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Secretion, cleavage and binding of complement component C3 by the human monocytic cell line U937

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Secretion of complement component C3 by U937 cells was studied. Preliminary evidence for a cell-associated proteolytic activity specific for C3 is given, as well as for a covalent-like binding of C3 fragments to the cell membranes. Secretion of C3, in the presence of 10 ng of phorbol 12-myristate 13-acetate/ml, is 120–140 ng/10^6 cells per 24 h on the third day after addition of the activator. As shown by SDS/polyacrylamide-gel electrophoresis, the intracellular pro-C3 (200 kDa) and the extracellular secreted C3 (α-chain 110 kDa and β-chain 75 kDa) are identical with the forms of C3 previously characterized from human serum. Incubation of U937 cells in the presence of exogenous radiolabelled C3 shows that membrane-bound proteinase(s), not related to the classical-pathway or the alternative-pathway C3 convertases, is (are) able to cleave C3; this cleavage leads to the binding of the resulting C3 fragments to the cell membrane through reaction of membrane acceptors with the carbonyl group of C3 revealed after disruption of the intramolecular thioester bond. The proteolysis appears to be fairly specific to C3, as C4, which also possesses an intramolecular thioester bond, is not cleaved and does not bind to the cells. *p*-Nitrophenyl *p'*-guanidinobenzoate (1 mM) and di-isopropyl phosphorofluoridate (2 mM) are potent inhibitors of the proteolysis, whereas soya-bean trypsin inhibitor (1 mM), leupeptin (0.1 mg/ml) and 1,10-phenanthroline (1 mM) were ineffective. Immunological characterization of the cell-bound C3 fragments with monoclonal antibodies shows an evolution of the proteolysis of the fragments from iC3b to C3dg epitopes. Extraction of membrane-bound fragments by detergent, followed by SDS/polyacrylamide-gel electrophoresis, shows two fragments, of 43 kDa and 46 kDa, with C3dg-like characteristics.

INTRODUCTION

The activation of complement component C3, which takes place in the course of humoral immune reactions, leads to the generation of fragments C3a and C3b and the derivatives of C3b, iC3b and C3dg, which are believed to play an important role in the cellular immune response (Morgan, 1986; Böttger & Bitter-Suermann, 1987). The generation of C3b is concomitant with the cleavage of an intrachain thioester bond, conferring to the molecule a transient capacity to form covalent-like bonds with nucleophilic acceptors such as amino or hydroxy groups (Sim *et al.*, 1981). C3b and its proteolytic fragments iC3b, C3dg and C3d are thus likely to remain attached to an acceptor site, while they exhibit a second binding site that is able to interact with receptors such as CR1, CR2, CR3 and CR4 through non-covalent bonds (Ross & Medof, 1985). Thus a bridging role can be assumed by C3b, iC3b, C3dg and C3d.

Previous results have shown that C3d polymers activate directly B cells (Erdei *et al.*, 1985; Melchers *et al.*, 1986) and that depletion of complement system with cobra-venom factor suppresses the antibody response in mouse (Pepys, 1974). Using C3-deficient guinea pigs, Böttger *et al.* (1986) showed that the secondary antibody response is impaired in the absence of C3: these animals were unable to react to low doses of injected antigen, and they had no amplification of the antibody titre as well as no switch from IgM to IgG, but when the concentration of the antigen was increased the immune response returned to normal.

In a human model system, Arvieux & Colomb (1988) showed that cross-linking of C3b to antigen stimulates the presentation of antigen by B lymphoblastoid cells, as analysed by the subsequent increased activation of human T cell clones.

Macrophages are known for their capacity to synthesize most proteins of the complement system, such as C1 (Bensa *et al.*, 1983), C2 (Littman *et al.*, 1983), Factor D (Barnum & Volanakis, 1985), $C\bar{1}$Inh (Hamilton *et al.*, 1987) and C3 (Zimmer *et al.*, 1982). They are also able to handle and present antigens to T cells. This last capacity is associated with the ability to cleave C3 and to fix C3 fragments on acceptor sites, as revealed by formation of rosettes between macrophages and erythrocytes (Fabry *et al.*, 1985).

U937 cells (Sundström & Nilsson, 1976) were chosen for the present experimentation as, in the presence of PMA, this cell line acquires the functions of normal human macrophages, such as expression of Fc-fragment receptors (Anderson & Abraham, 1980), complement receptors (Gilbert *et al.*, 1985), antibody-dependent

Abbreviations used: CR1, CR2, CR3 and CR4, receptors specific for the proteolytic fragments of C3; SAC1, *Staphylococcus aureus* Cowan 1; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide-gel electrophoresis.

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cellular cytotoxicity (Villiers *et al.*, 1987), interleukin 1 and other protein synthesis and secretion (Minta & Pambrun, 1985).

The purpose of the present work is to analyse the relationship between the synthesis of C3 and the capacity of membrane-bound proteinases to cleave C3 with a resulting binding of C3 fragments to the cell membrane.

MATERIALS AND METHODS

Materials

Tissue-culture media were from Eurobio, Paris, France; foetal-calf serum and mycoplasma detection kit were from Flow Laboratories, McLean, VA, U.S.A.; IgG-Sorb was from the Enzyme Center, Malden, MA, U.S.A.; trypsin [L-tosylphenylalanylchloromethane ('TPCK')-treated], soya-bean trypsin inhibitor, BSA, dithiothreitol and PMA were from Sigma Chemical Co., St. Louis, MO, U.S.A.; dinonyl phthalate and dibutyl phthalate were from Fluka, Buchs, Switzerland; [^{3}H]thymidine (5 Ci/mmol) was from C.E.A., Paris, France; [^{35}S]methionine (1106 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A.; Na^{125}I (100 mCi/ml) was from Amersham International, Amersham, Bucks., U.K.

Complement proteins and antisera

C3 was isolated from human plasma by the procedure of Al Salihi *et al.* (1982). C2 (Thielens *et al.*, 1982), C4 (Reboul *et al.*, 1979) and $C\bar{1}s$ (Arlaud *et al.*, 1979) were purified as described in the references cited. Antisera to human C3, C2 and C4 were raised in rabbit and antibodies were prepared by Na_2SO_4 precipitation of sera (Prahl & Porter, 1968). Rat monoclonal antibodies to C3 fragments were generously given by Professor P. J. Lachmann (Cambridge, U.K.) and used after Na_2SO_4 precipitation as above. Clone 3 and clone 4 were IgG γ_{2a}-κ and clone 9 was IgG γ_1-κ. Their specificities are respectively against C3d, C3c and a neoantigen of iC3b located in the C3g domain of C3 (Lachmann *et al.*, 1980, 1982).

The purified proteins were labelled with ^{125}I by using the Iodogen method (Fraker & Speck, 1978).

Cell line

U937 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated (56 °C for 30 min) foetal-calf serum and 2 mM-glutamine. Cells were maintained at 37 °C in a humidified atmosphere (5% CO_2 in air) at concentrations between 0.3×10^6 and 1×10^6 cells/ml in 75 cm^2 flasks (Falcon; Becton–Dickinson, Mountain View, CA, U.S.A.). The cell line was regularly checked for the presence of mycoplasma according to the manufacturer's instructions.

PMA stimulation of U937 cells

PMA (1 mg/ml) in dimethyl sulphoxide was stored at −70 °C and diluted in PBS (70 mM-NaCl/1.5 mM-KCl/0.75 mM-KH_2PO_4/4.1 mM-Na_2HPO_4 buffer, pH 7.5) just before use. U937 cells were washed with RPMI 1640 medium containing 10% (v/v) foetal-calf serum and adjusted to 5×10^5 cells/ml in the same medium; PMA was added to the cell suspension and the cells were cultivated at 37 °C in 5% CO_2 in air. After incubation, the culture medium was centrifuged and protein in the supernatant was determined. Cell proliferation in the presence of PMA was measured by incorporation of [^{3}H]thymidine: the cell culture was performed in a 96-well plate in the presence of PMA at the indicated concentrations; 48 h after the addition of PMA, [^{3}H]thymidine was added (20 μl, 0.4 μCi/well) and the cells were further incubated for 15 h. [^{3}H]Thymidine incorporation was measured after the cells had been harvested.

Radioimmunoassay for C3 and C2

Secreted proteins were quantified by a radioimmunoassay: 500 μl of culture supernatant was diluted with 500 μl of SAC1 buffer [150 mM-NaCl/0.25% (v/v) Nonidet P40/0.5 mM-EDTA/0.02% (w/v) NaN_3/0.5% (w/v) ovalbumin/50 mM-Tris/HCl buffer, pH 7.4] and incubated overnight at room temperature with 100 μl of ^{125}I-labelled antigen (C3 or C2) and 100 μl of dilutions of the corresponding antibodies (sufficient to precipitate 50% of the radiolabelled antigen). After incubation, 100 μl of a 10% (w/v) suspension of IgG-Sorb (heat-killed and formalin-fixed SAC1) in SAC1 buffer was added and the mixture was further incubated for 1 h. After centrifugation (9000 g at $r_{av.}$ 8.7 cm for 15 min), ^{125}I radioactivity in the SAC1 pellet was measured. A standard curve was obtained with 500 μl of RPMI 1640 medium supplemented with foetal-calf serum containing 0.625–20 ng of purified C3 or C2.

Preparation of C3b and C4b

C3b was obtained by proteolytic cleavage of purified C3 in the presence of trypsin. The mixture was incubated at 37 °C for 5 min (enzyme/substrate ratio 1:100, w/w). The reaction was stopped by addition of soya-bean trypsin inhibitor, with further incubation for 15 min at 37 °C (enzyme/inhibitor molar ratio 1:2). C4b was obtained by incubation of C4 in the presence of purified $C\bar{1}s$ (C4/$C\bar{1}s$ molar ratio 1:2.4) for 30 min at 37 °C. Formation of C3b or C4b was assessed from analysis of the reduced proteins by SDS/PAGE.

Determination of C3b acceptor sites

U937 cells were washed twice with PBS/BSA/glucose buffer [PBS supplemented with 0.5% (w/v) BSA and 2.5% (w/v) glucose]. Viability of the cells assessed by Trypan Blue exclusion was always more than 95%. A 100 μl portion of cell suspension (10^6 cells) was placed in each well of a 96-well (round-bottomed) plate and centrifuged (200 g at $r_{av.}$ 14.0 cm for 5 min). The cell pellet was incubated at 37 °C in the presence of 2 μg of ^{125}I-labelled C3 or C3b (2×10^6 c.p.m.) in 100 μl of the PBS/BSA/glucose buffer, and after the indicated time the cell suspension was centrifuged over 200 μl of an oil mixture of dinonyl phthalate/dibutyl phthalate (1:4, v/v). The radioactivities of the pellets and the supernatants were measured. At this stage only viable cells are considered for the binding, as dead cells do not sediment in the conditions used.

Analysis of C3 bound to U937 cells

After binding of ^{125}I-labelled C3, the U937 cell suspension was centrifuged (200 g ar $r_{av.}$ 14.0 cm for 5 min) and the cells were washed five times with the PBS/BSA/glucose buffer before lysis with 100 μl of 100 mM-KCl/10 mM-EDTA/0.5% (w/v) sodium deoxycholate/50 mM-Tris/HCl buffer, pH 7.4.

After 10 min at 0 °C, the mixture was centrifuged

(100000 *g* at $r_{av.}$ 6.5 cm for 30 min) in an L8 ultracentrifuge with an SW 60 rotor (Beckman) and the supernatant was diluted with an equal volume of 145 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.4, containing 1% (w/v) Triton X-100, 0.5% (w/v) SDS, 0.5% (w/v) sodium deoxycholate and 0.5% (w/v) human serum albumin.

The proteins were precipitated by addition of polyclonal rabbit anti-C3 antibody (20 μl), and after an overnight incubation at 0 °C the immune complexes were fixed on SAC1 for 30 min at 0 °C and centrifuged.

The pellets were dissolved in 50 μl of 0.1 M-Tris/HCl buffer, pH 6.8, containing 2% (w/v) SDS, 20% (w/v) glycerol and 50 mM-dithiothreitol and incubated for 1 h at 37 °C. Solubilized proteins were analysed by SDS/PAGE.

Biosynthetic labelling of U937 cells

U937 cells (5×10^5 cells/ml) stimulated by PMA (10 ng/ml) for 48 h were washed with RPMI 1640 medium containing 10% foetal-calf serum and incubated in the presence of methionine-free RPMI 1640 medium containing 10% of heat-inactivated foetal-calf serum from which methionine had been removed by exhaustive dialysis; the mixture was supplemented with 100 μCi of [^{35}S]methionine (1106 Ci/mmol)/ml.

After 18 h at 37 °C, the supernatant was harvested, centrifuged, dialysed against 145 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.4, and stored at −70 °C. The cells were extracted in 5 ml of lysis buffer (see above) followed by two cycles of freezing and thawing. The lysates were centrifuged at 100000 *g* ($r_{av.}$ 6.5 cm) for 45 min and stored at −70 °C.

The analysis of biosynthesized C3 was performed after addition of an equal volume of 145 mM-NaCl/20 mM-sodium phosphate buffer, pH 7.4, containing Triton X-100, human serum albumin, sodium deoxycholate and SDS as described above for the analysis of C3 bound to U937 cells.

SDS/PAGE analysis

Samples for electrophoresis were diluted with an equal volume of 0.1 M-Tris/HCl buffer, pH 6.8, containing 2% (w/v) SDS and 20% (w/v) glycerol. Protein reduction was performed in the presence of 50 mM-dithiothreitol. After 1 h incubation at 37 °C, the proteins were alkylated with 110 mM-iodoacetamide. SDS/PAGE was carried out as described by Laemmli (1970) in 10% (w/v) polyacrylamide slab gels. After electrophoresis, gels were dried and autoradiographed with Beta Max films (Amersham International) at −70 °C.

RESULTS

Effect of PMA on the proliferation of U937 cells

U937 cells (5×10^5 cells/ml) were incubated in the presence of PMA at different concentrations, and, after 2 days, [^{3}H]thymidine was added to the medium to analyse the cellular proliferation. Incorporation of radioactivity is inhibited by 42% in the presence of concentrations of PMA as low as 0.01 ng/ml; inhibition increases to 75% and 90% for PMA concentrations of 0.05 ng/ml and 0.1 ng/ml respectively.

Cell viability was checked in parallel experiments and found to be 93–95% after 3 days in the presence of 20 ng of PMA/ml and decreased only after 5 days (80%). For lower PMA concentrations the viability remained at a high level (93–95%) for at least 7 days after the addition of the activator.

Effect of PMA on the synthesis of C3 by U937 cells and comparison with C2 synthesis

U937 cells were cultivated in the presence of different concentrations of PMA; after 2 days the supernatants were collected and the amounts of secreted C3 and C2 were measured by radioimmunoassay as described in the Materials and methods section.

Maximum C3 secretion was obtained in the presence of 5–10 ng of PMA/ml: under these conditions 100–110 ng of C3 was secreted/10^6 cells per 24 h (a typical experiment is shown in Fig. 1*c*). This corresponds to a stimulation factor of 5-fold compared with the average basal secretion of C3 (15–25 ng/10^6 cells per 24 h). This basal secretion was determined on the same cell culture, in the absence of PMA, at the different times.

Maximum C2 secretion was observed at lower PMA concentrations (0.5–1 ng/ml): in these conditions 35 ng of C2/10^6 cells per 24 h was synthesized, which corresponds to a stimulation factor of 2–3-fold over the basal secretion. Increasing PMA concentrations over the optimum that gave the maximal secretion (10 ng/ml for C3 secretion and 1 ng/ml for C2 secretion) led to a decrease in the amount of secreted proteins. This phenomenon was more marked for C2 than for C3 (Fig. 1*c*).

Kinetics of C3 and C2 biosynthesis by U937 cells in the presence of PMA

U937 cells were incubated in the presence of different concentrations of PMA as indicated (Figs. 1*a* and 1*b*). The cell supernatant was changed after each period of 24 h, but PMA concentrations were maintained throughout the experiment. Controls in the absence of PMA indicated that C3 secretion remained between 10 and 20 ng/10^6 cells per 24 h and C2 secretion between 5 and 10 ng/10^6 cells per 24 h. Morphological changes in the cells (adhesion, vacuole formation) appeared during the first period of incubation with PMA, but the secretion remained at the control level for C2 or was slightly increased for C3 secretion. During the second and third periods of incubation in the presence of PMA, C3 secretion increased to 120–140 ng/10^6 cells per 24 h and maximal secretion was obtained on the third day after the first addition of PMA (Figs. 1*a* and 1*b*).

In these conditions maximal stimulation factors of 9-fold for C3 and 12-fold for C2 were obtained in the presence of 1–10 ng of PMA/ml and 0.5–1 ng of PMA/ml respectively.

When PMA was present only during the first 24 h of incubation a maximal secretion was also obtained on the third day in the presence of 10 ng of PMA/ml for C3 (stimulation factor 12-fold) and in the presence of 1 ng of PMA/ml for C2 (stimulation factor 14-fold). For low PMA concentration (0.5 ng/ml) a 24 h stimulation was ineffective. Biosynthesis of C3 and C2 in the presence or in the absence of PMA was completely inhibited by cycloheximide (1 ng/ml) incubated at the same time and was restored after removal of the inhibitor.

Characterization of biosynthesized C3

In order to characterize the biosynthesized C3 further, cells were incubated in the presence of PMA for 2 days and the supernatant was then replaced by a methionine-

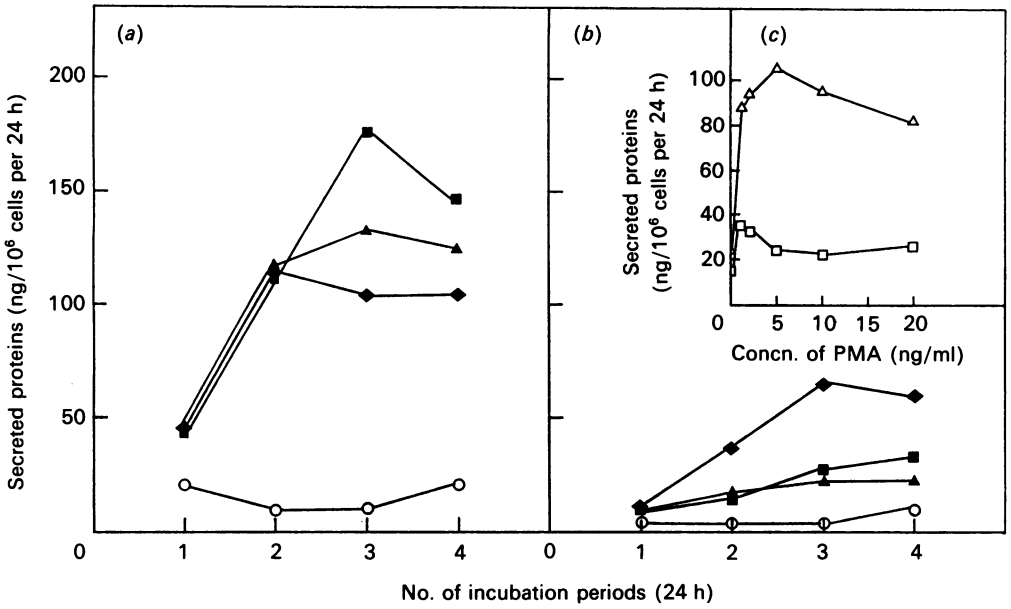

Fig. 1. Kinetics of C3 and C2 secretion by U937 cells in the presence of PMA

(*a*) and (*b*) U937 cells (5×10^5 cells/ml) were incubated alone (○) or in the presence of 1 ng (◆), 10 ng (■) or 50 ng (▲) of PMA/ml for periods of 24 h; at the end of each period the supernatants were collected and replaced by fresh medium supplemented with PMA. C3 (*a*) and C2 (*b*) were measured by radioimmunoassay as described in the Materials and methods section. The values correspond to the means for eight separate measurements. (*c*) U937 cells (5×10^5 cells/ml) were incubated in RPMI 1640 medium containing 10% foetal-calf serum in the presence of different concentrations of PMA. After 48 h C3 (△) and C2 (□) were measured in the supernatant by radioimmunoassay as described in the Materials and methods section.

free RPMI 1640 medium supplemented with [^{35}S]-methionine. The cells and the supernatants were harvested for analysis as described in the Materials and methods section.

C3 analysed from the cells (Fig. 2, lane B) shows the characteristic 110 kDa α-chain and 75 kDa β-chain of authentic C3 and a species of 200 kDa, likely to be pro-C3 (Brade *et al.*, 1977). C3 purified from the supernatant shows also the α- and β-chains (Fig. 2, lane C) with some contaminants of higher molecular mass and a very low amount of pro-C3.

As a reference, C2 analysis from the cells and from the supernatant (Fig. 2, lanes D and E) shows, in both cases, a single band corresponding to C2 purified from plasma (100 kDa).

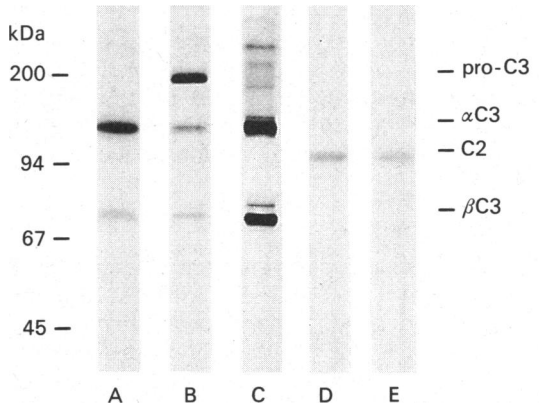

Fig. 2. SDS/PAGE analysis of ^{35}S-labelled C3 and C2 synthesized by U937 cells

After 2 days in the presence of 10 ng of PMA/ml, U937 cells were incubated in a methionine-free RPMI 1640 medium containing 10% foetal-calf serum supplemented with [^{35}S]methionine for 18 h. The supernatants and the cells were collected. C3 and C2 were precipitated by specific polyclonal antibodies. The immunoprecipitates were analysed by SDS/PAGE in reducing conditions. Lane A, C3 purified from serum; lanes B and C, C3 from the cells (lane B) and the supernatant (lane C); lanes D and E, C2 from the cells (lane D) and the supernatant (lane E).

Binding of C3 to U937 cells

As shown in Fig. 3(*a*), C3 binds to the cells in greater amount than does C3b. C3 binding increased continuously (278000 molecules of C3 per cell after 1 h, 370000 after 2 h), showing no saturation point, whereas in the same conditions C3b binding was saturable, reaching a maximum after 1 h (100000 molecules per cell).

C3 binding could be inhibited by prior treatment of C3 with 1 M-methylamine at 37 °C for 1 h at pH 9.0. In this case methylamine-treated C3 binding was lowered to the level of C3b binding. C3 binding was affected by different inhibitors: 69% and 67% inhibitions were observed when the cells were preincubated for 1 h in the presence of 1 mM-*p*-nitrophenyl *p'*-guanidinobenzoate and 2 mM-di-isopropyl phosphorofluoridate respectively whereas soya-bean trypsin inhibitor (1 mM) and leupeptin (0.1 mg/ml) were without effect; only 16% inhibition was observed in the presence of 1,10-phenanthroline (1 mM). EDTA (3 mM) lowered the binding of C3 to U937 cells by 97%.

The specificity of C3 binding was checked by incubation of U937 cells in the presence of C4 or C4b; in

these conditions C4 and C4b binding was very low (Fig. 3*b*). Fixation of C4 on the cells was increased by addition of exogenous $C\bar{1}s$, but even in these conditions binding remained low.

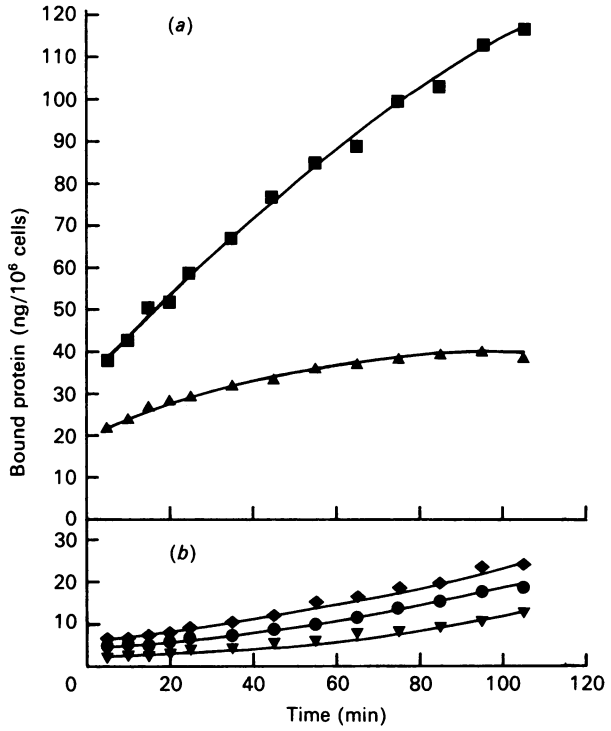

Fig. 3. Binding of C3 and C3b to U937 cells, and comparison with the binding of C4 and C4b

Portions (2 μg) of radiolabelled protein (C3 or C4) were incubated with 10^6 U937 cells in 100 μl. At the indicated times the mixtures were each centrifuged on an oil cushion as described in the Materials and methods section and the radioactivities of the pellet and the supernatant were counted. (*a*) U937 cells incubated with radiolabelled C3 (■) or C3b (▲). (*b*) U937 cells incubated with radiolabelled C4 (▼), C4b (●) or C4 in the presence of $C\bar{1}s$ (◆).

Characterization of C3 fragments bound to the cells

After binding of C3 to U937 cells (1 h at 37 °C), the cells were washed twice with the PBS/BSA/glucose medium and incubated in the presence of rat monoclonal antibodies to C3 fragments (clones 3, 4 and 9 described by Lachmann *et al.*, 1980). Incubation was in the presence of 0.5 mg of non-immune rabbit immunoglobulins/ml to saturate U937-cell Fc-fragment receptors. Binding of rat

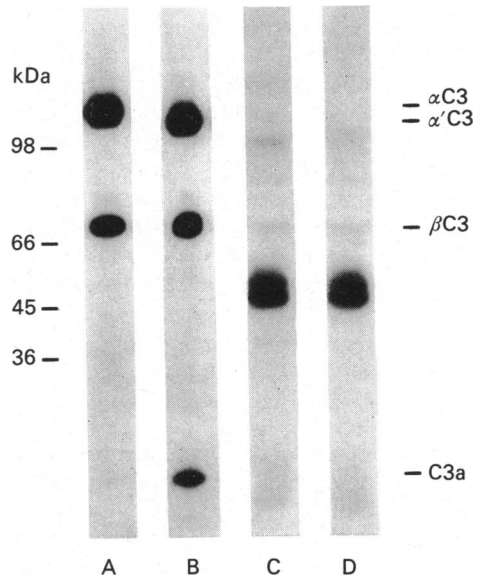

Fig. 4. SDS/PAGE analysis of C3 fragments bound to U937 cells

After incubation of ^{125}I-labelled C3 in the presence of U937 cells for 1 h, the cells were washed and membrane-bound C3 was precipitated with polyclonal antibodies against C3; portions of the resulting precipitate were adjusted to correspond to 30000 c.p.m. and analysed by SDS/PAGE after reduction. Lanes A and B, controls with purified C3 or C3b plus C3a respectively; lanes C and D, proteins extracted from the U937-cell membrane after binding of C3 (lane C) or C3b (lane D).

Table 1. Binding of monoclonal antibodies to U937 cells

In both experiments U937 cells (3×10^6 cells) were incubated with 100 μl of purified C3 (20 μg) for 1 h at 37 °C. After being washed, the cells were either mixed with the different monoclonal rat antibodies (Expt. I) or further incubated for 1 h at 37 °C without C3 before addition of the monoclonal rat antibodies (Expt. II). In the controls the cells were incubated as above but without C3. Binding of antibodies from clone 3, clone 4, clone 9 and non-immune rat immunoglobulins was performed at 0 °C (30 min) and quantified after a further incubation of U937 cells (30 min at 0 °C) in the presence of ^{125}I-labelled $(Fab')_2$ fragments of goat antibodies against rat immunoglobulins.

	Binding of ^{125}I-labelled goat anti-(rat immunoglobulin) antibodies (c.p.m.)			
	Clone 3	Clone 4	Clone 9	Non-immune rat immunoglobulins
Expt. I				
U937 cells + C3	67300	77492	76949	24144
Control	21850	16118	21039	30494
Expt. II				
U937 cells + C3 + incubation	61368	49835	61386	21000
Control	24279	24069	21323	25713

monoclonal antibodies was detected by further incubation with radiolabelled $(Fab')_2$ fragments of goat anti-(rat immunoglobulins) antibodies. As shown in Table 1, in a typical experiment, the three monoclonal antibodies bound to the cells, which, according to their specificities (clone 3, anti-C3d; clone 4, anti-C3c; clone 9, anti-C3g), indicated that a fragment with iC3b antigenicity was bound to the cells (Expt. I). Prolongation of the incubation of the cells at 37 °C led to a decrease of clone 4 binding, suggesting further proteolysis of these bound fragments (Table 1, Expt. II).

Radiolabelled C3 fragments were extracted from the cells by the lysis buffer and immunoprecipitated as described in the Materials and methods section; in these conditions, where 90% of the bound radioactivity was solubilized, two major fragments were identified in the extract by SDS/PAGE analysis (Fig. 4, lane C), with apparent molecular masses of 43 kDa and 46 kDa. As a control, the same protocol was applied to cells incubated with C3b. Fig. 4 (lane D) indicates a similar evolution of bound fragments, with two bands also at 43 kDa and 46kDa.

DISCUSSION

The biosynthesis of complement proteins by U937 cells has been described by several authors (Littman *et al.*, 1983; Randazzo *et al.*, 1985; Malhotra & Sim, 1985; Barnum & Volanakis, 1985; Bengio *et al.*, 1986; Minta & Isenman, 1987). The data presented here concern the covalent binding of C3 fragments to these cells, which appears to involve a cell-surface proteolytic activity as well as C3b acceptor sites. Stimulation of U937 cells for maximal C3 secretion was examined in order to use optimal conditions for a study of antigen presentation by these cells, in a long-term prospect.

The present results confirm that C3 secretion by U937 cells is stimulated in the presence of PMA by a factor of 12-fold. As a reference, C2 secretion by the same cells is less than C3 secretion, confirming previous results (Littman *et al.*, 1983), but is also sensitive to PMA. At high PMA concentration (50 ng/ml) C2 secretion declines whereas C3 secretion remains stable.

The data concerning C3 secretion are comparable with previously published results (Bengio *et al.*, 1986; Minta & Isenman, 1987), with minor differences associated probably with experimental conditions: the data reported by Minta & Isenman (1987) were obtained from cells cultivated in foetal-calf-serum-free medium; the results reported by Bengio *et al.* (1986) were observed with PMA concentrations higher than in the present study. Furthermore we have observed that Hepes buffer in the culture medium lessens C3 secretion and delays the maximal secretion to 5–6 days of culture in the presence of PMA.

Variations between reported secretion values are likely to be due also to contamination of U937 cells by mycoplasma, as, in our hands, contamination of the cells abolished all stimulation of C3 secretion even after 3 days in the presence of 10 ng of PMA/ml. All results reported here were obtained in mycoplasma-free U937 cells.

Examination of secreted C3 by SDS/PAGE indicated 110 kDa α-chain and 75 kDa β-chain corresponding to 'normal' C3, with minor amounts of pro-C3. Intracellular C3 was essentially pro-C3 with smaller amounts of C3.

Secreted C3 was stable in the supernatants (up to 17 h after secretion), in contrast with membrane-bound C3 fragments, as judged from incubation of the cells in the presence of exogenous C3.

Binding of exogenous C3, C3b or of C3b-like C3, obtained after incubation of C3 with methylamine, clearly showed a covalent binding of C3 fragments to U937 cells. This binding was not saturable, contrary to the binding of preformed C3b to complement receptors CR1 and/or CR3 and was increased by a factor of 2-fold when ionic strength was lowered to 0.075. Furthermore, recent experiments (results not shown) indicated that bound C3 radioactivity remained on plasma membranes upon subcellular fractionation and repeated washings; it was extracted only by detergent treatment. The covalent binding seems to result from the proteolysis of C3 close to the cell membrane, with a concomitant cleavage of the internal thioester bond of the ligand and the ensuing formation of ester or amide bond with hydroxy or amino groups on the surface of the cells. In the conditions used, no C3-specific proteolytic activity was observed in cell supernatants.

Bound C3 fragments were not stable, as stated above, undergoing further proteolysis, as shown by analysis with three monoclonal antibodies, which all react with cell-bound fragments. Extraction of bound C3 fragments by SDS followed by SDS/PAGE analysis revealed two fragments, of 43 kDa and 46 kDa, with characteristics close to those of C3dg. These values are also reminiscent of a 40 kDa C3 fragment extracted from PMA-stimulated U937 cells described by Erdei *et al.* (1988). In our case, the presence of C3c epitopes (reactivity with clone 4) on the bound fragments after incubation suggests that the 43 kDa and 46 kDa fragments are somewhat different from C3dg.

This observation also provides evidence for the absence of cell protein(s) bound to C3 fragments and a surprising homogeneity of the extracted C3 fragments. It is in favour neither of the formation of a stable amide bond between the ligand and a proteinic acceptor, nor of the formation of a less stable ester bond, as in this last case the presence of the detergent, used for the purification of the C3 fragments, stabilizes the ester (Venkatesh *et al.*, 1984). Observation of 43 kDa and 46 kDa fragments in the extracts might be in favour of a binding of the C3 proteolytic fragments to membrane lipids such as sphingomyelin or cholesterol. Nevertheless a cleavage of initial ester links with cell proteins by U937-cell esterases, as described for bound C3b by Venkatesh & Levine (1988), cannot be excluded.

Predominance of covalent-like binding of an activated C3 fragment to the cells over binding of C3b to receptors (Fig. 4) clearly illustrates the presence of a proteolytic activity on U937 cells. This activity is not due to complement convertases, as antibodies to Factor B or C4 were without effect on the binding of C3 fragments to the cells. This proteolytic activity and the resulting binding to membranes were sensitive to *p*-nitrophenyl *p'*-guanidinobenzoate (1 mM), di-isopropyl phosphorofluoridate (2 mM) or EDTA (3 mM), and fairly specific, as C4 was not cleaved in the conditions used for C3.

This activity was not due either to SVF proteinases adsorbed on the cell membranes, as control experiments (in the absence of U937 cells) on C3 and C3b incubated

in RPMI 1640 medium supplemented with 10% foetal-calf serum showed no cleavage of C3 and a proteolysis of C3b restricted to the iC3b stage.

Cleavage of C3 on the surface of macrophage-like cells is likely to provide these cells with bifunctional ligands involved in opsonization of antigens (covalent-like binding of C3 fragments to an acceptor antigen, non-covalent binding to U937-cell receptors) or cellular interactions (acceptors and receptors on different cells). This potential has been previously described by Ezekowitz *et al.* (1983), Ross & Medoff (1985) and Erdei *et al.* (1988) with endogenous C3.

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