Individual Cell-by-Cell Quantitation of Lymphocyte Surface Membrane Ig in Normal and CLL Lymphocytes and During Ontogeny of Mouse B Lymphocytes by Immunoperoxidase Assay

By Guillaume Dighiero, Enrique Bodega, Rosa Mayzner, and Jacques Louis Binet

A new quantitative immunoperoxidase method is presented for determining absolute amounts of peroxidase and, consequently, surface antigen densities of individual cells in B lymphocytes from normal individuals, from subjects with CLL and prolymphocytic leukemia, and during ontogeny of B lymphocytes in the mouse. The following results were observed: (1) The density of B antigenic sites was lower on CLL than on normal B lymphocytes. (2) The B antigenic density of leukemic lymphocytes varied less from cell to cell, forming a homogeneous peak on histograms. (3) In a very rare case of CLL, the antigen density was measured at the time of initial diagnosis (22,500 sites or 647 U) and during the development of a blastic crisis (135,000 sites or 2576 U). The cell by cell distribution changed from a homogeneous peak with a low number of antigenic sites per cell to a heterogeneous peak with a high number of antigenic sites per cell. (4) In prolymphocytic leukemia, the density of B antigenic sites was greater than on normal B lymphocytes and much more heterogeneous than on CLL lymphocytes. (5) During ontogeny of B lymphocytes in the mouse, maturation is associated with the appearance of a population of cells of intermediate to high SmIg density. The finding of a decrease in, and altered distribution of, surface markers in CLL is compared with these ontologic findings in the mouse, and the concept that a monoclonal B lymphocyte in CLL may be arrested at a particular stage in its differentiation is discussed.

In previous studies we have quantitated the surface immunoglobulins (SmIg) of normal human lymphocytes, of lymphocytes in chronic lymphocytic leukemia (CLL),1,2 and of splenic B lymphocytes in the Swiss mouse from birth up to the first month3 by the immunoperoxidase method.

We have established that: (1) the B lymphocytes that proliferate in CLL are not only monoclonal but also exhibit a very marked decrease in the number of antigenic membrane sites;4 (2) in “prolymphocytic leukemia” (PL), defined according to Galton et al.,4 and in very rare cases of acute transformation of CLL, the SmIg level observed is considerably greater than that found in classical CLL;5 (3) maturation of the B lymphocyte in the Swiss mouse is accompanied by an increase in SmIg density and the adult level is only attained between the second and third week.6

The technique employed in these studies permitted only a global measurement of all cells present, and the results obtained represented the mean of the whole cell population.6

By the same method, using a Leitz Classimat and a Zeiss microspectrophotometer, we have been able to measure SmIg on a cell-by-cell basis in normal subjects, in subjects with CLL and PL, and on the B lymphocytes of the Swiss mouse during ontogenesis.

The results of this study form the basis of this article.

MATERIALS AND METHODS

Patients

Peripheral blood lymphocytes from 4 normal controls and 8 patients with lymphoproliferative disorders (6 CLL: 1 stage 0, 2 stage I, 1 stage II, and 2 stage III according to Binet et al.7 and 2 prolymphocytic leukemias) were studied. All patients were investigated prior to treatment, and one case was studied both at the time of initial diagnosis and 9 mo later when a blastic crisis was diagnosed on the basis of clinical and cytologic criteria.

Animals

Conventionally raised Swiss mice (OFI strain) were obtained from IFFA CREDO laboratories, Fresnes, France. The pregnant females were housed in individual cages and the day of delivery (D-1) noted for each litter.

Antibodies

Purified anti-kappa and anti-lambda antibodies, coupled to peroxidase according to a method previously described, were used against human lymphoid cells.14 Specific peroxidase-labeled anti-mouse immunoglobulin was obtained from the Pasteur Institute. The antibodies used were tested by immunodiffusion to ensure that they reacted specifically with the κ, λ, γ, and μ chains of mouse immunoglobulin.

Isolation and Purification of Lymphoid Cells

Human lymphoid cells were isolated either simply by sedimentation or by centrifugation on a Ficoll-Triosil gradient. Residual polymorphs and monocytes were removed by incubation with carbonyl iron. Contaminating red blood cells were lysed by incubation in a 0.87% solution of ammonium chloride.

Spleens from newborn (1-2 days, 4 days, 9 days, 15 days, 21 days, and 28 days) and adult mice were rapidly removed under sterile conditions following sacrifice of the animals by decapitation and were gently separated in the Tenbroeck in vitro tissue crusher (2 ml). After filtration through a scoured metallic mesh, the cells were washed twice in Eagle’s medium (Pasteur Institute, Paris) at pH 7.4.

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and purified bovine serum albumin was added (final concentration 5%). The red blood cells were lysed by incubation with 0.87% ammonium chloride for 20 min at 4°C. Mouse cell suspensions were prepared by pooling cells from between 3 (adult) and 50 (1–2 day old mice) spleens. Before incubation with antibody, viability of the cell suspensions was determined by trypan blue exclusion, cell death never exceeding 10%.

**Incubation With Antibody**

Cells were washed in Eagle’s medium, pH 7.4, and purified bovine serum (final concentration 5%). The concentration was adjusted and the cells were divided into aliquots of 30–50 x 10⁶ cells/ml/tube. Human lymphoid cells were incubated for 1 hr at 4°C with 0.5 ml of antibody solution (anti-kappa and anti-lambda at a final concentration of 0.125 μg/ml) per tube. Control studies were performed by incubating the same number of cells with 0.5 ml of a mixture of anti-kappa and anti-lambda antiserum (0.125 μg/ml) under the same conditions in complement-depleted human serum.

Mouse cells were divided into 2 aliquots of 30 x 10⁶ cells and incubated for 1 hr at 4°C with 0.5 ml of peroxidase-labeled antimouse antibody, at a final concentration of 0.125 mg/ml. Negative controls were performed by incubating duplicate cultures of 30 x 10⁶ cells with 0.5 ml of peroxidase-labeled anti-mouse immunoglobulin (0.125 mg/ml) in complement-depleted mouse serum.

**Quantitation of Peroxidase Specifically Bound to Lymphocytes**

Following coupling, excess antibody in the supernatant was removed by washing four times, and the supernatant tested after the last washing to ensure the absence of peroxidase activity. The cells were then treated in two different ways.

**Spectrophotometry.** Peroxidase bound to the cell surface was revealed by orthodianizidine, a soluble substrate of peroxidase, and quantitated by spectrophotometric assay.⁸ Non-specific peroxidase activity was determined from the supernatant of tubes incubated with control reagents.

**Light microscopy.** SmIg-positive cells were determined after revealing the membrane-bound peroxidase-labeled antibodies with 3-amino-9-ethyl carbazole. An aliquot of 5 x 10⁶ cells was placed for 10 min in a solution of 3-amino-9-ethyl carbazole (2 mg) dissolved in 0.5 ml dimethyl formamide plus 9.5 of 0.05 M acetate buffer, pH 5, plus 1 drop of 3% hydrogen peroxide. The cells were then washed twice in phosphate-buffered saline (PBS), adjusted to a concentration of 300/cumm, and 0.2 ml of this suspension was centrifuged at 1000 rpm for 3 min in a Shandon E100 centrifuge. The slides were then washed twice with PBS, dried, mounted, and examined by light microscopy.

The mean number of antigenic sites per cell is easily calculated knowing the absorbence recorded on spectrophotometry (related to a standard peroxidase curve) and the percentage of SmIg positive cells.

**Cell-by-Cell Quantitation**

The SmIg-positive cells thus obtained were studied individually by two methods:

1. **The Leitz Classimat** is an instrument that has long been used in image analysis. The receptor is a Plumbicon tube with a linear response curve. The television image is composed of 625 lines. Discrimination selection is achieved by means of 10 bands ranging from black to white, and the intensity of peroxidase staining is sufficient to distinguish it from the rest of the cell. The surface marking was counted cell by cell at a 100x magnification, each cell being isolated by a mask. The results were fed into a computer, which automatically printed a histogram of 500 cells from each specimen.

2. **The Zeiss microspectrophotometer.** Cells were also analyzed using the Zeiss Automat Scanning Microspectrophotometer at 100x magnification with a wavelength of 490 nm and a scanning spot of 0.5 μ. The results were fed into a Wang computer.

**RESULTS**

Initial attention was directed to the comparison of the means and distributions given by the two instruments, and similar results were obtained (4 CLL, 1 PL, 10-day old mice, and adult mice): the mean showed a coefficient close to 2 (2.18; range 1.87–2.69) and the distribution histograms were very similar. On the basis of these findings, it was concluded that the results provided by the two instruments were comparable, and the remainder of the study was performed with the Leitz Classimat. The results obtained were expressed in optical units and corresponded to the digital expression of the optical activity detected by the machine for each cell. They are of course arbitrary units. The distribution histograms were plotted from the measurement of 500 cells. Concurrently, all the patients included in this study were assayed by spectrophotometry. This method provides a statistical appraisal of the overall population, and its results were expressed in terms of mean antigenic sites per cell.

In the 6 cases of CLL, activity varied between 25 and 1200 U. Categories of 100 U were thus established (Fig. 1). Maximum activity was between 100 and 560 U in nine patients, between 100 and 300 U in three patients, and between 500 and 600 U in the sixth patient. In these six patients, there seemed to be no correlation between the distribution obtained and the anatomicoclinical stage of the disease or the degree of lymphocytosis.

In the 4 normal control subjects, the activity detected varied between 400 and 2900 U. The maximum peak was 700 in 2 subjects, 1400 in the third, and 1500 in the last, although a certain number of lymphocytes exhibited as little immunoglobulin as those in CLL. (Fig. 2).

The distribution obtained was very different from that observed in CLL. In CLL, SmIg densities varied less from cell to cell, forming a histogram with an homogeneous peak; in the normal subjects the density detected varied in a much more heterogeneous fashion.

The results of the 6 cases of CLL and the 4 normal controls are combined in Fig. 2. While the surface densities in CLL ranged from 100 to 1300, with a peak between 100 and 300, they ranged from 400 to 2800 in the normals, with a peak between 700 and 1600. The homogeneous appearance in the CLL subjects...
contrasted with the heterogeneous aspect in the normals.

One case of CLL (case no. 1 ) was studied at the time of diagnosis and again 9 mo later at the time of a clinical deterioration when, on cytologic examination, more than 50% of the cells were overtly blastic in appearance. The diagnosis of a blastic crisis supervening in CLL was made at this time. The mean SmIg quantitation revealed 22,500 sites (or 647 U) at the time of initial diagnosis of CLL and 135,000 sites (or 2576 U) during the blastic crisis. This pattern confirmed our previous descriptions, but this was the first time we were able to test a patient both before and after a blastic crisis. Cell-by-cell quantitation confirmed this increase (Fig. 3) and revealed a greater heterogeneity in SmIg distribution.

In the two cases of PL, activity ranged from 500 to 3300 U with a maximum of 2100 in one case, and from 1900 to 4000 U with a maximum of 2900 in the other. The distribution of SmIg density was less uniform than in CLL (Fig. 4).

Distribution of SmIg in the mouse (Fig. 5) shows that (A) on the first day activity ranged from 100 to 1200 U with a peak between 400 and 600; (B) on the fourth day, the peak was between 400 and 500, but a small percentage of cells with a density of between 1200 and 1700 had appeared; (C) on the ninth day, the aspect was more heterogeneous and an increase in size of the population with a density of 1200–1700 was observed; (D) from the 15th day on, the distribution of SmIg density was comparable to that of the adult mouse and of normal humans.

### Table: Clinical and Immunologic Data

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<th>Case</th>
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<th>Lymphocyte Count</th>
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<th>Spleen Size</th>
<th>Leukocytes</th>
<th>Platelets</th>
<th>Prior Treatment</th>
<th>Follow Up Stage</th>
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| A | B | C | D |

**Fig. 1.** Clinical and Immunologic data for CLL patients and normal control donors.
Fig. 2. Cumulative cell-by-cell distribution of SmIg from 6 CLL patients and 4 normal control donors. The mean activity in optical units was 367 for CLL patients and 1377 for controls. Note the homogeneous pattern in CLL as compared to the heterogeneous pattern of control donors.

Fig. 3. Cell-by-cell distribution of SmIg at the time of initial diagnosis (2/78) and during blastic crisis (11/78). At the time of initial diagnosis the mean activity was 647 U and the pattern was homogeneous as in the other CLL patients. During blastic crisis, the mean activity detected by 2567 U and the pattern of distribution appeared much more heterogeneous.
DISCUSSION

These results present a number of problems regarding the techniques of cell marking and the interpretation of the results in terms of maturation of the B lymphocyte.

The immunoperoxidase method permits very accurate determinations, theoretically at the molecular level, and provides at least the same degree of accuracy as the antiglobulin consumption test, the isotope antiglobulin precipitation inhibition test, and the hemagglutination technique. It represents a simple and reproducible technique for the quantitation of antigens at a cellular level in whole populations. The immunoperoxidase method allows rapid quantitation, since the determination and counting of positive cells are performed in a single step. Furthermore, this technique facilitates examination by light microscopy and renders electron microscopy possible. The novel feature of this report is the possibility of quantifying antigenic binding sites on individual cells by this technique.

This method may be compared with other techniques that have been used for cell-by-cell immunoglobulin quantitation. The most widely employed of these is quantitative immunopaautoradiography at the cellular level; but despite the increasing use of autoradiography, all attempts to determine quantitative relationships between the number of grains and the amount of isotope present on the surface of the cells remain problematical because of the physical properties of iodine-125. Thiel al. overcame this problem by the use of standard sources of radioactivity exposed together with the specimen on the same autoradiograph. Nevertheless, this method is still very cumbersome and difficult to apply.

Sandquist et al. described another method, microfluorometry, which permits the investigation of dynamic features of surface antigens, but according to the authors' own indications, the method is restricted to a low number of measurable cells.

Rapid-flow microfluorometry permits rapid quantitation on a cell-by-cell basis. It is certain that this method has considerable potential for future applications, but it is difficult to compare these two methods which are based on very different procedures.

We have previously shown that during ontogenesis, maturation of the mouse B lymphocyte is accompanied by a decrease in mean SmIg density. Our cell-by-cell results confirm these data and suggest that the phenomenon is related to the appearance of an increasing percentage of cells having a greater SmIg density.

Unlike many other mammalian species, humoral immune competence against numerous antigens in mice is acquired only after birth, towards the end of
the third week. The density of surface Ig on individual B lymphocytes is thus critical to the understanding of antigen–B-lymphocyte interaction and activation. A number of investigations, some conflicting, on the development of B cells in the mouse spleen and on the evolution of SmIg density with age during the neonatal period have been reported. Osmond and Nossal \(^5\) concluded that a substantially greater heterogeneity in the amount of Ig per B cell existed in the immature than in the mature population, whereas Sidman and Unanue \(^6\) indicated that immature cells have, on the average, more Ig than mature cells. Scher et al. \(^7\) using rapid-flow microfluorometry, detected a large population possessing a relatively low to intermediate density of surface Ig in the spleen and lymph nodes of adult mice.

Our results contradict those of Osmond and Nossal, since we found a greater dispersion in adult than in neonatal mice. In contrast to Sidman and Unanue, we found that immature cells have, on the average, less Ig than mature cells. In agreement with Scher et al. we observed that B cells in the adult mouse are more heterogeneous and that, in addition to a high density population, there is also a population of low density. However, in contrast to these authors, we have found that this latter population is the only one present in neonatal mice and have observed the progressive appearance of a population of intermediate to high density during maturation. These differences may be explained by the different techniques used in these studies. Sidman and Unanue and Osmond and Nossal employed autoradiography, while Scher et al. used
microfluorometry and we used an immunoenzymatic method. The presence of a genetic factor controlling lymphocyte SmIg density may provide a further explanation for the discrepancy observed, since Sidman and Unanue employed C57BL/6 mice while we worked with Swiss mice. However, the results of Scher et al. using C57BL/6 mice differ from those of Sidman and Unanue.

It is now recognized that the vast majority of cases of CLL represents a B-cell disorder. The homogeneous character of SmIg and the restriction to the simple light and heavy chain, with the exception of IgD, suggest that the leukemic proliferation of B cells affects a single clone.\(^2\)\(^3\)\(^4\)\(^5\) In previous quantitative reports, we have also shown that these lymphocytes have a very low SmIg density compared with normal B lymphocytes. Our cell-by-cell results confirm these findings and show that the Ig density on single cells varies less on CLL lymphocytes than on normal B cells when measured by the quantitative immunoperoxidase assay. The appearance of the histograms obtained is comparable to that observed in neonatal mice up to the tenth day.

In contrast, the cell-by-cell distribution of SmIg was very different in the case of a blastic crisis supervening in established CLL and in two cases of PL. We report here for the first time a case of CLL in which the SmIg was measured both at the time of the initial diagnosis and during a blastic crisis (Fig. 3). In addition to an increase in the amount of SmIg (22,500 sites or 647 U before blastic crisis and 135,000 sites or 2576 U during blastic crisis), the cellular distribution appeared much more heterogeneous.

In the two cases of PL, we confirmed our previous descriptions that the cells in this disease have a large amount of SmIg. The histograms in both cases also show a more heterogeneous distribution than in CLL. Since the amount of SmIg increases during the immunologic maturation of the B lymphocyte, it seems likely that the cells in CLL are blocked at an earlier stage of maturation. However, the precise stage of B-cell maturation in CLL cells is still poorly defined. In mouse or human B-cell ontogeny, the early B lymphocytes bear only μ SmIg, δ chains appearing later at the cell surface. Since most B-cell clones bear μ and δ chains, the block in further maturation appears to occur at a later stage. On studying G6PD, Fialkow et al.\(^2\)\(^3\) postulated that CLL involves a committed B-cell progenitor, in contrast to chronic myelocytic leukemia and other myeloproliferative syndromes, all of which involve a multipotent hematopoietic stem cell.

In blastic crisis of CLL, and in PL, the cells carry a large amount of SmIg and thus appear to be arrested at a different stage of maturation. The fact that the amount of SmIg varies considerably from cell to cell producing an heterogeneous pattern is compatible with the concept of persistent maturation. Preud'homme and Seligmann\(^2\) postulated that in Waldenstrom's disease maturation may proceed uninterrupted to the stage of the Ig-secreting plasma cell, and this may explain the fact that the amount of SmIg varies from cell to cell in this disease, producing a heterogeneous pattern.

In the rare blastic crises supervening in CLL, the diagnosis is provided by the finding of a large number ( \(> 50\%\)) of very young cells in addition to cells identical to those of classical CLL. Similarly, PL is recognized by the presence of a variable percentage of prolymphocytes and blast cells. These cells could correspond to a clone committed to differentiation by an unknown agent. However, during ontogenesis in the mouse and in Waldenstrom's disease this cytologic picture is not observed.

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**REFERENCES**


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