Complement Factor H-binding protein of Raji cells and tonsil B lymphocytes

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Several reports have indicated that Factor H has specific effects on certain cell populations, suggesting that Factor H receptors may exist. Lambris & Ross [(1982) *J. Exp. Med.* **155**, 1400–1411] purified a protein from Raji B-lymphoblastoid cell culture supernatants, using Factor H–Sepharose affinity chromatography. This species appeared to consist of two disulphide-linked components each of M_r 50 000, with an additional 50 000- M_r chain attached non-covalently. The existence of cell-surface Factor H-binding proteins has now been re-investigated with ^{125}I surface-labelled Raji and tonsil B cells. Non-ionic-detergent extracts of the cells, in 0.1% Nonidet P40/10 mM-sodium phosphate buffer, pH 7.4, were incubated with Factor H–Sepharose in the presence of proteinase inhibitors. After the beads had been washed, bound components were eluted with 50 mM-NaCl. A single radioactive species was eluted from the resin, which migrates identically with Factor H (apparent M_r 170 000) in SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions. Biosynthetic radiolabelling studies confirmed that this species was synthesized by Raji cells. Examination of culture supernatants from biosynthetically radiolabelled Raji cells showed again the presence of a single soluble species that bound to Factor H–Sepharose, but this species was of lower M_r (approx. 105 000) than the membrane-derived protein. The soluble form may be produced by proteolysis of the membrane form, or may be of separate origin. The similarity in size of the cell-surface protein to Factor H was initially confusing, but it is distinct from cell-surface Factor H on the basis of three criteria: (1) it is not recognized by anti-(Factor H) monoclonal antibodies MRC OX23 and MRC OX24, nor by polyclonal F(ab')₂ anti-(Factor H); (2) it does not bind to Zn²⁺-chelate resin, whereas Factor H does; (3) cell-surface Factor H present on U937 cells does not bind to Factor H–Sepharose.

INTRODUCTION

Factor H is one of the control proteins of the complement system (Whaley & Ruddy, 1976). It is an abundant single-chain plasma glycoprotein (M_r approx. 150 000). The primary sequence of Factor H is made up of repetitive internally homologous units, each about 60 amino acid residues long, characterized by highly conserved cysteine, proline and aromatic residues. These repeat units are present in functional homologues of Factor H, such as CR1 and C4bp, and also in a wide range of other complement and non-complement proteins (Sim *et al.*, 1986; Ripoché *et al.*, 1986; Reid *et al.*, 1986).

The main function of Factor H is the regulation of the activity of C3b, the cleavage product of the most abundant complement component, C3. During activation of the alternative pathway of complement, Factor B binds to C3b. This complex is then activated to form the enzyme complex C3bBb, which can cleave native C3 molecules. The principal way of regulation of this feedback process is the inactivation of C3b. Factor H is one of the essential cofactors for the cleavage of C3b by the serine proteinase Factor I. Two membrane proteins, CR1 and 'gp 45-70', are also known to possess the same activity (Fearon, 1979; Seya *et al.*, 1986). In addition to

its cofactor activity, Factor H regulates the formation and decay of the C3 convertase and C5 convertase enzymes (C3bBb and C3b₂Bb respectively) of the alternative pathway by inhibiting the interaction between C3b and Factor B (Pangburn *et al.*, 1978).

Several reports suggest that lymphocytes and monocytes/macrophages have receptors for Factor H. Lambris *et al.* (1980) reported that B lymphocytes can be triggered by Factor H to release endogenous Factor I. Tsokos *et al.* (1985) have demonstrated that Factor H acts directly on B cells by blocking the differentiation but not the proliferation of these cells. Moreover, Factor H has been shown to trigger the oxidative metabolism of monocytes (Schopf *et al.*, 1982) and the release of prostaglandin E and thromboxane from macrophages (Hartung *et al.*, 1984). Lambris & Ross (1982) characterized a molecule of the lymphocyte membrane that reacted with an antibody to anti-(Factor H) idiotype. This species appeared to consist of two disulphide-linked components each of M_r 50 000 with an additional 50 000- M_r chain attached non-covalently. They identified a molecule with the same characteristics from the supernatants of Raji cell cultures, using Factor H–Sepharose affinity chromatography.

We have now re-investigated the existence of cell-surface Factor H-binding protein using Factor H–

Abbreviation used: RAM-F(ab')₂, Fab fragments of affinity-purified rabbit anti-(mouse IgG) antibodies.

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Sephacrose 4B affinity chromatography of detergent extracts of ^{125}I -labelled Raji and tonsil B cells. Here we report the presence of a single-chain Factor H-binding molecule of M_r 170000 in the membrane of B cells. This species has been found to migrate identically with Factor H on SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions, but has been proven to be distinct from soluble or cell-surface Factor H. The molecule is synthesized by the cells and inhibits Factor H-mediated rosette formation of Raji cells.

MATERIALS AND METHODS

Reagents and media

Nonidet P40, soya-bean trypsin inhibitor (type 1-S), Pepstatin A (pepsin inhibitor), 1,10-phenanthroline, bovine serum albumin, iodoacetamide and di-isopropyl phosphorofluoridate were all purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Iodogen was obtained from Pierce and Warriner (Chester, U.K.). Autofluor was from National Diagnostics (Somerville, NJ, U.S.A.). All tissue-culture materials were purchased from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.) unless otherwise stated.

Buffers

Phosphate-buffered saline was 8.2 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 buffer, pH 7.5, containing 139 mM- NaCl and 3 mM- KCl . Dulbecco's medium A and B was phosphate-buffered saline containing 1 mM- CaCl_2 and 1 mM- MgCl_2 .

Lysis buffer 'A' was 10 mM-sodium phosphate buffer, pH 7.5, containing 0.15 mM- CaCl_2 , 0.5 mM- MgCl_2 , 1% (w/v) Nonidet P40, 100 μg of soya-bean trypsin inhibitor/ml, 5 mM-iodoacetamide, 2.5 mM-di-isopropyl phosphorofluoridate, 20 μM -1,10-phenanthroline and 5 μg of pepstatin/ml. Lysis buffer 'B' was as lysis buffer 'A' but containing 0.1% Nonidet P40. Lysis buffer 'C' was as lysis buffer 'B' but containing 1 mM-EDTA instead of CaCl_2 and MgCl_2 .

Proteins and affinity adsorbents

Factor H and C1q were prepared as described by Sim & DiScipio (1982) and Reid (1981) respectively. Polyclonal rabbit antibodies to human Factor H were purified from antiserum by triple 14% (w/v) Na_2SO_4 precipitation, and the purified antibodies were digested to form the Fab fragments (Micklem *et al.*, 1984). Mouse monoclonal antibodies to Factor H, MRC OX23 and MRC OX24 (Sim *et al.*, 1983), were purified from ascites fluid by triple 18% (w/v) Na_2SO_4 precipitation. The control monoclonal antibody MRC OX1 [rat anti-(mouse leucocyte common antigen) (Sunderland *et al.*, 1979)] and ^{125}I -labelled RAM-F(ab')₂ were generously supplied by Dr. A. F. Williams, M.R.C. Cellular Immunology Unit, Oxford.

Proteins were coupled to CNBr-activated Sepharose 4B (Pharmacia, Milton Keynes, Bucks., U.K.) as recommended by the manufacturer. The coupling buffer was 0.1 M- NaHCO_3 , pH 8.3, containing 0.5 M- NaCl . The final density of coupled proteins (mg per ml of gel) was: Factor H, 0.8; C1q, 0.2; Fab anti-(Factor H), 4.0; MRC OX23 antibody, 1.0; MRC OX24 antibody, 1.0. Non-immune human IgG was coupled to CNBr-activated Sepharose 4B at a final concentration of 10 mg/ml. Gelatin-Sepharose 4B was obtained from Pharmacia.

Cells

Human tonsil cells were prepared and enriched for B lymphocytes as described previously (Micklem *et al.*, 1984).

Raji cells (Flow Laboratories) were grown in RPMI 1640 medium supplemented with 5% (v/v) foetal-calf serum, penicillin (100 i.u./ml), streptomycin (100 μg /ml), kanamycin (50 μg /ml), glutamine (2 mM) and sodium pyruvate (1 mM). Spent medium was collected when the cells had grown to approx. 10^6 /ml. U937 cells were grown as described previously (Malhotra & Sim, 1985).

Biosynthetic labelling of Raji cells with [^{35}S]cysteine

Raji cells harvested from the culture medium were washed twice with Dulbecco's medium A and B, followed by one wash in cysteine-free medium (RPMI 1640-Select-Amine Kit; Gibco, Grand Island, NY, U.S.A.). Then the cells were resuspended in 6 ml of cysteine-free medium containing 5% (v/v) foetal-calf serum at 1.6×10^6 cells/ml. L-[^{35}S]Cysteine (400 μCi ; 39.6 Ci/mmol; Amersham International, Amersham, Bucks., U.K.) in 250 μl of cysteine-free medium was added to the cells, and then they were cultured under normal conditions for 25 h. At the end of the culture period cells were pelleted by centrifugation. To the culture supernatant were added di-isopropyl phosphorofluoridate (2.5 mM), iodoacetamide (2 mM), soya-bean trypsin inhibitor (50 μg /ml) and Nonidet P40 (0.1%), and it was kept at -70°C until used. The cells were washed twice in Dulbecco's medium A and B, extracted in 2 ml of lysis buffer 'A' and kept at -70°C until used. Before use the preparations were cleared of detergent-insoluble material by centrifugation at 100000 g for 2 h.

Surface radioiodination of cells

Cells were washed three times with Dulbecco's medium A and B, and the concentration of the suspension was adjusted to 2×10^7 cells/ml. Labelling was carried out by the Iodogen method (Markwell & Fox, 1978) with 1 mCi (37 MBq) of Na^{125}I (carrier-free from Amersham International) for radioiodination of 1 ml of the suspension. After labelling, cells were washed three times in Dulbecco's medium A and B, then extracted in lysis buffer 'A'. After 15 min on ice the extract was centrifuged at 1000 g and gel-filtered on a PD-10 column [Sephadex G-25 (medium grade); Pharmacia] pre-equilibrated in lysis buffer 'B'. The extract was cleared of detergent-insoluble material and used for experiments on the day of preparation. The gel-filtration step resulted in a 2-fold dilution of the detergent extract.

Affinity chromatography on protein-bearing resins

Preparations used for affinity chromatography were first pre-cleared by incubating 1 ml of extract with 1 ml (packed vol.) of non-immune human IgG-Sepharose 4B on a rotary stirrer for 1 h at 4°C . Then 0.2 ml of solubilized pre-cleared membrane of radiolabelled cells or 2 ml of the culture supernatant of biosynthetically labelled cells was incubated with 0.5 ml of Factor H-Sepharose, or in control samples with 0.5 ml of C1q- or gelatin-Sepharose 4B. The resins were equilibrated with lysis buffer 'B' before use. After mixing for 2 h at 4°C on a rotary stirrer, beads were transferred into small columns (0.8 cm-diam. disposable columns; Pierce and

Warriner) and washed with lysis buffer 'B' until the eluted radioactivity was at a minimum. Then columns were eluted either with lysis buffer 'B' containing 50 mM-NaCl or with lysis buffer 'C' containing 50 mM-NaCl, as indicated.

Affinity chromatography on Zn^{2+} -chelate resin

The pre-cleared ^{125}I -labelled cell-membrane extract (0.2 ml) was dialysed into 150 mM-NaCl/20 mM-sodium phosphate buffer, pH 6, and then applied to small columns (2 cm $\times$ 0.8 cm diam.) of Zn^{2+} -chelate resin (Boehringer Mannheim, Lewes, Sussex, U.K.) equilibrated in the same buffer. Material that did not bind to the resin was collected, and after being desalted on a PD-10 column equilibrated in lysis buffer 'B' it was subjected to affinity chromatography on Factor H-Sepharose 4B as described above. The Zn^{2+} -chelate column was subsequently washed with 150 mM-NaCl/20 mM-sodium acetate buffer, pH 5, then with 200 mM-sodium EDTA, pH 7.4, to elute bound protein.

Binding of Factor H to Raji cells and B lymphocytes

To demonstrate the binding of Factor H to the cells, an indirect binding assay was performed in duplicate, as described by Mason & Williams (1986). For the dilution of the cells and proteins and also for washing, Dulbecco's medium A and B containing 0.5% bovine serum albumin and 0.01% NaN_3 was used throughout the assay, which was carried out on ice.

Serial dilutions of Factor H (25 μl) ranging from 78 $\mu\text{g}/\text{ml}$ to 1300 $\mu\text{g}/\text{ml}$ were added to 25 μl of Raji cells or tonsil B lymphocytes, at a density of 1×10^8 cells/ml. After 1 h incubation cells were washed, and 2×10^6 sheep erythrocytes in 25 μl of buffer were added to them together with 25 μl of 100-fold dilutions of the ascites fluids of MRC OX23 or MRC OX1 antibodies. Both monoclonal antibodies are of the IgG₁ subclass. Samples were incubated for 1 h, washed and then mixed with 25 μl of radiolabelled RAM-F(ab')₂ (200000 c.p.m./ μg to 2×10^7 c.p.m./ μg). After further incubation for 1 h cells were washed twice with 1 ml of buffer, and cell-associated radioactivity was measured.

Rosette formation of Raji cells with sheep erythrocytes bearing Factor H

Factor H was coupled to sheep erythrocytes by the CrCl_3 method by adding 1 ml of Factor H solution (1 mg/ml) to 1 ml of erythrocyte suspension (1%, v/v), as described by Johnstone & Thorpe (1982). Rosetting was performed by mixing 25 μl of Raji cell suspension (5×10^6 cells/ml) with 25 μl of Factor H/erythrocyte suspension (1%, v/v). After incubation at 37 °C for 10 min cells were gently centrifuged (80 g for 2 min) and put on ice. Rosette formation (i.e. binding of three or more indicator cells by the Raji cells) was evaluated by light microscopy 2 h later. A total of 200–250 Raji cells were counted.

The effect of Factor H-binding materials on rosetting was tested by preincubating 0.2 ml of Factor H-erythrocytes (1%, v/v, suspension) with 0.2 ml of different dilutions of the fractions specifically eluted from the Factor H-Sepharose resin. After incubation for 30 min at room temperature, the indicator cells were washed once with 1 ml of phosphate-buffered saline and mixed with the Raji cells, and rosette-forming cells were

Fig. 1. Binding of isolated Factor H to tonsil B lymphocytes

An indirect binding assay with Factor H, an anti-(Factor H) antibody and a second antibody was performed as described by Mason & Williams (1986). B lymphocytes were isolated from human tonsils, and incubated with Factor H diluted to final concentrations in the range 39–624 $\mu\text{g}/\text{ml}$, then with monoclonal anti-(Factor H) antibody (MRC OX23) and ^{125}I -labelled RAM-F(ab')₂ as described in the Materials and methods section. Data are not corrected for non-specific binding, and points are the average of duplicate determinations. The binding of RAM-F(ab')₂ to B cells preincubated with MRC OX23 but no Factor H is shown to indicate the level of non-specific binding (■). Further control samples show the binding of RAM-F(ab')₂ to B cells preincubated with Factor H and then treated with MRC OX1 instead of MRC OX23 (-----).

counted as described above. Erythrocytes treated with CrCl_3 , but without protein, were included as controls.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out in the system of Laemmli (1970). Samples for electrophoresis were prepared as follows. Samples eluted from the affinity column were precipitated with 10% (w/v) trichloroacetic acid at 4 °C. The precipitates were washed with cold acetone, dried and redissolved in 50 μl of 0.1 M-Tris/HCl buffer, pH 8, containing 4 M-urea and 0.1% SDS. Samples were divided and analysed under reducing and non-reducing conditions. Gels were stained with Coomassie Blue. M_r standards were purchased from Sigma Chemical Co. Radioiodinated and ^{35}S -labelled proteins were detected by autoradiography as described previously (Malhotra & Sim, 1985).

Immunoprecipitation with anti-(Factor H) antibodies

Raji-cell detergent extracts (200 μl), which had been pre-cleared with non-immune human IgG-Sepharose 4B as described above, were incubated with 50 μl (packed volume) of Sepharose 4B beads bearing covalently bound antibodies. After rotating on a mixer for 18 h at 4 °C, the beads were washed thoroughly with Dulbecco's medium A and B containing 0.1% Nonidet P40 and 0.5 M-NaCl, followed by water. Bound protein was then eluted with 100 mM-NaCl/50 mM-diethanolamine buffer, pH 11.5. Eluted material was precipitated with 10% trichloroacetic

Fig. 2. Affinity chromatography of ^{125}I -labelled Raji-cell and tonsil B-lymphocyte extracts on Factor H-Sepharose 4B

(a) Raji cells (2×10^7) were labelled with ^{125}I and extracted in lysis buffer 'A' as described in the Materials and methods section. A 0.2 ml portion of solubilized membranes, pre-cleared with non-immune IgG-Sepharose, was mixed with 0.5 ml (packed volume) of Factor H-Sepharose 4B coupled at a density of 0.8 mg of protein/ml of gel. After rotary mixing for 2 h at 4°C , beads were divided and transferred into two columns. Columns were washed with lysis buffer 'B'. One of the columns was then eluted with lysis buffer 'B' containing 50 mM-NaCl (track 1, non-reduced sample; track 2, reduced sample), and the other column was washed with lysis buffer 'C' containing 50 mM-NaCl (track 3, non-reduced sample; track 4, reduced sample). In tracks 5 and 6 the non-reduced and reduced samples of materials eluted from Clq-Sepharose with lysis buffer 'C' containing 50 mM-NaCl are shown, as controls. (b) Tonsil B lymphocytes were labelled with ^{125}I and treated as described for Raji cells: tracks 1 and 2 show the results of procedures identical with those for tracks 1 and 2 of (a). Trichloroacetic acid precipitates of column eluates were analysed on 7.5% polyacrylamide gels in the presence of SDS. The reduced standards for M_r calibration were: myosin (M_r 205000), Factor H (M_r 170000), phosphorylase *b* (M_r 92000), catalase (M_r 60000), ovalbumin (M_r 42000) and carboxypeptidase A (M_r 25000).

acid and analysed by SDS/polyacrylamide-gel electrophoresis.

RESULTS

Saturable binding of Factor H to tonsil B lymphocytes

Lambris *et al.* (1980) have demonstrated specific, dose-dependent and saturable binding of Factor H to the cells of the lymphoblastoid cell-line Raji, by the uptake of ^3H -labelled ligand. In an indirect binding test, with monoclonal anti-(Factor H) antibody, we have confirmed these results. Further, Lambris *et al.* (1980) showed in double-labelling experiments that the Factor H-receptor-bearing cells of human blood are among surface-Ig-bearing lymphocytes. Saturable binding of Factor H to human B lymphocytes, however, has not previously been demonstrated. In Fig. 1 we show that Factor H binds in a dose-dependent saturable manner to B cells isolated from human tonsils by depletion of erythrocyte-rosette-forming cells. Binding reaches a plateau at about $300\ \mu\text{g}$ of Factor H/ml. Essentially identical results were obtained with Raji cells, with the same cell and Factor H concentrations.

In order to examine the cell-surface proteins that might be responsible for the binding of Factor H, Factor H-Sepharose affinity-chromatography experiments were done with detergent extracts of surface ^{125}I -labelled Raji cells and tonsil B cells.

Affinity chromatography of Factor H-binding proteins

Detergent extracts of surface ^{125}I -labelled cells were subjected to chromatography on Factor H-Sepharose 4B as described in the Materials and methods section. Bound proteins were eluted with buffers of increased ionic strength. Tracks 1 and 2 in Fig. 2(a) show a protein band eluted with moderate-ionic-strength buffer (lysis buffer 'B' containing 50 mM-NaCl) from Factor H-Sepharose 4B after incubation of the matrix with detergent extracts of ^{125}I -labelled Raji cells. The migration pattern of the non-reduced and the reduced cell-membrane-derived protein is identical with that of plasma Factor H, revealing the characteristic mobility shift of the molecule on reduction. The apparent M_r of the non-reduced species is 140000 and that of the reduced one is 170000, both estimates being made in comparison with reduced standards (Fig. 2a). A possible role of bivalent cations in binding can be excluded, since an identical pattern was seen when the column was eluted with buffer containing 1 mM-EDTA (lysis buffer 'C') and 50 mM-NaCl (Figs. 2a, tracks 3 and 4).

In control experiments affinity columns of Clq- and gelatin-Sepharose 4B were used for the adsorption of Raji-cell extracts, and beads were eluted with lysis buffer 'C' containing 50 mM-NaCl. None of these resins bound the protein that was adsorbed by Factor H-Sepharose, proving the Factor H-specificity of the species with apparent M_r 170000. In tracks 5 and 6 of Fig. 2(a)

Fig. 3. Affinity chromatography of the extract of Raji cells biosynthetically labelled with [^{35}S]cysteine

Raji cells were biosynthetically labelled with [^{35}S]cysteine, extracted with detergent and adsorbed by Factor H-Sepharose (0.8 mg of protein/ml of gel) as described in the Materials and methods section. The column was eluted with lysis buffer 'C' containing 50 mM-NaCl. Samples were analysed by SDS/polyacrylamide-gel electrophoresis. Proteins containing [^{35}S]cysteine were detected after impregnation of the 6.5% polyacrylamide slab gels with Autofluor. Track 1 shows the non-reduced sample, track 2 the reduced one. M_r standards were as described in the legend to Fig. 2.

material eluted from Clq-Sepharose is shown. As a further control, U937 cells, which do not bind Factor H, were used. The 170000- M_r protein was not observed when detergent extracts of U937 cells were subjected to affinity chromatography on Factor H-Sepharose 4B.

The same species with apparent M_r 170000 was bound to and eluted from Factor H-Sepharose when using extracts of tonsil B lymphocytes. However, in this case the background of non-specific binding material (i.e. the binding of lower- M_r proteins, which bind to Clq- and gelatin-Sepharose as well) was higher (Fig. 2b).

It has been suggested by Lambris *et al.* (1980) that only an oligomeric form of Factor H binds in a stable manner to receptor-bearing cells. To check the possible effect of ligand density on the binding, Factor H was coupled to Sepharose 4B at final concentrations of 0.2 mg/ml, 0.8 mg/ml and 8 mg/ml of packed gel, and these beads were used for adsorption of the Raji cell extract. There was, however, no significant difference in the results obtained with the three different columns.

Biosynthesis of the Factor H-binding molecule by Raji cells

Despite thorough washing of the cells before surface radioiodination, the possibility could not be excluded that the Factor H-binding molecule observed has been only passively adsorbed by the Raji cells from the foetal-calf-serum-containing medium. To clarify this point Raji cells were cultured for 25 h in the presence of [^{35}S]cysteine in otherwise cysteine-free medium, and were then extracted in lysis buffer 'A'. The pre-cleared soluble

fraction of the preparation was then applied to the Factor H-Sepharose 4B resin and bound material was eluted with lysis buffer 'B' containing 50 mM-NaCl. Proteins containing [^{35}S]cysteine were detected by fluorography. In Fig. 3 it is demonstrated that the same species could be isolated from the biosynthetically labelled cell membrane as from the surface-labelled cells, proving that the Factor H-binding protein with apparent M_r 170000 is the product of the Raji cells. It is notable that the affinity chromatography of biosynthetically labelled cells produces a more complex picture than that seen with surface-labelled cells (cf. Fig. 2a). The additional lower- M_r bands on Fig. 3 may represent intracellular proteins, which would not be detected by surface labelling. Alternatively, these may be surface proteins that are detected with greater sensitivity by [^{35}S]cysteine-labelling.

Characteristics of the Factor H-binding molecule: differences from Factor H

The evidence that Factor H molecules isolated from serum readily bind to each other to form dimers and oligomers (Ripoche *et al.*, 1984; S. J. Perkins & R. B. Sim, unpublished work), and moreover the striking

Fig. 4. Immunoprecipitation of ^{125}I -labelled Raji-cell extracts with the monoclonal anti-(Factor H) antibody MRC OX23 covalently bound to Sepharose 4B

Immunoprecipitation of the radiolabelled Raji-cell extract was carried out by mixing 0.2 ml of the pre-cleared radiolabelled cell extract with 100 μl of the immuno-adsorbent overnight at 4 $^{\circ}\text{C}$. After non-specifically bound material had been washed off, specifically bound material was eluted with high-pH buffer, as described in the Materials and methods section. Eluted material was precipitated with 10% trichloroacetic acid, washed with cold acetone and applied to SDS/polyacrylamide gels (7.5%) for electrophoresis under reducing and non-reducing conditions. Tracks 1 and 2 show, for comparison, Factor H-binding protein eluted from Factor H-Sepharose 4B (as in Fig. 2) without and after reduction, and tracks 3 and 4 show material eluted from MRC OX23-Sepharose in non-reduced form and in reduced form respectively. M_r standards were as described in the legend to Fig. 2.

Fig. 5. Affinity chromatography of the supernatants of Raji cells biosynthetically labelled with [35 S]cysteine

Raji cells (1×10^7) were biosynthetically labelled with [35 S]cysteine as described in the Materials and methods section. After addition of 0.1% Nonidet P40 detergent and proteinase inhibitors as described in the text, 2 ml of the supernatant was mixed with 0.5 ml of Factor H–Sephacrose 4B. After incubation the beads were washed with lysis buffer 'B' and eluted with lysis buffer 'B' containing 50 mM-NaCl. Eluted samples were prepared for SDS/polyacrylamide-gel electrophoresis on 7.5% polyacrylamide gels as described in the Materials and methods section. Track 1 shows the non-reduced sample, track 2 the reduced sample. M_r standards were as described in the legend to Fig. 2.

identity of the apparent M_r value and mobility shift after reduction of the Factor H-binding membrane molecule and Factor H on SDS/polyacrylamide-gel electrophoresis, raised the possibility that the Factor H-binding membrane structure is Factor H itself. To clarify this point two monoclonal anti-(Factor H) antibodies, namely MRC OX23 and MRC OX24, and the Fab fragments of polyclonal rabbit anti-(Factor H) were coupled to Sepharose beads, and used for immunoprecipitation from the detergent extract of surface- 125 I-labelled Raji and tonsil B cells. No Factor H-like molecule could be eluted from these immunosorbents, indicating that there is no antigenic relationship between the Factor H-binding membrane component and Factor H. In Fig. 4 material specifically bound to the monoclonal antibody MRC OX23 is shown, and compared with material eluted from a Factor H–Sephacrose column.

In an alternative approach the Zn^{2+} -binding capacity of the cell-membrane-derived protein was tested, since it is known that Factor H binds to Zn^{2+} -chelate resins very strongly (Day & Sim, 1986). A detergent extract of Raji cells was passed through a Zn^{2+} -chelate column, and the unbound material was collected and re-equilibrated in lysis buffer 'B' by passage through a PD-10 (Sephadex

Table 1. Effect of Factor H-binding molecules from cell membranes and from culture supernatant on Factor H-dependent rosette formation

Indicator cells prepared by coupling Factor H molecules to sheep erythrocytes with $CrCl_3$ were preincubated with different dilutions of Factor H-binding material derived either from the cell membrane or from the culture supernatant of Raji cells. For rosetting, pretreated erythrocyte–Factor H complexes were mixed with Raji cells in a ratio of 50:1, as described in the Materials and methods section. In control experiments the rosetting of erythrocytes bearing Factor H, preincubated with fractions containing no Factor H-binding material, was tested; and was found to give similar results to the positive control, i.e. the percentage of rosettes was 23–26. Erythrocytes treated with $CrCl_3$ but no Factor H gave 2–3% rosetting. Dilutions indicate the final dilution of material during incubation with the Factor H-treated erythrocyte.

Indicator cells pretreated with fractions containing:	Percentage inhibition of rosetting
170000- M_r molecule (1:10 dilution)	86–92
170000- M_r molecule (1:20 dilution)	44–51
170000- M_r molecule (1:40 dilution)	0–5
105000- M_r molecule (1:10 dilution)	0–5
105000- M_r molecule (1:20 dilution)	0–5
105000- M_r molecule (1:40 dilution)	0–5

G-25) column equilibrated in the same buffer. The material was then incubated with Factor H–Sephacrose and eluted as previously described. The Factor H-binding molecule was recovered from the material that did not bind to the Zn^{2+} -chelate, providing further evidence of the lack of identity with Factor H.

As a further comparison with Factor H, tests were done with U937 cells (a monocytic cell line), which is known to express cell-surface-bound Factor H at a level of about 10^4 molecules/cell (Malhotra & Sim, 1985). The Factor H-binding species of Raji and B cells is distinct from surface Factor H of U937 cells in the following properties: (a) surface Factor H of U937 cells is recognized by MRC OX23, MRC OX24 and polyclonal Fab anti-(Factor H) (Malhotra & Sim, 1985), whereas the Raji and B cell protein is not (see above and Fig. 4); (b) surface Factor H of U937 cells binds readily to Zn^{2+} -chelate resin, whereas the Raji and B cell protein does not; (c) as noted above, surface Factor H of U937 cells does not bind to Factor H–Sephacrose under conditions identical with those in which the Raji and B cell protein binds. It therefore appears that cell-surface-bound Factor H does not serve as a Factor H receptor, and that the 170000- M_r protein of Raji and tonsil B cells is distinct from Factor H, although it is of the same apparent M_r .

Isolation of a Factor H-binding protein from the culture supernatant of [35 S]cysteine-labelled Raji cells

Many receptor structures are known to be released from cells *in vivo* and/or during culture (Neauport-Sautes *et al.*, 1979; Ishizaka, 1984; Micklem *et al.*, 1985; Yoon & Fearon, 1985). Further, the release of a Factor H-binding protein from Raji cells has been reported by Lambris & Ross (1982). This protein appeared to consist

of three components of M_r 50000, two of them linked by disulphide bridges and one attached non-covalently. To re-investigate the presence and nature of Factor H-binding protein(s) present in the culture supernatant of Raji cells, proteinase inhibitors, as described in the Materials and methods section, and 0.1% Nonidet P40 detergent were added to the spent culture medium of [35 S]cysteine-labelled cells. The material was dialysed against lysis buffer 'B' and then incubated with Factor H-Sepharose beads. After non-specifically bound material had been washed off, the affinity column was eluted with lysis buffer 'B' containing 50 mM-NaCl. Eluted proteins are shown in Fig. 5. The M_r of the Factor H-binding protein released by the cells is 95000 under non-reducing conditions, and after reduction of disulphide bridges a mobility shift can be observed revealing a molecule of M_r 105000. Both M_r estimates are made in comparison with reduced standards. As a control, a gelatin-Sepharose affinity column was also tested, but no protein in the same M_r range was eluted under the same conditions.

Effect of Factor H-binding proteins on rosette formation of Raji cells with erythrocytes bearing Factor H

In these experiments we tested whether Factor H-binding proteins isolated from the cell membrane and from the culture supernatant of the cells can affect Factor H-mediated binding of particles to Raji cells. The results summarized in Table 1 show that fractions containing the membrane-derived Factor H-binding protein, with apparent M_r 170000, exert a dose-dependent inhibitory effect on Factor H-dependent rosetting. In contrast, however, the material isolated from the supernatant, having apparent M_r 105000, has no effect on the same process. The percentage of rosette-forming cells in the control samples with erythrocytes lacking Factor H did not exceed 3%.

DISCUSSION

Tonsil B lymphocytes and cells of the B-lymphoblastoid cell line Raji express on their surface a protein of M_r about 170000 that interacts specifically with Factor H-Sepharose under conditions of low ionic strength (Fig. 2). The protein is synthesized by Raji cells (Fig. 3). The monocytic cell line U937 lacks this protein. Both Raji and tonsil B lymphocytes bind purified Factor H in a specific and saturable manner (Fig. 1; Lambris *et al.*, 1980; Lambris & Ross, 1982), and have been considered to possess a specific Factor H receptor. The 170000- M_r Factor H-binding protein isolated from Raji-cell membranes inhibits, in a dose-dependent manner, the rosetting of Factor H-bearing sheep erythrocytes to Raji cells (Table 1). The 170000- M_r membrane protein therefore has the properties of a Factor H receptor.

The 170000- M_r membrane protein is very similar in its migration properties in SDS/polyacrylamide-gel electrophoresis to both the soluble and membrane-bound forms of Factor H itself. However, we were unable to demonstrate antigenic cross-reactivity between Factor H and the Factor H receptor. Further, soluble Factor H or Factor H expressed on the cell surface of U937 cells does not bind to Factor H-Sepharose, and is therefore distinct from the Factor H receptor.

In a previous report Lambris & Ross (1982) described

the isolation, from B lymphoblastoid cell lines, of a cell-surface protein that was recognized by an antibody to an anti-(Factor H) idiotype. This protein appeared to be of M_r about 150000, and was made up of three polypeptide chains of M_r 50000, two of which were disulphide-linked. An apparently identical protein was isolated from Raji cell culture supernatant by using Factor H-Sepharose affinity chromatography (Lambris & Ross, 1982). The 170000- M_r Factor H-binding protein isolated in our study may be the same as that described by Lambris & Ross (1982), since the overall M_r is similar. Lambris & Ross (1982) made extensive use of the proteinase inhibitors phenylmethanesulphonyl fluoride, soya-bean trypsin inhibitor, benzamidine and 6-aminohexanoic acid in their preparations, although it is not clear whether these were present at all stages. In our own study, however, a wider range of proteinase inhibitors, including inhibitors of thiol, carboxy and metalloproteinases was used throughout. It is therefore possible that the species observed by Lambris & Ross (1982) is a proteolytically cleaved form of the 170000- M_r single-chain protein that we observe.

The 105000- M_r species isolated from tissue-culture supernatants (Fig. 5) is not similar in overall M_r to the 150000- M_r protein isolated from culture supernatants by Lambris & Ross (1982). The difference in overall M_r could be accounted for by the loss of the non-covalently attached segment (50000 M_r) of the protein described by Lambris & Ross (1982). Although both proteins were purified by Factor H-Sepharose affinity chromatography, conditions were different. Lambris & Ross (1982) bound the material to the affinity resin in the absence of detergent, and eluted it with 0.5 M-NaCl plus Nonidet P40 detergent, whereas in our study Nonidet P40 was present throughout, and elution was with 50 mM-NaCl. Differences in binding and elution conditions might result in variable recovery of non-covalently bound fragments of protein. High-salt elution conditions are also, however, less suitable for separation of specifically and non-specifically bound protein. At present, however, we have no other means (e.g. antisera) to demonstrate or exclude the possibility that either the membrane protein or the supernatant protein observed in our study is the same species as that described by Lambris & Ross (1982). The 105000- M_r protein isolated from culture supernatants may be a product of proteolysis of the 170000- M_r species, although this requires further investigation. The same mobility shift on reduction is seen with this form (Fig. 5).

The quantity of the Factor H-binding protein present on Raji cells appears to be very small. The 170000- M_r species is not visible on autoradiographs of SDS/polyacrylamide-gel electrophoretograms of unfractionated 35 S- or 125 I-labelled extracts, and the 170000- M_r protein is likely to represent less than 1% of the radiolabelled species in these preparations. Similarly, on analysis of SDS/polyacrylamide-gel electrophoretograms of Factor H-Sepharose column eluates, the 170000- M_r species is not readily detectable by silver-staining. Because of the low amount present, we have not attempted to quantify the yield of protein in column eluates.

The solubilised 170000- M_r species is effective in inhibiting Factor H-dependent rosette formation by Raji cells (Table 1). The 105000- M_r soluble species from culture supernatants was not effective in this test, although it was estimated to be at a similar concentration

to the 170000- M_r protein, as judged by ^{35}S radioactivity measurements. The 105000- M_r protein evidently does have affinity for Factor H, as it was isolated on Factor H-Sepharose, but its affinity may be lower than that of the intact species. A difference in affinity is likely to be exaggerated by the fact that rosetting is done at higher ionic strength than is the binding to Factor H-Sepharose.

The physiological role of a Factor H receptor is not yet clear, although, as noted in the Introduction, Factor H has been reported to have direct effects on B lymphocytes. Effects on monocytes and macrophages have also been reported, but there is no evidence to show whether or not the Factor H receptor of these cells is the same as that discussed here. Saturation of Factor H binding to tonsil B lymphocytes appeared to occur at the rather high concentration of about 300 μg of Factor H/ml (Fig. 1), which is close to the average plasma concentration of Factor H. Ripoche *et al.* (1984, 1985) have, however, reported that Factor H isolated from serum or plasma can be divided into two (conformationally different) populations on the basis of their surface hydrophobicity properties, and that only one of these populations binds to Raji cells. Nothing is known of the interconversion of these two populations *in vivo*, but it is possible that the tonsil lymphocyte Factor H receptor recognizes with high affinity a minor subpopulation of Factor H *in vivo*, and of the unfractionated Factor H used in the binding study (Fig. 1).

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