Binding of fluid-phase complement components C3 and C3b to human lymphocytes

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It is known that a population of B-lymphocytes has receptors for the third component of complement, C3, and that these lymphocytes may be identified by their ability to form rosettes with sheep erythrocytes coated with covalently bound fragments of complement component C3. Human tonsil lymphocytes, enriched for B-cells, form rosettes with sheep erythrocytes coated with antibody and complement components C1, C4b and C3b (EAC143b cells). Fluid-phase C3 will inhibit rosette formation between EAC143b and human tonsil lymphocytes over the same concentration range as fluid-phase C3b. C3 is not cleaved to C3b during incubation with lymphocytes or with lymphocytes and EAC143b cells. Fluid-phase ^{125}I -labelled C3 and ^{125}I -labelled C3b bind to lymphocytes in a specific manner. The characteristics of binding of both radioiodinated C3 and radioiodinated C3b are very similar, but the binding of C3 is again not a result of cleavage to C3b. Salicylhydroxamic acid does not inhibit binding of ^{125}I -labelled C3 to tonsil lymphocytes at concentrations that completely inhibit binding of ^{125}I -labelled C3 to EAC142 cells via the nascent binding site of C3b. It is concluded that C3 and C3b share a common feature involved in binding to lymphocytes bearing receptors for the third component of complement.

The serum protein, C3, is a major component of the complement system. When the complement system is activated, by contact with immune aggregates or micro-organisms, for example, C3 is cleaved specifically by proteolytic enzymes of the complement system into two fragments, C3a and C3b. The C3b fragment may become covalently bound to the material that stimulated complement activation (Law & Levine, 1977). Once formed, C3b is degraded by other proteinases of the complement system and then by other uncharacterized proteinases to fragments designated C3c and C3d (Porter & Reid, 1979).

The existence of cellular receptors for the third component of complement has been demonstrated by observation of the attachment of particles coated

in C3b or C3d to specific cell types either in suspension [see Bianco & Nussenzweig (1977) for a review] or in tissue slices (Schrieber & Penny, 1979). The earliest observation of this phenomenon was immune adherence, the binding of foreign organisms to primate erythrocytes, non-primate platelets and leucocytes in the presence of immune serum [reviewed by Nelson (1963)]. The binding of complement-coated particles to phagocytic cells (opsonization) is very likely to facilitate ingestion of these particles (Ehlenberger & Nussenzweig, 1977). A population of B lymphocytes (Parish, 1975) also have receptors for the third component of complement, as shown by rosette formation between these lymphocytes and particles coated with fragments of C3. The outcome of the binding of C3b-bearing particles to lymphocytes is not understood, although it has been suggested that the attachment of immune complexes to lymphoid cells via C3 receptor is involved in follicular localization of antigen (Klaus & Humphrey, 1977).

There is strong evidence that fluid-phase C3b, as well as C3b attached to particles, binds to complement receptor-bearing lymphocytes (Ross & Polley, 1975; Simonian *et al.*, 1976; Papamichail & Pepys,

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; VBS⁺⁺, 142 mM-NaCl/5 mM-sodium 5,5-diethylbarbiturate/0.5 mM-MgCl₂/0.15 mM-CaCl₂, pH 7.5; SDS, sodium dodecyl sulphate; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); the nomenclature of complement components is that recommended by the World Health Organisation (1968).

1979). Binding of fluid-phase C3b and C3b bound to particles, both of which are produced during complement activation, might suggest that lymphocyte C3 receptors have a function associated with complement activation. It would be expected, if this were the case, that complement receptor-bearing lymphocytes would distinguish between the complement activation products and non-activated C3. However, binding of uncleaved non-activated C3 to complement receptor-bearing lymphocytes has been described (Theofilopoulos *et al.*, 1974; Ross & Polley, 1975; Dierich & Landen, 1977; Frade & Strominger, 1980). The situation is confused because there are also reports indicating that fluid-phase C3 does not bind to lymphocytes (Simonian *et al.*, 1976; Papamichail & Pepys, 1979). To explain the binding of fluid-phase C3 to complement receptor-bearing lymphocytes, it has been suggested that C3 is cleaved to C3b during the binding reaction and it is C3b which then binds to lymphocytes (Frade & Strominger, 1980).

The chemistry of C3 and its activation is now beginning to be understood (Law *et al.*, 1979; Tack *et al.*, 1980; Sim *et al.*, 1981), and therefore we decided to reinvestigate whether or not fluid-phase C3 does bind to lymphocytes. This is important in view of the high concentration of C3 in plasma (1.2 mg/ml) and in determining whether C3 activation to the C3b form is required for binding to lymphocytes.

We present evidence to show that fluid-phase C3 does bind to lymphocytes and that it is not cleaved to C3b during binding.

Materials and methods

Tonsil lymphocytes

Fresh human tonsils, removed at routine tonsillectomy, were teased into McCoy's medium (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) and mononuclear cells were prepared by centrifugation of the tonsil cell suspension over Histopaque (Sigma Chemical Co., Poole, Dorset, U.K.) (Jondal *et al.*, 1972). The population of cells forming rosettes with sheep erythrocytes (Jondal *et al.*, 1972) was then removed, as described by Parish (1975). The final lymphocyte preparation, enriched in B-cells, showed less than 1% contamination with adherent cells.

In some cases, lymphocytes were stored by resuspending the cells in dimethyl sulphoxide [5% (v/v) in foetal-calf serum; Flow Laboratories] at 1×10^8 – 2×10^8 cells/ml and freezing gradually in 1 ml portions to -70°C . Frozen cells could be stored for up to 2 weeks. Just before use, the cells were thawed quickly and washed three times in either McCoy's medium or DGV⁺⁺ by centrifugation (400g, 5 min) at 23°C . Lymphocytes prepared in this way were 95% viable. Cell viability was

determined by exclusion of Trypan Blue. The minimum time between extraction of lymphocytes from tonsils and their use in binding assays was 4 h.

Trypsin treatment of lymphocytes

B-cell-enriched tonsil lymphocytes (10^7 cells/ml of McCoy's medium) were incubated with trypsin (treated with Tos-Phe- CH_2Cl), from Worthington Biochemical Corp., Freehold, NJ, U.S.A., at 0.75 mg/ml (20 min, 37°C). The digestion was stopped by addition of soya-bean trypsin inhibitor (Type I-S, Sigma) to a final concentration of 3.75 mg/ml. The mixture was incubated at 37°C for a further 5 min. Control samples were prepared by pre-mixing trypsin with soya-bean trypsin inhibitor before addition to cells. Cells were washed three times with DGV⁺⁺.

Proteins

C3 was prepared and stored as previously described (Sim *et al.*, 1981). C3b was generated from C3 by a fluid-phase alternative-pathway convertase, as described by Sim & Sim (1981) or else C3b was prepared by digestion of C3 with trypsin by using a ratio of 100:1 (w/w) of C3 to trypsin. After 1 min at 37°C the reaction was stopped by addition of a fivefold excess of soya-bean trypsin inhibitor over trypsin. The incubation was continued for 5 min at 37°C .

C3c was prepared by digestion of C3 (1 mg/ml) with trypsin (4%, w/w) in 25 mM-potassium phosphate buffer, pH 7.0, for 4 h at 37°C . The reaction was stopped by adding a twofold molar excess over trypsin of soya-bean trypsin inhibitor. C3c was separated from the C3d fragment by gel filtration on a column (80 cm $\times$ 2.5 cm diam.) of Ultrogel AcA 34 (LKB Instruments Ltd., Croydon, Surrey, U.K.) in the same buffer. If necessary, traces of undigested C3b and C3d were removed by salt-gradient elution from a column (20 cm $\times$ 1.5 cm diam.) of DEAE-Sephacel (Pharmacia, Hounslow, Middx., U.K.) equilibrated in the same buffer.

$\beta_1\text{H}$ was prepared as previously described (Sim & Sim, 1981).

Guinea-pig C1 was prepared by a single neutral euglobulin precipitation of guinea-pig serum (Linscott, 1968). Guinea-pig C2, prepared by the method of Kerr & Porter (1978) was kindly donated by Dr. M. A. Kerr (now at the Department of Pathology, University of Dundee, Dundee, Scotland, U.K.).

Bovine serum albumin used as competing protein was Cohn fraction V from Sigma.

Rosettes

Sheep erythrocytes coated with rabbit antibody to sheep erythrocyte membranes and complement component C4 (EAC4) were prepared by the method of Borsos & Rapp (1967) by using either

rabbit haemolysin (Mayer, 1961) or the 19S fraction of rabbit antibodies against sheep erythrocytes (Cordis, Miami, FL, U.S.A.) as antibody and human serum as the source of C4. EAC14 cells were formed by adding guinea-pig C1 (approx. 400 functional molecules/cell) to EAC4 cells in DGVB⁺⁺. Routinely, EAC143b cells were formed by incubating (15 min, 37°C) an equal volume of EAC14 cells (10⁹/ml in DGVB⁺⁺) and human C3 (500 µg/ml of VBS⁺⁺) in the presence of 100 functional units of guinea-pig C2/cell. Trace amounts of ¹²⁵I-labelled C3 were added to determine the number of C3 molecules bound per sheep cell. Controls for non-specific binding of C3 were done in the absence of C2. The number of molecules of C3 bound was 20 000–25 000 per erythrocyte, which gave maximal levels of rosette formation. EAC143b cells could be stored at 4°C for 3 days in DGVB⁺⁺ containing penicillin (40 i.u./ml) and streptomycin (40 µg/ml) and were washed into fresh DGVB⁺⁺ just before use.

To form rosettes, 50–100 µl of EAC143b cells at 1.6 × 10⁸/ml of DGVB⁺⁺ were mixed with an equal volume of B-cell-enriched tonsil lymphocytes at 4 × 10⁶/ml in DGVB⁺⁺ and incubated (30 min, 37°C) on a rotating wheel at 36 rev./min. For each experiment, rosette formation between EAC14 and lymphocytes was found to be no greater than 5%. Rosettes of three or more sheep cells per lymphocyte were scored after staining with Toluidine Blue (Hudson & Hay, 1976). At least 200 lymphocytes were counted in each test.

In experiments where inhibitors of rosette formation were tested, lymphocytes (100 µl of 4 × 10⁶ cells/ml) were preincubated in the presence of inhibitor (100 µl in VBS⁺⁺) (30 min, 37°C) and then centrifuged, the supernatant was removed and replaced by 100 µl of DGVB⁺⁺ and rosette formation was done in the usual way. If inhibitor was required to be present during rosette formation, 50 µl of EAC14 or EAC143b cells (3.2 × 10⁸/ml in DGVB⁺⁺), 50 µl of lymphocytes (8 × 10⁶/ml) and 100 µl of VBS⁺⁺ or inhibitor in VBS⁺⁺ were added. After rosettes were scored, samples from the unstained preparations were centrifuged and supernatants were removed for SDS/polyacrylamide-gel electrophoresis. In some cases the remaining pellet was also prepared for SDS/polyacrylamide-gel electrophoresis.

Radioiodination

Proteins were radioiodinated by the method of Fraker & Speck (1978) with 1,3,4,6-tetrachloro-3,6-diphenylglycoluril (Iodogen; Pierce and Warriner, Chester, U.K.). Typically, 200 µg of Iodogen was used with 1 ml of C3 (300 µg) in 100 mM-potassium phosphate/150 mM-NaCl/5 mM-EDTA, pH 7.5, and 1 mCi of carrier-free [¹²⁵I]iodide (The Radiochemical Centre, Amersham, Bucks., U.K.;

sp. radioactivity 15–17 mCi/µg of iodine). Protein was separated from free iodine by gel filtration on a column (10 cm × 0.8 cm diam.) of Sephadex G-25. The incorporation of ¹²⁵I into C3, C3b and C3c was, on average, 10⁶ c.p.m./µg. Counting for γ-radiation was done in an LKB 1270 Rackgamma counter with a counting efficiency of 40%.

Assays of binding of C3, C3b and C3c to lymphocytes

Lymphocytes were washed in DGVB⁺⁺ containing 10 mg of bovine serum albumin per ml and then resuspended in this buffer to 5 × 10⁷ or 10⁸ cells/ml. Unless otherwise stated, lymphocytes were added to start the reaction. Protein components were all dialysed into VBS⁺⁺ just before use. At the end of the incubation, duplicate samples of 75 µl of the mixture was layered over 200 µl of oil, as described by Stahl *et al.* (1980). The cells were separated from the aqueous phase by centrifugation for 30 s (9980 g) in an Eppendorf 5412 centrifuge. The supernatant samples were removed for SDS/polyacrylamide-gel electrophoresis and for determination of radioactivity. The tip of the tube, containing the cell pellet, was cut off and radioactivity was determined in this fraction.

SDS/polyacrylamide-gel electrophoresis

Samples of protein were reduced and alkylated at pH 7.0 for SDS/polyacrylamide-gel electrophoresis as described previously (Sim & Sim, 1981). In some cases, gel samples were prepared at pH 10.

When samples of lymphocytes were prepared for gel electrophoresis after binding assays, 50 µl of 8 M-urea/200 mM-sodium phosphate/2% (w/v) SDS, pH 7.0, was placed in a 400 µl microfuge tube. Oil (200 µl) was placed on top of this solution and the sample of lymphocytes was centrifuged through the oil directly into the denaturing buffer. The supernatant aqueous medium was removed and the region of the tube containing lymphocytes was cut off and incubated with 50 µl of VBS⁺⁺ and dithiothreitol (20 mM). These samples were treated in the same way as other gel samples.

Electrophoresis was performed, gels were stained with Coomassie Blue, destained and subjected to autoradiography as described previously (Sim *et al.*, 1981).

Results

State of C3b bound to EAC14 cells

Lymphocytes have been reported to have receptors both for the C3b and C3d portions of C3 (Ross *et al.*, 1973). Therefore the state of C3 bound to EAC14 cells used in rosette formation was determined. Polyacrylamide-gel electrophoresis of EAC143b cells prepared by using ¹²⁵I-labelled C3

was performed before and after treatment of EAC143b cells to destroy ester bonds (Sim *et al.*, 1981). The gel patterns show that C3 is bound as high-molecular-weight components or as C3b (Fig. 1, track 1) (Law *et al.*, 1979). When EAC143b-cell membranes were treated at high pH to break ester bonds, there is a decrease in the high-molecular-weight bands and a corresponding increase in the α' -chain of C3b (Fig. 1, track 4). This shows that there is negligible binding of intact C3 and that there has been no significant breakdown of bound C3b to other degradation products. The state of bound C3b is not altered during rosette formation with B-cell-enriched lymphocytes (Fig. 1, tracks 2 and 5).

Fig. 1. Characterization of ^{125}I -labelled C3b bound to EAC143b cells before and after rosette assay

EAC143b cells ($200\mu\text{l}$ of 3.2×10^8 cells/ml of DGVB $^{++}$) prepared with ^{125}I -labelled C3 (sp. radioactivity 2.5×10^6 c.p.m./nmol) were incubated (30 min, 37°C) either with $200\mu\text{l}$ of DGVB $^{++}$ (tracks 1 and 4) or with $100\mu\text{l}$ of human tonsil lymphocytes enriched for B-cells (8×10^6 /ml of DGVB $^{++}$) and $100\mu\text{l}$ of DGVB $^{++}$ (tracks 2 and 5) or with $100\mu\text{l}$ of DGVB $^{++}$ and $100\mu\text{l}$ of $\beta_1\text{H}$ (250 g/ml in DGVB $^{++}$) (tracks 3 and 6). Samples ($50\mu\text{l}$) of each incubation mix were removed for determination of rosette formation as described in the text and the remaining solution was centrifuged ($1000g$, 10 min, 4°C). The supernatant was removed and the resulting pellets were resuspended and divided between two tubes and centrifuged as described above. The pellets were drained and treated (1 h, 37°C) either with $25\mu\text{l}$ of 50 mM-sodium phosphate/100 mM-NaCl/1% SDS, pH 7.4 (tracks 1–3), or with $25\mu\text{l}$ of 50 mM-diethanolamine/100 mM-NaCl/1% (w/v) SDS, pH 11.0 (tracks 4–6) to break ester bonds. Samples were then prepared for gel electrophoresis on 7.5% polyacrylamide gels in the presence of SDS in the usual way. Track 7, $\beta_1\text{H}$; track 8, $\beta_1\text{H}$ ovalbumin and C3b as standards; track 9, the C3 used to prepare EAC143b cells. Tracks 1–6 show autoradiographs, whereas tracks 7–9 show Coomassie Blue staining.

$\beta_1\text{H}$ has been implicated in binding of complement-coated erythrocytes (EAC143b) to lymphocytes (Dierich & Schmitt, 1980; Okuda & Tachibana, 1980). In the present study, the C3 used to prepare EAC143b cells did not contain detectable $\beta_1\text{H}$ (Fig. 1, track 9). Rosette formation between lymphocytes and EAC143b cells was not increased in the presence of $\beta_1\text{H}$ under the conditions described in Fig. 1, and the state of bound C3b was unaltered after incubation with $\beta_1\text{H}$ (Fig. 1, tracks 3 and 6). The $\beta_1\text{H}$ used in the present studies (Fig. 1, track 7) was able to cleave C3b in the presence of C3b inactivator (Sim & Sim, 1981).

It has been reported that Raji cells (a lymphoblastoid B-cell line) do not form rosettes with EAC143b cells (Lambris *et al.*, 1980), contrary to earlier findings (Ross & Polley, 1975). Although the work presented here refers to tonsil lymphocytes, we found that Raji cells do form rosettes with EAC143b cells on which the bound C3b is in the condition shown in Fig. 1.

Inhibition of rosette formation between EAC143b and tonsil lymphocytes by C3 or C3b

As shown in Fig. 2, both C3 and C3b inhibit rosette formation between tonsil lymphocytes and EAC143b cells over the same concentration range. The results presented in Fig. 2 have been obtained with C3 or C3b present during rosette formation. However, the same result is produced if lympho-

Fig. 2. Inhibition of rosette formation between EAC143b cells and tonsil lymphocytes C3 and C3b

Samples ($50\mu\text{l}$) of human tonsil lymphocytes enriched for B-cells (8×10^6 /ml in DGVB $^{++}$) were incubated (30 min, 37°C) with $100\mu\text{l}$ of VBS $^{++}$ alone or with C3 (●) or C3b (○) at various concentrations in $100\mu\text{l}$ of VBS $^{++}$ together with $50\mu\text{l}$ of EAC143b cells or EAC14 cells (3.2×10^8 /ml in DGVB $^{++}$) as described in the text. Samples ($50\mu\text{l}$) were removed for determination of rosette formation. Control rosette formation with EAC14 cells was always less than 5% and has not been subtracted. The arrow indicates the concentration of C3b bound to EAC143b cells.

cytes are incubated with the competing protein immediately before rosette formation. The effect was specific, since bovine serum albumin was not inhibitory.

In order to determine if the effect of C3 was due to C3 being cleaved to C3b during rosette formation, samples of the supernatant obtained after rosette formation at each concentration of C3 were examined by polyacrylamide-gel electrophoresis in the presence of SDS. It is evident from the gel shown in Fig. 3 that the C3 in the supernatant is not degraded to C3b. The C3 that is associated with the cell pellet is also uncleaved, as will be discussed below (see Fig. 7).

It is known that C3 can exist in a haemolytically inactive state (von Zabern *et al.*, 1981). Haemolytically inactive C3 exhibits certain properties normally associated with C3b, e.g. it is cleaved by β_1 H and C3b inactivator (Parkes *et al.*, 1981). The loss of haemolytic activity may be due to hydrolysis of an internal thiol ester in C3 (Tack *et al.*, 1980; Sim *et al.*, 1981). The C3 used in the studies presented here was not 'C3b-like'. This was determined by three criteria: (a) it was haemolytically active; (b) it was shown to be completely cleavable by a fluid-phase alternative-pathway convertase (Parkes *et al.*, 1981); and (c) it underwent denaturation-induced cleavage to the same extent as fully active C3 (Sim & Sim, 1981). The extent of

denaturation-induced cleavage, which is characteristic of active, but not of inactive, C3 or C3b, was not altered after C3 had been incubated with either lymphocytes alone or with lymphocytes and EAC143b cells.

We have found that C3 deliberately inactivated by hydrazine or hydroxylamine treatment also inhibits rosette formation over the same concentration range as haemolytically active C3 and C3b.

Binding of 125 I-labelled C3 and 125 I-labelled C3b to lymphocytes

Both 125 I-labelled C3 and 125 I-labelled C3b bind to tonsil lymphocytes to the same extent (Fig. 4). The

Fig. 3. SDS/polyacrylamide-gel electrophoresis of C3 used to inhibit rosette formation

After measurement of rosette formation, as described in the legend to Fig. 2, the remaining mixture was centrifuged (1000g, 10min at 4°C) and samples (25 μ l) of supernatant solution with different amounts of C3 were prepared for gel electrophoresis (tracks 1–5). A sample of supernatant with no C3 added (track 6) and a sample of C3 incubated in DGVB⁺⁺ without either lymphocytes or EAC143b cells (track 7) was also prepared for gel electrophoresis. β_1 H, C3b and ovalbumin are shown as standards (track 8).

Fig. 4. Binding of 125 I-labelled C3, C3b and C3c to lymphocytes

Samples (100 μ l) of human tonsil lymphocytes enriched for B-cells (5×10^7 /ml in DGVB⁺⁺ containing 10mg of bovine serum albumin/ml) were incubated with 100 μ l of various concentrations of 125 I-labelled C3 (●), 125 I-labelled C3b (○) or 125 I-labelled C3c (□). Each protein was adjusted to a specific radioactivity of 2.5×10^6 c.p.m./nmol in VBS⁺⁺. Incubation was for 30min at 37°C, and samples of the cell pellets were counted for radioactivity as described in the text. The total amount of each protein bound per 5×10^6 cells is shown (a). Binding of C3 (●) and C3b (○) has also been corrected for binding of C3c (b).

binding of ^{125}I -labelled C3c is much lower, in agreement with a previous report (Theofilopoulos *et al.*, 1974).

The binding of ^{125}I -labelled C3 is specific in that it is decreased in the presence of unlabelled C3 (Fig. 5), but not in the presence of bovine serum albumin. Similarly, the binding of ^{125}I -labelled C3b is competed for by unlabelled C3b (results not shown), but is unaffected by bovine serum albumin. Further evidence for an identical mode of binding of C3 and C3b is that ^{125}I -labelled C3 binding is decreased in the presence of unlabelled C3b (Fig. 5). The same is true of ^{125}I -labelled C3b binding in the presence of C3 (results not shown).

A background amount of binding of ^{125}I -labelled C3 and C3b to trypsin-treated lymphocytes is

Fig. 5. Specificity of binding of ^{125}I -labelled C3 and C3b to lymphocytes

Samples (100 μl) of human tonsil lymphocytes enriched for B-cells ($5 \times 10^7/\text{ml}$ in DGVB⁺⁺, containing 10 mg of bovine serum albumin/ml) were incubated (30 min, 37°C) in the presence of ^{125}I -labelled C3 (6×10^5 c.p.m., sp. radioactivity 10^6 c.p.m./ μg) with different amounts of unlabelled C3 (●) or unlabelled C3b (○) in VBS⁺⁺, in a total volume of 200 μl . Lymphocytes were also incubated (30 min, 37°C) in the presence of ^{125}I -labelled C3c (6×10^5 c.p.m., sp. radioactivity 0.8×10^6 c.p.m./ μg) with different amounts of unlabelled C3c (□). Lymphocytes, in DGVB⁺⁺ with 10 mg of gelatin/ml, were also incubated with ^{125}I -labelled C3 with different amounts of bovine serum albumin (△). Samples of trypsin-treated lymphocytes ($5 \times 10^7/\text{ml}$ in DGVB⁺⁺ with 10 mg of bovine serum albumin/ml) were incubated with ^{125}I -labelled C3 and different amounts of unlabelled C3 (■). Radioactivity associated with the cell pellets was determined as described in the Materials and methods section and is expressed as the percentage of binding of ^{125}I -labelled C3 in the absence of any additional unlabelled component. This corresponds to 21 000 c.p.m./ 5×10^6 lymphocytes.

observed, but this is not decreased in the presence of an excess of unlabelled C3 (Fig. 5) or C3b. The characteristics of ^{125}I -labelled C3c binding to lymphocytes, as shown in Fig. 5, are not dependent on the concentration of unlabelled C3c and are very similar to the background binding to trypsin-treated lymphocytes. It is known that trypsin treatment of lymphocytes inhibits rosette formation between complement receptor-bearing lymphocytes and EAC143b cells (Bianco & Nussenzweig, 1977), and the binding of ^{125}I -labelled C3 and C3b to trypsin-treated lymphocytes can be considered as non-specific binding.

It has previously been reported that ^{125}I -labelled C3 and ^{125}I -labelled C3b bind to lymphocytes with the same concentration-dependence, but this has been attributed to proteolysis of C3 to C3b (Frade & Strominger, 1980). In view of the identical nature of the curves of C3 and C3b binding to lymphocytes (Figs. 4 and 5), it would suggest that if proteolysis were occurring, then most of the C3 would require to be cleaved to C3b to produce such a result. In all experiments of the type shown in Figs. 4 and 5, samples of the supernatant solutions after removal of lymphocytes were subjected to SDS/polyacrylamide-gel electrophoresis (Fig. 6).

In Fig. 6 the β -chain (mol.wt. 70 000) of C3 or C3b is concealed by the excess of bovine serum albumin (mol.wt. 68 000) that is present. Conversion of C3 to C3b does not affect the β -chain. No cleavage of the α -chain of C3 is detectable in either the Coomassie Blue-stained gel (Fig. 6) nor in the corresponding autoradiograph, proving that neither the unlabelled nor the radioiodinated C3 was proteolysed to C3b.

In these experiments, only a small percentage (1–4%) of the total ^{125}I -labelled C3 that is supplied binds to lymphocytes, and it could be argued that only a fraction of the ^{125}I -labelled C3 that is already in the form of C3b is the component responsible for binding. It could also be argued that the ^{125}I -labelled C3 that binds is cleaved by cell-surface proteinases and becomes attached as C3b via the nascent binding site. As shown in Fig. 7, however, the radioiodinated component that binds to lymphocytes incubated with ^{125}I -labelled C3 migrates on polyacrylamide gels identically with C3. The ^{125}I -labelled material that binds in a similar fashion when radioiodinated C3b is supplied is indistinguishable from C3b. There are no high-molecular-weight components that might be indicative of covalent attachment of ^{125}I -labelled C3b to cell-surface components. Fig. 7 should be compared with Fig. 1, where C3 is bound covalently as C3b to EAC143b cells.

It could also be argued that the small percentage of ^{125}I -labelled C3 that binds to lymphocytes corresponds to haemolytically inactive C3. How-

Fig. 6. SDS/polyacrylamide-gel electrophoresis of ^{125}I -labelled C3 and C3b incubated with lymphocytes

^{125}I -labelled C3 (500 μl of a 500 $\mu\text{g}/\text{ml}$ solution of specific radioactivity 2.5×10^6 c.p.m./nmol) or ^{125}I -labelled C3b (500 μl of a 500 $\mu\text{g}/\text{ml}$ solution of specific radioactivity 2.7×10^6 c.p.m./nmol) was incubated either with DGVB $^{++}$ (500 μl) containing 10 mg of bovine serum albumin/ml or with human tonsil lymphocytes enriched for B-cells (500 μl of 5×10^7 cells/ml in DGVB $^{++}$, containing 10 mg of bovine serum albumin/ml). After 90 min at 37°C, samples were centrifuged to pellet the lymphocytes and 25 μl of each supernatant was prepared for SDS/polyacrylamide-gel electrophoresis. Series a shows protein stain and series b shows the corresponding autoradiograph. Track 1, C3 + DGVB $^{++}$ and bovine serum albumin; track 2, C3b + DGVB $^{++}$ and bovine serum albumin; track 3, C3 + lymphocytes; track 4, C3b + lymphocytes; track 5, DGVB $^{++}$ and bovine serum albumin alone.

ever, the ^{125}I -labelled C3 that binds undergoes denaturation-induced cleavage to the same extent as fully haemolytically active C3. In Fig. 7, where gel samples were prepared under mild conditions (30 min, 37°C, pH 7.0) some denaturation-induced fragmentation of ^{125}I -labelled C3 has occurred. The small fragment (mol.wt. 46 000) is more heavily iodinated than the larger denaturation-induced fragment (mol.wt. 74 000) (E. Sim, unpublished work) and so is more obvious on autoradiography. As expected, no such fragmentation is observed for C3b.

Effect of salicylhydroxamate on ^{125}I -labelled C3 binding

It has been shown that hydroxamic acids inhibit the covalent binding of C3 via the nascent binding site (Twose *et al.*, 1980; Sim *et al.*, 1981). The effect of salicylhydroxamate on formation of EAC143b cells is shown in Fig. 8. Over the same concentration range of salicylhydroxamate, there is no detectable inhibition of ^{125}I -labelled C3 binding to

Fig. 7. SDS/polyacrylamide-gel electrophoresis of C3 and C3b bound to tonsil lymphocytes

Human tonsil lymphocytes, enriched for B-cells (100 μl of 5×10^7 cells/ml in DGVB $^{++}$ containing 10 mg of bovine serum albumin/ml) were incubated for 60 min at 37°C with either (a) ^{125}I -labelled C3 (6 μg in 100 μl of VBS $^{++}$) or (b) ^{125}I -labelled C3b (7.5 μg in 100 μl of VBS $^{++}$). Total radioactivity supplied was 3×10^6 c.p.m. in both cases. Samples of lymphocytes with bound C3 or C3b were prepared for polyacrylamide-gel electrophoresis as described in the Materials and methods section. An autoradiograph is shown.

lymphocytes. This again shows that C3 does not require to be cleaved to bind to lymphocytes.

Time- and temperature-dependence of ^{125}I -labelled C3 and C3b binding to lymphocytes

The specific binding of radioiodinated C3 and C3b to lymphocytes increases with time at 37°C (Fig. 9c). At 23°C, binding is slower (Fig. 9b) and at 0°C (Fig. 9a), little binding is observed above the non-specific-binding value. The characteristics of binding of both C3 and C3b appear identical at each temperature. The effects of time and temperature shown in Fig. 9 are very similar to those observed by

Fig. 8. Effect of salicylhydroxamate on binding of C3 to lymphocytes and EAC142 cells

Samples (200 μ l) of B-cell-enriched human tonsil lymphocytes (5×10^7 cells/ml in DGVB⁺⁺, containing 10 mg of bovine serum albumin/ml) were incubated (30 min, 37°C) in the presence of 100 μ l of VBS⁺⁺ containing ¹²⁵I-labelled C3 (280 μ g, 1.2×10^6 c.p.m.) and different concentrations of salicylhydroxamate. Triplicate samples (75 μ l) were removed and radioactivity associated with the cell pellet was determined after centrifugation through oil as described in the text (□). EAC14 cells (200 μ l of 10^9 /ml in DGVB⁺⁺) plus 100 μ l of C2 in DGVB⁺⁺ (final concn. 2.5 μ g/ml) plus 100 μ l of ¹²⁵I-labelled C3 in DGVB⁺⁺ (final concn. 175 μ g/ml, sp. radioactivity 30000 c.p.m./ μ g) were incubated for 20 min at 37°C in the presence of various concentrations of salicylhydroxamate. The radioactivity associated with the cell pellet was determined as described above, and is expressed as a percentage of the value obtained in the absence of salicylhydroxamate (▨). In both experiments, salicylhydroxamate was added from a stock solution of 100 mM-sodium salicylhydroxamate, pH 7.5. Results are expressed as the mean $\pm$ S.E.M.

Dierich & Landen (1977) for preincubation of lymphocytes in studies on inhibition of rosette formation. The binding of C3b to Raji cells, detected by immunofluorescence with antibodies to C3 (Theofilopoulos *et al.*, 1974; Ross & Polley, 1975) has also been shown to be temperature-dependent. It is clear from the other results presented here that the temperature-dependent phenomenon is not due to cleavage of C3 to C3b.

Discussion

The results presented here show that both fluid-phase C3 and C3b do bind to lymphocytes, indistinguishably, in an apparently specific and saturable manner and C3 is not converted into C3b on binding.

Fig. 9. Effect of time and temperature on binding of C3 and C3b to tonsil lymphocytes

Samples (500 μ l) of a suspension of B-cell-enriched human tonsil lymphocytes (10^8 /ml) or trypsin-treated tonsil lymphocytes (10^8 /ml), both in DGVB⁺⁺ containing 10 mg of bovine serum albumin/ml were incubated at (a) 0°C, (b) 23°C or (c) 37°C, in the presence of 2 nmol in 500 μ l of VBS⁺⁺ of either ¹²⁵I-labelled C3 (●) or ¹²⁵I-labelled C3b (○). In each case the specific radioactivity of the radioiodinated protein was 1.2×10^6 c.p.m./nmol. At various times, duplicate samples (75 μ l) were removed from the incubation mixture and radioactivity in the cell pellet was determined after centrifugation of the material through oil as described in the Materials and methods section. Binding to lymphocytes has been corrected for non-specific binding to trypsin-treated lymphocytes.

It is likely that C3 and C3b are binding to C3b receptors on lymphocytes in that the binding is sensitive to trypsin treatment of lymphocytes, which destroys C3b receptor activity (Bianco & Nussenzweig, 1977). Also, C3 or C3b binding inhibits rosette formation between EAC143b cells and lymphocytes, and it has been observed by others (Frade & Strominger, 1980) that binding of fluid-phase C3 or C3b only occurs with cell lines that possess C3b receptor activity. The binding of fluid-phase C4 by C4b-receptor-bearing cells has been reported as being indistinguishable from fluid-phase C4b binding (Ross & Polley, 1975), but these studies were performed before the existence of haemolytically inactive C4 was known (von Zabern *et al.*, 1981).

It can be seen from Fig. 4(b) that the binding of C3 approaches saturation at around 5×10^5 molecules of C3 or C3b bound per cell, after correction for non-specific binding. This number of C3 molecules bound per lymphocyte is relatively high. For example, the number of copies of the major cell-surface glycoprotein of thymocytes, Thy 1, is around 10^6 per cell (Mason & Williams, 1980). Such

high, or even higher, amounts of fluid-phase C3 binding have been reported previously for Raji cells (Theofilopoulos *et al.*, 1974; Dierich & Landen, 1977). However, at the time when these earlier studies were performed, less was known about the C3 molecule and it was not possible to determine unequivocally that C3 was not cleaved.

As discussed in the introduction, primate erythrocytes participate in immune adherence reactions and a molecule has been purified from human erythrocyte membranes which has the characteristics of a C3b receptor molecule (Fearon, 1979). An anti-serum to this molecule cross-reacts with lymphocytes, and in an indirect binding assay it has been calculated that there are 2.1×10^4 molecules of this protein per lymphocyte, which is clearly much less than the number of molecules detected by direct binding of C3 or C3b.

The problem posed by these observations is twofold.

(a) What is the reason for the discrepancy between the number of molecules of fluid-phase C3 and C3b that can bind per lymphocyte and the number of molecules of C3b receptor detected by the binding of antibodies to the molecule purified from human erythrocytes by Fearon (1979) and identified as C3b receptor (Fearon, 1980)?

(b) Is the binding of fluid-phase C3 or C3b to lymphocytes important compared with the binding of particle-bound C3b?

It has been reported that the binding of C3b to lymphocytes, as measured by indirect immunofluorescence, shows patches at 37°C but not at 0°C (Theofilopoulos *et al.*, 1974; Ross & Polley, 1975; Papamichail & Pepys, 1979). In studies where fluorescently labelled antibodies against C3b have been used, it is always a possibility that the clustering is due to the antibody-binding, but temperature-dependent clustering of lymphocyte-bound C3b has also been observed with fluorescent C3b itself (Papamichail & Pepys, 1979).

The high extent of C3 binding and the temperature-dependence of C3 binding may reflect a clustering of C3 receptors on the lymphocyte cell surface, followed by internalization, as has been described for α_2 -macroglobulin receptors (Goldstein *et al.*, 1979). It has been reported that C3 receptors on the lymphocyte cell surface do exist in clusters (Petty *et al.*, 1980), and it has been suggested previously that clustering of C3b receptors is a prerequisite for rosette formation (Landen *et al.*, 1978). What is clear from our studies is that C3, if internalized, is not degraded in up to 2 h.

We have preliminary evidence to suggest that C3, once bound by lymphocytes, is not readily exchangeable, even in the presence of a 100-fold excess of fluid-phase C3. The values that we have measured suggest that 25% of bound C3 is released/h. Other

workers have reported that extensive washing of lymphocytes after incubation with C3 does not affect the inhibition of rosette formation between EAC143b cells and lymphocytes (Dierich & Landen, 1977).

It appears that complement receptor-bearing lymphocytes do not distinguish between fluid-phase C3 and C3b and it may be that activation of the complement system does not have an effect on this system. However, complement activation also results in deposition of C3b on the activating particle. It is difficult to assess the relative affinity of bound C3b and fluid-phase C3 or C3b for lymphocyte C3b receptors. In Fig. 2 the concentration of C3b bound to EAC143b cells, assuming a uniform distribution in the incubation solution, is marked with an arrow. It is clear that a large excess of fluid-phase C3 or C3b is required to inhibit rosette formation. Similar observations have been made for inhibition of rosette formation between lymphocytes and EAC14b by fluid-phase C4b (Bokisch & Sobel, 1974). However, this is a simplistic approach to the concentration of bound C3b, because there is good evidence to favour the existence of clusters of about 30 molecules of C3b on the activating surface (Mardiney *et al.*, 1968; Sim *et al.*, 1981), which will give rise to localized areas of high concentration of C3b.

If fluid-phase C3 binds to complement receptor-bearing lymphocytes *in vivo*, it is important to determine the concentration of C3b receptors in blood. From the values of numbers of C3b receptor molecules on different cell types, determined by indirect radioimmunoassay (Fearon, 1980), the total number of C3b receptor sites/ml of blood is calculated to be approx. 5.3×10^{12} (see Table 1). However, the number of molecules of C3 is 2×10^{15} /ml of blood assuming a concentration of C3 as 0.6 mg/ml of blood ($3.2 \mu\text{M}$). Therefore the concentration of C3b receptor sites on white cells is very small compared with the number of C3 molecules that are present. At the concentration of C3 in blood, it can be estimated from Fig. 4(b) that at least 85% of C3 receptor sites on lymphocytes are occupied with C3.

It is likely that C3 in plasma interacts with C3 receptors on lymphocytes. It has been reported that rosette formation between EAC143b cells and lymphocytes can be inhibited by incubating lymphocytes with autologous serum (Dukor *et al.*, 1974; Jondal *et al.*, 1976). This effect is not observed if animals are treated with cobra venom factor to deplete C3 before removal of serum (Dukor *et al.*, 1974).

The role of lymphocyte receptors for the third component of complement is not understood. However, during development of lymphocytes there is evidence to show that C3b receptors are present on

Table 1. *Calculation of C3b receptor concentration in blood*

The number of receptor sites per cell were estimated from data of Fearon (1980). Cell numbers were from Dacie (1958). The molecular weight of C3 used to calculate the number of molecules was 186 000.

Cell type	Receptors/cell	Cells/ml of blood	Receptors/ml of blood
Erythrocytes	10^3	5×10^9	5×10^{12}
Leucocytes	4.5×10^4	7.5×10^6	3.4×10^{11}

C3	
$\mu\text{g/ml}$ of blood ... 600	
Molecules/ml of blood ... 1.9×10^{15}	

cells that develop into plasma cells capable of producing 19S antibody (Mason, 1976). It may be that it is the binding of fluid-phase C3, rather than particle bound or fluid-phase C3b, which is important for this pathway of maturation.

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