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Research Article

Human monocytes synthesized the third component of complement (C3) up to 5 wk in vitro. Evidence for net C3 synthesis was based on (a) incorporation of ¹⁴C-labeled amino acids into C3 protein, (b) identity of the allotype of C3 produced in vitro with that of the donor's serum C3, even in the presence of carrier C3 protein of a different allotype; (c) correspondence of electrophoretic mobility, size, and subunit structure of C3 protein produced in vitro with serum C3; (d) inhibition of C3 production with cycloheximide. Monocytes from two unrelated C3-deficient patients were studied under conditions that supported C3 synthesis by normal monocytes. Serum from each of the patients contained less than 1% of the normal C3 concentration, both their monocytes produced C3 at approximately equal to 25% of the normal rate when studied after 2 wk in vitro. The C3 produced in vitro by monocytes from one of the patients had the molecular weight of normal serum C3 and dissociated appropriately under reducing conditions. Monocytes from C3-deficient patients could not be distinguished from normals on the basis of morphology, rosetting with C3-coated erythrocytes, or rates of C2, and total protein synthesis.

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Biosynthesis of the Third Component of Complement (C3) In Vitro by Monocytes from Both Normal and Homozygous C3-Deficient Humans

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ABSTRACT Human monocytes synthesized the third component of complement (C3) up to 5 wk in vitro. Evidence for net C3 synthesis was based on (a) incorporation of ^{14}C -labeled amino acids into C3 protein, (b) identity of the allotype of C3 produced in vitro with that of the donor's serum C3, even in the presence of carrier C3 protein of a different allotype; (c) correspondence of electrophoretic mobility, size, and subunit structure of C3 protein produced in vitro with serum C3; (d) inhibition of C3 production with cycloheximide.

Monocytes from two unrelated C3-deficient patients were studied under conditions that supported C3 synthesis by normal monocytes. Serum from each of the patients contained <1% of the normal C3 concentration, but their monocytes produced C3 at $\approx 25\%$ of the normal rate when studied after 2 wk in vitro. The C3 produced in vitro by monocytes from one of the patients had the molecular weight of normal serum C3 and dissociated appropriately under reducing conditions. Monocytes from C3-deficient patients could not be distinguished from normals on the basis of morphology, rosetting with C3-coated erythrocytes, or rates of C2, and total protein synthesis.

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INTRODUCTION

Early studies of the biosynthesis of the third component of complement (C3) indicated that many tissues and cells were capable of incorporating radiolabeled amino acids into C3 antigen as detected in the presence of carrier serum by Ouchterlony and immunoelectrophoretic analysis (1). Significant limitations of the methods used prevented definitive conclusions regarding the site(s) of C3 synthesis. Subsequently, using a different approach, it was established that the liver is the principal site of C3 synthesis (2). After transplantation of the liver from a donor with a distinct C3 allotype, the recipient's C3 type disappeared from his serum and thereafter was entirely replaced by the C3 type of the donor. Immunofluorescence studies with anti-C3 antibody suggested that the hepatocyte and not the Kupffer cell was the site of C3 synthesis (3). More recent work has demonstrated production of functionally and immunochemically active C3 by human fetal liver as early as the first trimester of gestation (4), by a well-differentiated rat hepatoma cell line (5), and by primary rat liver cell cultures, highly enriched in hepatocytes.¹ Although it seemed that the liver, and specifically the hepatocyte, was the major site of synthesis of C3, the demonstration that human rheumatoid, but not normal, synovium produced functionally active C3 in vitro (6) indicated

¹ Breslow, J., P. Rothman, and H. R. Colten. Unpublished data.

that, under certain circumstances, extrahepatic C3 synthesis might occur.

In view of these considerations and the inaccessibility of liver for routine longitudinal study of human C3 synthesis in genetic and acquired abnormalities of C3 production, blood monocytes were examined for their capacity to synthesize C3 *in vitro*. A recently developed method (7) for the preparation and maintenance of human monocytes for up to 6 mo in culture was utilized for these studies. The cells can be obtained from small volumes of blood, synthesize lysozyme and the second component of complement (C2), phagocytose latex beads, rosette with IgG- or C3-coated erythrocytes, and kill *Listeria monocytogenes*.

Deficiency of the third component of complement in man is inherited as an autosomal codominant trait (8–11).² Family studies indicated in two kindred that C3 deficiency results from inheritance of a near silent gene (8, 12) and metabolic studies of C3 *in vivo* suggested slight hypercatabolism in homozygous C3 deficiencies (11, 12) not sufficient to account for the markedly reduced serum C3 concentrations (<1% of normal). In order to study directly the biochemical basis of C3 deficiency, the functional capacities of monocytes from affected patients were compared to monocytes from normal individuals.

METHODS

Preparation of monocyte monolayers. Long-term primary cultures of human monocytes were prepared using a previously described method (7). Preliminary studies indicated that hemolytically active partially purified C3 (Cordis Laboratories, Miami, Fla.) was stable for at least 48 h in Medium 199 (Microbiological Associates, Bethesda Md.) supplemented with 10% heat-inactivated (56°C, 2 h) fetal calf serum (HFCS), and 50 u/ml each of penicillin and streptomycin (M199-HFCS, Microbiological Associates; the medium used in all monocyte culture studies).

Incorporation of radiolabeled amino acids into protein. Established monocyte monolayers were washed with M199 lacking *l*-lysine, *l*-leucine, *l*-isoleucine, and *l*-valine (Special M199, SpM199, Microbiological Associates)³ and then incubated in 0.5 ml of SpM199 supplemented with 10% dialyzed heated fetal calf serum (dHFCS) and ¹⁴C-labeled *l*-lysine (270 mCi/mmol), *l*-leucine (270 mCi/mmol), *l*-isoleucine (270 mCi/mmol), and *l*-valine (225 mCi/mmol) (New England Nuclear, Boston, Mass.) at a final concentration of 10 μ Ci/ml. After 120 h at 37°C in a humidified 5% CO₂:95% air atmosphere, culture medium was removed, immediately centrifuged at 1,700 g maximum for 15 min, and stored at -90°C before further analysis.

² Davis, A. E., J. S. Davis, A. R. Rabson, S. G. Osofsky, H. R. Colten, F. S. Rosen, and C. A. Alper. Homozygous C3 Deficiency: Detection of C3 by radioimmunoassay. *Clin. Immunol. Immunopathol.* In press.

³ Abbreviations used in this paper: dHFCS, dialyzed heated fetal calf serum; GaHC3, goat anti-human C3 antibody; NGS, normal goat serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SpM199, Special medium 199.

Quantitation of complement proteins. The source and preparation of reagents for hemolytic titration of individual complement components is given in reference (13) and the assays were performed as described in reference 4. Immunochemical estimations of complement protein concentration in serum was carried out by electroimmunoassay (14) using rabbit antisera to C3 and Factor B.

Agarose electrophoresis of radiolabeled C3. Radiolabeled monocyte culture medium was dialyzed against Veronal-buffered saline dextrose ($\mu = 0.075$; pH 7.35) containing 1 mM sodium azide and 10 mM sodium EDTA at 0°C with three dialysate changes for a total of 12 h. The dialyzed medium was concentrated up to 100-fold in a collodion apparatus (Shleicher & Schuell, Inc., Keene, N.H.), and then subjected alone or together with carrier serum to prolonged agarose gel electrophoresis and immunofixation with anti-C3 antiserum (15). Gels were dried, stained for protein content, and stored for 2 mo in contact with X-ray film (Cronex 6 Dupont Inst., Newtown, Conn.) to develop autoradiographs.

Preparation of Sepharose-bound goat anti-human C3 immunoabsorbant. Goat antiserum to human C3 (GaHC3) was obtained from Atlantic Antibodies, (Westbrook, Maine) and gamma globulin fractions of the specific antiserum and of normal goat serum (NGS) were prepared by Pevikon block electrophoresis (Mercer Consolidated Corp. Yonkers N.Y.).

The anti-C3 and normal goat gamma globulins were bound to Sepharose 4B beads (Pharmacia, Inc., Piscataway, N.J.) by a previously described method (16). The gamma globulin fractions of GaHC3 (previously adjusted to ≈ 4.5 mg protein/ml) were each added to give final concentrations of ≈ 0.6 mg of protein/ml of Sepharose. Approximately 80–90% of the original protein was bound to the beads. The beads were stored for use at a concentration of 40% vol/vol in phosphate-buffered saline plus 1 mM sodium azide at 4°C.

Quantitative immunoabsorption of radiolabeled C3. Radiolabeled monocyte culture medium (previously stored at -90°C) was thawed at room temperature, diluted fourfold with M199-10% HFCS, and centrifuged at 15,000 g for 1 h at 0°C. 100- μ l portions of either Sepharose-NGS or Sepharose-GaHC3 were added to disposable plastic tubes and after centrifugation the supernate was removed and the washed Sepharose resuspended in 40 μ l of phosphate-buffered saline containing 2% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.), 2% sodium deoxycholate (Sigma Chemical Co.) and 50 mM each of lysine, leucine, isoleucine, and valine. 200- μ l aliquots from a single sample of ultracentrifuged radiolabeled culture medium was added to two tubes, one containing Sepharose-GaHC3 and the other containing Sepharose-NGS. These mixtures were incubated at 0°C with agitation and after 3 h the beads were washed three times in phosphate-buffered saline (containing 0.5% Triton, 0.5% deoxycholate), three times in phosphate-buffered saline, and then transferred to 10 ml of Instagel (Packard Instrument Co., Inc., Downers Grove, Ill.) for scintillation counting.

Radiolabeled C3 production per cell was calculated by subtracting nonspecific (Sepharose-NGS) from specific (Sepharose-GaHC3) radioactivity and dividing by the total DNA content of the cover slips from which the medium had been harvested.

Total protein synthesis. Total protein synthesis was estimated by mixing 20 μ l of the diluted culture medium with 2 ml of 10% trichloroacetic acid (TCA) (J. T. Baker Chemical Co., Phillipsburg, N.J.). The mixtures were incubated 1 h at 4°C and centrifuged at 4,500 g for 5 min at 0°C. The TCA was decanted and 0.5 ml of 1.0 N NaOH added to the precipitate. The tubes were vigorously mixed, incubated at 37°C for 5 min, and 2 ml of ice-cold TCA was added to each tube. After 1 h at 4°C the TCA-insoluble radioactivity was harvested

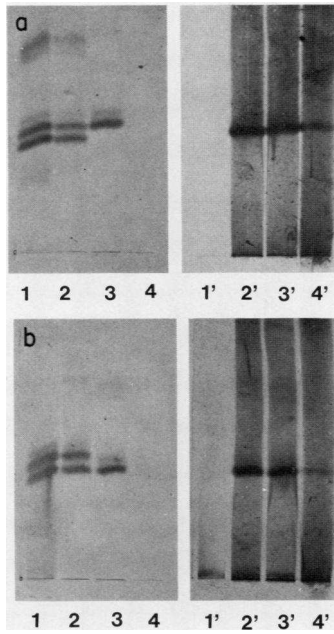


FIGURE 1 Prolonged agarose electrophoresis and immunofixation by monocytes in vitro. Left panels, protein stain; right panels, autoradiographs. (a) Carrier serum: slots 1 and 2, C3FS₁; slot 3, C3FF; slot 4, no serum. Monocyte medium: slot 1, none; slots 2-4, from donor with C3FF (b) Carrier serum: slots 1 and 2, C3FS₁; slot 3, C3SS, slot 4, no serum. Monocyte medium: slot 1, none; slots 2-4, from donor with C3SS.

and washed thoroughly with 10% TCA on glass filter disks (Whatman, GF C, 2.4 cm diam) then counted by scintillation spectrometry.

Preparation of purified C3 and ¹²⁵I-C3. C3 was purified from fresh plasma by the method of Nilsson and Müller-Eberhard (17) and labeled with ¹²⁵I by the iodine monochloride method (18).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The C3 produced in vitro was prepared and analyzed on SDS-PAGE exactly as previously described in detail (19) except that rabbit antiserum to human C3 (Atlantic Antibodies) and antiserum to ovalbumin (19) (to serve as control immunoprecipitate) were used. ¹²⁵I-labeled C3 was analyzed on SDS-PAGE. Parallel gels with standard molecular weight markers were run and stained with Coomassie Blue according to reference (20). Markers of known molecular weights included β-galactosidase (130,000, Worthington Biochemical Corp., Freehold, N.J.), phosphorylase (94,000-Sigma Chemical Co.), bovine serum albumin (68,000-Sigma Chemical Co.), ovalbumin (43,000-Schwarz/Mann Div. Becton, Dickinson & Co., Orangeburg, N.Y.) and Carbonic anhydrase (29,000-Worthington Biochemical Corp.).

Fluorimetric assay of DNA. The number of monocytes per cover slip was determined using a previously described fluorimetric assay for DNA (7).

Rosettes. Procedures for rosette formation with C3-coated sheep erythrocytes were as previously described (21).

Monocyte donors. The monocytes of nine normal subjects and two C3-deficient patients were studied after obtaining informed consent. The clinical and serological findings in these C3-deficient patients have been reported elsewhere (8-10).

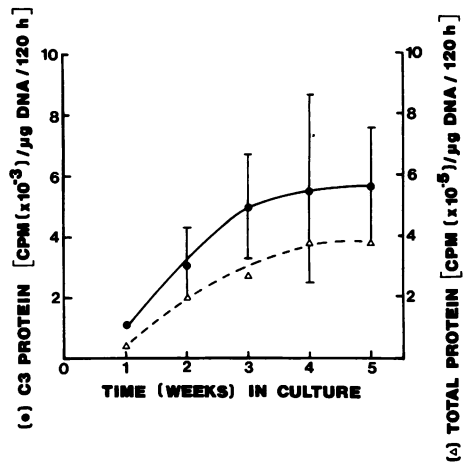


FIGURE 2 Kinetics of C3 (●) and total protein (Δ) synthesis by human monocytes in vitro. Symbols indicate mean, and bars ±1 SD of individual samples from nine normal subjects.

RESULTS

Agarose electrophoresis of ¹⁴C-labeled C3 produced by monocytes. Media from monocyte monolayers incubated with ¹⁴C-labeled amino acids contained radiolabeled material that precipitated in Ouchterlony gels with antibody to C3 in the presence of carrier protein, a finding in accord with previous reports (1, 22). In order to rule out the possibility of nonspecific coprecipitation of label, cultures of monocytes from donors of C3 allotype SS, FS, and FF, respectively, were prepared, and after 2 wk in vitro cells were incubated in the presence of radiolabeled amino acids for 5 days. The media were then harvested, dialyzed,

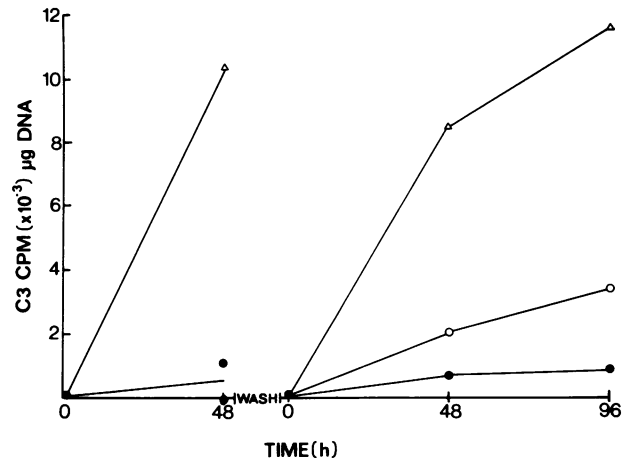


FIGURE 3 Effect of cycloheximide on C3 synthesis by normal monocytes in vitro. Cells incubated in medium alone (Δ, ○) in medium containing cycloheximide (0.5 μg/ml) (●). All cover slips washed at 48 h; samples obtained at timed intervals thereafter from the individual cultures.

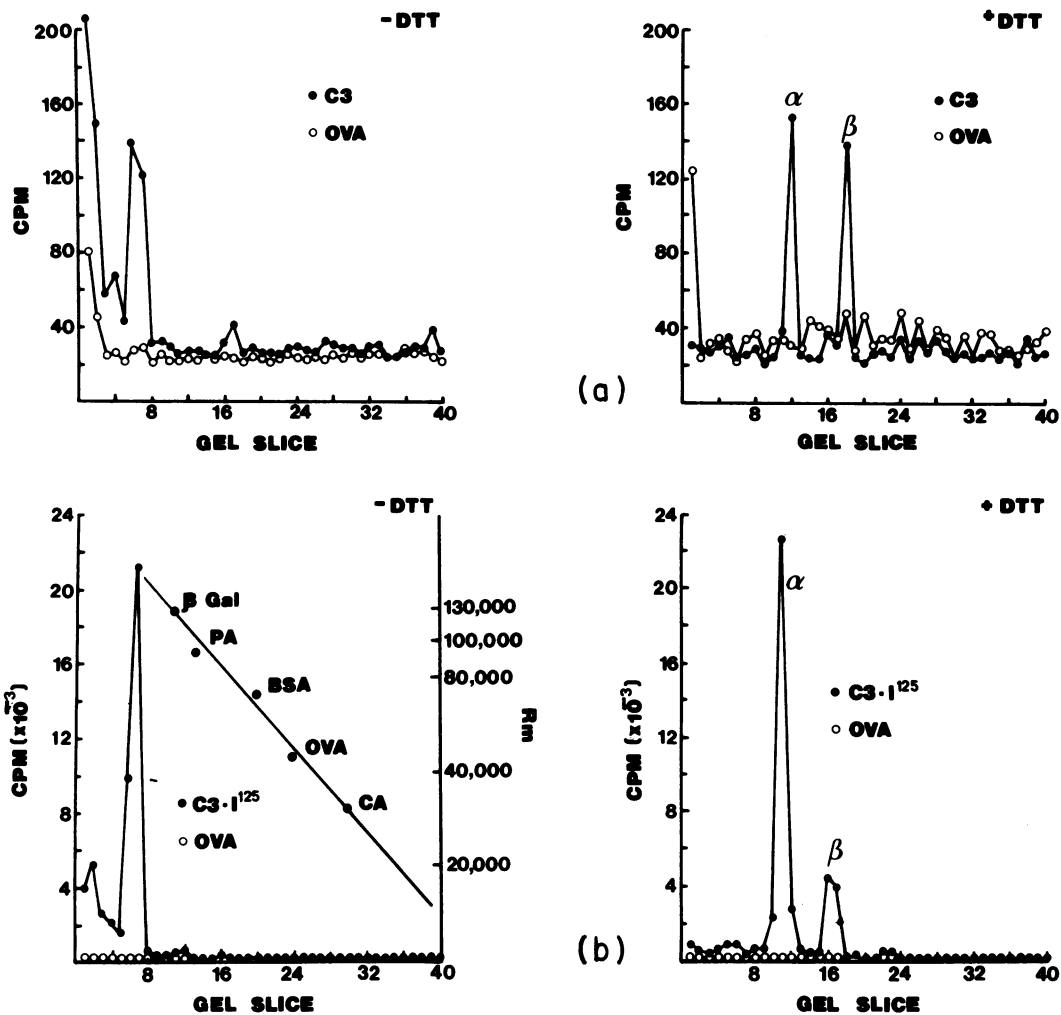


FIGURE 4 SDS-polyacrylamide gel electrophoresis: (a) C3 synthesized by normal monocytes in vitro (top panels) under nonreducing and reducing conditions (in 50 mM dithiothreitol). (b) Bottom panels, purified ^{125}I -labeled human C3 under nonreducing and reducing conditions (in 50 mM dithiothreitol). Standard molecular weight markers as given in the text.

concentrated, and subjected to agarose electrophoresis and immunofixation in the presence of carrier serum of either identical or distinct C3 allotype (15) or in the absence of carrier serum. In each instance, the radiolabeled C3 produced in vitro was of monocyte donor type (Figs. 1a,b); that is, the mobility of labeled C3 was independent of carrier serum C3 type or the presence of carrier. The amount of radiolabeled C3 detected was markedly reduced in medium harvested from monolayers incubated in the presence of cycloheximide (0.5 $\mu\text{g}/\text{ml}$) and absent in medium to which ^{14}C -labeled amino acids had been added after harvesting from the monocyte cultures (data not shown).

Kinetics of C3 and total protein synthesis. The kinetics of C3 production by monocytes were examined by quantitatively estimating radiolabeled C3 secreted into the media at timed intervals between 1 and 5 wk

in vitro. Monocyte monolayers from nine normal subjects were prepared; at some time intervals as many as eight monocyte cultures from a single subject were analyzed. These data (Fig. 2) indicated that C3 production per cell increased with time in culture. An increase in total protein synthesis paralleled the increase in rate of C3 production.

Reversible inhibition of C3 production by cycloheximide. Replicate monocyte monolayers from a single individual (2 wk in culture) were incubated in M199-HFCS containing cycloheximide (0.5 $\mu\text{g}/\text{ml}$) for 48 h at 37°C, after which each monolayer was rinsed. One of the preparations incubated in the presence of cycloheximide was fed with fresh M199-HFCS lacking the inhibitor and the other incubated again in medium with cycloheximide. C3 production was monitored with the immunoadsorption assay. As shown in (Fig. 3),

cycloheximide inhibited C3 production by $\approx 90\%$; C3 production was partially restored by removing the inhibitor.

SDS-PAGE of C3 synthesized in vitro. Monocytes (2 wk in vitro) were incubated with radiolabeled amino acids for 5 days and the size of reduced and unreduced immunoprecipitable C3 protein and purified ^{125}I -labeled C3 estimated by SDS-PAGE. Radiolabeled C3 produced by normal monocytes had an approximate 174,000-dalton mol wt and dissociated into two subunits (mol wt 118,000 and 77,000) under reducing conditions (Fig. 4a), a size and structure similar to purified serum C3 (Fig. 4b).

Biological function of C3 produced in vitro. A volume of medium containing ^{14}C -labeled C3 (produced by normal monocytes in culture) was mixed with an equal volume of normal human serum. One portion of this mixture was incubated at 0°C and another at 37°C for 16 h. Each was then subjected to prolonged agarose electrophoresis, immunofixation, and autoradiography. The results (Fig. 5) showed conversion of ^{14}C -labeled C3 at 37°C , but no significant conversion at 0°C (i.e., labeled C3 functioned as substrate in "aging" of fresh serum in vitro). When either medium alone or fresh serum alone were preincubated at 37°C , then mixed and immediately electrophoresed, no conversion of labeled C3 was observed.

None of the media harvested from monocyte cultures contained detectable hemolytically active C3 (limits of the detection system 7.5×10^6 effective molecules, under optimum conditions 2.25×10^{10} C3 protein molecules per ml). In addition, no conversion of Factor B was observed when medium containing C3

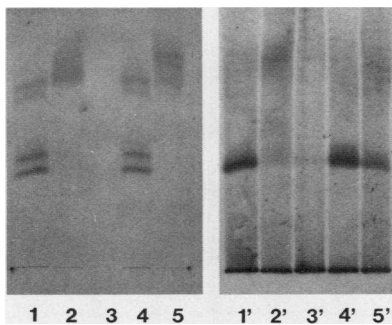


FIGURE 5 Conversion in normal serum of C3 produced by monocytes. Agarose electrophoresis and immunofixation with anti-C3 antiserum stained for protein (left panel) and autoradiograph (right panel). Medium from monocytes (C3FF) incubated with serum (C3FS₁) for 16 h at 0°C (slot 1) or at 37°C (slot 2); slot 3, medium (C3FF) alone incubated at 37°C for 16 h. Slot 4, exactly as 3, except carrier serum (C3FS) added after incubation and just before electrophoresis. Slot 5, carrier serum (C3FS₁) alone incubated at 37°C for 16 h then monocyte medium added just before electrophoresis.

TABLE I
Serum Levels and Synthesis of C3 by Monocytes from Two Unrelated Patients Homozygous for C3 Deficiency

| | Serum C3 | | Synthesis by monocytes in vitro† | | |
|-----------|--------------------|--------|----------------------------------|--------|------------------------|
| | Titer* | Normal | C3 | Normal | Total protein§ |
| | | % | cpm/ μg DNA | % | cpm/ μg DNA |
| Normal | 22,000 \pm 7,000 | 100 | 3,220 | 100 | 184,000 |
| Patient 1 | <10 | <0.01 | 667 | 21 | 128,000 |
| Patient 2 | <10 | <0.01 | 902 | 28 | 324,000 |

* Dilution of serum yielding 63% lysis (average of one lytic site per indicator cell).

† Production by monocytes during 120 h in vitro.

§ Incorporation of ^{14}C -labeled amino acids into TCA-precipitable material during 120 h in vitro.

produced by monocytes was mixed with C3-deficient serum.

Synthesis of C3 by monocytes from C3-deficient patients. The results given in Table I indicate that monocytes from the two C3-deficient individuals produced radiolabeled C3 protein at a rate of 20–30% of the mean normal rate. Serum obtained from each patient at the same time as the in vitro monocyte study contained 4–9 U/ml of hemolytically active C3 and there was no detectable C3 protein by immunochemical methods.

Radiolabeled C3 produced in vitro by monocytes from patient 1 was subjected to SDS-PAGE. Under nonreducing conditions, a single peak of radioactivity was detected with mobility corresponding to molecular size of $\approx 185,000$ daltons. Under reducing conditions, the radiolabeled C3 dissociated into two subunits, but the amount of radioactivity in each peak was too small to precisely estimate their molecular sizes. Monocytes from the C3-deficient patients could not be distinguished from normals on the basis of morphology, rosetting with C3-coated erythrocytes, or rates of C2 and total protein synthesis.

DISCUSSION

Previous investigators (22) suggested that mononuclear phagocytes were a site of C3 synthesis, based on the detection of radiolabeled C3 precipitin arcs on Ouchterlony or immunoelectrophoretic analysis of culture medium in the presence of carrier serum. However, it was recognized that net synthesis of C3 was not demonstrated and the possibility could not be ruled out that the radiolabeled immunoprecipitates were artifacts resulting from nonspecific coprecipitation of labeled proteins or amino acids with carrier C3. In the present study, this possibility was tested and ruled out by analysis on prolonged agarose electrophoresis of radiolabeled C3 produced in vitro. The results indi-

cated that incorporation of radiolabel into C3 protein was independent of carrier C3 allotype and was determined solely by the C3 allotype of the cell donor. Moreover, C3 production was reversibly inhibited by low concentrations (0.5 $\mu\text{g/ml}$) of cycloheximide, an inhibitor of protein synthesis. The radiolabeled C3 protein produced in vitro had the approximate molecular weight and subunit structure of purified human serum C3. The estimates of molecular size of intact C3 (both the purified serum C3 and that produced in vitro) were somewhat different from that (190,000) reported in the literature (23). This difference was not observed in analysis of the α - and β -subunits, the sizes of which corresponded closely to published figures (α -chain 120,000; β -chain 75,000) (23). Since the size of intact C3 exceeded that of the largest marker protein, a precise estimate of its molecular weight could not be determined under nonreducing conditions. C3 synthesized in vitro had the electrophoretic mobility of native C3, including the small charge differences determined by genetic allotype. Labeled C3 functioned as substrate in aging of fresh serum in vitro under conditions that led to similar conversion of normal serum C3 breakdown products. Monocyte culture medium alone did not support conversion of the labeled C3, indicating further that C3 was stable under the culture conditions employed and that the monocytes did not produce at least one of the components required for C3 conversion during aging of serum. No functionally active C3 was detected in these cultures by hemolytic assay, and the medium did not restore the ability of C3-deficient human serum to convert Factor B in the presence of zymosan. In view of the physicochemical similarities between native C3 and the C3 detected in the monocyte culture medium, it is probable that the failure to detect functionally active C3 is due to differences in sensitivity of the hemolytic and immunochemical assays. Alternatively, the monocyte product may be a precursor or dysfunctional C3 molecule with physicochemical differences too small to detect by the methods used in these studies.

The rate of C3 protein secreted into the medium per monocyte increased with time in culture. This increase was paralleled by a similar increase in total protein synthesis. Previous studies of monocytes in culture (7) revealed other significant changes in monocyte structure and function during the first several weeks in vitro, perhaps reflecting monocyte maturation. Local production of C3 by macrophages in an inflammatory exudate might increase C3 tissue concentrations at sites where complement consumption was in progress. A constant local supply of native C3 may well be of importance in supporting complement-dependent host defenses since activated C3 is rapidly catabolized.

Monocytes from two unrelated homozygous C3-deficient patients produced substantially more C3 in

vitro than would be predicted on the basis of their serum C3 levels or previous C3 turnover studies which demonstrated only a slightly increased fractional catabolic rate in homozygous C3 deficient. The discrepancy between the in vivo and in vitro data may be accounted for by at least two possible explanations: (a) The proportion of the total body C3 synthesized by monocytes may be small and the rate of C3 synthesis by the liver (specifically the hepatocyte) may be more directly proportional to the serum C3 level. Primary hepatocyte cultures would then be expected to reveal the true extent of the biosynthetic defect in these patients; (b) C3 producing cells may be suppressed in the C3-deficient patients by the presence or absence of regulatory factors. When placed in culture these cells may be released from inhibition or stimulated by culture conditions to produce C3 at the relatively high rates observed. Study of the response of normal monocytes to C3-deficient serum and studies of monocytes from heterozygous C3-deficient individuals may help to resolve these questions.

Monocytes from C3-deficient patients could not be distinguished from normals on the basis of morphology, rosetting with C3-coated erythrocytes, or rates of C2 and total protein synthesis. Thus, on the basis of these limited criteria, the 70–80% reduction in C3 synthesis rate appears to be specific. There was no apparent relationship between monocyte surface C3 “receptors” and the source of the monocytes or the C3 biosynthetic capacity of the cells in vitro.

The ability of monocytes from C3-deficient patients to produce C3 in vitro stands in contrast to studies of monocytes from C2-deficient humans (24) and macrophages from C4-deficient guinea pigs (25) in which specific and total biosynthetic defects for C2 and C4, respectively, persisted in vitro. In each case, the relevant complement component (C2 or C4) was functionally and immunochemically absent from serum, as well as from monocyte macrophage cultures. These observations suggest, but do not prove, a different genetic mechanism to account for human C3 deficiency as compared to guinea pig C4 deficiency and perhaps human C2 deficiency. The latter deficiency states may best be explained by structural gene defects while C3 deficiency may result from a defect in biosynthetic regulation.

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