Surface Bound Immunoglobulins as a Cell Marker in Human Lymphoproliferative Diseases

By J. L. Preud'homme and M. Seligmann

Membrane-bound Ig were studied by immunofluorescence methods in 116 patients with lymphoproliferative disorders. Surface Ig synthetized in vitro were studied in many patients. In 70 of 73 cases of chronic lymphocytic leukemia (49 without and 24 with monoclonal serum Ig), monoclonal surface Ig with a distribution for heavy and light chains similar to that of normal lymphocytes were found. In three cases, these surface Ig accumulated as crystals in the lymphocyte cytoplasm. Studies limited to staining of freshly drawn cells may lead to erroneous conclusions since serum antibodies may become bound to the cell surface and simultaneous positivity for \( \mu \), \( \gamma \), \( \kappa \), and \( \lambda \) due either to the attachment of immune complexes at the lymphocyte surface or to anti-IgG antibody activity of the monoclonal surface IgM, was not rare. IgM with anti-IgG activity was found on the surface of both lymphocytes and lymphoblasts in a patient with acute transformation of chronic lymphocytic leukemia. In a single case, \( \mu \) and \( \gamma \) chains were simultaneously found on some lymphocytes besides two single producer clones. A biclonal proliferation characterized by distinct surface Ig markers was demonstrated in three other cases of lymphocytic leukemia and in a patient with Waldenström's macroglobulinemia. In this latter condition (31 cases), most narrow lymphoid cells including plasma cells and the majority of blood lymphocytes carried IgM determinants which had the same light chain type as the serum IgM and which shared its eventual antibody activity. Similar results were obtained in a few patients with the hematological features of macroglobulinemia but with serum monoclonal IgG or IgA or with unreleased IgM. Studies on cases of \( \gamma \) and \( \alpha \) heavy chain diseases, cold agglutinin disease, leukemic reticuloendotheliosis, and Sézary's reticulosis are also recorded. The value of surface Ig as B cell markers in lymphoproliferative diseases is outlined.

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The presence of membrane bound immunoglobulins (Ig) on the surface of normal lymphocytes is well documented in different species, including man. Although thymus derived (T) lymphocytes presumably carry small amounts of Ig moieties on their surface which are directly detectable only by very sensitive methods, those lymphocytes which bear surface immunoglobulins (S. Ig) in sufficient amounts to be evidenced by rather insensitive techniques such as direct immunofluorescence have been shown in several species to be bone marrow or bursa derived (B) lymphocytes. The results obtained in the study of patients with immunodeficiency diseases lead to similar conclusions.

In our experience and in that of most other investigators, IgM is the main Ig class on peripheral blood lymphocytes of normal individuals. Our figures are the following: 5%-25% positive for μ chains (mean 13), 1%-7% positive for γ chains (mean 4), 0.5%-4% positive for α chains (mean 2); 10%-15% (mean 12.5) and 2%-7% (mean 4) positive for κ and λ chains, respectively.

Previous data obtained from a limited number of patients with lymphoproliferative diseases in this and other laboratories suggested that S.Ig provide useful markers of proliferating B lymphocytes, but the studies have led to somewhat contradictory results. The purpose of the present study was to characterize, by a direct immunofluorescence procedure on living cells, the S.Ig on the lymphocytes of more than 100 patients with lymphoproliferative disorders in order to evaluate their origin, significance and usefulness in the study of hematological diseases.

MATERIALS AND METHODS

Patients

The patients under study were followed in our Institute and were repeatedly submitted to the usual blood and marrow cytological examinations. Lymphangiography and marrow and lymph node biopsies were performed in most patients.

The diagnosis of Waldenström's macroglobulinemia (W.M.) was supported in 31 patients by the finding of pleomorphic lymphoid proliferation in the bone marrow and of a serum monoclonal IgM, with normal or slightly increased blood lymphocyte counts. In four patients the clinical and bone marrow features were similar to those found in W.M. However, in two of these patients the serum monoclonal Ig belonged to the IgG class and in another to the IgA class, whereas in the fourth patient no monoclonal Ig were detectable in serum or urine. The presence of well documented marrow (and lymph node) lymphocytic proliferation, consisting mainly of mature small lymphocytes, was the main criterion for the diagnosis of chronic lymphocytic leukemia (CLL), irrespective of the presence or absence of a serum monoclonal Ig component. These CLL patients had blood lymphocyte counts ranging from normal up to 500,000/cu mm, the majority showing a truly leukemic picture. We first studied patients with classical features of CLL and high lymphocyte counts. Then we selected CLL patients with serum monoclonal Ig, peculiar hematological, or other features which complicated diagnosis. Sixty of the 73 CLL patients who were studied had never received any treatment.

Other patients studied include three patients with heavy γ or α chain diseases, two patients with cold agglutinin disease, two patients with a leukemic form of histiolymphocytosis (or leukemic reticuloendotheliosis), and three patients (referred from the Dermatology Department of the Rothschild Foundation) with Sezary's reticulosis and numerous Sezary's cells in the blood.
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Methods

Serum studies included repeated electrophoretic and immunoelectrophoretic analyses, quantitation of Ig levels for the three main classes by radial immunodiffusion and a systematic search for various autoantibodies (antinuclear factors, anti-γ-globulin factors, Coombs test).

Rabbit antisera to purified κ or λ chains were selected for their reactivity with both Bence Jones proteins and myeloma proteins of the corresponding light chain type. Antisera to purified normal IgG and to monoclonal Ig of the three main classes or their heavy chains were selected for their ability to react both with the Fc fragment and with nonidiotypic determinants located on the Fd segment. The selected antisera to γ, μ, α, κ or λ chains were pooled and rendered monospecific by adsorption on suitable antigens coupled to Sepharose 4B by cyanogen bromide. Specificity was carefully assessed by double diffusion and, after labeling, by immunofluorescence staining of fixed smears of cells from patients with myeloma or macroglobulinemia of known class and type and on living cells of patients with CLL and immunodeficiency diseases. Antisera monospecific for the three heavy-chain classes and the two light-chain types were used in all instances.

The procedures used for the separation of blood lymphocytes and marrow lymphoid cells, for conjugation of the IgG fractions of the antisera with rhodamine or fluorescein, for the detection of S.Ig by staining in the cold of living cells in suspension, and for the labeling of intracytoplasmic Ig on fixed cell smears, have been previously described.15,16,20 In order to study both surface and intracytoplasmic Ig in the same cell preparation, the cells treated with rhodamine labeled antisera were flattened on slides, fixed, and stained again with fluorescein conjugates.

Combinations of antibody induced redistribution and double labeling experiments were used for studying the relationship between different S.Ig molecules. When cells stained in the cold by anti-Ig sera are incubated at 37°C, a polar clustering of labeled S.Ig (cap formation) occurs.11 The cells submitted to this antibody induced redistribution process were cooled at 0°C and stained again by antisera coupled to the opposite fluorochrome, as previously described.22 This procedure is very useful for investigating the molecular relationship of different cell surface determinants.22

To investigate if the S.Ig found on the surface of lymphocytes were or were not actually synthetized by these lymphocytes, the cells were treated with trypsin and incubated in a culture medium for 6–7 hr as previously described.15,23 Control experiments have shown that S.Ig thus detected were indeed Ig molecules synthetized by the cells during the incubation time.23

RESULTS

(1) Chronic Lymphocytic Leukemia

In most of the cases of CLL most of the circulating blood lymphocytes bore Ig molecules on their surface since immunofluorescence study of living cells in suspension showed positive reactions at the lymphocyte surface with antisera to Ig chains. After labeling in the cold, the positive cells were characterized by the presence of numerous small spots regularly distributed all around the cell surface. These positive lymphocytes were easily distinguishable from occasional labeled polymorphonuclears and monocytes by phase contrast examination of each microscopic field and also from killed cells which exhibited homogeneous intracytoplasmic staining. The fluorescence pattern of the positive lymphocytes was usually faint and uniform in a given patient, the number, size and brightness of the spots being very similar on all positive lymphocytes. In contrast, the fluorescence pattern varied from patient to patient. When the cells exhibiting a diffuse membrane fluorescence were
warmed, the staining pattern rapidly changed and a redistribution of labeled S.Ig to one pole of the cells (cap formation) occurred, involving 80%–90% of the positive cells after 1 hr at 37°C. Except in very rare instances (mentioned below) no Ig determinants were detected in the cytoplasm of the S.Ig bearing lymphocytes when fixed cells were studied.

**CLL Without Monoclonal Serum Ig:** The distribution of Ig classes and types on lymphocytes from 49 CLL patients without serum monoclonal Ig is shown in Table 1. In 71% of the cases the determinants of a presumably monoclonal Ig were found at the cell surface. IgM (or its monomeric subunit) was the main Ig class detected on the leukemic lymphocytes and a single light chain type was present (κ in 14 cases and λ in 6). The monoclonal S.Ig belonged to the IgG class in 14 patients and to IgA in a single one. In one of these patients, 28% of blood lymphocytes contained rod-shaped crystalline cytoplasmic inclusions which were brightly stained when the fixed cells were studied with antisera to µ and λ chains. The cytoplasm outside these crystals was unstained. Most lymphocytes in this case, including all those which contained the IgM crystals, carried surface IgM.

In 20% of cases, the simultaneous presence of γ, µ, κ, and λ chain determinants was observed. The reasons for this “mixed staining pattern” will be discussed below.

In one patient, a double proliferation was demonstrated since 1/3 of the blood lymphocytes were shown to carry µ and κ determinants whereas 2/3 bore γ and λ chains. Cytological examination showed that 1/3 of the proliferating cells were lymphoblasts and 2/3 lymphocytes. As these cells were not distinguishable by phase contrast examination, we cannot be certain that the two cell populations identified by their S.Ig marker did in fact correspond to the two populations found on Giemsa stained smears. In contrast to this finding, in another patient with a blood picture showing 75% small lymphocytes and 25% large lymphoblasts the same Ig chains (µ and λ) were seen on most cells including the lymphoblasts which were readily identified by their size. A third patient showed a lymphoblastic proliferation which corresponded to a true change in his disease from chronic to acute. For one year he had had classical CLL characterized by small lymphocytes and a good response to chlorambucil therapy. Then his clinical status suddenly deteriorated and the blood leukocyte count increased to 45,000. At that time, half of the proliferating cells in the bone marrow, lymph nodes, and blood were typical lymphoblasts (Fig. 1). The patient died shortly afterwards in spite of combined chemotherapy. Immunofluorescence study demonstrated the presence of the same IgM κ molecules with a well defined anti-IgG antibody activity (see below) on both lymphocytes and lymphoblasts.

Contrasting with these results, we did not find any positive lymphocytes in the blood from four untreated patients. In one of these cases, with normal blood lymphocyte counts, most bone marrow lymphocytes were positive for a monoclonal S.IgG. In the three other cases, blood lymphocyte counts were moderately but clearly increased and the results of immunofluorescence studies were negative in blood and bone marrow samples which contained numerous...
lymphocytes. The clinical features in one of these patients were unusual in that there were large compressing abdominal lymph nodes and a lymphangiographic pattern similar to that observed in sarcomas. The two other S.Ig negative patients showed no unusual features.

**CLL with Monoclonal Serum Ig:** Twenty-four patients with CLL and a monoclonal serum Ig were studied (Table 2). The levels of the monoclonal Ig were usually low (less than 10 mg/ml) except in one patient whose IgG peak reached 70 mg/ml without evidence of multiple myeloma.

In 7 of 10 patients with a serum IgM peak, a monoclonal IgM with the same light chain type as that of the serum IgM was present on the surface of the lymphocytes. The fluorescence pattern was less uniform than in CLL cases without serum monoclonal Ig. The three remaining cases were characterized by the previously mentioned "mixed staining pattern" (i.e., positivity for γ, μ, κ, and λ on the same cells).

In 11 of 12 CLL patients with a monoclonal serum IgG, immunofluorescence study of the freshly drawn lymphocytes showed S.IgG determinants with the same light-chain type as the serum IgG. The single patient with a high IgG peak and Bence Jones proteinuria (kindly referred by Dr. C. Sultan) showed no evidence of multiple myeloma after four years of follow-up and was greatly improved by chlorambucil therapy. Many blood lymphocytes,
Table 2. Lymphocyte Membrane Ig in 24 Chronic Lymphocytic Leukemia Patients With Serum Monoclonal Ig

<table>
<thead>
<tr>
<th>Serum Monoclonal Ig</th>
<th>Membrane Ig</th>
<th>Biclonal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k</td>
<td>μ</td>
</tr>
<tr>
<td>IgM</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>IgG</td>
<td></td>
<td>1*</td>
</tr>
<tr>
<td>IgA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*S. Ig determinants synthesized in vitro.

60% of the bone marrow lymphocytes, and virtually all of the plasma cells contained intracytoplasmic monoclonal IgG. All those positive lymphocytes also bore S. IgG.

Large lymphocytes with crystalline structures in their cytoplasm (Fig. 2A), surrounded by ergatoplasmic membranes were observed in the bone marrow and lymph nodes from a patient with monoclonal serum IgG k (kindly referred by Dr. Oberling). The blood lymphocytes did not contain such crystals. Staining of marrow fixed cells showed a strong positivity for γ and k chains restricted to the crystals (Fig. 2 B) and the presence of a few plasma cells containing IgG κ Most blood and bone marrow lymphocytes, including those which contained the crystals, carried S. IgG κ (Fig. 2C).

The twelfth patient with a monoclonal serum IgG (of the λ type) is of special interest. Small crystalline inclusions were found in 10%-15% of blood lymphocytes and were shown by intracytoplasmic staining to be positive for μ and λ chains (Fig. 3). By electron microscopy these crystals were seen to be surrounded by ergatoplasmic membranes. In addition 1% of the blood lymphocytes and a few plasma cells contained IgG λ. Double labeling experiments for cytoplasmic and membrane staining showed the presence of μ and λ chains on those lymphocytes which contained the crystals whereas γ determinants were not found on these cells. Surface immunofluorescence staining was performed twice. The blood lymphocyte count was 300,000 the first time and had spontaneously decreased to 90,000 at the time of the second examination 10 days later. The percentages of positive lymphocytes were 51% for μ, 50% for γ, and 95% for λ when first studied and 60%, 80%, and 98% for μ, γ, and λ respectively, at the second examination. Double labeling showed the presence of lymphocytes with surface positivity restricted to γ and λ or μ and λ and also of lymphocytes bearing μ, γ, and λ chains together. Most cells were single producers on first examination whereas more than 50% of the cells were double producers at the time of the second study.

In one patient with untreated CLL and a monoclonal serum IgA κ, without evidence of multiple myeloma, the bone marrow and lymph node plasma cells contained a and κ chains. A small percentage of lymphocytes from the bone marrow, lymph nodes, and blood (maximum 6%) carried a and κ determinants while the majority of lymphocytes were shown to bear μ and λ chains. In a second patient with serum IgA λ, all the features of multiple myeloma were
associated with those of CLL. When this patient was studied, he had received Melphalan and Chlorambucil. He was lymphopenic and his bone marrow no longer showed demonstrable lymphocytic proliferation. Ten per cent of the blood lymphocytes and most of the few bone marrow lymphocytes bore \( \mu \) and \( \lambda \) chains whereas very sparse marrow and blood lymphocytes carried \( \alpha \) and \( \lambda \) determinants which were also present inside all the bone marrow plasma cells.

Trypsin Experiments and Antibody Induced Redistribution: In the 73 patients with CLL, immunofluorescence study of living lymphocytes thus showed monoclonal S.IgM in 39\%, monoclonal S.IgG in 35\%, monoclonal S.IgA in 1\%, a biclonal pattern in 6\%, and a mixed pattern in 19\% of the

Fig. 2. Marrow lymphocytes containing crystalline IgG\( ^{\cdot} \). A, Giemsa stain. B and C, immunofluorescence (anti-\( \gamma \) conjugate). Intracytoplasmic (B) and membrane staining (C).
cases. However, the Ig detected on the surface of the lymphocytes may be different from those Ig actually being synthetized by the cells, as shown by trypsin experiments.

Five patients showing the mixed staining pattern (\(\mu\), \(\gamma\), \(\kappa\), and \(\lambda\)) were studied after removal of S.Ig by trypsin treatment and an incubation in a culture medium for 6–7 hr. Only \(\mu\) and \(\kappa\) determinants were then detected. In three of these five patients, several experiments showed that this newly synthetized IgM was able to specifically bind normal human IgG.\(^{23}\) These experiments were conducted with IgG preparations devoid of any aggregated material since we have found that aggregated normal IgG is able to bind to all normal or leukemic S.Ig bearing lymphocytes.\(^{23}\) Combinations of antibody-induced redistribution and double labeling experiments performed with freshly drawn cells indicated that the binding of normal IgG to the S.IgM

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**Fig. 3.** Blood lymphocytes containing crystalline IgM\(^{\lambda}\). A, Giemsa stain. B, electron micrograph. C and D, immunofluorescence (anti-\(\mu\) conjugate). Intra-cytoplasmic (C) and membrane staining (D).
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also occurred in vivo and was the reason for the mixed staining pattern. These and other findings provided evidence of an actual anti-IgG activity in the monoclonal surface IgM. In contrast, in the two remaining patients with similar mixed staining, the newly synthetized IgM was not able to bind normal IgG, and surface IgG and IgM on freshly drawn cells showed an independent behavior during the antibody-induced redistribution process. In one of these two patients multiple auto-immune abnormalities were found, including a high level of rheumatoid factor. In such cases we are presumably dealing with the attachment of immune complexes to the B lymphocyte surface.

Similar experiments were performed in seven patients with monoclonal S.IgG. In three cases with IgG κ (including two with a monoclonal serum IgG κ) the lymphocytes synthetized γ and κ determinants in vitro. Similarly, IgG λ was shown to be a cell product in two of four cases with S.IgG λ. In contrast, when lymphocytes from the two other patients (one of whom had a monoclonal serum IgG λ) were incubated in culture medium after removal of the S.IgG λ, γ determinants were no longer detectable and the cells carried newly synthetized μ and λ chains. When freshly drawn cells from these two patients were labeled by antiserum to γ chains and subsequently warmed, cap formation occurred on most lymphocytes and previously undetected μ chain determinants were found by double labeling all around the cell surface except at the site of the cap.

In several patients with monoclonal S.IgM and in the single patient with S.IgA, the trypsinized lymphocytes did synthetize the same Ig chains in vitro as found in vivo and, after polar clustering of the S.Ig, the remaining cell surface was not stained by any anti-Ig sera.

**Percentage of Positive Cells:** The percentage of positive blood lymphocytes (Table 3) was greater than 70% in the majority of cases. In all instances the same number of cells was labeled for the heavy and for the light chain in a given patient. Negative cells were present in all blood samples studied and such cells were not distinguishable from the positive lymphocytes by phase contrast examination. When blood lymphocyte counts were higher than 10,000, most of the lymphocytes were usually positive in untreated as well as in treated patients. Less than 30% of lymphocytes were labeled

| Blood Lymphocyte Counts | Negative | Percentage of Positive Blood Lymphocytes*<br>40%<br>50%<br>60%<br>70%<br>80%<br>90% |  |
|-------------------------|----------|---------------------------------|---|---|---|---|---|---|
| <5,000                   | 2†       | 6 (5)                           | 6 (1) | 5 | 3 | 0 |  |
| 5,000-10,000            | 2        | 0                               | 1 | 1 | 3 | 3 (1) |  |
| >10,000                 | 0        | 2 (1)                           | 1 | 2 (1) | 9 | 27 (4) |  |
| Total                   | 4        | 8                               | 8 | 8 | 15 | 30 |  |

†Most bone marrow lymphocytes positive for monoclonal S.Ig in one patient.
*The number of patients under treatment is shown in parenthesis.
Table 4. Immunofluorescence Data in Patients With Marrow Pleomorphic Lymphoid Proliferation

<table>
<thead>
<tr>
<th>Number of cases</th>
<th>Serum Monoclonal Ig</th>
<th>Marrow Plasma Cells</th>
<th>Blood Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytoplasm</td>
<td>Membrane</td>
</tr>
<tr>
<td>30</td>
<td>IgM</td>
<td>Monoclonal IgM</td>
<td>Monoclonal IgM</td>
</tr>
<tr>
<td>2</td>
<td>IgGκ</td>
<td>γκ</td>
<td>γκ</td>
</tr>
<tr>
<td>1</td>
<td>IgAκ</td>
<td>ακ</td>
<td>ακ</td>
</tr>
<tr>
<td>1</td>
<td>none</td>
<td>μκ</td>
<td>μκ</td>
</tr>
<tr>
<td>1</td>
<td>IgM + IgGκ</td>
<td>2 populations containing μκ or γκ</td>
<td>2 populations μκ and γκ</td>
</tr>
</tbody>
</table>

*Mixed staining patterns in three cases.

for the monoclonal marker in patients given Chlorambucil and having normal lymphocyte counts, whereas untreated patients with normal lymphocyte counts showed low or high percentages of positive lymphocytes. As a whole the percentage of positive blood lymphocytes was correlated with the lymphocyte counts (p < 0.01). Even when the positive cells represented a limited percentage of lymphocytes, the figures were clearly abnormal since the positivity was practically restricted to the determinants of one heavy and one light chain. In most cases, lymphocytes bearing Ig specificities other than those of the monoclonal S.Ig were very rare. Normal absolute numbers were found in only three patients who also had normal serum Ig levels.

(2) Waldenström's Macroglobulinemia and Related Conditions (Table 4)

Thirty-one patients with W.M. were studied and the results confirmed our previous data. Intracytoplasmic staining for monoclonal IgM was restricted to the bone marrow plasma cells and to a limited number of bone marrow and blood lymphocytes (except for a single case where 30% of lymphocytes contained detectable intracytoplasmic IgM). However most bone marrow proliferating lymphoid cells, including plasma cells, and 10%-80% (mean 50%) of blood lymphocytes from untreated patients exhibited a surface IgM with the same light-chain type as the serum IgM. When the proliferation was controlled by therapy, a low percentage of blood lymphocytes (mean 9%) carried the monoclonal S.IgM. The fluorescence pattern was strikingly heterogeneous in every patient. Control experiments ruled out the possibility of a passive coating of serum IgM on the lymphocytes and proved the actual synthesis of S.IgM by the cells.

A mixed staining pattern was observed in three patients with W.M. Studies of the newly synthetized S.IgM were undertaken as described above in one of these three patients because of the known anti-IgG antibody activity of the serum monoclonal IgM. The results clearly showed that the IgM molecules present at the cell surface shared the antibody activity of the patient’s serum IgM.

Similarly, the results obtained in two patients with cold agglutinin disease were suggestive of an antibody activity of the S.IgM. The patient’s sera
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contained a monoclonal IgM \( \kappa \) exhibiting the antierythrocytic antibody activity. The same Ig determinants were found on 60% of the blood lymphocytes in both cases and clumping of red cells occurred in vitro around a similar percentage of lymphocytes.

An additional patient with W.M. and a serum IgM \( \lambda \) had been followed and treated for several years when a second serum monoclonal Ig (IgG \( \kappa \)) appeared. In the bone marrow, two populations of plasma cells containing either \( \kappa \) and \( \lambda \) or \( \gamma \) and \( \kappa \) chains were found by intracytoplasmic staining. Surface immunofluorescence demonstrated two distinct lymphocyte clones in bone marrow and blood characterized by \( \mu \lambda \) and \( \gamma \kappa \) S.Ig markers, respectively.

Three patients showed a pleomorphic lymphoid proliferation in their bone marrow quite similar to that seen in W.M. but had a serum monoclonal Ig belonging to the IgG (two cases) or IgA (one case) class. The bone marrow plasma cells and a small percentage of lymphocytes contained intracytoplasmic IgG or IgA whereas 30%–60% of blood lymphocytes and most bone marrow lymphocytes carried the corresponding monoclonal S.Ig. The findings thus differed from W.M. only by the heavy chain class of the Ig and by the absence of S.Ig on plasma cells.

A presumptive diagnosis of W.M. was considered in a patient with the usual clinical, cytological, and histological features of this disease. However, neither a monoclonal serum Ig nor free \( \mu \) chains could be demonstrated in serum or urine. IgM \( \kappa \) molecules were found in the cytoplasm of 13% of the bone marrow cells including lymphocytes and 3% typical plasma cells, some of which showed positive intranuclear inclusions and some a morular cell pattern. In the surface immunofluorescence experiments, 57% of the blood lymphocytes were brightly stained by antisera to \( \mu \) and \( \kappa \) chains. These data suggest a "nonsecretory macroglobulinemia."

It should be emphasized that, as in CLL, very few lymphocytes carrying other Ig specificities than those of the monoclonal S.Ig were found in these conditions with bone marrow pleomorphic lymphoid proliferation.

(3) Miscellaneous

The bone marrow of two patients with \( \gamma \) heavy-chain disease contained a number of plasmacytes and lymphoid cells that showed intracytoplasmic immunofluorescence when stained by anti-\( \gamma \) but not by anti-light-chain reagents. Surface staining of the bone marrow and blood cells showed a decrease in Ig bearing lymphocytes but cells carrying \( \gamma \) chains without light chains could not be demonstrated. One of these patients was studied again during a complete clinical remission when the abnormal protein was no longer detectable in serum.\(^{25}\) At that time, the bone marrow contained a normal number of plasma cells with a normal distribution of heavy and light chains and the percentages of blood lymphocytes bearing the different heavy and light chains were in the normal range.

The cells from a surgically removed mesenteric node of a patient with \( \alpha \)-chain disease were studied. No Ig chains could be demonstrated at the surface of the numerous lymphoid cells and plasma cells which contained intracytoplasmic \( \alpha \) chains without detectable light chains.
In two patients with leukemic reticuloendotheliosis, two cell populations (lymphocytes and histiocyte-like cells) were seen by phase contrast microscopy. The histiocytes were stained by each of the five antisera to Ig chains and by rhodamine labeled polymeric human IgG. This finding was not further explored. No monoclonal lymphocytic population was found since the percentages of lymphocytes positive with each antiserum were at the lower limit of the normal ranges.

The patients with Sezary’s reticulosis were studied since recent data suggest that Sezary’s cells may be proliferating T lymphocytes. These easily distinguishable large cells were not stained by any reagent and the only abnormality was a moderate decrease of S.Ig bearing lymphocytes.

DISCUSSION

The results presented in this paper clearly show that surface immunoglobulins provide useful markers in the study of lymphoproliferative disorders. Our data provide evidence that the proliferative process affects B lymphocytes in W.M. and related conditions, and in most if not all cases of CLL Indeed, lymphocyte populations characterized by S.Ig markers were found in all W.M. and in 70 of 73 CLL patients. This finding correlates with several other observations. Phytohemagglutinin responsiveness is presently considered as a T cell property and most CLL and W.M. patients show a poor and/or delayed response when the blood lymphocytes are reacted with this mitogen. There is some correlation between phytohemagglutinin unresponsiveness and lymphocyte counts. Similarly the present data show a correlation between lymphocyte counts and the percentages of S.Ig bearing cells. The normal phytohemagglutinin responses which have been observed in treated patients with normal lymphocyte counts may be related to the low percentages of S.Ig bearing cells which were found in such patients. The ability of leukemic lymphocytes to bind polymeric IgG is in agreement with their B cell nature since B lymphocytes have been shown to possess surface receptors for IgG. Moreover the presence of the complement receptor, known to be a B cell feature, has recently been demonstrated on leukemic lymphocytes.

The possibility of a T lymphocyte proliferation should be considered in the 3 CLL patients whose lymphocytes did not bear detectable S.Ig, particularly in the one with peculiar clinical features. However before reaching this conclusion one should recall that in most CLL patients the immunofluorescence of positive lymphocytes was strikingly fainter than that observed in normal subjects. If the amount of S.Ig increases during the maturation process of B lymphocytes, proliferating B lymphocytes blocked at an early stage of maturation (see below) could carry subthreshold amounts of S.Ig, and this could account for the negative cases.

The finding that S.Ig of the proliferating B cells are usually restricted to a single light-chain and heavy-chain class strongly suggests that we are dealing with a monoclonal proliferation. That these S.Ig are monoclonal in nature is evidenced by the presence in the serum of all W.M. and many CLL patients of a monoclonal Ig similar to that found on the positive cells. The
demonstration in several cases of a defined antibody activity of the S.Ig synthetized in vitro on most of the lymphocytes (reflecting identical Ig variable regions) is further strong evidence that the S.Ig in CLL are structurally homogeneous.

In all of the patients studied we found both a heavy and a light chain on the surface of the positive lymphocytes. We were thus unable to confirm the finding of Grey et al., who detected only light chains on the lymphocyte surface in several CLL patients. We found no correlation between the immunochemical type of S.Ig and other CLL features such as lymphocyte counts, extent of the lymphocytic proliferation, disease activity, and serum polyclonal Ig levels.

IgM was the predominant heavy-chain class that was identified on the cells of CLL patients. Thus the distribution of heavy and light chains among our CLL patients clearly reflects the distribution of S.Ig on normal lymphocytes. It is analogous to the well-known situation in multiple myeloma where the incidence of various monoclonal serum Ig closely parallels the distribution of Ig in normal serum.

The incidence of S.IgM in CLL when the study is limited to immuno-fluorescence on freshly drawn cells is obviously an underestimate since we have shown by trypsin experiments that, in some instances of IgG positivity, and in all studied cases with the mixed staining pattern, the cells did in fact synthesize an S.IgM.

In two of the seven cases with IgG positive cells which were submitted to trypsin treatment, we have shown the actual synthesis of an S.IgM. We can conclude from the antibody induced redistribution experiments that these IgG molecules were not bound to the IgM receptors. The behavior of these IgG during the redistribution process was similar to that of antibodies directed against surface antigenic determinants and differed from the behavior of antigen-antibody complexes. The nature of the surface antigenic determinants which react with these IgG antibodies is unknown. We are currently attempting to establish if leukemic antigens are involved. In view of the high incidence of a positive Coomb’s test in cases of CLL with S.IgG, it is conceivable that these patients produce antibodies directed against a blood group antigen common to erythrocytes and lymphocytes.

Freshly drawn lymphocytes showed the simultaneous presence of μ, γ, κ, and λ determinants on the cells of 16 patients (13 with CLL and 3 with W.M.). Further experiments performed in six of these patients proved that this mixed staining pattern can be considered as a false polyclonal appearance since only μ and κ chains were detected on the cell surface after removal of S.Ig either by trypsin or after antibody induced redistribution followed by incubation for 6 hr. These results, showing that the actual cell product was monoclonal, should be considered when interpreting some previously published data. Moreover, in four of the six patients thus studied, we have demonstrated that the mixed staining pattern was due to an anti-IgG antibody activity of the S.IgM receptors. The incidence of this anti-IgG activity of membrane bound IgM appears to be relatively high among CLL patients. It is analogous to the strikingly high percentage of serum monoclonal IgM
possessing this antibody activity in W.M. It is noteworthy that two of the CLL patients with anti-IgG activity of S.IgM showed neither an Ig spike nor detectable rheumatoid factor activity in their serum.

The mixed staining pattern is not always due to an anti-IgG activity of the IgM receptor and in two CLL patients it was probably related to the attachment of immune complexes to the leukemic lymphocyte surface.

The single exception to the rule of class restriction of S.Ig in B lymphoproliferative disorders was the patient in whom some lymphocytes carried \( \mu, \gamma, \) and \( \lambda \) chains. This patient showed also a biclonal proliferation (see below) with either \( \mu \lambda \) or \( \gamma \lambda \) markers. Since normal lymphocytes may carry more than one heavy-chain class in the early stages of the immune response, whereas class restriction occurs in the later steps, this double producer clone may well represent the proliferating clone from which the single producer clones originated.

In view of the results of the present study, W.M. and CLL should be considered as closely related disorders both characterized by a monoclonal B lymphocyte proliferation but differing in the degree of maturation of B lymphocytes into plasma cells.

The present data strongly suggest that the pleomorphic lymphoid proliferation characteristic of W.M. represents the proliferation of a clone of B cells which continues to mature and differentiate into IgM secreting plasma cells. It is thus not surprising that the S.IgM shares the eventual anti-IgG antibody activity of the secreted serum IgM, and a similar situation appears likely in cold agglutinin disease. It is worth noting that S.IgM persists on IgM secreting plasma cells, as already shown in normal rabbit. The presence of S.Ig on IgM plasma cells may be of interest with respect to the chronology of the immune response. Conversely, we did not find S.Ig on the plasma cells of some of the studied cases of classical IgG or IgA myeloma, nor in the three cases of pleomorphic lymphoid proliferation with serum monoclonal IgG or IgA herein reported. These latter cases demonstrate that the syndrome of B cell proliferation with persistent maturation into plasma cells is not restricted to macroglobulinemia and can also occur for IgG or IgA carrying lymphocytes. Another situation, i.e., "nonsecreting macroglobulinemia," can also occur in this syndrome since the present study includes a patient with all the features of W.M., including the results of the immunofluorescence studies, but with no monoclonal serum IgM. This situation is similar to that of nonsecreting myeloma where we have previously demonstrated the presence of an apparently unreleased intracytoplasmic monoclonal Ig.

It should be emphasized that a high number of blood lymphocytes bearing the monoclonal marker was found in all untreated patients with W.M. or related conditions. Despite the usual absence of an increase in blood lymphocyte counts, these conditions can, therefore, be considered as almost a leukemic process. It is noteworthy that only the less differentiated cells appear to circulate.

As in W.M., the vast majority of blood and bone marrow lymphocytes from most patients with CLL synthesize monoclonal S.Ig. In the most common situation, without monoclonal serum Ig, the homogeneous Ig present on
the surface of the lymphocytes is detected neither in the rare bone marrow plasma cells nor in serum. These data and the strikingly homogeneous fluorescence pattern in a given CLL patient are consistent with the hypothesis of a monoclonal B lymphocyte proliferation with a block in the maturation process.\textsuperscript{10} It should be noted that such a block has recently been demonstrated in another situation, i.e., some cases of primary immunodeficiency.\textsuperscript{34,35}

Conversely the persistence of some degree of maturation appears likely in most cases of CLL with a monoclonal serum Ig since a monoclonal Ig characterized by the same light and heavy chain is found in the serum, in a few plasma cells and on the lymphocytes. This situation does not hold in those cases where the monoclonal lymphocyte Ig marker differs from the monoclonal serum Ig. In these latter cases, the monoclonal serum Ig is not directly related to the lymphocytic proliferation and may result from the conjunction of antigenic stimulation and a state of immunodeficiency usually observed in CLL.\textsuperscript{36} In one of our patients, the possibility exists that the monoclonal serum IgG is an antibody against leukemic lymphocytes since IgG molecules were bound to the lymphocyte surface but were independent of the IgM receptors.

In all but four cases of CLL with or without monoclonal serum Ig, intracytoplasmic Ig could not be demonstrated in those lymphocytes carrying S.Ig. This negative finding is probably due to the lack of sensitivity of the immunofluorescence technique on fixed cells, this procedure being less sensitive than the surface staining because of its unavoidable background. Exceptions to this rule were observed in a patient with a high level of monoclonal serum IgG in whom the cytoplasm of numerous lymphocytes was stained and in the three patients with intracytoplasmic crystalline inclusions.

Of particular interest is the finding of a biclonal proliferation in five patients. As shown in Table 5, various patterns can be found in these biclonal cases with regard to monoclonal serum Ig reflecting persistent clonal differentiation: absence of any homogeneous serum component, presence of a single monoclonal serum Ig corresponding to one of the proliferating clones or the presence of a biclonal pattern in the serum corresponding to both

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Lymphocyte S-lg Markers</th>
<th>Intracytoplasmic Staining</th>
<th>Serum Monoclonal Ig</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CLL</td>
<td>γ λ + μ κ</td>
<td>Negative</td>
<td>none</td>
</tr>
<tr>
<td>2</td>
<td>CLL Ig</td>
<td>μ λ + α κ</td>
<td>α κ (plasma cells)</td>
<td>IgA κ</td>
</tr>
<tr>
<td>3</td>
<td>CLL +</td>
<td>μ λ + α λ</td>
<td>α λ (plasma cells)</td>
<td>IgA λ</td>
</tr>
<tr>
<td></td>
<td>multiple myeloma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CLL</td>
<td>μ λ + γ λ + μ γ λ</td>
<td>γ λ (lymphocytes and plasma cells) + μ λ (crystals in lymphocytes)</td>
<td>IgG λ</td>
</tr>
<tr>
<td>5</td>
<td>W.M.</td>
<td>μ λ + γ κ</td>
<td>μ λ + γ κ (plasma cells and some lymphocytes)</td>
<td>IgG κ + IgM λ</td>
</tr>
</tbody>
</table>

Table 5. Summarized Data in Five Patients With Biclonal Lymphoid Proliferation
lymphocyte clones. Whereas in three patients, the two S.Ig markers differed in both their heavy and light chains, both S.Ig markers shared λ light chains in the two other patients with biclonal proliferation. This finding suggests that the two proliferating clones could have been derived from a common clone. This situation is similar to that observed in some instances of serum biclonal Ig.\textsuperscript{37,38} One of these patients was simultaneously affected with chronic lymphocytic leukemia and multiple myeloma.

In one of the cases of biclonal proliferation (case 1 of Table 5), the blood picture showed both lymphocytes and lymphoblasts and these two populations were possibly characterized by different S.Ig markers. Conversely in the patient affected with a true acute exacerbation and lymphoblastic transformation of CLL (an extremely rare event), the lymphoblastic population certainly belonged to the same clone as the lymphocytic population since both types of cells carried on their surface an IgM λ exhibiting anti-IgG antibody activity.

In closing, it is worth emphasizing that, in addition to its great interest for a better classification and understanding of lymphoproliferative diseases, the search for membrane bound Ig is, in our experience, of undoubtable value for the diagnosis of CLL at a relatively early stage.

\textit{Note Added in Proof:} In view of the presence of a monoclonal S.Ig on the “lymphoblasts” of three CLL patients herein reported, we have studied with Dr. G. Flandrin, three patients (two children and a young adult) having a tumoral form of “acute lymphoblastic leukemia” and a peculiar appearance of the leukemic cells similar to those found in nodes from patients with lymphoblastosarcomas (lymphosarcoma cell leukemia). In all three cases, the blast cells carried a monoclonal S.Ig (IgG kappa, IgM kappa and IgM lambda, respectively) and the in vitro synthesis of these S.Ig was proven in the single case where trypsinization experiments were performed. Such rare cases, usually labeled as acute lymphoblastic leukemia, presumably correspond to a B cell proliferation.

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