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## Concise Publication

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## CONCISE PUBLICATIONS

### Immunoglobulins on the Surface of Lymphoid Cells in Waldenström's Macroglobulinemia

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**A B S T R A C T** Immunofluorescence study of viable and fixed cells was performed on marrow and blood samples from 25 patients with Waldenström's macroglobulinemia. Whereas intracytoplasmic staining for IgM was restricted to the plasma cells and to a limited number of lymphocytic cells, the vast majority of the pleomorphic "lymphoid" proliferating cells in the marrow were shown to bear the monoclonal IgM on their surface. All IgM-secreting plasma cells displayed membrane positivity. Most lymphocytic proliferating cells carried the monoclonal IgM on their surface in the absence of detectable amounts of intracytoplasmic IgM. Despite the usual absence of increase in blood lymphocyte counts, a large number of circulating lymphocytes were shown to bear membrane-bound monoclonal IgM in patients with active disease. The number of fluorescent spots, their size and brightness varied greatly from cell to cell in marrow and blood samples of a given patient, and this finding is in contrast to the homogeneous fluorescent pattern observed in chronic lymphocytic leukemia. The data suggest that macroglobulinemia represents the proliferation of a clone of B cells which continues to mature and differentiate.

#### INTRODUCTION

The presence of immunoglobulin (Ig)<sup>1</sup> moieties on the surface of small lymphocytes has been shown by different methods. Those lymphocytes bearing surface Ig

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<sup>1</sup>*Abbreviations used in this paper: Ig, immunoglobulins; WM, Waldenström's macroglobulinemia.*

in sufficient amount to be evidenced by direct immunofluorescent staining of living cells (1-4) are presumably bone marrow- or bursa-derived (B) cells (5-7).

The main cytological feature of Waldenström's macroglobulinemia (WM) is the pleomorphic character of the "lymphoid" proliferation. Monoclonal IgM is demonstrated by immunofluorescence in the cytoplasm of several types of cells belonging to the lymphocytic and plasmacytic categories (8, 9). However we have found that in most patients a strikingly low percentage of the lymphoid proliferating cells displayed intracytoplasmic IgM positivity. Indeed, in most marrow and node samples of 38 studied patients, the majority of lymphocytic cells was not stained whereas most plasma cells were positive, and we have postulated that those lymphocytes without detectable intracytoplasmic IgM may bear membrane-bound monoclonal IgM (9). The present study was undertaken in 25 patients in order to evaluate this hypothesis.

#### METHODS

Rabbit antisera to purified  $\kappa$ - or  $\lambda$ -chains were selected for their reactivity with both Bence Jones proteins and IgG, IgA, and IgM 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  $\gamma$ ,  $\mu$ ,  $\alpha$ ,  $\kappa$ , or  $\lambda$ -chains were pooled and rendered monospecific by adsorption on suitable antigens coupled to Sepharose 4 B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) by cyanogen bromide, in order to avoid soluble complexes. Specificity was carefully controlled by double diffusion and, after labeling, by immunofluorescence on fixed smears of cells from pa-

tients with myeloma and macroglobulinemia of known class and type.

Peripheral blood lymphocytes were isolated by Ficoll-triosil (Pharmacia Fine Chemicals, Inc.) gradient centrifugation (10). The separation of the marrow white cells and the washing of cell suspensions were performed as previously described (9). The procedures used for the detection of intracytoplasmic Ig by fluorescein-conjugated antisera have been previously described (9). The preparation of rhodamine conjugates and the immunofluorescent study of living cells in suspension were performed according to Pernis, Forni, and Amante (2). In order to avoid pinocytosis, the staining was effected at 4°C and in the presence of 1 per 1000 sodium azide. Identification of lymphoid cells and counting of positive cells required alternative examination of each field in phase contrast and in ultraviolet light. In order to study both surface and intracytoplasmic IgM, the suspensions stained by rhodamine or fluorescein-labeled antiserum to  $\mu$  were flattened on slides using a cytocentrifuge, fixed, and stained again by the same antiserum coupled to the alternate fluorochrom. A Leitz Orthoplan microscope equipped with an Opak-Fluor vertical illuminator (E. Leitz GMBH, Wetzlar, Germany) was used with selective combinations of filters (2).

Two types of experiments were performed with trypsinized cells. (a) Marrow and blood lymphocytes were stained in the cold and washed as described above. A sample was examined and the cells were then treated with trypsin, washed three times, examined, and incubated at 37°C. After 1 hr and 5 hr of incubation, the cells were examined without further staining and stained as previously. (b) The cells were first treated with trypsin, washed, and incubated at 37°C. Samples were stained after the washings and after 1 hr and 5 hr of incubation. All washings and incubations were performed in Eagle's spinner minimal essential medium supplemented with 20% fetal calf serum. The treatment by trypsin was effected at a concentration of 2.5 mg/ml in the same medium for 20 min at 37°C. This relatively high concentration of trypsin was needed because the high protein content of the medium.

## RESULTS

In all bone marrow samples of the 25 studied patients, "lymphoid" cells were shown to carry monoclonal IgM on their surface. These cells were stained by antisera to  $\mu$ -chains and to  $\kappa$ - (19 patients) or  $\lambda$ - (6 patients) chains. The light chain type was identical to that of the serum IgM. The size and shape of these positive cells ranged from those of small lymphocytes to those of typical plasma cells with all intermediates (Fig. 1). Except in one patient with 20% plasma cells and without excess of lymphocytes, the cytological examination of the marrow showed the usual predominance of pleomorphic lymphocytes with a moderate increase in plasma cells. As previously found in most patients with WM (9), almost all plasma cells and only a small percentage of lymphocytes contained detectable intracytoplasmic IgM. On the other hand the staining for surface-bound IgM showed a large predominance of positive lymphocytic cells. Indeed, as shown by double labeling experiments, most lymphocytes bearing IgM on

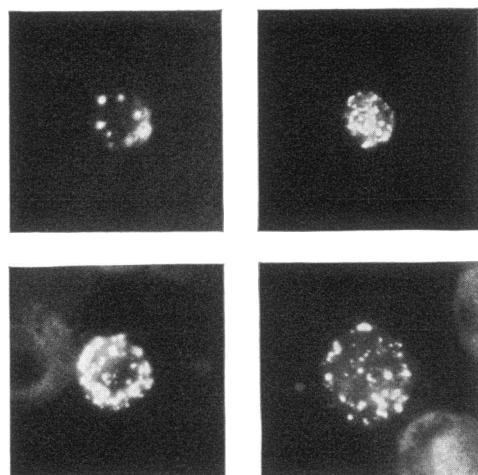


FIGURE 1 Different patterns of cells from the same marrow sample of a patient with macroglobulinemia stained in suspension by anti-IgM rhodamine conjugate.

their surface (particularly the small ones) had no detectable intracytoplasmic IgM whereas those cells with intracytoplasmic IgM, including all plasmocytes, did carry also membrane-bound IgM (Fig. 2).

Positivity for membrane IgM was expressed by the presence of numerous fluorescent spots which were distributed on the entire surface of cells. The number of fluorescent spots, their size and intensity of brightness varied greatly from cell to cell in a given marrow sample (Fig. 1). Even cells of similar size and shape exhibited this wide diversity of spots. In patients with active proliferative disease, whether untreated or in relapse, the vast majority of "lymphoid" cells (mean 90%) showed positivity for surface IgM. However in all cases some lymphocytes were not stained. In patients who were improved under chlorambucil therapy with subnormal marrow cellularity and mild lymphoid involvement, the percentage of "lymphoid" cells bearing monoclonal IgM ranged from 5 to 70% (mean 35%). The low figures represent likely an underestimate since these marrow samples were greatly diluted by blood.

In normal subjects IgM is the predominant Ig class occurring on the surface of peripheral blood lymphocytes. The percentage of IgM positive cells detectable in our experimental conditions ranges in 20 controls from 5 to 25% (mean 13%) whereas the percentage of lymphocytes positive for  $\gamma$ - and  $\alpha$ -chains were of 1-7% (mean 4%) and 0.5-4% (mean 2%), respectively. The incidence of cells stained by anti-light chain sera were of 10-15% (mean 12.5%) for  $\kappa$  and 2-7% (mean 4%) for  $\lambda$ . These figures were supported by experiments showing that the percentage of positive cells reached a plateau with concentrations of conjugates lower than those routinely used. Although periph-

eral blood lymphocyte counts were in the normal range in all untreated patients with WM except two who showed only a moderate increase (7600 and 6200/mm<sup>3</sup>, respectively), 50–80% of peripheral lymphocytes did bear monoclonal IgM on their surface. The single patient who was an exception to this rule was the one with the purely plasmacytic involvement of marrow since only 12% of his blood lymphocytes were positive for  $\mu$  and  $\lambda$  chains. All patients under chemotherapy were lymphopenic. Whereas the percentage of monoclonal IgM positive lymphocytes was high (around 40%) in the relapsing patients, it was remarkably low (mean 9%) when the disease was in satisfactory control, as gauged by clinical and hematological status and by the level of serum IgM. As in marrow, the pattern of the fluorescent spots varied in all patients from cell to cell in the same blood sample. Only very few peripheral cells were stained for intracytoplasmic IgM.

When purified normal blood lymphocytes were incubated for 30 min at 37°C in a WM serum containing 3.5 g/100 ml of monoclonal IgM, the number and pattern of surface IgM positive cells were unaffected by this procedure. The experiments with trypsinized cells were performed four times and gave similar results. They showed that the IgM on the cell surface was removable by treatment with trypsin and subsequently reappeared on cells kept in culture conditions. When the cells were first labeled by conjugated sera and then treated with trypsin, examination after trypsinization and after 1 hr and 5 hr of incubation as well as after staining of cells incubated for 1 hr in culture medium showed very sparse positive cells. Similarly unlabeled trypsinized cells were not stained by antiserum to IgM immediately after trypsin treatment nor 1 hr later. In contrast, trypsinized cells which had been incubated for 5 hr were shown to carry numerous IgM spots. The percentage of positive cells as compared to the number of cells stained for IgM before trypsin treatment varied from 60% to 90%.

In samples from marrow and blood, the number of cells bearing Ig chains other than those of the mono-

clonal IgM was extremely low. It should be noted that all the studied patients had low levels of serum IgG and IgA.

## DISCUSSION

The present immunofluorescence study of marrow cells in WM shows that whereas intracytoplasmic staining for IgM is restricted to the plasma cells and to a limited number of lymphocytic cells, the vast majority of proliferating cells do bear the monoclonal IgM (or its monomeric subunit) on their surface. In addition a high number of circulating lymphocytes were shown to carry membrane bound monoclonal IgM in patients with active disease.

The antisera used in this study were shown to be monospecific at the level of sensitivity of the immunofluorescence method and were devoid of detectable contaminants such as heterophile antibodies since they did not stain the surface of lymphocytes of patients with sex-linked agammaglobulinemia (11).

The IgM molecules detected on the lymphoid cells of the patients with WM were monoclonal in nature. In view of the predominance of IgM with  $\kappa$ -chains on the surface of normal lymphocytes, the results obtained in six cases with IgM of the  $\lambda$ -type were of interest in this respect. The hypothesis of a passive coating of the cells by the serum IgM has been ruled out. Although Ig detected at the surface of lymphocytes could result in some situations from the presence of antibodies to membrane determinants, the possibility appears most unlikely in WM. The simultaneous presence of monoclonal IgM in the cytoplasm and on the surface of some cells and the results obtained with trypsinized cells do strongly suggest actual synthesis of the membrane-bound IgM. However in most lymphocytic proliferating cells which carry the monoclonal IgM on their surface, we were unable to demonstrate with the same conjugates the presence of intracytoplasmic IgM. We have observed a similar discrepancy when studying Ig-bearing lymphocytes from normal subjects or patients with chronic lymphocytic leukemia (12) except in a patient in whom unreleased monoclonal IgM did accumulate in crystalline form in the cytoplasm of leukemic lymphocytes with membrane-bound IgM (13). The concentration of the intracytoplasmic IgM molecules which have been detected by other methods in IgM-bearing lymphoid cells (4, 14) is presumably beyond the sensitivity of the immunofluorescence technique on fixed cells. This procedure is less sensitive than the surface staining, probably because of the unavoidable background.

The presence of surface IgM provides a useful marker in order to demonstrate at the cellular level the monoclonal nature of the highly pleomorphic "lymphoid" proliferation characteristic of WM. The present data

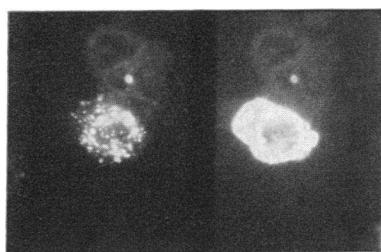


FIGURE 2 Fluorescence patterns of the same plasma cell stained in suspension by anti-IgM rhodamine conjugate (left) and subsequently labeled after fixation by an anti-IgM fluorescein conjugate (right).

strongly suggest that WM represents the proliferation of a clone of B cells with persistent maturation from the small lymphocyte to the IgM-secreting plasma cell. The striking heterogeneity of the fluorescent patterns on the cells of a same patient may well be related to this maturation process.

The percentage and absolute number of circulating lymphocytes bearing the monoclonal marker is remarkably high in patients with WM. Despite the usual absence of increase in blood lymphocyte counts, WM may therefore be considered as a leukemic process. While the mature cells of the proliferating clone are located in marrow and nodes, only the less differentiated cells appear to circulate. The presented data in treated patients indicate that alkylating agents presumably affect primarily those immature cells which do circulate.

Other immunoproliferative disorders such as Burkitt's lymphoma (15) and chronic lymphocytic leukemia (3, 12, 16, 17) are often featured by cell surface-bound monoclonal IgM. Although circulating leukemic lymphocytes from a few patients were found either to bear monoclonal IgG or IgA or to have no detectable membrane Ig, most cases of chronic lymphocytic leukemia studied in this laboratory were characterized by the presence of a monoclonal IgM marker (12). It is of interest to note that all of the IgM positive lymphocytes of a given leukemic patient showed fluorescent spots of similar size, brightness, and distribution. This finding is in contrast to the considerably heterogeneous pattern of membrane fluorescence observed among circulating cells of each patient with WM. This homogeneous pattern in chronic lymphocytic leukemia could possibly reflect a block in the maturation of the proliferating B lymphocyte clone.

The membrane positivity of all IgM-secreting plasma cells in WM is in agreement with the recent findings of Pernis in normal rabbit (3) and is in contrast with some negative results reported in mouse and human IgG plasmacytoma cells (18). This finding may be of interest with respect to the chronology of the immune response.

It appears likely that membrane IgM receptors share the antibody specificity of the Ig secreted by differentiated cells of the same clone and WM provides an interesting model for such studies. Indeed recent experiments performed in our laboratory have demonstrated that the IgM present on the surface of the lymphocytes did share the anti-IgG antibody activity of the patient's serum monoclonal IgM.<sup>2</sup>

<sup>2</sup> Preud'homme, J. L., and M. Seligmann. In preparation.

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