Biosynthesis of complement components by cultured rat hepatocytes

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Hepatocytes which had been isolated from the livers of Charles River rats were cultured *in vitro*. The cells were shown to synthesize albumin and the complement components C4, C2, C3 and B. Pulse-label studies with [³⁵S]methionine showed that C4 and C3 were synthesized as single polypeptide chains. Pro-C4 did not appear to be converted into the plasma form of C4 intracellularly, whereas cell lysates contained the α - and β -chains of plasma C3 as well as pro-C3. It is concluded that culture of rat hepatocytes *in vitro* provides a useful technique for studies of the synthesis of complement components.

INTRODUCTION

Although mononuclear phagocytes have been shown to synthesize C1 and its subcomponents, C4, C2, C3, C5, factors B, D, P, H and I, and C1-inhibitor [reviewed by Colten (1976) and McPhaden *et al.* (1985)], it is probable that the liver is the primary site of synthesis of complement components. This conclusion is based on observations of patients who have received orthotopic liver transplants. After transplantation the allotypes of C3, C6, C8 and B in the blood of these patients become those of the donor (Alper *et al.*, 1969; Alper & Rosen, 1976; Hobart *et al.*, 1977).

Liver tissue cultured *in vitro* synthesizes complement components (Colten, 1972; Colten & Frank, 1972), but, as the liver consists of hepatocytes, Kupffer cells and other cell types such as endothelial cells, the cell of origin is not apparent from these studies. Furthermore, although cell lines established from rat and human liver cell cancers synthesize complement components (Rommel *et al.*, 1970; Strunk *et al.*, 1975; Morris *et al.*, 1982), these cells may not behave as normal hepatocytes.

Further studies on complement synthesis by normal populations of purified hepatocytes *in vitro* are indicated. The technique of collagenase perfusion of the liver (Berry & Friend, 1969) permits the isolation of large numbers of viable hepatocytes, which can be cultured in monolayers. We have applied this technique to the study of the synthesis of the complement components C4, C2, C3 and factor B by rat hepatocytes.

MATERIALS AND METHODS

Reagents

Insulin, cortisol, cycloheximide, PMSF and collagenase (type IV) were from Sigma, Poole, Dorset, U.K.; WME, MEM (without L-methionine), gentamycin and Lux tissue-culture dishes (60 mm and 35 mm diameter) were from Flow Laboratories, Irvine, Ayrshire, Scotland,

U.K.; FCS was from Gibco-Biocult, Paisley, Renfrewshire, Scotland, U.K.; [³⁵S]methionine was from New England Nuclear Corp., Southampton, U.K.; guinea-pig C2 and C5 were from Cordis Laboratories, Miami, FL, U.S.A.; goat anti-(human C4) antiserum was from Atlantic Antibodies, Scarborough, ME, U.S.A.

Complement components and antisera

Guinea-pig C1 was purified from serum by euglobulin precipitation (Lachmann & Hobart, 1978). Human factor D was purified by the technique of Fearon & Austen (1975). Rat serum diluted 1:15 in VBS-EDTA (40 mmol·l⁻¹) served as a source of terminal components (C3–C9). Guinea-pig serum which had been depleted of C3, C4 and C5 (R345) by treatment with KSCN (1 mol·l⁻¹) and hydrazine (15 mmol·l⁻¹) was used in the C3 assays reconstituted with respect to C5 by the addition of purified C5 (50 units/ml).

Antiserum to rat C3 was generously donated by Dr. Mohammed Daha (Department of Nephrology, University Hospital, Leiden, The Netherlands).

Antiserum to rat albumin was prepared by immunizing rabbits with the purified antigen emulsified in Freund's complete adjuvant.

Hepatocyte isolation and culture

Hepatocytes were isolated from the livers of Charles River rats by the modified (Paine & Legg, 1978) collagenase perfusion technique of Berry & Friend (1969). The cells were washed three times in WME, the cell count determined, and the suspension adjusted to 2×10^6 cells/ml in WME-FCS. Cell viability was assessed by using a Trypan Blue (0.1%)–exclusion test. Cytochrome preparations were fixed with either formol/saline and stained with haematoxylin and eosin, or with Pararosanalin for staining for non-specific esterase (Horwitz *et al.*, 1977). The cells (2×10^6 /plate or 4×10^6 /plate) were then added to a series of culture plates and, when necessary,

Abbreviations used: FCS, foetal-calf serum (heat-inactivated, 2 h at 56 °C); lysis buffer, Tris/HCl buffer, pH 7.4 (50 mmol·l⁻¹), containing KCl (100 mmol·l⁻¹), EDTA (5 mmol·l⁻¹), PMSF (5 mmol·l⁻¹), 0.5% (w/v) bovine serum albumin, 0.5% (w/v) Triton X-100 and 0.5% (w/v) deoxycholate; MEM, (Dulbecco's) minimal essential medium; PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate; VBS-EDTA, iso-osmotic veronal-buffered saline containing sodium barbitone (0.375 g·l⁻¹), diethylbarbituric acid (0.575 g·l⁻¹), NaCl (8.5 g·l⁻¹) and EDTA (40 mmol·l⁻¹); WME, William's medium E; WME-FCS, WME containing 5% (v/v) FCS, insulin (10 μ mol·l⁻¹), cortisol (100 μ mol·l⁻¹) and gentamycin (100 mg·l⁻¹); the terminology for complement components is that recommended by the World Health Organisation (1968).

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extra WME-FCS was added to ensure that the total volume of culture medium in each plate was 2 ml. After incubation overnight at 37 °C in a humidified atmosphere of CO₂/O₂ (1:19), the culture supernatants were removed, and the adherent cells washed three times with fresh WME-FCS. The last wash was used for control samples and designated 'time 0'.

Culture supernatants were sampled by two procedures. In one series of cultures the entire supernatant was removed at timed intervals and replaced with fresh WME-FCS. The amount of a component in each supernatant was added to that present in the previous sample to obtain the cumulative synthesis. In a replicate series of cultures, 100 µl aliquots of each supernatant were removed at the same intervals with the volume being restored by the addition of an equal volume of WME-FCS. The samples of supernatants were frozen at -70 °C until required.

After 24 h in culture, hepatocyte monolayers were fixed in 1% (w/v) glutaraldehyde and processed for transmission electron microscopy.

Assays for complement components and rat albumin

Haemolytic assays were used to measure the concentrations of C4 (Gaither *et al.*, 1974), C2 (Rapp & Borsos, 1970), B (Fearon *et al.*, 1973) and C3 (Cooper & Muller-Eberhard, 1970) in the culture supernatants. Rat albumin was measured by a sandwich enzyme-linked immunosorbent assay with peroxidase-conjugated rabbit anti-(rat albumin) antiserum (Engrall & Perlmann, 1972). This assay could detect 4 ng of albumin/ml with confidence.

Internal labelling of newly synthesized proteins

After 16 h in culture, a series of hepatocyte monolayers (1×10^6 cells) was washed in methionine-free MEM. The

cells were then cultured for 1 h in methionine-free MEM to which [³⁵S]methionine (250 µCi) had been added. The supernatants of all the cultures were removed and replaced with methionine-containing MEM (corresponding to time 0 of culture). At time 0, and at hourly intervals up to 6 h, a culture supernatant was removed and centrifuged to remove non-adherent cells. The monolayer was lysed in lysis buffer. The supernatants and lysates were then immunoprecipitated with anti-C4, anti-C3 and anti-albumin, and the immunoprecipitates were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography (Yeung-Laiwah *et al.*, 1985).

RESULTS

Hepatocytes in culture

The average yield of cells was 1×10^8 per liver. Over 99% of these cells were viable, as shown by the Trypan Blue-exclusion test. Morphological studies on cyto-centrifuge preparations stained with haematoxylin and eosin, or Pararosanalin for non-specific esterase, revealed that over 99.5% of the cells were hepatocytes. The occasional contaminating cells appeared to be mononuclear phagocytes, most probably Kupffer cells. The hepatocytes retained their polygonal form, and electron-microscopic studies showed that, during the first 24 h of culture, junctional complexes were re-established and canaliculi were detected (Fig. 1).

Biosynthesis of complement components and albumin

Measurable amounts of C4, C2, C3, B and albumin were demonstrable in the culture supernatants. The patterns of cumulative synthesis of all four complement components and albumin were similar (Figs. 2 and 3). However, in those cultures from which only small

Fig. 1. Electron micrograph of rat hepatocyte monolayer 24 h after plating (magnification $\times 2925$)

Fig. 2 Cumulative (-----) and continuous (—) synthesis of C2 by isolated rat hepatocytes

Rat hepatocytes were cultured as described in the text in WME-FCS at 2×10^6 or 4×10^6 cells per culture plate (60 mm diameter). For the cumulative synthesis, the entire supernatant was replaced with fresh WME-FCS, whereas for the continuous synthesis only 100 μ l portions of the supernatant were removed and replaced at 3, 6, 9 and 24 h of culture. C2 was measured by standard haemolytic assay. Each point represents the mean value $\pm$ S.E.M. for triplicate cultures. The numbers of hepatocytes per plate are shown against the curves.

portions of medium were removed, the amount of all five proteins synthesized was considerably less after 24 h of culture. This difference was least marked with albumin and most obvious with C3 and C4. Concentrations of C3 and C4 increased between 0 and 9 h of culture, after which time the haemolytic activity of these components in the medium decreased. This pattern differed from that of C2 and B; concentrations of these components in culture supernatants increased between 9 and 24 h of continuous culture.

Within 9 h, the hepatocytes synthesized $7.5 \mu\text{g}$ of albumin/ 10^6 cells and, in cumulative experiments, $12.5 \mu\text{g}$ of albumin/ 10^6 cells. Assuming 1 g of liver wet weight contains 1.7×10^8 hepatocytes (Weibel *et al.*, 1969), the cultures synthesized $142 \mu\text{g}$ of albumin/h per g of liver in the continuous system and $236 \mu\text{g}$ of albumin/h per g in the cumulative method of sampling. In comparison, $470 \mu\text{g}$ was reported for the fasted-rat liver *in vivo* (Peters & Peters, 1972).

Cycloheximide inhibition studies

Adding cycloheximide (5 $\mu\text{g}/\text{ml}$) to the culture medium decreased the synthesis of C4 by 70%, C2 by 90%, C3 by 100%, factor B by 67% and albumin by 73%. Within 6 h of replacement of cycloheximide-containing medium with fresh WME-FCS, synthesis of complement components and albumin was restored.

Internal labelling of C3, C4 and albumin

Fluorographic analysis of C3 immunoprecipitates from the culture supernatants revealed two dense bands corresponding to the α (120 kDa)- and β (69 kDa)-chains of C3 (Fig. 4a). A band of 192 kDa was also visible and probably corresponded to pro-C3 (Brade *et al.*, 1977; Morris *et al.*, 1982). Another band, of 43 kDa, which increased in density throughout the chase period, is probably a cleavage product (C3dg) of the C3 α -chain. Another band, designated '170 kDa', was seen in the

Fig. 3. Cumulative (-----) and continuous (—) synthesis of C3, C4, factor B and albumin by isolated rat hepatocytes

Rat hepatocytes were cultured as described in the text in WME-FCS at 2×10^6 cells per culture plate (60 mm diameter). C3, C4 and factor B were measured by standard haemolytic assay and albumin by a sandwich enzyme-linked immunosorbent assay. Each point represents the mean value $\pm$ S.E.M. for triplicate cultures.

Fig. 4. SDS/7.5%-polyacrylamide-gel electrophoresis of extracellular (a) and intracellular (b) immunoprecipitates of [35 S]methionine-pulsed rat hepatocyte cultures (1×10^6 cells)

At time 0, and up to six 1-hourly intervals after pulsing, culture supernatants (extracellular) and cell lysates (intracellular) were immunoprecipitated with anti-C3. A and B represent M_r standards and control immunoprecipitates produced by rabbit anti-ovalbumin.

Fig. 5. SDS/7.5%-polyacrylamide-gel electrophoresis of extracellular (a) and intracellular (b) immunoprecipitates of [35 S]methionine-pulsed rat hepatocyte cultures (1×10^6 cells)

At time 0, and up to six 1-hourly intervals after pulsing, culture supernatants (extracellular) and cell lysates (intracellular) were immunoprecipitated with anti-C4. A and B represent M_r standards and control immunoprecipitates produced by rabbit anti-ovalbumin.

anti-C3 but not in the control immunoprecipitates. Anti-C3 immunoprecipitates of the lysates revealed three prominent bands. These corresponded to pro-C3 (190 kDa) and the α (120 kDa)- and β (70 kDa)-chains of C3. Other bands precipitated by anti-C3, but not by non-immune rabbit IgG, were also seen (designated a, b, c and d in Fig. 4b).

Analysis of the C4 immunoprecipitates from the supernatants revealed major bands of 93 kDa, 73 kDa and 31 kDa, which correspond to the α -, β - and γ -chains of C4 respectively (Fig. 5) (Chan & Atkinson, 1984). Other minor bands of 86 kDa and 79 kDa were precipitated specifically by anti-C4. These bands decreased in intensity with increasing length of chase period. In the lysates a single major band of 185 kDa corresponding to pro-C4 was seen (Fig. 5). Pro-C4 was present in the lysates up to 6 h of the chase period. Two additional minor bands (90 kDa and 78 kDa) were precipitated in the lysates only at zero time.

The molecular mass of intracellular albumin was

70 kDa, whereas that of the extracellular molecule was 67 kDa (results not shown).

Stability of components

Incubation (37 °C for 24 h) of C4, C2, C3 or B with hepatocyte culture supernatants resulted in a loss of

haemolytic activity. The proportion of activity lost was 69% for C2, 57% for C3, 74% for C4 and 39% for B (results not shown).

DISCUSSION

The liver is a major site of protein synthesis. In the rat, 19% of total body protein synthesis occurs in this organ (Kirsch, 1982). In the human, the liver synthesizes about 48 g of protein daily, half of which is for export. Albumin makes up 50% of the protein that is secreted from human liver (Kirsch, 1982). As the cell content of the liver includes hepatocytes, Kupffer cells and endothelial cells, the relative contribution of these cells to the hepatic synthesis of complement components can only be assessed by studying isolated cell populations. In experiments reported in the present paper, the isolation techniques produced a cell population in which the proportion of hepatocytes was more than 99%. By using primary cultures of isolated rat hepatocytes we have investigated the synthesis of complement components. The synthesis of C4, C2, C3, B and albumin by these cells is proved by the continued accumulation of these proteins in the culture medium over 24 h of culture, by the ability of cycloheximide to inhibit synthesis reversibly, and by the incorporation of [³⁵S]methionine into newly synthesized C4, C3 and albumin. We have not been able to study the incorporation of radiolabelled amino acids into B or C2 because of lack of suitable antisera.

The pulse-label studies of C4 and C3 indicate that both of these molecules are synthesized as single-polypeptide-chain precursors. Both proteins are rapidly secreted, as shown by the disappearance of the major part of the immunoprecipitable radioactivity after a chase period of 1 h. It appeared that conversion of pro-C3 into C3 occurred intracellularly, as demonstrated by the appearance of the α - and β -chains in the lysates. However, a proportion of the precursor molecules escaped intracellular processing. The anti-C3 immunoprecipitates of the cell lysates revealed bands other than pro-C3 and α - and β -chains, and these possibly represent intracellular degradation products of C3. Results of the immunoprecipitation experiment, using an antiserum to human C4, were similar to those previously reported by Chan & Atkinson (1984), who used a human hepatoma-derived cell line (Hep G2). We were able to demonstrate α -, β - and γ -chains of C4 in supernatants of rat hepatocyte cultures and pro-C4 in cell lysates. The absence of pro-C4 from the supernatants and of native C4 from the lysates suggests all the precursor molecule is converted into C4 as it is secreted from the hepatocyte. The anti-C4 immunoprecipitation of the supernatants revealed other major bands, which appeared to decrease in intensity during the chase period. The failure of these bands to increase in parallel with the C4 subunits suggest they may not simply be degradation products.

The results of the cumulative-synthesis studies revealed that, when the medium was changed at regular intervals, the quantities of all four components which were detected were greater than those which were measured in small samples. This was most marked in the case of C4. Possible explanations include (i) increased degradation of complement components by either complement activation in the culture medium or non-specific proteolysis, or (ii) decreased synthesis of components. As our experiments have shown that the haemolytic activities of C4, C2, C3

and B are decreased when these components are incubated in hepatocyte culture supernatants, it is probable that some of this difference is due to degradation. Degradation of complement components was observed in the supernatants of Hep G2 cell cultures (Morris *et al.*, 1982). In particular, a decrease in the levels of C4 and C2 may be due to cleavage by newly synthesized C1s.

In summary, our studies show that the culture of rat hepatocytes *in vitro* provides a useful model for the investigation of synthesis of complement components. An assessment of the contribution by each cell type to hepatic synthesis of complement components may be possible by using purified cell populations isolated from rat liver. The technique may also provide the basis for studying the mechanism(s) which regulate the synthesis of complement components.

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