THE SEPARATION OF DIFFERENT CELL CLASSES

FROM LYMPHOID ORGANS

IV. The Separation of Lymphocytes from Phagocytes

on Glass Bead Columns, and Its Effect on

Subpopulations of Lymphocytes and Antibody-Forming Cells

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ABSTRACT

Four separate effects can be demonstrated when lymphoid cell suspensions are passed through columns of siliconed glass beads. (a) A temperature-dependent "active adherence" of phagocytic cells, such as macrophages and polymorphs. (b) A temperature-independent and selective trapping by "physical adherence" of particular classes of lymphoid cells, including certain antibody-forming cells. (c) A "size-filtration" effect that traps larger cells, but only becomes significant with beads below 100 μ in diameter. (d) A selective retention of damaged cells, which occurs with all columns under all conditions tested. An active adherence column technique has been developed to separate phagocytes from lymphocytes while minimizing selection within the lymphocyte population by physical adherence or size filtration. In less than 10 min at 37°C it reproducibly produces a preparation of mouse spleen lymphocytes >500-fold depleted of active macrophages, and approximately 50-fold depleted of active polymorphs, with good over-all cell recoveries and cell viability. The lymphocyte fraction appears fully active in its ability to initiate immune responses to at least two different antigens, but is changed in over-all composition and selectively depleted in certain classes of antibody-forming cells.

INTRODUCTION

Techniques for the separation of individual cell types from the mixed populations of cells in animal tissues are being developed in a number of laboratories, and some have been presented in earlier papers of this series (Shortman, 1966; 1968; 1969 a, b; Shortman and Seligman, 1969 c). Many separation techniques have relied on the physical properties of the cells such as size, density, or electrophoretic mobility. However, such properties do not necessarily correlate with the functional capacities of the cell. Direct answers to many questions would be obtained if the separation technique depended on the actual functional properties of the cell. The previous paper of this series presented such a technique for separation of erythroid from lymphoid and other cells (Shortman and Seligman, 1969 c).

Phagocytic cells may be separated by a method

that seems to reflect cell function. Active adherence of cells to solid substrata seems correlated with phagocytic activity. Adherence depends on the metabolic activity of the cell, and in addition requires the presence of calcium and magnesium ions and a heat-labile factor in serum. This adherence effect has been exploited by running blood cells through glass bead, glass wool, or nylon columns, which retain monocytes and polymorphonuclear leukocytes but allow lymphocytes to pass through (Garvin, 1961; Rabinowitz, 1964). However, when we employed cell sources other than human blood, a number of technical difficulties were encountered, and principles additional to active adherence came into play. The major problem was that large numbers of lymphocytes were retained by the column, in addition to the phagocytes.

Other glass bead column procedures have been deliberately designed to retain classes of cells other than phagocytes. Earlier work from this laboratory (Shortman, 1966; Shortman, 1969 b) involved columns of fine beads, run in the cold. This procedure appeared to select cells on the basis of size, and removed the larger, dividing cells from the small lymphocytes appearing in the filtrate. Other workers (Plotz and Talal, 1967; Salerno and Pontieri, 1969) have reported retention of antibody-forming cells on columns similar to those used for phagocytic cell removal. Since antibody-forming cells would not be expected to display the active adherence properties of phagocytes, this observation suggested that yet another principle was involved.

The present report presents a modification of the method of Rabinowitz (1964), suitable for separating macrophages and polymorphs from lymphocytes in a wide range of situations. The performance of the column with the complex cell mixture in spleen is presented in detail, together with an examination of the principles involved and the limitations of the procedure.

MATERIALS AND METHODS

Animals

8–14-wk old CBA mice of either sex were used. Antigen-stimulated animals were given an intraperitoneal injection containing both 6×10^8 washed sheep erythrocytes (SRC)[1] and 10 μg polymerized

bacterial flagellin (POL) from *Salmonella adelaide* strain 1338, and the spleens were studied 5 days later.

Cells

Cell suspensions free of clumps and debris were required to prevent blockage of the columns. Spleen cells were teased out and cleared of debris as described elsewhere (Shortman, Diener, Russell, and Armstrong, 1970). All cell suspensions were kept at 0°–4°C. In more recent experiments the suspensions have been cleared of all damaged cells prior to fractionation, as described elsewhere (Shortman, in preparation).

Cell Counts

Total nucleated cell counts, damaged and non-damaged cell counts, and differential counts on smeared and stained preparations for morphological classification were all as described elsewhere (Shortman, Diener, Russell, and Armstrong, 1970).

Counts of Cells Showing Phagocytic Activity

$5–15 \times 10^6$ cells in 1 ml were mixed with the test substance and incubated 3–5 hr, by use of the medium, chambers, and conditions identical with those used for obtaining an immune response in tissue culture (Shortman, Diener, Russell, and Armstrong, 1970). The medium was pre-equilibrated to temperature and pH. After incubation the cells were dispersed and harvested in the culture medium, and any remaining adherent cells were removed by vigorous washing of the chamber with ethylenediaminetetraacetate (EDTA)-containing medium (Rabinowitz, 1964). The medium and washings were pooled, layered above fetal calf serum, and centrifuged at 350 g for 7 min. The pellet of cells was resuspended in 50% serum-salt solution and again layered above serum and centrifuged. Smears (narrower than the width of the slide) were prepared, fixed in methanol, subjected to radioautography if required, and finally stained with Giemsa's. The proportion of phagocytic cells was determined from a count of 20,000 cells and included all cells viewed by scanning directly across from one edge to the other, at many points along the length of the smear.

Phagocytosis of SRC was assessed after incubation with 4×10^6 washed sheep erythrocytes. Only cells showing intact erythrocytes within their cytoplasm were scored as phagocytic. To test the possibility that phagocytic cells would be missed because of complete lysis of ingested SRC, controls were run with SRC

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cell; EDTA, ethylenediaminetetraacetate; IgG, gamma immunoglobulin class G; IgM, gamma immunoglobulin class M; POL, polymerized bacterial flagellin; SRC, sheep erythrocytes.

labeled with ^{125}I, and phagocytosis was scored by radioautography, as for POL. All labeled cells were found to have visible SRC within their cytoplasm. It was concluded that the morphological assessment alone was satisfactory.

Phagocytosis of POL was assessed after incubation with 0.04 μg ^{125}I-labeled polymerized flagellin from *Salmonella adelaide* (strain 1338), prepared by direct iodination with chloramine-T to a substitution rate of ~1 molecule of iodine per 40,000 mol wt flagellin unit (Ada, Nossal, and Pye, 1964). Radioautographs of smears on gelatin-coated slides were prepared, with use of Kodak NTB2 liquid emulsion and a 1–2 day exposure. Cells with greater than five grains over them were counted as phagocytic. The method could not distinguish between surface-bound material and true phagocytosis.

Counts of Cells Showing Direct Interaction with Antigens In Vitro

Cells were reacted with ^{131}I-labeled POL or hemocyanin in the cold and in the presence of azide, and reactive cells were counted by radioautography as described by Byrt and Ada (1969).

Counts of Antibody-Forming Cells

For POL antigen, the assay normally depended on the adherence of motile bacteria to antibody-forming cells, by use of the technique of Diener (1968). This technique should detect all classes of antibody on the surface of cells. In some experiments, where stated, a hemolytic assay was used, to detect predominantly IgM antibody released by the cells. This assay was carried out by first conjugating POL to SRC, by using a technique similar to that of Golub, Mishell, Weigle, and Dutton (1968) except that the POL concentration in the reaction mixture was 1 mg/ml, and the entire process was repeated three times on the one batch of SRC to obtain adequate surface concentration of POL. The assay was then similar to that for SRC. This procedure had about half the sensitivity of the adherence assay.

For SRC antigen, the assay depended on the formation, in the presence of complement, of plaques of lysis in an SRC monolayer, by use of the technique of Cunningham and Szenberg (1968). This assay could detect antibody, predominantly IgM, released by the cells (direct plaques). To assay for other classes of noncomplement-fixing antibody, plaque formation was enhanced by including in the chambers the optimum concentration of a nonspecific rabbit anti-mouse globulin antiserum, provided by Dr. N. Warner. The additional plaque numbers obtained were calculated by difference and termed "enhanced plaques." In some experiments, where stated, a

cytoadherence assay for cells reacting to SRC was used. This assay was carried out by mixing cell suspensions with one-tenth the concentration of SRC used for the plaque lysis assay, and omitting complement. The mixture was incubated at 37°C for 30 min, then placed in the same chambers used for the previous assay and viewed under phase-contrast microscopy, 500-fold magnification. The number of "rosettes" or cells showing more than four adherent SRC was counted.

Assay for Capacity to Initiate Immune Responses In Vivo

This assay depended on the formation of foci of antibody-forming cells in the spleen of irradiated animals 7 days after the injection of test cells together with either POL or SRC, and was carried out as described elsewhere (Shortman, Diener, Russell, and Armstrong, 1970).

Glass Beads

The beads were obtained from Cataphote Australia Pty. Ltd., Melbourne. Prior to first use, they were soaked in 1 N HCl, rinsed in several changes of water, soaked in alcoholic potash, rinsed in several changes of water, then distilled water. The beads were then siliconed by immersion with intermittent shaking in Dow-Corning silicone Z-4141 diluted 1:100 with distilled water. The siliconed beads were washed once with deionized distilled water, drained, then dried in an oven at 110°C. Before use they were suspended in water in a conical side-arm flask, and autoclaved if sterile techniques were required. Before packing columns the flask was evacuated to remove air bubbles, and a small quantity of ethyl alcohol was added to the suspension to lower the surface tension. After use the beads were expressed from the column, the pad of glass wool was removed, and the beads were shaken with saline to remove residual cells. The beads were then boiled with diluted "Teepol" detergent, rinsed, washed with alcoholic potash, resiliconed, and dried as before. The size of the beads for active adherence columns ("standard columns") was 300–600 μ, with a median diameter around 450 μ and a spherical content of greater than 80%. Batches of beads with smaller median diameters or a high proportion of beads with surface "blebs" gave unsatisfactory results, too many lymphocytes being retained. The size of the beads for "size filtration" was 105–150 μ in diameter for the first column, 60–90 μ in diameter for the second. For the "cold, physical adherence" columns, the bead size for removing only the most adherent cells was 300–600 μ and the bead size for obtaining maximum trapping of adherent cells was 290–360 μ.

Columns Designed to Separate by Active Adherence

Two sizes of columns have been generally employed, the large size (2.4 cm in diameter $\times$ 14 cm high) for total nucleated cell numbers ranging from 10^8 to 10^9, the smaller size (1.8 cm in diameter $\times$ 14 cm high) for numbers ranging from 2×10^7 to 2×10^8. All columns were jacketed to permit temperature control with circulating water at 37°C. The columns were sealed with a rubber bung. A pad of coarse, siliconed glass wall was placed in the base of the column to retain beads on subsequent packing. The dry column was sterilized, if necessary, then filled with water. The column was packed by introducing the bead suspension under the liquid surface and allowing the beads to fall as a continuous shower. 30 min before use the column was brought to temperature equilibrium and washed with medium 199 to pH equilibrium.

Media for Active Adherence Separation

The basic medium was medium 199, obtained from Commonwealth Serum Laboratories, Melbourne. This medium offered the advantage of being a tissue culture medium, but the disadvantage of becoming alkaline due to CO_2 loss on standing. Care was taken to maintain pH at all times in the range 6.8–7.3, as judged by the phenol red indicator and a set of standards. This was accomplished by starting with medium, gassed if necessary, at pH $\sim$6.5; by pre-equilibrating the column; by working rapidly; and by sealing all tubes and the column when not in operation. Effective separation was not achieved in one experiment with alkaline medium. The serum used for CBA mouse cells was normal, nonheat inactivated, CBA mouse serum, stored frozen in sealed tubes, and diluted with medium 199 before use. (In experiments with rat cells rat serum was used, and with human cells, human serum). A shortage of mouse serum prevented the use of 50% CBA mouse serum–medium 199 throughout. Accordingly, fetal calf serum (Commonwealth Serum Laboratories) was used in the initial pre-equilibration of the column, and the mouse serum level was reduced to 25% in the later washings. Although CBA mouse cells were always directly suspended in CBA mouse serum, serum from other mouse strains was frequently used for the later washings, without affecting the results. The Ca^{++}- and Mg^{++}-free EDTA (ethylenediaminetetraacetate) medium used for eluting adherent cells was as described by Rabinowitz (1964). To help conserve viability and to reduce the physical adherence effect that occurs in the absence of serum (Shortman, 1966), 2% fetal calf serum was included in the EDTA medium.

Active Adherence Separation

A column of size appropriate for the number of cells was pre-equilibrated to temperature (37°C) and pH, (6.8–7.3), then washed through with 1 column vol of 60% fetal calf serum–199. Just before applying the cells, 2 ml of 60% mouse serum–199 was run onto the top of the column. The use of 60% serum provided a density "step," which maintained a sharp boundary by preventing the suspension of dense cells in 50% serum from mixing into the preceding solutions. The debris-free mouse spleen cells were suspended in 50% mouse serum, 4–5 ml for the large column, 2–3 ml for the small, in a sealed, siliconed glass or plastic tube. The suspension was incubated at 37°C for 5 min (to ensure that the cells were fully active immediately on contact with the glass beads) with intermittent stirring with a Vortex mixer (Scientific Industries, Inc., Queens Village, N.Y.) (to minimize aggregation and adherence to the tube). The suspension was then run onto the bead column, followed by an equal volume of 50% mouse serum–199, and washed through with 1 column liquid vol of 25% mouse serum–199. Just after the first 1 ml washing with 50% mouse serum, the upper few millimeters of the column bed were gently stirred, to reduce nonspecific blocking in this area. Care was taken to ensure continuous, uninterrupted flow, and to avoid running the column dry. The flow rate was adjusted so that the residence time of the main band of cells in the column was 8–10 min. The initial flow rate while the cells were entering the column was about twice that employed during the washing stages, in order to reduce blocking in the upper regions of the column. (When different sources of cells or beads were employed, the residence time was sometimes adjusted by ±2 min around these values. Slower flow rates were used if all phagocytes failed to adhere, and faster rates were used if excessive numbers of lymphocytes were retained.) To displace adherent cells by chelation of Mg^{++} and Ca^{++}, 2 column vol of EDTA medium–2% serum were then passed through the column at approximately double the previous flow rate. Not all adherent cells were removed by this process alone, and additional agitation was needed for full recovery. This was accomplished by almost filling the column with EDTA medium to well above the bead surface, sealing the column with a rubber bung, tilting the column until almost horizontal, and gently rolling and shaking it until all but the last few centimeters of the bead bed was disrupted. The column was returned to a vertical position and the bead bed was allowed to reform. Washing with the displacing medium was then continued. During this process most of the viable adherent cells were released and appeared in the washings, but most damaged cells reattached to the reconstituted column and were thereby removed. Four separate fractions were usually collected from

such a column. Fraction I consisted of the initial band of nonadherent cells appearing in an effluent volume of three times the input band. Fraction II consisted of the column washings prior to the elution with EDTA medium, but not including the interface. Fraction III consisted of the shower of cells directly released by EDTA medium alone, and included the interface with the last mouse serum-199 washings. No attempt was made to subdivide fraction III, as in the technique of Rabinowitz (1964). Fraction IV included all cells released by the final column disruption and washing step. In the experiments reported in this paper, these were pooled to give only two fractions: fraction I was pooled with fraction II to give "nonadherent cells," fraction III with fraction IV to give "adherent cells."

Size Filtration

The technique designed to separate by size filtration was similar to that described previously (Shortman, 1966), but was modified for spleen cell preparations. Columns were 3.7 cm in diameter × 4 cm high, "loosely packed," and pre-equilibrated at 5°-7°C in 16% fetal calf serum–Dulbecco's salt solution (20% serum). Approximately 5 × 10^8 nucleated spleen cells were suspended in 3 ml of 16% serum and passed through a column of 105–150 μ beads at 5°-7°C. The cells were washed through with 16% serum. The upper 0.5 cm of the bead bed was gently stirred at the beginning of the washing step to reduce blockage. The residence time of the main cell band in the column was 10–15 min. The cells in the filtrate were recovered, suspended in 1 ml 16% serum, and passed through a 60–90 μ bead column, under similar conditions except for a residence time of 25–30 min. The final effluent was the size-filtered, "small lymphocyte" preparation.

Physical Adherence Separation

To study the physical adherence effects occurring under the conditions of active adherence separation, the "standard columns" were simply transferred to a cold room at 5°-7°C. Two further columns were developed from this procedure.

To fully deplete a cell suspension of all cells showing significant physical adherence, the columns were 2.4 cm in diameter × 14 cm high, of 290–360 μ beads, and pre-equilibrated at 5°-7°C with 25% fetal calf serum in Dulbecco's salt solution (25% serum). The cells (approximately 5 × 10^8 nucleated cells) were suspended in 4 ml 25% serum, and passed through the column at 5°-7°C. The cells were washed through with 25% serum, the upper few millimeters of the column bed being stirred gently after the first 1 ml of washings. The residence time of the main cell band was 30 min. The effluent represented the "least adherent" elements.

To obtain a preparation of the "most adherent" elements, the columns were 2.4 cm in diameter × 5 cm high of 300–600 μ beads, and pre-equilibrated at 5°-7°C with 50% fetal calf serum in Dulbecco's salt solution (50% serum). The columns were run as above, except that the residence time of the main cell band passing through the column was about 3 min. After washing with several column volumes of 50% serum, the beads were expressed from the column and gently shaken in the same medium. After the beads settled out, the supernatant was removed and extraction was repeated. The combined extracts represented the most adherent elements.

RESULTS

Changes from the Rabinowitz Technique

In the original procedure of Rabinowitz (1964) human blood leukocytes in 50% human serum were run onto a dry column, the column outlet was closed, and the column was incubated at 37°C for 30 min. After this period free lymphocytes were washed out, and the polymorphs and monocytes were eluted with an EDTA-containing medium. The active adherence column technique described in this paper was developed by a series of modifications of the Rabinowitz method, in order to obtain efficient separation with mouse spleen. The aims were: (a) to improve the efficiency of trapping of phagocytes; (b) to minimize trapping of lymphocytes; (c) to improve over-all recovery and especially the recovery of macrophages; (d) to eliminate damaged cells. These aims would require opposing conditions, and the final procedure was to some extent a compromise. The major changes were: (a) the bead size was increased, reducing the trapping of lymphocytes. Still larger beads allowed some phagocytes to penetrate the column. (b) Mouse serum was used with mouse cells. Other serum sources (e.g., fetal calf serum) gave some active adherence, but some phagocyte penetrated the column. Heat-inactivated (56°C, 30 min) serum was ineffective. (c) The cells were pre-equilibrated to 37°C, so they were active for the entire residence time in the column. (d) The cells were moved rapidly and continuously, as a band, through a pre-equilibrated column, to allow efficient active adherence but minimum trapping of lymphocytes. (e) The column was disrupted at the end of the procedure to recover cells, especially macrophages not directly eluted by the EDTA medium.

The Separation of Mouse Spleen Cells

A series of test runs was performed to assess the performance and reproducibility of the method when applied to mouse spleen. The distribution of nucleated cells, both intact and damaged (as differentiated by dye exclusion), is given in Table I. One advantage of the procedure was the selective elimination of damaged cells, which showed a strong tendency to adhere to the column, even in the presence of EDTA, and after disruption of the column and rapid elution. The over-all recovery of intact cells was good, and could be increased if needed by a second extraction of the bead bed. A recovery of 50–60% of intact cells in the filtrate was the maximum compatible with complete elimination of active macrophages. A relatively high proportion of cells were in the adherent fraction, higher than could be accounted for by the phagocyte content.

The morphological composition of the two fractions is summarized in Table II. Recoveries of all classes of cells were satisfactory. As expected from the total counts, lymphocytes were the dominant cell class in both fractions. The adherent fraction was enriched for polymorphs and macrophages. The filtrate fraction represented a lymphocyte

TABLE I

The Distribution of Intact and Damaged Mouse Spleen Cells on Columns Designed to Separate by Active Adherence

	Total cells recovered in fraction		Percentage of intact cells
	Intact cells	Damaged cells	
	% of input ± SD		% of total nucleated cells ± SD
Original suspension	100	100	75 ± 7
Filtrate fraction	52 ± 14	10 ± 5	94 ± 4
Adherent fraction	32 ± 15	11 ± 5	90 ± 5
Over-all recovery	84 ± 13	21 ± 7	

Results are means of 12 experiments.

TABLE II

The Separation of Lymphocytes from Phagocytes on Columns Designed to Separate by Active Adherence

	Cells per 1000 intact nucleated cells				
	Morphological classification			Phagocytic activity	
	Lymphocytes	Polymorphs	Macrophages	SRC	POL
Original suspension	951	28	18	1.19	14.1
Filtrate fraction	997	0.6	1.3	0.00	0.5
Adherent fraction	876	68	52	1.22	15.5
Over-all recovery, % of input	84	79	96	33	37

Results are the means of eight experiments in the case of morphological classification, three in the case of phagocytic activity. In the latter case a total of 300,000 cells were scanned in the filtrate fraction. The lymphocyte count included all classes of lymphocytes, small, medium, and large. All cells of the granulocyte series, mature and immature, are included in the polymorph count. The macrophage count includes cells with the appearance of blood monocytes. Recoveries were calculated by first determining the total number of each cell type in the fractions (differential count × total intact nucleated cells in fraction) and comparing this to the total input of that class of cell onto the column.

fraction markedly depleted (∼ 24-fold) of phago-
cytes. Two points should be noted concerning the
residual "phagocytes" in the lymphocyte prepara-
tion. Firstly, Rabinowitz (1964) has shown that
any cells that have lost phagocytic activity, because
of damage or other reasons, fail to adhere; these
would still be scored as phagocytic elements by
morphological criteria. Secondly, although distinc-
tion between macrophages and lymphocytes was
straightforward for most cells, there existed a small
proportion with intermediate characteristics. It is
possible that some of the cells scored as "macro-
phages" in the filtrate fraction were in reality
lymphocytes.

The Distribution of Mouse Spleen Cells Showing Phagocytic Activity

Functional tests for phagocytic activity provided
a far more objective and critical test of the behavior
of the column than morphological criteria. Cells in
the column fractions were assayed for their ability
to phagocytose two quite different substances
commonly used as antigens, namely sheep erythro-
cytes (SRC) and polymerized bacterial flagellin
(POL). These were handled by different sets of
phagocytic elements in the unfractionated spleen
under our test conditions. All cells found ingesting
SRC were macrophages. Of the cells scored as
"phagocytic" for POL, 91% were polymorphs,
5% macrophages, and 4% appeared to be lympho-
cytes.

Assays of fractions for phagocytic activity are
given in Table II. It is clear that the filtrate frac-
tion was more depleted of actively phagocytic ele-
ments than the simple morphological count sug-
gested. The depletion of cells phagocytosing SRC
was at least 500-fold, to one cell in 100,000 or less.
Of the small numbers of POL-labeled cells in the
filtrate fraction, none were macrophages, 53%
were polymorphs, and 47% were lymphocytes.
Since the assay for POL could not distinguish label
on the cell surface from label within cells, these few
lymphocytes may represent the nonphagocytic
antigen-binding cells considered later. The two to
three active polymorphs per 10,000 lymphocytes
probably represented true "leakage" of phago-
cytes through the column.

The Distribution of Antigen-Binding Lymphocytes

The results of Table II show that the adherence
column filtrate represented a lymphocyte prepara-

tion markedly depleted of phagocytic elements.
Such a preparation is required for many immuno-
logical studies, such as investigation of the role of
phagocytes in initiation of immune responses
(Shortman, Diener, Russell, and Armstrong,
1970). However, it was first essential to determine
if this lymphocyte preparation was immunologi-
cally equivalent to the starting material, since only
50–60% of all lymphocytes were recovered in the
filtrate. One class of cell of current interest is the
lymphocyte that binds large amounts of antigen to
the surface of the cell membrane, as distinct from
ingestion of material by phagocytosis (Byrt and
Ada, 1969; Naor and Sulitzeanu, 1967). Only a
small proportion of all lymphocytes interact with
any one antigen. There is evidence that at least
some of these cells play a crucial role in immune
responses (Ada and Byrt, 1969). Accordingly the
distribution of these reactive lymphocytes was
investigated.

The distribution of lymphocytes binding two
different antigens, POL and hemocyanin, was
followed. Assays for antigen-binding cells were
carried out in the cold, and in the presence of
azide, to inhibit phagocytosis. The results in Table
III demonstrate that antigen-binding lymphocytes
penetrated the column, but somewhat less exten-
sively than lymphocytes in general. The result was
a slight depletion of these cells in the filtrate (to
70–80% of the starting levels) and a corresponding
small enrichment in the adherent fraction.

The Distribution of Cells Initiating Adoptive Immune Responses

The most crucial test of the functional integrity
of the lymphocyte population was its ability to
initiate an immune response when transferred to

TABLE III

The Level of Antigen-Binding Lymphocytes in Mouse Spleen Fractions from Columns Designed to Separate by Active Adherence

	Antigen-binding cells per 1000 intact nucleated cells	
	POL	Hemocyanin
Original suspension	0.13	2.4
Filtrate fraction	0.09	1.9
Adherent fraction	0.20	4.8
Over-all recovery, %	86	106

The results represent a single experiment.

animals whose own immune system was crippled by X-irradiation. Responses to two different antigens, SRC and POL, have been studied and the results have been presented in detail in a previous paper (Shortman, Diener, Russell, and Armstrong, 1970). Briefly, the lymphocytes in the filtrate fraction were capable of initiating responses to both antigens tested. There was some suggestion of an enhanced response to SRC and a reduced response to POL, but differences between these results and the results obtained with the original material were of marginal significance. The adherent fraction gave a response in accordance with its lymphocyte content.

The Distribution of Antibody-Forming Cells

The preceding results showed that the column filtrate represented a phagocyte-free lymphocyte preparation with an adequate complement of those lymphocytes needed to initiate immune responses, leading to the production of antibody-forming cells. An additional question was whether the end product of the immune response, the antibody-forming cells, would also pass through the column. Two facts made this question more pertinent. Firstly, morphological studies had indicated that the filtrate fraction was about 6-fold depleted in plasma cells (see Table VII), and that the adherent fraction was correspondingly enhanced. Although only a proportion of all antibody-form-

ing cells would be of this morphological class, the results suggested some selectivity not immediately obvious from a study of lymphocytes alone. Secondly, Plotz and Talal (1967) and later Salerno and Pontieri (1969) showed that passage of spleen cells through glass bead columns (not necessarily under optimum conditions for active adherence of phagocytes) produced a relative depletion of cells forming antibody against SRC.

To test for the distribution of antibody-forming cells, mice were injected with SRC or POL, and the spleens were removed 5 days later (near the peak of the response) and fractionated on columns designed to separate by active adherence. The distribution of active cells was initially determined by a cytoadherence assay for POL, and a hemolytic plaque assay for SRC. The results (Table IV) showed a distribution quite different from that of lymphocytes in general, and marked differences between different antibody-forming cells.

Cells forming antibody against SRC were almost completely retained by the column, to a more marked extent than the previous investigators reported (Plotz and Talal, 1967; Salerno and Pontieri, 1969). This applied to both direct and to anti–mouse globulin antisera-enhanced plaques, so adherence was probably common to both IgG and IgM antibody producers. In contrast a significant proportion of cells forming antibody against POL appeared in the filtrate, although this frac-

Table IV

The Distribution of Mouse Spleen Antibody-Forming Cells (AFC) on Columns Designed to Separate by Active Adherence

Antigen	SRC			POL		
AFC assay	Direct hemolytic plaques	En-hanced hemolytic plaques	Cytoad-herence rosettes	Adherence colonies	Direct lysis plaques	En-hanced lysis plaques
Level of AFC in original suspension (AFC/10^6 viable nucleated cells)	900	5000	7400	1000	490	590
Percentage of input AFC in:						
Filtrate fraction	3	0	2	29	22	13
Adherent fraction	24	66	32	41	20	44
Over-all recovery, % of input	27	66	34	70	42	57

The results for anti-SRC hemolytic plaques, and for POL cytoadherence colonies, are means of four experiments on animals injected with both SRC and POL. The "reverse" assays, cytoadherence for SRC and hemolysis for POL, are each a single experiment on separate animals injected with only one antigen. All spleens were obtained 5 days postinjection.

	Total intact nucleated cells		Macrophages		Polymorphs		Cells forming antibody against SRC			
							Direct plaques		Enhanced plaques	
	Distribution as % of total input									
Temperature, °C	37	6	37	6	37	6	37	6	37	6
Filtrate fraction	54	53	2	22	1	27	3	6	<1	1
Adherent fraction	28	24	82	66	76	45	24	54	66	102
Over-all recovery	82	77	84	88	77	72	27	60	66	103

Results are the means of four experiments at 37°C, two experiments at 6°C. In all cases the spleens were from mice injected with antigen 5 days previously. Macrophage and polymorph distributions were obtained from total cell counts and smeared and stained preparations, using morphological criteria.

tion was still about 2-fold depleted compared to the original material.

It seemed possible that this difference between antigens could reflect the method of assay, since the hemolytic technique required actual release of antibody from the cell, whereas the cytoadherence technique would require only antibody on the cell surface. Accordingly, the assay principles were reversed, adopting a cytoadherence rosette assay for SRC, and a hemolytic plaque assay for POL. This gave results basically like those of the previous assay techniques (Table IV). It was concluded that the differences in behavior of different antibody-forming cells reflected in some way the nature of the antigen, but not the assay technique or, in any obvious way, the class of antibody produced.

The Distribution of Cells on Columns Run in the Cold

The almost complete retention of certain antibody-forming cells on the columns posed the question of whether they were displaying active adherence, in the manner of phagocytes, or whether some other phenomenon was occurring. A simple test for active adherence was its temperature dependence: as demonstrated by Garvin (1961), active adherence drops off rapidly below 20°C. Accordingly, experiments were performed with the standard columns and were identical in all respects with the active adherence separation, except that the columns and cell suspensions were maintained at 5°–7°C in a cold room. The results are summarized in Table V.

It was of interest that the total nucleated cell distribution was unaffected by the temperature change. This indicated that those lymphocytes retained by the column were not exhibiting active adherence. By contrast, lowering the temperature had a marked effect on the distribution of macrophages and polymorphs, a significant proportion appearing in the filtrate fraction. Many phagocytes were, however, retained by the column even at low temperature. This may have occurred because of residual cell activity at 5°–7°C, or alternatively because factors additional to active adherence affect the retention of these cells. In contrast to the results with phagocytes, the distribution of cells forming antibody against SRC was unaffected by the temperature drop, the only significant change being an improved over-all recovery. The adherence of such cells to columns run in the cold was termed "physical adherence" to distinguish it from active adherence.

The Distribution of Lymphocytes on Columns Designed to Show Physical Adherence in the Cold

The standard columns used for active adherence separation, operated at 37°C or at 6°C, gave a significant proportion of spleen lymphocytes (~ 30%) in the adherent fraction. Initially, this was

TABLE VI

Fractionation and Refractionation of Cells on Columns Designed to Separate by
Physical Adherence

	First fractionation			Second fractionation of spleen	
Cell preparation	Blood erythrocytes	Thymus suspension	Spleen suspension	Least adherent	Most adherent fraction
% of input cells in filtrate	64	47	20	58	9

The results are the means of two experiments. The columns used were those designed
to maximize the trapping of adherent elements, and were run at 6 °C. Full details are
in Materials and Methods. The filtrate from first fractionation represented the least
adherent fraction for the refractionation experiments. The most adherent fraction
(16% of the initial nucleated cells) was obtained on separate columns, as described in
Materials and Methods.

simply ascribed to nonspecific trapping or block-
age in the column, since the filtrate fraction ap-
peared to retain full immunological competence.
However, the specific adherence of a class of anti-
body-forming cells raised the possibility that
lymphocytes might be divisible into two classes,
one showing physical adherence like certain anti-
body-forming cells, the other not. To examine this
possibility, two different cold, physical adherence
columns were devised, as described in Materials
and Methods. One was designed to maximize
trapping of adherent cells, and involved smaller
beads, slower flow rates, and lower serum levels
than the standard column. The second was de-
signed only to trap the most adherent elements,
and involved the same beads and serum levels as
the standard column, but a shorter column length
and faster flow rate. With these techniques it was
possible to test for differences between lympho-
cytes, as in Table VI.

Since most columns would probably display
some degree of nonspecific trapping of cells, mouse
erythrocytes, a homogeneous and presumably non-
adherent population, were used in an attempt to
monitor this effect in the columns designed to
maximize trapping of adherent elements. When
applied to these cold physical adherence columns,
an average of 64% of the input cells were found in
the filtrate. This was taken as the maximum degree
of penetration expected from a nonadherent popu-
lation. Mouse thymus lymphocytes penetrated the
column about 75% as effectively as erythrocytes
(Table VI). By contrast mouse spleen cells were
extensively retained by the column, only 20%
penetrating.

The question of possible separation within the
population of spleen lymphocytes was tested by
refractionation experiments. The 20% of cells
from spleen originally penetrating the columns
designed to maximize removal of adherent cells
were rerun through the same type of column, when
they penetrated more extensively than thymus
cells, and almost as well as erythrocytes. Con-
versely, a fraction selected to represent the most
adherent elements was very largely trapped when
tested on these columns. It was concluded that
different lymphocyte populations showed different
degrees of physical adherence, and that spleen con-
tained both adherent and nonadherent elements.

A Comparison of Three Column Techniques

The physical adherence of lymphocytes to sili-
coned glass beads in the cold recalled earlier work
from this laboratory in which cells were separated,
apparently on the basis of size, by passage through
very fine (50–90 μ diameter) siliconed glass beads
at 5°–7°C (Shortman, 1966, 1969 b). A form of
physical adherence of lymphocytes was observed at
this stage, but it was assumed that this property
was shared by all cells, fractionation depending
only on size. In support of this, size fractionation
was ineffective with a bead size of about 100 μ in
diameter. However, since the conditions used must
have produced the type of physical adherence
separation described in the present report, it
seemed important to check if size filtration effects
really did exist or if fractionation merely reflected
the fact that large cells coincidentally showed more
extensive physical adherence than small. In addi-

TABLE VII

The Composition of Cell Preparations after Passage through
Different Siliconed Glass Bead Columns

Separation principle on which column technique was based	Relative depletion or enrichment $\left(\dfrac{\text{Differential count of filtrate fraction}}{\text{Differential count of original suspension}}\right)$						
	Cell preparation	Lymphocyes			Plasma cells	Macro-phages	Poly-morphs
		Small	Medium	Large			
Active adherence	Mouse spleen	1.1	0.89	0.48	0.17	0.03	0.01
Physical adherence	Mouse spleen	1.1	0.43	0.25	0.00	0.48	1.1
	Mouse thymus	0.98	1.34	0.97	—	—	—
	Rat thoracic duct	1.0	1.0	1.1	—	—	—
Size filtration	Mouse spleen	1.1	0.19	0.03	0.00	0.09	1.1
	Rat thoracic duct	1.0	0.21	0.00	—	—	—

Results are the means of two to eight experiments. Details of the separation procedures are given in Materials and Methods. The physical adherence columns were those designed to give maximum depletion of adherent cells. Over-all recoveries of intact nucleated cells in the filtrate fraction of mouse spleen averaged 51% for active adherence, 14% for physical adherence, and 10% for size filtration.

tion to this, it was necessary to check if the active adherence or cold physical adherence columns themselves exhibited a size-based selection in any way. The three bead-column separation techniques were, therefore, compared by a study of the morphological composition of the filtrate fraction from each column. The results are summarized in Table VII.

The columns designed to separate by active adherence produced, as well as removal of phagocytes, a marked depletion of plasma cells and some depletion of the larger lymphocytes in mouse spleen preparations. The type of columns designed to produce a maximum depletion of cells showing physical adherence in the cold gave, with mouse spleen, a more extensive depletion of plasma cells and larger lymphocytes, compared to active adherence separation. This is in contrast to the results with macrophages, where the depletion was very much less than with active adherence, and to the results with polymorphs, where no depletion at all occurred in the cold. The depletion of larger lymphocytes by physical adherence columns was not obtained when mouse thymus or rat thoracic duct cells were tested. This suggested that it was not a consequence of cell size as such, but reflected

the physical adherence characteristics of a particular class of spleen cells.

The final type of column, designed to select by size filtration, and using very small beads at 5°–7°C, produced a filtrate fraction which differed from that of the previous two techniques. Size filtration of mouse spleen cells produced a much more extensive depletion of larger lymphocytes than could be accounted for by physical adherence alone. This depletion of large cells was obtained with all sources of lymphocytes, including rat thoracic duct cells where physical adherence alone had no such effect. The effect of size filtration on phagocytes also emphasized this difference from the other columns; more than 10-fold depletion of macrophages was obtained without depleting the smaller polymorphs.

Overall the results of Table VII would be in accordance with the following:

(a) The distribution of intact lymphoid cells depended on three types of fractionation principles operating to different degrees in the three types of columns.

(b) The standard active adherence column was the only one to display metabolism-dependent adherence, assuming this to be inoperative at 5°–

7°C. However, it also involved a physical adherence effect independent of metabolic activity.

(c) The relatively large-sized beads used in the physical adherence columns (and in the standard active adherence columns) did not produce selection by size. These columns run at 5°–7°C produced fractionation exclusively by the ill defined physical adherence effect.

(d) Extensive physical adherence effects must have occurred in the small-bead size filtration columns, but an additional effect of selection by size was also introduced.

DISCUSSION

Advantages of the Active Adherence Column Technique

The column technique, if run as described in this paper, produces in less than 10 min at 37°C a lymphocyte preparation reproducibly and very extensively depleted of actively phagocytic elements, with good over-all cell recovery and cell viability and minimum depletion of lymphocytes. A careful "balance sheet" approach has been adopted, so that the relative immunological capacity of the separated lymphocytes can be assessed in a quantitative way. This technique has produced clear-cut experimental results when applied to the problem of the role of "accessory" cells in the initiation of immune responses in tissue culture (Shortman, Diener, Russell, and Armstrong, 1970). The response to one antigen (SRC) was found to be absolutely dependent on the presence of some accessory cell in the adherent fraction, while the response to another antigen (POL) was independent. The antibody-forming cells for SRC antigen were shown to originate from precursor cells in the nonadherent fraction. The accessory cell had many of the properties of a macrophage. These results were obtained over a range of cell concentrations, and did not require a limiting dilution approach to show the effects of removal of adherent elements.

This column technique offers the advantages of speed, efficiency, and control, compared to two alternative approaches which depend on the same phenomenon of active adherence. The original Rabinowitz column technique (Rabinowitz, 1964) produces too marked a loss of lymphocytes and a distortion of the lymphocyte population to be useful for mouse spleen. Separation of adherent and nonadherent fractions of mouse spleen has also

been obtained by culturing the cells for 30–60 min in plastic dishes, or in plastic dishes containing glass beads (Mosier, 1967; Hartmann, Dutton, McCarthy, and Mishell, in press). This procedure has also produced a nonadherent fraction that requres addition of adherent cells to obtain a response in culture to SRC antigen. However, this technique is more difficult to control, and seems less efficient in that repetitive separations or use of limiting dilutions were needed to show the effects of loss of adherent elements. The precise efficiency of this technique and its effects on the lymphocyte population have not been well documented.

Disadvantages of the Active Adherence Column Technique

Although lymphocytes are effectively depleted of phagocytes by the active adherence columns, the converse is not the case. Although enriched for phagocytes, the major cell type in the adherent fraction is still lymphocytes, and this complicates the interpretation of any biological activity shown by this fraction.

A major disadvantage of the technique is the physical adherence selection within the lymphocyte population. In the present study this was minimized and care was taken to establish that the phagocyte-depleted population was similar to the original lymphocytes by a number of criteria, including the ability to initiate immune responses. Nevertheless, the technique produced an almost complete removal of a certain distinct class of very adherent lymphocytes, and of particular antibody-producing cells. It is clear that several control experiments such as those used in this paper have to be run before it can be assumed that any biological effect is related to the removal of phagocytes, as opposed to elimination of particular classes of lymphocytes. These difficulties are probably shared by all the current techniques for separating by active adherence.

The Physical Adherence of Lymphocytes

In this study the adherence of lymphocytes has been regarded as a problem to be minimized when running columns aimed at separation by active adherence. It may be, however, that this phenomenon can be exploited to produce a useful separation of different classes of cells. For example, it is of considerable interest that thymic lymphocytes showed extensive penetration of the column while

certain antibody-forming cells in spleen were completely trapped. The least adherent and most adherent lymphocytes from spleen might correspond to the "thymus-derived" and "antibody-forming cell precursor" populations, respectively. Development of such a physical adherence separation method would require a more detailed and thorough background study than is presented here.

Size-Filtration of Lymphocytes

It is fortunate that the earlier column technique for separation of small lymphocytes (Shortman, 1966; Shortman, 1969 b) has now been displaced by sedimentation rate separation (Miller and Phillips, 1969; Peterson and Evans, 1967) since the column method was not only inefficient but also must have produced selection by parameters additional to size. Although a genuine size-based selection existed, the conditions were such that extensive physical adherence selection must also have occurred. These size filtration columns have produced intriguing functional selection between the antigen-sensitive cells in thoracic duct lymph. The column-purified small lymphocyte preparations were fully active in transferring the ability to respond to SRC antigen but showed very low responsiveness to POL antigen (Lewis, Mitchell, and Nossal, 1969; Nossal, Shortman, Miller, Mitchell, and Haskill, 1967). This may have reflected a size difference in the initiating cells, but as is now clear, and was in fact suggested by Lewis, Mitchell, and Nossal (1969), it could equally well have reflected the physical adherence selection effect.

Antigen-Coated Columns

In earlier work from this laboratory (Shortman, unpublished data) many attempts were made to purify specific antibody-forming cells by passage through various types of columns containing immobilized forms of the particular antigen. Although specific bindings of cells did occur, no useful purification was ever obtained because the effect was overwhelmed by nonspecific trapping of cells. Similar results have recently been published by Wigzell and Andersson (1969). This nonspecific trapping is clearly the physical adherence effect we have described. Wigzell and Andersson also demonstrated a specific depletion of memory cells by passage through the antigen-coated columns. It is obvious that the same precautions would be needed

in exploiting this effect as are needed in separating by active adherence: controls would always be needed to ensure that any result was attributable to immune-adherence, and not just to distortion of the lymphocyte population by physical adherence effects.

Application of Active-Adherence Columns to Other Cell Sources

Although the active adherence columns were developed because of the particular problems associated with spleen cells, they have now been successfully applied to a wide range of tissues. Some sources in fact give much clearer separation and a marked enrichment for phagocytes, because the level of adherent lymphocytes is much lower (e.g., blood, peritoneal cells). The technique gives separations comparable with the Rabinowitz method (Rabinowitz, 1964) when applied to human, rat, or mouse blood. Efficient separation of macrophages from lymphocytes is obtained with rat and mouse peritoneal fluid or peritoneal exudate. When applied to the complex cell mixture of mouse bone marrow, a column effluent consisting only of small to medium sized lymphocytes and erythroid cells is obtained, and there is substantial selection between "stem-cells" that form colonies in irradiated animals and those that form colonies in vitro on agar-plates. Full details of these applications will be presented elsewhere.

REFERENCES

ADA, G. L., and PAULINE BYRT. 1969. Specific inactivation of antigen-reactive cells with ^{125}I-labelled antigen. *Nature (London).* **222**:1291.

ADA, G. L., G. J. V. NOSSAL, and J. PYE. 1964. Antigens in immunity. III. Distribution of iodinated antigens following injection into rats via the hind footpads. *Aust. J. Exp. Biol. Med. Sci.* **42**:295.

BYRT, PAULINE, and G. L. ADA. 1969. An *in vitro* reaction between labelled flagellin or haemocyanin and lymphocyte-like cells from normal animals. *Immunology.* **17**:503.

CUNNINGHAM, A. J., and A. SZENBERG. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology.* 14:599.

DIENER, E. 1968. A method for the enumeration of single antibody-producing cells. *J. Immunol.* 100:1062.

GARVIN, J. E. 1961. Factors affecting the adhesiveness of human leucocytes and platelets in vitro. *J. Exp. Med.* 114:51.

GOLUB, E. S., R. I. MISHELL, W. O. WEIGLE, and R. W. DUTTON. 1968. A modification of the hemolytic plaque assay for use with protein antigens. *J. Immunol.* 100:133.

HARTMANN, K., R. W. DUTTON, M. M. McCARTHY, and R. I. MISHELL. 1970. Cell components in the immune response. II. Cell. attachment separation of immune cells. *Cell. Immunol.* 1: 182.

LEWIS, HEATHER, JUDITH MITCHELL, and G. J. V. NOSSAL. 1969. Subpopulations of rat and mouse thoracic duct small lymphocytes in the Salmonella flagellar antigen system. *Immunology.* 17:955.

MILLER, R. G., and R. A. PHILLIPS. 1969. Separation of cells by velocity sedimentation. *J. Cell Physiol.* 73:191.

MOSIER, D. E. 1967. A requirement for two cell types for antibody formation *in vitro*. *Science (Washington).* 158:1573.

NAOR, D., and D. SULITZEANU. 1967. Binding of radioiodinated bovine serum albumin to mouse spleen cells. *Nature (London).* 214:687.

NOSSAL, G. J. V., K. D. SHORTMAN, J. F. A. P. MILLER, G. F. MITCHELL, and J. S. HASKILL. 1967. The target cell in the induction of immunity and tolerance. *Cold Spring Harbor Symp. Quant. Biol.* 32:369.

PETERSON, E. A., and W. H. EVANS. 1967. Separation of bone marrow cells by sedimentation at unit gravity. *Nature (London).* 214:824.

PLOTZ, P. H., and N. TALAL. 1967. Fractionation of splenic antibody-forming cells on glass bead columns. *J. Immunol.* 99:1236.

RABINOWITZ, Y. 1964. Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns including tissue culture observations. *Blood.* 23:811.

SALERNO, A., and G. M. PONTIERI. 1969. Heterogeneity of populations of antibody-forming cells fractionated by glass bead columns. *Clin. Exp. Immunol.* 5:209.

SHORTMAN, K. 1966. The separation of different cell classes from lymphoid organs. I. The use of glass bead columns to separate small lymphocytes, remove damaged cells and fractionate cell suspensions. *Aust. J. Exp. Biol. Med. Sci.* 44:271.

SHORTMAN, K. 1968. The separation of different cell classes from lymphoid organs. II. The purification and analysis of lymphocyte populations by equilibrium density gradient centrifugation. *Aust. J. Exp. Biol. Med. Sci.* 46:375.

SHORTMAN, K. 1969 a. Equilibrium density gradient separation and analysis of lymphocyte populations. *In* Progress in Separation and Purification. Symposium on Modern Separation Methods of Macromolecules and Particles. Theo Gerritsen, editor. John Wiley and Sons Inc., New York. 2:167.

SHORTMAN, K. 1969 b. The separation of lymphocyte populations on glass bead columns. *In* Progress in Separation and Purification. Symposium on Modern Separation Methods of Macromolecules and Particles. Theo Gerritsen, editor. John Wiley and Sons Inc., New York. 2:91.

SHORTMAN, K., E. DIENER, PAMELA RUSSELL, and W. D. ARMSTRONG. 1970. The role of non-lymphoid accessory cells in the immune response to different antigens. *J. Exp. Med.* 131:461.

SHORTMAN, K., and KATHRIN SELIGMAN. 1969 c. The separation of different cell classes from lymphoid organs. III. The purification of erythroid cells by pH-induced density changes. *J. Cell Biol.* 42:783.

WIGZELL, H., and B. ANDERSSON. 1969. Cell separation on antigen-coated slides. Elimination of high rate antibody-forming cells and immunological memory cells. *J. Exp. Med.* 129:23.