The Ultrastructural Localization of Immunoglobulins in Human B Cells of Immunoproliferative Diseases

By M. F. Gourdin, J. P. Farcet, and F. Reyes

The cellular distribution of immunoglobulins in human malignant and normal B cells was investigated by immunoelectron microscopy by direct incubation of fixed cells with peroxidase-coupled antibody. These conjugates penetrated into the cell, resulting in the simultaneous detection of surface and cytoplasmic immunoglobulins. The latter were seen as specific intracisternal staining of the perinuclear space and endoplasmic reticulum and occasionally of the Golgi complex. Plasma cells were frequently characterized by a heterogeneity of reactivity of the endoplasmic reticulum. Minute amounts of cytoplasmic immunoglobulin were demonstrated in cells without developed secretory organelles, such as lymphoma cells and lymphocytes from chronic lymphocytic leukemia (CLL). The method allowed us to define several subsets of cells according to the expression of surface and cytoplasmic immunoglobulins and thus to determine the stage of maturation of cells involved in monoclonal proliferation.

SYNTHESIZED IMMUNOGLOBULINS are expressed at the surface or in the cytoplasm of B cells according to the maturational stage of the cell. Immunofluorescence and immunoperoxidase techniques have been widely used for the demonstration of both surface and cytoplasmic immunoglobulins in human malignant B cells by light microscopy. Immunoelectron microscopy methods have also been developed in order to localize cellular immunoglobulins at the ultrastructural level, usually using an immunoperoxidase procedure. However, technical limitations have been encountered in the detection of cytoplasmic immunoglobulins by immunoelectron microscopy; in contrast, surface immunoglobulins can be detected more easily. For this reason, few studies of human proliferative B cells by immunoelectron microscopy have been done so far. We report the results of an ultrastructural study using a one-step immunoperoxidase procedure that was initially developed for detecting surface immunoglobulins on human B lymphocytes. This procedure allowed the simultaneous detection of both surface and cytoplasmic immunoglobulins in specimens from patients with various immunoproliferative diseases.

MATERIALS AND METHODS

Cell Samples

Blood samples (listed in Table I) were obtained from patients with chronic lymphocytic leukemia (CLL) and its polylymphocytic variant (pro-CLL), plasma cell leukemia (myeloma), leukemic phase of lymphoma, Waldenström’s disease, acute lymphoblastic leukemia Burkitt-type (ALL). Marrow cells were obtained from patients with myeloma and a case of α-chain disease with bone marrow involvement. Spleen cells were obtained by surgery in one case of pro-CLL and one of lymphoma. Blood cells from patients with Sézary’s syndrome, T-ALL, and T-CLL served as controls.

B-cell lines under study were Burkitt-derived Raji and Daudi; the lymphoma derived (CEM) T-cell line was used as control.

Normal spleen cells were obtained by surgery in 3 cases of traumatic rupture.

Cell Suspensions

The preparation of blood and marrow cell suspensions was performed as already described. Briefly, blood buffy coat was aspirated in plasma, centrifuged, and resuspended in Hank’s balanced salt solution (HBSS) for 1 hr at room temperature before further washing in HBSS; aspirated marrow particles were dissociated and washed in HBSS. For spleen cell suspensions, small fragments were passed through a stainless steel sieve and subsequently centrifuged on Ficoll-Hypaque (density 1.077 g/cm³); after 3 washes, viability was checked by exclusion of Trypan blue dye.

Cell Cultures

Normal spleen cells were stimulated in vitro by pokeweed mitogen (GIBCO Lab, Grand Island, N.Y.). Final cell concentration was adjusted at 10⁶ cells/ml in Iscove’s modified Dulbecco medium (medium (GIBCO Lab) supplemented with 10% fetal calf serum and 10 μl/ml pokeweed mitogen. Cultures were carried out in 15-ml plastic flasks incubated for 8 days at 37°C with 6.5% CO₂ and 100% humidity. Cells were harvested on days 4 and 7.

Fixation

The various cell suspensions were extensively washed in HBSS and fixed by resuspending the cell pellet in glutaraldehyde (Taab Lab, Emmer Green, Reading, England) 1.25% in phosphate-buffered saline 0.1 M, pH 7.4, for 30 min at room temperature. After 2 additional washes, the fixed cells were stored in cold saline until required.

Anti-immunoglobulin Antibodies (Anti-Ig)

Anti-Ig specific for heavy and light chains were affinity-purified from rabbit antiserum by affinity chromatography in a solid immunoadsorption procedure. Immunoadsorbents were obtained by polymerizing human purified monoclonal IgM, IgG, and IgA. Anti-μ, anti-γ, and anti-α were purified by a procedure previously described. Anti-κ, and anti-λ were purified from monospecific antiserum (C.D.T.S Bois Guillaume, Rouen, France) using a polymer of appropriate light chain. Polyvalent anti-Fab Ig antibodies were...
## Table 1. IEM Staining Experiments

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*Indicates incubations performed with the corresponding conjugate.
†First determined by optical immunostaining of slg or clg.
‡Staining experiments repeated 6 times at intervals during the study.
NS, nonsecreting.

Purified from antiserum (kindly provided by Dr. Ph. Druet) using a polyclonal IgG immunoabsorbent. Normal rabbit IgG (Miles Lab, Elkhart, Indiana) was used in control experiments.

### Conjugates

Rabbit anti-Ig and normal IgG were coupled to horseradish peroxidase (RZ3, Sigma, St. Louis, Mo.) by the two-step procedure using glutaraldehyde as a coupling agent. Noncoupled peroxidase was removed by ammonium sulphate precipitation of the conjugate. Conjugates were stored at 4°C at a concentration of 1 mg/ml in PBS; before use they were centrifuged at 48,000 g for 30 min at 4°C.

### Immunelectron Microscope (IEM) Staining

The number and the origin of the specimens stained by the various conjugates are given in Table 1.

The pellet of 1 ml of a 3% (v/v) fixed cell suspension was resuspended in 1 ml conjugate for 1 hr at room temperature. Working concentration of conjugate was 1 mg/ml; in some experiments, the conjugate was used at lower concentrations (0.2–0.02...
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Fig. 1. Pro-CLL spleen cells: anti-μ conjugate. Several slg+ lymphocytes and a slg- plasma cell are seen. The latter has an eccentric nucleus and a fully developed positive ER. The Golgi apparatus is also positive (G, see inset). This plasma cell is slg-. E is an erythrocyte whose reactivity results from the peroxidatic activity of hemoglobin. (x6000; inset x12,000.)

mg/ml). Subsequent steps of glutaraldehyde postfixation, incubation with diaminobenzidine, and embedding were as previously described.4

Controls

Affinity-purified anti-Ig were tested before coupling by immunodiffusion. The activity and the specificity of conjugates were checked in several ways: (1) staining of T cells; (2) optical immunoperoxidase staining of surface Ig and cytoplasmic Ig of cells with a known monoclonal phenotype;2 (3) IEM staining of surface Ig of B-CLL cells with a known phenotype. The specificity of the immunoelectron microscopic detection of cytoplasmic Ig was further established in the present study by “intrinsic” controls, i.e., by comparing the results given by anti-heavy-chain and anti-light-chain conjugates in malignant cells (Table I; Figs. 13 and 14); the monoclonality of the cells was first determined by optical immunoperoxidase or by immunofluorescence kindly performed by Dr. Ch. Andre. For the study of polyclonal pokeweed mitogen activated B cells, the conjugate of normal IgG was used as a control of the anti-IgM staining.

RESULTS

The localization of cytoplasmic Ig was detected directly in fixed cells by immunoelectron microscopy on a wide variety of cells from patients with diseases characterized by a monoclonal proliferation of B cells. The cells examined are believed to represent the malignant counterparts of the normal B-lymphocyte maturation.8 Surface immunoglobulin was simultaneously detected by the staining of the cell surface (Fig. 1).

Cytoplasmic immunoglobulin was detected by immunoperoxidase within the lumina of cisternae of the endoplasmic reticulum (ER) and/or within the perinuclear space (PNS) (Fig. 2). The intensity of the intracisternal staining varied from dense and diffuse to heterogeneous and granular.

When the plasmacytic cells of patients with monoclonal immunoglobulins present in the serum were examined with appropriate polyclonal or monoclonal antibodies to heavy or light chain antigens, cytoplasmic immunoglobulins were detected in the abundant endoplasmic reticulum and, to a lesser extent, in the perinuclear space. Frequently, stained and unstained strands of the endoplasmic reticulum were randomly distributed in the cytoplasm of the same cell (Figs. 3 and 4). The Golgi apparatus was usually not stained, even in fully developed plasma cells. This pattern of cytoplasmic immunoglobulin described above was present in the majority of the plasmacytic cells from patients with myeloma and among the cells of patients with Waldenström’s macroglobulinemia and α-chain disease.
Fig. 5. Blood cells from a pro-Cu sample with a serum monoclonal Ig; anti-'L-conjugate. All lymphocytes are slg+. Some having in addition a PNS reactivity (arrow). (x 6000.)

Fig. 7. Lymphoma blood cell; anti-'L-conjugate. This immature cell is slg- and has clg restricted to the PNS. L. lipid inclusion; Gr. part of a granulocyte with myeloperoxidase-containing granules. (x 18.000.)

Fig. 4. Blood plasma cell from myeloma; anti-Fab'y conjugate. This sig-cell has positive ER cisternae seen as elongated (single arrow) or round vacuoles (double arrow); they are randomly distributed among numerous ER cisternae that are negative. N. nucleus. (x 12.000.)

Cytoplasmic immunoglobulin was also detected in nonplasmacytic cells with a poorly developed endoplasmic reticulum. In about one-third of the cells of patients with lymphoma without a monoclonal component in the serum, immunoglobulin was detected in the endoplasmic reticulum and perinuclear space (Figs. 6, 7, and 15); some cells had surface immunoglobulins and others did not. In the cells of two patients with CLL who had a monoclonal serum component, about 20% of the cells showed this pattern of distribution of cytoplasmic immunoglobulin; most of these cells also had surface immunoglobulin (Fig. 5), as did the remaining 80% of the lymphocytes.

The cells of patients with CLL who did not have a serum component and of patients with ALL usually did not have detectable cytoplasmic immunoglobulin (Fig. 9); however, a few cells in these cases did have cytoplasmic immunoglobulin, usually with, but occasionally without, surface immunoglobulin.

When cell lines maintained in culture were examined, it was found that the cells of the Raji line did not have surface immunoglobulin but did have cytoplasmic immunoglobulin in the perinuclear spaces and/or in elongated strands of endoplasmic reticulum in two-thirds of the cells (Fig. 8). The vast majority of the cells of the Daudi cell line, on the other hand, exhibited only surface immunoglobulins.

Thus, three patterns of staining for immunoglobulin were found: surface immunoglobulin only was present, cytoplasmic immunoglobulin only was present, or both surface and cytoplasmic immunoglobulins were present. Usually the majority of cells in a given specimen displayed one of these patterns; however, the other two
patterns were frequently seen in a small proportion of the cells. Thus, in the cells of patients with common CLL and the cells from the Daudi cell line, surface immunoglobulin was seen in most cells but a few cells had cytoplasmic immunoglobulins with and without surface immunoglobulin. The majority of cells from patients with CLL and a monoclonal serum immunoglobulin protein and from patients with Waldenström’s disease had surface immunoglobulins, but a significant number of cells had cytoplasmic immunoglobulins, a much larger proportion than in other clinical settings.

Cytoplasmic immunoglobulins were also detected in normal spleen cells activated by pokeweed mitogen (Figs. 10–12). The pattern of occurrence of immunoglobulin in these cells was very similar to that seen in cells from patients with malignant disease described above; large blast cells were seen with cytoplasmic IgM in the perinuclear space at one end of the spectrum of reactive cells. At the other end, plasma cells with fully developed endoplasmic reticulum that contained immunoglobulin were also seen. The Golgi complex in these cells was infrequently stained. In addition to these two types of cells, cells with surface immunoglobulin only and cells with both surface and cytoplasmic immunoglobulin were found as minor populations.

Generally, the concentration of conjugate resulted in distinct intracisternal staining without significant cytoplasmic background. Some cells, however, exhibited a diffuse extracisternal reactivity apparently located at the level of ribosomes. When present in a given sample, this diffuse “ribosomal” staining was present in only a few cells; occasionally, in some of the experiments performed with Raji cell line, PWM-activated cells, and with one myeloma sample, this
pattern was present in up to 40% of the cells that were undamaged. In these cases the intracisternal staining could hardly be distinguished. Dilution of the conjugates from 0.2 to 0.02 mg/ml abrogated the diffuse "ribosomal" reactivity, whereas the intracisternal staining of the endoplasmic reticulum and perinuclear zone appeared clearly, as shown by experiments performed with Raji cells and one myeloma sample.

**DISCUSSION**

Immunoelectron microscopy, using a direct procedure that minimizes both the number of manipulations and the loss of cells, is adequate to demonstrate intracellular synthesized Ig, as shown in this study of a wide spectrum of malignant and normal samples consisting of various subsets of Ig-synthesizing cells. Thus, this procedure provides us with a useful tool for studies of immunoproliferative diseases in relation to cell maturation.

Immunoperoxidase methods have been used in the study of neoplastic B cells with light microscopy, but technical limitations have restricted their use at the ultrastructural level. The first studies of antibody-producing cells, using immunoperoxidase methods and electron microscopy, were performed in animals using peroxidase as immunogen and tracer; they demonstrated the packaging of Ig in the perinuclear space, endoplasmic reticulum, and Golgi apparatus. Subsequently, difficulties have been encountered when peroxidase-coupled conjugates of a larger size have been used. Although the postembedding technique has not been useful, the preembedding technique has permitted intracellular staining; however, this staining was not constant, and it has been suggested that the cell membrane might act as a barrier, fixation and conjugate penetration being opposite requirements. By using smaller sized conjugates, penetration has been found to be enhanced or unmodified.

The influence of aldehyde fixatives upon penetration of conjugate, has also been investigated; however, it is difficult to draw a clear conclusion from these data. From our present observations and our initial studies of surface immunoglobulin, as well as from other preliminary studies of various fixatives (unpublished), we concluded that fixation by 1.25% glutaraldehyde does not significantly destroy the antigenicity of surface and intracisternal Ig; some alteration may occur that would explain that intracellular immunoglobulin becomes undetectable if present in too low amounts.

The concept of a "membrane barrier" has also led to attempts at modifying the cell permeability, but enzymatic digestion of the cell coat has failed to improve the staining. The addition of saponin to fixative has recently proposed and found to enhance the conjugate penetration within suspended B cells, but saponin treatment resulted in a loss of the surface Ig staining. In our system, the various aspects of intracisternal staining we have observed establish that acceptable cell preservation and penetration of conjugate are achieved by using entire antibody molecules coupled to peroxidase and the conventional glutaraldehyde fixative. The mechanism of penetration of the conjugates within fixed suspended cells remains unknown; it does not rely on an abnormal permeability of malignant cells since we observed a similar intracisternal staining in PWM-activated normal lymphocytes. Moreover, our finding of cells with a simultaneous surface and PNS staining indicates that surface immunoglobulin crosslinked with glutaraldehyde does not constitute a barrier to the conjugate penetration.

In addition to the intracisternal cytoplasmic staining, we observed a diffuse cytoplasmic reactivity apparently located on ribosomes in a variable number of cells. A similar extracisternal staining has been reported in some B cells from immunized animals regardless of the immunoperoxidase procedure used; we have previously reported it in a case of human leukemia expressing an Ig light chain restriction. Others have attributed a diffuse reactivity to the artefactual staining of necrotic cells, but in the present study, this pattern was independent of any cellular damage. However, on one hand, we found that this pattern was dependent on the concentration of the conjugate as shown in Raji and myeloma samples. On the other hand, a similar cytoplasmic reactivity occurred in occasional cells from monoclonal samples in the presence of unrelated anti-heavy or light chain conjugates, and in some PWM-activated cells in the presence of normal conjugated IgG. This, taken together with other results obtained in non-B blast cells (to be published) establish that the "ribosomal" pattern of staining may in some cases result either from an artefactual diffusion of the immunocytochemical reaction product (oxidized diaminobenzidine) or from a nonspecific fixation of conjugates. Therefore, such a pattern does not appear to be reliable unless rigorous controls of staining are performed. The need for such controls in interpreting any cytoplasmic Ig staining pattern in a given sample has to be emphasized, the best control being the staining of monoclonal cells with unrelated anti-heavy or light chain conjugates (Figs. 13 and 14).

Based on such controls, the patterns of intracisternal cytoplasmic immunoglobulin that we observed in neoplastic and normal human B cells are in accordance
with those described in immunized animals\textsuperscript{10,13,20,23} whose Ig-producing cells reflect different maturational stages ranging from large cells with immunoglobulin exclusively in the perinuclear space ("immunoblasts") to plasma cells with fully developed endoplasmic reticulum containing immunoglobulins.

We frequently observed a mixture of reactive and unreactive cisternae of the endoplasmic reticulum that did or did not contain immunoglobulin in individual plasma cells. The different cisternae were randomly distributed in the cytoplasm, suggesting that the absence of staining of some did not result from an inadequate penetration of the conjugate in some cell areas; this was further substantiated by the finding of cells in which segments of endoplasmic reticulum did not contain immunoglobulin, but the perinuclear space and peripheral endoplasmic reticulum did. The coexistence of immunoglobulin positive and negative cisternae of endoplasmic reticulum has already been reported in antibody-producing cells by using different immunoperoxidase procedures;\textsuperscript{13,15,18,22,23} in some of these studies,\textsuperscript{13,15,18} problems of penetration were minimal or nonexistent, since peroxidase was used as antigen and tracer. These studies also found patchy staining of endoplasmic reticulum. Thus, it is difficult to ascribe this in our studies to a failure of penetration of the conjugate and therefore probably reflects a functional state of the ER.

The relative rarity of staining of the Golgi apparatus that we observed can be discussed in a similar way. Plasma cells with a negative Golgi have been repeatedly found in tissues stained by the various immunoperoxidase procedures cited above;\textsuperscript{15,17,18,26,32} in one such study, this feature characterized many of the antibody-producing cells harvested at the peak of a secondary response in animals immunized with peroxidase. Although a selective permeability of the membranes of the Golgi body has to be considered, these data and our observations raise the question of the role of the Golgi complex in intracellular transport and secretion of Ig.

Immunoelectron microscopy allowed us to detect minute amounts of cytoplasmic immunoglobulins when it was restricted to the perinuclear space; this was particularly well illustrated in two patients with lymphoma in whom a sizeable number of cells had demonstrable immunoglobulin in the perinuclear space that was not evidenced by light microscopy. It is possible that the restricted staining of the perinuclear space of endoplasmic reticulum as seen in ultrathin sections might not reflect the true Ig content of the whole cell; however, samples from patients with monoclonal proliferation demonstrate large numbers of identical cells so that the examination of several sections makes it unlikely that cells with immunoglobulin evidence in the perinuclear space might be packed with immunoglobulin in other cytoplasmic areas.

Thus, these observations confirm the value of immunoelectron microscopy in localizing limited amounts of cytoplasmic Ig.\textsuperscript{32} In this respect, however, our findings in the cells of patients with CLL and B-ALL and in Daudi cells have to be considered. These cells synthesize Ig molecules\textsuperscript{18,33} that we detected only at the surface of the cell, with the exception of a minor population of cells with cytoplasmic immunoglobulin, which we interpret as secretory cells (see below). Since the synthesis of membrane Ig must occur somewhere within the cell, the absence of any cytoplasmic staining in the vast majority of these surface Ig+ cells indicates that there is a threshold of sensitivity of the IEM technique. Membrane Ig are biochemically distinct
from secretory Ig, but it is not yet known whether they share the same mechanism of intracellular transport (ribosomes → ER → Golgi) or if they segregate into distinct cytoplasmic organelles. Whatever the pathway is, we were unable to detect newly synthesized membrane Ig in any cytoplasmic compartment; this may result from their low amount at the site of synthesis in conjunction with some degree of fixative-induced alteration, as discussed above.

Finally, we would like to stress that the simultaneous detection of surface and cytoplasmic immunoglobulin allows us to discriminate between several subsets of cells in a given sample. The assumption that such subsets reflect sequential maturation stages stems from IEM studies of developing B cells in immunized animals and from the current schemes of B-cell differentiation. As an example, the following cell subsets coexisted in a lymphoma sample with a monoclonal phenotype μ: (1) surface only IgM, (2) both surface IgM and cytoplasmic IgM, (3) cytoplasmic IgM only present in the perinuclear space and a few strands of endoplasmic reticulum, as detected by both anti-μ and anti-κ conjugates (Fig. 15). This demonstrates that cells involved in a monoclonal proliferation can be located at different maturation stages. This was also illustrated in common CLL by the finding of a minor population of cytoplasmic Ig-positive lymphocytes diluted among the surface Ig-positive cells. Recent biochemical studies indicate that resting B lymphocytes, in addition to membrane Ig, synthesize secretory IgM, which is mostly retained within the cell. Moreover, it has been demonstrated that lymphocytes from patients with common CLL (i.e., without serum monoclonal Ig) secrete small amounts of pentameric IgM. Whether this secretory potential is a property of every cell or of a subpopulation of cells has not been determined; our observations would favor the latter hypothesis.

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