MEMBRANE IMMUNOGLOBULINS OF B LYMPHOCYTES

Inability to Detect Certain Characteristic IgM and IgD Antigens*

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Evidence from studies of normal human peripheral blood lymphocytes and leukemia lymphocytes of patients with chronic lymphocytic leukemia (CLL)[1] indicates that IgM and IgD are the predominant receptor Igs on the human lymphocyte surface (1–3). They occur together on the same cell surface in many but not all instances. Little is known about their interrelationship although it has been demonstrated that they form caps independently (3). In the course of analyzing these surface Igs and studying their relationship to each other it was observed that not all IgM and IgD antisera with well defined antibodies to the serum Igs detected them on the lymphocyte surface. The present report represents a detailed investigation of this phenomenon and demonstrates that a part of the Fc fragment is unavailable for reaction with specific antibodies.

Materials and Methods

Preparation of IgM and Its Fragments. Two IgM κ and two IgM λ proteins were isolated from defibrinated plasma of patients with Waldenström's macroglobulinemia by Pevikon (Mercer Corp. Yonkers, N. Y.) block electrophoresis. One IgM κ and one IgM λ proteins further purified by gel chromatography on Sephadex G-200 (Pharmacia Chemical Co., Uppsala, Sweden). Partial reduction and alkylation of the IgM κ and the IgM λ were made by reduction with 0.02 M dithiothreitol followed by alkylation with 0.03 M iodoacetamide at pH 8.0. Complete reduction and alkylation of these two proteins were carried similarly in 5 M guanidine HCl. The completely reduced and alkylated IgM λ was soluble in phosphate-buffered saline (PBS) whereas the completely reduced and alkylated IgM κ was insoluble. $F(ab)_{\mu}$(pep) and $F(ab')_{2\mu}$(pep) fragments were prepared from the IgM κ according to Schrohenloher and Bennett (4). The IgM was incubated with $1/100$ weight of pepsin (Worthington Biochemical Corp., Freehold, N. J.) in 0.1 M sodium acetate buffer (pH 4.1) for $2\frac{1}{2}$ h at 37°C. Digestion was stopped by neutralization to pH 8. The resulting mixture containing the fragments was concentrated and applied to a Sephadex G-100 column. $F(ab)_{\mu}$(pep) fragments were obtained as the second peak after the peak of the void volume of the column. The void volume peak contained

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[1] *Abbreviations used in this paper:* CLL, chronic lymphocytic leukemia; PBS, phosphate-buffered saline.

$F(ab')_{2\mu}$(pep) fragments and undigested IgM. Purified $F(ab')_{2\mu}$(pep) fragments were obtained by repeated gel chromatography on Sephadex G-150.

$F(c)_{5\mu}$(tryp) fragments were obtained essentially according to the method of Plaut and Tomasi (5). Trypsin (Worthington) digestion of IgM was performed at 60°C for 3 h with a substrate-to-enzyme ratio 50:1 in 0.05 M Tris-HCl buffer, 0.015 M calcium chloride (pH 8.1). An equivalent amount of soybean trypsin inhibitor was added to the reaction mixture. The $F(c)_{5\mu}$ and $F(ab)_{\mu}$ were separated by gel chromatography on Sephadex G-200. $F(c)_{5\mu}$(tryp) was obtained from the IgM κ and the IgM λ.

$F(ab')_{2\mu}$(pep) and $F(ab)_{\mu}$(pep) gave single precipitin lines in the gamma region with antisera to kappa type light chains on immunoelectrophoresis. F(ab) (pep) did not react with anti-μ specific antisera whereas $F(ab')_{2\mu}$(pep) reacted with some anti-μ antisera. $F(c)_{5\mu}$(tryp) formed a precipitin line in the beta region with anti-μ antisera but not with antisera to kappa type light chains or antisera specific for Fab of IgG. By agar diffusion analysis with anti-μ antiserum all three fragments were deficient antigenically when compared to the whole IgM and $F(ab')_{2\mu}$(pep) and $F(c)_{5\mu}$(tryp) showed reaction of nonidentity. $F(ab)_{\mu}$(pep) showed partial inhibition of the precipitin line formed by anti-μ antiserum and $F(ab')_{2\mu}$(pep).

Anti-Immunoglobulin Antisera. Antisera against isolated IgM and IgD were raised in New Zealand Red rabbits. For immunofluorescence, $F(ab')_2$ fragments made from gammaglobulin fraction of these antisera according to Nisonoff et al. (6) were conjugated with tetramethylrhodamine isothiocyanate. $F(ab')_2$ fragments specific for μ (Rho-anti-μ) and $F(ab')_2$ fragments specific for δ (Rho-anti-δ) were made by appropriate absorptions and the specificity of these antisera was ascertained as previously reported (2). F(ab') fragments of Rho-anti-μ were obtained by mild reduction with 0.01 M cysteine. Absorption with 0.1 mg $F(ab')_{2\mu}$(pep) per 1 ml of Rho-anti-μ abolished its ability to agglutinate $F(b')_{2\mu}$(pep)-coated red cells and its titer against $F(c)_{5\mu}$-coated red cells remained unaltered. Similarly, absorption with 0.1 mg $F(c)_{5\mu}$(tryp) per 1 ml of Rho-anti-μ abolished its ability to agglutinate $F(c)_{5\mu}$-coated red cells and its titer against $F(ab')_2$-coated cells remained unchanged. Absorption of Rho-anti-μ and Rho-anti-δ with packed CLL cells was carried out at room temperature for 45 min. Antisera were absorbed at a ratio of 1 ml antiserum per 2.5×10^9 cells. Absorption of F(ab') fragments of Rho-anti-μ by CLL cells was carried out in a similar manner. These fragments did not agglutinate IgM-coated cells but they interfered with the agglutination of IgM-coated red cells by an anti-μ antiserum. For hemagglutination inhibition, the anti-μ antisera were used after absorption with Fraction II human gamma globulin.

Lymphocyte Preparation and Cell Fractionation. Mononuclear cells were isolated from the peripheral blood of patients with CLL with WBC greater than 25,000/mm^3 and normal subjects by Ficoll-Hypaque discontinuous gradients. The cells were washed six times with 50 ml PBS. Viability of lymphocyte preparations was greater than 95% by trypan blue exclusion. For cell fractionation, lymphocyte suspensions were subjected to sonication. After sonication, no intact lymphocytes were observed by microscopic examination. The homogenate was centrifuged at 133,000 *g* for 60 min. The sediment was resuspended in an original vol of PBS. The homogenate, the supernate, and the sediment were used for hemagglutination inhibition or absorption experiments.

Hemagglutination Inhibition and Antiserum Absorption. Hemagglutination and hemagglutination inhibition experiments were carried out as described previously (7). Purified proteins were coated onto human red cells by the bizdiazotized benzidine method (BDB). A lymphocyte suspension was incubated with an equal volume of the appropriately diluted antiserum for 45 min and after centrifugation, 0.1 ml of the supernate was used to test its ability to agglutinate indicator red cells. More than 90–95% lymphocytes were viable after absorption. Cell homogenate and sediment fractions were used for absorption of the diluted antiserum in an identical manner and the supernatant fraction was used as an inhibitor directly. By comparison of the absorption efficiency of the cell suspensions in serial dilution and known amount of inhibitory proteins, the gross amount of surface Ig was estimated. Determinations of inhibitor protein concentration were done by the modified Folin-Ciocaulteau method.

Immunofluorescence. Immunofluorescent staining of lymphocyte surface Ig was performed as described by Pernis et al. (8). Since $F(ab')_2$ fragments of Rho-anti-μ and Rho-anti-δ were used, no attempt was made to remove aggregates before use. Immunofluorescent staining of intracellular Ig of cells from the lymphoblast line RPMI 1788 was done according to Hijmans et al. (9). The cell line was obtained from Associated Biomedic Systems Inc. (Buffalo, N. Y.) and maintained in RPMI 1640 culture medium supplemented with 10% fetal calf serum, penicillin, and streptomycin.

Results

Hemagglutination Assays. One of the major procedures employed in the present study of the surface Igs was essentially an antiserum absorption technique utilizing viable lymphocytes. The degree of absorption of the specific antiserum was then determined by hemagglutination using IgM, IgD, or their fragments as coat proteins on the red cells. It was initially observed in the case of IgM that not all the antibodies giving specific hemagglutination could be absorbed out by normal or CLL lymphocytes known to have IgM on their surface. Table I shows the results with one of the IgM systems employed. The upper portion of the table shows the specificity of the hemagglutination reaction using whole IgM as the red cell coat and an antiserum specific for IgM as the agglutinator. It can be seen that whole isolated IgM inhibited the agglutination as did the Fc fragments made by the trypsin procedure. However, Fab fragments did not inhibit nor did other Igs. The lower portion of Table I shows the results with this system when IgM-bearing lymphocytes or their fractions were employed to absorb out or inhibit the antiserum. The whole cells had no effect on the hemagglutination even when high concentrations were used despite the fact that fluorescent antibody analyses clearly showed surface IgM. However, the homogenate of the cells and the supernate fraction inhibited the antiserum even at low

TABLE I

Hemagglutination System Consisting of Red Cells Coated with Whole IgM and Anti-IgM Antiserum

The upper portion shows the specificity of the system which is only inhibited by whole IgM or the Fc fragment. The lower part shows the effect of cells or their fractions in absorbing out or inhibiting the agglutinating antiserum. Whole cells had no effect while the homogenate was strongly inhibitory.

Protein	Inhibitor protein concentration (μg/ml)					
	100	10	1	0.25	0.062	0.016
IgM	0	0	0	0	0	2
$F(c)_{5\mu}$(tryp)	0	0	0	0	0	2
$F(ab')_{2\mu}$(pep)	2	2	2	2	2	2
$F(ab)_{\mu}$(pep)	2	2	2	2	2	2
Pooled IgG	2	2	2	2	2	2
IgD	2	2	2	2	2	2
IgA	2	2	2	2	2	2
Source of cells	10^8 cells/ml					
	1	0.5	0.25	0.125	0.062	0.031
RF(CLL) Cells	2	2	2	2	2	2
Homogenate	0	0	0	0	0	2
Sediment	0	2	2	2	2	2
Supernate	0	0	0	0	0	2
SF(normal) cells	ND	2	2	2	2	2

Antiserum dilution 1/3,200. Coating protein whole IgM.

concentrations. Lymphocytes and their fractions from eight patients with CLL behaved in the same manner. Similar results were obtained with three different antisera and a variety of isolated IgM proteins as red cell coats. Agar diffusion analysis indicated that all of these antisera contained antibodies primarily directed to the Fc portion of the molecule and were negative or weakly reactive to the various Fab fragments.

Four of ten IgM specific antisera were found to react significantly with $F(ab')_{2\mu}$(pep) fragments in agar diffusion. When these antisera were employed and the $F(ab')_{2\mu}$(pep) fragment was used as the red cell coat, strong agglutination was obtained. This system is shown in Table II. Whole IgM and the $F(ab')_{2\mu}$ fragments inhibited the reaction but the Fc fragments were entirely negative. However, in contrast to the previous results, whole lymphocytes effectively removed the antibodies so that the agglutination reaction became negative. Similar results were obtained when whole IgM was used as the red cell coat provided that the antiserum had the additional absorption with Fc fragments. In each case anti-IgM antibodies directed against Fab sites probably near the hinge region of the molecule were involved and these were readily removed by the lymphocytes. These systems could be used to quantitate the IgM on the lymphocytes of normal individuals and CLL cases. Levels up to 1,000 ng/10^8 cells were obtained for some of the 13 CLL cases studied.

If IgM systems using Fc fragments as the coat were employed with whole antisera or antisera specific for the Fc region, the whole lymphocytes failed to

TABLE II

Hemagglutination System Consisting of Red Cells Coated with $F(ab)_{2\mu}(pep)$ and Anti-IgM Antiserum

In this system the CLL lymphocytes absorb out the agglutinating antibodies and IgM is detected on the cell surface.

Protein	Inhibitor protein concentration (μg/ml)					
	100	10	1	0.25	0.062	0.016
IgM	0	0	0	0	1	2
$F(c)_{5\mu}$(tryp)	2	2	2	2	2	2
$F(ab)_{2\mu}$(pep)	0	0	0	0	0	2
$F(ab)_{\mu}$(pep)	0	0	2	2	2	2
Pooled IgG	2	2	2	2	2	2
IgD	2	2	2	2	2	2
IgA	2	2	2	2	2	2
Source of cells	10^8 cells/ml					
	1	0.5	0.25	0.125	0.062	0.031
RF(CLL)	0	0	0	0	0	2
SF(normal)	ND	0	1	1	2	2

Antiserum dilution 1/800. Coating protein $F(ab')_{2\mu}$ (pep).

remove enough antibodies to alter the hemagglutination. Evidence was obtained that some Fc antibodies were removed by the lymphocytes but enough antibodies always remained to give agglutination. IgMs obtained on partial reduction and alkylation behaved very similar to the whole IgM and reacted with antibodies that the lymphocytes failed to remove. IgMs after complete reduction and alkylation failed to react with antibodies specific for Fc and Fab fragments. Studies with IgG and IgA systems in a fashion similar to the above work with IgM did not detect any antigens belonging to these classes in the CLL cells examined. Lymphocytes and their fractions of one of the CLL patients did not absorb either Fc or Fab specific antibodies. However, immunofluorescent study confirmed that these lymphocytes possessed no surface IgM.

Immunofluorescence. In the initial studies, IgM was readily detected on the lymphocyte surface of the normal and CLL cells by fluorescence even though the first hemagglutination system described above failed to show surface IgM. This discrepancy was one of the findings that raised the possibility of buried IgM determinants on the lymphocyte surface. Both the anti-IgM Fc and the anti-IgM Fab antisera described above gave strong fluorescence of IgM-bearing cells. Some of these data are shown in Table III. The percentage of cells showing surface staining with the rhodamine-conjugated anti-IgM antiserum is indicated both for two normal and two CLL lymphocyte preparations. The IgM-specific antiserum

TABLE III

Effect of Absorption With CLL Cells on the Detection of Surface and Intracellular IgM

Surface IgM staining is virtually eliminated but antibodies giving intracellular staining and agglutination remain.

	Surface immunofluorescence				Intracellular immunofluorescence lymphoblastoid line RPMI 1788	Hemagglutination titer against IgM-coated red cells
	Normal		CLL			
	SF	CD	RF	GO		
	% positive cells				*no. positive cells*/200 cells*	
Rho-anti-μ	10	10	91	64	21	1/256
Rho-anti-μ absorbed by:						
$F(ab')_{2\mu}$(pep)	11	9	92	67	21	1/256
$F(ab')_{2\mu}$(pep) and once with CLL cells	11	11	86	42	20	1/128
$F(ab')_{2\mu}$(pep) and twice with CLL cells	<1	4	<1	<1	21	1/128
$F(ab')_{2\mu}$(pep) and three times with CLL cells	<1	2	<1	<1	19	1/64
$F(ab')_{2\mu}$(pep) twice with CLL cells and $F(c)_{5\mu}$(tryp)	ND	ND	ND	ND	0	0

* Positive cells were those with bright intracellular staining typical of plasma cell types.

shows staining of approximately 10% of normal lymphocytes and a high percentage of the CLL cells. Similar staining was obtained after absorption of the antiserum with $F(ab')_{2\mu}$(pep) fragments. After additional absorptions with IgM-bearing CLL cells, the surface fluorescence reaction on normal and CLL cells essentially disappeared. However, antibodies remained in the antiserum which gave strong agglutination and stained the intracellular Ig in the cells of a lymphoblastoid line. Approximately 10% of the cells of this line stained strongly with the unabsorbed antiserum. A similar percentage stained with the antiserum absorbed with viable lymphocytes. This staining and the agglutination only disappeared after absorption with the IgM Fc fragments. Similar results were obtained with lymphocyte absorptions of the F(ab') fragments of the specific IgM antibodies. These data and those from other similar experiments added further evidence that a portion of the Fc determinants of the lymphocyte membrane Ig is unavailable for reaction with the anti-Fc antiserum.

Experiments were also carried out on the IgD system. Serial absorptions of IgD-specific antisera with IgD-bearing CLL cells abolished the surface staining of IgD-bearing lymphocytes but left strong hemagglutinating activity just as was the case for the IgM system. In one experiment, after three absorptions with lymphocytes, the fluorescence was completely abolished but a 1/320 titer in hemagglutination remained. The hemagglutinating activity that could not be absorbed out with lymphocytes was shown to be specific for IgD in inhibition experiments.

Discussion

The observations described above indicate that viable lymphocytes with surface IgM fail to react with a population of anti-IgM antibodies found in most IgM-specific antisera. These antibodies are directed against the Fc part of the molecule and react strongly with isolated serum IgM as well as the intracellular IgM of both lymphocytes and plasma cells. Absorption experiments with lymphocytes failed to remove these antibodies as demonstrated both by hemagglutination and fluorescent antibody studies. Antisera made specific for the Fab portion of the IgM molecule were completely removed by such lymphocyte absorptions. It also was apparent that not all of the antibodies directed against Fc antigens were unavailable for reaction with the surface IgM; some clearly were absorbed out with lymphocytes. Some evidence has been obtained that the C-terminal part of the Fc is involved. Preliminary studies utilizing a CH4 fragment obtained from IgM Fc by limited enzymatic digestion (10) indicates that this C-terminal area has at least part of the antigenic determinants that are not detectable on the lymphocyte surface.

Results similar to those for IgM were obtained for IgD, the other major membrane Ig of human peripheral blood lymphocytes. Somewhat less extensive studies were carried out with this system but clear evidence for an absence of Fc antigens was obtained in absorption experiments where lymphocytes failed to remove a population of IgD antibodies.

The exact interpretation of these findings remains unclear. It was apparent

that a portion of the Fc of the surface IgM and IgD molecule was different from those of the serum IgM and IgD and the most likely possibility is that it is buried in the lymphocyte membrane so that the specific antibodies or their Fab fragments were unable to reach it. Other possibilities also require consideration. Additional carbohydrate may be present on the membrane-bound IgM which might change the availability of certain antigenic sites. Some differences in carbohydrate of surface IgM has been observed by Melchers and associates (11). A third possibility is that the amino acid structure is different. Most studies with lymphocyte surface radioiodination by the procedures developed by Uhr and associates (12) have indicated that the μ-chains were very similar for membrane and intracellular IgM. However, there have been some reports of differences in the size of the μ-chain (13). It will be of interest to see if the membrane Ig after release from the cell with nonionic detergents will then react with the lymphocyte-absorbed antisera. Such experiments should be feasible.

Studies have been carried out by Fröland and Natvig (14) on IgG2 on lymphocytes that are relevant to the present observations. They found, by the use of genetic markers, that Gm(n) which has been localized to the pfc′ fragment of the IgG molecule was not detectable. Pernis et al. (8) had similar results regarding surface IgG on rabbit lymphocytes. Other workers using fluorescent antibody analyses in the IgA system have noted that not all IgA antisera reveal IgA on the membrane[2] (15). In the present studies all IgM and IgD antisera tested showed the surface Igs by the fluorescent antibody technique but it was only when other techniques were utilized that the differences from serum Igs became apparent. Some previous observations (16) by techniques similar to the absorption-hemagglutination method employed in the present study failed to detect IgM on the CLL and normal lymphocyte surface. These results are now readily explainable as due to antibodies to the absent lymphocyte determinants which were not absorbed out and the agglutination was not altered.

Recent findings on the amino acid sequence of the IgM heavy chains indicate that, except for a carbohydrate group attached to residue 563, the C-terminal area (CH4 homology region) is free of carbohydrate and is very rich in hydrophobic amino acids (17). This may relate to the postulated attachment and partial burial of the C-terminal portion of the IgM heavy chains in the lipid moieties of the membrane. It is certainly clear that the Fab portion of the molecule is on the outer surface of the membrane and is available for antigen binding as well as for all the Fab-specific antibodies utilized in the present study. In addition the sites reacting with idiotypic antibodies are clearly available (18).

Summary

Hemagglutination and fluorescent antibody studies have provided strong evidence for the unavailability or absence of specific antigenic sites on membrane-bound IgM which are present in serum and intracellular IgM. Antisera specific for different parts of the molecule indicated that a portion but not all of the Fc

[2] Lars Hanson. Personal communication

was involved. Absorption experiments with normal and leukemic viable B lymphocytes failed to remove a population of Fc antibodies found in IgM-specific antisera. Similar findings were made for IgD, the other major membrane immunoglobulin of human peripheral blood B cells. Various interpretations of these observations are discussed. The most likely possibility appears that the C-terminal portion of the heavy chains of the immunoglobulin molecule is buried in the membrane.

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