Isolation from human chronic-lymphocytic-leukaemia cells of membrane glycoproteins associated with Fc-receptor functions

Physical parameters and production of polyclonal antibodies

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Two membrane glycoproteins that bound immune complexes and inhibited Fc-receptor- (FcR-)mediated functions *in vitro* were purified from human FcR⁺ chronic-lymphocytic-leukaemia cells. A multi-step purification was developed, consisting essentially in: (i) Tween 40 extraction of crude cell membranes; (ii) solubilization of membrane fragments by Renex-30; (iii) isolation of glycoproteins by affinity chromatography on *Lens culinaris* haemagglutinin–Sephrose; (iv) papain treatment of the eluted glycoproteins followed by gel-filtration chromatography; (v) purification by polyacrylamide-gel electrophoresis of two molecular species from the protein-size fraction enriched for immune-complex-binding activity. The two electrophoretically isolated components displayed apparent molecular masses of 70 and 45 kDa by SDS/polyacrylamide-gel electrophoresis and restricted charge heterogeneity by two-dimensional analysis. Two-dimensional peptide mapping revealed the presence of many peptides in common between the two proteins and the absence of a number of peptides in the 45 kDa component. These two polypeptides were used as immunogens to produce polyclonal antibodies that cross-reacted with both proteins and specifically inhibited FcR-mediated reactions *in vitro*. Furthermore, FcR-related components from detergent-extracted lysates of the human K562 and U937 cell lines or human placental membranes were revealed by the putative anti-FcR antibodies adsorbed on Protein A–Sephrose.

INTRODUCTION

FcRs have been detected on cells such as B and T lymphocytes (Lydyard & Fanger, 1982), K and NK cells (Herberman & Ortaldo, 1981), macrophages (Unkeless & Eisen, 1975), monocytes (Anderson, 1982) and polymorphonuclear cells (Kulczycki & Metzger, 1974). They are reportedly involved in processes such as antibody-dependent cellular cytotoxicity, phagocytosis, release of soluble mediators such as prostaglandins (Suzuki *et al.*, 1980), allergic response mediated by basophils and mast cells (Segal *et al.*, 1977) and trans-placental materno-fetal IgG transport (Brambell, 1966). FcRs have been also found to mediate cytotoxicity of human tumour cells *in vitro* (Steplewski *et al.*, 1983) and other related mechanisms, and in general to take part in both positive and negative feedback regulatory mechanisms of antibody production, by recognizing the expression of various IgG isotypes (Lübeck *et al.*, 1985; Daeron & Fridman, 1985).

It is widely accepted that FcRs borne by various mammalian cell types show a striking heterogeneity (7ème Forum d'Immunologie, 1985). Study of FcR structure and heterogeneity may elucidate the mechanism of their binding specificity and their role in modulating immune response. However, progress in isolation and characterization of FcRs has been hampered by their low cell-surface density and relatively low affinity for monomeric IgG. Numerous techniques have been

applied by our laboratory and others in attempts to isolate FcRs. These methods include affinity chromatography over monomeric or aggregated IgG (or Fc) coupled to a solid matrix, or alternatively immune co-precipitation with antibody–antigen complexes of radiolabelled FcR from detergent-solubilized cell lysates.

Reported structural information about FcRs isolated from different cell sources is widely divergent (for review see Unkeless *et al.*, 1981). Several factors may account for this: susceptibility to proteolysis during purification (Bourgeois *et al.*, 1977; Kulczycki, 1984), structural polymorphism (Baum *et al.*, 1985; 7ème Forum d'Immunologie, 1985) and co-isolation of unrelated membrane proteins due to non-specific interactions with IgG (Unkeless *et al.*, 1981).

In the present study efforts were made to isolate human FcRs by means of different techniques in attempts to obtain reliable molecular and functional data and explore possible human FcR heterogeneity (Fleit *et al.*, 1982; Anderson *et al.*, 1985). A multi-step procedure was employed for the purification from human CLL cells of plasma-membrane glycoproteins that displayed FcR activity. Two proteins that bound immune complexes were isolated and further purified by non-denaturing PAGE. The functional characterization of polyclonal antisera raised against the putative FcR glycoproteins and their capacity to recognize FcR expressed on different human cells were also investigated.

Abbreviations used: CLL, chronic lymphocytic leukaemia; FcR, Fc receptor; PMNC, peripheral mononuclear cells; SRBC, sheep erythrocytes; SaCl, *Staphylococcus aureus* Cowan I; Sh, sheep; Rb, rabbit; S, serum; Ia, MHC class II antigens; EA-B and EA-H, antibody-coated bovine erythrocytes and antibody-coated human erythrocytes respectively; PAGE, polyacrylamide-gel electrophoresis; LcH, *Lens culinaris* haemagglutinin.

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MATERIALS AND METHODS

Cells

FcR⁺ CLL cells were obtained by leucopheresis. Lymphocytes and PMNC were purified by a standard Ficoll/Hypaque gradient centrifugation (Boyum, 1968).

A human erythromyeloid line (K562) and a human monocytic line (U937) were gifts from Dr. Robert Painter (Department of Biochemistry, University of Toronto, Toronto, Ont., Canada).

Murine macrophage cell line P388D₁ was a gift from Dr. V. Colizzi (Institute of Microbiology, University of Pisa, Pisa, Italy).

The cells were maintained in conventional RPMI 1640 medium (GIBCO, Grand Island, NY, U.S.A.) containing 10% (v/v) fetal-calf serum, penicillin (10 units/ml) and streptomycin (100 µg/ml), all from GIBCO.

Full-term normal placentas were obtained from the Women's College Hospital (Toronto, Ont., Canada) and processed within 30 min of delivery.

Preparation of crude cell membranes

Frozen peripheral lymphocytes (1×10^8 – 8×10^9 nucleated cells) were thawed and resuspended at a cell concentration of 0.3% in buffer A (78 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl, 2 mM-MgSO₄ and 2 mM-CaCl₂) containing 1 mM-tosylphenylalanyl-chloromethane ('TPCK'), 0.2 mM-tosyl-lysylchloromethane ('TLCK'), 2 mM-phenylmethanesulphonyl fluoride and Tween 40 (4%, v/v). The cell suspension was kept for 60 min on ice, and then the cells were disrupted by homogenization. Insoluble debris was cleared from the homogenate by centrifugation at 3000 g for 30 min at 4 °C, and the plasma-membrane fraction was sedimented at 105000 g for 80 min at 4 °C.

Solubilization of cell membranes

The crude cell-membrane pellet was washed twice with buffer A and resuspended in buffer B (75 mM-Tris/HCl buffer, pH 7.8, containing 0.5 M-NaCl) containing 2.5% (v/v) Renex-30 (Ruger Chemical Co., Irvington, NJ, U.S.A.), a non-ionic detergent, homogenized and centrifuged at 105000 g for 80 min at 4 °C.

LcH-Sepharose affinity chromatography

The solubilized membrane material was applied to an LcH-Sepharose (Pharmacia) column (1.2 cm × 15 cm) pre-washed with buffer C (75 mM-Tris/HCl buffer, pH 7.8, containing 0.5 M-NaCl, 0.2% Renex-30 and 0.02% bovine serum albumin). The unretarded material was washed out with buffer C, and the bound fraction was eluted with the same buffer containing 5% (w/v) methyl α-mannoside (Sigma Chemical Co.).

Radioiodination

[¹²⁵I]Iodination was done by the chloramine-T technique as described by Greenwood *et al.* (1963) with a labelling efficiency of about 70%. The radiolabelled material was fractionated on a column (1 cm × 80 cm) of Bio-Gel A1.5m (200–400 mesh) (Bio-Rad Laboratories) in buffer C. Samples (20 µl) of the eluate were monitored for radioactivity and for binding to immune complexes (Fig. 1).

Human K562 and U937 cells and human placental membranes were surface-labelled with ¹²⁵I by the

lactoperoxidase/glucose oxidase method (Hynes, 1973) with the use of Enzymobeads (Bio-Rad Laboratories). After the labelling cells or membranes were washed in phosphate-buffered saline (75 mM-sodium phosphate buffer, pH 7.2, containing 0.1 M-NaCl), resuspended in phosphate-buffered saline containing 10 mM-EDTA, 58 mM-6-aminohexanoic acid, 0.001% (w/v) pepstatin, 2 mM-phenylmethanesulphonyl fluoride and 10 mM-iodoacetamide, and extracted for 1 h at 4 °C with 0.5% Nonidet P-40. After 30 min centrifugation at 100000 g, 10 µl samples were removed to determine the radioactivity precipitable by trichloroacetic acid (10%, w/v).

Papain treatment

A portion of glycoprotein fraction (0.02 A₂₈₀ unit) was radiolabelled. It was then treated with papain (Worthington Biochemical Corp.) in the presence of bovine serum albumin (as a carrier protein) in an enzyme/carrier ratio of 1:40. Digestion was carried out for 30 min at 37 °C in the presence of 1 mM-EDTA and 10 mM-cysteine hydrochloride. The reaction was stopped by addition of 10 mM-iodoacetamide. The digest was applied to a Bio-Gel A1.5m column (1 cm × 80 cm) and eluted with buffer C, and the collected fractions were monitored for radioactivity.

Immunization and preparation of IgG and (Fab')₂ fragments

Antibodies directed against the isolated A and B components of FcR were raised in two rabbits immunized with 0.050 A₂₈₀ unit of either protein. The rabbits were injected five more times at 30-day intervals, and bled once a week during the immunization regime. IgG fractions were isolated from each antiserum by QAE-Sepharose chromatography (Joustra & Lundgren, 1969) and further purified by affinity chromatography on Protein A-Sepharose CL-4B (Pharmacia).

(Fab')₂ fragments were obtained by pepsin digestion of purified IgG proteins followed by gel filtration on Sephadex G-150, as previously described (Gorini *et al.*, 1969).

Binding assay

Fc receptor. The basic procedure for isolation of Fc-binding material was the direct binding assay based on the co-precipitation of labelled FcR with immune complexes obtained by mixing normal rabbit serum and Sh anti-(Rb IgG) serum or Protein A-bearing SaCI. In a standard reaction 15000 c.p.m. of labelled preparation in a volume of 20 µl was added to 3 µl of normal rabbit serum and 30 µl of 75 mM-Tris/HCl buffer, pH 7.8, containing 0.2% Renex-30 and incubated at 37 °C. After 60 min, 50 µl of Sh anti-(Rb IgG) serum or 20 µl of 10% SaCI bacterial suspension was added and the mixture was incubated for 60 min at 37 °C. In control experiments (Fab')₂ fragments of Rb IgG (30 µg in 3 µl) and (Fab')₂ fragments of Sh anti-(Rb IgG) antibodies (750 µg in 50 µl), or (Fab')₂ fragments of Rb anti-Ia antibodies (3 µl) and (Fab')₂ fragments of Sh anti-(Rb IgG) antibodies (50 µl), were used to form immune complexes. After incubation, the mixtures were spun down at 200 g for 10 min at room temperature. The immune precipitates were washed twice with borate-buffered saline (50 mM-sodium borate buffer, pH 8.0, containing 0.1 M-NaCl) and the radioactivity was monitored with a γ-radiation counter. The results obtained were expressed

as the percentage binding relative to the total radioactivity (c.p.m.) added to the samples. In several binding experiments heat-aggregated human IgG was also used, in place of normal rabbit serum, in order to estimate the overall yield of receptor activity.

Anti-receptor. The extent of binding and specificity of anti-FcR antibody was assayed by the double-antibody technique. Immune complexes were formed between labelled FcR fractions (15000 c.p.m.) and anti-FcR sera (3 μ l), or related (Fab')₂ (80 μ g in 3 μ l), or normal rabbit serum (NRS) (3 μ l) as a control, and immunoprecipitated with Sh anti-Rb serum (60 μ l) or 10% SaCl (20 μ l), as described above. The percentage binding was calculated as follows:

$$\text{Binding (\%)} = \frac{(\text{c.p.m. bound by anti-FcR}) - (\text{c.p.m. bound by NRS})}{\text{total c.p.m. added}} \times 100$$

Inhibition of EA rosette formation

Receptor. Bovine erythrocytes were sensitized by incubation with a sub-agglutinating dose of IgG from rabbit anti-(bovine erythrocyte) serum at 37 °C for 10 min, centrifuged at about 300 *g* for 5 min and incubated, at a concentration of 0.5% (v/v), overnight at 4 °C with human PMNC (5 × 10⁶ cells/ml), in a ratio of 1:1 (v/v). After sedimentation at 300 *g*, the mixture was resuspended and the number of rosette-forming cells was counted. The biological activity of the isolated FcR was determined by the degree of the inhibition of EA rosette formation. Cells with at least five bound erythrocytes were scored as rosettes.

Anti-receptor. Rh⁺ human erythrocytes coated with high-titre human anti-D IgG (EA-H, 0.5%) were tested with P388D₁ cells (5 × 10⁶/ml) preincubated for 60 min, at 3 °C, with and without anti-receptor sera, used as inhibitors.

Agglutination assay of placenta microvilli

SRBC were sensitized with Rb IgG to SRBC, 1 h at 37 °C, and used immediately in the assay. Placenta microvilli plasma membranes were prepared by the procedure of Booth *et al.* (1980). Optimal agglutination conditions were determined by titrating placenta plasma membranes with 10 μ l of a 2.5% suspension of IgG-coated SRBC in U-shaped micro-titre wells. After incubation at room temperature for approx. 4 h, the diameters of the agglutinations were measured to the nearest 0.5 mm. Inhibition assays were performed similarly by adding 25 μ l of 5-fold serial dilutions of anti-FcR or anti-vimentin sera to mixtures containing 100 μ l of membranes and 10 μ l of IgG-coated SRBC. Anti-vimentin was used as control antiserum and was generously contributed by Kursad Turksen.

PAGE

Analytical slab (7 cm × 12 cm)-gel electrophoresis was carried out in accordance with Laemmli (1970) in 10% or 12% polyacrylamide gels containing 0.1% SDS. The ¹²⁵I-labelled samples were run in non-reducing or reducing conditions. Phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya-bean trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa) were used as molecular-mass markers. After the electrophoresis the

gels were dried and exposed to Trimax 3M X-ray films double-screened for autoradiography.

Preparative disc-gel electrophoresis was carried out by separating the samples on 10% polyacrylamide rod gels in the absence of SDS.

Two-dimensional electrophoresis was carried out in 3 mm × 100 mm cylindrical gels in the first dimension (isoelectric focusing), and on an SDS/12.5%-PAGE slab gel in the second dimension, essentially as described by O'Farrell (1975).

Peptide mapping

The method used was the same as described by Sorrentino *et al.* (1983), with minor modifications. In

the first dimension the peptic digests (about 20000–30000 c.p.m.) were subjected to high-voltage electrophoresis for 5 h at 700 V on silica-gel plates (20 cm × 20 cm) in pyridine/acetic acid/water (1:10:89, by vol.). Ascending chromatography was performed in the second dimension in pyridine/butane/acetic acid/water (10:15:2:8, by vol.). Peptides were detected by autoradiography.

Extraction of human placenta plasma-membrane proteins

Human plasma membranes were prepared as described by McNabb *et al.* (1976) and extracted with lithium di-iodosalicylate (Sigma Chemical Co.) by using the procedure of Mikulska *et al.* (1982).

Immunoabsorption

Portions of ¹²⁵I-labelled detergent-solubilized proteins extracted from either human cell lines (K562 and U937) or human placenta equivalent to approx. 3 × 10⁵ c.p.m. of trichloroacetic acid-precipitable radioactivity were mixed with 15 μ l portions of immune sera. After the addition of 50 μ l of a 50% (w/v) Protein A–Sepharose suspension in phosphate-buffered saline containing 5% (w/v) bovine serum albumin, the mixtures were incubated at 4 °C for 18–24 h with head-over-head rotation. The mixtures were then washed ten times with phosphate-buffered saline containing 0.1% Nonidet P-40, eluted with SDS/PAGE sample buffer (50 mM-sodium phosphate buffer, pH 7.0, containing 2% SDS, 0.1 M-iodoacetamide and 10% glycerol) and analysed by SDS/PAGE.

Immunological reagents

Rb anti-Ia serum was produced by immunization with human Ia antigens purified from Daudi cells (Tanigaki *et al.*, 1983). Sh anti-Rb serum was obtained by using Rb IgG as purified antigen. Rb anti-SRBC serum was obtained by immunizing animals with a 1% suspension of erythrocytes. Rb IgG fractions and (Fab')₂ fragments were obtained by the methods described above.

SaCl (heat-killed and formalin-fixed) bearing Protein A (Pansorbin) was purchased from Calbiochem–Behring Corp. and used as a 10% bacterial suspension.

Fig. 1. Gel filtration of radioiodinated human FcR preparation from CLL cells

An FcR preparation (0.020 A_{280} unit) obtained by non-ionic detergent solubilization and partially purified by LcH-Sepharose affinity chromatography was labelled with 500–600 μCi of ^{125}I , treated with papain for 1 h at 37 °C with 1 mg of enzyme/40 A_{280} units of bovine serum albumin as a carrier protein, and fractionated on a column (1.5 cm $\times$ 80 cm) of Bio-Gel A1.5m. The volume of each fraction was 0.65 ml. The graphs are plots of radioactivity ($\bullet$) and FcR-binding activity (Δ) for 20 μl fractions. The binding activity was tested by the direct binding assay with Rb IgG and Sh anti-(Rb IgG) antibodies as immune complexes. The fraction indicated by the arrow was purified by LcH-Sepharose affinity chromatography and used in the further experiments.

RESULTS

Isolation of FcR-related glycoproteins from a plasma-membrane lysate of human CLL lymphocytes

CLL cells were suspended in Tris buffer containing Tween-40 and disrupted by homogenization. The membrane fraction was resuspended in Tris buffer containing 2.5% Renex-30. The solubilized material was applied to an LcH-Sepharose column, and the bound glycoprotein fraction was eluted with 5% methyl α -mannoside. Approx. 0.020 A_{280} unit of the glycoprotein fraction was labelled with ^{125}I (500–600 μCi) and then fractionated by chromatography on a Bio-Gel A1.5m column. A heterodisperse broad peak and a minor peak of unbound radioactive iodine were obtained.

The protein peak was subjected to a mild papain treatment and again fractionated by gel-exclusion chromatography on a Bio-Gel A1.5m column (Fig. 1). The eluate was monitored for relative radioactivity and for immune-complex binding. The immune-complex binding pattern exhibited a distinct activity peak at tube fractions 122–140 corresponding to the descending shoulder of a broad peak observed in the radioactivity profile. When papain treatment was omitted no distinct peak with Fc-binding activity was obtained. Small traces

Fig. 2. Autoradiograph of SDS/PAGE of FcR-containing material after affinity chromatography on LcH-Sepharose

A sample of retarded fraction (about 10000 c.p.m.) that contained FcR activity was co-precipitated with Rb IgG-Sh anti-(Rb IgG) antibodies and the precipitate and the supernatant were subjected to a SDS/PAGE on a 12% gel, either in non-reducing (N.R.) or in reducing (R.) conditions. The gel was dried and autoradiographed. Lanes 1 and 5, supernatants (N.R. and R. respectively); lanes 3 and 4, precipitates (N.R. and R. respectively); lane 2, FcR before precipitation. The positions of molecular-mass markers are indicated.

Fig. 3. Disc-gel electrophoretic separation, in the absence of SDS, of FcR-containing glycoprotein fraction

A sample of retarded fraction from LcH-Sepharose affinity chromatography (120×10^3 c.p.m.) with FcR activity was subjected to PAGE on a 10% rod gel (6 mm $\times$ 85 mm). After electrophoresis the gel was sliced into 1 mm segments from the cathode and the radioactivity was determined. The values were plotted with the top of the gel on the left-hand side of the Figure.

of class II antigens were detected by a sensitive radioimmunoassay with two different specific rabbit antisera.

The localized FcR-containing fraction was concen-

Fig. 4. Autoradiographs of O'Farrell two-dimensional gel analysis of two FcR components

Radioiodinated 70 kDa (a) and 45 kDa (b) components, yielding 4×10^5 c.p.m. of trichloroacetic acid-precipitable radioactivity, eluted from disc-gel electrophoresis, were subjected to isoelectric focusing on cylindrical gels (first dimension) and then subjected to PAGE on 12.5% slab gels (second dimension). Basic ends of isoelectric-focusing gels are on the left; the direction of electrophoresis is from top to bottom. The positions of molecular-mass markers are indicated.

trated and further purified by LcH-Sepharose affinity chromatography. The fraction eluted with methyl α -mannoside was subjected to an SDS/12% PAGE analysis. As shown in Fig. 2, the FcR material gave essentially two component peptides, one of an apparent molecular mass of 70 kDa and another of 45 kDa after co-precipitation with immunocomplexes, when run under reducing or non-reducing conditions. Two other lighter, polydisperse, components, which are present in the glycoprotein fraction before immunoprecipitation (lane 2), are also faintly detectable in the precipitate patterns (lanes 3 and 4) at positions corresponding to α - and β -chains of human class II antigens.

Disc-gel electrophoresis run in the absence of SDS (Fig. 3) showed two major components (designated 'A' and 'B'). When eluted and subjected to SDS/PAGE they migrated as a 70 kDa protein and a 45 kDa protein respectively. Components A and B were analysed separately by the immune-complex binding test, and both displayed binding capacity.

Two-dimensional analysis (Fig. 4) by isoelectric focusing (in the first dimension) and electrophoresis in the presence of SDS (in the second dimension) revealed one single asymmetrical spot for each component. The

Fig. 5. Autoradiographs of peptide maps of two FcR components

Peptic digests of the 70 kDa (a) and 45 kDa (b) polypeptides, containing 2×10^4 – 3×10^4 c.p.m. of trichloroacetic acid-precipitable radioactivity, were first subjected to high-voltage electrophoresis on silica-gel plates and then to ascending chromatography at right-angles. Electrophoresis (e) was from bottom to top, and chromatography (c) from left to right, as indicated by the arrows. The map (c) shows peptides common to the two components.

isoelectric points calculated from the first dimension were 6.11 for component A and 6.37 for component B.

The comparison of the spots in the peptide mapping confirms the existence of two protein components, having various peptides in common, as shown in Fig. 5 by the autoradiographs of micro fingerprinting. A considerable number (about 30) of spots located in the hydrophilic as well as in the hydrophobic region in the

Table 1. Inhibition of rosette formation between EA-B and human PMNC

Inhibitor was the isolated FcR glycoprotein at decreasing concentrations from an initial solution of 1.5 A_{280} units/ml in Tris buffer, pH 7.8, containing 2.0% Renex-30. The 50 μ l inhibitor dilutions containing 2.5×10^6 PMNC were incubated with 50 μ l of 1% EA-B for 60 min at 4 °C. Stated inhibitor dilutions are relative to 100 μ l final volume. EA-B were bovine erythrocytes sensitized by rabbit anti-(bovine lymphocytes) IgG. The percentage of PMNC forming rosettes in the absence of inhibitors was 33%. The first dilution at which the detergent alone did not lyse erythrocytes and did not inhibit rosette formation was 1:100. The inhibition values given are the average for triplicate experiments with S.E.M. less than 10%.

Inhibitor dilution	Inhibition of rosette formation (%)
None (control)	0
1:100	90.4
1:125	86.6
1:200	79.2
1:400	77.0
1:625	79.1
1:800	67.4
1:1000	50.5

peptide map of the heavier component did not appear in the map derived from the 45 kDa component.

Functional identification of the isolated FcR glycoprotein

The specific Fc-binding activity of the fraction enriched by LcH-Sepharose chromatography was assayed as the ability to inhibit EA-B rosette formation with human PMNC at an initial concentration of 1.5 A_{280} units/ml. As shown in Table 1, a 1:100 protein dilution produced a 90% inhibition of rosettes, and at a 1:1000 dilution a 50% inhibition persisted.

Induction and characterization of polyclonal antisera against the isolated FcR glycoproteins

Rabbit antibodies were raised against the two components A and B isolated by disc-gel electrophoresis (see Fig. 3) in non-denaturing conditions. Both antisera and respective (Fab')₂ fragments of purified IgG, when tested in the binding assay, were co-precipitated by the homologous inducing antigen and cross-reacted with each other, suggesting that the proteins shared epitopes. When the precipitates and corresponding supernatants were analysed by SDS/PAGE (Fig. 6a), two proteins, of approx. 70 and 45 kDa, were observed. Two lower-molecular-mass contaminants were also present in the supernatants. The cross-reactivity of the two antisera is clearly shown in Fig. 6(b), which shows that either antiserum bound both A and B proteins.

Effect of antibodies on FcR functional activities

The receptor-binding activity of purified IgG from the two antisera and derived (Fab')₂ fragments was evaluated by the degree of inhibition of EA-H rosette formation. The results shown in Table 2 demonstrate the specificity of the antisera against Fc receptor activity. Strong inhibition (more than 60%) is observed even at the equivalent of a 1:1250 serum dilution. The inhibition by

Fig. 6. Autoradiographs of SDS/PAGE of anti-FcR immunoprecipitates in non-reducing conditions

Immunoprecipitates, formed as described in the Materials and methods section, containing 125 I-labelled unseparated components of FcR, i.e. the methyl α -mannoside eluate from LcH-Sepharose affinity chromatography, or 125 I-labelled separated components (A or B), were washed twice with borate-buffered saline and dispersed in 50 mM-sodium phosphate buffer, pH 7.0. Samples were solubilized by heating for 2–5 min in 2% SDS dissolved in 50 mM-sodium phosphate buffer, pH 7.0, containing 100 mM-iodoacetamide and analysed in SDS/PAGE in 10% gels. (a) Co-precipitates with both components of labelled FcR. Anti-A serum: lane 1, precipitate; lane 3, supernatant. Anti-B serum: lane 2, precipitate; lane 4, supernatant. Lane 5, standard molecular-mass markers. (b) Co-precipitates with separated components of labelled FcR. Anti-A serum: lane 1, precipitated with A; lane 2, precipitated with B. Anti-B serum: lane 3, precipitated with A; lane 4, precipitated with B. Lane 5, standard molecular-mass markers.

(Fab')₂ fragments was considerably lower, suggesting a supplementary role in the binding of the receptor by the Fc region of the antibody molecule. Control inhibition values, either for IgG from an unrelated hyperimmune whole antiserum (Rb anti-SRBC) or for the respective pepsin fragment, remained low. Therefore the two anti-FcR sera seem to have the same behaviour when their functional activities are compared.

Reaction of anti-A serum with putative FcR molecules expressed on human myeloid-derived cell lines and human placenta

In an effort to investigate the capacity of the anti-FcR antiserum to recognize FcR antigens on different human

Table 2. Inhibition of rosette formation between EA-H and the murine macrophage cell line P388D₁

Inhibitors were IgG or (Fab')₂ fragments prepared from anti-receptor sera (anti-A or anti-B), and from a hyperimmune rabbit serum (anti-SRBC) as a control. (2.5×10^5) P388D₁ cells in 50 μ l of phosphate-buffered saline were incubated with 50 μ l of inhibitor solution for 30 min at 4 °C. The first two columns give the inhibitor final concentrations. Cells were pelleted, resuspended in 50 μ l of a 0.5% EA-H suspension and further incubated for 30 min at 4 °C. EA-H were human erythrocytes coated with high-titre anti-D IgG. Rb IgG were isolated from normal rabbit serum. The formation of rosettes in the absence of inhibitors was 70%. Inhibition values are the averages for triplicate experiments with s.e.m. less than 10%.

Concn. of inhibitor (μ g/100 μ l)		Inhibition (%)						
IgG	(Fab') ₂	Anti-A IgG	Anti-B IgG	Anti-SRBC IgG	Anti-A (Fab') ₂	Anti-B (Fab') ₂	Anti-SRBC (Fab') ₂	Rb IgG
100	67.5	96.7	85.2	20.4	48.1	44.1	10.2	25.0
20	13.5	80.1	75.7	26.1	31.0	22.1	2.3	5.1
4	2.7	65.3	68.0	13.8	15.4	17.3	0	0
0.8	0.55	64.2	51.3	1.1	2.0	3.2	0	0

Fig. 7. SDS/PAGE analysis of cell-surface proteins immunoadsorbed with anti-A antibodies

Samples of proteins extracted from surface-labelled human cell lines (K562 or U937) or placental plasma membranes, yielding 3×10^5 c.p.m. of trichloroacetic acid-precipitable radioactivity, were mixed with 10–25 μ l of anti-A serum and 50 μ l of a 50% Protein A–Sepharose suspension in phosphate-buffered saline, and incubated overnight at 4 °C. After ten washings with phosphate-buffered saline containing 0.1% Nonidet P-40, proteins bound to Protein A–Sepharose were eluted with SDS/PAGE sample buffer, and subjected to SDS/PAGE in a separating 10% polyacrylamide gel. Proteins were detected by autoradiography. Lanes 1 and 3 contain proteins immunoadsorbed from detergent-extracted lysates of surface-labelled U937 and K562 cells respectively. Lane 2 contains proteins immunoadsorbed from lithium diiodosalicylate extracts of human placental plasma membranes.

cell lines and to detect a possible tissue FcR polymorphism, immuno-adsorption experiments were carried out with protein extracts from human FcR⁺ cells. The human cell lines K562 and U937 and placental plasma membranes were surface-labelled with ¹²⁵I. Proteins were extracted and immuno-adsorbed with the antiserum

Fig. 8. Inhibition of agglutination of human placental membranes and IgG-coated SRBC by anti-receptor serum

Samples (100 μ l) of placental plasma membranes and 10 μ l portions of IgG-coated SRBC were distributed in U-shaped micro-titre wells. Then 25 μ l portions of 5-fold serial dilutions of anti-A (Δ) or anti-vimentin ($\bullet$) antibodies in phosphate-buffered saline were added. The optimal agglutination is 5–6 mm diameter. The degree of inhibition was established by the decrease in the diameter of the agglutination pellet.

immobilized on Protein A–Sepharose. Bound proteins were eluted and analysed by SDS/PAGE (Fig. 7). Two components of about 70 kDa and 45 kDa for U937 and one component of 70 kDa and a heavier component at the origin of the gel for K562 were detected. Anti-FcR antibodies also revealed one surface protein of about 55 kDa when adsorbed with a placental lithium diiodosalicylate extract, but no bands of the same molecular masses as the immunogenic FcR components were found.

Furthermore, the agglutination of IgG-coated erythrocytes with placental membrane extracts was strongly inhibited by anti-A serum (anti-B serum was not tested), whereas anti-vimentin antibodies, used as a control, were ineffective as inhibitors (Fig. 8). This supports the specificity of anti-A serum for cell-surface proteins involved in FcR-mediated functions.

DISCUSSION

Numerous authors have reported the isolation and characterization of human FcR. However, the reported biochemical properties of the isolated proteins vary widely (Unkeless *et al.*, 1981; Dorrington, 1984). The conflicting data may result from factors such as human FcR heterogeneity (Fleit *et al.*, 1982) or susceptibility to proteolysis (Kulczycki, 1984; Looney *et al.*, 1986). These factors and the relatively low IgG-binding affinity of FcR expressed on human granulocytic and lymphoid cells may account for the variable results obtained by conventional IgG-ligand affinity chromatography.

To circumvent some of these problems, a procedure originally described by Tanigaki & Tosi (1982) for the isolation of class II antigens was utilized. Among the membrane proteins isolated from CLL cells by this procedure, a fraction was observed to be co-precipitated with IgG-anti-IgG immune complex. The same protein fraction did not react with anti-Ia antiserum. On the basis of these findings, a protocol was devised (see the Materials and methods section) for the isolation of glycoproteins that displayed FcR activity when assayed by immune-complex co-precipitation or inhibition of rosette formation.

After papain treatment and non-denaturing isoelectric separation, putative FcR proteins of molecular mass approx. 70 and 45 kDa (designated 'A' and 'B' respectively) were identified by SDS/PAGE.

Two possible interpretations might account for the effect of papain. Firstly, the 45 kDa protein might be proteolytically derived from the 70 kDa protein by papain hydrolysis. This hypothesis is supported by the observation that antisera raised against the individual proteins cross-reacted with both proteins (Fig. 6). Further, peptide maps of the 45 kDa protein contained fewer peptides than those of the 70 kDa protein, but there were many common peptides. Comparing the FcR isolated in the present work with those purified by different procedures from human myelo-monocytic and erythroblast cells (Anderson *et al.*, 1985; Lübeck *et al.*, 1985), a consensus emerges, relating a protein of approx. 70 kDa with FcR activity. This receptor is distinct from another 40–43 kDa FcR of lower Fc-binding affinity that is expressed on similar cells (Jones *et al.*, 1985; Looney *et al.*, 1986). A third type of human FcR that is expressed on lymphoid and granulocytic cells also has a low affinity for IgG and is distinguished by its heterogeneous SDS/PAGE profile ranging from about 50 to 70 kDa and by the fact that it is recognized by monoclonal antibodies exclusive of the other FcR types (Fleit *et al.*, 1982; Perussia & Trinchieri, 1984).

In the light of evidence for FcR polymorphism (Baum *et al.*, 1985; Tax *et al.*, 1985) and for an 'isotypic' network (Daeron & Fridman, 1985), the antisera obtained in the present study may provide useful tools for investigation of inter-relatedness of FcR expressed on different cell types. Anti-A serum isolated an approx. 70 kDa protein from K562 cells. This finding contrasts with that of Anderson (1982), who used an antiserum raised in goat against a 72 kDa FcR expressed on U937 cells and was unable to isolate a protein of this size from K562 cells. The reasons for this discrepancy are unknown. However, in accordance with reported FcR activity *in vitro* being associated with proteins of approx. 72 kDa and 40 kDa expressed on U937 cells (Anderson, 1982; Anderson

et al., 1985; Lübeck *et al.*, 1985), anti-A serum bound proteins of similar size from U937 detergent lysates.

Likewise, anti-A serum produced potent inhibition of FcR activity *in vitro* of placental membranes (Fig. 8) and adsorbed a protein of approx. 55 kDa from glycoprotein extracts of placental membranes similar to the FcR protein (51 kDa) isolated by Mikulska *et al.* (1982).

The present observations provide possible evidence for different molecular forms of antigenically related FcR. It remains to be demonstrated whether the antisera described recognized epitopes common to genetically polymorphic FcR or a single FcR type that underwent different processing during either expression or isolation. In this regard, it is noteworthy that Vaughn *et al.* (1985) have described the production of a monoclonal antibody that recognized FcR on U937 cells, B cells and PMNC. The SDS/PAGE profiles of the FcR isolated by this antibody showed distinct size heterogeneity.

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