Analysis of C3-receptor activity on human B-lymphocytes and isolation of the complement receptor type 2 (CR2)

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The rosetting of defined C3-fragment-coated sheep erythrocytes to B-cell-enriched tonsil lymphocytes was measured. The rosetting lymphocytes were homogeneous with respect to expression of C3b, iC3b and C3d receptors. Isolation of receptors for C3 fragments from surface-radioiodinated lymphocytes by affinity chromatography on immobilized C3u, iC3b and C3d,g produced two proteins with partially overlapping specificities. A protein of 240 000 M_r , recognized by the monoclonal antibody To5 and identified as CR1 (complement receptor type 1), had affinity for C3u and iC3b. A protein of 145 000 M_r , recognized by the monoclonal antibody B2, had affinity for all three C3 fragments. Inhibition of rosetting by antibodies to these proteins indicates that CR1 is responsible for C3b-mediated rosetting and that the 145 000- M_r receptor (CR2) is responsible for C3d-mediated rosetting. Partial inhibition by both anti-CR1 and anti-CR2 antibodies of iC3b-mediated rosetting indicates that both receptors are involved in iC3b-mediated rosetting. No other protein appears to be involved in tonsil B-cell rosetting to C3-fragment-coated cells. A method for preparing CR2 from tonsil lymphocytes based on affinity chromatography on C3d,g-Sepharose has been developed. Forty tonsil pairs (2×10^{10} B-cells) yield about 40 μ g of pure protein equivalent to a purification of 6500-fold from a detergent extract.

The third component of human complement (C3), which is the major complement component, binds covalently to molecules and particles that activate the complement sequence (Law & Levine, 1977), as the C3b fragment (178 000 M_r). The C3b

Abbreviations used: DGVB⁺⁺, 140 mM-glucose/71 mM-NaCl/2.5 mM-sodium 5,5-diethylbarbiturate/0.5 mM-MgCl₂/0.15 mM-CaCl₂, pH 7.5, containing 1 g of gelatin/litre; phosphate-buffered saline, 8.2 mM-Na₂HPO₄/1.5 mM-KH₂PO₄/139 mM-NaCl/3 mM-KCl/5 mM-glucose/1 mM-CaCl₂/1 mM-MgCl₂, pH 7.5 (Dulbecco & Vogt, 1954); SDS, sodium dodecyl sulphate; DFP, diisopropyl phosphorofluoridate; PMSF, phenylmethanesulphonyl fluoride; SBTI, soya-bean trypsin inhibitor; NP40, Nonidet P40; the nomenclature of complement components and of C3 and its fragments is as recommended by the World Health Organisation (1968, 1981); further C3 fragments are as defined by Davis *et al.* (1984); IgG, immunoglobulin G; CM-, carboxymethyl.

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fragment is subsequently degraded to iC3b (175 000 M_r) (Harrison & Lachmann, 1980; E. Sim *et al.*, 1981) and then to C3d,g (36 000–41 000 M_r) and C3d (approx. 27 000–34 000 M_r) which all remain covalently bound to the activating surface (Freedman *et al.*, 1977; Harrison & Lachmann, 1980; Lachmann *et al.*, 1982; Davis *et al.*, 1984).

Human B-lymphocytes have been shown to bind to particles coated with C3b, iC3b and C3d,g (or C3d), and these receptor activities have been referred to as CR1, CR3 and CR2 respectively (Ross, 1982). The receptor activity CR1 is due to a cell-surface glycoprotein that is also present on human erythrocytes, polymorphonuclear leucocytes and monocytes (Fearon, 1980) and was initially described as having an M_r of 205 000 (Fearon, 1979). This protein is now regarded as being polymorphic, with variants of M_r ranging from 160 000 to 260 000 (Dykman *et al.*, 1983, 1984; Wong *et al.*, 1983). Although CR1 protein is responsible for the interaction of lymphocytes with particles bearing C3b, there is controversy as to whether this molecule participates in CR3 activity

(i.e. the binding of iC3b-bearing particles) on B-lymphocytes (Fearon & Wong, 1983). A two-polypeptide-chain molecule has been identified on monocytes, macrophages, neutrophils and natural killer cells which has CR3 activity (Beller *et al.*, 1982; Wright *et al.*, 1983). However, this molecule is not present on tonsil B-lymphocytes.

A molecule of 72000 M_r has been purified from culture supernatants of Raji lymphoblastoid cells and has been identified as a C3d receptor (CR2) (Lambris *et al.*, 1981). It has been suggested that this molecule acts as the receptor for iC3b (Ross & Lambris, 1982). Another glycoprotein, of 140000 M_r , has been reported in human B-lymphocytes as the C3d (CR2) receptor (Iida *et al.*, 1983) and this has been identified as a molecule previously known as B2 (Nadler *et al.*, 1981).

To investigate which proteins are responsible for CR2- and CR3-receptor activities in human tonsil B-lymphocytes, affinity chromatography was carried out on detergent extracts of human tonsil B-lymphocytes. We present evidence that the CR2 activity resides on a molecule of 145000 M_r , identical with that previously described (Iida *et al.*, 1983), whereas the iC3b-binding activity is due to both the 250000- M_r molecule known as CR1 and to the 145000- M_r protein. There does not appear to be a third distinct molecule on human tonsil B-lymphocytes responsible for their interaction with particles coated with iC3b.

Materials and methods

Proteins and affinity adsorbents

Factor H was prepared as described previously (Sim & DiScipio, 1982). Factor H was coupled to CNBr-activated Sepharose 4B (Pharmacia, Milton Keynes, Bucks., U.K.) as described by Cuatrecasas (1970), at a density of 2mg of factor H/ml of Sepharose. The coupling buffer was 100mM-potassium phosphate/100mM-KCl, pH 7.5. Factor I was prepared as described by Hsiung *et al.* (1982) and was generously provided by Mr. V. Malhotra of this unit. C2 was prepared as described by Kerr *et al.* (1978). C3 was prepared (Hammer *et al.*, 1981; R. B. Sim *et al.*, 1981) and converted to C3b by treatment with trypsin (R. B. Sim & E. Sim, 1981). The iC3b fragment was prepared by digestion of C3b (0.8mg/ml) in 20mM-potassium phosphate/0.5mM-EDTA, pH 7, for 1.5h at 37°C with factor H-Sepharose (10 μ l of Sepharose/ml) and factor I (10 μ g/ml). The factor H-Sepharose was then removed by centrifugation. C3d,g was isolated from serum (5litres) aged for 5 days at 37°C as follows. Proteins precipitating with 14% (w/v) poly(ethylene glycol) 4000 (Sigma, Poole, Dorset, U.K.) were removed from the serum, and the remaining soluble proteins, equilibrated

in 10mM-potassium phosphate/0.5mM-EDTA, pH 7.0, were loaded on to a DEAE-Sephadex (Pharmacia) column (5cm diam. $\times$ 90cm) in the same buffer. C3d,g was eluted by applying a 4-litre linear gradient of NaCl in the column buffer, to a final concentration of 0.5M-NaCl. C3d,g was released from the column between NaCl concentrations of 0.15M and 0.24M, and was detected by double radial immunodiffusion using anti-C3d antiserum (Dakopatts a/s, Glostrup, Denmark). Fractions containing C3d antigen were pooled, equilibrated in 20mM-Tris/HCl, pH 7.5, and applied to a column of Matrex Red A (Amicon, Stonehouse, Glos., U.K.) (1.5cm diam. $\times$ 20cm) in the same buffer. The column was washed extensively with the starting buffer, and C3d,g was eluted by applying a linear gradient of KCl to a final concentration of 2M-KCl. C3d,g was released from the column between KCl concentrations of 0.55M and 1.4M. The resulting preparation contained 242mg of a mixture of C3d,g and C3d, as discussed below, with no other detectable protein contaminants. C3 was converted into the haemolytically inactive form, C3u (Parkes *et al.*, 1981) [also termed C3i or C3(H₂O)] by incubation with 100mM-hydroxylamine, pH 9, for 1h at 37°C. C3u has 'C3b-like' properties (von Zabern *et al.*, 1981) and is used as a ligand in this study as if it were C3b. C3u-Sepharose was made by coupling C3u to CNBr-activated Sepharose at pH 8.3 in 100mM-borate buffer. The final ligand density was 7.5mg/ml of Sepharose. The C3u-Sepharose was a gift from Mr. V. Malhotra. iC3b-Sepharose and C3d,g-Sepharose were made by coupling iC3b and C3d,g to CNBr-activated Sepharose at pH 9.3 in 100mM-bicarbonate buffer. The final ligand concentration was 3.4mg/ml for iC3b and 2.4mg/ml for C3d,g. The preparation of C3d,g used for making the affinity adsorbent contained 50% (w/w) C3d,g, identified by comparison with the N-terminal sequence found by Davis *et al.* (1984) and 50% (w/w) of a C3d molecule that lacks the N-terminal 27–29 amino acids of C3d,g (R. B. Sim, unpublished work).

Trypsin [tosylphenylalanylchloromethane ('TPCK')-treated] was purchased from Worthington, Freehold, NJ, U.S.A. Soya-bean trypsin inhibitor (SBTI) (type 1-S) was purchased from Sigma, Poole, Dorset, U.K., and coupled to CNBr-activated 4B at a density of 4mg/ml of Sepharose as described by Cuatrecasas (1970).

Tonsil lymphocytes

Tonsil lymphocytes were prepared by teasing human tonsils in phosphate-buffered saline containing Aprotinin (100 μ g/ml) (Sigma) and subsequent centrifugation over Histopaque 1077 (Sigma). Lymphocytes were enriched for B-cells as

previously described (E. Sim & R. B. Sim, 1981). Cells were prepared and used on the same day.

Radioiodination

Tonsil lymphocytes were surface-labelled by the method of Markwell & Fox (1978). Tonsil cells (10^7) in 1 ml of phosphate-buffered saline in a glass liquid-scintillation vial coated with 100 μ g of Iodogen (Pierce and Warriner, Chester, Cheshire, U.K.) were incubated with 0.5 mCi of Na^{125}I (NEZ-033A, carrier-free; New England Nuclear, Southampton, U.K.) for 20 min at room temperature. The cells were washed three times in phosphate-buffered saline and resuspended in 1 ml of the same buffer. Approx. 30% of the ^{125}I was taken up by the cells. The cells were immediately solubilized by adding 1 ml of 10 mM-sodium phosphate, 1% (w/v) NP40 (Sigma) containing 10 μ g of SBTI/ml, 5 mM-DFP (Sigma), 2 mM-PMSF (Sigma), pH 7.4. After 5 min at room temperature the extract was centrifuged at 1000g. The extract was applied to a prepacked Sephadex G-25M column (PD-10; Pharmacia) equilibrated with the same buffer and inhibitors to remove low- M_r ^{125}I -labelled material and stored at -70°C . Approx. 5% of the initial radioactivity remained in this extract.

Soluble proteins were radioiodinated as described previously (R. B. Sim., *et al.*, 1981).

Complement-coated sheep erythrocytes

Sheep erythrocytes were coated with IgM, C1 and C4 (EAC14) by the method of Thompson (1978), except that zymosan-treated human serum (as the source of C1 and C4) was used immediately after preparation.

EAC14 cells were coated with ^{125}I -labelled C3 fragments as follows.

EAC14 (10^9 /ml) were incubated (30 min at 37°C) with 100 μ g of C3/ml in 142 mM-NaCl/5 mM-sodium barbitone/HCl/0.5 mM-MgCl₂/0.15 mM-CaCl₂, pH 7.5, ^{125}I -labelled C3 (approx. 2 μ Ci/ml) and 100 functional units of guinea-pig C2/cell. The resulting EAC3b cells were washed four times with DGVB⁺⁺. EAiC3b were prepared by incubating EAC3b (10^9 /ml) in DGVB⁺⁺ with factor H (50 μ g/ml) and factor I (5 μ g/ml) for 1.5 h at 37°C in the presence of SBTI (10 μ g/ml) and 2 mM-PMSF. EAiC3b were then washed four times with DGVB⁺⁺. EAC3d were prepared by incubating EAiC3b at 10^9 /ml in 142 mM-NaCl/5 mM-sodium barbitone/HCl/10 mM-EDTA, pH 7.5, with 4 μ g of trypsin/ml for 10 min at 37°C and then a 3-fold molar excess of SBTI was added to stop the reaction. Cells were washed four times in DGVB⁺⁺ to remove the C3c released during the trypsin treatment. All complement-coated sheep cells were

stored at 4°C at 10^9 cells/ml in DGVB⁺⁺ containing 10 μ g of SBTI/ml, 2 mM-PMSF, penicillin (40 i.u./ml) and streptomycin (40 μ g/ml).

The C3 fragment that was present on the sheep cells was confirmed by two methods.

(a) *Haemolytic assay.* Complement-coated sheep erythrocytes in DGVB⁺⁺ (100 μ l at 10^8 /ml) were incubated with serial dilutions (100 μ l) of C3-deficient human plasma (from a patient with homozygous C3 deficiency; Roord *et al.*, 1983) in DGVB⁺⁺ for 20 min at 37°C . The cell suspensions were diluted with 2 ml of 150 mM-NaCl and centrifuged for 5 min at 2000g. The A_{412} of the supernatant was measured and lysis expressed as a percentage of the absorbance of cells diluted in water. Less than 10% lysis at a serum dilution of 1:2 was observed for EAC14, EAiC3b and EAC3d cells. A 50% lysis of EAC3b was observed at a serum dilution of 1:200. This indicates that only EAC3b expressed complement-activating C3b on their surface. This assay method was used to monitor the time course of conversion of EAC3b cells to EAiC3b cells during incubation with factors H and I.

(b) *Release of covalently bound radioactive C3 fragments from the erythrocyte surface and subsequent analysis.* A suspension of complement-coated sheep cells (100 μ l of 10^9 cells/ml) was diluted with 1.4 ml of 10 mM-sodium phosphate, pH 7.5, containing 10 μ g of SBTI/ml and 2 mM-PMSF and the sheep-cell ghosts pelleted by centrifugation at 13000g for 5 min. Ghosts were washed once in the same buffer and re-pelleted. The pellet was resuspended in 200 μ l of 1 M-hydroxylamine (pH 10.5)/0.5% (w/v) SDS and incubated for 1 h at 37°C . This treatment causes hydrolysis of the ester bonds between C3 fragments and the cell surface (Law & Levine, 1977). Protein was precipitated by the addition of 1.3 ml of cold (-20°C) ethanol and pelleted by centrifugation at 13000g for 5 min. The pellet was resuspended in 100 mM-Tris/HCl/4 M-urea/1% (w/v) SDS/20 mM-dithiothreitol, pH 8, and then, after alkylation with 41 mM-iodoacetamide, subjected to SDS/polyacrylamide-gel electrophoresis. The radioactive protein bands detected by autoradiography were compared with known standards.

Rosettes

Rosetting was assessed by two methods.

(a) *Separation of rosetted and non-rosetted lymphocyte populations by centrifugation* (Parish, 1975). B-cell-enriched tonsil lymphocytes (5 ml at 2×10^7 /ml) in phosphate-buffered saline were incubated with 50 μ Ci of [^{51}Cr]chromate (500 mCi/mg; Amersham International, Amersham, Bucks., U.K.) for 20 min at 37°C , washed in phosphate-buffered saline to remove extracellular

radioactivity and resuspended in DGVB⁺⁺. Then 100 μ l of ⁵¹Cr-labelled tonsil lymphocytes at 10⁷/ml was mixed with 10 μ l of sheep erythrocytes coated with C3 fragment (10⁹/ml) and incubated for 20 min at 37°C. The cell mixture was layered on 0.5 ml of Histopaque 1083 (Sigma) in a 5 mm-diameter tube and centrifuged for 10 min at 1000g. The interface and pellet were removed and the radioactivity associated with each was measured in a LKB 1275 γ -radiation counter. Rosetting was expressed as the percentage of radioactivity in the pellet.

(b) *Microscopic examination.* Lymphocytes, mixed with C3-fragment-coated erythrocytes and incubated as described above, were stained with Acridine Orange [final concn. 0.005% (w/v)] (Hopkins & Williams, Chadwell Heath, Essex, U.K.) and rosetting was measured with a dark-field fluorescence microscope.

To prepare B-lymphocytes depleted of cells which rosette with a particular type of C3-fragment-coated erythrocyte, B-cell-enriched lymphocytes (0.5 ml of 10⁷/ml) were rosetted with 50 μ l of a 10⁹/ml suspension of a specified type of C3-fragment-coated erythrocyte as described above. The mixture was layered on to 1 ml of Histopaque 1083 and centrifuged as described above. The non-rosetting lymphocytes were recovered from the interface and washed in DGVB⁺⁺. Rosetting of this population with each type of complement-coated cell was determined as described above.

Antibodies and immunoprecipitation

Rabbit anti-(human C3) was raised by multiple injections of C3 in Freund's adjuvant at 2–4-week intervals into the popliteal-node regions of rabbits. The antisera were strongly reactive against both C3c and C3d. Rabbit anti-CR1 was made in the same way. The CR1 used for immunization was isolated from human erythrocytes by modifications of the method of Fearon (1979).

Rabbit anti-CR2 and another preparation of anti-CR1 were prepared as follows. The soluble extract from approx. 10¹⁰ B-lymphocytes was passed over a column of C3u-Sepharose (1.4 cm diameter $\times$ 7 cm) in 10 mM-sodium phosphate/1% (w/v) NP40/2 mM-PMSF. The column was washed extensively with the starting buffer and then the bound material was eluted in 10 mM-sodium phosphate/130 mM-NaCl (pH 7.4)/1% (w/v) NP40. The eluate, which contained both CR1 and the 145000-*M_r* C3-fragment-binding protein (CR2, as discussed below), was taken and precipitated with trichloroacetic acid [10% (w/v), 1 h, 0°C]. SBTI (50 μ g/ml) was added as a carrier for the precipitation. The pellet was washed twice with ice-cold acetone and then prepared for SDS/polyacrylamide-gel electrophoresis. The material was fully

reduced with dithiothreitol (20 mM) and alkylated with iodoacetamide (41 mM). Gels were stained with Coomassie Blue and the sections of stained gel corresponding to CR1 and to the 145000-*M_r* band (CR2) were excised and emulsified in Freund's complete adjuvant. These preparations were then injected into the popliteal-node regions of rabbits (three injections at 10-day intervals) and the rabbits bled at 14-day intervals after the first injection. The total CR1 injected was less than 2 μ g/rabbit and the total 145000-*M_r* protein (CR2) was less than 5 μ g/rabbit.

γ -Globulin fractions from rabbit antisera were prepared by triple (NH₄)₂SO₄ precipitation as described previously (E. Sim *et al.*, 1983). The Fab fragment of rabbit IgG was obtained by papain digestion of the purified γ -globulin followed by CM-cellulose (CM-32; Whatman, Maidstone, Kent, U.K.) chromatography (Dower *et al.*, 1975). The Fab fractions contained no detectable uncleaved IgG, as assessed by SDS/polyacrylamide-gel electrophoresis.

Mouse monoclonal antibodies used were (i) To5 (anti-CR1; Gerdes *et al.*, 1982), generously provided by Dr. D. Y. Mason, Department of Haematology, John Radcliffe Hospital, Oxford, U.K., (ii) B2, purchased from Coulter Electronics, Luton, Beds., U.K., and (iii) A5/15, directed against human complement component C2 (C. M. Anderson & E. Sim., unpublished work) was used as a control.

Immunoprecipitation with mouse monoclonal antibodies was done using rabbit antibody to mouse γ -globulin (RAM) coupled to Sepharose as the solid-phase reagent. The general procedure was as described previously (E. Sim *et al.*, 1983), except that an additional wash with 0.05% (w/v) SDS was carried out and the material released from rabbit anti-mouse IgG-Sepharose by treatment at pH 2.2 was precipitated with trichloroacetic acid before analysis by SDS/polyacrylamide-gel electrophoresis.

Polyacrylamide-gel electrophoresis

Large volume samples (more than 100 μ l) and samples containing NP40 were precipitated with ice-cold 10% (w/v) trichloroacetic acid in the presence of 10 μ g of SBTI and the precipitate was washed twice in ice-cold acetone. The washed precipitate was dissolved in 100 mM-Tris/HCl/4 M-urea/1% (w/v) SDS/20 mM-dithiothreitol, pH 8, at 80°C for 10 min.

Samples were subjected to polyacrylamide-gel electrophoresis in the presence of SDS as described previously (R. B. Sim *et al.*, 1981). The gels were either stained with Coomassie Blue or by the silver-staining method of Wray *et al.* (1981). Radioactivity was detected by autoradiography of the

dried gel using Industrex CX film (Kodak, Hemel Hempstead, Herts., U.K.).

Preparation of C3d receptor

Tonsil lymphocytes (10^8 /ml) were treated with 3% (w/v) Brij 96 in 150 mM-NaCl containing 2.5 mM-DFP for 15 min at 4°C. Nuclei were removed by centrifugation at 1000g for 5 min and Triton X-100 (Sigma) was added to the supernatant to a final concentration of 0.1% (w/v).

The extracts were stored at -70°C until material equivalent to 40 tonsil pairs (approx. 2×10^{10} B-lymphocytes) was accumulated. The pooled extracts (200 ml) were dialysed against 10 mM-sodium phosphate/1% (w/v) NP40/2 mM-PMSF and applied to a C3d,g-Sepharose affinity column (1.4 cm diameter $\times$ 7 cm) in the same buffer at 6 ml/h at 5°C. The column was washed with 50 ml of 20 mM-NaCl/10 mM-phosphate/1% (w/v) NP40, pH 7.4, and the bound material was eluted with three successive washes of 30 ml of 80 mM-NaCl, 80 ml of 130 mM-NaCl and 30 ml of 190 mM-NaCl in 10 mM-sodium phosphate/1% (w/v) NP40, pH 7.4. The 145 000- M_r protein was detected by SDS/polyacrylamide-gel electrophoresis of the column fractions after silver staining and was found only in the 130 mM-NaCl eluate. The C3d,g-Sepharose column was regenerated by washing with 400 mM-NaCl and re-equilibrating with 10 mM-sodium phosphate/1% (w/v) NP40, pH 7.4.

The pooled fractions containing the 145 000 M_r material (40 ml) were dialysed against 10 mM-sodium phosphate/1% (w/v) NP40, pH 7.4, and applied to a DEAE-Sephacel (Pharmacia) column (7 mm diameter $\times$ 2 cm) equilibrated in the same buffer. The column was washed with 20 ml of buffer and the bound material eluted with 20 ml of 150 mM-NaCl in the same buffer. The column fractions were analysed by SDS/polyacrylamide-gel electrophoresis and silver staining and the fractions containing the 145 000- M_r protein were pooled.

Results

Rosetting between tonsil B-lymphocytes and C3-coated sheep erythrocytes

The structure of the C3 fragment that was covalently bound to the sheep erythrocytes was confirmed by preparing the erythrocyte intermediates with ^{125}I -labelled C3. Subsequent release of the bound C3 fragments with hydroxylamine for SDS/polyacrylamide-gel electrophoresis permits examination of the nature of the C3 fragment bound to the cell both before and after rosetting. It also provides a means of calculating the number of C3b molecules initially bound per EAC3b cell.

Typical values are $(1-2) \times 10^4$ molecules/erythrocyte.

Examples of the analysis of each type of C3-fragment coated sheep erythrocyte used is shown in Fig. 1. It is clear that the sheep erythrocytes are

Fig. 1. Structure of covalently bound C3 fragments on coated sheep erythrocytes before and after rosetting. Sheep erythrocytes coated with ^{125}I -labelled C3 fragments were prepared as described in the Materials and methods section. C3 fragments covalently bound to the erythrocyte membrane were then released by incubation with hydroxylamine and examined by SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. The samples were reduced and alkylated; the gels contained 7.5% (w/v) acrylamide. Autoradiographs of the gels are shown. EAC3b (tracks 1 and 2), EAC3b (tracks 3 and 4) and EAC3d (tracks 5 and 6) are shown before (tracks 1, 3 and 5) and after (tracks 2, 4 and 6) rosetting with tonsil B-lymphocytes as described in the Materials and methods section. The gels of C3b (tracks 1 and 2) show the radioactive α' -chain (108 000 M_r) and the β -chain (70 000 M_r). iC3b can be recognized by the cleavage of the α' -chain of C3b into a radioactive N-terminal portion of 68 000 M_r that migrates close to the β -chain (tracks 3 and 4). The remaining C-terminal fragment of the α' -chain (40 000 M_r) is barely visible in this system, since it contains little of the radiolabel. C3d (tracks 5 and 6) is a heterogeneous α' -chain fragment of about 30 000 M_r that contains only about 10% of the radioactivity of the C3b from which it is derived (E. Sim *et al.*, 1981).

coated with the designated C3 fragment. The state of each of the three C3 fragments is not changed during rosetting.

Rosetting of B-cell-enriched tonsil lymphocytes with C3-fragment-coated erythrocytes is shown in Table 1. The extent of rosetting with EAiC3b is reproducibly greater than that of EAC3b and EAC3d (Table 1, column 1). Since the erythrocyte intermediates are prepared sequentially, this is not due to differences in the number of C3 fragments on the erythrocytes.

In an attempt to determine whether the receptors for C3b, iC3b and C3d are expressed on different subpopulations of lymphocytes, cell suspensions were pre-absorbed (by rosetting) with each type of C3-fragment-coated erythrocyte before rosetting. The results (Table 1, columns 2-4) indicate that depletion of the lymphocytes by rosetting with a particular C3-fragment-coated erythrocyte uniformly decreases the extent of rosetting to all of the three C3-fragment-coated erythrocytes. Thus there appears to be no significant differences in receptor expression, detectable by rosetting, within the tonsil B-cell population, in agreement with a previous report for tonsil lymphocytes (Dobson *et al.*, 1981), whereas peripheral blood lymphocytes have been separated into populations rosetting predominantly with C3b-coated cells or with iC3b- and C3d-coated cells (Perlmann *et al.*, 1981).

Affinity chromatography of membrane proteins on C3u-, iC3b- and C3d,g-Sephadex

Surface-¹²⁵I-labelled lymphocytes were solubilized with 1% (w/v) NP40 and applied to C3-fragment-Sephadex columns in low-ionic-strength buffer as described in the legend to Fig. 2 and in the Materials and methods section. After extensive

washing, addition of 130mM-NaCl to the buffer results in the elution of the peptides shown in Fig. 2.

Two peptides of apparent M_r 240000 and 145000 bind to C3u-Sephadex and iC3b-Sephadex. Only the 145000- M_r peptide binds to C3d,g-Sephadex. These results are expressed quantitatively in Table 2, row 1. These polypeptides do not bind to a control column of SBTI-Sephadex. The smaller of the two peptides is just visible in the total radioiodinated cell extract when compared with unbound material (Fig. 2, tracks 1 and 2)

Fig. 2. Affinity chromatography of ¹²⁵I-labelled lymphocyte extracts

Tonsil lymphocytes were prepared and labelled with ¹²⁵I as described in the Materials and methods section. A 1ml portion of the extract (10⁶ c.p.m.) in 10mM-sodium phosphate/1% (w/v) NP40, pH 7.4, was applied to each affinity column (7mm diameter × 1cm), equilibrated with the same buffer and washed extensively in the column buffer. Bound proteins were then eluted by washing with 3ml of 130mM-NaCl in the column buffer. The total cell extract, the material which did not bind to the column and the eluted proteins were analysed by SDS/polyacrylamide-gel electrophoresis. The acrylamide concentration was 6.5% (w/v). Autoradiographs are shown of the total cell extract (track 1), unbound material from the C3u column (track 2) and bound proteins eluted from C3u-Sephadex (track 3), iC3b-Sephadex (track 4) and C3d,g-Sephadex (track 5). The M_r markers are myosin (200000 M_r), phosphorylase *b* (92000), catalase (60000), ovalbumin (42000) and carboxypeptidase A (25000).

Table 1. Rosetting of B-lymphocytes with sheep erythrocytes coated with C3 fragments

Rosetting between ⁵¹Cr-labelled B-cell enriched tonsil lymphocytes and C3-fragment-coated sheep erythrocytes was measured by separating and counting the rosetting and non-rosetting populations of labelled lymphocytes as described in the Materials and methods section. Lymphocytes were depleted of cells rosetting with a particular type of C3-fragment-coated erythrocyte by absorption as described in the Materials and methods section.

Coated sheep erythrocytes	Percentage rosetting of lymphocytes adsorbed with:			
	None	EAC3b	EAiC3b	EAC3d
EAC3b	55	11	8	12
EAiC3b	77	32	23	28
EAC3d	58	25	12	7

Table 2. *Affinity chromatography of ^{125}I -labelled lymphocyte extracts and identification of the 240000- M_r protein with To5* Surface ^{125}I -labelled tonsil lymphocytes were solubilized, applied to C3u-, iC3b-, C3d,g- and SBTI-Sepharose and the bound proteins were eluted as described in the legend to Fig. 2. The eluates were analysed by SDS/polyacrylamide-gel electrophoresis and the portions of the gels corresponding to the 240000 M_r proteins counted for radioactivity. Analysis of the To5 immunoprecipitate and the To5 precleared eluate was performed as described in the text.

	Column ligand ...	^{125}I radioactivity (c.p.m.) corrected for background			
		C3u	iC3b	C3d,g	SBTI
Total eluate		1284	1112	858	308
240000 M_r		228	160	42	35
145000 M_r		630	648	750	114
To5 immunoprecipitate					
240000 M_r		173	127	66	17
To5 precleared					
240000 M_r		74	60	67	25
145000 M_r		670	452	580	134

and is thus a major radioiodinated cell-surface component.

Identification of the C3-fragment-binding proteins

Samples of the surface- ^{125}I -labelled tonsil lymphocyte extract (10^5 c.p.m. each) were applied to C3u-, iC3b-, C3d,g- and SBTI-Sepharose columns and the bound material was eluted as described above. The amount of radioactivity eluted in each case is shown in Table 2, row 1. This corresponds to a specific binding of 1% of the applied material in the case of C3u- and iC3b-Sepharose and 0.5% in the case of C3d,g-Sepharose. After SDS/polyacrylamide-gel electrophoresis of this material and localization of the labelled bands by autoradiography, the portions of the gel track containing the 240000- M_r and the 145000- M_r bands were cut out and counted for radioactivity. The results, (Table 2, rows 2 and 3), indicate that approx. 30% of the eluted radioactivity from the C3u and iC3b columns, and an insignificant amount from the C3d,g column, migrates to a position corresponding to 240000 M_r . Identical column eluates were immunoprecipitated with To5 monoclonal antibody as described in the Materials and methods section and the precipitated material was analysed by SDS/polyacrylamide-gel electrophoresis. The radioactivity at the 240000- M_r position of these gels was measured and the results are shown in Table 2, row 4. The antigen recognized by To5 is present in the C3u-Sepharose and iC3b-Sepharose column eluates, but not in the material eluted from C3d,g-Sepharose.

Surface- ^{125}I -labelled tonsil lymphocyte extract was pre-cleared by immunoprecipitation with To5 and samples of this material were applied to the affinity columns. The eluates were analysed by SDS/polyacrylamide-gel electrophoresis and the

240000- M_r and the 145000- M_r regions of the gel were counted for radioactivity. The results (Table 2, rows 5 and 6) show that the 240000- M_r protein had been removed from the extract. This confirms that the 240000 M_r protein is CR1.

Surface- ^{125}I -labelled tonsil lymphocyte extract was passed down a C3u-Sepharose column and the bound material was eluted as described above. This material was immunoprecipitated with B2 monoclonal antibody as described in the Materials and methods section. The immunoprecipitate and the unprecipitated material were analysed by SDS/polyacrylamide-gel electrophoresis and the radioactive proteins detected by autoradiography. The results are shown in Fig. 3 together with a control immunoprecipitation with A5/15. Only the 145000- M_r protein is precipitated with B2. This indicates that the 145000- M_r protein is identical with the protein described previously (Iida *et al.*, 1983).

Rosette inhibition by antibodies

To confirm that the behaviour of these two proteins identified after affinity chromatography reflects their function on the cell surface, the inhibition of rosetting by antibodies to these proteins was examined. The results are shown in Table 3.

The Fab fragment of antibody to CR1 inhibits 82% of EAC3b rosette formation with human tonsil B-lymphocytes. There is some inhibition of rosetting with EAiC3b but not with EAC3d. Rabbit anti-CR1 prepared by both of the procedures described in the Materials and methods was equally effective. The Fab fragment of a polyclonal antibody prepared against the 145000- M_r protein (anti-CR2 in Table 3) was found to inhibit 39% of rosetting between human tonsil B-lymphocytes

Fig. 3. Immunoprecipitation with B2 monoclonal antibody. ^{125}I -labelled lymphocyte extract was subjected to affinity chromatography on C3u-Sepharose and the bound material was eluted in buffer containing 130mM-NaCl as described in the Materials and methods section and the legend to Fig. 2. The eluate was immunoprecipitated with B2 and A5/15 monoclonal antibodies and the immunoprecipitates analysed by SDS/polyacrylamide-gel electrophoresis and detected by autoradiography. Tracks 1 and 2, eluate from C3u-Sepharose; track 3, immunoprecipitation with A5/15; track 4, immunoprecipitation with B2. M_r markers are as shown in the legend to Fig. 2.

and EAC3d and 21% of the rosetting with EAiC3b cells, but gave no inhibition with EAC3b.

Neither of the monoclonal antibodies To5 or B2 caused marked inhibition alone, but in the presence of a second antibody (rabbit anti-mouse γ -globulin: RAM in Table 3), To5 inhibited 52% of rosetting with EAC3b cells and gave slight inhibition with EAiC3b but no inhibition of rosetting with EAC3d cells. B2, in the presence of second antibody, inhibits 85% of rosette formation between lymphocytes and EAC3d cells. It also gives 75% inhibition of rosetting with EAiC3b cells but no inhibition with EAC3b cells. The second antibody alone did not inhibit when used in the absence of To5 or B2.

The Fab fragment of a polyclonal antibody against C3 almost completely inhibited all types of rosette formation that were tested. This confirms

Table 3. Inhibition of B-lymphocyte rosetting with C3-fragment-coated sheep erythrocytes by antibodies to CR1 and CR2

B-cell-enriched tonsil lymphocytes were incubated with buffer alone, or with the stated Fab fragments of rabbit antibody or with monoclonal antibody for 30 min at 37°C. C3-fragment-coated sheep erythrocytes were added as indicated and the cells incubated for rosetting as described in the Materials and methods section. The rosetting was measured by Acridine Orange staining and visual observation as described in the Materials and methods section. The polyclonal anti-CR1 and anti-CR2 Fab fragments were used at a concentration equivalent to a 2-fold dilution of the original antiserum. The polyclonal anti-C3 was used at a concentration equivalent to a 10-fold dilution of the original antiserum. The polyclonal antibodies were not affinity-purified. To5 monoclonal anti-CR1 was used as a 50-fold diluted ascites fluid. B2 antibody was used as a 10-fold dilution of the manufacturer's stock solution. Rabbit anti-mouse γ -globulin (RAM) was used at approx. 50 μg of specific antibody/ml. Rosetting in the absence of antibodies with EAC3b, EAiC3b and EAC3d was 66, 78 and 72% respectively. All values are corrected for EAC14 background rosetting, which was 2%.

Antibody	Percentage inhibition of rosetting with:		
	EAC3b	EAiC3b	EAC3d
Fab anti-CR1	82	13	5
Fab anti-CR2	0	21	39
To5	15	6	0
To5/RAM	52	10	0
B2	1	2	10
B2/RAM	2	75	85
RAM	1	0	1
Fab anti-CR3	95	98	100

that no other interaction, e.g. Fc-Fc-receptor, contributed significantly to the rosetting observed.

These results indicate that the interaction of human B-lymphocytes with EAC3d is mediated entirely by the 145000- M_r protein. The CR3 receptor activity (i.e. iC3b binding), appears to be due to the recognition of iC3b by both 240000- M_r and the 145000- M_r proteins. Antibodies against each of these proteins partially inhibit rosette formation between EAiC3b and tonsil B-lymphocytes. Although both the 240000- M_r and the 145000- M_r proteins are purified on C3u-Sepharose, antibodies recognizing the 145000- M_r protein do not inhibit rosetting between tonsil lymphocytes and EAC3b cells.

Isolation of CR2

The 145000- M_r protein (CR2) was prepared as described in the Materials and methods section.

Fig. 4. Affinity chromatography of lymphocyte extract on C3d,g-Sepharose

A detergent-soluble fraction was prepared from lymphocytes as described in the Materials and methods section and subjected to affinity chromatography on C3d,g-Sepharose. After washing the column as described in the Materials and methods section the bound proteins were eluted by a discontinuous NaCl gradient also as described in the Materials and methods section. The column eluate was collected in 3 ml fractions. A 30 μ l sample of these fractions was precipitated with trichloroacetic acid and analysed by SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. The acrylamide concentration was 6.5%, and the silver-stained gel is shown. The changes in NaCl concentration are as indicated. The material at 70000 and 74000 M_r are contaminants of the gel running buffer and appear when no sample is applied. The M_r markers are as shown in the legend to Fig. 2.

The ionic-strength profile and the silver-stained SDS/polyacrylamide-gel electrophoresis gels of the column fractions of the affinity purification on C3d,g-Sepharose are shown in Fig. 4. The 145000- M_r protein was eluted in the 130 mM-NaCl fractions.

In order to purify further and concentrate the 145000- M_r protein, ion-exchange chromatography on DEAE-Sephacel (Pharmacia) was used. To establish conditions under which the 145000- M_r protein could be eluted at high concentration with a step gradient, a preliminary experiment was undertaken, as follows.

125 I-labelled 145000- M_r protein prepared from a surface-labelled lymphocyte extract on C3d,g-Sepharose as described above was desalted on a Sephadex G-25M column and eluted with 10 mM-sodium phosphate/1% (w/v) NP40, pH 7.4. This was passed on to a DEAE-Sephacel column equilibrated with the same buffer. The column was eluted with steps of increasing concentrations of NaCl and the eluate was analysed by counting 125 I radioactivity and by SDS/polyacrylamide-gel electrophoresis. Further counting of 125 I radioactivity in slices of the gels (Fig. 5) established that 150

mM-NaCl would elute the 145000- M_r protein. An elution procedure based on this experiment with radiolabelled material was then applied to the unlabelled preparation as described in the Materials and methods section. Fig. 6 shows the silver-stained SDS/polyacrylamide gels of pooled material from the C3d,g-Sepharose column (track 1) and the pure protein pool from the DEAE-Sephacel (track 2).

The original cell extract of 200 ml had a protein concentration of 1.3 mg/ml, a total of 260 mg of protein from the equivalent of 2×10^{10} B-lymphocytes. Approx. 40 μ g of pure 145000- M_r protein was recovered, corresponding to a recovery of 8300 molecules per tonsil B-lymphocyte and equivalent to a 6500-fold purification from the solubilized cell extract.

Discussion

We have isolated the proteins of tonsil B-lymphocytes that recognize fragments of C3. The two proteins isolated by affinity chromatography have been identified as CR1 (Fearon, 1980), by immunoprecipitation with To5 (Gerdes *et al.*,

Fig. 5. DEAE-Sephacel chromatography of ^{125}I -labelled lymphocyte proteins eluted from C3d,g-Sephacel. ^{125}I -labelled proteins from lymphocyte extracts eluted from C3d,g-Sephacel as described in the legend to Fig. 2 were desalted on a Sephadex G-25 column equilibrated with 10mM-sodium phosphate/1% (w/v) NP40, pH 7.4, and passed on to a DEAE-Sephacel column (7mm diameter $\times$ 1cm) equilibrated with the same buffer. The column was sequentially eluted with 10ml of column buffer, 10ml of 150mM-NaCl in column buffer and 10ml of 300mM-NaCl in the same buffer. Fractions (1 ml) of the eluate were counted for radioactivity in a γ -radiation counter. The ^{125}I elution profile is shown (a). Fractions were pooled as indicated and analysed by SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. The acrylamide concentration used was 6.5%. The gel was dried and tracks of the gel were cut up and counted for radioactivity. The ^{125}I profiles of the C3d,g-Sephacel column eluate (b), the unbound material (pool 1, panel c), the 150mM-NaCl eluate (pool 2, panel d) and the 300mM-NaCl eluate (pool 3, panel e) are shown. All the labelled 145000- M_r protein eluted in the 150mM-NaCl pool.

Fig. 6. 145000 M_r C3-binding protein

The 145000- M_r protein (CR2) was prepared as described in the Materials and methods section. The pooled material containing the 145000- M_r protein from the C3d,g-Sephacel column and the pooled 150mM-NaCl eluate from the DEAE-Sephacel column were analysed by SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. The acrylamide concentration was 6.5% (w/v). The gels were silver-stained, dried down and photographed. Track 1, C3d,g-Sephacel eluate; track 2, DEAE-Sephacel eluate. The markers are as shown in the legend to Fig. 2.

1982) and CR2 recognised by B2 antibody (Iida *et al.*, 1983). Although B-cells exhibit receptor activity towards the three different fragments C3b, iC3b and C3d (C3d,g), only two receptor molecules appear to be involved. Each of these two lymphocyte proteins appears to be able to bind to iC3b, and no other, additional, molecule mediates CR3 activity.

The interaction of CR1 with iC3b is shown clearly by its binding to iC3b-Sephacel (Fig. 2) at low ionic strength. Antibodies against CR1 also show some inhibition of EAiC3b rosetting (Table 3), although their effect on iC3b-dependent rosetting is much less than on C3b-dependent rosetting. This interaction may be significant for rosetting only in the absence of other receptors for iC3b, as was suggested for monocytes from a patient deficient in CR3 (Dana *et al.*, 1984).

Solubilized CR2 binds to both iC3b- and C3d,g-Sepharose (Fig. 2). Antibodies to CR2 inhibit both iC3b- and C3d-dependent rosetting (Table 3). The ability of CR2 alone to mediate EAiC3b rosetting is demonstrated by the rosetting of Raji lymphoblastoid cells (which do not express CR1; Dobson *et al.*, 1981) with EAiC3b cells (Scheiner *et al.*, 1982) through a molecule closely related antigenically (Iida *et al.*, 1983) and in size (Barel *et al.*, 1981) to the receptor from normal B-cells.

Solubilized CR2 also interacts with immobilized C3u (used here as a substitute for C3b) (Fig. 2), but antibodies to CR2 do not significantly influence C3b-dependent rosetting in this system (Table 3). Although CR2 is not involved in the C3b-dependent rosetting of tonsil B-lymphocytes, the binding of solubilized CR2 to C3u may have a functional counterpart in the rosetting of Raji cells with EAC3b (E. Sim *et al.*, 1981; Scheiner *et al.*, 1982).

The interaction of solubilized receptors with affinity columns at low ionic strength is clearly an artificial system, but it appears it can be an accurate reflection of the biological activities of the receptor molecules. The use of fragments C3b, iC3b and C3d,g on affinity supports has provided information on receptor specificity and extends the work already reported on the use of C3 species as ligands (Fearon, 1979; Schneider *et al.*, 1981; Iida *et al.*, 1983).

The preparation of CR2 using C3d,g-Sepharose involves only two steps. It appears to be a major cell-surface protein of tonsil B-lymphocytes and can be isolated in quantities adequate for further study.

While this paper was in preparation a report was published (Weiss *et al.*, 1984) confirming the identity of the C3d receptor with a monoclonal antibody, HB-5.

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