The generation of active fragments of complement receptor type 2 by trypsin digestion

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B lymphocytes and Raji cells express the complement receptor type 2 (CR2) of 145 kDa which recognises the C3d fragment of C3. When intact cells are treated with trypsin, CR2 is degraded. There is a parallel loss in C3d-mediated rosetting and in proteins which bind to C3d-Sepharose. Initially 97 and then 83 kDa fragments of CR2 are produced which retain C3d binding activity. These fragments are associated with the cell surface and mediate rosetting. Purified ^{125}I-labelled CR2, solubilised in detergent, produces fragments of apparently identical size on treatment with trypsin. The 83 kDa fragment produced by trypsin treatment closely resembles the major C3d binding protein spontaneously released into Raji cell culture medium.

Complement C3 receptor *CR2* *Tryptic fragmentation* *B lymphocyte* *Raji cell*

1. INTRODUCTION

The third component of the complement, C3, binds covalently to surfaces which activate the complement system, as the C3b fragment (M_r 178000) [1]. This fragment acts as a component of the complex enzymes, C3 convertase and C5 convertase, which activate C3 and C5, respectively, in the complement sequence. C3b is inactivated by cleavage to iC3b (M_r 175000) [2]. Subsequently iC3b is degraded to C3d,g (M_r 38000) and C3d (M_r 33000) [3]. These fragments of C3 are recognised by cell surface receptors which mediate phagocytosis [4] and are possibly involved in control of the immune response [5].

Human B-lymphocytes rosette with particles coated with C3b, iC3b or C3d. The C3b-rosetting activity is due to a 250 kDa membrane glycoprotein [6] termed complement receptor type 1 (CR1) [7], also present on human erythrocytes, polymorphonuclear leucocytes and monocytes.

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The C3d binding activity (CR2) [7] is due to a protein of 145 kDa [8], identical to the B lymphocyte differentiation antigen recognised by the monoclonal antibody B2 [8,9]. Rosetting with iC3b coated cells is mediated by both CR1 and CR2, each of which has an affinity for iC3b [10].

Although the molecules on tonsil B lymphocytes which mediate rosetting with C3 fragments have been identified, the situation with Raji and other lymphoblastoid cells is less clear. Raji cells rosette with C3b-coated particles although they do not express the CR1 protein [11]. It has been suggested that C3b-binding activity is due to a 140 kDa protein [12] or to a Factor H-like molecule (150 kDa) [13]. The protein responsible for the C3d-binding activity of Raji cells was initially identified as a 72 kDa protein prepared from the supernatants of Raji cell cultures [14]. Antigenic cross-reactivity has been observed between the 72 kDa protein and the Epstein-Barr virus (EBV) receptor [15]. Reports that the 145 kDa CR2 is the EBV receptor [16] and that antibodies to the 72 kDa protein inhibit CR2 activity [17] strengthen earlier sugges-

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tions [8,18] that the 72 kDa molecule is a fragment of CR2.

This report describes the proteolytic fragmentation of CR2, from B lymphocytes and Raji cells, both on the cell surface and solubilised in non-ionic detergent. The C3d-binding fragments have been compared with the C3d binding proteins from Raji culture medium. The relationship between CR2 and the molecule from the culture medium is discussed.

2. MATERIALS AND METHODS

2.1. *Cells*

Raji cells (Flow Laboratories, Irvine, Ayrshire, England) were cultured in RPMI 1640 supplemented with 10% (v/v) foetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Spent medium was collected when the cells had grown to approx. 10^6/ml.

Biosynthetically labelled Raji cell products were prepared from cells cultured in RPMI 1640 without methionine (Flow Laboratories) supplemented with 5% (v/v) foetal calf serum, penicillin and streptomycin in the presence of 10 μCi/ml [^{35}S]methionine (Amersham International, Amersham) for 3 days.

B lymphocytes were prepared from human tonsils obtained from routine tonsillectomy [10].

Cells were surface-radioiodinated by incubating a suspension (5×10^6 cells) in 0.5 ml PBS (8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 139 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.2) with 0.5 mCi ^{125}I (Amersham) in polyethylene tubes coated with Iodogen (Pierce and Warriner, Chester, England) for 10 min at room temperature. The cells were washed 4 times in PBS at room temperature to remove free iodide.

Cells were suspended at 2×10^7/ml in $DGVB^{2+}$ (140 mM glucose, 71 mM NaCl, 2.5 mM sodium barbitone, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH 7.5 containing 0.1% gelatin) and treated with trypsin (TPCK-treated, Worthington, Freehold, NJ) at 100 μg/ml at 37°C. Samples were removed at the times indicated and proteolysis stopped by addition of soy bean trypsin inhibitor (Type 1S, Sigma, Poole, England) at a 3-fold weight excess over trypsin. Incubation was continued for 5 min at 37°C.

2.2. *Rosetting*

Sheep erythrocytes coated with IgM, C1 and C4 (EAC14) and with C3d (EAC3d) were made and rosette measurements were performed as before [10]. Rosetting was measured by incubating 10^6 trypsin-treated Raji cells with 10^8 EAC14 or EAC3d cells for 20 min at 37°C. Rosetting was estimated by microscopic examination. The rosetting of EAC14 was insignificant in all cases.

2.3. *Affinity chromatography*

Radioiodinated or biosynthetically labelled cells (2×10^7/ml) were lysed by addition of an equal volume of lysing buffer (2%, w/v) Nonidet-P40 (NP40, Sigma), 10 mM sodium phosphate, pH 7.4, containing 10 μg/ml soy bean trypsin inhibitor, 5 mM iodoacetamide and 2 mM phenylmethylsulphonyl fluoride (PMSF), and mixed on ice for 20 min. The extract was centrifuged (50000 $\times$ *g* for 10 min) and applied to a C3d-Sepharose column [10] (1 ml containing 2.2 mg C3d/ml Sepharose) equilibrated with 1% (w/v) NP40 in 10 mM sodium phosphate buffer, pH 7.4, at 6 ml/h. The column was washed in the same buffer and the C3d-binding proteins were eluted with a linear NaCl gradient (total volume 40 ml) up to 500 mM NaCl in the starting buffer. The method used is similar to that described [10].

2.4. *SDS-polyacrylamide gel electrophoresis*

Samples for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared by precipitation with 10% (w/v) trichloroacetic acid at 5°C, using 50 μg soy bean trypsin inhibitor as carrier protein. The precipitate was washed once with 5% (w/v) trichloroacetic acid and twice with ice-cold acetone before solubilization in 1% (w/v) SDS, 4 M urea, 100 mM Tris-HCl, pH 8, at 80°C for 10 min. Samples were reduced with 3 mg/ml dithiothreitol and alkylated with 16 mg/ml iodoacetamide. SDS-PAGE was performed according to Laemmli [19] using a 6.5% gel. Marker proteins were visualised by Coomassie blue staining and the gels were dried for autoradiography using Fuji RX film and a Cronex 'lightning plus' intensifying screen (DuPont, Stevenage, Herts, England) exposed at −70°C.

2.5. *Preparation of radiolabelled CR2*

^{125}I-labelled CR2 was prepared from human

spleen. Spleen membranes, a gift from Dr S.-K. Law, were prepared by homogenising chopped spleen suspended in 2.5% (w/v) Tween 40 (Sigma), 10 mM Tris-HCl, pH 8, containing 10 mM iodoacetamide, 2.5 mM di-iodopropyl phosphorofluoridate (Sigma) and 0.2 mM PMSF with a Potter homogeniser (6 strokes, 100 rpm) on ice. This material was centrifuged at 3000 × *g* for 15 min and the supernatant centrifuged at 40000 × *g* for 20 min. The pellet produced was washed twice in 10 mM Tris-HCl, pH 7.4, and resuspended at a protein concentration of 20 mg/ml in lysing buffer. This preparation was mixed with a solubilised extract of 2×10^7 surface-radioiodinated Raji cells in the same buffer. The mixture was applied to a C3d-Sepharose column (5 ml), as described in section 2.3. The bound material was eluted with a gradient to 500 mM NaCl (total volume 100 ml) in 10 mM sodium phosphate, 1% (w/v) NP40, pH 7.4. The fractions were analysed by SDS-PAGE and autoradiography and the CR2-containing fractions were pooled. The pooled material was reiodinated using 1 mCi $Na^{125}I$ in the presence of one Iodobead (Pierce and Warriner) for 10 min on ice. Free iodide was removed by gel filtration on a Sephadex G25 column, equilibrated in 1% (w/v) NP40, 10 mM sodium phosphate, pH 7.4. The preparation was further purified by a second passage and elution from a C3d-Sepharose column as described above.

2.6. *Purification and labelling of proteins from spent medium*

Raji cells (10^6/ml) in culture medium (250 ml) were centrifuged at 1000 × *g* for 5 min and the culture supernatant was then centrifuged at 100000 × *g* for 1 h. The 100000 × *g* pellet was solubilized in lysing buffer for 20 min on ice. The solubilized extract of the 100000 × *g* pellet was applied to a C3d-Sepharose column (1 ml) as described above. The C3d-binding proteins were eluted with 5 ml of 100 mM NaCl, 1% (w/v) NP40, 10 mM sodium phosphate, pH 7.4 and labelled with 0.5 mCi $Na^{125}I$, using one Iodobead. Free iodide was removed by gel filtration as above (section 2.5), and repurified on C3d-Sepharose as before.

The 100000 × *g* supernatant of Raji cell medium was dialysed against 10 mM sodium phosphate, pH 7.4, and run on a C3d-Sepharose column (5 ml) as described in section 2.5, but without detergent (NP40). The C3d-binding proteins were eluted with 25 ml of 100 mM NaCl in 10 mM phosphate (pH 7.4) and labelled with ^{125}I. The iodinated proteins were then repurified on C3d-Sepharose in the absence of detergent.

Medium from cells cultured in the presence of [^{35}S]methionine was centrifuged as above. The detergent extract of the 100000 × *g* pellet was applied to a C3d-Sepharose column and the eluted proteins were analysed by SDS-PAGE. The gel was impregnated with Autofluor (National Diagnostics, Aston Clinton, Bucks, England) prior to drying down and autofluorography at −70°C.

3. RESULTS

3.1. *Trypsinization of cells*

To establish whether active fragments of CR2 could be produced, B lymphocytes or cultured Raji cells were treated with trypsin and the degradation of CR2 was measured.

Surface ^{125}I-labelled Raji cells were trypsinized as described in section 2.1 and a small sample of the cells was assessed for rosetting to EAC3d at each time point. Fig.1 shows the progressive loss of

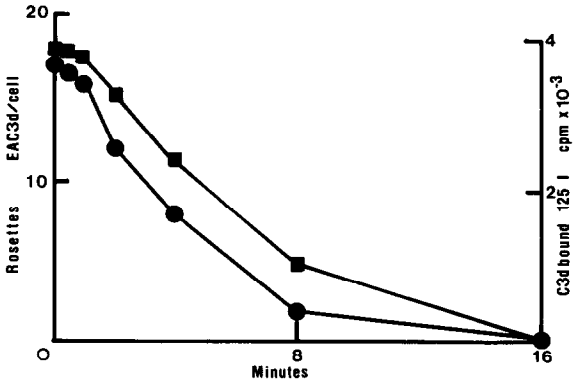

Fig.1. Loss of rosetting with EAC3d cells and loss of C3d-binding proteins during trypsin treatment. Rosetting is expressed as the average number of erythrocytes per Raji cell (—●—) (100 Raji cells were counted). ^{125}I-labelled Raji cells were treated with trypsin, solubilised, and extracts applied to a C3d-Sepharose column in 1% (w/v) NP40, 10 mM sodium phosphate, pH 7.4. Bound proteins were eluted as described in section 2.3. The radioactivity associated with bound proteins is shown (—■—).

rosetting activity during trypsin treatment. Cell pellets at each time point were solubilized with lysing buffer and extracts were applied to C3d-Sepharose columns. The columns were washed and the C3d-binding proteins were eluted by increasing the ionic strength as described in section 2.3. The radioactivity in the C3d-binding proteins is shown in fig.1. It can be seen that the loss of the ^{125}I radioactivity bound to C3d-Sepharose closely follows the loss of rosetting.

The C3d-Sepharose column eluates were precipitated with trichloroacetic acid and analysed by SDS-PAGE. Fig.2a shows an autoradiograph of the gel tracks corresponding to 0, 4 and 16 min of trypsin treatment. It can be seen that CR2 of 145 kDa in track 1 is broken down in 4 min to 2 major fragments of 97 kDa and 83 kDa which still bind C3d. By 16 min only a small amount of the 83 kDa fragment remains.

The sections of the gel containing the 145, 97 and 83 kDa bands were cut out and the radioactivity measured. The counts are shown in fig.2b. The 97 kDa species appears transiently in the early stages of trypsin treatment. The 83 kDa species appears after the 97 kDa protein and persists longer. After 4 min the predominant C3d binding species is the 83 kDa molecule which appears to mediate a reduced but significant level of rosetting (cf. fig.1). Rosetting has dropped to half its original value after 4 min of treatment with trypsin although less than 10% of the intact CR2 remains. Because of the purification procedure, the proteins visible on the autoradiograph are cell-surface-associated C3d-binding proteins and, as such, are likely to mediate EAC3d rosetting.

Similar results have been obtained from surface labelled tonsil B lymphocytes.

3.2. *Trypsinization of solubilized CR2*

Trypsin treatment of ^{125}I-labelled, solubilized CR2 from spleen results in breakdown of the 145 kDa CR2 with sequential appearance of the 97 and 83 kDa fragment (fig.3). Breakdown of the 83 kDa fragment is slow under the conditions used. By 8 min it is the major fragment visible, and incubation for up to 2 h does not result in further breakdown.

Digests of solubilized CR2 from spleen were subjected to C3d-Sepharose chromatography (section 2.3) to determine which fragments retain the

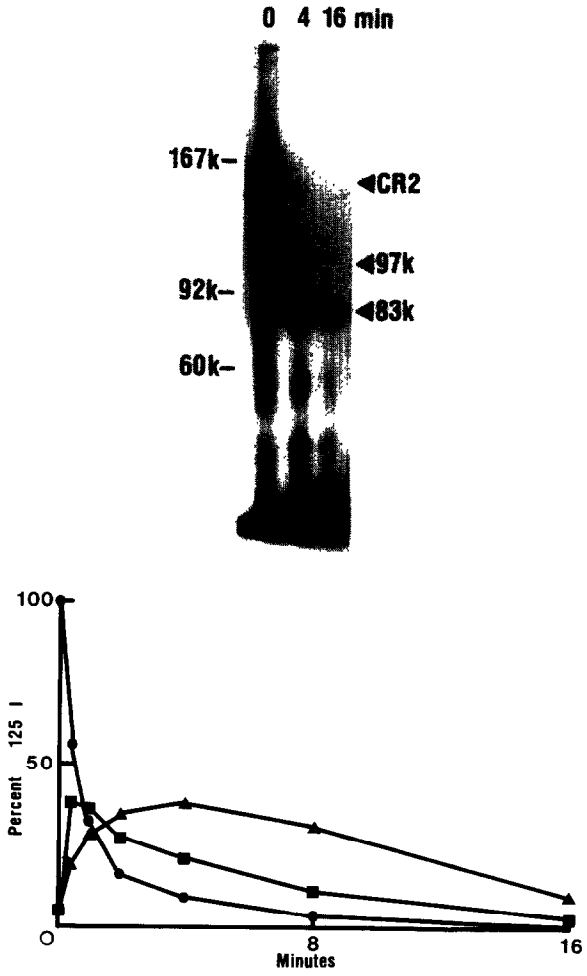

Fig.2. Analysis of C3d-binding proteins following trypsin treatment of Raji cells. (a) Autoradiographs of polyacrylamide gel tracks of C3d-binding proteins from ^{125}I-labelled Raji cells following trypsinization for the indicated times. Positions of CR2 and its fragments are shown by arrows. Positions of M_r standards α_2M (M_r 167000), phosphorylase (M_r 92000) and catalase (M_r 60000) are indicated. (b) Distribution of radioactivity between different C3d-binding proteins following trypsinization. (—●—) Intact CR2, M_r 145000; (—■—) M_r 97000; (—▲—) M_r 83000. Radioactivity is expressed as a percentage of the radioactivity in the CR2 band before trypsinization.

C3d binding activity. Intact CR2 purified in this way rebinds to C3d-Sepharose and runs at an apparent M_r of 130000 on SDS-PAGE in non-reducing conditions. The 83 kDa fragment also

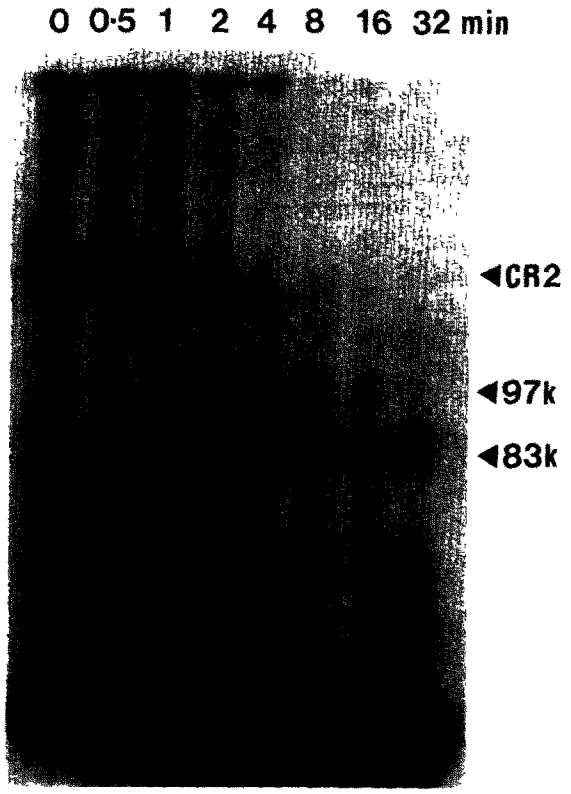

Fig.3. Trypsinization of detergent-solubilized CR2. Autoradiograph of a polyacrylamide gel of ^{125}I-labelled CR2 treated with trypsin (10 μg/ml in 1% NP40, 0.1% gelatin, 10 mM sodium phosphate, pH 7.4, containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$) for the times indicated as described in section 2. CR2 and the molecular masses of the radioactive bands are shown, calculated from standards as in the legend to fig.2a.

Fig.4. Comparison of C3d-binding proteins from spent Raji culture medium and from tryptic digest of purified CR2. Autoradiographs of polyacrylamide gels of ^{125}I-labelled C3d-binding proteins prepared from spent culture medium (track 1); [^{35}S]methionine labelled C3d-binding proteins from spent culture medium (track 2) and ^{125}I-labelled C3d-binding tryptic fragments of purified CR2 (track 3). Samples were prepared as discussed in the text.

binds to C3d-Sepharose (fig.4, track 3). This fragment runs at 65 kDa in non-reducing conditions and is not apparently disulphide-linked to any other major fragment.

3.3. *C3d binding proteins from spent culture medium*

Spent Raji culture medium was centrifuged at 100000 × *g* to produce a pellet fraction including membranous debris which was subsequently extracted with detergent, and a supernatant of soluble proteins. These 2 fractions were subjected to affinity chromatography to identify C3d-binding proteins. These preparations were labelled with ^{125}I and repurified as described in section 2.6.

When analysed on SDS-PAGE (fig.4), the 100000 × *g* pellet preparation shows bands at 145, 120, 83 and 45 kDa (track 1). Greater than 90% of the radioactivity is in the 83 kDa band. The 100000 × *g* supernatant showed no recognisable bands (not shown). The prominent 83 kDa band has the same R_f as the principal fragment from partial trypsinization of CR2 (fig.4, track 3). No C3d-binding proteins are present in unused culture medium processed in the same way.

To determine whether these C3d binding proteins were synthesized by the cells, Raji cells were cultured in the presence of [^{35}S]methionine. The medium from such cultures was fractionated into 100000 × *g* pellet and supernatant and both

analysed for C3d-binding proteins as before. A similar labelled 83 kDa protein is found in the medium pellet preparation (fig.4, track 2), indicating that this protein is produced by the cells. For comparison C3d-binding proteins in a tryptic digest of ^{125}I-labelled CR2 is shown (fig.4, track 3).

4. DISCUSSION

Tonsil B lymphocytes and Raji cells both express CR2 molecules which are indistinguishable from each other on SDS-PAGE analysis and are the same as the CR2 prepared from whole spleen. This is in contrast to CR1 which exhibits a change in apparent size when expressed in different cell types [20].

Raji cell rosetting with EAC3d is sensitive to trypsin treatment, and the loss of ^{125}I-labelled proteins which bind to a C3d affinity column exactly follows the loss of rosetting (fig.1). The loss of the intact 145 kDa CR2 molecule is more rapid than the loss of rosetting activity indicating that the remaining C3d-binding proteins, principally the 83 kDa fragment, can mediate rosetting (fig.2). This fragment can be produced by trypsin treatment of whole cells or by trypsin treatment of affinity purified CR2 in non-ionic detergent (fig.3).

It was reported that a C3d-binding molecule of approx. 72 kDa could be isolated from spent Raji cell culture medium [11]. To determine whether intact CR2 or an active fragment of CR2 was released, spent culture medium was examined for proteins which would bind to C3d-Sepharose. Particulate and soluble material was separated by centrifugation at 100000 × g for 1 h. The supernatant contained no C3d-binding proteins. A detergent extract of the particulate pellet contained traces of intact 145 kDa CR2 (fig.4) together with a major 83 kDa C3d-binding protein. This molecule migrates identically on SDS-PAGE to the 83 kDa tryptic fragment of CR2 (fig.4). These results indicate that CR2 fragments which recognise C3d are released into Raji culture medium possibly in a form associated with membranous vesicles. Although no direct comparison of the 72 kDa molecule previously reported [14] and the 83 kDa fragment reported here is possible, these species have very similar characteristics, and are likely to be the same. Some variation in molecular mass as estimated by SDS-PAGE is not unexpected, as the 72 kDa fragment is glycosylated [8]. If these fragments are closely related then the speculation [8,17,18] that the first-reported 72 kDa protein is a fragment of CR2 is confirmed.

The characteristics of the 83 kDa fragment reported here are consistent with it being membrane bound as it is not released from trypsin-treated cells, and it can be sedimented from spent culture medium and subsequently solubilised by detergent. Similarly sized fragments of CR2 have been reported after V8 protease treatment of Raji cells [21]. The production of distinct stable proteolytic fragments of CR2 may be useful in subsequent structural analysis as the C3 binding site appears to be preserved.

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