CULTURED HUMAN MONOCYTES SYNTHESIZE AND SECRETE $\alpha_2$-MACROGLOBULIN*

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Alpha$\alpha$-macroglobulin is a wide-spectrum protease inhibitor (1) present in normal human plasma in high concentrations, especially in children and young adults (2, 3). The site(s) of its synthesis in vivo is not known; liver has been considered to be the most plausible candidate organ for this activity (4). In vitro, diploid fibroblast-like cell strains are known to synthesize and secrete $\alpha_2$-macroglobulin (5), and there is evidence that tissue slices from several organs may produce the protein (6).

Association of $\alpha_2$-macroglobulin with the surface of a subpopulation of mononuclear leukocytes, probably B lymphocytes, has been reported in rabbits (7), mice, and men (8, 9). Cultures of human embryonic blood (10) and of adult leukocytes (11) were reported to incorporate amino acid precursors into protein precipitable by anti-$\alpha_2$-macroglobulin. Here we report experiments which suggest that most, if not all, of the $\alpha_2$-macroglobulin produced in human leukocyte cultures is synthesized by cells of the monocyte-macrophage lineage.

Materials and Methods

Cultures of Mononuclear Leukocytes and Their Subpopulation. Mononuclear leukocytes were isolated from the buffy coat fraction of blood from healthy donors, kindly provided by the Finnish Red Cross Blood Transfusion Center, Helsinki. The standard dextran sedimentation (12) and subsequent Ficoll-Isopaque centrifugation (13) were used as described previously (14). Finally the cells were suspended in prewarmed growth medium: RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 2 mM glutamine, 100 IU/ml penicillin, 50 $\mu$g/ml streptomycin, and 10% fetal calf serum. Mononuclear leukocytes comprised more than 90% of all living (trypan blue-excluding) cells in these suspensions.

Populations enriched in T lymphocytes or B + T lymphocytes were prepared by passing mononuclear leukocyte suspensions through nylon wool (NW)† (15) or cotton wool (CW) (16, 17), respectively. The resulting nonadherent cell populations will later be referred to as NW$^-$ and CW$^-$ cells, respectively. Mononuclear leukocytes, NW$^-$ cells, and CW$^-$ cells were cultured at 37°C in 30 ml Falcon plastic flasks (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) at 2–3 × 10^6 cells/ml of growth medium.

Monocyte-enriched cell cultures were prepared by incubating mononuclear leukocyte suspensions in medium 199, supplemented with 2 mM glutamine and antibiotics, on glass coverslips for

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Abbreviations used in this paper: CW, cotton wool; CW$^-$ cells, cells not retained by cotton wool; NW, nylon wool; NW$^-$ cells, cells not retained by nylon wool; SDS, sodium dodecyl sulfate; SmIg, surface membrane immunoglobulin.
**Percentages of Lymphocytes and Monocytes in Different Subpopulations of Peripheral Blood Leukocytes**

| Subpopulation                      | Cell type marker* | SRBC-rosetting† | SmIg§ | Phagocytic cells¶ |
|------------------------------------|-------------------|-----------------|-------|------------------|
|                                    |                   | T lymphocytes   | B lymphocytes | Monocytes |
| Mononuclear leukocytes (unfractionated) | %                | %               | %     | %                |
| CW− cells                          | 70-80             | 10-15           | 1-5   |                  |
| NW− cells                          | 85-90             | 1-2             | 1-5   |                  |
| Adherent cells                     | 0-1               | 1-3‡            | 85-95 |                  |

* Tests for the markers were performed before starting the cultures.
† Sheep erythrocytes (SRBC) rosetting was carried out as recommended by Aiuti et al. (17).
§ Cells were washed three times with phosphate-buffered saline and stained for surface membrane immunoglobulin (SmIg) at +4°C, by using fluorescein-isothiocyanate-conjugated swine anti-human immunoglobulin (17). Small lymphocyte-like cells with bright granular fluorescence in epi-illumination were considered as B cells.
¶ Phagocytic cells were identified by incubating for 60 min at 37°C in RPMI + 50% fetal calf serum containing polystyrene particles (diameter 1.01 μm; Polysciences, Inc., Warrington, Pa.), and examining in a phase contrast microscope after washing with phosphate-buffered saline.
‡ By incubating the culture for 3 days in medium 199 practically all SmIg+ cells disappeared.

1-2 h at 37°C. After washing carefully four times with phosphate-buffered saline the adherent cell cultures were refed with the serum-free medium 199, and incubated at 37°C. The medium was changed daily or every 2nd day. Alternatively the cells were refed with medium 199 supplemented with 10% fetal calf serum, and incubated without medium changes. In some later experiments a 1:1 mixture of medium 199 and RPMI-1640 medium (Grand Island Biological Co.) was used, as it was found that this mixture was optimal for the survival of monocyte-macrophage cultures. Percentages of different types of mononuclear leukocytes in these preparations are shown in Table I.

### Radioimmunoassay of α₂-Macroglobulin

Alpha₂-macroglobulin was purified from human plasma by a modification of the procedure described by Roberts et al. (18) and iodinated by the method of Krohn et al. (19) by using minimal concentrations of chloramine-T. Antiserum against highly purified α₂-macroglobulin was purchased from Behringwerke, AG, Marburg-Lahn, W. Germany, absorbed with lyophilized fetal calf serum (40 mg/ml), and tested for monospecificity towards human α₂-macroglobulin by double immunodiffusion and immunoelectrophoresis. The absorption removed all detectable antibodies against bovine α₂-macroglobulin. Of the iodine label in α₂-macroglobulin, 89% was precipitated by the absorbed antiserum in a double antibody immunoprecipitation. The dose-response curve for α₂-macroglobulin in a double antibody type radioimmunoassay was linear between 2.4 and 200 ng/ml. Details of purification and labeling of α₂-macroglobulin, as well as of the radioimmunoassay are reported elsewhere.²

### Immunofluorescence

Cells on coverslips or in suspension were fixed with 3.5% formaldehyde (20 min, +20°C) and cold acetone (20 min, -20°C). Alternatively, the cells were fixed with the formaldehyde alone (20). Indirect immunofluorescence studies for α₂-macroglobulin were carried out by using calf serum-absorbed rabbit anti-α₂-macroglobulin serum and fluorescein-isothiocyanate.

² Mosher, D., O. Saksela, and A. Vaheri. Manuscript submitted for publication.
nate-conjugated anti-rabbit-γ-globulin. Rabbit antiserum against human plasma fibronectin (5), a
glycoprotein present in plasma and on cultured fibroblasts (21), was used in a similar immunofluo-
rescence technique to identify possibly contaminating fibroblasts in the culture. The tests with
fixed cells were carried out at room temperature and those with living cells at +4°C.

Metabolic Labeling. Adherent cell cultures were transferred to a modified basal medium of
eagle for diploid cells containing 1/10th of the normal methionine concentration and supplemented
with antibiotics as above plus 0.05% bovine serum albumin (or plus 10% fetal calf serum thor-
oughly dialyzed against phosphate-buffered saline). The cells were then labeled with 10 µCi/ml
35S-methionine (Radiochemical Centre, Amersham, England, sp act 200-400 Ci/mmol) for 24 h at
37°C.

Analysis of Radiolabeled Proteins. Supernates of radiolabeled cell cultures were clarified by
centrifugation for 10 min at 800 g at 4°C and supplemented with phenylmethyl sulfonlfuoride, 1
mM, and Trasylol (aprotinin, Bayer AG, Leverkusen, W. Germany), 100 U/ml. Cells were
dissolved in a mixture of 6 M urea, 0.1% Triton X-100, 0.01% NaNa, and 2 mM phenylmethyl
sulfonlfuoride, and subsequently mixed with 1 vol of normal human serum.

For Ouchterlony double diffusion-autoradiography portions of the supernates were concen-
trated 10-fold by precipitation with 50% saturated (NH4)2SO4 together with carrier human serum.
Concentrated supernates and cell extracts were analyzed by double diffusion in agarose gel by
standard techniques (22). Radioactivity in the precipitates was localized by autoradiography.

For double antibody precipitation, 1-ml portions of the supernates were shaken for 2 h at room
temperature with 15 µl of specific antisera against α2-macroglobulin, or fibronectin, or with 15 µl
of normal rabbit serum. The antigen-antibody complexes were precipitated at 4°C by a 16-h
incubation with 375 µl of sheep anti-rabbit globulin mixed with an equal volume of 20 mM Tris-
HCl buffer, pH 8.0, 2 mM phenylmethyl sulfonlfuoride, 200 U/ml Trasylol, and 1% sodium
deoxocholate. The precipitate was collected by centrifugation (20 min, 15,000 g), washed three
times with 10 mM Tris-HCl buffer, pH 8.0, and once with water. The above protease inhibitors and
deoxocholate were present through the buffer washes. The final precipitate was dissolved in 250 µl
of 4% SDS, 10% 2-mercaptoethanol, and buffered to pH 6.8 with Tris-HCl.

Electrophoresis in discontinuous polyacrylamide slab gels containing SDS (24) was used to
identify the labeled polypeptides in immunoprecipitates. Radioactivity in the migrating bands
was assayed by autoradiography. The following molecular weight standards were used: plasma
fibronectin (Mr = 2.0 × 106), α2-macroglobulin (Mr = 1.6 × 106), phosphorylase a globulin (Mr = 9.3
× 104), bovine serum albumin (Mr = 6.8 × 104), and ovalbumin (Mr = 4.3 × 104), all labeled with
14Cformaldehyde according to Rice and Means (25), and dissolved in 4% SDS and 10% 2-
mercaptoethanol in Tris-HCl buffer, pH 6.8.

Commercial Reagents and Chemicals. Dextran T500 and Ficoll 400 were obtained from Phar-
macia Fine Chemicals, Uppsala, Sweden; Isopaque was supplied by Medica Ltd, Helsinki; nylon
wool (Leukopak) was from Fenwall Laboratories, Inc., Morton Grove, Ill. Media and fetal calf
serum were from Gibco-BioCult, Paisley, Scotland, tissue culture vials were from Falcon Plastics,
Ltd., Oxford, Calif. Fluorescein-isothiocyanate-conjugated globulin fraction of swine antiserum
wards human immunoglobulin was from Meloy Laboratories, Inc., Springfield, Va. Rabbit
antiserum against human β,C/β,A was from Behringwerke.

Results

Release of α2-Macroglobulin by Cultured Mononuclear Leukocytes. Cultures of
mononuclear leukocytes, NW leukocytes, and CW leukocytes were incubated at 37°C in medium RPMI + 10% fetal calf serum, and portions of cell-free supernates were collected daily and kept at −20°C until assay. Initial cell concentra-
tions in different cultures were identical within each experiment. Radioim-
Production of \( \alpha_2 \)-macroglobulin by human leukocyte cultures. A. \( 15 \times 10^6 \) leukocytes were cultured in 5 ml of RPMI-medium (Grand Island Biological Co.) supplemented with 10% fetal calf serum. Samples of culture medium were drawn as indicated and assayed for \( \alpha_2 \)-macroglobulin by radioimmunoassay. Symbols: ■—■, unfractionated mononuclear leukocytes; ▼—▼, CW-; △—△, NW- cells, and ○—○, blank medium. B. 1.5–2.0 \( \times 10^6 \) adherent cells were cultured in 5 ml medium 199 without serum. Arrows indicate complete medium change. ●—●, \( \alpha_2 \)-macroglobulin in the cell supernate.

Radioimmunoassays of the samples showed increasing concentrations of \( \alpha_2 \)-macroglobulin in supernates of unfractionated mononuclear leukocytes (Fig. 1A). Low concentrations of \( \alpha_2 \)-macroglobulin were also found in both NW- and CW- cultures (but only at the later stages of the incubation [Fig. 1A]).

This result suggested that monocytes, which were removed by passage through nylon wool or cotton wool, might be responsible for the appearance of \( \alpha_2 \)-macroglobulin in the culture medium of unfractionated mononuclear leukocytes. Therefore, adherent cell cultures enriched in monocytes (Table I) were incubated at 37°C in serum-free medium 199, and portions were assayed for \( \alpha_2 \)-macroglobulin. Relatively large amounts of \( \alpha_2 \)-macroglobulin appeared in the medium (Fig. 1B). It should be noted that the cell number per milliliter medium in the adherent cell cultures was only about 10% of that in the unfractionated mononuclear leukocyte cultures.

Metabolic Labeling of Protein(s) Precipitable by Anti-\( \alpha_2 \)-Macroglobulin. Adherent cell cultures of mononuclear leukocytes were labeled overnight with \(^{35}\)S-methionine. Supernatant protein was coprecipitated with human se-
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FIG. 2. Ouchterlony double diffusion assay for immunoreactive $\alpha_2$-macroglobulin and fibronectin synthesized in adherent cell cultures. Left panels: protein staining with ponceau red. Right panels: autoradiography. The respective wells contained: A. Center well, anti-$\alpha_2$-macroglobulin; 1 and 4, cell extract; 2, anti-C$_3$'; 3 and 6, cell supernate; 5, anti-human serum. B. Center well, anti-fibronectin; 1 and 4, cell extract; 2, anti-$\alpha_2$-macroglobulin; 3 and 6, cell supernate; 5, anti-human serum.

The carrier human serum globulins formed several precipitation lines with anti-human serum but only a single line when tested against anti-$\alpha_2$-macroglobulin or anti-fibronectin. Autoradiography revealed radioactivity in the single precipitation line between the cell supernate and anti-$\alpha_2$-macroglobulin (Fig. 2). The respective line between the cell supernate and anti-human serum also was radioactive. A faint line of radioactivity (not visible in the photograph) was seen at the precipitate formed by the cell supernate and anti-human-C$_3$' serum. Radioactivity from the cell extract was precipitated by anti-human-C$_3$' and in several lines by anti-human serum but not by anti-$\alpha_2$-macroglobulin. Radioactivity was not precipitated when the cell extracts or the cell supernates were tested against anti-fibronectin (Fig. 2). These results indicate that $\alpha_2$-macroglobulin and a number of other serum proteins, including C$_3$' but probably not fibronectin, are synthesized in adherent cell cultures prepared from human blood mononuclear leukocytes.

Labeled Polypeptides Precipitated by Anti-$\alpha_2$-Macroglobulin. Adherent cell cultures of human peripheral blood mononuclear leukocytes were incubated at 37°C in serum-free medium with daily medium change, or in medium supplemented with 10% fetal calf serum without medium change. In both types of the cultures morphological transformation of the initially round cells to enlarged extension-sending macrophages was seen. The proportion of altered cells in-
increased with increasing incubation time (days). Parallel cultures were labeled with \(^{35}\)S-methionine at 1-2 or 4-5 days of incubation. Supernates were collected, and portions were treated with anti-\(\alpha_2\)-macroglobulin, anti-fibronectin, or normal rabbit serum and subsequently with anti-rabbit immunoglobulin. The precipitates were dissolved and electrophoresed in discontinuous polyacrylamide gel slabs containing SDS.

In addition, samples of concentrated supernates were reacted against anti-\(\alpha_2\)-macroglobulin or anti-fibronectin in Ouchterlony double diffusion, the precipitates were cut out, dissolved, and electrophoresed as above. Autoradiography of the gels showed a major radioactive band comigrating with authentic subunit of \(\alpha_2\)-macroglobulin, in channels containing material precipitated by anti-\(\alpha_2\)-macroglobulin (Fig. 3, channels 2, 5, and 7). Greater amounts of label migrating at this band were seen in supernates from cultures incubated for 4 days before labeling than in those from cells labeled after 1 day in culture (channels 2 and 5). Cells incubated in the serum-containing medium produced greater amounts of this material than cultures in the serum-free medium (not shown).

Material precipitated by anti-fibronectin contained a weak band migrating at \(\alpha_2\)-macroglobulin but never a band of \(2.2 \times 10^6\) daltons, the size of fibronectin subunit (21). Several weak bands with lower molecular weight were produced by precipitates of both antisera (barely visible in Fig. 3). Precipitates formed with normal rabbit serum did not show any reproducible radioactive bands in electrophoresis.

Analysis of supernates and cell extracts of labeled monocytes by electrophoresis showed that \(\alpha_2\)-macroglobulin is not a major labeled cellular protein under these conditions (Fig. 3, channels 9 and 10). Staining of the gels for proteins (not shown) resulted in a similar conclusion.

**Immunofluorescence Studies.** Suspensions of mononuclear leukocytes or adherent cell cultures on coverslips, either unfixed or fixed with formaldehyde or with formaldehyde-acetone, were stained for \(\alpha_2\)-macroglobulin and fibronectin by the indirect technique. The results were repeatedly negative while tests for fibronectin on human skin fibroblasts were regularly positive (21).

**Discussion**

These results show that \(\alpha_2\)-macroglobulin is synthesized in human leukocyte cultures. When the leukocyte cultures were depleted of monocytes it was found that less \(\alpha_2\)-macroglobulin appeared into the medium. Conversely, adherent cells enriched in monocytes produced much greater quantities of \(\alpha_2\)-macroglobulin per cell than any of the populations more rich in lymphocytes. These results suggest that monocytes or a subpopulation of the monocyte-macrophage lineage is responsible for the production of \(\alpha_2\)-macroglobulin in this system. Metabolic labeling of supernatant \(\alpha_2\)-macroglobulin in adherent cell cultures proved that the protein was a product of synthesis by the cells rather than a result of release of previously endocytosed material. SDS-polyacrylamide gel electrophoresis of the immunoprecipitates showed that anti-\(\alpha_2\)-macroglobulin specifically precipitates a polypeptide from the supernate which migrates at the position of authentic \(\alpha_2\)-macroglobulin subunit. This indicates that the material precipitated by the antiseraum is true \(\alpha_2\)-macroglobulin and not, e.g., labeled protease-unlabeled \(\alpha_2\)-macroglobulin complex.
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Skin fibroblasts have been shown to synthesize small amounts of α₂-macroglobulin in culture, and it is theoretically possible that fibroblasts contaminate leukocyte cultures as a result of skin wounding by venipuncture. However, skin fibroblasts also synthesize large amounts of fibronectin (21). Lack of metabolic
labeling of fibronectin indicates that contamination by fibroblasts cannot be responsible for the synthesis of α₂-macroglobulin in adherent blood cell cultures. In addition, our cultured adherent cells did not contain fibronectin detectable by indirect immunofluorescence while skin fibroblasts are readily positive in the test.

Surface α₂-macroglobulin has been suggested for a marker for B lymphocytes (7-9). We could show very little synthesis and secretion of α₂-macroglobulin by the lymphocyte preparations. The NW- cells produced as much α₂-macroglobulin as CW- cells, making it unlikely that the small amount of α₂-macroglobulin in the lymphocyte preparations originated from B cells. In addition, we could not detect α₂-macroglobulin on cultured lymphocytes in the immunofluorescence assays. Our results do not, however, exclude the possibility that B cells bind α₂-macroglobulin from the plasma to their surface or synthesize but do not secrete α₂-macroglobulin.

There are several reasons to believe that α₂-macroglobulin synthesized and secreted by adherent cells may have an important function. Studies of in vitro cultures of fibroblast-like cells suggest a positive correlation between the secretion of proteases and α₂-macroglobulin. Activated macrophages are known to shed plasminogen activator (26), lysosomal hydrolases (27), elastase (28), and collagenase (29), all of which are inhibited by α₂-macroglobulin (1). Rabbit peritoneal macrophages have been shown to specifically phagocytose α₂-macroglobulin protease complexes (30). It is interesting to note that the monocytes were found to release significant amounts of α₂-macroglobulin only after some days in culture. During this incubation period, there was a partial transformation to macrophages as judged by morphology. Similarly, Gordon and co-workers (31) reported that only activated monocytes produce plasminogen activator. Simultaneous secretion of proteases and protease inhibitors and phagocytosis of protease-inhibitor complexes may help in controlling the active protease levels in the microenvironment of cell surface and allow both spatial and temporal differences in the protease levels to occur. Protease activity is considered to be important in migration of macrophages in tissues (32). In addition, there is evidence (33-34) that α₂-macroglobulin may participate in the regulation of in vitro phenomena thought to be representative of portions of the in vivo immune response.

Summary

Alpha₂-macroglobulin levels in the supernates of cultures of different subpopulations of human peripheral blood mononuclear leukocytes were assayed by a radioimmunooassay. Unfractionated mononuclear leukocytes produced greater amounts of the macroglobulin (4.0 vs. 0.8 ng/10⁶ cells) than did subpopulations enriched in T or B + T lymphocytes, by passage through nylon wool or cotton wool columns, respectively. Still higher concentrations of α₂-macroglobulin (40 ng/10⁶ cells) were measured in the supernates of glass-adherent mononuclear leukocyte cultures. These results suggest that cells of monocyte-macrophage lineage are mainly, if not exclusively, responsible for the appearance of α₂-macroglobulin in the supernate of human peripheral blood leukocyte cultures.

The de novo synthesis and release of α₂-macroglobulin by cultured monocytes was demonstrated by immunoprecipitation of radioactivity from supernates of
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$\text{S}$-methionine-labeled glass-adherent cells. Antiserum against purified $\alpha_2$-macroglobulin was used in both Ouchterlony double diffusion and double antibody precipitation tests. SDS-polyacrylamide gel electrophoresis of immunoprecipitates showed that most of the radioactivity comigrated with authentic $\alpha_2$-macroglobulin subunit at about 160,000 daltons.

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