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Isolation and Characterization of C4-Synthesizing Cells from Guinea-Pig Spleen

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Summary. Separation of C4-synthesizing cells from the spleen has been achieved through isopycnic density gradient centrifugation of spleen cells on albumin gradients. Cells which synthesize C4 are low-density, large mononuclear cells, probably macrophages. C4-synthesizing cells clearly differ in density from cells which synthesize antibody to sheep erythrocytes 7 days following immunization with erythrocytes. These cells are much denser than cells which synthesize C4. Spleen cells which transfer delayed hypersensitivity to tuberculin are separated in the same region of the gradient as cells which synthesize C4, but it is considered unlikely that the two functions are associated with the same cell type.

INTRODUCTION

A large proportion of spleen cells are so similar in morphology that it is often difficult to precisely identify the cells involved in particular immunological reactions. Recently, however, the use of density gradient centrifugation has permitted isolation of functionally distinct cells from lymphoid organs despite their morphological similarity, and has provided another descriptive characteristic for these cells (Szenberg & Shortman, 1966; Shortman, 1968; Saunders, 1970; Bossen and Rowlands, 1972). On this basis, we have used isopycnic density gradient centrifugation of guinea-pig spleen cells in order to isolate the cells which synthesize the fourth component of complement (C4). C4-synthesizing cells were then compared on the basis of density with cells which synthesized antibody to sheep erythrocytes or which transferred delayed hypersensitivity to tuberculin.

MATERIALS AND METHODS

The haemolytic assay used in this study was essentially the same as described previously (Littleton, Kessler and Burkholder, 1970). Basically, EAC1 (sensitized sheep erythrocytes reacted with the first component of complement) were mixed in agar media with each of the cell fractions recovered from an albumin density gradient following isopycnic density gradient centrifugation of a suspension of spleen cells. Each suspension in agar of EAC1 and spleen cells was incubated and the synthesis of C4 by spleen cells was assayed by counting haemolytic plaques formed after the stepwise addition of purified guinea-pig C2 and EDTA-treated rat serum (Rat C-EDTA).

Animals

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

Spleen cell preparations

Suspensions of spleen cells were prepared in Shortman's balanced salt solution essentially the same as outlined earlier (Shortman, 1968; Bossen and Rowlands, 1972), except that foetal calf serum was replaced by heat-inactivated (56°, 30 minutes) autologous serum *. Using a polypropylene stirrer, cells were gently teased from the surface of the spleen into 25–50 ml of Shortman's buffered balanced salt solution containing serum and 0·2 units of heparin per ml. This cell suspension was held in an ice bath for 10 minutes in a 50-ml centrifuge tube in order to allow large particulate material to settle. The supernatant suspension was aspirated, and cells which passed through a 230 mesh wire screen were washed in buffered balanced salt solution, adjusted to a standard volume, and counted.

Complement reagents and buffers

Functionally pure guinea-pig C1 and C2 (Cordis Corporation, Miami, Florida) were used throughout this study. Fresh-frozen rat serum was obtained locally (GIBCO Microbiology Laboratories, Madison, Wisconsin) and stored at −70° until used. Veronal buffered saline (Mayer, 1961) with 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. Veronal-buffered saline containing 0·1 per cent gelatin (GVB) was used to dilute a stock solution of 0·2 M ethylenediaminetetraacetic acid (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.

Haemolytic intermediates

Optimally sensitized sheep erythrocytes (EA) were prepared from commercial anti-sheep haemolysin (Baltimore Biological Laboratory, Baltimore, Maryland) and fresh sheep erythrocytes (Robbin Laboratories, Chapel Hill, North Carolina and GIBCO Microbiology Laboratories, Madison, Wisconsin) (Mayer, 1961). EAC1 were prepared fresh daily by incubating equal volumes of EA (5×10^8 cells per ml) and purified guinea-pig C1 (500 units per ml) at 4° for 10 minutes. The cells were then washed three times by centrifugation at 4° and resuspended in cold GVB–G1. EAC1 were held at 4° and used within a few hours of preparation.

Albumin density gradients, centrifugation and fractionation

Linear density gradients of 15–30 per cent bovine serum albumin (BSA) were prepared in 10-ml thin wall polypropylene tubes as described previously (Bossen and Rowlands, 1972), and $3–5 \times 10^8$ washed spleen cells suspended in 0·5 ml of 10 per cent BSA were layered onto the gradient. The gradients were spun at 3800 ***g*** at 4° for 45 minutes in a Sorvall RC2B centrifuge (Sorvall, New Haven, Connecticut) using an HB4 rotor, or in an International B20 centrifuge (International Equipment Company, Needham Hts., Massachusetts) using a 947 head. Initially, twenty-four fractions of seven drops each were collected from the top of the tube using an LKB fractionator. In later experiments, four

* Control experiments demonstrated that the inclusion of serum at this step was not responsible for subsequent plaque formation.

fractions of forty-two drops each were collected. The fractions were numbered from the top of the gradient. To determine the density of each fraction, a 100 lambda sample was weighed using a Sartorius model 2400 balance (Sartorius-Werke, W. Germany) and the density was calculated from this value. The cells in each fraction were washed three times in a chilled wash solution consisting of three parts Ringer's Lactate Solution (RL) (Baxter) and one part Eagle's Minimal Essential Medium (MEM) (Grand Island Biological Company, Grand Island, New York). They were then counted, cell viability was determined by exclusion of Trypan Blue dye, and Wright-stained smears were made. Finally, 0·3 ml aliquots from each fraction were assayed for synthesis of C4 using the modified Jerne haemolytic plaque technique.

Haemolytic plaque technique

The haemolytic plaque technique described in an earlier communication (Littleton, Kessler and Burkholder, 1970) was used to assay cells from each fraction of the gradient for synthesis of C4. The assay was slightly modified in that purified guinea-pig C2 and Rat C-EDTA were used to complete the complement sequence subsequent to fixation of C4. After the plates containing EAC1 and spleen cells were incubated for 30 minutes at 37° in a 5 per cent CO_2–95 per cent air atmosphere, C2 (250 CH_{50} units per 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 (0·4 M EDTA) (Wyatt, Rapp and Borsos, 1971). The plates were incubated at 37° and haemolytic plaques counted in 20–30 minutes when plaque formation was completed.

RESULTS

When spleen cells were subjected to isopycnic density gradient centrifugation on preformed 15–30 per cent albumin density gradients, fractions were obtained which were enriched in C4-producing activity. A total of nine spleen cell suspensions have been fractionated using this technique, and the results from four representative gradient runs are presented in Table 1. Fig. 1 illustrates the typical patterns of distribution of nucleated cells and C4-synthesizing activity on the gradients.

In each of the spleen suspensions subjected to density gradient centrifugation, C4 synthesis measured by haemolytic plaque formation was low or absent in the starting suspension but was recovered from the top six fractions (2·5 ml) of the gradient. The mean density value for this portion of the gradient was 1·053, a visible band of cells being recovered at a density of 1·058. In the remaining fractions from the gradient (the bottom 7·5 ml), there was no C4-synthesizing activity. When the cells in the top 2·5 ml were assayed by the haemolytic plaque technique for C4 synthesis, the number of detectable C4-synthesizing cells varied among different spleens and ranged from eighty to four thousand active cells per million viable cells, or one C4-synthesizing cell per 250 to 10,000 cells. The top portion of the gradient, which contained only 1·3–5 per cent of the total cells recovered, was thus several hundred to several thousand times enriched in cells which synthesized C4.

As was reported earlier (Bossen and Rowlands, 1972), this separation procedure was also carried out on spleen cells from guinea-pigs immunized seven days previously with a single dose of sheep erythrocytes. In this case, single cells which synthesized anti-sheep

Table 1

Representative distribution of plaque-forming activity (due to C4 synthesis) following separation of spleen cells by albumin density gradient centrifugation

Fraction	Plaque-forming cells per 10^6 viable cells			
	Animal number 35	Animal number 101	Animal number 112	Animal number 116
1–2	55*			
3–4	80			
5–6	60	105†	72	640
7–8	0			
9–10	0			
11–12	0	0	0	0
13–18	0	0	0	0
19–24	0	0	0	0
pre-gradient suspension	0	0	NT‡	0

* In order to provide at least 10^5 cells for assay, every two fractions pooled prior to assay.

† With fractions from spleens of animals 101, 112 and 116, every six fractions were pooled and concentrated X6 prior to assay.

‡ Not tested.

erythrocyte antibody were identified using the direct Jerne haemolytic plaque technique (Jerne and Nordin, 1963). As is illustrated in Fig. 2, the distribution on the gradient of antibody-producing cells was entirely different from that of cells which synthesized C4. Most of the antibody-synthesizing cells were contained in fractions 7–17, or in an area falling between 2·5 and 7 ml from the top of the gradient. This area corresponds to a density of 1·060 to 1·083; although most of the antibody-synthesizing activity was located in a region with a density close to 1·063.

Comparison of Fig. 2 with Fig. 1 reveals that antibody synthesis, at least at the point sampled in this study, is associated with a population of cells distinctly different from those

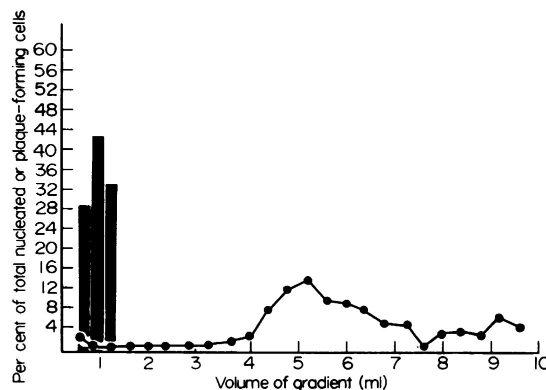

Fig. 1. Representative distribution of (●) nucleated and (■) C4-producing cells on a 15–30 per cent continuous albumin density gradient. The lower numbers correspond to the less dense portions of the gradient.

cells which synthesize C4. In contrast, when fractions containing cells from spleens of tuberculin-sensitive animals were assayed for transfer of delayed hypersensitivity, all of the activity was contained in the top 2·5 ml of the gradient (Bossen and Rowlands, 1972), the same region where C4-synthesizing cells were located.

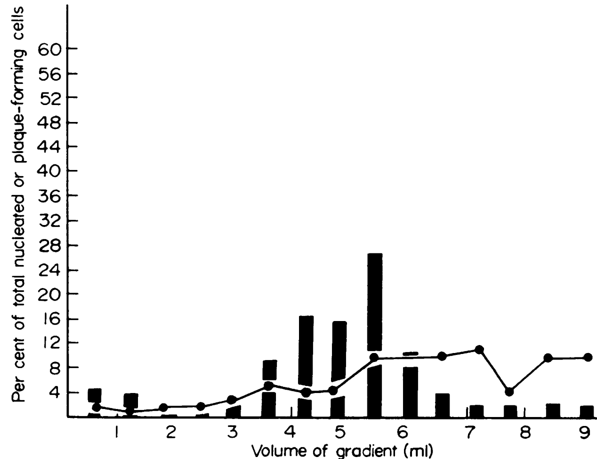

FIG. 2. Representative distribution of (●) nucleated and (■) antibody-producing cells on a 15–30 per cent continuous albumin density gradient. The lower numbers correspond to the less dense portions of the gradient.

Examination of Wright-stained smears revealed that each of the fractions recovered from the gradient was heterogeneous in cell type although fractions in different parts of the gradient were enriched in particular cell types. Fifty to sixty per cent of the cells in the top 2·5 ml of the gradient were small lymphocytes and the remaining 40–50 per cent of the cells were large lymphocytes, blast cells, stem cells and phagocytic cells. The fractions

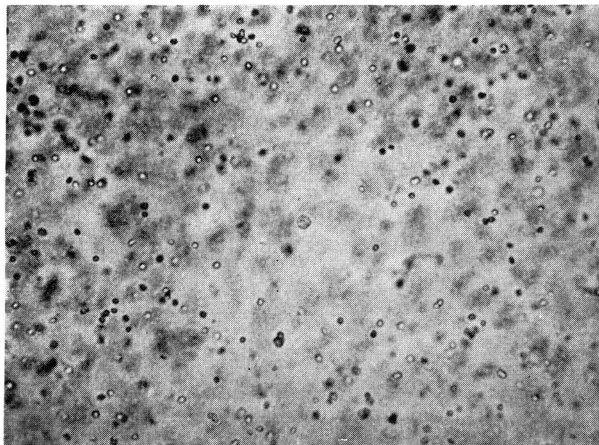

FIG. 3. Haemolytic plaque with central C4-producing cell (cell diameter = 15 μm) from pool of fractions 5 and 6 recovered from spleen cell separation on albumin density gradient. (Magnification × 125.)

containing antibody-producing cells included a predominance of small lymphocytes (80 per cent) and 15 per cent large lymphocytes or blast cells.

Individual C4-synthesizing cells observed in the centres of plaques were similar in morphology to C4-synthesizing cells described in a previous communication (Littleton *et al.*, 1970; Littleton, 1970). They were large mononuclear cells 15–30 microns in diameter. If colloidal carbon (32 mg) or EA were presented to the cells prior to assay, phagocytized material was occasionally visualized in the cytoplasm of the C4-synthesizing cells. A representative C4-synthesizing cell isolated from the gradient is illustrated in Fig. 3.

DISCUSSION

Cells which synthesize C4 have been isolated from the spleen through isopycnic density gradient centrifugation of spleen cells on albumin gradients. Spleen cells which synthesize C4 were low density cells comprising a very small proportion of the total cells in the suspension. They were associated with a region of the gradient enriched in large mononuclear cells and containing a high level of phagocytic activity. When assayed by the haemolytic plaque technique for C4 synthesis, C4-synthesizing cells in the centres of plaques appeared as large mononuclear cells 15–30 microns in diameter, with abundant cytoplasm. Under the appropriate experimental conditions, phagocytized material was occasionally visualized in the cytoplasm of these cells.

Cells which synthesized C4 were separated on the gradient from antibody-synthesizing cells. Cells which synthesized antibody to sheep erythrocytes were higher in density than C4-synthesizing cells, and they appeared nearer the middle of the gradient. They were associated with a portion of the gradient enriched in small lymphocytes. Cells which transferred delayed hypersensitivity to tuberculin, however, were separated from the remainder of the spleen cells in the same region of the gradient as were C4-synthesizing cells. Thus, it is possible that cells which synthesize C4 and cells which transfer delayed hypersensitivity to tuberculin are identical. However, the bulk of information on cells which react in delayed hypersensitivity argues against this point, and we have concluded that the cell in the spleen which synthesizes C4 is a reticuloendothelial cell (macrophage), whereas the cell which transfers delayed hypersensitivity is a low density lymphoid cell.

A major advantage of this separation procedure is that it is highly reproducible, for C4-producing activity has always been recovered from the same region of the gradient. Similarly, in other investigations using this same method, spleen cells functioning in antibody synthesis *in vitro* (Bossen and Rowlands 1972), the primary immune response *in vivo* and *in vitro* (Haskill, Bryt and Marbrook, 1970), graft-versus-host reactions (Szenberg and Shortman, 1966) and passive transfer of delayed hypersensitivity (Bossen and Rowlands, 1972) have been consistently localized to defined regions of the gradient.

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