

Anti-Human Immunoglobulin G Activity of Membrane-Bound Monoclonal Immunoglobulin M in Lymphoproliferative Disorders

(lymphocyte/receptors/surface immunoglobulin/rheumatoid factor/lymphatic leukemia)

J. L. PREUD'HOMME AND M. SELIGMANN

Research Institute on Blood Diseases, Hôpital Saint-Louis, Paris 10e, France

Communicated by Jacques Monod, May 22, 1972

ABSTRACT Lymphocytes from a patient with Waldenström's macroglobulinemia and known anti-immunoglobulin IgG activity of serum monoclonal IgM and lymphocytes from selected patients with chronic lymphocytic leukemia were studied by the membrane immunofluorescence procedure. Freshly drawn lymphocytes were shown to bear simultaneously μ , γ , κ , and λ chain determinants. Experiments combining redistribution induced by antibody and double labeling proved that IgG was bound to surface IgM. After removal of surface immunoglobulins by treatment with trypsin followed by incubation in culture medium, or after redistribution induced by antibody, the exclusive presence of a newly synthesized monoclonal IgM was demonstrated. Several experiments showed that this surface IgM does specifically bind normal human IgG molecules devoid of aggregated material. The IgG molecules could be removed from the cell surface by lowering the pH. In addition to its high incidence among serum monoclonal macroglobulins, anti-IgG activity of membrane-bound monoclonal IgM is not uncommon in patients with chronic lymphocytic leukemia, a disease that provides homogeneous populations of lymphocytes derived from bone marrow, with receptor sites of defined antibody activity.

The presence of receptor molecules of immunoglobulin on the surface of lymphocytes derived from bone marrow (B lymphocytes) is well documented in different species including man (1, 2). Since lymphocytes from many patients with chronic lymphocytic leukemia (1, 3, 4) and from patients with Waldenström's macroglobulinemia (5) are characterized by a monoclonal surface IgM marker, these immunoproliferative disorders may provide suitable models for the study of antibody activities of surface immunoglobulins on homogeneous B-lymphocyte populations. In view of the high incidence of antibody activity against IgG among serum monoclonal IgM (6), it appeared logical to search for such an antibody activity of surface monoclonal IgM. We have studied the cells of a patient with Waldenström's macroglobulinemia and known rheumatoid-factor activity of serum monoclonal IgM. Moreover, we have found by immunofluorescence study of living cells the simultaneous presence of γ -, κ -, λ -, and usually μ -chain determinants on the lymphocytes from several patients with chronic lymphocytic leukemia. One of the possible explanations for this unexpected finding is the anti-IgG activity of surface immunoglobulins. The results presented here clearly show that, in some instances, the monoclonal IgM on the surface of lymphocytes does specifically bind normal human IgG.

Abbreviation: B lymphocytes, bone marrow-derived lymphocytes.

MATERIALS AND METHODS

The methods used for isolation of lymphoid cells from blood or bone marrow, immunofluorescence study of living cells in suspension, and preparation and characterization of reagents stained with fluorescein or rhodamine that are monospecific for γ , μ , α , κ , and λ chains have been described (4, 5). Pooled normal human IgG was purified by chromatography on diethylaminoethyl (DEAE)-cellulose columns and coupled to tetramethylrhodamine isothiocyanate (Baltimore Biological Laboratories, Cockeysville, Md., lot 105.1345). IgG was aggregated by heating to 60° for 30 min. "Native" IgG refers to a purified IgG preparation processed just before use through a Sephadex G-200 column in order to remove any aggregated material. All immunofluorescence staining and subsequent washings were performed at 4°. Eagle's minimal essential medium, supplemented with 20% fetal-calf serum, was used for washing and as incubation medium. The various preparations of human IgG and IgA were used at concentrations of 1-2 mg/ml.

For trypsinization experiments, living lymphocytes from blood and bone marrow of patients with Waldenström's macroglobulinemia and from blood of patients with chronic lymphocytic leukemia were stained by rhodamine-conjugated antisera to the 5 immunoglobulin polypeptide chains (μ , γ , α , κ , or λ) and by rhodamine-conjugated aggregated and native IgG. After three washings, aliquots were examined by immunofluorescence and the remaining cells were incubated for 30 min at 37° in the culture medium with 2.5 mg of twice crystallized trypsin (Nutritional Biochemical Corp., Cleveland, Ohio)/ml. After three further washings, the cells were incubated at 37° for 6 hr. These cells were examined by immunofluorescence after trypsinization and after 1 and 6 hr of incubation in order to control the efficiency of the treatment with trypsin and insure the absence of a possible transfer of removed immunoglobulin to acceptor cells. After 6 hr of incubation, the cells were stained by the same reagents. Since the conjugation of native IgG could result in some degree of aggregation, the cells were also incubated with native unlabeled IgG (and IgA) for 30 min at 4°, washed three times, and stained by anti- γ , anti- μ (and anti- α) conjugates.

Redistribution of surface immunoglobulins induced by antibody occurs when cells labeled at 4° are warmed to 37°, and cap formation is observed on 80-90% of cells (7, 8). Lymphocytes previously stained by reagents coupled to rhodamine (listed above) were incubated at 37°. After 1 hr of incubation, aliquots were stained in the cold by antisera to μ or

γ chains conjugated to fluorescein. This double-labeling procedure provides an interesting model for investigation of molecular relationship of different membrane determinants (8). The remaining cells were incubated 5 hr more. Labeled surface immunoglobulins were then no longer detected on the cells, and experiments identical to those performed after trypsinization were done in order to demonstrate and characterize the newly synthesized surface immunoglobulins.

For study of redistribution of surface immunoglobulins induced by antigen, the cells that had been stained with native IgG coupled to rhodamine in the last steps of the trypsin and capping experiments were incubated at 37° for 1 hr and examined for cap formation. Similarly, cells that had been incubated with native unlabeled IgG and washed in the cold, were incubated at 37° for 1 hr and subsequently cooled and stained with anti- γ conjugate. Another approach was to incubate washed untreated lymphocytes at 37° for 1 hr. The cells were then cooled to 0° and stained by the antiserum to γ chains. For the latter experiments, in order to avoid any redistribution induced by the antiserum to γ chain, staining and subsequent washings were performed at 0°; the stained cells were quickly smeared, air dried, and fixed in absolute ethanol for 5 min before examination.

In another set of experiments, freshly drawn, purified, and washed lymphocytes were incubated in 0.01 M citric acid-0.15 M NaCl (pH 3) for 2 min and centrifuged. After three washings in culture medium, the cells were stained by conjugated antisera to the various immunoglobulin polypeptide chains.

RESULTS

Immunofluorescence study of freshly drawn lymphocytes showed membranes positive for γ , κ , and λ chains in the five patients studied and also positive for μ -chain determinants in four of these patients. As most cells were stained by all reagents, the four immunoglobulin chains were necessarily present on the same cells. The lymphocytes were also stained by heat-aggregated IgG coupled to rhodamine but were not stained by "native" IgG that is coupled to rhodamine. The immunofluorescence pattern was made of numerous small spots uniformly scattered on the cell surface, as usually observed after staining at 4°. The results of the following experiments were essentially the same with the cells of four of the five patients (three with chronic lymphocytic leukemia and one with Waldenström macroglobulinemia).

Experiments with trypsinized cells

Trypsinization removed all detectable surface immunoglobulins. In the absence of further staining, the cells remained negative after 1 and 6 hr of incubation. In contrast, immunofluorescence staining performed after 6 hr of incubation exclusively showed newly synthesized μ and κ determinants on 60-100% of the lymphocytes. μ chains were detected in all four patients, including the one that gave negative results with the anti- μ conjugate before trypsinization. The trypsinized cells were also stained by conjugated "native" IgG. Moreover, after incubation with the uncoupled native IgG (ascertained to be free of aggregated material), staining by anti- γ conjugate revealed IgG binding. This incubation with native IgG reproduced the *in vivo* situation, since the results after staining by the anti- μ conjugate were identical to those obtained with freshly drawn cells. The cells remained negative after incuba-

tion with purified IgA and subsequent staining by anti- α conjugate.

Redistribution experiments

Redistribution of surface immunoglobulins in caps was induced by the anti- μ conjugate. After 1 hr of incubation at 37°, these cells were subsequently stained by antiserum to γ chains coupled to the opposite fluorochrome (fluorescein or rhodamine). Under these conditions the caps showed a mixed staining, and the cell surface outside the caps was devoid of any stain. Similarly, staining by the anti- μ conjugate after induction of redistribution of surface immunoglobulins by antiserum to γ chains did not reveal μ determinants outside the caps that were stained in most instances by both reagents. The same clustering of surface immunoglobulins was observed after incubation with conjugated aggregated IgG. The results of double-labeling experiments performed with the anti- μ conjugate were identical to those obtained when the redistribution had been induced by the antiserum to γ chains.

Previously labeled surface immunoglobins were not detectable anymore, after 6 hr of incubation at 37°, on lymphocytes on which cap formation had been induced by antisera to γ or μ chains. At that time, the same experiments done after trypsinization were performed. The results were identical, showing the appearance of newly synthesized μ and κ chains and the binding of native IgG.

In these latter experiments, as well as in the experiments with trypsinized cells, most lymphocytes that were stained in the cold by "native" IgG coupled to rhodamine and kept at 37° for 1 hr showed a redistribution in caps. The same redistribution was observed when cells previously incubated with unconjugated native IgG and washed in the cold were incubated at 37° for 1 hr and subsequently stained at 0° by the anti- γ conjugate. This result is in contrast to the spotty immunofluorescent pattern found after staining by the anti- γ conjugate of freshly drawn lymphocytes with bound IgG. In order to explain this discrepancy and to test if this was not due to the excess of IgG bound to surface immunoglobulins *in vivo*, untreated cells were washed several times at room temperature (20-24°) and incubated at 37° for 1 hr. The cells were then cooled and stained with the anti- γ conjugate at 0°. Under these conditions, redistribution of surface immunoglobulins occurred. However, only 10% of the positive lymphocytes showed true caps, and the main staining pattern was made of large patches irregularly distributed on the cell surface.

Additional studies

After a 2-min incubation in citric acid the cells were severely damaged, since 20-30% of the lymphocytes did not exclude trypan blue and exhibited intracytoplasmic staining with the conjugated antisera. However, the results of the subsequent immunofluorescent staining were clear since those cells excluding the dyes showed a membrane that was strongly positive for μ and κ determinants but became negative for γ and λ chains.

A series of control experiments were performed with circulating lymphocytes from normal subjects and from patients with chronic lymphocytic leukemia with membrane-bound monoclonal IgM but without any staining by the anti- γ conjugate. The trypsinized cells were not able to bind native IgG. The anti- μ conjugate was able to induce cap formation on fresh cells bearing IgM on the surface, whereas no re-

distribution was observed on cells carrying IgM after incubation with the conjugated antiserum to γ chains. Native IgG from humans or rabbits coupled to rhodamine constantly yielded negative results. In contrast to this finding, heat-aggregated, conjugated human IgG stained all normal or leukemic lymphocytes with surface immunoglobulins. However, when redistribution in caps induced by anti- μ conjugate was followed by staining with aggregated IgG coupled to the alternate fluorochrome, mixed staining of the caps was not observed, and the conjugated aggregated IgG labeled the remaining cell surface.

The findings in the fifth patient exhibiting a positive membrane for γ , μ , κ , and λ chains can also be considered controls. This patient with chronic lymphocytic leukemia showed multiple autoimmune abnormalities, including a high concentration of serum rheumatoid factor. Double-labeling experiments showed that the repartition of surface IgG on freshly drawn cells was unaffected by the redistribution of surface IgM induced by antibody. The anti- γ conjugate did not induce cap formation, although some degree of clustering was observed. Treatment with acid did not remove the surface IgG. In the late stages of trypsin or capping experiments, the presence of newly synthesized surface immunoglobulins exclusively made of μ and κ chains was demonstrated, but these cells were unable to bind conjugated or unconjugated native IgG.

DISCUSSION

The present findings provide strong evidence for anti-IgG activity of the monoclonal IgM bound to membranes of lymphocytes from four patients with lymphoproliferative diseases. Immunofluorescence study of freshly drawn lymphocytes showed the simultaneous presence of μ , γ , κ , and λ determinants on the cells of these patients. However, after removal of surface immunoglobulins either by treatment with trypsin or after redistribution induced by antibody followed by incubation in culture medium at 37° for 6 hr, only μ and κ chains were detected on the cell surface. Several control experiments have ascertained that these monoclonal immunoglobulin molecules were synthesized *de novo*. This newly synthesized IgM was able to bind normal human IgG. Moreover, double-labeling experiments with freshly drawn cells indicated that this binding occurred *in vivo* since the redistribution of surface immunoglobulins induced by the anti- μ conjugate did involve the γ determinants and the reverse.

Several findings strongly suggest that we are dealing with an anti-IgG antibody activity of the surface IgM. In the patient with Waldenström's macroglobulinemia, the serum monoclonal IgM exhibited all the features of an antibody to human IgG. The fact that this antibody activity is shared by surface immunoglobulins on lymphocytes is expected since Waldenström's macroglobulinemia represents the proliferation of a clone of B cells with persistent maturation and secretion (5). In all patients studied, the binding activity was specific for the IgG class, and neither rabbit nor calf IgG were bound by IgM on the cell surface. IgG molecules were dissociated from the surface IgM by treatment with acid. It appears likely that IgG is bound to the Fab portion of the receptor IgM, since the C-terminal part of the Fc region is poorly accessible to immunofluorescent labeling (9). In accordance with its high affinity for rheumatoid factor, heat-aggregated IgG was able to displace the native IgG combined

with IgM on freshly drawn cells. Indeed, aggregated IgG induced cap formation, and these caps were shown by double-labeling to contain the μ determinants. Native IgG was also able to induce redistribution of IgM molecules on the surface of cells carrying newly synthesized IgM. The ability of unaggregated IgG to induce cap formation is presumably due to its antigenic bivalency. However, this capping phenomenon did not occur *in vivo*. This discrepancy may be due to an *in vivo* excess of antigen. One should recall here that redistribution of surface determinants induced by antibody is inhibited by antibody excess (7).

The presence of receptor sites for the Fc portion of IgG molecules on the surface of mouse and human B lymphocytes has recently been demonstrated (10, 11). However, the results presented above clearly indicate that the present findings are due to an actual binding of IgG to surface IgM and not to such receptor sites. Our control experiments provided no evidence of staining of normal or other leukemic B lymphocytes by conjugated native IgG. This negative result may be explained by the fact that the bridge between IgG not combined to antigen and Fc receptor sites on B lymphocytes is loose enough to be disrupted by a single washing (10). In contrast, we have observed that heat-aggregated human IgG is able to bind to any normal or leukemic B lymphocyte, presumably through interaction with Fc receptors. We should emphasize here that our experiments with the cultured cells bearing newly synthesized IgM were performed with unconjugated IgG devoid of aggregated material.

The results obtained in the study of the cells from the fifth patient showing "mixed" (μ , γ , κ , and λ) staining of freshly drawn lymphocytes were clearly different from those discussed above. In this patient, we are presumably dealing with an attachment of circulating antigen-antibody complexes either to the Fc receptor site (10) or to the complement receptor (12) of the B lymphocyte surface. This mixed-staining pattern is not uncommon in lymphoproliferative disorders since we have encountered it in 20% of 65 random patients with chronic lymphocytic leukemia. Rheumatoid-factor activity of surface IgM accounts for this confusing finding in four of the five patients studied. In addition to the attachment of immune complexes to the lymphocyte surface, other situations (such as the simultaneous presence of monoclonal surface immunoglobulins and of antibody molecules directed against membrane determinants of leukemic lymphocytes) could result in this mixed staining observed with truly monospecific antisera. Since we have demonstrated in all five patients studied that these leukemic lymphocytes did in fact synthesize a single type of heavy and light chains, such immunofluorescence patterns do not dispute the monoclonal nature of surface immunoglobulins in patients with chronic lymphocytic leukemia (3, 4).

The incidence of anti-IgG activity of membrane-bound IgM appears to be relatively high among patients with chronic lymphocytic leukemia. This is not surprising in view of the strikingly high percentage of serum monoclonal IgM possessing this activity in patients with Waldenström's macroglobulinemia (6). It is worth noting that two of the three patients with chronic lymphocytic leukemia, with anti-IgG activity of surface immunoglobulins, showed neither spike nor detectable rheumatoid-factor activity in their serum. This finding is consistent with the hypothesis that chronic lymphocytic leukemia represents in most instances a monoclonal proliferation of B

lymphocytes with a block in the maturation process (4). As in the case of myeloma proteins in the past, the homogeneous populations of B lymphocytes from patients with chronic lymphocytic leukemia should provide immunologists with surface-antigen receptors with individual antibody activities.

We thank Sylvaine Piat for excellent technical assistance. This work was supported by Grants from D.G.R.S.T. (71.7.2857 and 71.7.3065), I.N.S.E.R.M. (71.10.502) and C.N.R.S. (E.R.A. 239).

1. Pernis, B., Ferrarini, M., Forni, L. & Amante, L. (1971) in "Progress in Immunology," *First International Congress of Immunology*, ed. Amos, B. (Academic Press, New York), pp. 95-106.
2. Nossal, G. J. V., Warner, N. L., Lewis, H. & Sprent, J. (1972) *J. Exp. Med.* **135**, 405-428.
3. Grey, H. M., Rabellino, E. & Pirofsky, B. (1971) *J. Clin. Invest.* **50**, 2368-2375.
4. Preud'Homme, J. L., Klein, M., Verroust, P. & Seligmann, M. (1971) *Eur. J. Clin. Biol. Res.* **16**, 1025-1031.
5. Preud'Homme, J. L. & Seligmann, M. (1972) *J. Clin. Invest.* **51**, 701-705.
6. Metzger, H. (1969) *Amer. J. Med.* **47**, 837-844.
7. Taylor, R. B., Duffus, N. P., Raff, M. C. & De Petris, S. (1971) *Nature New Biol.* **233**, 225-229.
8. Preud'Homme, J. L., Neauport-Sautes, C., Piat, S., Silvestre, D. & Kourilsky, F. M. (1972) *Eur. J. Immunol.*, in press.
9. Froland, S. S. & Natvig, J. B. (1972) *Scand. J. Immunol.* **1**, 1-12.
10. Basten, A., Sprent, J. & Miller, J.F.A.P. (1972) *Nature New Biol.* **235**, 178-180.
11. Wigzell, H., Sundqvist, K. G. & Yoshida, T. O. (1972) *Scand. J. Immunol.* **1**, 75-87.
12. Nussenzweig, V., Bianco, C., Dukor, P. & Eden, A. (1971) in "Progress in Immunology," *First International Congress of Immunology*, ed. Amos, B. (Academic Press, New York), pp. 73-82.