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Biosynthesis of normal and low-molecular-mass complement component C1q by cultured human monocytes and macrophages

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High levels of low-molecular-mass complement component C1q (LMM-C1q), a haemolytically inactive form of C1q, are found in serum of individuals with inherited complete (functional) C1q deficiency and in serum of patients with systemic lupus erythematosus, whereas lower levels are present in normal serum [Hoekzema, Hannema, Swaak, Paardekooper & Hack (1985) J. Immunol. **135**, 265–271]. To investigate whether LMM-C1q is a (by-)product of C1q synthesis or the result of degradation of Clq, cultures of blood monocytes and of alveolar macrophages, which secrete functional C1q, were studied. A considerable portion of C1q-like protein secreted by these cells was found to be LMM-C1q. In contrast with the C1q fragments that resulted from degradation of normal C1q during phagocytosis, culture-derived LMM-C1q appeared to be identical with LMM-C1q found in serum, as judged by sedimentation behaviour, subunit structure and recognition by poly- and mono-clonal antibodies raised against C1q. The presence of LMM-C1q in cytoplasmic organelles compatible with the Golgi apparatus and the inability to generate LMM-C1q by impeding hydroxylation and triple-helix formation of C1q further argues against degradation as its source. Monocyte cultures of homozygous probands from two families with complete functional C1q deficiency reflected the abnormalities in serum, i.e. absence of functional C1q, but increased levels of LMM-C1q. By contrast, secretion of C1q and LMM-C1q by cells from healthy individuals was clearly co-ordinate, indicating that LMM-C1q in serum may provide a unique marker of C1q synthesis *in vivo*.

INTRODUCTION

Low or even undetectable serum levels of complement component C1q are often found in patients experiencing a major exacerbation of systemic lupus erythematosus (SLE) and are generally considered to reflect accelerated catabolism of this complement component, caused by immune complexes that fix C1 *in vivo* and activate the classical pathway of the complement system (Schur, 1975, 1982). On the other hand, individuals who lack serum C1q because of an inherited defect of synthesis and who have no functional classical pathway show a clear predisposition to develop SLE or SLE-like syndromes (Loos & Heinz, 1986).

Furthermore, patients with active SLE who are not genetically C1q-deficient share a typical serological abnormality with these C1q-deficient individuals: their serum contains considerable amounts of low-molecular-mass C1q (LMM-C1q) regarded as 'non-functional' on account of its inability to participate in classical-pathway activation (Hannema *et al.*, 1984; Hoekzema *et al.*, 1985*a*). Interestingly, low levels of LMM-C1q are also detectable in normal sera (Hoekzema *et al.*, 1985*a*). These observations raise the obvious question whether LMM-C1q is the result of degradation of normal C1q or synthesized as such. The finding that LMM-C1q contains apparently intact A, B and C polypeptide chains with abnormal disulphide bonding, i.e. absence of the CC dimer and presence of an abnormal AC dimer, argues against proteolysis as the source of LMM-C1q (Hoekzema *et al.*, 1985*b*).

If LMM-C1q is synthesized, the most likely candidate for its primary site of production would be the cell responsible for levels of functional C1q in serum. However, the primary site of synthesis for serum C1q in man has not been established, although many different tissues and cell types secrete functional C1q *in vitro*, including cells of the intestinal and urogenital tract (Colten *et al.*, 1968; Bing *et al.*, 1975; Morris *et al.*, 1978), spleen cells (Kohler, 1973; Lai A Fat & van Furth, 1975), fibroblasts (Al-Adnani & McGee, 1976; Reid & Solomon, 1977; Morris *et al.*, 1978; Skok *et al.*, 1981), peritoneal macrophages (Stecher *et al.*, 1967; Müller *et al.*, 1978) and cultured monocytes (Morris *et al.*, 1978; Bensa *et al.*, 1983; Reboul *et al.*, 1985; Tenner & Volkin, 1986).

Since both cultured (human) monocytes and (guinea-pig peritoneal) macrophages have been shown to synthesize C1q with a subunit structure identical with that of plasma C1q (Reboul *et al.*, 1985; Rabs *et al.*,

Abbreviations used: SLE, systemic lupus erythematosus; FCS, fetal-calf serum; McAb, monoclonal antibody; FITC, fluorescein isothiocyanate; NHS, normal human serum; r.i.a., radioimmunoassay; PBS, phosphate-buffered saline (10 mM-sodium phosphate/150 mM-NaCl), pH 7.4; RT, room temperature; VBS^{++}, veronal-buffered saline (5 mM-sodium 5,5-diethylbarbiturate/0.145 M-NaCl), containing 1 mM-$MgCl_2$ and 0.15 mM-$CaCl_2$, pH 7.4; $VBsucr^{++}$, veronal buffer, containing 5.8% (w/v) sucrose, 0.5% (w/v) albumin, 1 mM-$MgCl_2$ and 0.2 mM-$CaCl_2$ of conductivity 7 mS and pH 7.4; SDS/PAGE, polyacrylamide-gel electrophoresis in the presence of SDS; LMM-C1q, low-molecular-mass complement component C1q; PDI, protein disulphide-isomerase.

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1986; Tenner & Volkin, 1986), we tested whether human blood monocytes and alveolar macrophages in culture also secrete LMM-C1q.

The results presented here show that LMM-C1q is indeed an important product of C1q-synthesizing cells and not the result of degradation or impaired hydroxylation of normal C1q. The significance of this finding with regard to SLE and inherited C1q deficiency is discussed.

MATERIALS AND METHODS

Percoll and CNBr-activated Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden). L-Proline, L-azetidine-2-carboxylic acid, 2,2'-dipyridyl and soybean-trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Medium lacking proline/hydroxyproline (RPMI-1640 Select-Amine kit) and fetal-calf serum (FCS) (High Clone) were from Gibco (Grand Island, NY, U.S.A.). Medium lacking methionine (RPMI-1640) was purchased from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). Ascorbic acid was obtained from Merck (Darmstadt, Germany) and IgG-coated latex beads (Rapi-Tex-RF) were from Behringwerke (Marburg, Germany). Benzamidine was purchased from Janssen Chimica (Beerse, Belgium) and (4-amidinophenyl)methanesulphonyl fluoride from Boehringer (Mannheim, Germany).

Human C1q was purified as described by Tenner *et al.* (1981) and radiolabelled by the chloramine-T method, essentially as described by Sobel *et al.* (1975), except that 1 μCi of ^{125}I was added/μg of C1q and that dialysis was followed by ultracentrifugation in isokinetic 5–25%-(w/v)-sucrose gradients to separate monomeric ^{125}I-C1q from ^{125}I-C1q aggregates.

Samples (250 ng) of ^{125}I-C1q showed 95% binding to aggregated IgG in the C1q-binding test, performed essentially as described by Zubler *et al.* (1976). Purification of rabbit antibodies against human C1q from antiserum (batch KH43-05-PO2; Department of Immune Reagents, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) as well as radiolabelling of these antibodies by the chloramine-T method, were performed as described by Hack *et al.* (1981). Rabbit anti-(human C1q)–Sepharose was prepared by coupling 50 μl of the antiserum to 100 mg of CNBr-activated Sepharose 4B.

The procedures of preparation and characterization of monoclonal antibodies (McAb) against C1q are reported elsewhere (Hoekzema *et al.*, 1988). Table 1 summarizes the properties of the antibodies against C1q that were used in the present study.

Fluorescein isothiocyanate (FITC)-labelled goat antibodies against mouse immunoglobulins were obtained from the Central Laboratory Department of Immune Reagents (GM-17-F01).

Blood

Blood was collected either in 500 ml plastic bags, containing 70 ml of acid-citrate/dextrose [2.7% (w/v) disodium citrate and 2.3% (w/v) glucose], or in silicone-treated Vacutainer tubes (Becton–Dickinson, Meylan, France), containing 0.38% (w/v) trisodium citrate. All blood samples were processed within 4 h of collection.

Alveolar macrophages

Alveolar macrophages, obtained with informed consent by broncheolar lavage of a healthy volunteer, were kindly provided by Dr. E. V. van de Graaf (Clinical Immunology Laboratory, Academic Medical Center, Amsterdam, the Netherlands).

Table 1. Recognition of C1q and LMM-C1q by the antibodies used in the present study

The ability of antibodies to bind normal and/or LMM-C1q was assessed by several techniques not discussed in the present paper including immunoabsorption and radioimmunoassays with serum/plasma after separation of proteins by ultracentrifugation.

Anti-C1q antibody	Detection of: C1q	LMM-C1q
Rabbit	+++	++
McAb130	+++	–
McAb101	+	+++

Separation and culture of monocytes

Monocytes were isolated by two different methods. When buffy coats from 500 ml samples of blood were available, cells were separated by centrifugation over Percoll followed by elutriation centrifugation, essentially as described by de Boer & Roos (1986), with the exception that the final Percoll-centrifugation step for the separation of monocytes (±90%) from basophils (5–10%) was omitted. When smaller (30–50 ml) samples of blood were used as the starting material, monocytes were isolated by two-step density-centrifugation method of de Boer *et al.* (1981), with the exception that Ficoll was replaced by Percoll. The purity of monocytes obtained by these procedures ranged from 90 to 95% for the elutriation method and from 70 to 80% for the two-step centrifugation method. Unless indicated otherwise, cells were cultured in 24-well Multidishes (Nunc, Roskilde, Denmark) at 5.0×10^6 cells/well in Iscove's modified Dulbecco's medium, supplemented with 10% (v/v) heat-inactivated (1 h, 56 °C) FCS, 10 μg of L-(+)-ascorbic acid/ml, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Culture supernatant was harvested and medium replenished after 24 h and then twice a week.

C1q-haemolytic assay

Serial dilutions of culture supernatant were tested for functional C1q by C1q-haemolytic assay, as previously described (Hoekzema *et al.*, 1985*a*). Dilutions of pooled normal human serum (NHS) were used as a standard for the determination of functional C1q in culture supernatants. The assay permitted detection of C1q at concentrations as low as 100 pg/ml.

R.i.a. for LMM-C1q

Before the r.i.a. for LMM-C1q, culture supernatants (2 ml) were dialysed (24 h) against 4 mM-sodium phosphate buffer, pH 6.0, at 4 °C to precipitate euglobulins, including functional C1q. The precipitate, containing the bulk of normal C1q, was removed by centrifugation (2700 *g*, 60 min, 4 °C).

Serial dilutions of euglobulin supernatants from culture medium were incubated in rotating polystyrene tubes with 1.5 mg of Sepharose 4B to which 30 μg of an

anti-C1q monoclonal antibody (McAb 101) had been coupled; this McAb preferentially binds LMM-C1q (Hoekzema *et al.*, 1985*b*). The incubation was performed in 1 ml of phosphate-buffered saline (PBS), pH 7.4, containing 0.1% (w/v) Tween 20, 10 mM-EDTA and 800 mM-NaCl for 16 h at room temperature (RT). After extensive washing of the Sepharose beads with PBS/0.1% (w/v) Tween 20, a second incubation was performed (16 h at RT) with 50 μl of ^{125}I-labelled rabbit antibodies against C1q (2 ng $\sim$ 20000 c.p.m.) in the same buffer. After washing of the beads, bound radioactivity was measured with a γ-radiation counter. The amount of LMM-C1q in culture supernatants was then calculated by reference to a standard curve of euglobulin supernatant of NHS, arbitrarily defined as containing 100% LMM-C1q.

Sucrose-gradient ultracentrifugation

Monocyte culture supernatant was analysed by ultracentrifugation in isokinetic gradients of sucrose [25–5% (w/v)], prepared in veronal-buffered saline containing 1 mM-$MgCl_2$ and 0.15 mM-$CaCl_2$, pH 7.4 (VBS^{++}), essentially as described previously (Hoekzema *et al.*, 1985*a*). Conditions are given in the legends to the Figures.

Immunofluorescence with monoclonal anti-C1q

After 20 days of culture, blood monocyte-derived macrophages were harvested by vigorous pipetting of 1 ml culture wells, containing 2×10^5 cells, with ice-cold PBS, pH 7.4. This procedure detached about 90% of the adherent cells, which were subsequently spun down on glass slides and fixed in acetone (5 min). The slides were incubated for 30 min at RT with 200 μl of anti-C1q hybridoma culture supernatant, containing 10–50 μg of anti-C1q McAb/ml, diluted 1:5 in PBS.

The slides were washed (3×10 min) with PBS and incubated (30 min, RT) with 1 ml of a 1:20 dilution in PBS of FITC-labelled goat antibodies against mouse immunoglobulin. The slides were washed again, mounted with a coverglass and photographed through a standard fluorescence microscope. As controls, several McAbs of different subclass against unrelated antigens such as human IgG and horseradish peroxidase as well as normal mouse serum were used. None of these stained the cells after incubation with FITC-labelled goat anti-mouse immunoglobulin.

Phagocytosis of ^{125}I-C1q by culture-derived macrophages

^{125}I-C1q (50×10^6 cpm $\sim$ 500 μg) was incubated (16 h, RT) with 680 μl of a 1:10 dilution of IgG-coated latex beads in veronal buffer, containing 5.8% (w/v) sucrose, 0.5% (w/v) human serum albumin, 1 mM-$MgCl_2$ and 0.2 mM-$CaCl_2$, pH 7.4, and a conductivity of 7 mS at 20 °C ($VBsucr^{++}$). The latex was washed extensively in $VBsucr^{++}$ to remove unbound ^{125}I-C1q, and 10^6 c.p.m. of ^{125}I-C1q-IgG–latex complexes were added to culture wells, containing approx. 2×10^5 culture-derived macrophages. To control wells, ^{125}I-C1q without IgG latex was added. After 20 h of culture, the cells had phagocytosed most of the latex, as judged by microscopy. The supernatants were obtained and the cells were harvested as described under 'Immunofluorescence with monoclonal anti-C1q' above. The cells were then washed in PBS, pH 7.4, by repeated centrifugation (10 min, 400 *g* at RT) to remove extracellular latex beads and ^{125}I-C1q. The cells were then lysed in 250 μl of PBS containing 1% (w/v) Nonidet P40, 5 mM-EDTA, 1 mM-(4-amidinophenyl)methanesulphonyl fluoride, 10 mM-benzamidine and soybean-trypsin inhibitor (0.02 mg/ml).

Lysate samples were analysed by non-reducing SDS/polyacrylamide-gel electrophoresis (SDS/PAGE), using a 1.5 mm-thick 13%-(w/v)-polyacrylamide slab gel in a Tris/HCl, pH 8.8, system containing 0.1% SDS. After drying the gel, autoradiography was performed at −70 °C with Kodak XAR-5 X-ray film. Culture supernatants containing ^{125}I-Clq (20000 c.p.m.) were incubated with McAb-101– or polyclonal-anti-C1q–Sepharose to test recognition of ^{125}I-C1q (fragments) by these antibodies.

Metabolic labelling of culture-derived macrophages

After 20 days of culture, 2×10^6 cells were incubated for 1 h in RPMI-1640 medium (2×10^5 cells/ml) lacking methionine and containing 1% (v/v) of heated (1 h, 56 °C) FCS and 36 mM-$NaHCO_3$. Next, [^{35}S]methionine (40 μCi; The Radiochemical Centre, Amersham, Bucks., U.K.) was added to the cells together with fresh ascorbic acid (10 μg/ml). After 5 days of culture, supernatants were collected, adjusted to 0.1% (w/v) Tween-20, 10 mM-EDTA and 500 mM-NaCl and immunoabsorbed by incubation (16 h, 4 °C) with 20 mg of Sepharose to which 600 μg of McAb 130 (which recognizes normal C1q but not LMM-C1q) had been coupled. The Sepharose was separated from the unbound fraction by centrifugation, and the latter was immunoabsorbed by incubation (16 h, 4 °C) with 20 mg of Sepharose to which 600 mg of McAb 101 had been coupled. After extensive washings in PBS, both batches of Sepharose were eluted with sample buffer for SDS/PAGE, and eluates were analysed under non-reducing and reducing (1% mercaptoethanol) conditions in a 13% gel essentially as described above, except that this gel was incubated with EN^{3}HANCE (New England Nuclear) and the fluorophore was precipitated with distilled water before drying and autoradiography.

RESULTS

Normal and LMM-C1q in supernatant of cultured monocytes

To test whether the supernatant of cultured monocytes contains LMM-C1q, a sample obtained after 20 days of culture was analysed by ultracentrifugation in a sucrose gradient as described in the Materials and methods section. Fig. 1 shows that, although the gradient was prepared in VBS^{++}, allowing association of C1q with C1r and C1s to yield C1, which has a sedimentation coefficient ($s_{20,w}$) of 16 S, all C1q-haemolytic activity (stippled) was recovered at 11 S, the position of free C1q. Next to this peak of functional C1q, a second peak lacking haemolytic activity was detected by rabbit anti-C1q r.i.a. (●) at 4 S, the $s_{20,w}$ of LMM-C1q in serum (Hoekzema *et al.*, 1985*a*,*b*). The r.i.a. with McAb 101 (○) confirmed the presence of LMM-C1q at 4 S and clearly demonstrated the lack of significant binding of normal (11 S) C1q by this antibody (in this experiment normal C1q was not removed before testing the fractions with McAb 101).

Next, the appearance of normal and LMM-C1q was monitored in monocyte cultures from ten healthy controls and two homozygous individuals (G and V) with an

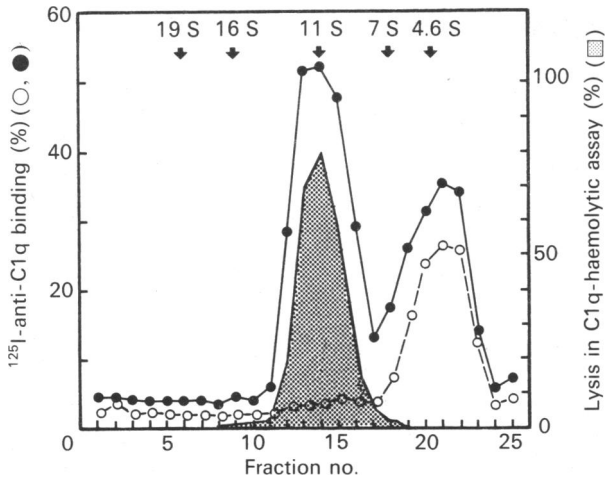

Fig. 1. Sedimentation behaviour of forms of C1q present in monocyte culture supernatant

Culture supernatant (500 μl) of culture-derived macrophages (day 20 of culture) was ultracentrifuged (26000 rev./min, 20 h, 20 °C) in a 5–25% isokinetic sucrose gradient, prepared in VBS^{++}, pH 7.4. The positions of marker proteins IgM (19 S), C1 (16 S), C1q (11 S), IgG (7 S) and albumin (4.6 S) in separate runs of NHS were determined by nephelometry. Samples of collected fractions were tested in (*a*) the rabbit-anti-C1q sandwich r.i.a. (100 μl, ●), (*b*) the McAb-101 r.i.a. for LMM-C1q (200 μl, ○) and (*c*) the C1q–haemolytic assay (10 μl, stippled area), as described in the Materials and methods section, except that euglobulin precipitation before the McAb-101 r.i.a. was omitted.

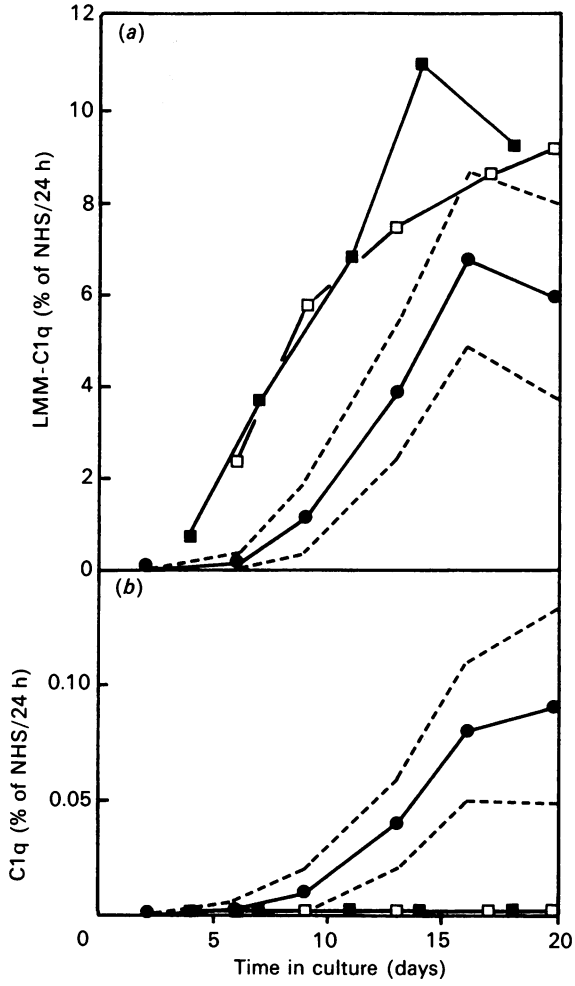

Fig. 2. Secretion of functional C1q and LMM-C1q by monocytes in culture

Levels of LMM-C1q (*a*), assessed by McAb-101 r.i.a., and functional C1q (*b*), assessed by C1q-haemolytic assay, obtained at various times during the maturation of monocytes into macrophages were measured and expressed as amounts (percentage of the level present in NHS) that had been secreted into 1 ml of culture medium in 24 h. The mean (●) ± 2 S.D. (----) of 10 healthy controls is indicated. The squares indicate values obtained in monocyte cultures from two homozygous C1q-deficient patients, G (■) and V (□).

inherited deficiency of plasma C1q and high serum levels of LMM-C1q (660–700% that of NHS). Since culture medium was replenished at various time intervals, the amounts of normal and LMM-C1q that had accumulated in the supernatant were expressed as levels (% of NHS) secreted into 1 ml of culture medium in 24 h. As judged from this 'rate of secretion', cells from healthy controls progressively developed the ability to produce LMM-C1q (Fig. 2*a*) and functional C1q (Fig. 2*b*) while maturing into macrophage-like cells. After 20 days of culture, the rate of secretion for functional C1q reached a steady state at 0.1% C1q of NHS (about 100 ng)/24 h per ml, corresponding to $(1.5–3) \times 10^4$ functional molecules/h per cell.

The rate of secretion for LMM-C1q increased in a similar manner, although Fig. 2(*a*) shows that the amounts of LMM-C1q secreted throughout the culture period were excessive when compared with those of functional C1q. As a result, the LMM-C1q/C1q ratios in cultures were 30–100 times higher than the ratio in NHS. Since we estimated that, in NHS, LMM-C1q accounts for 1–5% of 'C1q-antigen', the number of LMM-C1q molecules secreted by these cultures probably exceeded the number of functional C1q molecules.

The secretion of LMM-C1q was even more impressive in the cultures from C1q-deficient patients G and V (■, □, Fig. 2*a*), amply surpassing the upper limit [mean + (2 × S.D.)] of the control cultures. Normal C1q was not detectable in these cultures either by C1q-haemolytic assay (Fig. 2*b*) or by a r.i.a. with McAb-130–Sepharose and radiolabelled rabbit anti-C1q (results not shown).

In the ten monocyte cultures from healthy individuals, the production of functional and LMM-C1q was clearly co-ordinated: amounts of functional C1q correlated significantly with amounts of LMM-C1q ($P < 0.001$) whether compared at a single moment for the whole group or longitudinally for individual cultures.

Degradation of ^{125}I-C1q by cultured monocytes

To test whether LMM-C1q in supernatants of cultured monocytes results from ingestion and subsequent degradation of secreted normal C1q, cells were allowed to digest radiolabelled normal C1q, followed by analysis of both cell lysates and supernatants. The ^{125}I-C1q was presented to the cells after binding of the molecule to IgG-coated latex beads to facilitate phagocytosis, or without latex beads. Because preliminary experiments

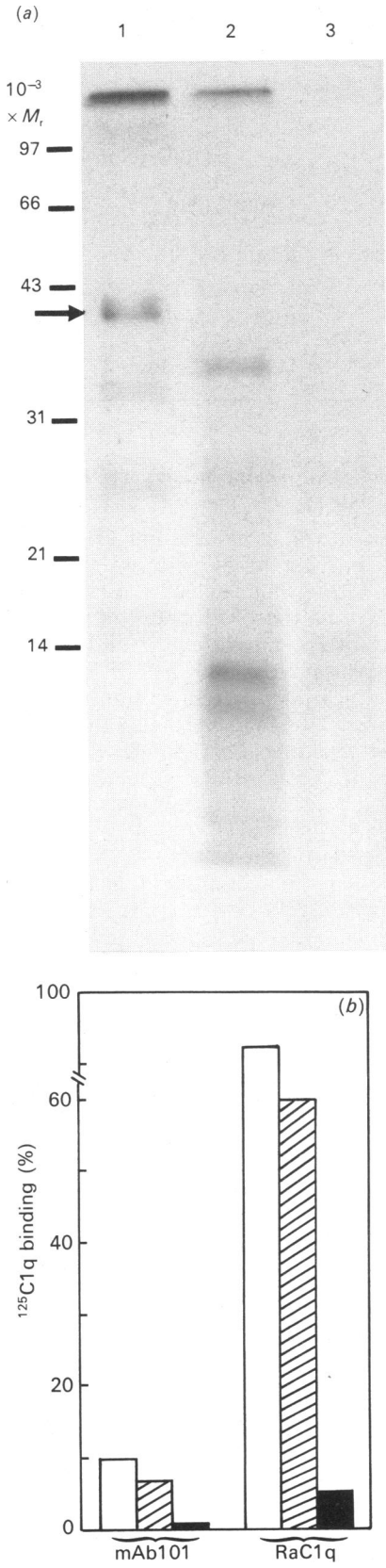

Fig. 3. Degradation of ^{125}I-C1q by culture-derived macrophages

(*a*) Autoradiogram of non-reduced SDS/13%-PAGE of ^{125}I-C1q (lane 1) and of cell lysate containing ^{125}I-C1q that had been offered to macrophages (20 h), either as a complex with IgG latex (lane 2) or without IgG latex (lane 3). The arrow indicates the position of the CC dimer of normal C1q in protein-stained gels. Positions of low-M_r markers are indicated. (*b*) Ability of anti-C1q McAb 101–Sepharose 4B (McAb 101) and rabbit anti-C1q–Sepharose 4B (RaC1q) to recognize ^{125}I-C1q (□) and ^{125}I-C1q from culture supernatant after incubation (20 h) with macrophages without IgG-latex (▨) or as a complex with IgG-latex (■).

showed that the cells required several hours to engulf most of the latex, the incubation was performed overnight (20 h). Next, cells and supernatants were harvested for analysis of ingested and secreted ^{125}I-C1q (fragments).

Fig. 3(*a*), lane 2, shows the behaviour in non-reducing SDS/PAGE of radiolabelled fragments from lysates of ^{125}I-C1q–IgG–latex-treated cells. The band corresponding to the M_r −41000 CC-dimer of ^{125}I-C1q (arrow in lane 1) had disappeared to yield major fragments with M_r values of 35000, 13000 and 11000. No radioactive band was observed at 48000, which is the position expected for ^{125}I-LMM-C1q (Hoekzema *et al.*, 1985*b*; R. Hoekzema, M. C. Brouwer & C. E. Hack, unpublished work). No significant amount of ^{125}I-C1q, intact or fragmented, was present in the lysate from cells that had been incubated with ^{125}I-C1q without IgG latex (lane 3 in Fig. 3*a*).

Fig. 3(*b*) shows the ability of McAb 101–Sepharose and rabbit-anti-C1q–Sepharose (both added in excess) to bind ^{125}I-C1q or fragments of ^{125}I-C1q from culture supernatant of the same cells. As judged by binding to McAb-101–Sepharose, digestion of ^{125}I-C1q by the cells had not generated ^{125}I-LMM-C1q in the culture supernatant; instead, the weak binding of ^{125}I-C1q to McAb 101 (maximal 10% of the input) was abolished. The same was observed with rabbit-anti-Clq–Sepharose: the radiolabelled fragments that had been secreted by the cells were no longer recognized by the antibodies.

Other experiments, not shown here, demonstrated that shorter incubations (30 min to 4 h) of ^{125}I-C1q with cultured monocytes did not yield intra- or extra-cellular ^{125}I-LMM-C1q either.

Immunofluorescence of cultured monocytes

Fig. 4 demonstrates intracellular C1q and LMM-C1q in fixed culture-derived macrophages by indirect immunofluorescence with monoclonal anti-C1q and fluorescent goat antibodies to mouse Ig. When normal C1q was revealed in cells from healthy controls by McAb 130, characteristic patterns of perinuclear fluorescence were observed, compatible with the stack of cisternae that forms the Golgi apparatus (Geller Lipsky & Pagano, 1985; Willingham & Pastan, 1985). In some cells, the organelle containing C1q was dense, with little vacuolation (Fig. 4*a*), whereas in others the structure was more reticular and vesiculated (Fig. 4*b*). In cells from C1q-deficient patient G, no fluorescence was observed with McAb 130 against normal C1q (results not shown). However, when cells from this patient were incubated with McAb 101 against LMM-C1q, patchy fluorescence was observed, scattered throughout the cytoplasm (Fig. 4*c*). In cells from healthy controls, the fluorescence obtained with McAb 101 was much weaker, whereas the cytoplasmic distribution was variable (results not shown). Control McAbs or normal mouse serum did not stain the cells from patients or healthy individuals.

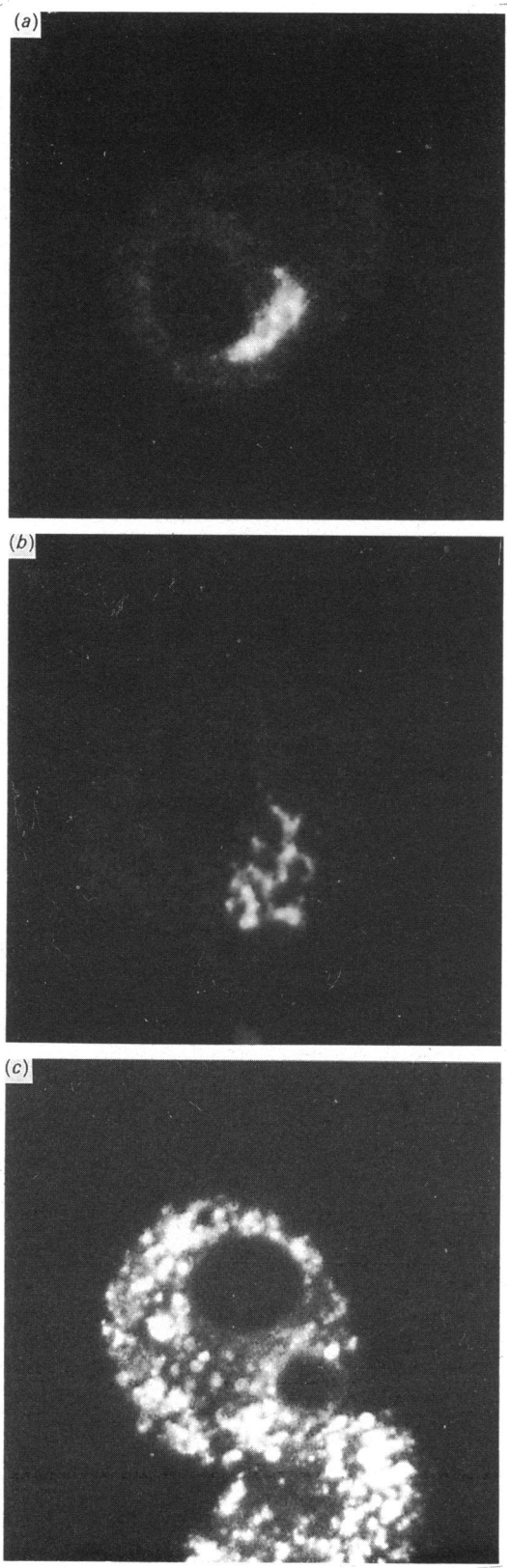

Fig. 4. Immunofluorescence of culture-derived macrophages with anti-C1q McAb

Intracellular C1q and LMM-C1q were revealed by immunofluorescence of fixed culture-derived macrophages (day 20 of culture) with monoclonal antibodies and FITC-labelled goat anti-mouse Ig as described in the Materials and methods section. Magnification × 500. (*a*) and (*b*), Two types of representative staining patterns, obtained with McAb 130 that binds normal C1q, in cultured cells from a healthy individual; (*c*) staining of cells from C1q-deficient patient G with McAb 101, which binds LMM-C1q.

Table 2. Ascorbic acid does not stimulate secretion of normal or LMM-C1q by cultured monocytes from C1q-deficient patient V

	C1q or LMM-C1q secreted (% of level in NHS/24 h)			
	Control (day 20)		Patient V (day 20)	
Ascorbic acid	C1q	LMM-C1q	C1q	LMM-C1q
Present (10 μg/ml)	0.26	4.70	0	9.20
Absent	0.14	3.20	0	9.37

Effect of underhydroxylation on secretion of functional and LMM-C1q

To investigate whether the high levels of LMM-C1q present in supernatant of cultured monocytes are the result of an intracellular defect in hydroxylation and/or triple-helix formation of C1q, cells were cultured in (*a*) the presence of 2,2′-dipyridyl, an inhibitor of prolyl and lysyl hydroxylases (Müller *et al.*, 1978), (*b*) the presence of azetidine-2-carboxylic acid, a homologue of L-proline that is incorporated into collagen and precludes normal helix formation (Lane *et al.*, 1971; Bienkowski, 1978), (*c*) the absence of additional ascorbic acid, which serves as an important cofactor of prolyl hydroxylase and (*d*) the presence of ascorbic acid (10 μg/ml) oxidized by exposure to daylight. Fig. 5 shows that 2,2′-dipyridyl at 12.5–50 μM inhibited the secretion of haemolytically active C1q, but not of LMM-C1q, in a dose-dependent manner. At 100–200 μM, when the secretion of functional C1q was totally inhibited, levels of LMM-C1q were also drastically reduced. When cells were cultured in the presence of azetidine, in the absence of fresh ascorbic acid or with ascorbic acid that had been oxidized by light, again the secretion of both functional C1q and of LMM-C1q was impaired, although the effect on functional C1q was somewhat more pronounced. Thus none of the culture conditions expected to impede hydroxylation and triple-helix formation of C1q resulted in an increase in LMM-C1q, as judged by the McAb-101 r.i.a.

Table 2 shows the effect of fresh ascorbic acid on the synthesis of functional and LMM-C1q by monocytes of a C1q-deficient patient (V) and of a healthy control at day 20 of culture. Ascorbic acid stimulated secretion of normal and LMM-C1q by control cells, but did not yield detectable levels of functional C1q or an increase in LMM-C1q in the culture of patient V. However, in the absence of ascorbic acid, LMM-C1q levels in this culture already were about 300% of those of the control culture.

Metabolic labelling of culture-derived macrophages

After culturing cells in the presence of [^{35}S]methionine, [^{35}S]C1q and [^{35}S]LMM-C1q were immunoabsorbed from the culture medium by using McAb 130–Sepharose

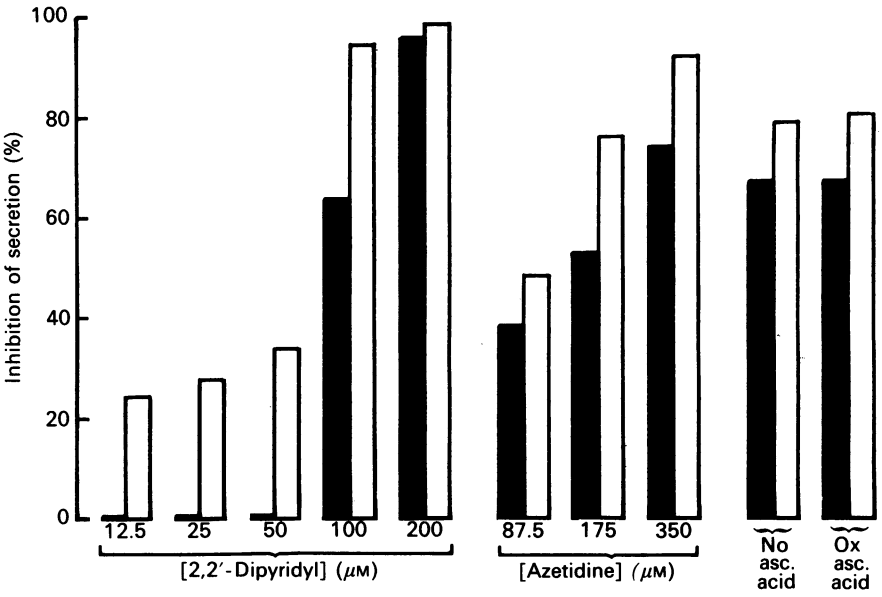

Fig. 5. Effects of inhibition of hydroxylation and triple-helix formation of C1q

Culture-derived macrophages were cultured either in the presence of 2,2'-dipyridyl or azetidine-2-carboxylic acid (azetidine), or in the absence of fresh ascorbic acid (No asc. acid) or in the presence of oxidized ascorbic acid (Ox. asc. acid). The amounts of functional C1q and LMM-C1q secreted in the culture medium were measured. The effect of the different conditions is expressed as percentage inhibition of the secretion of functional C1q (□) and LMM-C1q (■) in comparison with control cultures under normal conditions.

and McAb 101–Sepharose respectively. Next, immunoabsorbed material was analysed by SDS/PAGE and fluorography. Fig. 6 (lane 1) shows that, in the absence of reducing agent, [^{35}S]LMM-C1q demonstrated a major band of M_r 55000 migrating at the position of the AB dimer of normal plasma C1q. A second, rather broad, band of M_r 44000–49000 was present, in agreement with the smaller subunit of LMM-C1q isolated from serum (Hoekzema *et al.*, 1985*a*,*b*; R. Hoekzema, M. C. Brouwer & C. E. Hack, unpublished work). No band was observed at M_r 41000, the position of the CC dimer of normal C1q. In contrast, ^{35}S-labelled normal C1q (lane 2) demonstrated the expected AB (M_r 55000) and CC (M_r 41000) subunits.

Under reducing conditions, both [^{35}S]LMM-C1q (lane 3) and [^{35}S]C1q (lane 4) demonstrated A (M_r 32000), B (M_r 31000) and C (M_r 27000) polypeptide chains with normal electrophoretic mobility. The fourth band at M_r 25000, designated C′ and present both in the radiolabelled molecules and in protein-stained C1q isolated from plasma, was found to represent a C chain with intact intrachain disulphide bonds, attributable to insufficient reduction before SDS/PAGE.

Normal and LMM-C1q in the supernatant of cultured alveolar macrophages

Alveolar macrophages and blood monocytes, obtained from the same healthy volunteer, were cultured and assessed for production of functional and LMM-C1q as described in the Materials and methods section. Fig. 7 shows that, throughout the culture period, the rate of C1q and LMM-C1q secretion increased proportionally for both cell types, although the alveolar cells that had matured *in vivo* appeared 5–6 days 'ahead' of the monocytes that were maturing *in vitro*. The ratio LMM-C1q/C1q was also very similar for the two cultures and showed the same 5–6 day 'time-lag' for the monocytes.

DISCUSSION

The results of the present study demonstrate that cultured macrophages of healthy donors, whether matured *in vivo* or *in vitro*, secrete excessive amounts of an LMM-C1q that has apparent identity with the molecule in serum. Others have shown that cultured human monocytes secrete functional C1q with a subunit structure identical with that of serum C1q. Reboul *et al.* (1985) found apparently normal AB and CC dimers and A, B and C polypeptide chains in immunoprecipitates from the supernatant of metabolically radiolabelled monocytes that had been cultured for 12–15 days. However, those authors also observed an unidentified subunit that migrated between the AB and CC dimer. Tenner & Volkin (1986) confirmed the identity of monocyte and serum C1q, but they also noticed a marked discrepancy between levels of C1q quantified haemolytically or immunochemically; a greater amount of C1q was detected by goat antibodies to C1q than by the C1q-haemolytic assay. These findings can be explained by the presence of significant amounts of LMM-C1q in the supernatant of cultured monocytes. It is likely that the polyclonal antibodies that were used in the studies mentioned above had also detected LMM-C1q, which contains all three polypeptide chains of normal C1q. The abnormal AC dimer of LMM-C1q at M_r 48000 (Hoekzema *et al.*, 1985*a*,*b*; R. Hoekzema, M. C. Brouwer, E. R. de Graeff-Meeder, H. P. T. van Helden & C. E. Hack, unpublished work) probably corresponds to the unidentified subunit observed by Reboul and co-workers.

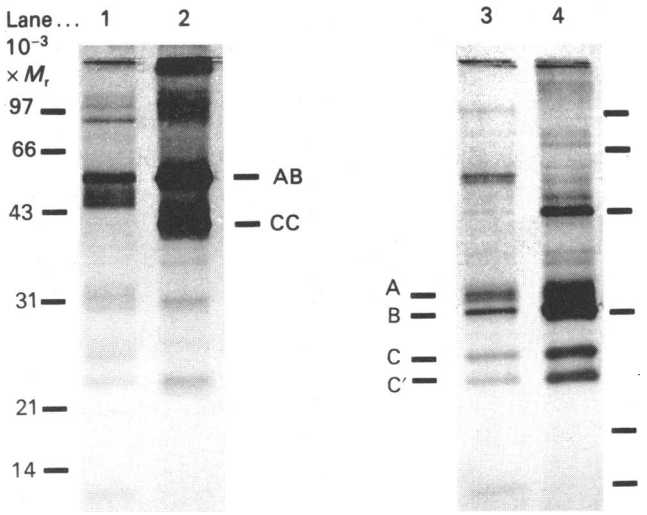

Fig. 6. Fluorography of SDS/PAGE of metabolically radiolabelled and immunoabsorbed C1q and LMM-C1q

Metabolically ^{35}S-labelled C1q and LMM-C1q were immunoabsorbed on anti-C1q McAb–Sepharose from culture supernatant and analysed in a 13% gel as described in the Materials and methods section. Lane 1, LMM-C1q immunoabsorbed on McAb-101–Sepharose, non-reducing conditions; lane 2, C1q immunoabsorbed on McAb-130–Sepharose, unreduced conditions; lane 3, as lane 1, but reducing conditions; lane 4: as lane 2, but reducing conditions. The positions of subunits of plasma C1q and of low-M_r markers, determined by protein staining, are indicated.

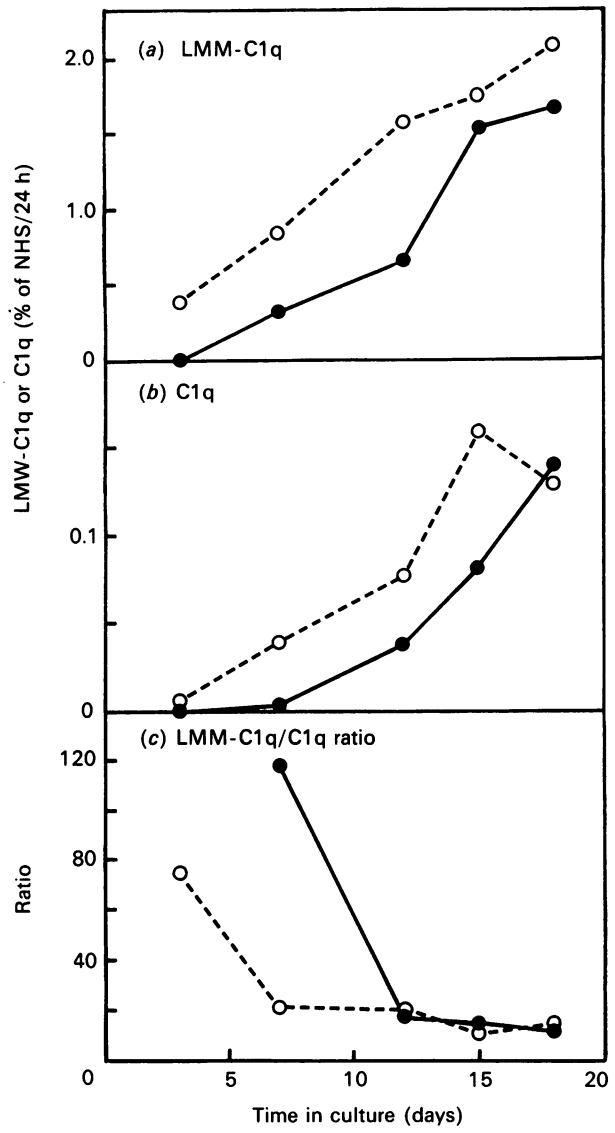

Fig. 7. Secretion of functional C1q and LMM-C1q by monocytes and alveolar macrophages in culture

Levels of LMM-C1q (assessed by McAb-101 r.i.a.) (*a*), functional C1q (assessed by C1q-haemolytic assay) (*b*) and ratios LMM-C1q/C1q in culture supernatant (*c*), obtained at different times during culture of blood monocytes (●) or alveolar macrophages (○) of the same healthy donor. Levels of C1q and LMM-C1q are expressed as the amount (% of NHS) in 1 ml of culture medium secreted by 10^5 cells in 24 h.

Adding radiolabelled C1q to monocyte cultures, either in fluid phase or in complex with IgG-latex, did not yield fragments compatible with LMM-C1q as judged by SDS/PAGE and binding studies with poly- or monoclonal anti-C1q. Therefore LMM-C1q is clearly different from the degradation products obtained after phagocytosis of C1q (Veerhuis *et al.*, 1985, 1986). The presence in LMM-C1q of apparently intact A, B and C polypeptide chains and the accumulation of LMM-C1q in cytoplasmic organelles compatible with the Golgi apparatus further argue against proteolysis as its source. One could speculate that, for some unknown reason, cells in culture are unable to properly hydroxylate C1q. Analogous to the situation in collagen synthesis by fibroblasts (Prockop *et al.*, 1976), this could lead to underhydroxylated C1q with defective triple-helix conformation, as has been proposed by others (Müller *et al.*, 1978; Loos, 1982). A defective triple helix of the collagenous portion of subunits of C1q could prevent subsequent assembly of the intact molecule and result in secretion of LMM-C1q instead. Although we did not measure the hydroxyproline content of LMM-C1q, additives such as 2,2′-dipyridyl and azetidine-2-carboxylic acid, which impede hydroxylation and triple-helix formation (Prockop *et al.*, 1976; Bienkowski *et al.*, 1978), did not increase LMM-C1q levels in monocyte cultures, whereas ascorbic acid, which clearly stimulates hydroxylation (Morris & Paz, 1980) and secretion (Tenner & Volkin, 1986) of C1q, equally stimulated secretion of LMM-C1q. Therefore it is unlikely that LMM-C1q merely represents underhydroxylated C1q. However, ascorbic acid could have an additional stimulatory effect on the synthesis of normal and LMM-C1q not related to its role as a cofactor of hydroxylation. Such a direct effect of ascorbic acid has been demonstrated for the synthesis of collagen (Murad *et al.*, 1981, 1983; Geesin *et al.*, 1986) and is accompanied by an increase in procollagen mRNA (Tajima & Pinnell, 1982). A recent study in guinea pigs (Johnston *et al.*, 1987) suggests a similar effect on the synthesis of C1q: a high dietary intake of ascorbic acid resulted in a 30–50% increase in levels of plasma C1q.

Interestingly, although ascorbic acid clearly stimulated secretion of LMM-C1q in monocyte cultures from healthy controls, it had no effect on cells from C1q-deficient patients in whom secretion of LMM-C1q

already seemed to be stimulated, as judged by the increased intra- and extra-cellular levels of LMM-C1q. Clearly, an understanding of the genetic defect that underlies this particular form of C1q deficiency is complicated by the fact that LMM-C1q is a normal constituent of human plasma. Therefore it does not represent a genetically defective protein. Two other possibilities have to be considered. First, LMM-C1q could represent a different form of C1q, as has been demonstrated for the C1q-like molecule that is synthesized by fibroblasts (Reid & Solomon, 1977; Skok *et al.*, 1981). Analogous to fibroblast C1q, LMM-C1q could be the product of a gene distinct from, but closely related to, that which codes for serum C1q. If this is the case, one could speculate that LMM-C1q has a specific function of its own, possibly restricted to the vicinity of the macrophage. Since LMM-C1q does not incorporate C1r and C1s and therefore has an unoccupied collagen-like region, which is essential for binding of normal C1q to C1q receptors (Tenner & Cooper, 1980), it could interact with the many different cells that appear to have these receptors on their membrane (Tenner & Cooper, 1981, 1982; Bobak *et al.*, 1987).

A second possibility regarding the origin of LMM-C1q would be that it results from incorrect assembly of C1q from its composing subunits. Considering the abnormal disulphide bonds in LMM-C1q, i.e. the absence of CC and the presence of AC dimers, the process of interchain disulphide-bond formation would be a likely candidate for such a post-translational defect. The enzyme responsible for correct disulphide formation in multi-chain proteins, including collagen, is protein-disulphide-isomerase (PDI) (Freedman, 1987). Whether secretion of LMM-C1q by cultured cells, including those of C1q-deficient patients, can be attributed to a dysfunction of PDI, remains to be determined.

The secretion of functional and LMM-C1q by alveolar macrophages increased during the time in culture in a manner very similar to the secretion of these molecules by blood monocytes maturating *in vitro*. Furthermore, LMM-C1q/C1q ratios were also comparable for alveolar and culture-derived macrophages. These findings allow two important conclusions. First, they argue against a defect of maturation itself as the cause of excessive LMM-C1q production by monocytes in culture. Secondly, they indicate that, once in culture, alveolar macrophages, and probably also macrophages from other tissues, do not represent 'resting' cells, but are rapidly activated by the '*in vitro*' situation. This should be considered by investigators who study the properties of macrophages in culture.

In conclusion, LMM-C1q is synthesized by the same cells that produce functional C1q of normal structure, and the synthetic rate of the two molecules appears to be highly co-ordinated, at least in non-C1q-deficient individuals. Although these results indicate that serum levels of LMM-C1q may provide a unique marker of C1q synthesis in diseases with an accelerated metabolism of this complement component, such as SLE, C1q turnover studies in patients are required to determine this.

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