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Characterization of the Fc Receptors of the Murine Leukemia L1210

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A glycoprotein extract prepared from the plasma membranes of L1210 cells was passed over columns of Sepharose 4B to which either heat-aggregated human IgG or $F(ab')_2$ fragments had been coupled. The intact IgG column bound 35.7% of the applied counts, whereas the $F(ab')_2$ columns bound 2.8%. The bound glycoproteins were eluted with citrate buffer (pH 3.2) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Three peaks with apparent molecular weights of 65,000, 45,000, and 28,000 daltons were identified and purified by electroelution from polyacrylamide gels. The isolated proteins were able to bind to the same subclasses of mouse IgG myeloma proteins as the intact L1210 cells, indicating that these molecules are related to L1210 surface Fc receptors. Amino acid analyses of the 3 proteins were markedly similar suggesting that the observed molecular heterogeneity might be due to carbohydrate differences. Neuraminidase digestion of the isolated proteins resulted in mobility shifts on polyacrylamide gel electrophoresis which were consistent with the interpretation that either the isolated proteins have considerably different sialic acid contents, or that removal of the sialic acid results in disaggregation of an Fc receptor molecule.

Key words: Fc receptors, membrane glycoproteins, mouse leukemia

Surface receptors for the Fc portion of the IgG molecule have been identified on a wide variety of cells (1). Cells bearing Fc receptors are identified by their ability to bind heat-aggregated IgG or antigen-antibody complexes, or to form rosettes with IgG coated erythrocytes (2, 3). It has been suggested that Fc receptors identified by aggregated IgG or immune complexes are identical (4), but a recent report demonstrated that a single cell line might possess separate Fc receptors with different IgG subclass affinities and different binding properties for aggregated versus monomeric IgG (5). The molecular identity of Fc receptors remains uncertain largely due to the lack of structural studies concerning these membrane components.

Abbreviations: PBS – phosphate-buffered saline; SDS – sodium dodecyl sulfate; PAGE – polyacrylamide gel electrophoresis; GP – glycoprotein.

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L1210 is a carcinogen-induced leukemia derived from DBA/2 mice that lacks surface immunoglobulin but bears an Fc receptor. L1210 is apparently a bone-marrow derived (B) lymphocyte since it also carries a B cell alloantigen (6). We have previously shown that redistribution of L1210 Fc receptors results in the selective release from the cell surface of a 45,000 dalton Fc-binding protein (7). In this report we demonstrate that 3 proteins which bind to the Fc region of aggregated IgG can be isolated from the plasma membranes of L1210 cells. Biochemical studies indicate that the observed molecular heterogeneity of the isolated molecules may be due to differences in their carbohydrate content or to aggregation of a basic subunit.

MATERIALS AND METHODS

L1210 cells were grown in stationary culture in RPMI 1640 supplemented with 10% fetal calf serum (Flow Laboratories, Rockville, Maryland). Five $\times 10^{10}$ cells were labeled with ^{125}I (Radiochemical Centre, Amersham, England) by the lactoperoxidase technique described by Marchalonis et al. (8). The cells were swelled in hypotonic buffer consisting of 2 mM $NaHCO_3$, 0.2 mM $CaCl_2$ and 5 mM $MgCl_2$ (pH 6.8), and ruptured in a tight fitting Dounce homogenizer. The plasma membranes of the cells were prepared by a partition of the cell lysates in a 2-phase system of polyethylene glycol and dextran according to the method of Hourani et al. (9).

A glycoprotein extract of the plasma membrane preparation was obtained by the lithium diiodosalicylate technique described by Marchesi and Andrews (10). Briefly, the plasma membrane fraction was vigorously resuspended in 10 ml of 0.05 M Tris-HCl buffer (pH 7.4). Recrystallized lithium diiodosalicylate (Eastman, Rochester, New York) was added to a final concentration of 0.3 M and the solution stirred vigorously at room temperature for 15 min. Two volumes of cold distilled water were added and the solution stirred for an additional 10 min. The sample was centrifuged at 48,000 × g for 40 min and to the supernatant was added an equal volume of freshly prepared 50% phenol. After stirring for 30 min the aqueous and phenol phases were separated by centrifugation at 4,000 × g for 1 h in a swinging bucket rotor. The aqueous phase was carefully removed and extensively dialyzed against 4 changes of distilled water over a period of 72 h. In some experiments the glycoprotein extract was labeled with ^{125}I by the chloramine T method (11).

Purified human IgG (Miles Laboratories, Kankakee, Illinois) was heat aggregated at a concentration of 40 mg/ml in phosphate-buffered saline at 63°C for 15 min. An $F(ab')_2$ fraction of human IgG was prepared by pepsin digestion (12), and heat aggregated at 63°C for 30 min, at which point the solution became faintly turbid. The aggregated proteins were coupled to activated Sepharose 4B (Pharmacia, Piscataway, New Jersey) as previously described (7).

The labeled glycoprotein extract, dissolved in PBS[1] was passed over columns of Sepharose 4B coupled to either aggregated human IgG or $F(ab')_2$ fragments. The columns were washed with PBS until the radioactivity in the eluate was equal to the background radioactivity and the percentage of radioactivity bound to each column was calculated. The bound radioactivity was eluted with 0.1 M citrate buffer, pH 3.2, and analyzed by SDS-PAGE as previously described (7). The radioactive peaks were isolated by electroelution from polyacrylamide gels (13).

Columns containing Sepharose 4B coupled to nonaggregated mouse myeloma proteins of different IgG subclass (Bionetics Laboratories, Kensington, Maryland) were prepared and the isolated glycoproteins were subjected to affinity chromatography on the different columns. After the columns were washed with PBS the percentage of applied counts

bound to each column was calculated. The ability of L1210 to bind the same nonaggregated myeloma proteins was determined by indirect immunofluorescence with the use of fluorescein isothiocyanate conjugated goat antimouse immunoglobulin (Antibodies, Inc., Davis, California).

The isolated glycoproteins were dissolved in 25 μl of 50 mM acetate buffer (pH 5.5) containing 20 mg/ml of NaCl and 1 mg/ml of $CaCl_2$ and incubated at 37°C for 1 and 2 h with 5 μl (2.1 international units) of neuraminidase (Type V, Sigma Chemical Company, St. Louis, Missouri). Reactions were stopped by the addition of 9 M urea to bring the total urea concentration in the sample to 6 M, and the digested and undigested samples reanalyzed by SDS-PAGE. Ovalbumin was incubated for 2 h under identical conditions and analyzed by SDS-PAGE in order to detect any proteolytic activity in the enzyme preparation. In some control tubes 5 μl of diisopropylfluorophosphate was also added to the incubation mixture.

Fc receptor activity in the glycoprotein extract was determined by incubating varying amounts of the labeled extract with either untreated sheep erythrocytes or sheep erythrocytes which had been incubated with a subagglutinating titer of rabbit IgG antisheep red cell antibody (Cordis Laboratories, Miami, Florida). Fc receptor activity represents the amount of radioactivity bound by the IgG coated erythrocytes minus the radioactivity bound by the untreated erythrocytes.

RESULTS

The glycoprotein extract prepared from 5×10^{10} L1210 cells contained approximately 8 mg of protein as determined by the method of Lowry et al. (14). The extract appeared to have Fc receptor activity since erythrocytes coated with IgG antibody bound 32% of the applied radioactivity while uncoated erythrocytes bound only 11%. In addition, when increasing amounts of the GP extract were added to a mixture of L1210 cells and IgG-coated erythrocytes we noted a progressive decrease in the number of EA rosette-forming cells, indicating a competitive inhibition between the cell-bound Fc receptors and the solubilized Fc receptors in the GP extract.

The GP extract was subjected to affinity chromatography on columns of Sepharose 4B to which either heat-aggregated human IgG or $F(ab')_2$ fragments had been covalently coupled. After washing with PBS the intact IgG column bound 35.7% of the applied labeled preparation, whereas the $F(ab')_2$ column bound only 2.8% (Table I). Approximately 75% of the radioactive material bound to the IgG column was eluted with citrate buffer.

TABLE I. Binding of Glycoprotein Extract to Sepharose 4B Columns Coupled to Heat-Aggregated IgG or $F(ab')_2$ Fragments

	% cpm Bound	
	Sepharose 4B+ aggregated IgG	Sepharose 4B+ aggregated $F(ab')_2$
Elution buffer[a]		
PBS, 0.01 M, pH 7.2	35.7	2.8
Citrate buffer, 0.1 M, pH 3.2	8.4	1.6

[a] Equal counts of glycoprotein extract were applied to the columns and eluted with PBS until the cpm in the eluate was equal to background. After the % cpm bound was calculated, the columns were then eluted with citrate buffer, pH 3.2.

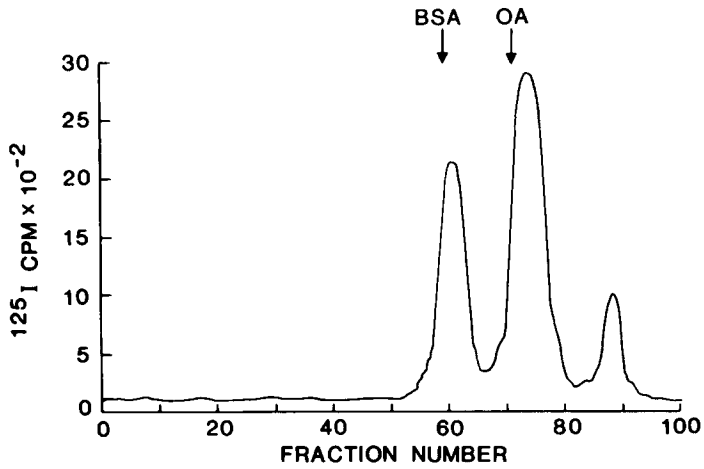

Fig. 1. SDS-PAGE analysis of the citrate eluate from the Sepharose 4B-heat aggregated human IgG column. The 5% polyacrylamide gels were sliced after electrophoresis. BSA (bovine serum albumin) and OA (ovalbumin) refer to the mobility of the marker proteins. The citrate eluate from the $F(ab')_2$ column revealed no peaks.

TABLE II. Similar IgG Subclass Affinities of Intact L1210 Cells and Isolated Fc Binding Glycoproteins

Sample	Mouse myeloma proteins		
	IgG1	IgG2a	IgG2b
L1210 cells[a]	++	++++	+
FI[b]	26.2	41.7	22.6
FII[b]	17.0	56.8	5.3
FIII[b]	10.8	19.3	10.3

[a]L1210 cells were incubated with one of the mouse myeloma proteins, washed, and then stained with fluorescein conjugated antimouse Ig. The intensity of the fluorescence was graded from + (barely visible) to ++++ (most intense).

[b]Equal numbers of counts of FI, FII, and FIII were passed over Sepharose 4B columns to which one of the myeloma proteins had been coupled. The columns were washed with phosphate buffered saline until the cpm in the eluates were equal to background. The numbers refer to the percentage of counts bound by each column.

The citrate eluates of both columns were analyzed by SDS-PAGE (Fig. 1). No distinct components were detected in the eluate from the $F(ab')_2$ column. As shown in Fig. 1, 3 distinct peaks, designated FI, FII, and FIII, were found in the eluate from the intact IgG column. This pattern was not altered when the proteolytic inhibitor, diisopropylfluorophosphate, was present throughout the plasma membrane and glycoprotein extraction procedure. FI, FII, and FIII were purified by electroelution from SDS-polyacrylamide gels (13) and each fraction contained less than 8% cross-contamination from the other fractions.

The ability of mouse myeloma proteins of different IgG subclasses to bind to intact L1210 cells was tested by indirect immunofluorescence. As judged by the intensity of the fluorescence, Table II shows that L1210 binds mouse IgG2a > IgG1 > IgG2b. Columns of Sepharose 4B coupled to the same myeloma proteins were prepared and the percentage of labeled FI, FII, and FIII bound by these columns determined (Table II). All 3 fractions revealed the same binding pattern for the mouse myeloma proteins as did the intact cells, although the actual percentage of counts bound varied considerably.

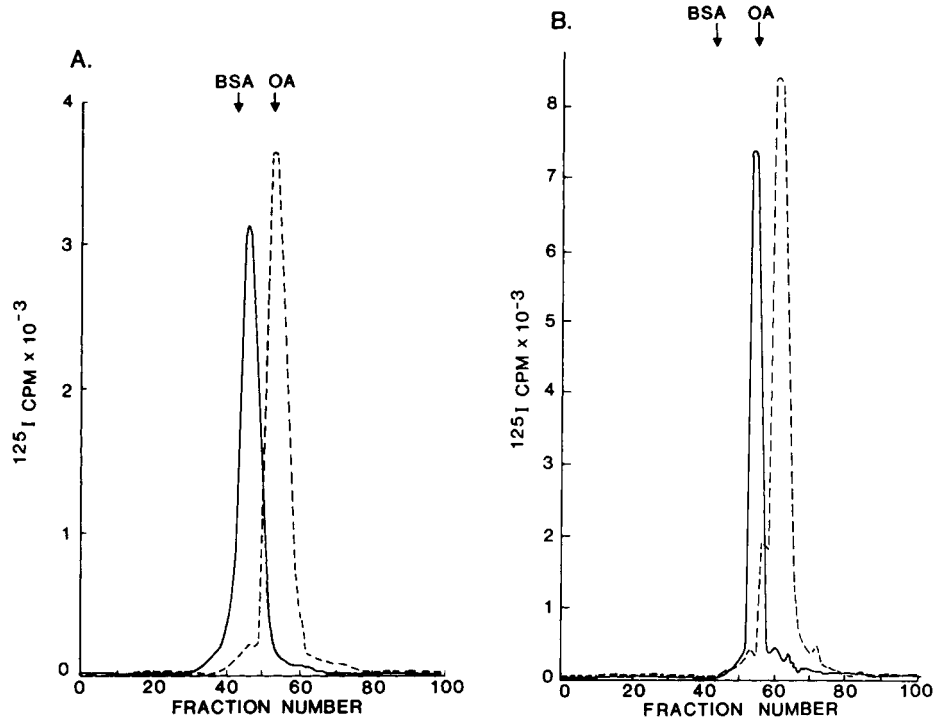

Fig. 2. Shift in mobility on 7.5% polyacrylamide gels of FI and FII after incubation with neuraminidase for 1 h. A) FI untreated (——), and after treatment with neuraminidase (---). B) FII untreated (——), and after treatment with neuraminidase (---). Incubation for 2 h did not reveal any further changes in mobility.

Reduction of FI, FII, and FIII with 2-mercaptoethanol revealed no change in molecular weight indicating that each consisted of a single polypeptide chain. Furthermore, amino acid analyses of FI, FII, and FIII showed remarkable similarity suggesting that the differences between the 3 fractions might reside primarily in the carbohydrate portions of the molecules. To explore this possibility, FI, FII, and FIII were digested for 1 h with neuraminidase and analyzed by SDS-PAGE (Fig. 2). Figure 2A shows that neuraminidase digestion of FI results in a mobility shift such that FI now migrates in the same position as FII. Upon neuraminidase digestion FII has a shift in mobility to a position intermediate between FII and FIII (Fig. 2B). No change in mobility was noted in FIII after neuraminidase digestion. Incubation with neuraminidase for 2 h did not result in any further shifts in mobility of FI and FII. Incubation of ovalbumin with neuraminidase for 2 h did not result in any degradation and the addition of diisopropylfluorophosphate to the incubation mixture did not alter the results.

DISCUSSION

These studies indicate that 3 proteins which bind to the Fc region of aggregated IgG can be isolated from the plasma membrane of the murine leukemia L1210. Since these 3 proteins exhibit the same spectrum of IgG subclass affinity as the intact cell it demonstrates that the isolated molecules are related to the Fc receptors of the cell. However at this point we do not know if each of the fractions represents a separate and distinct Fc receptor or if they are structurally related to each other.

Biochemical analyses of the isolated molecules indicate that they are composed of single polypeptide chains of similar amino acid composition, yet they differ considerably in their apparent molecular weights. Preliminary carbohydrate analyses of FI and FII have confirmed their glycoprotein nature, however FIII has not been subjected to such analysis due to insufficient material.

The data from the neuraminidase digestion suggests that the apparent molecular weight differences may be due in part to differences in the sialic acid moieties of the molecules. It is unlikely, however, that the large differences in molecular weight can be solely ascribed to differences in sialic acid content. Another interpretation of the observed heterogeneity may be that during the isolation procedure on SDS gels aggregates are formed. It has been reported that reversible aggregation of glycophorins occurs when these molecules are treated with organic solvents or run on SDS gels (15). If the heterogeneity of the Fc receptors on SDS gels is due to aggregate formation, it is possible that the neuraminidase digestion may result in disaggregation and the observed mobility shifts.

We recently reported that a 45,000 dalton Fc-binding protein is released from the surface of L1210 cells after redistribution of the cell Fc receptors by aggregated IgG and anti-IgG (7). The relationship of this molecule to the proteins described in this study is still to be determined, but it is of interest that of the 3 components isolated, it was found that FII, with an apparent molecular weight of 45,000 daltons, was the major component.

Rask and co-workers have isolated from crude membrane fractions of mouse spleen cells 3 polypeptides with molecular weights of 65,000, 18,000, and 15,000 daltons which had affinity for aggregated human IgG (16). They proposed that proteolytic degradation of the largest component gives rise to the observed molecular heterogeneity. The finding by Walker that a single cell line may possess separate Fc receptors for aggregated or monomeric IgG of different subclasses strongly suggests that Fc receptors will be found to exhibit molecular heterogeneity (5). We have preliminary data which indicates that L1210 possesses a large, complex Fc receptor which binds to IgG which is bound to antigen, while the 3 proteins described in this study bind to native IgG (17). Although the relationship of these proteins to each other has yet to be determined, it is possible that their arrangement and linkage on the cell surface may account for different binding specificities.

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