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Isolation of C4-synthesizing Cells from Guinea-Pig Liver by Ficoll Density Gradient Centrifugation

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Summary. Cells which synthesize the fourth component of complement have been isolated from guinea-pig liver through rate zonal density gradient centrifugation of suspensions of liver cells on continuous density gradients (2 to 15 per cent) of Ficoll. This procedure separates non-parenchymal cells from parenchymal cells on the basis of size. Cells which synthesized C4 were identified as low density, non-parenchymal cells which comprised only a small proportion of the cells added to the gradient. Phagocytic cells were restricted to the same portion of the gradient as cells which synthesized C4, and there is evidence that cells which synthesize C4 are also phagocytic.

INTRODUCTION

As a result of many studies on complement synthesis during the past decade (see review by Burkholder and Littleton, 1971; also Rommel, Goldlust, Bancroft, Mayer and Tashjian, 1970; Geiger, Day and Good, 1972; Wyatt, Colten and Borsos, 1972), it has become apparent that synthesis of most complement components is associated with a variety of tissues, and in particular with both macrophages and the liver. Because of its size, the liver probably makes the largest contribution to circulating levels of many complement components, but the identity of the cell type which synthesizes components of complement in the liver and other tissues has remained elusive.

The purpose of the present investigation was to more precisely characterize the cell or cells in the liver which synthesize C4. On the basis of results obtained following haemolytic analysis of tissue culture supernatants, Jensen (1969) concluded that the liver parenchymal cells was probably the site of C4 synthesis. However, in studies conducted in this laboratory (Littleton, Kessler and Burkholder, 1970), when liver cell suspensions were assayed by the modified Jerne haemolytic plaque technique for C4 synthesis, C4-synthesizing cells observed in the centres of haemolytic plaques were found to be similar in morphology to C4-synthesizing cells from numerous other tissues and were therefore thought to be liver macrophages or Kupffer cells.

In the present investigation, suspensions of dissociated cells prepared from guinea-pig liver were subjected to rate zonal centrifugation on Ficoll density gradients prior to assay of the cells by the plaque technique previously described for the detection of C4 synthesis. Density gradient centrifugation permitted fractionation of the heterogeneous starting

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suspension of liver cells into distinct cell populations which were then characterized morphologically and evaluated for their capacity to synthesize C4.

MATERIALS AND METHODS

Animals

Male albino guinea-pigs weighing 500–800 grams (Dublin Laboratories, Dublin, Virginia and Marvin O'Brien, Oregon, Wisconsin) were used in this investigation.

Preparation of liver cell suspensions

Suspensions of liver cells were prepared in Garvey's reticuloendothelial medium (Garvey, 1961) (REM) (Grand Island Biological Company, Grand Island, New York), essentially the same as described previously for spleen cells (Ilgen, Bossen, Rowlands and Burkholder, 1973).

Ficoll density gradients, centrifugation and fractionation

Linear density gradients of 2–15 per cent Ficoll (Pharmacia, Piscataway, New Jersey) were prepared by a modification of the method described by Pretlow and Boone (1969). A stock solution of 20 per cent Ficoll in REM was appropriately diluted with REM to prepare linear gradients with a concentration range of 2–15 per cent. The gradients, 16-ml quantities in 16 × 114 thin wall polyallomer tubes (Scientific Products, Chicago, Illinois) were generated on a gradient marker similar to the one described by Pretlow and Boone. Ninety million liver cells were added to the top of the gradient which was then centrifuged at 100 *g* for 10 minutes at 0° in either a Sorvall RC-2B centrifuge (Ivan Sorvall, Norwalk, Connecticutt) using an HB-4 rotor, or in an International PR6 centrifuge (International Equipment Company, Needham Heights, Massachusetts) using a 269 head. Four 2·5-ml fractions were collected from the top of the gradient using a Pasteur pipette and the fractions were numbered in order from top to bottom as fractions 1–4. Each of the fractions was washed twice in a wash solution of Ringer's lactate solution and REM (3:1) without heparin or serum and resuspended in the same. Cell counts, viability determinations (Trypan Blue exclusion), and morphological evaluations were made on each of the fractions, which were then assayed for C4 production.

Complement reagents, buffers, and haemolytic intermediates

Functionally pure guinea-pig C1 and C2 (Cordis Corporation, Miami, Florida) were used throughout the investigation. Fresh frozen rat serum (rat C) was obtained locally (GIBCO Microbio Labs, Madison, Wisconsin) and stored at −70° until used. Veronal-buffered saline, (Mayer, 1961), containing 0·1 per cent gelatin and 5 per cent glucose (GVB-G1) was used as a diluent for C1 and C2 and was employed in the preparation of haemolytic intermediates (EA, EAC1). Veronal-buffered saline containing 0·1 per cent gelatin (GVB) was used to dilute a stock solution of 0·2 M ethylenediaminetetraacetate (EDTA), pH 7·4, to a concentration of 0·08 M EDTA. The resulting GVB-EDTA buffer was used as a diluent for rat serum. Preparation of EA and EAC1 have been described (Ilgen *et al.*, 1973).

Haemolytic plaque technique

The haemolytic plaque technique described by Ilgen *et al.* (1973) was used to assay each fraction of liver cells for synthesis of C4. The agar plates containing EAC1 and liver cells

were incubated for 30 minutes at 37°; then C2(250 CH_{50} units/ml) was pipetted over the top surface of the agar. Fifteen minutes later, the liquid was aspirated and C2 added again. After an additional incubation period of 15 minutes, the C2 was aspirated and the surface of the plate flooded with rat C-EDTA (final concentration 0·04 M EDTA). The plates were incubated at 37° and haemolytic plaques counted after 20–30 minutes when plaque formation was completed.

RESULTS

Rate zonal centrifugation of liver cell suspensions on 2–15 per cent Ficoll gradients has permitted physical separation of different cell populations from the liver. C4 plaque-forming activity and high cell viability were maintained, and single cells were separated from cellular aggregates. Moreover, cell populations isolated from different regions of the gradient differed from each other morphologically and in their capacities to synthesize C4.

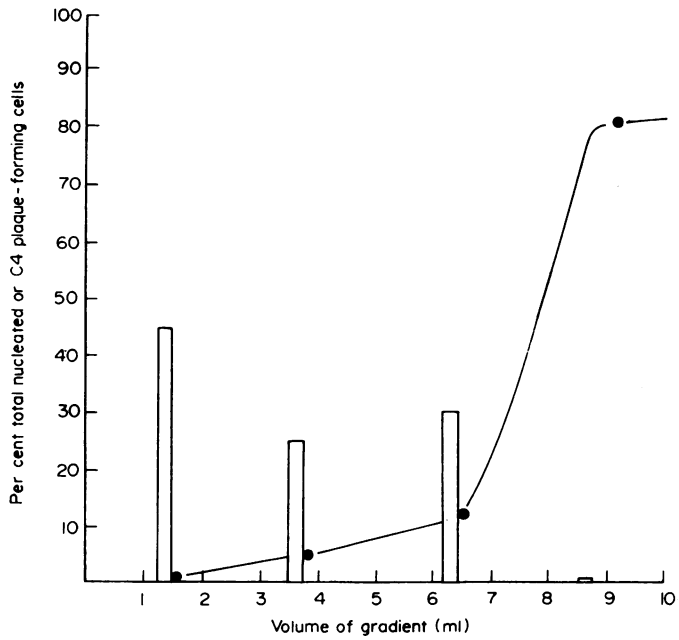

FIG. 1. Representative distribution of (●) cells and (□) C4-synthesizing activity following rate zonal density gradient centrifugation of guinea-pig liver cells on a 2–15 per cent Ficoll density gradient.

In this investigation, liver cell suspensions from a total of eleven animals were subjected to rate zonal centrifugation. In each case, nearly 100 per cent of the cells were recovered in the four fractions collected from the gradient. Of those cells recovered, 80–90 per cent were located in the bottom 2·5 ml of the gradient (fraction 4). Fraction 1 (the top 2·5 ml) contained about 1 per cent of the cells or less; fraction 2, 4 to 8 per cent; and fraction 3, 6 to 11 per cent. However, when cells from each gradient fraction were assayed by the haemolytic plaque technique for C4 synthesis, all of the C4-synthesizing activity was restricted to the top 3 fractions. A representative distribution on the gradient of cells from

one animal, and the location of C4-synthesizing activity on that gradient are illustrated in Fig. 1. Only the cells recovered in fractions 1–3 (11–20 per cent of the cells) contained any C4-synthesizing activity.

Table 1 summarizes the results obtained following fractionation and assay for C4 synthesis of cells from the livers of six representative animals. In each case, C4-plaque-forming activity was low or absent in the starting suspension of liver cells but was recovered from fractions 1 through 3. No plaque-forming activity was located in fraction 4. The number of C4-synthesizing cells detected in fractions 1–3 was variable, ranging from 100 to 2000 plaque-forming cells per million viable cells plated, or one plaque-forming cell per 500 to 10,000 cells.

TABLE 1

REPRESENTATIVE DISTRIBUTION OF C4-SYNTHESIZING (PLAQUE-FORMING) CELLS FOLLOWING RATE ZONAL DENSITY GRADIENT CENTRIFUGATION OF GUINEA-PIG LIVER CELLS ON 2–15 PER CENT FICOLL DENSITY GRADIENTS

Cell suspension	Animal number					
	98	99	100	101	108	117*
Fraction 1	640	1000	0	3200	0	57
Fraction 2	400	1125	4500	1970	570	137
Fraction 3	220	1666	3600	1070	0	0
Fraction 4	0	0	0	0	0	0
Pre-gradient suspension	NT	0	0	0	NT	0

* Animal received 32 mg colloidal carbon intravenously 15 minutes prior to killing.
NT = not tested.

Examinations of Wright-stained smears from each of the fractions revealed that fraction 1 was composed of very few cells; those present were mononuclear non-parenchymal cells. Fraction 2 contained predominately mononuclear cells; and fraction 3, erythrocytes, mononuclear cells and occasional polymorphonuclear leukocytes. Fraction 4 was composed of erythrocytes and parenchymal cells. When each of the fractions from the gradient was incubated in a 2 ml volume at 37° for 20 minutes with either 5×10^7 EA or 1 mg/ml of colloidal carbon, then washed three times in RL-REM wash solution and examined as Wright-stained smears, cells phagocytic for EA or carbon were identified only in the top 3 fractions.

C4-synthesizing cells which were observed in the centres of single-cell plaques in the agar were elongated or irregularly-shaped mononuclear cells ranging in length from three to six times the diameter of surrounding red cells. They rarely resembled parenchymal cells. The phagocytic capacity of plaque-forming cells was evaluated by administering colloidal carbon (32 mg) intravenously to an animal (animal No. 117) 15 minutes prior to killing. The liver was then processed as usual and plaques were produced by cells in the top two fractions. Aggregates of phagocytized carbon were occasionally visible in the cytoplasm of these cells. A few of these cells were so large that they might easily have been identified as liver parenchymal cells; however, histological examination of sections from the same liver (Fig. 2) failed to reveal any phagocytosis by parenchymal cells although active phagocytosis by Kupffer cells was apparent. The variable morphology of phago-

cytic plaque-forming cells is illustrated in Fig. 3 in which an aggregate of cells in the centre of a plaque produced by cells from a carbon-treated animal is illustrated. Cellular aggregation was a problem with cells recovered from the livers of animals which received carbon intravenously prior to killing.

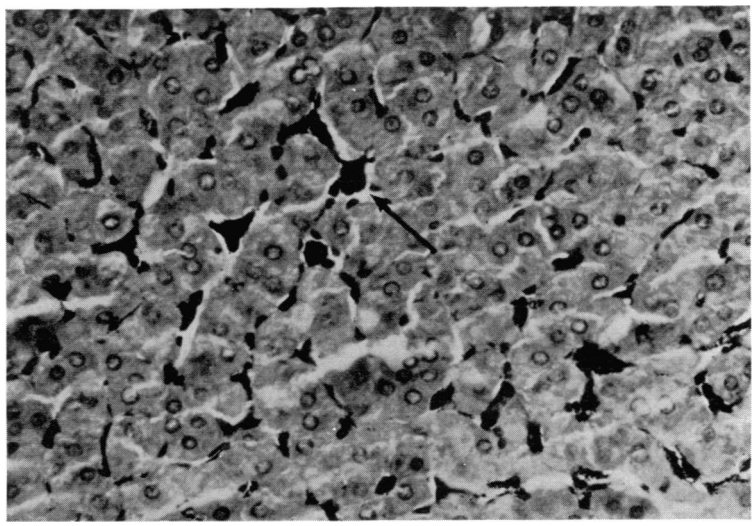

FIG. 2. Histological section taken from liver of animal which received 32 mg of colloidal carbon i.v. 15 minutes prior to killing. Phagocytosed carbon is localized in cells lining liver sinusoids (arrow); no phagocytosed carbon is apparent in liver parenchymal cells. (Magnification ×245.)

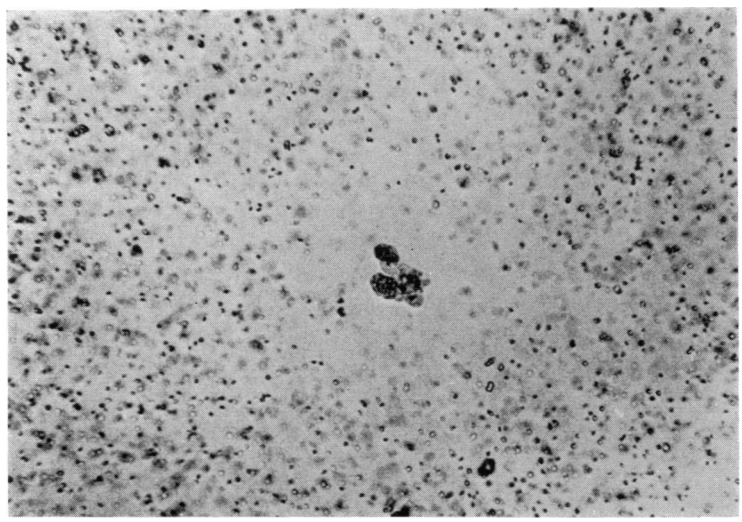

FIG. 3. Haemolytic plaque with central aggregate of phagocytic liver cells; at least one of these cells has synthesized C4. Animal received 32 mg of colloidal carbon i.v. 15 minutes prior to falling. (Magnification ×154.)

DISCUSSION

Cells which synthesize C4 have been isolated from guinea-pig liver by rate zonal centrifugation of suspensions of liver cells on 2–15 per cent continuous Ficoll density gradients. This procedure separates non-parenchymal cells from parenchymal cells on the basis of size, while high cell viability and functional activity are maintained. C4-synthesizing cells which were isolated in this manner were low density, non-parenchymal cells recovered from the top three fractions (fractions 1–3) of the gradient. By either *in vitro* or *in vivo* assays of phagocytic activity, phagocytic cells were likewise identified only in the top three fractions of the gradient. No C4-synthesizing or phagocytic activity was associated with the bottom fraction of the gradient (fraction 4), which contained 80–90 per cent of the total cells and a predominance of parenchymal cells.

The reason for the high degree of variation in the number of plaque-forming cells detected in the livers of different animals is not apparent. Similar variation among animals in the number of detectable C4-synthesizing cells in a given tissue has been noted previously (Littleton, Kessler and Burkholder, 1970), when an assay less well standardized than the present assay was used. Previously, variation in the sensitivity of the assay due to the R4 reagent could have been the basis for variation in the number of C4-synthesizing cells detected; but in the present investigation, R4 was replaced by standard amounts of purified C2 and rat C-EDTA.

It is somewhat surprising that, in the present investigation, synthesis of C4 was not detected among cells in the starting suspension. The starting suspension in this case corresponds to the liver cell suspension used in a previous investigation in which C4-synthesizing, plaque-forming cells were frequently detected. This unexpected finding cannot be attributed to use of a less sensitive assay in the present study; in fact, replacement of R4 in the terminal step in the assay with purified C2 and rat C-EDTA increased the sensitivity of the assay for C4-synthesizing cells. Similarly, the possibility of an inhibitor of C4 or of C4 synthesis produced by cells present in the starting suspension but separated from C4-synthesizing cells on the gradient, is highly unlikely, because such an inhibitor has never been described in the abundant literature on C4 and C4 synthesis.

Characterization of the cell in the liver which synthesizes C4, as a low-density, non-parenchymal cell lends further insight into the question of the C4-synthesizing cell in the liver. Since phagocytic activity was occasionally evident in cells observed in the centres of plaques, the results obtained in this investigation further support the concept that C4 synthesis in the liver occurs primarily if not exclusively in a reticuloendothelial cell, the Kupffer cell. These results are in accordance with a previous study conducted in this laboratory on C4 synthesis and do not support the concept that C4 synthesis in the liver occurs primarily in the liver parenchymal cell.

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