BIOSYNTHESIS OF THE COMPLEMENT COMPONENTS AND THE REGULATORY PROTEINS OF THE ALTERNATIVE COMPLEMENT PATHWAY BY HUMAN PERIPHERAL BLOOD MONOCYTES*

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Activation of the classical complement pathway occurs when immune complexes interact with the first component, C1, to convert it from its inactive precursor form to an active esterolytic enzyme, $C\bar{1}$.[1] $C\bar{1}$ by producing limited proteolysis of C4 and C2 produces the classical pathway C3 convertase, $C\overline{42}$ (1). The alternative pathway is activated when the complex surface polysaccharides of bacteria or yeasts come into contact with serum (2). As a consequence of this contact, C3b, the major cleavage product of C3; factors B (B)[2] and $\bar{D}$ ($\bar{D}$); and properdin (P) interact to assemble the alternative pathway C3 and C5 convertases (3, 4). Activation of the complement system results in the generation of phlogistic molecules from C2, B, C3, and C5 which act on the membranes of lymphocytes, macrophages, polymorphonuclear leukocytes, mast cells, platelets, and smooth muscle cells (5), all of which may be involved in the inflammatory response.

Modulation of complement activation is achieved by at least three plasma proteins: $C\bar{1}$-inhibitor which stoichiometrically inhibits C1 (6), C3b inactivator (C3BINA), which enzymatically inactivates C3b (7, 8), and β1H globulin which by binding to C3b, displaces Bb from the alternative pathway C3 and C5 convertases, prevents C3b from interacting with B and C5, and potentiates the inactivation of C3b by C3bINA (9, 10).

In human serum there is usually good correlation between concentrations of the

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[1] A bar over a symbol indicates that the component is in its active form.

[2] *Abbreviations used in this paper:* B, factor B; C3bINA, C3b inactivator; C-rat, rat serum diluted 1 to 15 in 0.086 M EDTA diluted in isotonic veronal-buffered saline containing 0.01% gelatin; $\bar{D}$, factor $\bar{D}$; $D5W^{++}$, 5% dextrose containing 0.0005 M Mg^{++} and 0.00015 M Ca^{++}; DFP, diisopropyl fluorophosphate; $DGVB^{++}$, equal volumes of $D5W^{++}$ and veronal-buffered saline containing 0.01% gelatin, 0.0005 M Mg^{++} and 0.00015 M Ca^{++}; $DGVB^{-}$, three volumes 5% percent dextrose and one volume isotonic veronal-buffered saline containing 0.01% gelatin; E^{rab}, rabbit erythrocytes; 0.01 M $EDTA.DGVB^{-}$, 0.086 M EDTA diluted in $DGVB^{-}$; 0.04 M $EDTA.GVB^{-}$, 0.086 M EDTA diluted in isotonic veronal-buffered saline containing 0.01% gelatin; 0.02 M $EDTA.GVB^{-}$, 0.04 M $EDTA.GVB^{-}$ diluted in isotonic veronal-buffered saline containing 0.1% gelatin; 0.01 M $EDTA.GVB^{-}$, 0.02 M $EDTA.GVB^{-}$ diluted in isotonic veronal-buffered saline containing 0.01% gelatin; GVB^{++}, isotonic veronal-buffered saline containing 0.01% gelatin, 0.0005 M Mg^{++}, and 0.00015 M Ca^{++}; GVB^{-}, GVB^{++} without Mg^{++} and Ca^{++}; MEM, Eagle's minimum essential medium; Mg.EGTA, one volume 0.1 M EGTA diluted in nine volumes of $DGVB^{-}$, containing 0.005 M Mg^{++}, pH 7.4; P, properdin; PBS, phosphate-buffered saline; RD, serum depleted of $\bar{D}$; RPMI.FCS, RPMI-1640 containing 20% heat-inactivated fetal calf serum.

regulatory proteins, C3bINA and β1H with each other and with the other components of the alternative pathway (11–14). These correlations have been used to emphasize the importance of serum concentrations of these proteins in the modulation of turnover of the alternative pathway. However, it is uncertain whether these correlations are accounted for by catabolic mechanisms or whether the synthetic rates of these proteins are governed by common factors.

In recent years it has become obvious that at least three cell types synthesize complement proteins, the hepatocytes, the epithelial cells of the gastrointestinal and genitourinary tracts, and cells of the monocyte-macrophage series (15). Human monocytes and/or macrophages have been shown to synthesize C1 and its subcomponents (16), C4 (17, 18), C2 (18–20), and C3 (17, 18, 21).

In this paper, evidence is presented to show that the human peripheral blood monocyte in short-term tissue culture synthesizes all the components of the alternative pathway and its control proteins and, in addition, C4, C2, and C5.

Materials and Methods

Preparation of Monocyte Monolayers. Peripheral blood mononuclear cells were isolated by centrifugation on Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) -Hypaque (Winthrop Laboratories, New York) cushions (22). The cells were washed three times with Eagle's minimum essential medium (MEM; Gibco-Biocult Ltd., Paisley, Scotland) and the cells finally resuspended in RPMI-1640 (Gibco-Biocult Ltd.) containing 20% heat-inactivated (2 h, 56°C) fetal calf serum (RPMI.FCS) to a concentration of 1×10^7/ml. Purified monocyte monolayers were prepared by layering 1 ml of cell suspensions onto a 22-mm square glass coverslip contained in a 35- × 10-mm plastic Petri dish (Nunc Laboratories, Oslo, Norway). The Petri dishes were incubated at 37°C in a humidified atmosphere of 5% CO_2 and air for 2 h. After incubation, the coverslips were washed vigorously with warm MEM to remove nonadherent cells and then overlayed with 2 ml RPMI.FCS and reincubated at 37°C in a humidified 5% CO_2 and air atmosphere. Using this technique, between five and seven cultures were prepared from each blood donation.

All the cells contained in monolayers prepared in this manner stained positively for nonspecific esterase (23), and most phagocytosed *Candida albicans*. None of the cells rosetted with unsensitized sheep erythrocytes (24), but >90% rosetted with EA (7S) (25) and EA43 (19S) (26) showing that they possessed both Fc and C3b receptors. Using fluorescein-labeled anti-Ig (Burroughs-Wellcome Ltd., Beckenham, Kent, England), unfixed monolayers were stained for surface Ig, but none of the cells showed positive staining.

Sampling of Cultures and Storage of Samples. To demonstrate the presence of complement proteins in the culture, 200-μl samples of supernate were removed on days 1–9, samples from cultures from the same donor were pooled, and on day 12, the total supernate was harvested. The removed culture fluid was replaced by an equal volume of fresh RPMI.FCS. In one experiment, three cultures were set up and the culture medium changed daily for 10 d to study cumulative complement protein synthesis.

Inhibition of Protein Synthesis. Cycloheximide (Sigma Chemical Co., Poole, Dorset, England) was dissolved in RPMI.FCS to a final concentration of 1.0 μg/ml. Cycloheximide-containing control cultures were sampled on days 1, 3, and 5. On day 5, the cycloheximide-treated cultures were washed and their culture medium replaced by normal RPMI.FCS, cultures were again sampled on day 7.

Buffers for Functional Assays. Isotonic veronal-buffered saline (pH 7.4), containing 0.01% gelatin, 0.0005 M Mg^{++}, and 0.00015 M Ca^{++} (GVB^{++}), 5% dextrose containing 0.0005 M Mg^{++} and 0.00015 M Ca^{++} ($D5W^{++}$), isotonic EDTA (0.086 M, pH 7.4), $GVB^{=}$ (same as GVB^{++} but without Ca^{++} and Mg^{++}), $DGVB^{++}$ (equal volumes of $D5W^{++}$ and GVB^{++}), $DGVB^{=}$ (three volumes 5% dextrose without Mg^{++} and Ca^{++} and one volume $GVB^{=}$), 0.04 M EDTA.$GVB^{=}$ (0.086 M EDTA diluted in $GVB^{=}$), 0.02 M EDTA.$GVB^{=}$ (0.04 M EDTA.$GVB^{=}$ diluted in $GVB^{=}$), 0.01 M EDTA.$GVB^{=}$ (0.02 M EDTA.$GVB^{=}$ diluted in $GVB^{=}$), 0.01 M

EDTA.DGVB$^-$ (0.086 M EDTA diluted in DGVB$^-$; pH 6.0), Mg.EGTA (one volume 0.1 M EGTA diluted in nine volumes DGVB$^-$, containing 0.005 M Mg^{++}, pH 7.4) were used in the various hemolytic assays.

Complement Reagents. Human C1 (27), C2 and C4 (28), C3 (29), C5 (30), B (31), P (32), $\bar{D}$ (33), C3bINA (34), and β1H (35) were purified and antisera to C4, C3, C5, B, P, C3bINA, and β1H produced by immunization of rabbits with the purified proteins in complete Freund's adjuvant. Anti-C2 was a gift from Dr. Hans J. Müller-Eberhard, Scripps Clinic and Research Foundation, La Jolla, Calif. Functionally purified C3, C5, C6, C7, C8, and C9 (Cordis Laboratories Inc., Miami, Fla.) were used in C3 and C5 hemolytic assays. Rat serum diluted 1 to 15 in 0.04 M EDTA.GVB$^-$ (C-rat) was used as a source of C3–C9 in the C4, C2, B, P, $\bar{D}$, C3bINA, and β1H hemolytic assays. Serum depleted of $\bar{D}$ (RD) was prepared by the gel filtration of fresh human serum over a 5.0- × 100-cm column of Sephadex G75 superfine (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). The exclusion peak was rechromatographed over the same column, concentrated to the original serum volume, and was unable to lyse rabbit erythrocytes (E^{rab}) in Mg.EGTA (36). Lysis was restored by the addition of purified $\bar{D}$, but not P or B. E^{rab} were separated from rabbit blood collected in Alsever's solution and stored at 0°C until used. EAC4 were prepared by the method of Borsos and Rapp (37).

Functional Assays. C2 (38), C4 (28), and C3 and C5 (39) were measured by standard stoichiometric hemolytic assays.

B. EAC43b were prepared by incubating EAC142 (1 × 10^8/ml DGVB^{++}) with an equal volume of DGVB^{++} containing 10 μg/ml C3 at 37°C for 30 min. Conversion to EAC43b was achieved by incubating EAC1423b in 0.01 M EDTA.GVB$^-$ for 2 h at 37°C. EAC43b were standardized to 1 × 10^8/ml DGVB^{++}. 100 μl of DGVB^{++} containing $\bar{D}$ (10 U/ml) and P (250 ng/ml), 50 μl of culture supernate, and 100 μl of EAC43b were mixed and incubated at 30°C for 30 min. 300 μl of C-rat were added to each tube, followed by a further incubation at 37°C for 1 h. The reaction was stopped by the addition of 2 ml 0.15 M saline, and after centrifugation, the degree of lysis was measured spectrophotometrically at 414 nm. Controls included cell blank (100 μl EAC43b + 450 μl DGVB^{++}), reagent blank (100 μl EAC43b, 100 μl DGVB^{++} containing $\bar{D}$ and P, 50 μl RPMI.FCS, and 300 μl C-rat), 100% lysis (same as reagent blank by lysed in 2 ml distilled water) and complement color (250 μl DGVB^{++} and 300 μl C-rat). Results were expressed as number of hemolytic sites (Z) = $-\ln(1 - y)$; where y = proportion of cells lysed.

P. EAC43b (1 × 10^8/ml DGVB^{++}) prepared as described for the B assay, were incubated at 30°C for 30 min with an equal volume of DGVB^{++} containing $\bar{D}$ (10 U/ml) and sufficient B to produce five hemolytic sites. The cells, EAC$\overline{43bBb}$ were centrifuged and resuspended 1 × 10^8/ml in ice-cold 0.01 M EDTA.GVB$^-$. 100 μl of EAC$\overline{43bBb}$ were incubated with an equal volume of supernate diluted 1 to 2 in 0.02 M EDTA.GVB$^-$ at 30°C for 15 min. After the addition of 0.3 ml C-rat and a further 1-h incubation at 37°C, 2 ml of 0.15 M saline was added to each tube to stop the reaction, and after centrifugation, the degree of lysis was measured spectrophotometrically at 414 nm. Controls included cell blank (100 μl EAC$\overline{43bBb}$, 400 μl DGVB^{++}), reagent blank (100 μl EAC43b, 100 μl supernate diluted 1 to 2 in 0.02 M EDTA.DGVB$^-$, and 300 μl C-rat), solo (100 μl EAC$\overline{43bBb}$, 100 μl RPMI.FCS diluted 1 to 2 in 0.02 M EDTA.GVB$^-$, and 300 μl C-rat), 100% lysis (same as control, except lysed in 2 ml distilled water), complement color (200 μl DGVB^{++}, 300 μl C-rat). The number of hemolytic sites per tube (Z) was calculated, and the number resulting from P activity was calculated by subtracting the control result from the test result.

The kinetics of decay of C$\overline{3bBb}$ in the presence of monocyte culture medium and RPMI.FCS was investigated. EAC$\overline{43bBb}$ (1 × 10^8/ml 0.01 M EDTA.GVB$^-$) prepared as described, were mixed with an equal volume of monocyte culture medium or RPMI.FCS diluted 1 to 2 in 0.02 M EDTA.GVB$^-$ at 30°C. 200-μl samples were taken at 5, 10, 15, and 30 min, added to 300 μl C-rat, and incubated for a further 1 h at 37°C. After termination of the reaction, hemoglobin release was measured spectrophotometrically.

β1H. EAC$\overline{43bBb}$, prepared as described for the P assay, were suspended to 1 × 10^8/ml in 0.01 M EDTA.GVB$^-$. The convertase was stabilized by the addition of P (250 ng/ml). The resultant EAC$\overline{43bBbP}$ (100 μl) were incubated at 30°C for 15 min with 100 μl of supernate diluted 1 to 2 in 0.02 M EDTA.GVB$^-$. The residual EAC$\overline{43bBbP}$ hemolytic sites were developed by the further incubation for 1 h in the presence of 300 μl C-rat. The reaction was

stopped by the addition of 2 ml 0.15 M saline, and after centrifugation the degree of hemoglobin release was determined spectrophotometrically. Controls included cell blank (100 μl EAC$\overline{43b\overline{Bb}P}$, 400 μl DGVB^{++}), reagent blank (100 μl EAC43b, 100 μl RPMI.FCS diluted 1 to 2 in 0.02 M EDTA.GVB$^{=}$, and 300 μl C-rat), control (100 μl EAC$\overline{r43b\overline{Bb}P}$, 100 μl RPMI.FCS diluted 1 to 2 in 0.02 M EDTA.GVB$^{=}$, and 300 μl C-rat), 100% lysis (as for control, but lysed in 2 ml distilled water). The results were expressed in Z′ units, where Z′ = ln of (1 − the percentage of inactivation) which is:

$$-\ln \frac{\text{(the percentage of cells lysed in test sample)}}{\text{(the percentage of cells lysed in control [control])}} \quad (40).$$

C3bINA. EAC43b, carrying limited C3b hemolytic sites were prepared using an R3 reagent as previously described (12). 100 μl EAC43b (1 × 10^8/ml 0.01 M EDTA.DGVB$^{=}$, pH 6.0) were incubated with 50 μl culture supernate in 100 μl 0.01 M EDTA.DGVB$^{=}$, pH 6.0, for 1 h at 37°C. After two washes in DGVB^{++}, the cells were resuspended in 100 μl DGVB^{++} and incubated with 100 μl DGVB^{++} containing B (2 ng/ml) and $\bar{D}$ (10 U/ml) for 30 min at 30°C. C$\overline{3b\overline{Bb}}$ sites were developed by incubation with 300 μl C-rat for 1 h at 37°C. 2 ml of 0.15 M saline were added to stop the reaction, and after centrifugation the degree of lysis was measured spectrophotometrically at 414 nm. All controls were incubated with 50 μl RPMI.FCS in 100 μl 0.01 M EDTA.DGVB$^{=}$ (pH 6.0) during the first incubation, and during the second incubation as follows: cell blank (100 μl EAC43b, 400 μl DGVB^{++}), reagent blank (100 μl EAC43b, 100 μl DGVB^{++}, and 300 μl C-rat), control (100 μl EAC43b, 100 μl DGVB^{++} containing B [2 μg/ml] and D [10 U/ml], and 300 μl C-rat), 100% lysis (same as reagent blank, but lysed in 2 ml distilled water). Results were expressed as Z′ units in the same way as the β1H results (above).

$\bar{D}$. 100 μl of E^{rab} (1 × 10^8/ml Mg.EGTA) were incubated for 30 minutes at 30°C with 25 μl culture supernate and 100 μl RD diluted 1 to 5 in Mg.EGTA. Excess C3–C9 was introduced by the addition of 300 μl C-rat followed by a further incubation of 1 h at 37°C. The reaction was stopped by the addition of 2 ml 0.15 M saline, and after centrifugation, the degree of lysis was measured spectrophotometrically at 414 nm. Controls included cell blank (100 μl E^{rab}, 425 μl Mg.EGTA), reagent blank (100 μl E^{rab}, 25 μl RPMI.FCS, 100 μl RD diluted 1 to 5 in Mg.EGTA, and 300 μl C-rat), 100% lysis (as for reagent blank, but lysed in 2 ml distilled water), complement color (25 μl RPMI.FCS, 100 μl RD, 150 μl Mg.EGTA, and 300 μl C-rat). Results were expressed as effective molecules of $\bar{D}$. In a separate experiment, monocyte culture fluid was incubated with 10^{-3} M diisopropyl fluorophosphate (DFP) (Sigma Chemical Co.) to inhibit $\bar{D}$ activity (41).

Radioimmunoassay for β1H. β1H (1 μg) was radiolabeled with I^{125} (IBS3; Radiochemical Centre, Amersham, Buckinghamshire, England) by the method of Hunter and Greenwood (42). The monomeric radiolabeled protein was separated from aggregated β1H, and from the free iodide gel by filtration on an Ultrogel AcA 34 (LKB Ltd., London, England) column (1.0 × 50 cm). Fractions containing ^{125}I were tested for their ability to precipitate with anti-β1H, and the fractions containing immunoprecipitable protein were pooled, aliquoted, and stored at −70°C. Sp act of ^{125}I-labeled β1H preparations ranged from 30 to 70 μCi/μg.

Concentrations of β1H in monocyte culture supernates were measured using the double antibody technique (43). The diluent buffer used was phosphate-buffered saline (PBS; containing 0.01 M phosphate, 0.3 M NaCl, 1% bovine serum albumin, pH 7.4). 100 μl of supernate was incubated with 100 μl of rabbit anti-β1H (diluted 1 to 5,000 in PBS), 100 pg ^{125}I-β1H in 100 μl PBS, and 300 μl PBS at 4°C overnight. Antibody-bound ^{125}I-β1H was precipitated by the addition of 100 μl normal rabbit serum (Burroughs-Wellcome Ltd.) diluted 1 to 25 in PBS, followed by a further incubation for 24 h at 0°C. Samples were centrifuged at 4°C for 30 min at 3,000 rpm, the supernates were removed, and the radioactivity present in the precipitate was measured in an automatic gamma counter (Nuclear Chicago, High Wycombe, England). A standard curve was constructed by including unlabeled β1H at concentrations ranging from 200 ng/ml to 20 pg/ml. The assay was significantly sensitive to detect 500 pg/ml of β1H in the culture fluid. RPMI.FCS did not influence the assay. All samples were treated in duplicate, and duplicate samples differing by >10% of each other were repeated.

Incorporation of Radiolabeled Amino Acids into Immunoprecipitable Protein. Radiolabeled (^{14}C)

valine and leucine (Radiochemical Centre) were added to RPMI.FCS to a final concentration of 25 μCi/ml. Culture fluids were harvested on day 5, pooled, extensively dialyzed against isotonic veronal-buffered saline (pH 7.4), lysophilized and redissolved in normal human serum. This serum was then tested by double immunodiffusion in 1.5% agarose plates, against antisera to C4, C3, C5, β1H, B, C3bINA, for 2 d followed by washing for 1 d in deionized water. Plates were air-dried, covered in plastic wrap (Perfawrap, High Wycombe, England), applied to x-ray film (Curix, RP1; Agfa Gevaert, Antwerp, Belgium) for 6 wk at room temperature in the dark, the film developed (Kodak D19, Eastman Kodak, Manchester, England) and the incorporation of radiolabel into immunoprecipitable protein studied.

Immunoperoxidase Localization of Complement Protein in Monocytes. Cultures of monocytes were prepared and incubated for 7 d as described above. The culture fluid was removed, the monolayers washed in warm MEM, fixed in 2.5% glutaraldehyde for 30 min at room temperature, and washed in Tris-buffered saline (0.15 M, pH 7.2). After blocking of endogenous peroxidase activity with acidified methanol containing hydrogen peroxide, monolayers were stained for C2, C4, C3, C5, B, P, C3bINA, or β1H by the immunoperoxidase bridge method (44). Negative controls were included in each run, and specificity of staining was confirmed by blocking experiments using purified protein preparations. Swine anti-rabbit IgG and peroxidase-anti-peroxidase immune complexes were obtained from Burroughs-Wellcome Ltd.

Results

Kinetics of Synthesis of Complement Proteins by Monocytes. Culture supernates were examined for C2, C4, C3, C5, B, $\bar{D}$, P, C3bINA, and β1H functional activity. C4, C3, and β1H were never detected, but it appeared that small quantities of functionally active C5 were produced in some cultures. Evidence for the production of C2, B, $\bar{D}$, P, and C3bINA was obtained by functional assay, and for β1H production by radioimmunoassay. The kinetics of production of all proteins were similar (Fig. 1), a slight delay in the onset of synthesis was less marked for $\bar{D}$ and C3bINA, followed by a rapid increase in synthesis between days 1 and 4, and finally, a plateau after day 5. On day 6, the following mean concentrations were: for C2 (29×10^{-7} effective molecules/culture $\pm$ 3.2×10^{-7} SD), B (133×10^{-7} effective molecules/culture $\pm$ 37 $\times 10^{-7}$ SD), $\bar{D}$ (85×10^{-7} effective molecules/culture $\pm$ 7.5×10^{-7} SD), C3bINA (45 Z′ U/culture $\pm$ 11.6 SD), and β1H (9.8 ng/culture $\pm$ 2.2 ng SD).

In the assays for C2, B, $\bar{D}$, and P, the number of hemolytic sites (Z) produced varied linearly with input of supernate added (Fig. 2). The volume of supernate also showed a linear relationship to the number of Z′ units produced in the C3bINA assay (Fig. 2).

Monocyte culture fluid retarded the decay of $\overline{C3bBb}$, the half-life of the convertase exposed to RPMI.FCS was 4 min and that exposed to monocyte culture fluid was 8 min (Fig. 3). $\bar{D}$ activity in monocyte culture fluid was completely inhibited by 10^{-3} M DFP.

Cumulative Synthesis of Complement Proteins. In three cultures set up in duplicate, the culture medium was changed daily (Fig. 4). The quantities of C2, B, $\bar{D}$, P, C3bINA, and β1H synthesized per day were added, and it was found that after day 1, synthesis continued at a constant rate up to day 10 without a plateau being reached. The slight flattening of the curve between days 9 and 10 probably reflects death of cells in the cultures. The slopes of the six graphs are very similar.

Cycloheximide-induced Inhibition of Synthesis. Incorporation of cycloheximide (1.0 μg/ml) into the culture medium produced inhibition of C2, B, $\bar{D}$, P, C3bINA, and β1H synthesis (Fig. 5). When the cycloheximide-containing medium was removed the

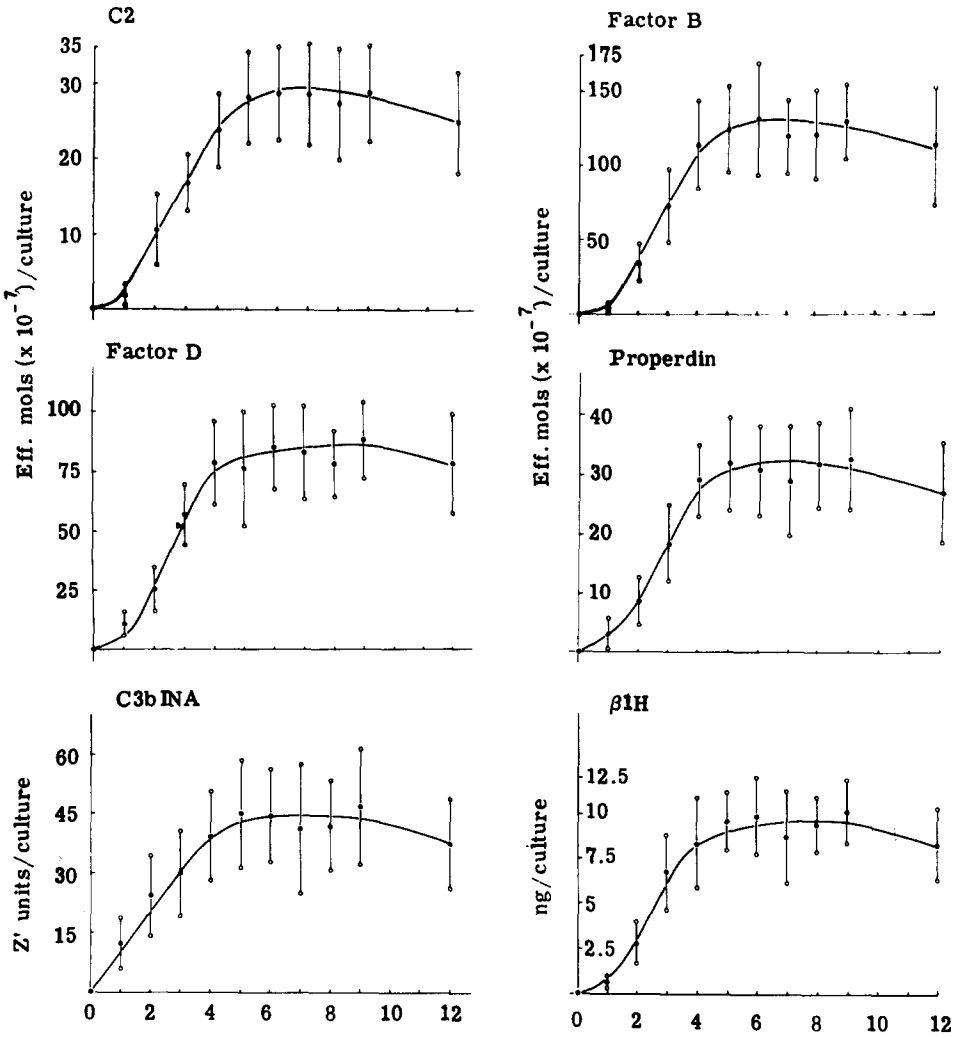


FIG. 1. Kinetics of synthesis of C2, B, D̄, P, C3bINA, and β1H. Concentrations of C2, B, D̄, and P are expressed as effective molecules (Eff. mols) ($\times 10^{-7}$) per culture; C3bINA concentrations are expressed as Z' units per culture (Materials and Methods); β1H concentrations are expressed as nanograms of β1H per culture. The results are the mean (●) ± SD (○) of six cultures.

monolayers washed in RPMI and recultured in fresh RPMI.FCS on day 5, synthesis of all six proteins were detected.

Incorporation of ^{14}C-labeled Amino Acids into Immunoprecipitable Protein. As can be seen in Fig. 6, ^{14}C-labeled amino acids were incorporated into C4, C3, C5, B, P, C3bINA, and β1H. Culture fluids containing cycloheximide (1.0 μg/ml) failed to show incorporation of ^{14}C-labeled amino acids into protein.

Immunoperoxidase Localization of Complement Proteins. All the monocytes in each culture stained for C4, C2, C3, C5, B, P, C3bINA, and β1H (Fig. 7). Virtually all the cells in each culture stained positively for all the proteins studied. The cytoplasm of monocytes which had retained their rounded shape throughout the period of culture stained densely, whereas monocytes which had spread showed a dense granular

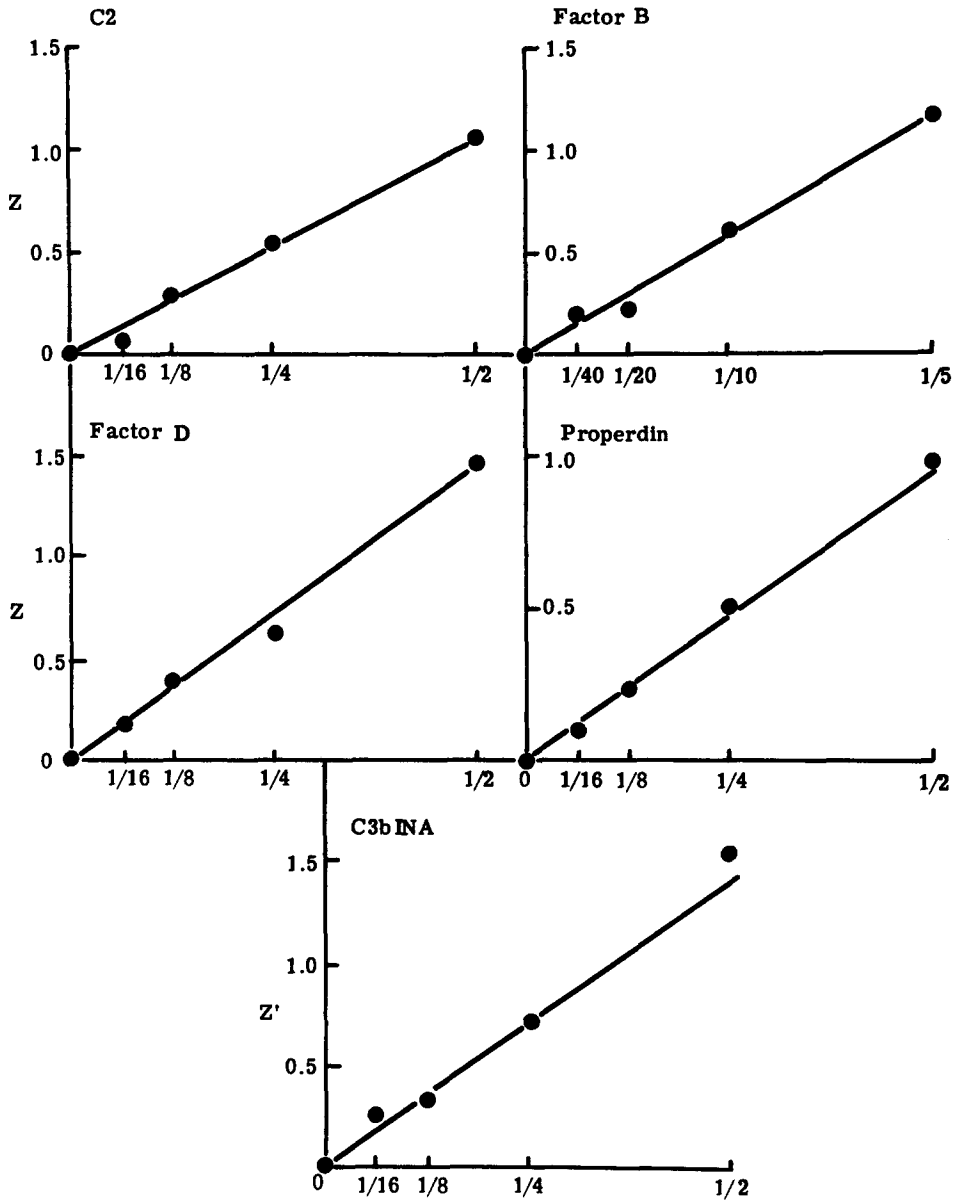


FIG. 2. Titration of supernates for C2, B, $\bar{D}$, P, and C3bINA. The number of effective molecules (Z) of C2, B, $\bar{D}$, and P, and the number of Z′ units (Materials and Methods) of C3bINA are plotted on the ordinates, and the input of supernate on the abscissas.

perinuclear stain associated with fine granular cytoplasmic staining. Staining with each antigen was blocked by prior absorption with the purified antigen; replacing the antiserum by nonimmune serum resulted in background staining only.

Discussion

The evidence that the cultured cells stained positively for nonspecific esterase,

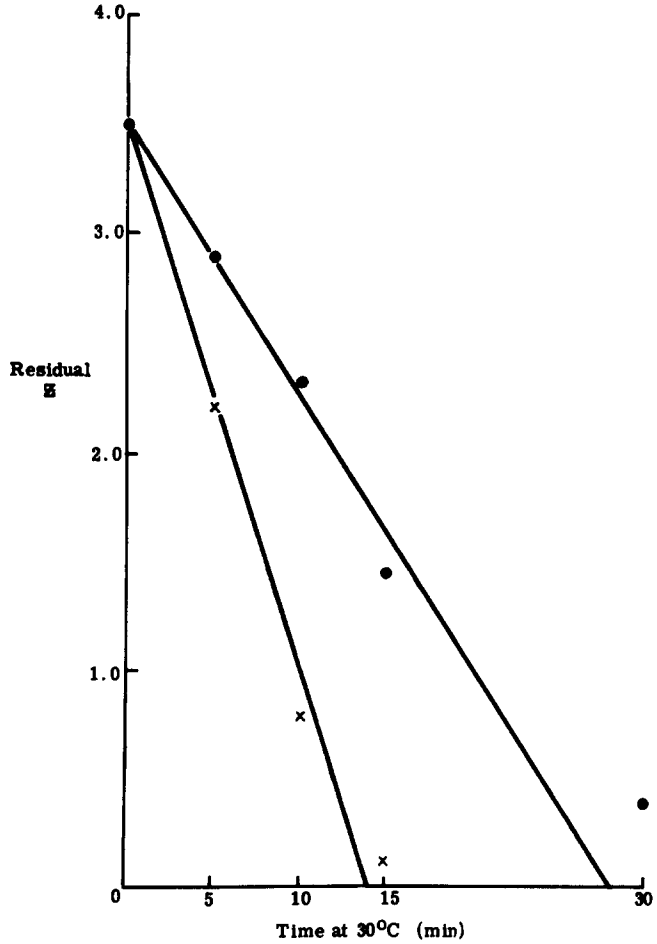


FIG. 3. Kinetics of decay of EAC$\overline{43bBb}$ in fresh RPMI.FCS (control) (×) and in monocyte culture fluid (O) at 30°C. The number of hemolytic sites (Z) (Materials and Methods) is plotted on the ordinate and time (in minutes) on the abscissa. The monocyte culture fluid slows the rate of decay of EAC$\overline{43bBb}$ ($t_{1/2}$ = 8.0 min vs. 4.0 min for RPMI.FCS [control]).

possessed Fc and C3b receptors, and phagocytosed *C. albicans*, coupled with the observations that they lacked sheep erythrocyte receptors and surface Ig, and their morphological appearances all suggested that the cells were monocytes free from lymphocytes and polymorphonuclear leukocyte contamination.

Previous studies (18–20) have shown that human monocytes in culture are capable of synthesizing C2, an observation we have used in these experiments to test the ability of our cultures to synthesize a complement protein, which also verifies these previous reports.

Evidence has been provided (Table I, summary) that monocytes in addition to synthesizing C2, also synthesize C4, C3, C5, B, $\bar{D}$, P, C3bINA, and β1H. Using functional assays, B, $\bar{D}$, P, and C3bINA activity were found (Fig. 1) whereas C4, C3, and β1H activities could not be detected, although the assays used would detect 200,000 effective molecules of C4 and 40,000 effective molecules of C3 per ml of a

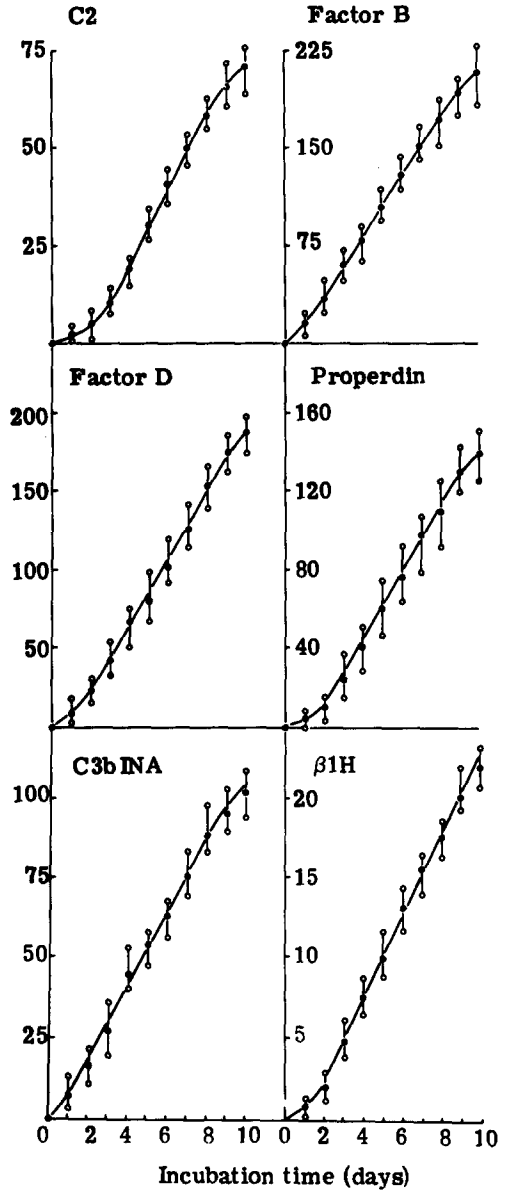


FIG. 4. Cumulative synthesis of C2, B, $\bar{D}$, P, C3bINA, and β1H by monocytes. Culture fluid was replaced with fresh RPMI.FCS daily and the number of effective molecules (Z) (Materials and Methods) of C2, B, $\bar{D}$, and P, Z′ units of C3bINA and nanograms of β1H synthesized each day were added together (shown on the ordinate). No plateau was seen after day 5. The results are the mean (●) and range of (○) of three cultures.

standard pool of human serum, and 20 ng/ml of β1H. However, using a radioimmunoassay, increasing concentrations of β1H were found in the culture supernates (Fig. 1). In addition to the functional assays described, P activity was capable of stabilizing $\overline{C3b\bar{B}b}$ (Fig. 3) and $\bar{D}$ activity inhibited by DFP (10^{-3} M). Incorporation of cycloheximide (1.0 μg/ml) into the culture medium resulted in reversible inhibition

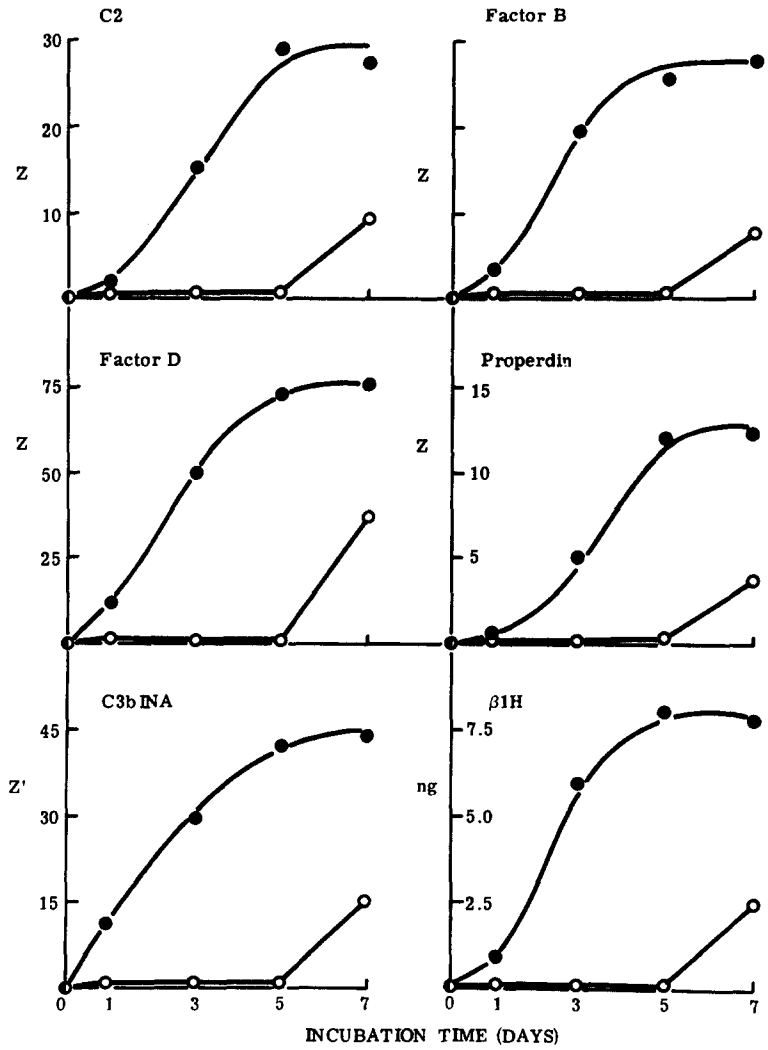


FIG. 5. The reversible nature of cycloheximide-induced inhibition of C2, B, D̄, P, C3bINA, and β1H synthesis. Monocytes were incubated in RPMI.FCS (●) or RPMI.FCS containing cycloheximide 1.0 μg/ml (○). On day 5, the cycloheximide-containing cultures were washed with RPMI and fresh RPMI.FCS was added. By day 7, synthesis of all proteins was restored. Results for C2, B, D̄, and P (effective molecules [× 10^{-7}]/culture), C3bINA (Z' units [Materials and Methods]/culture) and β1H (nanograms/culture) are shown on the ordinates, and incubation time (days) shown on the abscissas.

of C2, B, D̄, P, C3bINA, and β1H synthesis (Fig. 5). ^{14}C-labeled amino acids were incorporated into C4, C3, C5, B, P, C3bINA, and β1H protein (Fig. 5), but incorporation into D̄ could not be tested for because of lack of suitable antiserum.

Finally, using the immunoperoxidase technique, C2, C4, C3, C5, B, P, C3bINA, and β1H were found in all the cultured cells (Fig. 7) which is in agreement with findings of Einstein et al. (18) who found lysozyme was present in all human monocytes in culture. This may not mean that all monocytes are synthesizing these proteins; it is equally possible that only subpopulations have this property and the

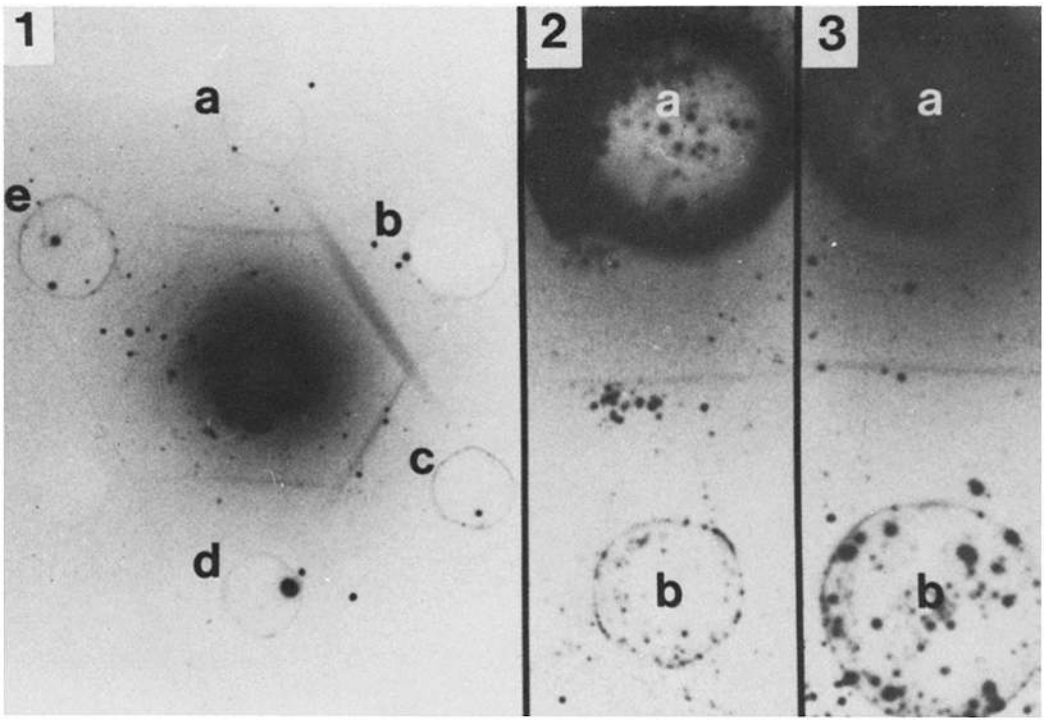
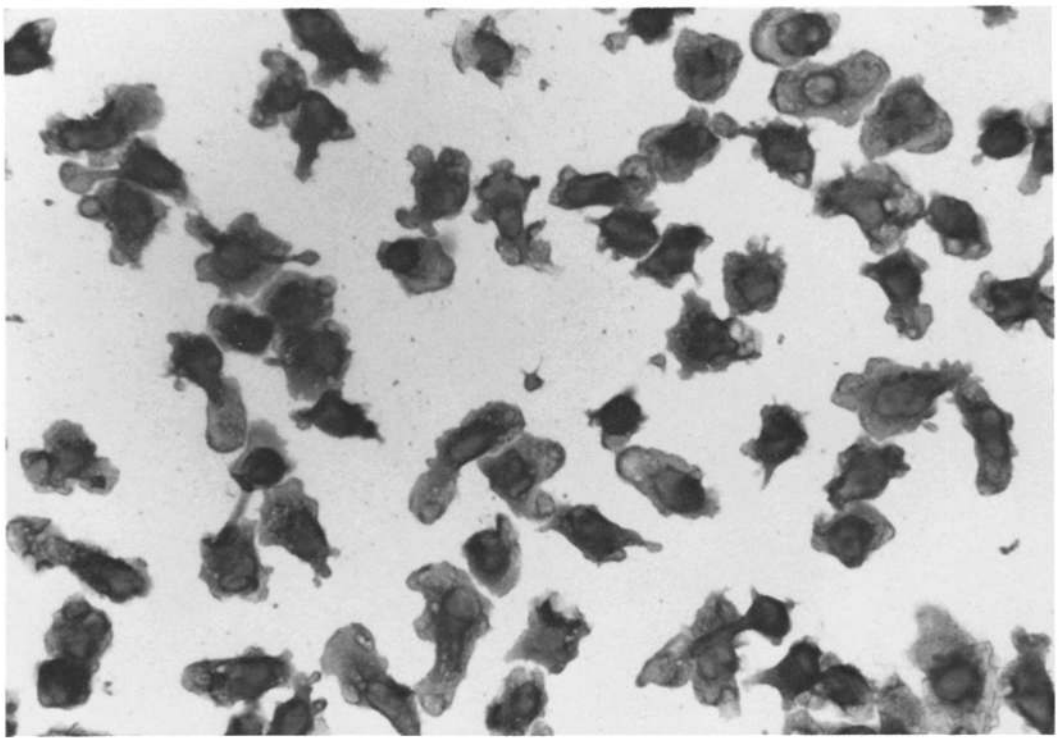


FIG. 6. Incorporation of ^{14}C-labeled amino acids into immunoprecipitable protein. (1) The concentrated monocyte culture fluid is in the center well and antisera are in the peripheral wells: a, anti-C4; b, anti-C3; c, anti-β1H; d, anti-B; and e, anti-C5. (2) The concentrated monocyte culture fluid is in well a, and anti-P in well b. (3) The concentrated monocyte culture fluid is in well a, and anti-C3bINA is in well b.

remainder pinocytose, or adsorb onto their surfaces, the newly synthesized proteins. Ultrastructural localization studies will discriminate among these possibilities.

From the failure to detect functionally active C4, C3, or β1H and the irregular detection of C5 activity, it could be argued that the detection of ^{14}C-labeled amino acids in the immunoprecipitable proteins could represent nonspecific binding to the carrier proteins (15). However, the immunoperoxidase localization studies, the cycloheximide-induced inhibition of ^{14}C-labeled amino acids into immunoprecipitable protein, and the detection of β1H by radioimmunoassay, all argue against this possibility. Three other explanations are possible: (*a*) monocytes synthesize and secrete precursor molecules which require proteolytic cleavage to be converted to the active molecule (45), (*b*) molecules are synthesized in active form, but are enzymatically degraded in the culture fluid, and (*c*) their concentrations may be too low to permit detection in the functional assays employed. C4 is apparently synthesized by monocytes as the single polypeptide chain, pro-C4, whereas C3 is present as a double polypeptide chain, and therefore has presumably been degraded in the culture fluid after synthesis (H. R. Colten. Personal communication.). The explanation why β1H was not detected functionally in the assay described here, could be a result of the low concentrations of the protein found (5 ng/ml) in the culture fluids (Fig. 1). In experiments investigating the mechanism of destabilization of $\overline{\text{C3bBbP}}$ by β1H, it

A



B

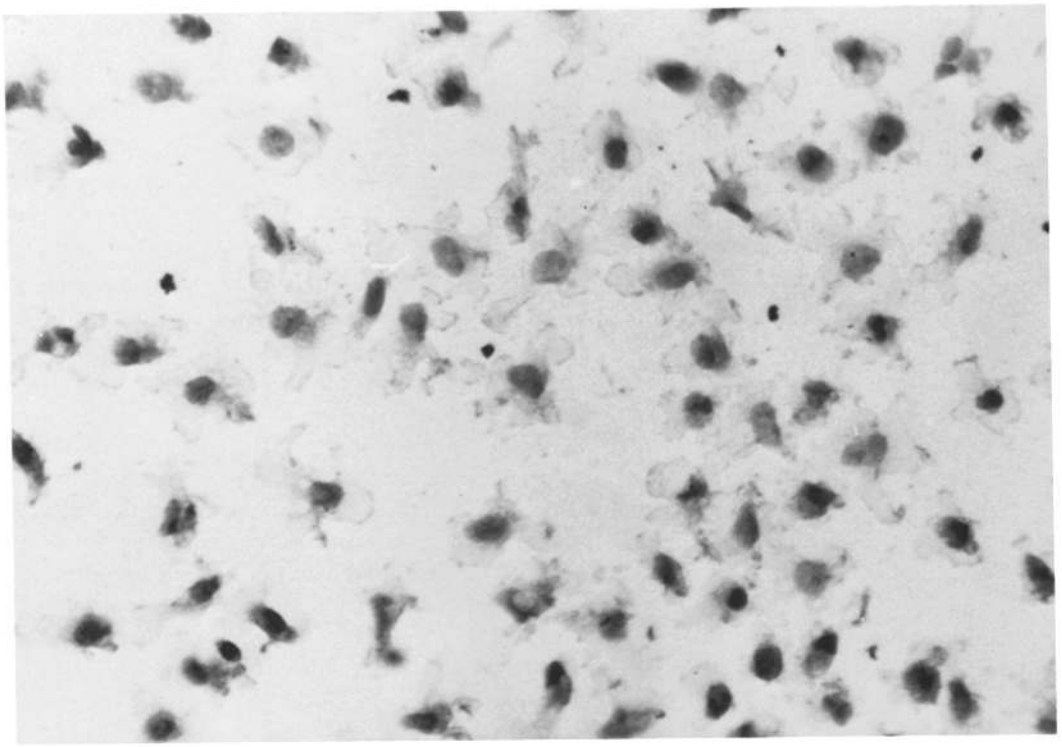


FIG. 7. Immunoperoxidase localization of complement proteins. (A) Anti-β1H. The rounded monocytes show an extremely dense granular staining, whereas the monocytes with spreading cytoplasm show dense perinuclear granules with fine granular cytoplasmic staining. × 400. (B) Anti-β1H absorbed with purified β1H. The staining pattern, shown above is completely inhibited. × 400.

TABLE I
Summary of Procedures Used to Detect Components Studied

	C2	C4	C3	C5	B	$\bar{D}$	P	C3bINA	β1H
Functional assay	+	−	−	±	+	+	+	+	−
Radioimmunoassay	ND	ND	ND	ND	ND	ND	ND	ND	+
Cycloheximide inhibition	+	+	+	+	+	+	+	+	+
Incorporation of ^{14}C-amino acids	ND	+	+	+	+	ND	+	+	+

ND, not done.

was difficult to detect concentrations of β1H <20 ng/ml (10, 35). It is also possible that β1H is secreted into the culture fluid as an inactive pro-β1H molecule: this possibility is currently under investigation.

The detection of hemolytically active B in these experiments is at variance with results obtained using guinea pig macrophage culture supernates, in which all the B is inactivated after its synthesis in the presence of functionally active $\bar{D}$ and C3, (46). The inactivation process probably depended upon cleavage of B by $\bar{D}$ during the assembly of the alternative pathway C3 convertase in the culture fluid. The ability to detect hemolytically active C3 in guinea pig macrophage cultures must be because the amount of $\overline{C3bBb}$ formed in the culture fluid is limited by the availability of B, when $\bar{D}$ and C3 are in excess. In contrast, in the human monocyte system described here, the limiting factor must be C3 as B and $\bar{D}$ are detected: either insufficient C3 is synthesized to turnover all the B produced, or the C3 is incapable of participating in the formation of a C3 convertase because it is in the form of an inactive precursor, or has been somehow degraded after synthesis.

It has previously been shown that proteolytic enzymes can substitute for $\bar{D}$ in the formation of $\overline{C3bBb}$ (47) and therefore, in the absence of immunoperoxidase localization with specific anti-$\bar{D}$ antibody, it is possible that other monocyte-derived enzymes could be responsible for the $\bar{D}$-like activity measured. Likewise, it is conceivable that certain lysozomal hydrolases could inactivate C3b; and thus the C3bINA activity observed could be a result of a combination of the actions of C3bINA and/or other enzymes. Molecular characterization of $\bar{D}$ and C3bINA activities in monocyte culture fluid should resolve these problems.

The recent observations that human peritoneal macrophages synthesize hemolytically active $C\bar{1}$ and its three subcomponents (16), together with the data presented in this communication, demonstrate that cells of the human monocyte-macrophage series are capable of synthesizing all the components which should ultimately be capable of forming the C3 and C5 convertases of both the classical and alternative pathways. Macrophages from laboratory animals are also capable of synthesizing the components of the classical and alternative pathways (15, 16, 46, 48) but have not been shown to produce C3bINA or β1H. By synthesizing C5, they produce the three proteins, C3, C5, and B, which are currently thought to provide most of the major phlogistic cleavage products of complement activity. The mobile nature of this source of complement components could have important consequences on the development of the local inflammatory reaction. The synthesis of the regulatory proteins C3bINA and β1H may be important in modulating the extent of complement activation at such sites.

The similarity of the observed rates of synthesis of C2, B, $\bar{D}$, P, C3bINA, and β1H (Fig. 2) suggest that action of a common controlling factor or factors, which could account for the correlation between the serum levels of these proteins.

Summary

Short-term cultures of human peripheral blood monocytes were shown to synthesize the alternative pathway complement components C3, factors B (B) and $\bar{D}$ (D), and properdin, the regulatory proteins C3b inactivator (C3bINA) and β1H, in addition to C2, C4, and C5. B, $\bar{D}$, properdin, C3bINA, and C2 were detected by functional assays, whereas β1H, C4, C3, and C5 could only be detected using immunochemical procedures. Immunoperoxidase localization studies showed that all the cells in each culture contained each component, so it is possible that all monocytes synthesize each component.

It is concluded that cells of the monocyte-macrophage series form a mobile source of complement components and regulatory proteins which can be concentrated at sites of inflammation.

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