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Enzymic assay of C3b receptor on intact cells and solubilized cells

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The C3b receptor of human erythrocytes is known to act as a cofactor for the cleavage of the complement protein C3b by the serine proteinase C3b/C4b Ina. The same cofactor activity is shown to be present on human tonsil B-lymphocytes. The cofactor activity of the C3b receptor can be assayed, on intact cells or in solubilized extracts of cells, by determining the rate of C3b cleavage in the presence of fixed concentrations of C3b and of C3b/C4b Ina. This assay method was used to compare the characteristics and relative quantities of C3b receptors on erythrocytes and lymphocytes. The cofactor activities associated with these two cell types resemble each other, but are distinct from the serum cofactor proteins, C4bp and Factor H, in antigenicity and in pH- and ionic-strength-dependence, and are distinct from Factor H in substrate specificity. Assay of cofactor activity in intact cells indicates that there are about 80-fold more receptors per cell on the lymphocyte surface than on erythrocytes. Assays with cells made permeable by detergent show that, whereas essentially all of the receptors on erythrocytes are on the cell surface, B-lymphocytes contain a large internal receptor pool, which makes up more than 80% of the total cofactor activity of the cell.

The complement system, which consists of about 20 proteins, is found in blood plasma and is a major defence against invasion by foreign material (Reid & Porter, 1981; Whaley & Ferguson, 1981). Activation of the complement system can occur by two routes, namely the classical pathway, which consists of components C1, C2 and C4, and the alternative pathway, which involves the serine proteinases Factor B and Factor D. Both of these activation mechanisms result in the formation of 'C3 convertase' enzymes, which in turn activate the most abundant complement protein, C3.

An activity has been purified from human erythrocyte membranes that acts as a regulator of the C3 convertase enzyme of the alternative pathway (C3bBb) (Fearon, 1979) and of the corresponding enzyme of the classical pathway (C4b2a) (Iida & Nussenzweig, 1981). The regulation of the activity of the C3bBb and C4b2a convertase enzymes was found to reside partly in the ability of the protein derived from erythrocyte membranes to act as an essential cofactor in the C3b/C4b-Ina-mediated cleavage of C3b or C4b, and partly in its ability to displace the Bb or C2a moieties of the convertase enzymes from their binding sites on C3b or C4b.

These activities are also found in plasma or serum associated with the proteins Factor H (formerly called β_1H) and C4b-binding protein (C4bp). These proteins are also essential cofactors for the cleavage of soluble C3b (Whaley & Ruddy, 1976) and C4b (Fujita *et al.*, 1978) respectively by C3b/C4b Ina. In these proteolytic reactions, the serine proteinase C3b/C4b Ina will not catalyse the cleavage of C3b or C4b alone, but will only degrade a complex formed between C3b or C4b and the corresponding cofactor. The cofactors themselves are not cleaved during the degradation of C3b or C4b. The serum cofactors C4bp and Factor H also regulate C3 convertases partly by displacing the C2a or Bb subunits of these enzymes.

The C3b/C4b-Ina-cofactor activity derived from human erythrocyte membranes is considered to be a property of a C3b receptor (known as complement receptor 1, or CR1) (Fearon, 1980). It has been suggested on the basis of immunological cross-reactivity that the C3b receptor from human

Abbreviations used: the nomenclature of complement components and fragments is, where appropriate, as recommended by the World Health Organisation (1968, 1981) (note: the World Health Organisation recommends simply 'I' for C3b/C4b Ina); other abbreviations are: C3u, C4u and C5u, haemolytically inactive forms of C3, C4 and C5 respectively; S-α_2M and F-α_2M, the active and the inactive forms of α_2-macroglobulin (α_2M) respectively.

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B-lymphocytes is related to the human erythrocyte C3b receptor (Fearon, 1980), and it has been shown that a monoclonal antibody that recognizes the C3b receptor of human lymphocytes also inhibits C3b receptor activity on human erythrocytes (Gerdes *et al.*, 1982).

Conventional methods for measuring C3b receptor activity on intact cells include (*a*) rosetting or haemagglutination techniques for observing the binding of indicator particles coated with C3b to C3b-receptor-bearing cells (see, e.g., Christensson & Biberfeld, 1978; Stein *et al.*, 1978) and (*b*) quantitative measurement of the binding of radioactively labelled monomeric (Theofilopoulos *et al.*, 1974; E. Sim & R. B. Sim, 1981) or oligomeric (Arnaout *et al.*, 1981) ligand to the receptors. These methods have the disadvantage that they are difficult to adapt to the assay of the detergent-solubilized receptor. In the present paper we report the characteristics of an enzymic assay method for C3b receptor, which is suitable for examination of the receptor in soluble or membrane-bound form. The assay is based on the use of the receptor as an obligatory cofactor in the C3b/C4b-Ina-mediated proteolysis of C3b (Fearon, 1979). This assay method was used to compare the cofactor activities detected on intact and detergent-solubilized human erythrocytes and human tonsil B-lymphocytes. These activities are immunologically distinct from the serum cofactors C4bp and Factor H, and show different pH-dependence in the assay.

Materials and methods

Proteins and sera

Human C3 and C3b were isolated and radioiodinated as described previously (R. B. Sim *et al.*, 1981; R. B. Sim & E. Sim, 1981). C4 and C4b were prepared by the method of Bolotin *et al.* (1977). C4c was prepared from C4b (Press & Gagnon, 1981) and coupled at pH 9.5, in 0.15 M-NaCl/0.2 M-$NaHCO_3$, to CNBr-activated Sepharose 2B (Cuatrecasas, 1970). The resulting affinity resin contained 1.2 mg of C4c/ml of packed Sepharose. Factor H (Sim & DiScipio, 1982), C3b/C4b Ina (Hsiung *et al.*, 1982) and α_2M (Sim & DiScipio, 1982) were prepared by established methods. C5, prepared as described by Hammer *et al.* (1981), was generously provided by Mr. A. W. Dodds (of this Unit). C3b receptor solubilized from human erythrocytes was prepared as described by Fearon (1979).

C4bp, obtained from Sepharose 6B chromatography of the neutral euglobulin precipitate from 1 litre of human serum (Sim, 1981), was purified to homogeneity by salt-gradient elution from a column (30 cm × 2.0 cm diam.) of C4c–Sepharose equilibrated in 5 mM-EDTA/25 mM-potassium phosphate buffer, pH 7.0. The column was developed in the same buffer containing a 500 ml linear gradient of NaCl from 0 to 2 M. C4bp was eluted at concentrations greater than 0.8 M-NaCl, and was separated from the major contaminant, immunoglobulin M. In cases where the concentration of immunoglobulin M was very high (greater than 30% of the total protein) before affinity chromatography, it was necessary to perform the C4c–Sepharose chromatography twice to obtain pure C4bp. The C4c–Sepharose affinity-chromatography step is based on, and the results are consistent with, the C4bp–C4c interaction described by Ferreira & Nussenzweig (1979).

Haemolytically inactive C3 (C3u), haemolytically inactive C4 (C4u) and inactive α_2M (F-α_2M) were produced by incubation of the active forms of these proteins in 100 mM-hydroxylamine hydrochloride/5 mM-EDTA/25 mM-potassium phosphate buffer, pH 9.0, for 1 h at 37°C (R. B. Sim & E. Sim, 1981; von Zabern *et al.*, 1981; Barrett, 1981). Inactive forms of C3, C4 and C5 (R. B. Sim & E. Sim, 1981; DiScipio, 1981) were also produced by incubation of these proteins in half-saturated KBr for 18 h at 4°C, as described by Müller-Eberhard *et al.*, (1966). The proteinase-cleaved form of α_2M (Barrett, 1981) was produced by incubating α_2M (1 mg/ml in 140 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.5) with a 6-fold molar excess of trypsin (Sigma Chemical Co., Poole, Dorset, U.K.) for 30 min at 37°C. Soya-bean trypsin inhibitor (type I-S; Sigma Chemical Co.) (at molar equivalence with trypsin) was then added to inactivate remaining untrapped trypsin.

Cell preparation and solubilization procedures

Human erythrocytes were obtained from the Regional Blood Transfusion Service, John Radcliffe Hospital, Oxford, U.K. Sheep erythrocytes were purchased from Tissue Culture Services, Slough, Berks., U.K. All erythrocytes were washed thoroughly in 140 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.5, and the buffy coat was completely removed before use.

Human erythrocyte membranes were prepared by osmotic lysis and were washed exhaustively in 0.5 mM-EDTA/5 mM-sodium phosphate buffer, pH 7.4. To solubilize C3b receptor, membranes were resuspended in 150 mM-NaCl/0.5 mM-EDTA/5 mM-sodium phosphate buffer, pH 7.4, to a concentration of 5 mg of protein/ml. The suspension was then made 1% (w/v) with respect to Triton X-100 and 1% (w/v) with respect to Nonidet P40, and incubated for 40 min at 37°C. Insoluble residue was pelleted by centrifugation at 100000 ***g*** for 1 h.

Tonsil lymphocytes were prepared and enriched for B-cells, as described previously (E. Sim & R. B.

Sim, 1981). Where indicated, cells were treated with trypsin by incubating (for 20min at 37°C) B-cells (10^7/ml) in RPMI 1640 medium (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) containing 0.06% (w/v) trypsin. A 5-fold molar excess of soya-bean trypsin inhibitor over trypsin was then added, and incubation was continued for 5min at 37°C. Cells were washed in $DGVB^{++}$ buffer [72.5mM-NaCl/2.5% (w/v) D-glucose/0.1% gelatin/0.15mM-$CaCl_2$/0.5mM-$MgCl_2$/2.5mM-sodium Veronal buffer, pH7.5] by centrifugation (at 400*g* for 5min) three times before use.

Solubilized extracts of lymphocytes were prepared by incubating B-cells (10^8/ml) in 3% (w/v) Brij 96/150mM-NaCl/2.5mM-di-isopropyl phosphorofluoridate for 15min on ice. This method gives 75% recovery of nuclei with complete loss of cell viability. The nuclei were sedimented by centrifugation (at 1000*g* for 5min), and then Triton X-100 was added to a final concentration of 0.1% (w/v) to the solubilized extract. In certain experiments, lymphocytes were rendered permeable with 1% (w/v) Triton X-100 and were used directly without removal of nuclei, as described in the appropriate Figure legends.

Immunological cross-reactivity

Samples of Sepharose (0.5ml packed volume) to which were bound covalently rabbit antibodies against Factor H or C4bp, or human immunoglobulin G as control, were incubated (for 16h at 4°C) with 2ml of isolated C4bp, Factor H or solubilized extracts of human erythrocyte membranes or tonsil lymphocytes. Samples were centrifuged at 10000*g* for 5min, and the C3b/C4b Ina-cofactor activity in the supernatants was measured.

Assay of C3b/C4b-Ina-cofactor activity

Cofactor activity was measured in a total volume of 25–200μl containing pure C3b/C4b Ina (0–50μg/ml), ^{125}I-labelled C3b or ^{125}I-labelled C3u at 1.5–4.5μg/ml (10^6c.p.m./μg), and a source of cofactor. To limit the possibility of proteolysis arising from release of intracellular proteinases, assays were performed in the presence of final concentrations of 0.25mM-di-isopropyl phosphorofluoridate (Sigma Chemical Co.) and 20μg of soya-bean trypsin inhibitor/ml.

Controls in which ^{125}I-labelled C3b or ^{125}I-labelled C3u was incubated with cofactor alone or with C3b/C4b Ina alone were performed in all experiments.

The mixtures were incubated at 37°C, and at various times samples were withdrawn and added to an equal volume of 2% (w/v) sodium dodecyl sulphate/6M-urea/0.2M-sodium phosphate buffer, pH7.0, to stop the reaction. After reduction and alkylation of samples, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as previously described (E. Sim *et al.*, 1981). The cleavage pattern of the radio-iodinated proteins was observed by radioautography of the dried and stained gel. The gel tracks were cut into 1mm strips, and the radioactivity associated with each strip was measured in an LKB Wallac 1270 Rackgamma counter. The rate of degradation of the α'-chain of C3b or the α-chain of C3u was calculated as shown previously (E. Sim *et al.*, 1981), by measuring the proportion of the total radioactivity associated with the α-chain or α'-chain position on the gels. The decrease of radioactivity in the α-chain or the α'-chain was plotted as a function of time, and the initial rate of degradation was determined graphically.

Results

Degradation of C3b in the presence of intact cells

When ^{125}I-labelled C3b is incubated with purified C3b/C4b Ina alone (Fig. 1*a*), no degradation of C3b is observed within 1h at 37°C. If, however, intact human erythrocytes (Fig. 1*a*) or intact human B-enriched tonsil lymphocytes (Fig. 1*b*) are added to the mixture of C3b and C3b/C4b Ina, the C3b is degraded rapidly. Incubation of ^{125}I-labelled C3b with either cell type alone, in the absence of C3b/C4b Ina, does not cause detectable degradation of C3b (Figs. 1*a* and 1*b*). The occurrence of degradation only when both C3b/C4b Ina and cells are present is consistent with the presence of a C3b/C4b Ina cofactor in the cell preparations possessing an activity like that of Factor H. When sheep erythrocytes or intact trypsinized B-lymphocytes are incubated with C3b/C4b Ina plus ^{125}I-labelled C3b, no degradation of C3b is observed (Figs. 1*a* and 1*b*).

Extraction of the C3b/C4b-Ina-cofactor activity from membranes

After osmotic lysis of human erythrocytes and washing of the membranes to remove all visible haemoglobin coloration, as described in the Materials and methods section, no C3b/C4b-Ina-cofactor activity was detectable in the supernatant from lysed cells or in the low-ionic-strength EDTA/phosphate washes. Almost 90% of the activity was present in the erythrocyte-'ghost' fraction. Subsequent solubilization of the erythrocyte 'ghosts' with Triton X-100 and Nonidet P40, as described in the Materials and methods section, resulted in selective solubilization of about 27% of the membrane protein, but greater than 95% of the C3b/C4b-Ina-cofactor activity.

Pattern of degradation of C3b or C3u

C3b, when incubated with C3b/C4b Ina and

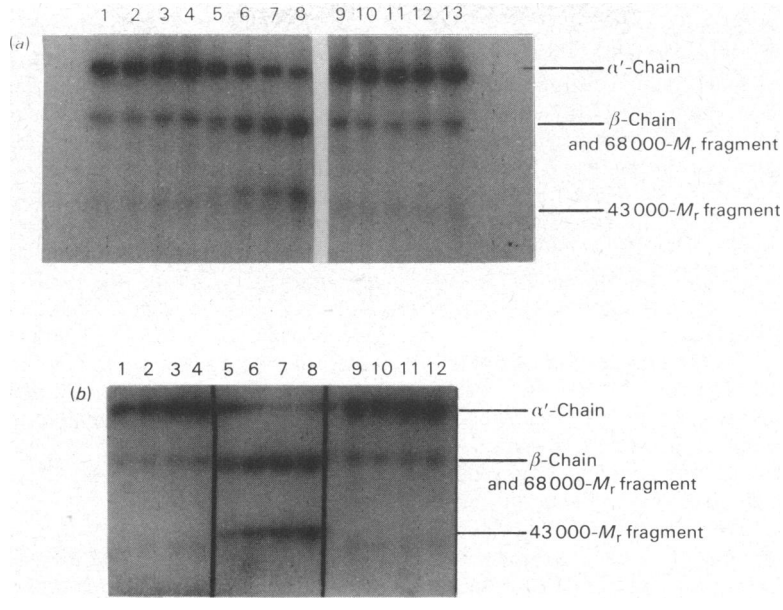

Fig. 1. *C3b/C4b-Ina-cofactor activity of intact cells*

(*a*) Human erythrocytes (2.5×10^8) were incubated with ^{125}I-labelled C3b (0.3 μg) only (tracks 1–4) or with C3b/C4b Ina (2 μg) and ^{125}I-labelled C3b (0.3 μg) (tracks 5–8). Sheep erythrocytes (2.5×10^8) were incubated with ^{125}I-labelled C3b (0.3 μg) amd C3b/C4b Ina (2 μg) (tracks 9–12). As a control, ^{125}I-labelled C3b was incubated with C3b/C4b Ina without cells (track 13). All incubations were done at 37°C in a total volume of 150 μl of DGVB^{++} buffer. Samples (20 μl) were removed after 5 min (tracks 1, 5 and 9), 15 min (tracks 2, 6 and 10), 30 min (tracks 3, 7 and 11) and 60 min (tracks 4, 8, 12 and 13). The reaction was stopped, and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described in the Materials and methods section. A radioautograph is shown. (*b*) Human tonsil B-lymphocytes (5×10^6) were incubated with ^{125}I-labelled C3b (0.3 μg) only (tracks 1–4) or with C3b/C4b Ina (2 μg) and ^{125}I-labelled C3b (0.3 μg) (tracks 5–8). Trypsinized tonsil B-lymphocytes (5×10^6) were incubated with ^{125}I-labelled C3b (0.3 μg) and C3b/C4b Ina (2 μg). All incubations were done at 37°C in a total volume of 150 μl of DGVB^{++} buffer. Samples (20 μl) were removed after 5 min (tracks 1, 5 and 9), 15 min (tracks 2, 6 and 10), 30 min (tracks 3, 7 and 11) and 60 min (tracks 4, 8 and 12). The reaction was stopped, and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described in the Materials and methods section. A radioautograph is shown.

human erythrocytes, erythrocyte 'ghosts' (Fig. 2) or B-lymphocytes or solubilized extracts from these cells, is cleaved to form the defined product, iC3b. The α'-chain (apparent M_r 108000) of C3b is cleaved to form two fragments of apparent M_r 68000 and 46000. The 46000-M_r fragment is then further cleaved to form a 43000-M_r fragment. In Fig. 2 the end products of apparent M_r 68000 and 43000 are clearly seen, with smaller quantities of the transient 46000-M_r intermediate. This pattern of cleavage of C3b to iC3b by C3b/C4b Ina is identical with that found when Factor H is supplied as cofactor (E. Sim *et al.*, 1981; Harrison & Lachmann, 1980). The cleavage of ^{125}I-labelled C3u in the presence of C3b/C4b Ina plus the cofactor from human lymphocytes or erythrocytes is again identical with the pattern seen when Factor H is supplied as cofactor: the α-chain of C3u is first cleaved to form fragments of apparent M_r 76000 and 46000, and the 46000-M_r fragment is then trimmed to apparent M_r 43000 (E. Sim *et al.*, 1981).

Dependence of the rate of C3b cleavage on the concentration of cofactor or enzyme

The rate of cleavage of C3b by C3b/C4b Ina is dependent on the amount of the cofactor supplied. The effect of incubating fixed concentrations of ^{125}I-labelled C3b and C3b/C4b Ina with increasing numbers of intact human erythrocytes or intact human B-lymphocytes is shown in Fig. 3. The rate of C3b cleavage increases linearly with cell number over the range illustrated. Similarly, the rate of C3b cleavage in the presence of a fixed quantity of cell-associated cofactor increases with C3b/C4b-Ina

concentration. Fig. 4 shows this concentration dependence for C3u cleavage in the presence of a fixed quantity of erythrocyte 'ghosts'.

Effect of cell lysis on cofactor activity

If human erythrocytes are incubated with C3b and C3b/C4b Ina in the presence of detergent to render the cells permeable, then the rate of C3b cleavage remains essentially the same as is observed with an equivalent number of intact cells (Fig. 5*a*). If, however, B-lymphocytes are rendered permeable by detergent, the cofactor activity detected is much greater than that found with intact cells (Fig. 5*b*). In six experiments, with batches of lymphocytes prepared over a period of 3 months, the increase in

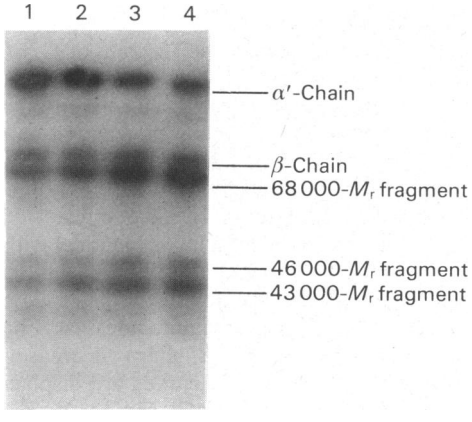

Fig. 2. *C3b-cleavage pattern by erythrocyte C3b/C4b Ina cofactor*

Human erythrocyte 'ghosts' (300 μg of protein) were incubated at 37°C with ^{125}I-labelled C3b (0.5 μg) and C3b/C4b Ina (4.5 μg) in a total volume of 150 μl of 140 mM-NaCl/5 mM-sodium phosphate buffer, pH 7.5. Samples (20 μl) were removed at 2 min (track 1), 10 min (track 2), 30 min (track 3) and 60 min (track 4). The reaction was stopped, and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described in the Materials and methods section. A radioautograph is shown.

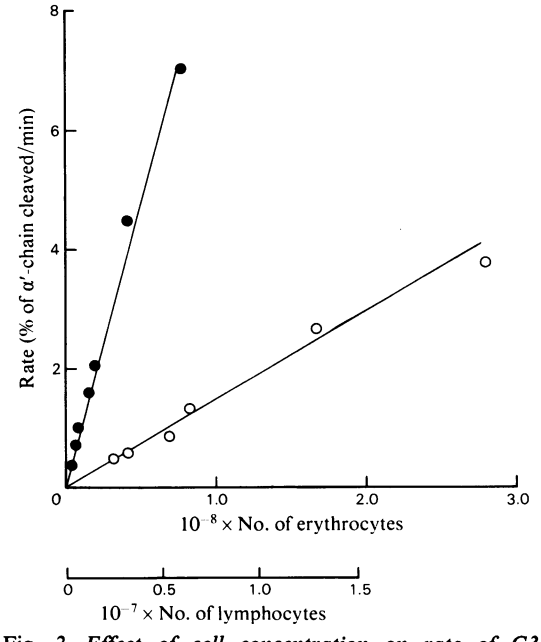

Fig. 3. *Effect of cell concentration on rate of C3b cleavage*

Human erythrocytes (○) or human tonsil B-lymphocytes (●) were incubated with ^{125}I-labelled C3b (0.75 μg) and C3b/C4b Ina (4.5 μg) in a total volume of 200 μl of DGVB^{++} buffer at 37°C. Samples (20 μl) were removed at various times, and the initial rate of cleavage of the α'-chain of C3b was determined as described in the Materials and methods section. The abscissa shows the number of cells per 200 μl of incubation mixture. Controls without C3b/C4b Ina showed no cleavage of the α'-chain.

Table 1. *Comparison of immunological cross-reactivity of C3b/C4b Ina cofactors*

Solubilized extract of human tonsil B-lymphocytes (800 μg total protein), solubilized extract of human erythrocyte membranes (1.6 mg total protein), Factor H (270 μg) or C4bp (232 μg) were each incubated (for 16 h at 4°C) in a total volume of 2.5 ml with Sepharose-bound antibody. The activity remaining unbound to the antibody–Sepharose was measured and is shown relative to the amount recovered from Sepharose-bound human immunoglobulin G, expressed as 100% recovery.

	C3b/C4b-Ina-cofactor activity remaining (%)		
Antibody coupled to Sepharose ...	Rabbit anti-(Factor H) antibody	Rabbit anti-C4bp antibody	Human immunoglobulin G
Cofactor			
Factor H	2	94	100
C4bp	95	3	100
Solubilized B-lymphocytes	92	96	100
Solubilized erythrocyte 'ghosts'	90	94	100

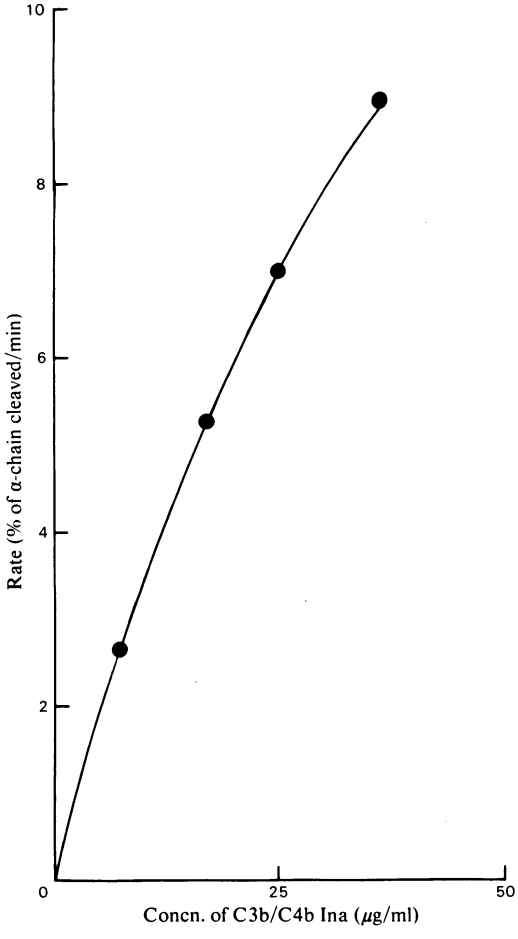

Fig. 4. *Effect of concentration of C3b/C4b Ina on rate of C3u cleavage*

Human erythrocyte 'ghosts' (equivalent to 3×10^8 cells) were incubated at 37°C with ^{125}I-labelled C3u (0.4 μg) in a total volume of 100 μl of 10 mM-sodium phosphate buffer, pH 7.5, containing various concentrations of C3b/C4b Ina. The initial rate of cleavage of the α-chain of C3u was determined as described in the Materials and methods section.

detectable cofactor activity found after detergent treatment was always in the range 6.5–8.5-fold.

Comparison of cell-associated C3b/C4b Ina cofactors with those from plasma

Recognition by antisera. The C3b/C4b-Ina-cofactor activities from human erythrocytes or from lymphocytes are not immunologically cross-reactive with either Factor H or C4bp. As shown in Table 1, when solubilized extracts from either human erythrocytes or lymphocytes are incubated with antibodies against either Factor H or C4bp coupled to Sepharose there is no decrease in cofactor activity, even though the capacity of the antibody–Sepharose preparations is sufficient to deplete at least 270 μg of Factor H or 232 μg of C4bp respectively.

pH-dependence. The pH-dependence of the cleavage of C3b by C3b/C4b Ina in the presence of Factor H has already been reported to show an optimum below pH 6 (Crossley & Porter, 1980), and this is illustrated in Fig. 6. However, the pH profile for the cleavage of C3b by C3b/C4b Ina in the presence of cellular cofactors from both lymphocytes and erythrocytes has a broad pH optimum between 7 and 8 (Fig. 6), which is distinct from the profile seen with Factor H.

Ionic-strength-dependence. The rate of C3b/C4b-Ina-mediated cleavage of C3b with both plasma cofactors and solubilized cellular cofactors is progressively inhibited by increasing NaCl concentration (Fig. 7). There is a sharp fall in activity with increasing salt concentration over the range 25–150 mM-NaCl for Factor H. The effect of ionic strength with C4bp as cofactor is identical with that of Factor H, but the results have been omitted for clarity. Although the reaction in the presence of the cofactors from either lymphocytes or human erythrocytes also shows a sharp decrease with increasing salt strength, the curves are displaced to higher concentrations of NaCl compared with the serum C3b/C4b Ina cofactors. The same pattern of inhibition by increasing salt concentration is seen for both the solubilized purified erythrocyte cofactor and for cofactor activity in erythrocyte 'ghosts'.

Specificity of the C3b/C4b Ina cofactors

The purified plasma cofactors, C4bp and Factor H, and the solubilized cofactor activities from lymphocytes and erythrocytes were incubated with C3b/C4b Ina and a range of possible substrates in order to determine the cleavage specificity of the enzyme-plus-cofactor system. The results are shown in Table 2. In agreement with the work of others, Factor H mediates cleavage of C3b and C3u (Whaley & Ruddy, 1976; Crossley & Porter, 1980), but not of C4b or C4u. C4bp participates in cleavage of C4b and C4u (Fujita *et al.*, 1978; Press & Gagnon, 1981) and also of soluble C3b and C3u (Nagasawa & Stroud, 1977; E. Sim *et al.*, 1981). The solubilized cofactor from erythrocytes, as found by Iida & Nussenzweig (1981), mediates C3b/C4b-Ina-dependent cleavage of C3b, C3u, C4b and C4u. The lymphocyte cofactor has the same cofactor specificity range as the erythrocyte material and C4bp (Table 2). Despite the structural similarities between C5, α_2M, C3 and C4 (R. B. Sim & E. Sim, 1981; DiScipio, 1981), the inactivated or cleaved forms of C5 and α_2M do not serve as

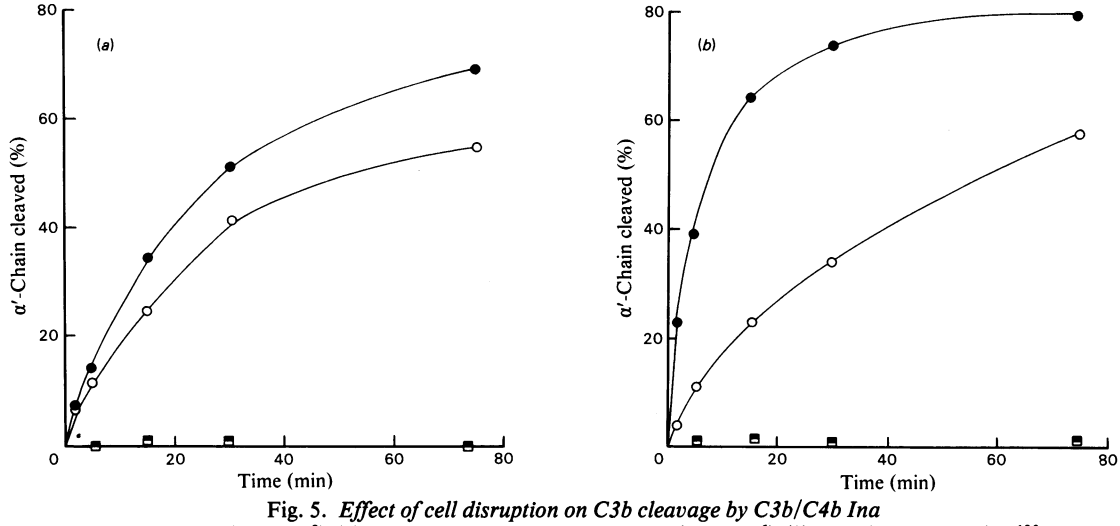

Fig. 5. *Effect of cell disruption on C3b cleavage by C3b/C4b Ina*

Human erythrocytes (2.5×10^8) (*a*) or human tonsil B-lymphocytes (3.0×10^6) (*b*) were incubated with ^{125}I-labelled C3b (0.5 μg) (□ and ■) or with ^{125}I-labelled C3b (0.5 μg) and C3b/C4b Ina (3.5 μg) (○ and ●) in a total volume of 150 μl of DGVB^{++} buffer (□ and ○) or in the same buffer containing 1% (w/v) Triton X-100 (■ and ●). All incubations were performed at 37°C. At various times, samples (20 μl) were removed, the reaction was stopped and the cleavage of the α'-chain of C3b was determined as described in the Materials and methods section.

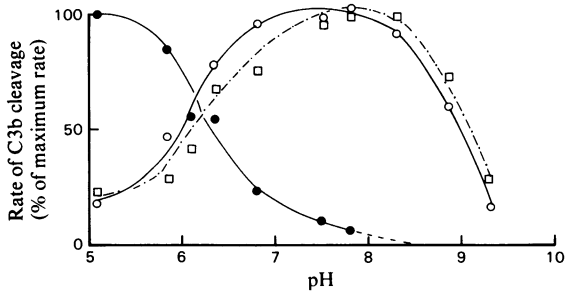

Fig. 6. *Effect of pH on C3b/C4b-Ina-cofactor activities*

Factor H (22.2ng) (●), solubilized extract from human tonsil B-lymphocytes (100 μg of protein) (□) or purified C3b/C4b Ina cofactor from human erythrocyte membranes (5.2 μg) (○) was incubated at 37°C with ^{125}I-labelled C3b (2.6 μg) and C3b/C4b Ina (3.5 μg) in a total volume of 160 μl of 100mM-NaCl/0.7mM-EDTA/0.7mM-di-isopropyl phosphorofluoridate/soya-bean trypsin inhibitor (13.3 μg/ml)/25mM-Mes (4-morpholine-ethanesulphonic acid)/25mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/25mM-Tris/25mM-glycine adjusted at 37°C to various values of pH. At different times, samples (20 μl) were removed from the incubation mixture and the initial rate of cleavage of the α'-chain of C3b was determined after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described in the Materials and methods section. The rate of cleavage of the α'-chain is expressed as a percentage of the maximum value obtained with each C3b/C4b Ina cofactor.

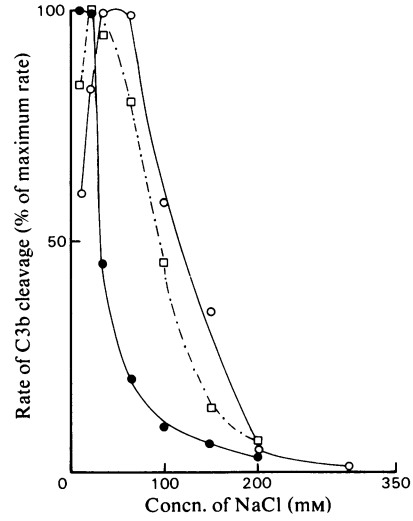

Fig. 7. *Effect of ionic strength on C3b/C4b-Ina-cofactor activities*

Factor H (14.8ng) (●), solubilized extract from human tonsil B-lymphocytes (50 μg of protein) (□) or purified C3b/C4b Ina cofactor from human erythrocyte membranes (3 μg) (○) was incubated at 37°C with ^{125}I-labelled C3b (0.7 μg) and C3b/C4b Ina (1.6 μg) in 150 μl of 0.7mM-EDTA/0.7mM-di-isopropyl phosphorofluoridate/soya-bean trypsin inhibitor (13.3 μg/ml)/2.5mM-potassium phosphate buffer, pH 7.4, containing different concentrations of NaCl. The initial rate of cleavage of the α'-chain of C3b was measured and is expressed as a percentage of the maximum value obtained with each C3b/C4b Ina cofactor.

Table 2. *Specificity of C3b/C4b Ina cofactors*
Substrates (0.5 mg/ml) in 10 mM-NaCl/0.5 mM-EDTA/10 mM-Tris/HCl buffer, pH 7.0, were incubated for 2 h at 37°C with C3b/C4b Ina (50 μg/ml) in the presence of one of the cofactors, namely C4bp (100 μg/ml), Factor H (20 μg/ml), solubilized extracts of lymphocytes (equivalent to 10^8 cells/ml) or solubilized erythrocyte membranes (equivalent to 10^9 cells/ml). The final reaction volume was 50 μl. Samples were then reduced and alkylated and examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Gels were stained with Coomassie Blue, and the extent of specific proteolytic cleavage of each substrate was determined. + indicates greater than 90% cleavage; – indicates no detectable cleavage. Appropriate controls containing either no cofactor, or no C3b/C4b Ina, were examined for each substrate, and in all of these cleavage was not detectable.

	Observation of substrate cleavage in the presence of C3b/C4b Ina plus:			
Substrate	Factor H	C4bp	Solubilized B-lympho-cyte extract	Solubilized erythrocyte 'ghosts'
C3	–	–	–	–
C3u	+	+	+	+
C3b	+	+	+	+
C4	–	–	–	–
C4u	–	+	+	+
C4b	–	+	+	+
C5 or C5u	–	–	–	–
F-α_2M, S-α_2M or trypsin-cleaved α_2M	–	–	–	–

substrates for C3b/C4b Ina in the presence of any of the cofactors.

Discussion

The results obtained in the present work show that there is an activity associated with the surface of both human erythrocytes and B-lymphocytes that will act together with C3b/C4b Ina to cleave C3b, C3u, C4b and C4u. This activity has previously been identified as being associated with human erythrocyte membranes and has been equated with C3b receptor, a glycoprotein of M_r 195000–205000 (Fearon, 1979, 1980; Dobson *et al.*, 1981). The C3b receptor of B-lymphocytes has been suggested to be identical with the C3b receptor of human erythrocytes on the basis of cross-reactivity with polyclonal (Fearon, 1980) or monoclonal antibodies (Gerdes *et al.*, 1982; Iida *et al.*, 1982). Consistent with the demonstration of cross-reactivity between C3b receptors of erythrocytes and lymphocytes, we have found that the C3b/C4b-Ina-cofactor activity associated with erythrocytes and that associated with B-lymphocytes have the same characteristics of pH- and ionic-strength-dependence (Figs. 6 and 7) and mediate the action of C3b/C4b Ina on the same range of substrates (Table 2). In contrast, neither cell-associated cofactor is recognized by rabbit antibodies to the plasma cofactors, Factor H and C4bp (Table 1). The cell-associated cofactor activities are distinct in ionic-strength-dependence from Factor H and C4bp (Fig. 7) and distinct in pH-dependence (Fig. 6) and substrate range (Table 2) from Factor H. The cell-associated and plasma cofactors, however, all mediate the same pattern of C3b/C4b-Ina-dependent proteolysis, namely cleavage at two sites in the α′- or α-chains of C3b (Fig. 2), C3u, C4b and C4u.

The method of assaying C3b receptor described in the present paper relies on kinetic measurement of C3b/C4b-Ina-cofactor activity. The initial rate of specific proteolysis of the α′-chain of C3b in the presence of a fixed quantity of C3b/C4b Ina is proportional to the amount of cell-associated C3b/C4b Ina cofactor present (Fig. 3). Similarly, in the presence of a constant quantity of cofactor, the rate of C3b cleavage is directly related to the concentration of C3b/C4b Ina (Fig. 4). The absence of proteolysis of C3b when C3b is incubated without added C3b/C4b Ina in the presence of erythrocytes or lymphocytes (Fig. 1) or with cells made permeable by detergent treatment (controls to Figs. 5*a* and 5*b*) clearly indicates that there is no significant endogenous or passively adsorbed C3b/C4b Ina present in the cell preparations.

The lack of C3b/C4b-Ina activity associated with B-lymphocytes, and especially with detergent-lysed lymphocytes, is in contrast with the results reported by Lambris *et al.* (1980), who have claimed that tonsil B-lymphocytes secrete C3b/C4b Ina when Factor H is present. Wilson *et al.* (1982) have also been unable to detect C3b/C4b Ina associated with human lymphocytes.

Factor H has been reported to be associated with a small proportion of peripheral-blood B-lymphocytes, probably by passive adsorption (Wilson *et al.*, 1982), but it is unlikely that this contributes significantly to the C3b/C4b-Ina-cofactor activity detectable on intact lymphocytes. A small proportion (8%) of lymphocyte-associated cofactor activity is lost on treatment with anti-(Factor H)–Sepharose (Table 1), but it is probable that this represents only a small handling loss.

Measurement of the rate of cleavage of C3b by C3b/C4b Ina in the presence of cofactor is a useful method for quantifying cofactor activity. With intact erythrocytes or lymphocytes as the source of cofactor, the activity measurements, expressed as activity per cell, show that approximately 80-fold more cofactor is present on B-lymphocytes than is present

on erythrocytes (Fig. 3). By the use of immunoassays, it has been estimated that there are 950–1400 C3b receptor molecules per erythrocyte (Fearon, 1980; Iida *et al.*, 1982). Immunoassay methods have also indicated 21000 to 60000 C3b receptor sites on the surface of lymphocytes (Fearon, 1980; Fearon & Abrahamson, 1981). Our finding of an 80-fold difference in cofactor activity between intact erythrocytes and intact B-lymphocytes is consistent with estimates of about 1000 and 60000 C3b receptor molecules on the surfaces of erythrocytes and lymphocytes respectively.

On solubilization of human erythrocytes, routinely we find that the rate of C3b/C4b-Ina-catalysed cleavage of C3b increases slightly (by up to 20%) (Fig. 5*a*). This is likely to be due to the increased diffusion rate of the soluble, as compared with the membrane-bound, receptor. However, the rate of C3b/C4b-Ina-catalysed cleavage of C3b in the presence of solubilized lymphocytes increases 6.5–8.5-fold compared with cleavage rates in the presence of intact lymphocytes (Fig. 5*b*). Thus it appears that the amount of C3b/C4b Ina cofactor that is present on the surface of B-lymphocytes represents only 12–15% of the total amount of C3b/C4b Ina cofactor that is present in lymphocytes.

Results of studies on the binding of C3b and C3 to B-lymphocytes at 37°C have suggested that these ligands are internalized (E. Sim & R. B. Sim, 1981), and it has also been shown that C3b receptors on human polymorphonuclear leucocytes can undergo internalization on binding of soluble multivalent ligand (Fearon *et al.*, 1981). In the case of C3b receptors on polymorphonuclear leucocytes, it has been suggested that the receptors are present within coated pits on the cell surface and can undergo internalization via coated vesicles. Coated vesicles and coated pits have also been reported in lymphoblastoid cells (Salisbury *et al.*, 1981). Cell types (mainly fibroblasts and phagocytic cells) in which receptor-mediated endocytosis via a coated-vesicle system has been most actively studied do not appear to contain large internal pools of receptor molecules. However, a hepatocyte system has been described in which only 10% of the asialoglycoprotein receptor content is on the cell surface, the remaining 90% being localized on internal membranes (for discussion see Kaplan, 1981). From the results described above we also suggest that B-lymphocytes contain a large internal pool of C3b receptors.

Iida *et al.* (1982) have estimated by immunoassay that B-lymphocytes contain about 360000 C3b receptor molecules per cell. This is 6–18-fold higher than the estimates made by Fearon and colleagues (Fearon, 1980; Fearon & Abrahamson, 1981; Fearon *et al.*, 1981) discussed above. Iida *et al.* (1982) made their estimate with solubilized lymphocytes but assumed that all of the receptor molecules detected were present on the surface, since pretreatment of the intact cells with trypsin (30min, 37°C) destroyed all the receptor molecules. However, the phenomenon of membrane recycling, as discussed by Kaplan (1981), may account for the exposure of the entire internal and external pool of receptors to trypsin during the relatively long incubation period used. Similar trypsin treatment of lymphocytes completely removes C3b/C4b-Ina-cofactor activity from intact cells (Fig. 1*b*). No cofactor activity is detectable if these trypsinized lymphocytes are subsequently solubilized.

We suggest that the estimate of 360000 receptor molecules per cell (Iida *et al.*, 1982) represents both the cell surface and internal content of C3b receptors. The discrepancy between receptor numbers produced by Fearon and colleagues (Fearon, 1980; Fearon & Abrahamson, 1981; Fearon *et al.*, 1981) (20000–60000 per cell) and Iida *et al.* (1982) (360000 per cell) is consistent with the 6.5–8.5-fold increase in detectable C3b/C4b-Ina-cofactor activity that we observe on detergent-solubilization of lymphocytes.

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