CLASS, SUBCLASS, AND ALLELIC EXCLUSION OF MEMBRANE-BOUND Ig OF HUMAN B LYMPHOCYTES

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Membrane-bound immunoglobulins (Ig) can be demonstrated on a population of human peripheral lymphocytes by several techniques, including immunofluorescent staining of viable lymphocytes in suspension (1–3). It is highly probable that these Ig-positive lymphocytes are B lymphocytes, i.e. homologous to bursa or bone marrow-derived cells in other species (2–4), and that the membrane-bound Ig molecules function as receptors for antigen (5). B lymphocytes are precursors for immunoglobulin-secreting cells, and there is overwhelming evidence that the immunoglobulin product of a single immunoglobulin-secreting plasma cell is homogeneous with regard to both antibody specificity and to Ig class, subclass, and allotype (6). It is, however, unknown at which stage during the differentiation of the B cells this phenotypic exclusion occurs.

The object of the present work is to investigate by means of double immunofluorescence staining whether the exclusion has already occurred in the B lymphocyte, i.e., if the membrane-bound Ig on any one B lymphocyte belongs to a single class, IgG subclass, and Gm type. Previous experiments where the relative distribution on B lymphocytes of Ig classes and subclasses was studied, indicated that this is the case (7).

Materials and Methods

Lymphocyte Donors.—Venous heparin blood (10 IU/ml of heparin) was drawn from healthy donors with known Gm type. Lymphocytes were obtained by the Isopaque-Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) centrifugation method described by Böyum and later used in our laboratory for preparation of cells for lymphocyte culture and for demonstration of membrane-bound Ig (8). The separated cells were washed three times in Hanks' balanced salt solution (Hanks' BSS, Grand Island Biological Co., Grand Island, N. Y.), counted in a Bürker hemocytometer counting chamber, and resuspended in Medium 199 (Grand Island Biological Co.).

Antisera Against Ig Classes, Subclasses, and Gm Allotypes.—Antisera against IgM, IgG, the four IgG subclasses, the pFc' fragment, and against Gm(f), Gm(z), Gm(n), Gm(g), Gm(a), and the "non a" marker related to the Gm system, were raised, absorbed, and tested for specificity as described elsewhere (9, 10). Anti-Gm(n), anti-Gm(g), anti-Gm(a), and anti-"non a" were raised in subhuman primates, while the other antisera were of rabbit origin.

Fluorochrome Labeling.—Isolated IgG from antisera was labeled either with fluorescein isothiocyanate (Baltimore Biological Laboratories, Baltimore, Md.), or lissamine rhodamine B (Imperial Chemical Industries, England), prepared according to Fothergill (11). Free fluorochrome was then removed and purification of conjugates was performed by chromatography on diethylaminoethyl cellulose columns (12). The conjugates had a molar fluorescein to protein (F/P) ratio of 2–3 (7) and were tested for specificity as described earlier (9).

Immunofluorescence Staining and Microscopy.—Staining was performed on viable lympho-

cytes in suspension (7). For double staining, cells were incubated with one conjugate, washed twice with Hanks' BSS, and then incubated with the second conjugate, washed, and resuspended in Medium 199. Microscopy was performed with a Leitz Orthoplan Microscope (E. Leitz, Inc., Rockleigh, N. J.) with an Osram HBo-200 mercury arc lamp and a Leitz vertical illuminator. Fluorescein-isothiocyanate fluorescence was detected with the following filter combination: 1.5 mm BG 12, KP 490, TK 495, K 495, and secondary filter AL 525 or 530, and rhodamine fluorescence with 4 mm BG 38, 2 mm BG 36, AL 546, TK 580, and secondary filter 530. An oil immersion objective Fl 54/0.95 was used.

RESULTS

Ig Classes and Subclasses.—Double staining experiments were performed with a fluorescein-labeled anti-IgG Fc and a rhodamine-labeled anti-IgM. In all experiments lymphocytes positive for either IgM or IgG were found. No lymphocytes bearing both IgM and IgG on the surface were present in any of the cases. Double staining experiments were also performed with a rhodamine-labeled antiserum directed against IgG2, and with fluorescein-labeled antisera against IgG1, IgG3, and IgG4. In these experiments, lymphocytes bearing either IgG1, IgG2, or IgG3 were found. However, no lymphocytes staining for more than one IgG subclass were found with anti-IgG2 *versus* anti-IgG1, or with anti-IgG2 *versus* anti-IgG3. In the experiments performed with double staining for IgG2 and IgG4, no cells positive for IgG4 were detectable. Table I shows results from double staining experiments with antisera against IgG subclasses.

TABLE I

Double Staining Experiments with Fluorescein-Labeled Anti-IgG2 and Rhodamine-Labeled Anti-IgG1, Anti-IgG3, or Anti-IgG4

Donor	Percentage of lymphocytes staining for						
	IgG1	IgG2	IgG3	IgG4	IgG1 and IgG2	IgG2 and IgG3	IgG2 and IgG4
OD	1	2	1	1	<0.1	<0.1	<0.1
050	2	5	—*	n.t.‡	<0.1	<0.1	n.t.
137	1	5	—	n.t.	<0.1	<0.1	n.t.
140	—	—	n.t.	n.t.	<0.1	n.t.	n.t.
124	1	4	1	1	<0.1	<0.1	<0.1

* Positive cells present, but percentage not determined.

‡ n.t. = not tested.

Gm Allotypes.—Gm(f) and Gm(z) are allotypic markers in the Fd part of IgG1 chains. Since these markers are exposed on the lymphocyte surface (7) and behave as being controlled by homoalleles (13), they were suitable for the study of possible restriction of Gm allotypes of the membrane-bound Ig. Double staining experiments were performed with a fluorescein-labeled anti-Gm(z) and a rhodamine-labeled anti-Gm(f). In the individuals tested, a small proportion of lymphocytes bearing these Gm types on the surface was detected. The pres-

ence or absence of Gm(z) or Gm(f) on the lymphocytes, however, always corresponded to their presence or absence in the respective sera. In addition, in heterozygous individuals carrying both allelic markers, no lymphocytes were found to be positive both for Gm(f) and for Gm(z). Representative results are shown in Table II.

Restriction of Ig on Chronic Lymphocytic Leukemia Lymphocytes.—19 patients with chronic lymphocytic leukemia were investigated, and lymphocytes from all patients showed restriction to one Ig class on the surface. 18 of them had IgM on lymphocytes, while one patient had IgG on the tumor cells. Lymphocytes from this patient were further studied for possible restriction of IgG subclasses and genetic markers. It was found that the leukemia cells were positive for IgG2, but negative for the other IgG subclasses. The lymphocytes were also tested for the IgG2 genetic marker Gm(n), and no reaction with anti-Gm(n)

TABLE II

Double Staining Experiments with Fluorescein-Labeled Anti-Gm(z) and Rhodamine-Labeled Anti-Gm(f)

Donor	Serum Gm type		Gm type on lymphocyte surface	
	f	z	f	z
976	+	−	+	−
338	−	+	−	+
B.P.	+	+	+	+*
B.V.	+	+	+	+*
J.N.	+	+	+	+*

* No double staining cells (<0.1%) presenting both Gm(f) and Gm(z) were observed.

was observed. Since the patient was Gm($a^-g^-b^+f^+n^+$) and probably of genotype $Gm^f\ Gm^b\ Gm^n/Gm^f\ Gm^b\ Gm^{n-}$, the lymphocytes lacked an IgG2 allelic marker which is expressed on normal lymphocytes (see later) and which is present in the serum of that individual. This finding therefore also indicated allelic exclusion of IgG on the lymphocyte surface.

Further Studies on Genetic Markers Localized to Different Portions of the Fc Fragment of IgG.—Surface-bound Ig is detected by antisera against light chains or against genetic markers of the Fd part of the heavy chain such as Gm(f) and Gm(z). Furthermore, a reaction is readily obtained by anti-IgG Fc. Since various genetic markers are localized to each of the two main homology regions, CH2 or CH3 (corresponding to the pFc′ subfragment) of the Fc fragment (10), these markers were utilized to study which part of Fc was available on the lymphocyte surface.

Staining of normal peripheral blood lymphocytes was performed with antisera directed against the Gm(a) and "non a" markers and against pFc′, all corresponding to the CH3 homology region. No reaction was seen with anti-

Gm(a) or anti-"non a" indicating that these antigens were not exposed on the lymphocyte surface. Correspondingly, only very few and sometimes no lymphocytes reacted with anti-pFc′. In controls with myeloma bone marrow cells these antisera gave a positive reaction. In addition, the IgG2 leukemia cells mentioned above were stained for "non a" which is present in IgG2 and with anti-pFc′, but no positive staining was obtained.

In contrast, normal lymphocytes stained well with anti-IgG Fc and also gave a reaction with anti-Gm(g) and anti-Gm(n) detecting antigens in the CH2 homology region.

DISCUSSION

The present data show that surface-bound immunoglobulins of human lymphocytes in peripheral blood are restricted with regard to class, IgG subclass, and Gm allotypes, and no "double stainers" were detected with the immunofluorescence technique. These results are in agreement with the relative distribution of Ig classes and IgG subclasses on lymphocytes (7), showing that the sum of the percentages for each Ig class or IgG subclass usually equaled the percentage of Ig-positive cells or IgG-bearing cells, respectively. A similar restriction was also found in a chronic lymphocytic leukemia patient with IgG2 Gm(n^-) bearing tumor cells. This latter observation, together with restriction concerning Ig classes and light chain types on cells in chronic lymphocytic leukemia reported by various groups (4, 14),[1] supports the conclusion that chronic lymphocytic leukemia represents a proliferation of B cells which at a given time only express the product of one Ig class or subclass cistron and one allelic gene on the lymphocyte surface.

This is in agreement with data showing allelic exclusion of surface Ig of rabbit lymphocytes (1, 15). Also, very recently Abdou (16) has obtained results indicating that human B lymphocytes from peripheral blood are restricted concerning class of surface-bound Ig.

There are, however, also reports which are in conflict with the concept of exclusion of surface-bound Ig on lymphocytes, obtained by stimulation of rabbit lymphocytes with antisera against Ig classes and allotypic markers (17). Evidence for more than one Ig class on the surface of guinea pig lymphocytes utilizing formation of rosettes with heterologous erythrocytes (18), or the so-called reverse immune cytoadherence test (19), has also been reported.

It is possible that these discrepancies are due to methodological factors; they might also be due to variable differentiation stage of the cells studied. However, our data from newborn babies indicate that restriction of surface-Ig apparently exists already on fetal lymphocytes (20).

No staining was obtained with anti-Gm(a) and anti-"non a" and very little

[1] Fröland, S. S., J. B. Natvig, and P. Stavem. 1972. Immunological studies of patients with lymphoproliferative diseases. Restriction of classes, subclasses and Gm allotypes of membrane-bound Ig. *Scand. J. Immunol.* In press.

or no staining with anti-pFc′ reacting with the C terminal (CH3) region of Fc. On the other hand, staining was seen with antisera against Gm(f) and Gm(z) within the Fd portion (CH1) of IgG and with antisera against the Gm(n) and Gm(g) antigens localized in the N-terminal part of Fc (CH2). These results indicate that parts of the C terminus of the Fc fragments of membrane-bound IgG molecules are not available on the cell surface, in contrast to the F(ab) portion and the N-terminal part of Fc. This orientation of the molecule is compatible with its function as receptor for antigen.

SUMMARY

Membrane-bound immunoglobulins of peripheral blood B lymphocytes and of lymphocytes from an IgG chronic lymphocytic leukemia showed restriction to one immunoglobulin class, one IgG subclass, and one Gm allotype. Certain antigens in the C-terminal part of IgG Fc were not exposed on the cell surface.

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