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Localization of Functionally Different Guinea-Pig Spleen Cells in Albumin Gradients

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Summary. Buoyant density centrifugation of linear albumin gradients was used to fractionate suspensions of guinea-pig spleen cells. The fractions obtained were studied with respect to morphology and function of their cells. Using this technique it was shown that cells responsible for the production of sheep cell haemolysin were located in fractions distinct from those which could be used for the passive transfer of delayed tuberculoprotein hypersensitivity.

INTRODUCTION

Several studies have demonstrated that morphologically similar lymphoid cells can perform separate immunological functions (Gowans and McGregor, 1965). The methods of separation which have been used are varied and range from electrophoretic mobilities (Sundaram, Phondke and Sundaresan, 1967) to separation by glass beads (Shortman, 1968). Recently, density gradient centrifugation has been used to separate lymphoid cells and the functions of these cells have been tested by various assay methods (Szenberg and Shortman, 1966).

The cells responsible for cellular and humoral immunity are thought to be derived from or influenced by different tissues, i.e. the thymic and bursa equivalent organs. It seemed possible, therefore, that the cells capable of passively transferring cellular immunity might differ in some physical parameters from the cell population responsible for anti-SRBC activity.

We have used the technique of albumin density gradient centrifugation in an effort to determine whether guinea-pig spleen cells with differing immunological functions can be separated on the basis of differences in density.

METHODS

Animals

Adult male guinea-pigs weighing 600–800 g were used throughout the study.

Immunizations

Sheep red blood cells (SRBC) preserved in Alsever's solution were obtained from

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Robbins Laboratories, Chapel Hill, North Carolina. Single intravenous doses of 5×10^8 SRBC were used for immunization. Initially, a study was conducted to determine the time of maximum anti-SRBC activity in the guinea-pig. On days 1, 3, 5, 7, 9 and 11 following SRBC injection, three experimental and a control animal were killed and the anti-SRBC activity of nonfractionated spleen cells evaluated by the Jerne plaque technique (Jerne, Nordin and Henry, 1963). Following these experiments, all anti-SRBC activity was evaluated on the 7th day following sensitization, the time of maximum anti-SRBC activity. Delayed hypersensitivity immunization was induced in other guinea-pigs by injecting 0·25 ml amounts of H37R1 or H37RA strains of *Mycobacterium tuberculosis* (*M tb*) in each testicle and subcutaneously in each groin area. 4–6 weeks after sensitization, animals demonstrated to have a positive reaction to intermediate strength PPD were used as spleen cell donors in the passive transfer experiments.

Spleen cell preparations

Spleens were finely minced with scissors and pushed and rinsed through No. 100 and No. 230 mesh metal sieves into a chilled Petri dish. The rinse solution was a buffered balanced salt solution (BBSS), pH 7·4, with foetal calf serum solution prepared as described by Shortman (1968). The cell suspension was then washed in buffered balanced salt solutions without foetal calf serum, adjusted to a standard volume, and the total cells counted.

Albumin preparations

Bovine serum albumin (BSA) was prepared as described by Shortman (1968). Briefly, a 20 per cent solution of BSA (Fraction V, Sigma, St Louis, Mo.) in 0·001 M ethylene–diamine tetracetate was prepared. A 1:5000 dilution of an antibiotic solution composed of 2·5 per cent penicillin G, sodium salt, and 2·5 per cent streptomycin sulphate was added. The solution was then dialysed against at least six changes of distilled water over a 2-day period. The solution was centrifuged at 7000 ***g*** for 1 hour at 4° and the supernatent lyophilysed. The powder was dissolved in a sterile balanced salt solution (294 milliosmoles) to produce a stock 40 per cent w/w solution at pH 5·1. Further dilutions were made from this stock solution It was necessary to prepare more than one batch of albumin during the course of this study, but no significant differences were noted in control gradients from different batches.

Centrifugation and fractionation

$3–5 \times 10^8$ washed spleen cells were resuspended in 10 per cent bovine serum albumin, prepared as described by Shortman (1968). These cells were layered onto preformed linear albumin gradients with a concentration range of 30–15 per cent albumin. The gradients were centrifuged at 3800 ***g*** at 4° for 45 minutes in a Sorvall RC2B centrifuge (Sorvall, New Haven, Conn.) using 10 ml thin wall polypropylene tubes and an HB4 rotor.

For the SRBC experiments, twenty-four fractions of seven drops each were collected using an LKB fractionator and expelling the gradient through the top of the tube. Four fractions of forty-two drops each were collected for the delayed hypersensitivity experiments. The cell fractions were washed twice in BBSS, counted, and Wright-stained smears were made.

Passive transfer

Spleens were removed from guinea-pigs sensitized to *M tb* and fractioned as described

above. After counting, an equal number of cells in BBSS from each of the four fractions were injected intraperitoneally into guinea-pigs previously shown to give no reaction to second strength PPD. Four hours after cell transfer the animals were challenged with second strength PPD and induration of the skin test site was evaluated at 8, 12, 18 and 24 hours after challenge. Induration greater than 5 mm was considered positive.

RESULTS

The results of a typical experiment for anti-SRBC activity are illustrated in Fig. 1.

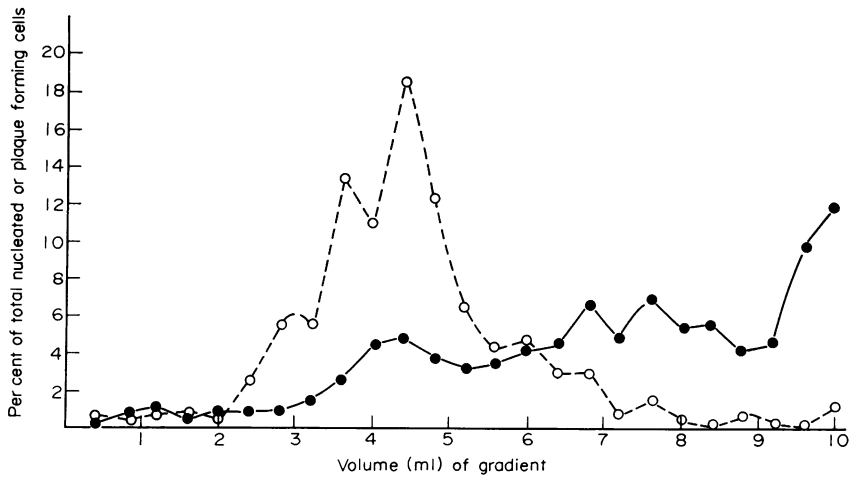

FIG. 1. Representative gradient 15–30 per cent albumin of spleen cells from a guinea-pig given a single injection of 5×10^8 SRBC 7 days before killing. The lowest numbers correspond to the least dense (15 per cent albumin) portions of the gradient. ●, Nucleated cells; ○, plaque-forming cells.

Approximately 90 per cent of the nucleated cells added to the gradients were recovered and 75–85 per cent of the cells in each fraction were viable as determined by exclusion of 0·1 per cent trypan blue dye. There was no significant difference between the distributions of total nucleated cells from normal or immunized animals. Although there was morphological heterogeneity of each fraction, certain ones were enriched with one or more cell types. Sixty per cent of the blast cells were in the upper one-half of the gradient, with 35–40 per cent in the upper 2·5 ml. Sixty per cent of the large lymphocytes were in the 3·2–5·2 ml levels and 75 per cent of the small lymphocytes were in the lower one-half of the gradient. Approximately 75 per cent of the polymorphonuclear leucocytes were in the bottom quarter of the gradient (7·6 ml and lower).

The plaque-forming cells appeared as one to three peaks in our gradients (Fig. 1) but they were always located between 2·5 and 7 ml fractions. In the experiment illustrated in Fig. 1 the cells in the zone of maximum plaque-forming activity were 82 per cent small lymphocytes, 9 per cent large lymphocytes, 5 per cent blast forms, with 4 per cent polymorphonuclear leucocytes.

PASSIVE TRANSFER EXPERIMENTS

Preliminary studies with unfractionated spleen cells indicated that a minimum of 5×10^7

spleen cells were required for the passive transfer of delayed hypersensitivity in guinea-pigs. Because of this, the gradients were divided into four large equal (2·5 ml) fractions to provide adequate numbers of cells for passive transfer. Equal numbers (10^7 to 4×10^7) spleen cells from each of the four fractions obtained from tuberculin sensitive guinea-pigs were injected intraperitoneally into individual animals shown to be unresponsive to second strength purified protein derivative (PPD) tuberculin.

Each of the guinea-pigs receiving cells from the upper 2·5 ml of the gradient showed positive responses to PPD. Those reactions which were interpreted as positive showed such a response by 8 hours and changed little during the following 16 hours of observation.

Recipients of cells from other fractions always gave negative responses to PPD. The cells in the upper 2·5 ml were 15–20 per cent blast cells, 50–60 per cent small lymphocytes, and 15–20 per cent large lymphocytes.

DISCUSSION

It is well known that there are different populations of lymphoid cells that perform different functions, e.g. thymic-dependent cells responsible for delayed hypersensitivity, and thymic-independent cells involved in humoral forms of immunity (Cooper, Percy, Peterson, Gabrielsen and Good, 1968). Many of the studies showing these separate functions have been carried out in either naturally-occurring or surgically-induced immunological deficiencies (e.g. experimental thymectomy). These showed that separate zones of lymph nodes were affected by absence of the thymus or, in chickens, the bursa, and correlations with absent cellular or humoral immunity were made.

Because of this, attempts have been made to fractionate lymphoid organs and bone marrow (Dicke, Tridente and van Bekkum, 1969) into subpopulations performing different immunological roles. Various types of density gradient centrifugation have been used for this purpose. Raidt, Mishell and Dutton (1968), using discontinuous albumin gradients, showed that after *in vivo* antigenic stimulation there was a decrease in the density of precursor and antibody producing cells of the mouse spleen. Haskill (1969) demonstrated the separation of antigen-sensitive and plaque-forming cells. L'Age-Stehr and Herzenberger (1970) using a method similar to Raidt, showed that the density gradient profiles of cells producing different classes of immunoglobulins were dissimilar. These authors also showed that memory cells were not identical with cells secreting antibodies by depleting gradient fractions of PFC by glass-bead columns and demonstrating retention of the ability to transfer memory for all classes of cells studied. Möller and Hiesche (1970) using mouse spleen cells reported that antibody-producing cells are enriched in low density fractions, while antigen-sensitive cells and cells which mediated GVH were enriched in the more dense fractions.

Our studies, in contrast to those above, used guinea-pigs instead of mice. This was done because the guinea-pig was known to be easily sensitized to *M tb*, skin testing was easier than in the mouse, and enough cells could be obtained from a single spleen so that as few as two animals were required to furnish enough cells for the passive transfer experiments.

The results of our studies show that plaque-forming activity is clearly not a function of the distribution of total nucleated cells since 75 per cent of the plaque-forming cells were found in a portion of the gradient containing only 25 per cent of the nucleated spleen cells (Fig. 1). Gorczynski, Miller and Phillips (1970) compared Ficoll and BSA gradient separation and found that in the mouse and rat spleen a homogeneous population of PFC was

found using Ficoll while heterogeneity was produced in BSA gradients. They suggest that the heterogeneity in BSA was due to changes in osmolality that exist in BSA gradients. We do not know of a similar analysis of guinea-pig spleen cells. We have demonstrated, however, that the density profile of cells responsible for transfer of delayed hypersensitivity was distinctly different from that of anti-SRBC antibody-producing cells with the experimental conditions utilized in our study. The morphology of those fractions with peak anti-SRBC activity was 80 per cent small lymphocytes and 15 per cent large lymphocytes or blast cells, while those fractions transferring delayed hypersensitivity were 50–60 per cent small lymphocytes and 30–40 per cent blasts or large lymphocytes.

It should also be noted that there was enrichment of ability to transfer delayed hypersensitivity in our light fractions since as few a 10^7 of the cells from the upper quarter of the gradient could transfer delayed hypersensitivity, while a minimum of 5×10^7 unfractionated cells were required for transfer.

We cannot exclude the possibility that earlier in the immune response in the guinea-pig, as in the mouse, one may find anti-SRBC cells in the lightest fractions of the gradient. This study does, however, show that at some time in the immune response, those fractions of the albumin gradient which are enriched in those cells capable of transferring delayed hypersensitivity differ from those with anti-SRBC activity. It is hoped that further purification of cell types will enable one to study a relatively homogeneous population of cells responsible for a given immunological function.

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