PROPERTIES OF AN Fcγ-BINDING PROTEIN
ISOLATED FROM HUMAN LEUKEMIC B CELLS*

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Receptors that specifically recognize and bind the Fc region of the IgG molecule have been described on a large number of cells. These receptors, which are called Fcγ receptors, have been identified on B lymphocytes (1), some T lymphocytes (2), polymorphonuclear cells (3), macrophages (4), mast cells (5), herpes virus-infected cells (6), and placental syncitiotrophoblasts (7). With the exception of antibody-dependent cell-mediated cytotoxicity (8) and immunophagocytosis (9), the biological role of Fcγ receptors is still unclear. Knowledge of the physicochemical properties of Fcγ receptors might (a) clarify whether the Fcγ receptors of the different types of cells are uniform or different in structure, (b) open up new possibilities for elucidating yet unknown functions of Fcγ receptors, and (c) shed light on the relationship of Fcγ receptors to other membrane proteins (10). Therefore, several attempts have been made to define the physicochemical structure of Fcγ receptor molecules. The studies performed so far, however, have not yielded consistent data. In particular, the mol wt estimated by various authors (11-18) varied between 15,000 and 130,000. These inconsistent estimates of the molecular weight might reflect an actual structural heterogeneity of Fcγ receptors on different types of cells or from different species, or they might be just technical artifacts. In an attempt to clarify this question, we have started to obtain data on the solubilization and physicochemical properties of Fcγ receptors of B cells. To be sure of investigating structurally uniform Fcγ receptors, we decided to first look at the Fcγ receptors in a B-type prolymphocytic leukemia and two cases of B-type chronic lymphocytic leukemia.

In this paper, we report on the solubilization, isolation, and some characteristics of a protein with the properties of Fcγ receptors that was obtained from the B-type lymphoid leukemia cells. Because some of the physicochemical properties of the Fcγ-binding protein we isolated were comparable to those of C1q or its subunits (19, 20), experiments were performed to clarify whether the isolated Fcγ receptor-like protein is identical with C1q or parts of it.

Materials and Methods

Leukemic Cells. Large numbers of leukemic cells were obtained by leukopheresis from one patient with chronic lymphocytic leukemia (CLL)1 of the B type, one patient with prolympho-

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1 Abbreviations used in this paper: CLL, chronic lymphocytic leukemia; CNBr, cyanogen bromide; DOC, sodium deoxycholate; E, erythrocyte; Ehu, human erythrocyte; Eox, ox erythrocyte; E?, sheep erythrocyte; EA, antibody-coated erythrocyte; EhuA, Rh-positive human type-O erythrocytes coated with anti-D
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cytic leukemia (PLL) of the B type, and one patient with CLL of the T type. In a second case of B-type CLL, the leukemic cells were prepared from the patient's spleen (1,500 g), which was heavily infiltrated by leukemic cells, by pressing the splenic tissue through a nylon mesh, followed by Urografin-(E. R. Squibb & Sons, New York) Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) gradient centrifugation of the cells (21). The lymphocytes from the two cases of B-type CLL expressed small amounts of surface immunoglobulin (SIg), bore receptors for erythrocytes coated with IgM antibodies and C3d (EAC3d) and mouse erythrocytes (E), but lacked receptors for sheep E and E coated with IgM antibodies and C3b (EAC3b). The PLL cells showed a thick layer of IgM/E and C1q on their surface and receptors for EAC3b and EAC3d, but lacked receptors for mouse E and sheep E. The cells from all three B-type leukemias bound IgG aggregates and ox E coated with anti-E antibodies of the IgG type (IgG-EoxA) in a percentage ranging from 40 to 95%. Sheep E coated with anti-E antibodies of the IgM type (IgM-EA) were not bound by the leukemic B cells. The T-type CLL cells were SIg negative and IA-like-antigen negative and did not bind aggregated IgG-fluorescein isothiocyanate (FITC) or IgG-EoxA, but formed rosettes with sheep E at 4°C.

IgG Aggregates. Human IgG was prepared from pooled human sera by fractionation with ammonium-sulfate precipitation followed by chromatography on DEAE-cellulose equilibrated with 0.0175 M phosphate buffer, pH 7.0. The IgG (20 mg/ml) was aggregated by heating at 63°C for 28 min (22). Soluble IgG aggregates with a mol wt between 2.5 × 10^7 and 3 × 10^7 were obtained by chromatography on a Sepharose 2B column (Pharmacia Fine Chemicals, Inc., 100 × 1.5 cm) calibrated with IgG, IgM, and dextran blue, and equilibrated with phosphate-buffered saline (PBS).

F(ab')2 Aggregates. F(ab')2 fragments were prepared by cleaving IgG with pepsin and purification on a Sephadex G-150 column (Pharmacia Fine Chemicals, Inc., 100 × 2.5 cm) (23). Soluble F(ab')2 aggregates were obtained by the same procedure as that described for IgG aggregates.

Soluble Peroxidase-Anti-Peroxidase-IgG (PAP-IgG) and -F(ab')2 (PAP-F(ab')2) Complexes. Soluble PAP-IgG complexes consisting of three molecules of horseradish peroxidase and two molecules of rabbit anti-peroxidase IgG-type antibodies were obtained from Dakopatts A/S, Copenhagen, Denmark. Fc fragments were cleaved from PAP-IgG with pepsin. The resulting PAP-F(ab')2 complexes were purified on a calibrated Ultragel AcA 22 column (LKB Produkter, Bromma, Sweden, 100 × 2.5 cm).

Preparation of Uncoated E Membrane Sheets and those Coated with IgG. 1 × 10^9 sheep E (Ea) were incubated at 37°C for 30 min in 3 ml of the IgG fraction of a rabbit anti-Ea serum with an agglutination titer of 1:32,000. The strongly agglutinated Ea were washed twice in PBS. The agglutinated Ea and 1 × 10^9 untreated Ea were homogenized by nitrogen cavitation (1,400 lb/in^2, 20 min). The resulting membrane sheets were then washed several times and homogenized with a Potter-Evhehm homogenizer until all contaminating hemoglobin was removed.

Coupling of Proteins to Sepharose 4B. Proteins were coupled to cyanoem bromide (CNBr)-activated Sepharose 4B as described by Cuatrecasas et al. (24).

Radioiodination. Proteins and purified cell membranes were labeled with 125I (Amersham Buchler Radiochemicals, Braunschweig, West Germany) using chloramine T (25). The cell membranes of intact cells were enzymatically iodinated with lactoperoxidase (1 mCi 125I/1 × 10^8 cells) (26). Unbound 125I was removed by passage over a Sephadex G-25 column or by exhaustive washing.

antibodies; EAC3d, erythrocytes coated with antibodies and C3b; EAC3d, erythrocytes coated with antibodies and C3d; EDTA-ME, a mixture of Na-EDTA and 2-mercaptoethanol; FITC, fluorescein isothiocyanate; GAR-R, rhodamine-labeled goat anti-rabbit IgG y-globulin; IgG-EA, erythrocytes coated with anti-erythrocyte antibodies of the IgG type; IgG-EoxA, Rh-positive human type-O erythrocytes coated with human anti-erythrocyte antibodies of the IgG type; IgG-EoxA, ox erythrocytes coated with anti-erythrocyte antibodies of the IgG type; IgG-EoxA, sheep erythrocytes coated with rabbit anti-erythrocyte antibodies of the IgM type; ME, 2-mercaptoethanol; NP-40, Nonidet P-40; PAP-F(ab')2, soluble complex of horseradish peroxidase and F(ab')2 fragments; PAP-IgG, soluble complex of horseradish peroxidase and anti-peroxidase antibodies of IgG type; PBS, phosphate-buffered saline; pl, isoelectric point; PLL, prolymphocytic leukemia; SDS, sodium dodecyl sulfate; SIg, surface immunoglobulin.
Isolation of Cell Membranes. Cells were homogenized by nitrogen cavitation (800 lb/in², 20 min), and membranes were pelleted at 105,000 g for 60 min after differential centrifugation at 600 g and 20,000 g for 30 min (27).

Preparation of Cell-Membrane Lysate. Purified membranes were incubated in 80 mM Na-EDTA/50 mM 2-mercaptoethanol (ME) (EDTA-ME) in 20 mM Tris-HCl, pH 7.3, or 0.1% Nonidet P-40 (NP-40, Shell Chemical Co., New York) in 20 mM Tris-HCl, pH 7.3, or 0.5% sodium deoxycholate (DOC) in 20 mM Tris-HCl, pH 7.3, at 4°C for 20 min. Insoluble material was removed by centrifugation at 105,000 g for 60 min. The supernate was used immediately.

Isolation of Fcy-binding Protein. Iodinated membrane lysates obtained from 2 × 10⁸ cells were routinely incubated with IgG aggregates or, in some experiments, with soluble PAP-IgG complexes at 37°C for 30 minutes and then coprecipitated with a sheep anti-human IgG serum or a sheep anti-rabbit IgG serum, respectively. The coprecipitates were exhaustively washed. In some experiments, IgG aggregates or F(ab')₂ aggregates were incubated with ¹²⁵I-labeled membrane lysates and separated from unbound membrane proteins by chromatography on a calibrated Sepharose 2B column. In other experiments, Fcy-binding proteins were isolated by incubating iodinated membrane lysates with IgG-coated E₈ membrane sheets at 37°C for 30 minutes, followed by exhaustive washing in 0.1 M Tris-HCl, pH 7.2. The material obtained by all of these methods was boiled in sodium dodecyl sulfate (SDS) sample buffer for 2 min. In a third set of experiments, iodinated membrane lysates were incubated at 37°C for 30 min with 1 mg of either aggregated IgG, aggregated F(ab')₂, PAP-IgG, or PAP-F(ab')₂ coupled to 1 ml Sepharose 4B. After exhaustive washing in PBS, the pelleted Sepharose 4B was boiled in SDS sample buffer for 2 min. The absorbed material was separated from the Sepharose 4B matrix by passage through a Whatman GF/A filter (Whatman Chemicals, Div. of W. & R. Balston, Maidstone, Kent, England), followed by 10-fold concentration with Ultrathimbles (UH 100/25; Schleicher & Schüll GmbH, Dassel, West Germany).

Isolation of Fcy-binding protein by means of column affinity chromatography was performed as follows. Aggregated IgG was coupled to CNBr-activated Sepharose 4B (24). After equilibration with PBS, ¹²⁵I-labeled membrane lysate from 1 × 10⁹ cells was applied to the column and incubated at 22°C for 30 min. The column was washed in PBS until radioactivity decreased to the baseline; retained proteins were eluted with 0.1 M glycine-HCl buffer, pH 2.8. Eluates were neutralized and incubated with 0.1 mg aggregated IgG at 37°C for 30 min. The incubated, aggregated IgG was isolated by coprecipitation as described above.

SDS-Polyacrylamide Gel Electrophoresis. SDS electrophoresis was performed on cylindrical gels according to the method described by Laemmli (28). The stacking and separating gel combinations used were either 3.5% and 7.5%, or 3.5% and 10% polyacrylamide concentration. Samples were pretreated by heating for 2 min in 2% (wt/vol) SDS sample buffer, either with or without 100 mM ME. By adding 1 M iodoacetamide, the reduced samples were alkylated to a final concentration of 100 mM. Unreduced human IgG, reduced bovine serum albumin, γ-chains, and reduced myeloma light (λ) chains were routinely used as internal markers. After electrophoresis, the gels were sliced into 1-mm segments and radioactivity was determined in a γ-counter (BF 5300, Berthold GmbH, Wildbad, West Germany).

Isoelectric Focusing. Isoelectric focusing was performed in cylindrical 5% polyacrylamide gels in the presence of 6 M urea. The gels contained 5% Ampholine (LKB Produkter) in the pH ranges 3.5–10 (four parts) and 4–6 (one part). The pH gradient was established at 150 V for 2 h. Coprecipitates dissolved in 8 M urea were applied to gels and focused for 16 h at 150 V and 0.4–0.5 mA per gel. After focusing, the gels were cut into 1-mm segments and radioactivity was determined. For determination of the final pH gradient, two parallel gels were cut into 20 segments, and the pH was measured in the distilled water eluates of each segment with a conventional pH meter.

Determination of NH₂-Terminal Amino Acid. Membranes from 1 × 10¹⁰ lymphocytes were solubilized with EDTA-ME, and Fcy-binding proteins were isolated from membrane lysates by incubation with IgG aggregates and coprecipitation with sheep anti-human IgG serum. After exhaustive washing in 0.15 M Tris-HCl, pH 7.5, the complexes were dissociated by incubation in 0.1 M citrate buffer, pH 2.8, for 20 min at room temperature, followed by chromatography on an Ultragel AcA-34 column. Fractions corresponding to a mol wt of 28,000 were collected, dialyzed against water, and lyophilized. Determination of the NH₂-terminal amino acid was performed by reaction of dansyl chloride with the protein, followed by hydrolysis and thin-
layer chromatography of amino acids (29). Dansylated amino acids were used as references (Serva Feinbiochemica, Heidelberg, West Germany).

**Complexes of E and Anti-E Antibodies of the IgG Type (IgG-EA).** Various IgG-EA complexes were prepared. IgG-EoxA was prepared by incubating ox E (Eo) in a hyperimmune rabbit serum, which had a low Eox-agglutination titer and a high hemolysis titer, at 37°C for 30 min. Complexes of Rh-positive human type-O E (Ehu) and human anti-E antibodies of the IgG type (IgG-EhuA) were prepared by incubating Ehu in a human serum containing a high titer (indirect-Coombs-test positive to a final dilution of 1:2,000) of nonagglutinating anti-D antibodies. Complexes of Eo and rabbit anti-E antibodies of the IgG type (IgG-EoA) were prepared by incubating Eo in the IgG fraction of a rabbit anti-Eo serum at a barely nonagglutinating titer. The highest percentage of rosettes and the most-stable rosettes were obtained with IgG-EoxA. IgG-EhuA yielded 20-60% and IgG-EoA, 40-95% lower percentages of rosetting cells. Thus, IgG-EoxA was routinely used in our experiments.

**IgM-EA.** IgM-EA was prepared by incubating Eo in the IgM fraction of an anti-Eo serum at a barely nonagglutinating titer. The IgM fraction was obtained by fractionation of the antiserum by means of ammonium-sulfate precipitation followed by passage through an Ultrogel AcA-34 column. The IgM nature of the sensitizing antibodies was ascertained by titration of the IgM-EA against monovalent anti-rabbit IgM and anti-rabbit IgG sera (obtained from Nordic Immunological Laboratories, Tilburg, The Netherlands).

**Rosette Formation.** 2 × 10^6 leukemic cells were incubated with 8 × 10^7 of IgG-EA complexes or IgM-EA in small plastic conical tubes (Eppendorf GmbH, Hamburg, West Germany) at 37°C for 5 min, followed by centrifugation at 200 g for 5 min and a second incubation at 37°C for 20 min. Afterwards, the cells were gently resuspended and kept on ice until counting.

**Rosette-inhibition Assay.** For detection of soluble Fcγ-binding protein, IgG-EoxA and/or IgG-EhuA were preincubated in EDTA-ME membrane lysate. The rosette assay was then performed.

For detection of agents blocking membrane receptors, the rosette tests were performed either in the presence of the agents (monomeric IgG, IgG aggregates, PAP-IgG, 19S IgM, carrageenin, or polyinosinic acid; the latter two obtained from Sigma Chemical Co., St. Louis, Mo.) or after preincubation of the leukemic cells with the agents (anti-C1q serum or anti-Fcγ-receptor sera), followed by two washings. The inhibition of rosette formation was calculated with the method of Dierich and Reisfeld (30).

**Binding of PAP-IgG.** 5 × 10^6 leukemic cells were incubated at 37°C for 30 min with 50 μl PAP-IgG at various dilutions. After three washings, centrifuge slides were prepared and fixed in 1% PBS-buffered glutaraldehyde solution for 1 min, followed by two washings in 0.05 M Tris-HCl buffer, pH 7.6. The slides were then incubated at 22°C for 10 min in Tris-HCl buffer containing 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemie, Munich, West Germany) (0.5 mg/ml, pH 7.6) and 0.001% H2O2. After several washings, the slides were counterstained with hemalum and mounted with Eukitt (Odelie Kindler, Freiburg, West Germany).

**Antisera.** Anti-Fcγ-receptor sera were prepared as follows. IgG-EoxA was incubated in EDTA-ME membrane lysate from the case of PLL and washed twice. The coating of the IgG-EoxA with Fcγ-binding protein was monitored by reduction of rosette formation. Three rabbits were immunized i.v. weekly with 5 × 10^8 of the incubated IgG-EoxA, which showed rosette formation reduced by at least 60%. Blood was obtained weekly, starting 1 mon after the first immunization. The antisera were exhaustively absorbed with Eox, Ehu, Eo, and glutaraldehyde-insolubilized serum protein, and partly absorbed with human brain homogenate and C1 (Cordis Laboratories Inc., Miami, Fla.). The absorbed and diluted (1:30) anti-Fcγ-receptor sera showed IgG-EA-rosette inhibition of 60-80% when compared with 1:30-diluted normal rabbit serum, whereas they did not suppress the binding of EAC3b and neuraminidase-treated Eo to appropriate cells. The anti-Fcγ-receptor sera stained Fcγ-receptor-positive leukemic cells, but not cells from T-type CLL, thymocytes, T cells of peripheral blood, erythrocytes, or Fcγ-receptor-negative EU-I-eel line cells (kindly donated by Dr. U. Schneider, Erlangen, West Germany) in indirect immunofluorescence.

Sheep anti-human IgG serum and sheep anti-rabbit IgG serum were produced by immunizing sheep with purified human IgG or rabbit IgG by means of the procedure of Hijmans et al. (31). The anti-human IgG serum was absorbed with Eox, human brain homogenate, PLL cells,
and glutaraldehyde-insolubilized purified IgM. This antiserum agglutinated only E coated with human IgG and not E coated with other proteins. The anti-rabbit IgG serum was absorbed with glutaraldehyde-insolubilized human serum polymer, Ehu, and PLL cells.

Anti-Eox sera containing a large amount of IgG antibodies were obtained by immunizing rabbits i.v. weekly with $1 \times 10^{10}$ Eox for 3 mon. Only the anti-Eox sera showing a low agglutination titer (1:4–1:8) and a high hemolysis titer (1:2,000, demonstrated by adding 1:64-diluted guinea pig serum) were used.

Anti-Ea sera with a high content of IgM antibodies were prepared by immunizing rabbits weekly with $1 \times 10^{10}$ Ea three times per wk for 3 wk. The first blood sample was taken and tested on the 14th d after the first immunization. The sera with the highest ME- (final concentration of 0.1 M) sensitive agglutination titers were pooled.

Anti-E8 sera with a high content of IgG antibodies were prepared by immunizing rabbits i.v. with $1 \times 10^{10}$ E8 three times per wk for 3 wk. The IgG fraction was isolated as described for human IgG.

The nonagglutinating human anti-D serum was a generous gift from Dr. Sachs, Blutspendezentrale, University of Kiel, Kiel, West Germany. Anti-C1q serum was obtained from Behring Werke AG, Marburg/Lahn, West Germany.

**Immunofluorescent Staining.** IgG aggregates were conjugated with FITC according to the method of Clark and Shepard (32). Goat anti-rabbit IgG-rhodamine isothiocyanate conjugate (Nordic Immunological Laboratories) was additionally absorbed with human tonsil cells before use in the indirect procedure. The staining procedure described by Vossen (33) was used. Control stainings performed with normal rabbit serum absorbed with Ehu, tonsil cells, brain and serum polymer, and an anti-a-fetoprotein serum (Dakopatts A/S) were completely negative at dilutions higher than 1:15. The anti-Fcy-receptor sera were usually used at a dilution of 1:20 for indirect immunofluorescence.

**Results**

**Properties of the Cell-bound Fcy Receptor.** A large proportion (40%, 70%, and 95%, respectively) of cells from the three B-type leukemias bound FITC-labeled IgG aggregates, IgG-EoxA, and PAP-IgG; a smaller proportion bound IgG-EhuA and IgG-EaA, but none bound IgM-EA. Cells from the case of T-type CLL and Ehu, both of which were used as control cells, neither bound detectable amounts of IgG aggregates labeled with FITC, nor of PAP-IgG, nor formed rosettes with IgG-EoxA. As shown in Fig. 1, the binding of IgG-EoxA to the leukemic B cells decreased with the addition of increasing amounts of IgG aggregates and monomeric IgG. A 200-fold molar excess of monomeric IgG was needed to achieve an inhibitory effect on IgG-EoxA-rosette formation similar to that of IgG aggregates. IgG-EoxA-rosette inhibition by monomeric IgG did not exceed 80%, even with 1.5 mg monomeric IgG. Nearly complete inhibition of IgG-EoxA-rosette formation could be obtained only with IgG aggregates. A similarly marked rosette inhibition was observed with PAP-IgG. IgG-EoxA-rosette formation was not affected by the presence of purified IgM, carrageenin, or polyinosinic acid, or by preincubation of the leukemic cells with anti-C1q serum. Preincubation of the leukemic cells with diluted (1:30) anti-Fcy-receptor sera, however, decreased IgG-EoxA-rosette formation by $>70\%$ when compared with normal rabbit serum, whereas it did not affect rosette formation with EAC3b.

**Solubilization of Fcy Region-specific Protein and Properties of the Solubilized Molecules.**

First, we solubilized membranes of the leukemic B cells, obtained by rupturing the cells by means of the nitrogen cavitation method and differential centrifugation, with NP-40. After iodination, the membrane lysates were incubated with IgG aggregates and then coprecipitated with an anti-IgG serum. SDS-polyacrylamide gel electrophoresis of the coprecipitates revealed six major peaks with mol wt ranging from 15,000
to 115,000 (Fig. 2, solid line). A basically similar SDS pattern was obtained with F(ab')2 aggregates (Fig. 2, dotted line). An Fcγ region-specific protein could not be coprecipitated from DOC-solubilized membranes either. These disappointing results encouraged us to try nondetergent agents. Good results were obtained with a mixture of EDTA and ME. EDTA-ME membrane lysates dialyzed against PBS strongly inhibited the binding of IgG-EoxA to leukemic B cells. The inhibition was dose-dependent (Fig. 3). Even highly concentrated EDTA-ME lysates, however, did not agglutinate IgG-EoxA. Absorption of the membrane lysates with IgG-EoxA, but not with uncoated Eox, removed the capacity of the membrane lysates to inhibit the binding of IgG-EoxA to leukemic B cells.

Isolation of the IgG-binding material from EDTA-ME lysates of the leukemic B cells was performed by (a) coprecipitation with IgG aggregates, (b) coprecipitation with PAP-IgG complexes, (c) absorption with E membrane sheets coated with IgG-type antibodies, (d) absorption with aggregated IgG and PAP-IgG complexes coupled
FIG. 2. SDS polyacrylamide gel profiles of iodinated membrane proteins solubilized with 0.1% NP-40 in 20 mM Tris-HCl and isolated with IgG aggregates (—) or F(ab')2 aggregates (⋯).

to Sepharose 4B, and (e) affinity chromatography on aggregated IgG-Sepharose 4B columns. SDS preparations from all types of material revealed identical patterns in SDS-polyacrylamide gel electrophoresis, viz.: one small-based peak with an apparent mol wt of 28,000 (Figs. 4 and 5, solid lines). The highest peak was obtained with PAP-IgG (not shown). An identical protein peak was isolated from membranes that had been prepared from surface-iodinated viable leukemic B cells, which confirmed that the isolated protein was a cell-surface protein. Reduction and alkylation of the Fcγ-binding protein did not affect its relative mobility; its NH2-terminal amino acid was determined to be glycine. Thin-layer chromatography of the dansylated receptor protein not subjected to acid hydrolysis did not show a fluorescent spot representing noncovalently bound glycine.

Material with binding affinity to aggregated or complexed IgG could not be isolated from lysates of iodinated and isolated membranes of cells from a human leukemia of the T type or Eμμ (Fig. 6, dotted and solid lines). Furthermore, no electrophoretic peak at all could be obtained with EDTA-ME lysates of the membranes of the B-type leukemia cells (Figs. 4 and 5, dotted lines) when F(ab')2 aggregates were used instead of IgG aggregates, when PAP-F(ab')2 complexes were used instead of intact PAP-IgG molecules, or when uncoated E membrane sheets were used instead of IgG-coated E membrane sheets.

Attempts to elute Fcγ-binding protein, without denaturation, from aggregated IgG-Sepharose 4B columns with 0.1 M glycine-HCl, pH 2.8, yielded only small amounts of iodinated material when compared with eluates obtained with SDS. Coprecipitation of the glycine-HCl eluates with IgG aggregates gave a small, but distinct peak corresponding to a mol wt of 28,000.

Isolation of the 28,000-dalton protein with IgG aggregates was not inhibited by
carrageenin (500 μg/ml) or polyinosinic acid (500 μg/ml).

Significant amounts of Fcγ-receptor-like protein were solubilized only with a mixture of EDTA and ME (Fig. 7, dotted line). With EDTA (not shown) or ME alone, no, or only very small amounts of, Fcγ-receptor-like protein was solubilized (Fig. 7, solid line), showing that both the removal of divalent cations and dissociation of disulfide bridges are necessary for liberation of the Fcγ-receptor molecules from the membranes.

To obtain data on the Fcγ-receptor-like molecules under nonreducing conditions, we dialyzed EDTA-ME lysates against oxygenated PBS supplemented with protease inhibitors. Coprecipitates obtained from the dialysate with IgG aggregates displayed two new peaks in SDS-polyacrylamide gel electrophoresis performed under nonreducing conditions, viz.: a large peak with an apparent mol wt of 115,000 and a smaller one with an apparent mol wt of 18,000 (Fig. 8). The 115,000-dalton component could be readily dissociated into a 28,000-dalton protein by adding ME.

The isoelectric-focusing pattern of total EDTA-ME membrane lysates is shown in Fig. 9 A. Twelve distinct peaks are discernible. After absorption of the lysate with IgG aggregates followed by precipitation with anti-IgG serum, one major peak with an
Fig. 4. SDS polyacrylamide gel profiles of iodinated membrane proteins solubilized with EDTA-ME and isolated with IgG aggregates (——) or F(ab')2 aggregates (⋯⋯). H, heavy chains of IgG. L, light chains of IgG.

Fig. 5. SDS polyacrylamide gel profiles of iodinated membrane proteins from B-type prolymphocytic leukemia cells solubilized with EDTA-ME and isolated with IgG-coated Eα membrane sheets (——) or with uncoated Eα membranes (⋯⋯).

Isoelectric point (pI) of 5.5 was missing (Fig. 9B). The missing peak, representing the Fcy-affined protein, with a pI of 5.5 could be recovered by isoelectric focusing of the coprecipitate (Fig. 9C).

Electrophoretic Pattern of C1q Solubilized with EDTA-ME and Isolated with IgG Aggregates. Because some of the properties of the Fcy-binding protein resembled those of C1q, we analyzed the SDS electrophoretic pattern of C1q isolated from C1 under the
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Fig. 6. SDS polyacrylamide gel profiles of iodinated membrane proteins from T-type chronic lymphocytic leukemia cells (——) and Ehu (····) solubilized with EDTA-ME and isolated with IgG aggregates.

same conditions as those for isolation of the Fcy receptor. As demonstrated in Fig. 10, C1q bound to IgG aggregates showed a major peak of 38,500 daltons, four very small peaks of 41,000, 34,000, 28,000, and 20,000 daltons, and a large peak of <10,000 daltons.

Detection of C1q and Fcy-Receptor Protein in EDTA-ME Membrane Lysates. To determine whether the EDTA-ME lysates contain C1q or its subunits with the capacity to bind complexed IgG, we incubated Ehu and Ehu densely coated with incomplete human anti-D antibodies (EhuA) in EDTA-ME lysates. The incubated Ehu and EhuA were stained for C1q by means of indirect immunofluorescence. To show that Fcy-receptor protein is bound by EhuA, we stained the incubated Ehu and EhuA with three different anti-Fcy-receptor sera prepared as described in Material and Methods; in addition, we analyzed the reduction of binding of the incubated EhuA to leukemic cells in comparison with the binding of nonincubated EhuA. The results are shown in Table I. The binding of Fcy-binding protein to the EhuA is evidenced by (a) the strong inhibition of rosette formation and (b) the positive staining of the incubated EhuA with all three anti-Fcy-receptor sera. The capacity of the anti-Fcy-receptor sera to stain incubated EhuA could not be removed by absorption of the antisera with C1q. Anti-C1q serum did not stain IgG-EA complexes loaded with Fcy-binding protein. This suggests that the Fcy-affined protein solubilized with EDTA-ME is not related to C1q.

Discussion

The Fcy receptors on the cells from the three B-type leukemias we investigated were detectable with FITC-labeled IgG aggregates, PAP-IgG, and IgG-EA complexes. Studies of inhibition of IgG-EA-rosette formation by IgG aggregates, PAP-IgG, and monomeric IgG showed that IgG aggregates and PAP-IgG strongly inhibit IgG-EaA-rosette formation and that a >200-fold molar excess of monomeric IgG is needed to cause rosette inhibition comparable to that obtained with IgG aggregates. This suggests that (a) the Fcy receptors on the cells from the three cases of B-type leukemia were detectable with IgG aggregates, PAP-IgG, and IgG-EaA; and (b) these Fcy receptors preferentially bound complexed IgG.

In isolation studies of the Fcy receptor, we first used NP-40 as the solubilization
agent. We were not able to select an Fcγ region-specific protein, however, from the NP-40 membrane lysates of leukemic B cells. Six SDS-electrophoretically identical proteins were isolated with IgG aggregates and F(ab')2 aggregates. The mol wt of the six proteins ranged from 115,000 to 15,000. This finding indicates that NP-40 liberates proteins that can be bound to both F(ab')2 and IgG aggregates. F(ab')2-affined membrane proteins were recently described (34, 35). It is not clear why similar proteins were isolated from NP-40 lysates with IgG and F(ab')2 aggregates. A possible explanation is that the six isolated proteins are associated by NP-40 to a complex, or that they chiefly represent a membrane complex that includes proteins with an affinity to bind to aggregates of IgG and F(ab')2. This heterogeneous complex precipitates with IgG and F(ab')2 aggregates as a single entity and is reduced to its individual protein components when the precipitates are prepared for SDS electrophoresis. Because we obtained similar nonspecific results with DOC lysates, we concluded that detergents are not suitable for the solubilization and isolation of Fcγ-receptor molecules. We therefore tried nondetergent solvents. These have the advantage that they do not lyse antibody-coated indicator cells (EA). Using the mixture of EDTA and ME described by Rask et al. (11) as a potent solvent of Fcγ-receptor molecules, we could solubilize material from membranes of the leukemic B cells that strongly inhibited the binding of IgG-E oxA to Fcγ-receptor-positive leukemic cells. The inhibitory activity was readily removed by exhaustive absorption of the EDTA-ME solubilisate with IgG-E oxA, but not with E ox. This eliminates the possibility that EA-rosette formation was suppressed either nonspecifically or by contaminating amounts of cytophilic IgG. The findings justify the conclusion that the inhibitory activity of the lysate actually represents solubilized Fcγ-binding protein.

Because of the reported susceptibility of solubilized Fcγ-receptor-like material to proteolysis (17), we started the isolation procedure with EDTA-ME membrane lysates immediately after iodination, without removing the unbound iodine by dialysis. The free iodine was removed by exhaustive washing in PBS after absorption or coprecipitation of the lysates with the substrates. Various methods and substrates were used for isolation of proteins with the capacity to bind to the Fc portion of IgG, viz.: coprecipitation with aggregated IgG and antigen-antibody complexes (PAP-IgG), absorption with IgG-coated E membrane sheets, absorption with aggregated IgG and
PAP-IgG complexes attached to Sepharose 4B, and affinity chromatography on aggregated IgG-Sepharose 4B columns. Fundamentally identical results were obtained with all methods and substrates used: SDS electrophoresis revealed one peak corresponding to a mol wt of 28,000. The highest peaks were obtained with PAP-IgG complexes. Affinity chromatography with glycine-HCl elution of the bound proteins yielded less significant peaks. When uncoated E membrane sheets, IgG-F(ab')2 aggregates, or PAP-F(ab')2 complexes were used for absorption, no peak could be found in SDS electrophoresis, showing the specific binding of the isolated protein to IgG and within the IgG molecule to the Fc region. Neither with IgG aggregates nor with PAP-IgG complexes were significant amounts of Fcy-affined protein detectable in the EDTA-ME extracts of membranes from an Fcy-receptor-negative T-cell leukemia or Ehu.

Isoelectric focusing of the isolated Fcy-affined protein showed a single peak with a pI of 5.5, indicating that the isolated 28,000-dalton protein was composed of homogeneous polypeptide chains. Because the relative mobility of the isolated protein in SDS electrophoresis was not altered after reduction and alkylation, we concluded that the protein consisted of one polypeptide chain. Another finding in favor of this
conclusion was that only one NH₂-terminal amino acid (glycine) was detected on isolated Fcy-receptor protein. Even concentrated EDTA-ME lysates did not agglutinate FcyA, suggesting that the Fcy-receptor protein we solubilized has only one binding site.

The failure to isolate any peaks discernible in SDS-polyacrylamide gel electrophoresis or isoelectric focusing with F(ab')₂ aggregates demonstrates that proteins with affinity to F(ab')₂ are not liberated by EDTA-ME, whereas they are liberated by NP-40.

It is interesting that significant amounts of Fcy-receptor protein were solubilized only with a mixture of EDTA and ME. With EDTA or ME alone, no, or only small amounts of, Fcy-receptor protein was solubilized. That suggests that divalent cations and disulfide bridges are involved in the linkage of the Fcy-receptor protein to the cell membrane. Although the specificity of the isolated protein for the Fc region of aggregated IgG was clearly demonstrated, we cannot conclude that the protein was a complete Fcy-receptor molecule. The isolated Fcy-receptor-binding protein might be bound to integral membrane proteins, which have yet to be identified, via disulfide bonds and divalent cations. Thus, we may merely conclude that the isolated protein represented at least the part of the Fcy receptor of leukemic B cells that is responsible for binding to the Fc portion of IgG.

There have been only two other reported attempts to solubilize Fcy-receptor molecules with nondetergent agents. In the first study (11), a mixture of EDTA and ME was used. The authors isolated IgG-binding molecules from murine spleen cells by absorption and desorption on a Sephadex column to which IgG aggregates were attached. The eluted protein differed, however, in molecular weight (68,000, 18,000, and 15,000) from the protein we isolated. In the second study (14), cell membranes were solubilized with lithium diiodosalicylate. The authors isolated three proteins with mol wt of 65,000, 45,000, and 28,000 from murine leukemia cells with monomeric IgG, and an additional protein with a mol wt of 125,000 with antigen-complexed IgG. Only the 125,000-dalton protein was dissociated into two smaller subunits with mol wt of 74,000 and 60,000.
The literature contains reports on four studies (12, 16–18) using detergents to solubilize IgG-binding proteins. The proteins isolated in those experiments differed greatly in mol wt (24,000–125,000) and in their susceptibility to reduction. It is notable, however, that in two of those studies the Fcy-binding proteins revealed apparent mol wt of 110,000 and 120,000, respectively, under nonreducing conditions.

Two research groups (13, 15) looked for Fcy-binding material either spontaneously released from cells or released by shedding with IgG aggregates and anti-IgG antibodies. One group (13) reported on the isolation of a 45,000-dalton protein from a murine leukemia cell line (L1210) and the other (15) on a 100,000-dalton protein from human lymphoid cell lines.

On the whole, the data in the literature on Fcy-binding proteins are very confusing. Because the mentioned studies differed chiefly in cell sources and solvents, it is not clear which of these parameters is responsible for the differences in the results. Furthermore, there is also the possibility of nonspecific interaction during the isolation procedure. For these reasons, we performed a large number of control experiments in the present study. As long as it is not possible to measure the biological effect induced by binding of ligands to Fcy receptors, the only way to test the specific binding of IgG to Fcy receptors is to perform control experiments with F(ab')2 fragments of IgG. Such control experiments do not, however, rule out the possibility that aggregated or complexed IgG is bound to cell-membrane proteins that do not act as Fcy receptors, but rather capture the IgG via nonspecific hydrophobic interaction. On the other hand, this problem also arises with Fcy-receptor assays of intact lymphoid cells.

As far as we know, none of the published studies contains a report on the isolation of a single polypeptide chain with a mol wt of 28,000 under reducing conditions. To enable a comparison of the isolated Fcy-binding protein not only with the reduced Fcy-receptor-like molecules described by other authors but also with nonreduced
molecules, and to obtain data on the aggregation properties of the IgG-binding protein that we isolated, we dialyzed EDTA-ME lysates against oxygenated PBS. With this procedure, the Fcγ-binding protein changed its mol wt to 115,000, probably by forming tetramers. The 115,000-dalton protein was reminiscent of the 125,000-dalton peak of Cooper et al. (14), the 120,000-dalton peak of Bourgeois et al. (17), the 110,000-dalton peak of Frade and Kourilsky (16), and the 100,000-dalton peak of Molenaar et al. (15). The Fcγ-binding proteins described by those authors might correspond to the tetrameric Fcγ-affined proteins that we isolated. This assumption would explain why the 100,000-dalton protein described by Molenaar et al. (15) was able to agglutinate EA and our 28,000-dalton peak was not. In its tetrameric form, the IgG-binding protein should be multivalent and thus capable of agglutinating EA. We have started investigations of these possibilities.

It has been reported that isolated C1q binds firmly to human lymphocytes (36) and thus might be responsible for the binding of IgG aggregates. To study the possibility of the Fcγ-receptor molecule being identical with C1q, we investigated one of the three B-type lymphoid leukemias that we used as sources of IgG-binding material, for the presence of C1q on the cell membrane. All of the cells from this case (B-type PLL) showed strong staining for C1q in double-layer immunofluorescence. From the following findings, we concluded that C1q and the Fcγ-binding protein isolated in this study are different structures: (a) The cells from all three cases of B-type leukemia formed rosettes only with IgG-EA complexes (40–95%) and not with IgM-EA (0–3%). If the Fcγ-receptor activity of the leukemic cells were mediated by C1q, both IgG-EA complexes and IgM-EA would be bound, because C1q reacts with the Fc fragments of both IgG and IgM (37). (b) EA-rosette formation by the C1q-bearing leukemic cells was not depressed by anti-C1q serum, but was remarkably inhibited by anti-Fcγ-receptor sera. (c) Carrageenin or polyinosinic acid did not inhibit the binding of the 28,000-dalton protein to IgG aggregates; these substances are known to inhibit the binding of C1q to complexed IgG (37, 38). (d) In SDS-polyacrylamide gel electrophoresis, C1 fragments isolated with IgG aggregates under the conditions used for the isolation of the Fcγ-binding membrane protein showed one large peak of

### Table I

| Method of detection                        | Ema* | EmaA* |
|-------------------------------------------|------|-------|
| Rosette inhibition                        | ND   | 70%   |
| Staining                                  |      |       |
| Anti-Fcγ-receptor serum 1 + GAR-R‡        | –    | +†    |
| Anti-Fcγ-receptor serum 2 + GAR-R         | –    | +     |
| Anti-Fcγ-receptor serum 3 + GAR-R         | –    | +     |
| Anti-Fcγ-receptor serum 3§ + GAR-R        | –    | +     |
| Anti-C1q serum + GAR-R                    | –    | –     |
| GAR-R                                     | –    | –     |

* Incubated in EDTA-ME lysates.
‡ GAR-R, rhodamine-labeled goat anti-rabbit IgG y-globulin.
§ Anti-Fcγ-receptor serum 3, additionally absorbed with C1.
ND, not done.
† +, positive fluorescence.
58,500 daltons and no predominant peak in the 28,000-dalton region. (e) EA prein-
cubated in EDTA-ME lysates displayed brilliant fluorescence with antisera directed
against the Fcγ-binding protein, but not with anti-C1q sera.

The data presented here show that the Fcγ-binding protein that we isolated from
leukemic B cells consists of a single polypeptide chain and has an apparent mol wt of
28,000 and a pH of 5.5. This structure has only one binding site, and its linkage to the
cell membrane is apparently mediated by divalent cations and disulfide bridges.
These features suggest that the isolated Fcγ-binding protein is not an integral
membrane protein, as defined by Singer et al. (39), but rather, an associated
membrane protein; it represents either a complete receptor molecule or at least the
fragment of Fcγ receptors that is responsible for binding to the Fc portion of IgG. The
28,000-dalton Fcγ-binding protein tends to aggregate, probably to tetramers, in the
absence of ME and might be expressed on the cell membrane in the form of tetramers.

Summary

A selectively Fcγ-binding protein was isolated from purified and radioiodinated
cell membranes from two cases of B-type chronic lymphocytic leukemia and one case
of B-type prolymphocytic leukemia by binding to IgG aggregates, horseradish per-
oxidase-anti-peroxidase IgG complexes, and sheep erythrocyte membrane sheets
densely coated with IgG. This protein could not be isolated from the cell membranes
of an Fcγ-receptor-negative chronic lymphocytic leukemia of the T type or from
membranes of human erythrocytes. The Fcγ-binding protein was efficiently solubi-
lized by a mixture of Na-EDTA and 2-mercaptoethanol, but not with one of these
agents alone, indicating that both divalent cations and disulfide bridges are involved
in the linkage of the Fcγ-binding protein to the cell membrane. In sodium dodecyl
sulfate-polyacrylamide gel electrophoresis, the Fcγ-binding protein revealed an ap-
parent mol wt of 28,000, and in isoelectric focusing it showed an isoelectric point of
5.5. The electrophoretic mobility of the 28,000-dalton protein did not change after
reduction and alkylation. It was determined that the NH2-terminal amino acid of the
protein was glycine. The isolated protein was unable to agglutinate antibody-coated
erthrocytes. These findings suggest that the 28,000-dalton IgG-affined protein was
composed of one polypeptide chain with one Fcγ-region-specific binding site. When
exposed to O2-enriched buffer lacking reducing agents, the 28,000-dalton protein
aggregated to a 115,000-dalton molecule. The isolated Fcγ-binding protein proved to
be different from C1q or its subunits.

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