Increased Expression of a Normal Lymphocyte Membrane Antigen on Chronic Lymphatic Leukemia Cells

Annemarie Hekman and Maria H. G. Melis

Department of Immunology, Antoni van Leeuwenhoekhuis, The Netherlands Cancer Institute, Amsterdam, The Netherlands

SUMMARY

Rabbit antisera against a 3 M KCl extract from lymphocytes isolated from tonsils were found to detect an antigen of the normal lymphocyte membrane that on chronic lymphatic leukemia cells is increased in quantity. This increase seems to be specific for chronic lymphatic leukemia.

A discrepancy was found between the results obtained with complement-dependent techniques and with methods wherein no complement is used. In complement-dependent methods, the antisera showed a much weaker reaction with normal lymphocytes than would be expected from the amount of antigen present, as established by other methods.

INTRODUCTION

Evidence that human leukemia cells may possess tumor-associated antigens has been obtained in recent years (1, 4, 8–11, 14, 15, 17, 24). When such investigations are performed with the use of heterologous antisera, which usually need extensive absorptions, the possibility of a quantitative difference in certain antigens on normal and leukemia cells, rather than the presence of a new antigen, must be taken into account and is sometimes difficult to disprove conclusively. This paper presents evidence that such quantitative changes do occur on CLL cells.

In the course of an investigation of the membrane antigens of leukemia cells, we attempted to produce leukemia-specific antisera by immunizing rabbits with various preparations of leukemia cells known to contain membrane antigen. As controls for the leukemia-specificity of the antisera after absorption, etc., antisera were also raised against similarly prepared extracts of nonmalignant lymphoid cells. Some of these were derived from tonsils. Although lymphocytes from tonsils removed because of chronic tonsillitis may be chronically stimulated and possibly different from peripheral lymphocytes in some respects, they may be assumed not to contain tumor-specific antigens in the strict sense.

This report concerns the unexpected reaction patterns of antisera against 3 M KCl extract from tonsillar lymphocytes when tested with normal peripheral lymphocytes versus CLL cells. It shows that normal antigens can be present on the membrane of CLL cells in increased concentrations and that this phenomenon might be specific for CLL.

MATERIALS AND METHODS

Cells. Lymphocytes were isolated from heparinized venous blood by centrifugation on Isopaque-Ficoll (2) after sedimentation of most of the erythrocytes with dextran (MW 200,000; 5%; Poviet, Amsterdam, The Netherlands). The lymphocytes were washed twice with HBSS. Cells not used immediately were frozen in HBSS containing 15% fetal calf serum (Flow Laboratories, Irvine, Scotland) and 10% dimethyl sulfoxide in a controlled cooling rate apparatus (Cryoson; Midden Beemster, The Netherlands) and were stored in liquid nitrogen. Slow dilution with HBSS containing 10% fetal calf serum after thawing (21) yielded usually more than 95% viable cells as judged by trypan blue exclusion.

Tonsils were obtained from children undergoing tonsillectomy because of chronic tonsillitis or recurrent angina. Lymphocytes were isolated by gently mincing the tissue in HBSS and centrifuging the cell suspension on an Isopaque-Ficoll gradient.

Extraction. Three M KCl extraction was performed according to the method described by Reisfeld et al. (19) and Gutterman et al. (8). Ten ml of 3 M KCl in phosphate buffer, pH 7.2, were added to 3 x 10^8 cells. The mixture was slowly stirred for about 16 hr at 4°C and was centrifuged at 140,000 x g for 1 hr. The supernatant was dialyzed against distilled water for 20 hr and centrifuged at 105,000 x g for 30 min. The clear, no longer viscous supernatant was lyophilized; 10^8 cells yielded 1 to 10 mg of lyophilized material.

Immunization. Rabbits received 3 weekly i.m. injections of 5 or 10 mg lyophilized 3 M KCl extract with complete Freund's adjuvant. This schedule was repeated after 1 month and, if necessary, once again after another month. The sera were inactivated at 56°C for 30 min and were stored at −20°C.

Cytotoxicity Test. This test was performed by a modification of the microdroplet technique (13). One µl of the
lymphocyte suspension (10 × 10⁶/ml in human complement) was added to 1 μl antiserum. After incubation for 60 min at 37°, 5 μl rabbit complement (undiluted, unabsorbed, pooled rabbit serum) were added and incubation was continued for 60 min. After the addition of 5 μl trypan blue (1% in 0.9% NaCl solution) and incubation for 20 min at room temperature, the percentage of viable cells was estimated. The end point of titrations was taken as the last dilution yielding 30% or more dead cells.

Immunoadherance. This was performed by methods described by Cormain et al.⁴

Immunofluorescence on Viable Cells. Lymphocytes (4 × 10⁶) in 20 μl HBSS were incubated with 20 μl antiserum in a round-bottom Microtiter plate (Cooke Engineering Co., Alexandria, Va.) for 30 min at room temperature. Cells were washed 3 times with HBSS, incubated with 40 μl fluorescein-conjugated swine anti-rabbit immunoglobulin (Nordic Pharmaceuticals, Tilburg, The Netherlands) (diluted 1:70) for 30 min at room temperature, and again were washed 3 times. Cells were resuspended in 50% glycerol in PBS and examined with a Leitz-Orthoplan microscope equipped with epillumination and interference filter combination for fluorescein isothiocyanate fluorescence according to the method of Poelm (18). The immunofluorescence test with anticomplement conjugate was essentially the same, except for the addition of 20 μl rabbit complement after the cells had been incubated with antiserum for 15 min, and the use of fluorescein-conjugated goat anti-rabbit β₁C (Nordic Pharmaceuticals) diluted 1:10.

Immunoelectron microscopy. The γ-globulin fraction of a goat anti-rabbit immunoglobulin antiserum was labeled with ferritin (Nutritional Biochemicals Corp., Cleveland, Ohio) using glutaraldehyde (Ladd Research Industries, Burlington, Vt.), as described by Micheil (12). Cells were incubated with antiserum and ferritin conjugate and were prepared for electron microscopy, as described by Calafat et al. (3).

Separation of T- and Non-T-Lymphocytes. Normal lymphocytes were separated in T- and non-T-lymphocytes by the formation of E rosettes or EAC rosettes, followed by centrifugation on Isopaque-Ficoll, as described by De Vries et al. (5).

Cultured Human Lymphoid Cell Lines. Raji and EB-3 cells were kindly provided by Dr. T. Splinter and Dr. J. Collard. Both lines are originally derived from Burkitt lymphoma cells.

PHA-stimulated Lymphocytes. These lymphocytes were kindly made available by Dr. V. P. Eysvoogel, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service.

RESULTS

The serum from 2 of the 3 rabbits immunized with 3 M KCl extract from tonsil lymphocytes showed consistently higher titers when tested on CLL cells than on normal lymphocytes, without any absorption being required. The strongest antiserum (Rabbit 518) was tested in the cytotoxicity test against lymphocytes from 14 healthy donors. As illustrated in Chart 1, 6 of these were negative and, on 5 others, only undiluted serum had a cytotoxic effect. On 2 occasions a titer of 1:5 was seen and, once, a titer of 1:10 was seen. In contrast, titers on 14 samples of CLL cells from 9 patients with leukocyte counts from 7,500 to 130,000/μl varied from 1:20 to 1:80, with only 1 case giving a titer of 1:5. No correlation existed between the cell count and the titer. Two CLL patients in clinical remission after chemotherapy, with leukocyte counts of 3,800 and 4,300/μl, respectively, yielded titers of 1:1 and 1:5. The latter patient had also been tested 2 months previously and, at that time, a cytotoxic titer of 1:80 had been obtained. His leukocyte count then was 7,500/μl mm, having fallen during 3 months of chemotherapy from an initial value of 180,000.

The 2nd antiserum (Rabbit 544) showed the same phenomenon, although the titers on CLL cells were somewhat lower (Chart 1), but the serum of the 3rd rabbit never showed any reactivity.

Since CLL cells are known to show anomalous reactions in the cytotoxicity test (4, 6, 25), rabbit antisera against whole lymphocytes or CLL cells or against other membrane preparations of normal lymphocytes were used as controls. These sera showed no difference in titer on the 2 types of cell. All the following experiments were performed with the

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<th>Cytotox. Titer</th>
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Chart 1. Cytotoxicity (cytotox.) titers of rabbit antisera against 3 M KCl extract from tonsil lymphocytes tested on lymphocytes from CLL patients and healthy controls. •, Rabbit 518, individual blood samples obtained from 9 CLL patients, of whom 5 were tested once, 3 twice, and 1 tested 3 times at 2- to 10-month intervals; x, Rabbit 544, individual blood samples from 4 CLL patients. neg, negative.

antiserum against tonsillar 3 M KCl extract that gave the highest titer on CLL cells.

Lymphocytes from 3 normal and 3 CLL blood samples were tested with the immunoadherence technique, which produced a similar pattern of reactivity. Titers on normal lymphocytes varied between zero and 1:4 and, on CLL cells they varied from 1:128 to 1:256.

To obtain more quantitative data on the difference in the amount of antigen on both types of cell, the antiserum was absorbed with increasing amounts of CLL cells or lymphocytes. A suspension containing different numbers of cells (Chart 2) was added to an equal volume of the inactivated antiserum (diluted 1:5) and incubated for 1 hr at room temperature. Cells were removed by centrifugation, and the absorbed serum was tested in the cytotoxicity test on CLL cells. Although the lymphocytes used for absorption had been previously shown to be negative in the cytotoxicity test, yet they were able to absorb the antiserum. As illustrated in Chart 2, 2 to 4 times as many normal cells were needed to obtain a given level of reduction of the cytotoxic activity. A correction should be applied to account for the larger size of the CLL cells. By measuring the diameter of 100 viable cells of each type, the ratio of their diameters was found to be 1.1. Assuming a spherical shape for both cell types, this yields a ratio of 1.2 for their surface areas, a value too small to apply to the rather inaccurate data obtained from the absorption experiments.

That the antigen is present on normal lymphocytes even when it cannot be detected in the cytotoxicity test was confirmed by immunofluorescence and immunoelectron microscopy. Eleven samples of normal lymphocytes were tested in the indirect immunofluorescence test with antiimmunoglobulin conjugate, and all showed a clear fluorescent staining, including cells that did not react in a simultaneously performed cytotoxicity test. Virtually 100% of the lymphocytes were stained. The type of staining was the same on CLL cells and lymphocytes, a fluorescent ring around the periphery of the cell, that on dilution of the antiserum disintegrated in evenly distributed patches. The titers were higher on CLL cells than on control lymphocytes, but as shown in Chart 3, the average difference is only 2-fold, with an appreciable degree of overlap between the 2 groups. Yet the difference is statistically significant (Wilcoxon test, p < 0.005).

Electron microscopy of cells treated with antiserum and ferritin-labeled antirabbit immunoglobulin showed a distribution of the ferritin in patches of varying size (Figs. 1 and 2). Control cells incubated with normal rabbit serum instead of antiserum were not labeled. The amount of ferritin per cell was rather variable; nevertheless, the CLL cells seemed to be more heavily labeled. As a semiquantitative measure of this difference, the percentage of the cell surface covered with ferritin was measured on photographs of 26 CLL cells and 20 normal lymphocytes. On the average, 30% of the surface of the CLL cells was labeled, whereas for normal lymphocytes the average value was 18%. The distribution of the extent of labeling is shown in Chart 4. Both cell types had an average of 12 ± 6 labeled patches, and the increased labeling of the CLL cells was due to the larger size of the labeled areas, rather than to an increase in their number.

Because of the discrepancy between the results obtained with complement-dependent methods and with techniques without complement, immunofluorescence was also performed with an anti β1C conjugate to detect the binding of complement factors to the cells independent of the cytotoxic effect. Titration of the antiserum in the presence of a
constant amount of complement (20 μl undiluted rabbit serum) yielded a titer of 1:32 on CLL cells, whereas normal lymphocytes were weakly stained only at a 1:2 dilution (Chart 3). Controls with heat-inactivated complement were negative. The reverse experiment, in which a series of dilutions of the complement were tested at antiserum dilutions of 1:2 and 1:4 showed a staining of the CLL cells with complement dilutions up to 1:8. Only the combination of undiluted complement and 1:2-diluted antiserum weakly stained the normal lymphocytes. Particularly in the weak reaction observed with normal lymphocytes, these results resemble those of cytotoxicity and immunoadherence tests, rather than of immunofluorescence with anti-immunglobulin conjugate.

Attempts to increase the expression of the antigen on normal lymphocytes have failed so far. Stimulation with PHA did not increase the susceptibility to the cytotoxic effect. The immunofluorescent staining on the PHA blasts was concentrated in a small area of the cell, in contrast to both CLL cells and unstimulated lymphocytes, which were evenly stained. Treatment with trypsin (0.25 mg/ml) for 20 min at 37°C did not change the reaction with the antiserum. Cells treated with neuraminidase (2 to 10 units/ml) were not viable enough to be used in the cytotoxicity test, but their absorption capacity was very similar to that of untreated lymphocytes.

As it was considered possible that the antiserum reacted with serum proteins adsorbed to the cells, the antiserum was absorbed with human serum. Addition of 10, 25, or 50 mg lyophilized normal human serum per ml antiserum, followed by incubation at room temperature for 60 min, did not affect the titer in cytotoxicity or immunofluorescence tests of either kind of cell. Gel double-diffusion tests did not show a precipitin line between the antiserum and normal human serum or serum from CLL patients.

Lymphocytes from normal blood were separated in T- and non-T-cells to investigate whether the B-cell character of CLL cells is involved in the difference in reactivity. Normal non-T-lymphocytes, mainly B-lymphocytes contaminated with monocytes, isolated as non-E-rosette-forming cells or after lysis of EAC rosettes, yielded cytotoxicity titers of 1:1 and 1:4, respectively. T-lymphocytes obtained after lysis of E rosettes did not react in the cytotoxicity test; when isolated as non-EAC-rosette-forming cells, they gave a titer of 1:4. These titers are comparable to those obtained with unfractionated lymphocytes.

A comparison of tonsil lymphocytes with peripheral lymphocytes could not be performed. Even when the tonsil cell suspensions were more than 85% viable initially, cytotoxicity tests were unsuccessful because negative controls showed unacceptably high levels of stained cells after incubation. The discrimination provided by immunofluorescence was not clear enough to establish whether tonsillar lymphocytes are more similar to CLL cells or to peripheral lymphocytes.

To study the occurrence of the antigen on other cells, immunofluorescence was performed on WBC from normal blood obtained after removal of most of the erythrocytes by sedimentation with dextran. Cells were identified under phase contrast before reading of the fluorescence. Only the lymphocytes were specifically stained by the antiserum. Other cells were unstained except for granulocytes, which showed the same weak aspecific staining with both normal rabbit serum and the antiserum. Among the lymphocytes used for the immunoferritin labeling, an occasional polymorphonuclear cell was seen. These cells were not labeled, again showing the specificity of the antiserum for lymphoid cells.

As shown in Table 1, a number of other lymphoid cells were tested in the cytotoxicity test and found to be similar to normal lymphocytes. For Raji and EB-3 cells this was also found by means of immunofluorescence and absorption experiments.

DISCUSSION

Unexpectedly, antisera against a 3 M KCl extract from tonsil lymphocytes detected an antigen of the membrane of normal lymphocytes that is present on the CLL cell membrane in increased amounts. Results from absorption experiments, immunofluorescence, and immunoelectron microscopy were in reasonable agreement. In these tests, the reaction of CLL cells was approximately twice as strong as that of normal cells. Complement-dependent methods, however, showed a much larger difference in reactivity between the 2 cell types. This phenomenon resembles the cytotoxicity-negative, absorption-positive effect observed with some HL-A typing sera (7, 26). As this larger difference was found in 3 complement-dependent methods based on different reaction mechanisms, it seems unlikely that the larger cytotoxic effect on CLL cells is due to an increased aspecific susceptibility of CLL cells to the conditions of the cytotoxicity test.

It appears that normal lymphocytes, while binding about one-half the number of immunoglobulins, compared with CLL cells, do bind substantially less than the corresponding amount of complement. It may be hypothesized that this is a reflection of a spatial configuration of the antigen on normal cells in which IgG doublets necessary for complement fixation can be formed only to a lesser extent.
Immunoferritin labeling did not show a conspicuously different localization pattern. It is possible, however, that differences in configuration too small to be visualized by ferritin labeling are sufficient to interfere with complement action, or the use of the indirect technique may have changed the distribution.

The possibility may be considered that there is no actual difference in amount of antigen, but that, on normal lymphocytes, part of the antigen is covered or present in an otherwise inaccessible form. This seems unlikely, however, since treatment with trypsin or neuraminidase did not increase the reactivity, nor did stimulation by PHA. Immunofluorescence on PHA blasts revealed a rearrangement of the antigen from an even distribution to a small area, but this was not paralleled by a change in the cytotoxic reaction. Although, on cultured lymphoid cell lines, changes in the amount of HL-A antigens (16, 20) and the occurrence of new antigens (22, 23) have been found, Raji and EB-3 cells were undistinguishable from normal lymphocytes in regard to the antigen described here.

The increased reactivity of CLL cells does not seem to be connected with the B-cell character of these cells. Neither the suspensions of normal lymphocytes enriched in B-cells nor B-cell lines (Raji and EB-3) showed a cytotoxic reaction comparable to CLL cells. Also, the nearly 100% staining in immunofluorescence and the fact that no completely unlabeled lymphocytes were seen in normal lymphocytes in regard to the antigen described here.

The material used to immunize the rabbits was derived from tonsils. Although this does not affect the conclusions drawn from the difference in the reactions of these antisera with CLL cells and peripheral lymphocytes, the question arises whether extracts from tonsils are unique in eliciting these antibodies. However, the number of rabbits immunized with extracts from different cells is too small to solve this problem. One rabbit immunized with a 3 M KCl extract from CLL cells remained negative after 3 courses of immunization, as did 1 of the 3 rabbits that received injections of the extract of tonsil cells. Of 2 rabbits given injections of the same extract of peripheral lymphocytes, 1 produced antibodies detectable by immunofluorescence but no cytotoxic antibodies. The other rabbit had very low-titered cytotoxic antibodies that did react slightly stronger with CLL cells. All rabbits produced antibodies to cytoplasmic antigens, as shown in gel diffusion tests (unpublished data). The apparently low immunogenicity of the antigen, combined with the variable yields of the 3 M KCl extraction, could be the cause of the lack of response of most of the rabbits.

It remains to be determined whether there is any correlation between the phenomenon of increased antigen expression and clinical aspects of the disease. All patients, except one, with leukocyte counts over 10,000 showed cytotoxicity titers of 1:20 or higher, but no correlation between titer and cell count was evident. In only 1 case a dramatic change in reactivity was seen; in a period of 2 months the cytotoxicity titer fell from 1:80 to 1:5, whereas the leukocyte count decreased from 7000 to 4300/cu mm. It is tempting to speculate that the high reactivity found with an already nearly normal cell count may indicate that the cells had still retained malignant properties. A series of patients will have to be followed during chemotherapy to substantiate this hypothesis.

ACKNOWLEDGMENTS

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A. Hekman and M. H. G. Melis

Table 1

<table>
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<th>Target cells</th>
<th>Total</th>
<th>Negative or 1:1</th>
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<td>11</td>
<td>3</td>
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</tr>
<tr>
<td>CLL cells</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes from CLL patients in remission</td>
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<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Normal non-T-lymphocytes</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>Normal T-lymphocytes</td>
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<td></td>
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<td>Raji cells</td>
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<td>2</td>
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<td></td>
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<tr>
<td>EB-3 cells</td>
<td>2</td>
<td>2</td>
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<tr>
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<sup>a</sup> The last dilution yielding 30% or more dead cells.
<sup>b</sup> Fourteen blood samples obtained from 9 CLL patients.
<sup>c</sup> Untreated or in relapse.
REFERENCES


Fig. 1. a, thin section of CLL cell treated with antiserum (diluted 1:10) and ferritin-labeled antiimmunoglobulin, stained with uranyl acetate and lead hydroxide. Forty % of the cell surface is labeled. × 20,000; b, higher magnification of enclosed area in a. × 55,000.

Fig. 2. a, thin section of normal lymphocyte treated with antiserum (diluted 1:10) and ferritin-labeled antimmunoglobulin stained with uranyl acetate and lead hydroxide. Areas labeled with ferritin are marked, together forming 17% of the cell surface. × 20,000; b, higher magnification of enclosed area in a. × 55,000.

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