline; AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia; WGA, wheat germ agglutinin; Con A, concanavalin A; WGA-P, wheat germ agglutinin-peroxidase conjugate; ConA-P, concanavalin A-peroxidase conjugate.

The hematopoietic system represents a unique opportunity to study alterations in glycoconjugates during differentiation. Originating from the pluripotent hematopoietic stem cell, differentiation results in morphologically identifiable end stage formed elements including lymphocytes, monocytes, granulocytes, erythrocytes, megakaryocytes and platelets. Within the bone marrow, there are at least 15 morphologically identifiable distinct intermediates of several lineages. Some human leukemias are representative of particular intermediate stages of hematopoietic differentiation (eg. promyelocytic leukemia).

A current goal of understanding hematopoietic differentiation is analysis of cellular glycoprotein patterns in phenotypically distinct cell subsets. In this manner, a "map" could be constructed of the appearance and disappearance of particular glycoconjugates serving important hematopoietic functions. We describe the analysis of cellular lectin-binding glycoproteins after separation by electrophoresis in SDS and transfer to nitrocellulose in a simple, rapid, one-step procedure using peroxidase-conjugated lectins. We term this analysis "WELLA" (Western enzyme-linked lectin analysis). WELLA has several advantages over previous techniques: (a) it allows higher resolution; (b) it avoids the use of radioactivity; (c) the conjugates are commercially available and stable for extended periods of time; (d) the steps are rapidly accomplished, allowing visualization of bands within 24 h of preparing cell extracts; (e) the development of the color reaction can be controlled visually and stopped at the appropriate intensity; (f) no darkroom or developing equipment is required; and (g) the number of bands obtained with any particular lectin is a distinct subset of the total glycoproteins, and this defined subset can be isolated subsequently by lectin affinity chromatography.

Using the WELLA we have detected WGA- and ConA-reactive glycoprotein differences on differentiated human hematopoietic elements, and we have detected polymorphic differences in lymphocyte glycoproteins from normal donors. We have also found differentiation-related similarities between particular leukemias and hematopoietic cells. In addition, WELLA detects distinct glycoproteins on these leukemias which are absent from hematopoietic counterparts.

Materials and Methods

Cell Isolation

Leukemic samples are obtained from the cell pheresis unit at M.D. Anderson Hospital and are at least 85% blasts. Normal peripheral blood samples are collected in heparinized syringes, diluted 1:2 in phosphate buffered saline (0.01 M sodium phosphate and 0.15 M NaCl pH 7.2, PBS) and layered on to Ficoll-Hypaque (density 1.077; Pharmacia, Uppsala, Sweden) and centrifuged for 20 min at 600 × *g*. The upper layer and the interface between it and the Ficoll-Hypaque are collected and centrifuged for 5 min at 500 × *g*. The resulting supernatant fraction is centrifuged at 1000 × *g* for 25 min at room temperature to collect platelets. Cellular contamination of the platelet fraction represented less than 0.5% of the material loaded on the gels. Mononuclear cells in the pellet from the 500 × *g* spin are washed, resuspended in RPMI 1640 (Gibco, USA) and 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA, USA) and allowed to adhere to the surface of a polystyrene flask at 37°C. After 2-4 h lymphocytes are decanted and the adherent cells in the

flask are washed gently two times. Separated subpopulations were examined microscopically after staining with Wright's stain (Sigma Chemical Co., St. Louis, MO, USA). The non-adherent mononuclear cells were morphologically greater than 80% lymphocytes and were less than 20% monocytes as judged by nonspecific esterase staining (NSE) [1]. The adherent monocytes are collected by scraping the flask with a rubber scraper. The adherent cells were greater than 80% monocytes as judged by NSE and less than 20% lymphocytes by morphology.

The cells which pellet through the Ficoll-Hypaque are red cells with a buffy coat of granulocytes. The buffy coat is collected and granulocytes are prepared by resuspending the buffy coat in 0.83% ammonium chloride for 20 min at 37°C to lyse contaminating red cells. The granulocyte fraction was approximately 90% pure morphologically, with about 10% contamination with lymphocytes and monocytes. Erythrocytes from beneath the buffy coat contained no other cell type.

Bone marrow is fractionated according to the method of Ellis *et al.* [2]. Buffy coat cells are layered on to discontinuous Percoll (Pharmacia) gradients of four layers (40%-70%) of discontinuous osmalarity (360-320 mosM). The gradients are centrifuged at 750 × *g* for 20 min and five fractions are collected. The 50%-60% interface yields a fraction enriched in progenitor cells by as much as 10 fold as determined by GM-CFC (granulocyte, macrophage colony forming cells).

Preparation of Cell Extracts

Leukemia or normal cells are washed three times in PBS and resuspended in extraction buffer (0.5 M mannitol, 0.5% Triton X-100, 5.0 mM $CaCl_2$, 0.02% NaN_3, 100 μM phenylmethylsulfonylfluoride added fresh). Ten volumes of extraction buffer are used for every vol of packed cells. If there are too few cells to measure packed volume, 10^7 cells are resuspended in 100 μl of extraction buffer. Cells are extracted for 20 min on ice and then nuclei are removed by centrifugation for three min at 13 000 × *g*. The supernatant extract is then solubilized in three-fold concentrated SDS sample buffer (final concentration 2% SDS, 0.063 M Tris-phosphate pH 6.7, 1% 2-mercaptoethanol, 0.5 mM EDTA, 10% glycerol, 0.003% bromophenol blue) and boiled for five min before being loaded on to gels. Between 25 and 50 μl per lane of extract is loaded on the gels.

Gel Electrophoresis and Western Transfer

Ten per cent SDS-polyacrylamide slab gels or 5-15% gradient slab gels are prepared according to the procedure of Maizel [3] as modified by Kramer and Canellakis [4]. A 3% stacking gel is prepared just before use. Western transfer is performed according to a modification of the method of Towbin *et al.* [5]. Proteins are transferred for 20 h at 40 mA followed by one hour at 150 mA constant current or for 3.5 h at 150 mA constant current to MicronSep nitrocellulose paper (Fisher Scientific, Pittsburgh, PA, USA) using Tris-glycine-methanol buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3). Prestained molecular weight standards (Bethesda Research Labs, MD, USA) are included on each gel. These include myosin heavy chain (M_r 200 000), phosphorylase B (M_r 92 500), bovine serum albumin (BSA, M_r 68 000), ovalbumin (M_r 43 000), α-chymotrypsinogen

(M_r 25 700), β-lactoglobulin (M_r 18 000) and cytochrome C (M_r 12 300). Transfers are stained for protein using 2% Amido Black (Sigma) in 2% acetic acid for 15 min at room temperature followed by destaining in 2% acetic acid.

Staining of Western Transfers with Peroxidase-conjugated Lectins

After electrophoretic transfer, the nitrocellulose sheets are allowed to dry for at least 30 min at room temperature. The dried sheets may be stored for at least a week at room temperature. Before staining, nonspecific conjugate binding to the sheets is blocked with 1% BSA, 1% polyvinylpyrrolidone (PVP, Sigma) in PBS for at least 30 min at room temperature.

The lectin-conjugate is diluted in the blocking solution to the appropriate concentration (usually 1-5 μg/ml). Each conjugate must be titrated to determine the optimum concentration for staining without increasing background staining. The Con A-horseradish peroxidase (ConA-P) and wheat germ agglutinin-horseradish peroxidase (WGA-P) were obtained from Sigma). As a specificity control, an aliquot of the conjugate solution should be preincubated with a lectin-specific sugar inhibitor for 15 min before staining a duplicate Western transfer.

After decanting the blocking solution, the transfers are incubated with a sufficient volume of the lectin conjugate solution to cover the nitrocellulose sheet (usually 10-30 ml) for 60 min at room temperature with gentle rocking. The conjugate is then removed by washing ten times with 25 ml 0.05% Triton X-100 (Sigma) and decanting. Bands are visualized by incubating with 0.06% 4-chloro-1-naphthol (Sigma) for 5-15 min at room temperature. The powdered substrate must be stored under nitrogen below 0°C. A stock solution of 0.3% 4-chloro-1-naphthol is prepared in methanol. The working solution is prepared by diluting the stock solution 1:5 in PBS containing 0.02% hydrogen peroxide. When the bands are sufficiently dark, the reaction is stopped by rinsing the sheets three to four times with deionized water. A purplish-gray precipitate is formed wherever the enzyme is present. The stained sheet is then allowed to dry, and the dried transblot can be photographed or scanned directly using a reflectance densitometer (e.g., Quick Scan R & D, Helena Labs., Beaumont, TX, USA).

Results

Both ConA-P and WGA-P stain many bands in hematopoietic cell extracts. Fig. 1 is a photograph of WGA-P-stained cell extracts from the K562 human erythroleukemic cell line and from cells from patients with acute leukemia. WELLA allows identification of the molecular weight of lectin-binding glycoproteins that differ from one sample to another. For example, prominent bands were observed at 20 kDa and 25 kDa in the immature myeloid fraction from bone marrow (lanes 5 and 6), and similar bands in the acute myelogenous leukemia (AML) cells (lane 10), classified M5 (monocytic leukemia).

Fig. 2 is a comparison of identical samples stained for protein with Amido Black and for reactivity with WGA-P and ConA-P to show the differences between protein staining and specific lectin staining. There are obvious differences in the glycoproteins bound

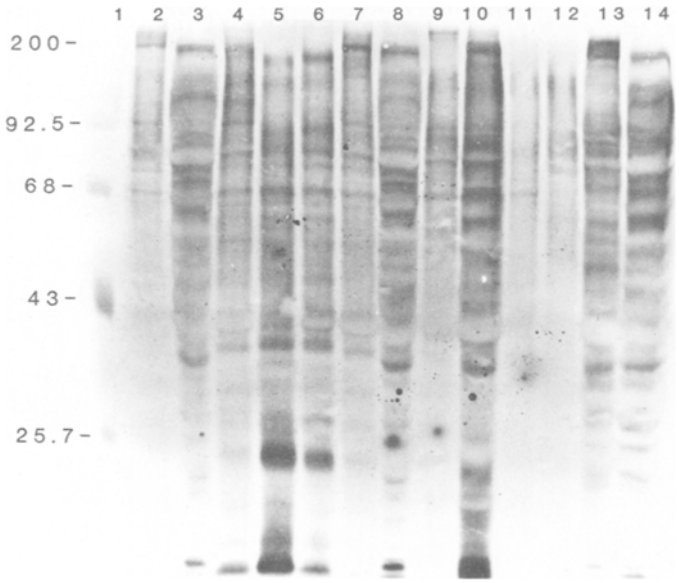

Figure 1. WELLA of WGA-P-stained cell extracts. Transfer of 10% SDS gel was stained as described in the text with 5 μg/ml WGA-P in PBS pH 7.2, 1% BSA, and 1% PVP. Lane 1 prestained molecular weight standards; lane 2, monocytes; lanes 3, 8 and 14, K562 cell extracts; lanes 4 and 7, bone marrow progenitor-enriched fraction from Percoll-gradient separation from two normal individuals; lanes 5 and 6, bone marrow immature granulocyte fraction from Percoll gradient, two normal individuals; lane 9, AML no. 1 (M5, monocytic leukemia) peripheral blood mononuclear cell fraction; lane 10, AML no. 2 (M5) peripheral blood mononuclear cell fraction; lane 11, AML no. 3 (M4, myelomonocytic leukemia) bone marrow mononuclear cell fraction; lane 12, AML no. 4 bone marrow mononuclear cells; lane 13, chronic lymphocytic leukemia (CLL) peripheral blood mononuclear cells.

by Con A and WGA in the same sample, and both are distinct from the major protein bands. For example, WELLA with WGA-P demonstrates two major bands at 200 kDa and 250 kDa in the acute lymphocytic leukemia (ALL) sample (lane 11), whereas only a single major band at 250 kDa is seen with ConA-P (lane 5). Neither of these bands is a major protein staining band (lane 8). There are both similarities and differences between the T-cell ALL sample and isolated lymphocytes with either lectin. For instance, neither of the above-mentioned high molecular weight glycoproteins yields prominent bands in the lymphocyte fraction (lanes 6 and 12). However, numerous similarities can be seen for the Con A-reactive bands in lanes 5 and 6 and for the WGA reactive bands in lanes 11 and 12. Also shown is the hapten inhibition of the WELLA for Con A with sucrose (lanes 2 and 3) and for WGA using *N,N',N''*-triacetylchitotriose purified from hydrolysed chitin (lanes 14 and 15) indicating the specificity of the reaction. No inhibition was obtained when inappropriate sugars were added (data not shown).

Figs. 3 and 4 show WELLA analysis of various cell types separated from the peripheral blood of three donors using two lectin-enzyme conjugates showing similar staining of

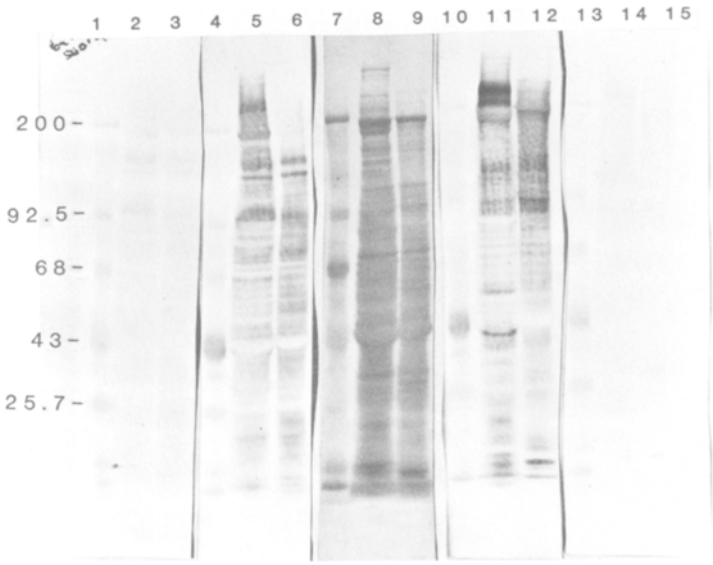

Figure 2. Comparison of WELLA-staining with Amido Black staining for protein of identical lanes. Lanes 1, 4, 7, 10, 13, prestained standard; lanes 2, 5, 8, 11, 14, T-cell ALL; lanes 3, 6, 9, 12, 15, isolated normal lymphocytes. Lanes 1-3 are stained with ConA-P + 0.2 M sucrose (specificity control); lanes 4-6 are stained with ConA-P as noted in the text; lanes 7-9 are stained for protein with 2% Amido Black; lanes 10-12 are stained with WGA-P; lanes 13-15 are stained with WGA-P and N, N', N''-triacetylchitotriose (100 μM).

the same cell type with the same lectin but different staining patterns of different cell types. In Fig. 3, the transfer is stained with ConA-P. In Fig. 4, the transfer is stained with WGA-P. For the most part, the same cell type from different donors has the same staining pattern with a particular lectin. For instance, platelets from three donors stained with Con A (Fig. 3, lanes 2-4) appear identical. There appear to be some individual differences within given cell types from different donors. For example, lymphocytes from the three normal donors stained with ConA-P (Fig. 3, lanes 5-7) display major differences in the 95 kDa to 110 kDa region but are similar outside this range. Differences between various cell types from the same donor are easily detected. For instance, platelets (lanes 2-4) and lymphocytes (lanes 5-7) show few similarities. Fewer differences are detected between lymphocytes (lanes 5-7) and monocytes (lanes 8 and 9).

Major differences are also seen when the same cell types are stained with different lectins. As an extreme example, erythrocytes do not stain with ConA-P (Fig. 3, lanes 10-12) but stain intensely with WGA-P (Fig. 4, lanes 13-15). From Figs. 3 and 4 it is clear that WELLA can be used to detect differentiation-related changes.

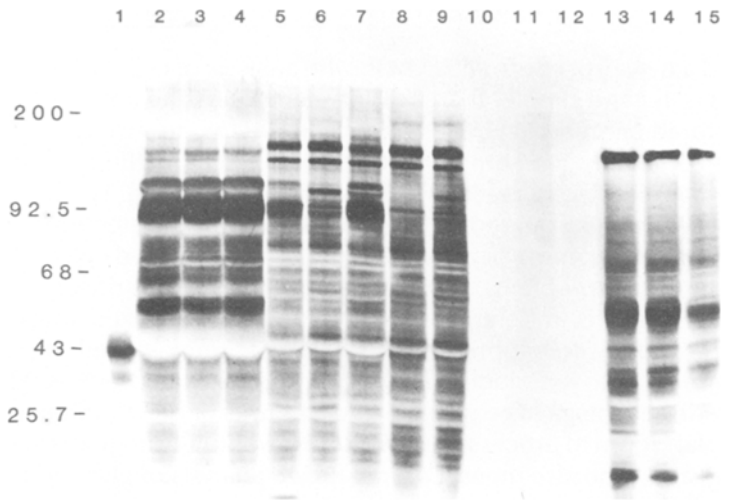

Figure 3. WELLA of ConA-P-stained cell extracts. Transfer was stained as described in the text with 2.5 μg/ml ConA-P. Lane 1, standards (ovalbumin is stained by the lectin enzyme); lanes 2-4, extracts of normal platelets; lanes 5-7, extracts of normal lymphocytes; lanes 8 and 9, extracts of normal monocytes; lanes 10-12, extracts of normal red blood cells; lanes 13-15, extracts of normal granulocytes.

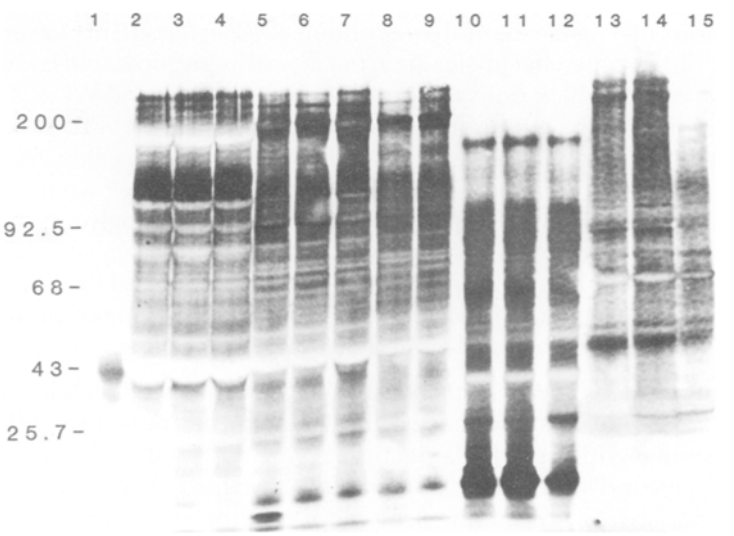

Figure 4. WELLA of WGA-P-stained cell extracts. Transfer stained as described in the text with 5 μg/ml WGA-P. Lanes 1-9, same as in Fig. 3; lanes 10-12, extracts of normal granulocytes: lanes 13-15, extracts of normal erythrocytes.

Discussion

We have described a new procedure (WELLA) for the analysis of cellular lectin-reactive glycoproteins. It is faster and simpler than existing techniques and avoids the use of radioiodine and autoradiography. WELLA is applicable to a wide variety of lectins for which peroxidase conjugates are commercially available. Lectin affinity chromatography has been used to bind lectin-reactive cellular glycoproteins [6]. The lectin-bound molecules were released and analyzed by SDS-polyacrylamide gel electrophoresis and detected either by protein staining or autoradiography of prelabeled cell extracts. This procedure suffers from two problems: the yields from the affinity purification are often low, and immobilized lectins in detergent solutions can have relaxed specificity and decreased stability [7]. Previous approaches using glycoproteins fixed in polyacrylamide gels and "staining" with radioiodinated [8-11] or [^{3}H]-acetylated lectins [12] are sensitive but have the disadvantage of requiring large amounts of radioiodine or other radioactive tracer; a darkroom to process the autoradiograms or fluorograms and long incubation, washing, and exposure times. Enzyme-linked analysis of glycoproteins reactive with Con A has been performed on Western transfers [13, 14] using a two-step procedure with Con A bridging the lectin-reactive glycoproteins and the Con A-reactive glycoenzyme horseradish peroxidase (HRP). This bridging procedure which was previously used with Con A and HRP in polyacrylamide gels [15], is not applicable to lectins which do not bind to HRP. Analysis of reactive glycoproteins using other lectins has been reported that employs multiple steps with lectin, antilectin antibody, radioiodinated protein A, and autoradiography [14], or biotinylated lectins followed by avidin-biotinyl-peroxidase complexes and then by substrate [16].

WELLA is a sensitive procedure, depending on the number of carbohydrate moieties to which a specific lectin will bind. For example, using 2 μg/ml ConA-P, ovalbumin is detectable at concentrations of 1 μg/lane and thyroglobulin at 0.2 μg/lane. This lower level of staining for ovalbumin is somewhat misleading, because this glycoprotein is composed of multiple species, one of which does not bind Con A but does bind WGA [17]. Commercial thyroglobulin contains a number of ConA-P-staining bands with a major species at about 240 kDa. The minimum level of detection for ovalbumin using WGA-P is 0.3 μg/lane. Results of WELLA of human erythrocyte ghosts using WGA-P were compared with the results of Robinson *et al.* [8]. They detected the major sialoglycoproteins PAS 1, 2, and 3 by labeling gels with ^{125}I-WGA followed by autoradiography. WELLA detects the same bands but requires less protein per lane (20-80 μg compared to 250 μg for ^{125}I) indicating that WELLA may be more sensitive. Also, WELLA detects many bands at high protein concentrations which are not detected by other methods.

We have detected differences in WELLA using WGA-P to stain isolated platelets, erythrocytes, lymphocytes, granulocytes, and monocytes. The same cell type from different individuals appears similar with a particular lectin-enzyme conjugate, whereas different cell types from the same individuals display distinctive glycoprotein patterns. Since the hematopoietic cells arise from a common progenitor cell, these are clearly differentiation-related changes. Similar results with different bands were obtained with WELLA using ConA-P. Using WELLA, we have detected glycoprotein similarities and differences between human acute leukemic cells and normal hematopoietic cells. WELLA can detect glycoproteins in acute leukemia cells with patterns similar to particular hematopoietic lineages. We found numerous similarities in the staining of T-cell ALL cells with

Con A to the staining of lymphocytes with Con A (Fig. 2, lanes 5 and 6) although the lymphocytes are a mixture of T and non-T cells, they are comprised of at least 70% T cells and the T lymphocyte bands should be well represented. Using WGA, we detected other similarities between the T-cell ALL cells and the lymphocytes (Fig. 2 lanes 11 and 12). WGA also detects major glycoproteins at 20 kDa and 25 kDa in normal granulocytes (Fig. 1, lanes 5 and 6) which are also prominant in an acute leukemia sample (Fig. 1, lane 10).

In addition, WELLA detects interesting differences between acute leukemia samples and normal related cell types. We have detected major reactive bands at 200 kDa and 250 kDa in a T-cell ALL sample (Fig. 2, lane 11) that are absent from normal lymphocytes (Fig. 2, lane 12). With Con A, prominent glycoproteins are detected in the T-cell ALL sample (Fig. 2, lane 5) at 180 kDa and 250 kDa that are missing from the normal lymphocytes (Fig. 2, lane 6). WELLA allows facile identification of glycoproteins of interest that can be isolated by lectin-affinity chromatography and used to produce monoclonal antibodies. The relevance of these differences will become clear only after investigation of a large number of acute leukemia samples properly classified by morphological criteria (e.g., FAB classification [18]).

A more thorough understanding of glycoconjugate alterations during hematopoietic differentiation will require isolated intermediates in specific lineages. With this goal in mind, WELLA should be able to display the specific point in differentiation at which specific glycoproteins are gained or lost, and also allow an investigation of the possibility that the glycosylation pattern changes on the same glycoproteins at particular stages of human hematopoietic differentiation. The differences detected between cells of different lineages with the WELLA could be due to different glycoproteins or to similar proteins with altered glycosylation. The latter case has been reported for T200 [19] and for murine I-A glycoproteins [20, 21]. Separated intermediates in hematopoietic differentiation would also allow a more meaningful WELLA analysis of the relationship of leukemia cell glycoproteins to their normal counterparts.

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