THE ROLE OF NONLYMPHOID ACCESSORY CELLS IN THE IMMUNE RESPONSE TO DIFFERENT ANTIGENS*

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There are at present two different and apparently contradictory theories concerning the role of nonlymphoid cells, particularly phagocytic macrophages, in the initiation of an immune response. One view is that macrophages have an essential role in “processing” antigens and “priming” lymphocytes. It has been suggested that macrophages produce either a modified form of the antigen or some form of ribonucleic acid which triggers or even directs the proliferation and differentiation of lymphoid cells. There is considerable evidence suggesting this type of role (10–12). The other and equally popular view is that macrophages have no specific role in initiating the immune response, their function being limited to that of a scavenger cell. The essential event in initiating an immune response is held to be the direct interaction of antigen with specific, antibody-like receptors on the lymphocyte surface. Again there is experimental evidence favoring this viewpoint (e.g., 7, 8, 13).

We are attempting to directly test these alternative theories, by combining cell separation procedures with a tissue culture study of the immune response. Following the work of Mischell and Dutton (1), Marbrook (2), and Diener and Armstrong (3) we have established tissue culture systems whereby dissociated mouse spleen cells develop an immune response to two different antigens, sheep erythrocytes (SRC) and polymerized bacterial flagellin (POL). Both responses lead to the development of antibody-forming cells, enumerated by the appropriate assays. We have also developed methods of segregating different classes of cells on the basis of density (5), size (4), and active adherence (6), as modified by Shortman et al. (manuscript in preparation). The approach was therefore to separate mouse spleen cells into various fractions and to assess the ability of these, alone or in combination, to give an immune response in culture. A major assumption was that the tissue culture response is a valid reflection of the non-

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eral events in the intact animal. Where possible we have checked this by a com-
parison of the in vitro response with an in vivo response measured by transfer
of cells to an irradiated recipient. The main strength of our approach was the
close comparison of two different antigens, SRC and POL. These were a fortu-
nate choice, since they have provided information relating directly to the two
opposing views on the role of "accessory" cells.

Materials and Methods

Animals.—Nonimmunized, inbred CBA mice aged 90-110 days were the normal source of
spleen cells. Male and female mice were used interchangeably. In some early experiments a
CBA line bearing the T6T6 marker was also used, without apparent effect on the results.
Where C57 mice were used as an alternative source of "adherent" cells, these were inbred
C57BL/Brad males or females.

Preparation of Spleen Cells.—The
animals were heart-bled, and the spleens were removed
aseptically into ice-cold saline. All subsequent procedures were performed aseptically in the
cold. Cells were gently released from the cut end of the capsule by stroking the spleen with
curved forceps. The cells were dispersed with a wide-bore Pasteur pipette into a medium con-
taining 50% fetal calf serum-50% Dulbecco’s salt solution. The following procedure was
adopted to reduce the level of aggregates and fine debris in the suspension. The suspension was
layered above 100% fetal calf serum in a tube, and it was set aside for 5 min to allow large
clumps to settle. The upper zone, containing cells now clear of larger aggregates, was trans-
ferred onto a second 100% fetal calf serum layer in a tube and centrifuged at 400 g for 7 min in
a swing-out head centrifuge. The supernatant was removed (the upper zones containing fine
debris first) leaving a relatively clean pellet of spleen cells.

Density Distribution Analysis.—Full
details of the method and control experiments have
been presented elsewhere (5). Briefly about 5 X 10^9 nucleated spleen cells were dispersed in an
isotonic gradient of bovine plasma albumin (Fraction V; Armour Pharmaceutical Co.; Chicago,
Ill.; pH 5.1, 15-27% w/w) and centrifuged at 4000 g for 45 min at 0-4°C. 15-25 fractions were
collected, the densities of each were determined, and the cells were recovered by dilution and
centrifugation. Total cell counts, smears, and assays for biological activity were then performed
on samples of each fraction. All results are expressed as density distribution functions, plotting
the total number of cells of a given type (or the total biological activity) per density increment
against density. In all cases, the results have been expressed relative to the peak value of 100%,
and the curves have been normalized to the same peak height for each profile. Recoveries of
viable cells were in the range 80-102%.

Size Filtration Through Fine Glass Beads.—This procedure selects cells by size and involves a
weak, physical interaction of cells with glass bead surface at 0°C, as distinct from active ad-
herence. The procedure was less efficient for spleen than for thoracic duct or lymph node cells,
because of the additional debris and damaged cells. The original procedure (4) was modified for
spleen preparations, and full details will be given elsewhere (Shortman et al., manuscript in
preparation). Briefly, after a prior filtration through ~ 150 μ diameter beads, the spleen cells in
16% serum were passed through columns of ~ 75 μ diameter siliconed beads at 0°C at a
relatively fast flow rate (~ 0.5 ml/min). The effluent was the "small lymphocyte" preparation
and represented 5-10% of the nucleated cells from the original cell suspension. In some ex-
periments more extensive depletion of large cells was obtained by lowering the flow rate until
only ~ 3% of the input cells passed through the column.

Removal of Erythrocytes from Small Lymphocyte Preparations.—The effluent from the size
filtration columns were 2-5-fold richer in erythrocytes than the starting material. To reduce the
erythrocyte level, use was made of the fact that at pH 7.0, mature erythrocytes are much more
dense than nucleated cells (14, 25). All procedures were carried out at 4°C. The cells of the effluent fraction were centrifuged, the supernatant was removed, and the pellet was well dispersed in a 3 ml isotonic albumin-salt solution, pH 7.0, density 1.089 g/cm³. 1 ml of the same solution was layered below the cell suspension, 1 ml balanced salt solution was layered above, and the interphases were stirred to produce a more diffuse zone. The sample was spun at 3000 g for 15 min in a swing-out head centrifuge. Mature erythrocytes sedimented to the bottom of the tube, leaving nucleated cells in suspension. The supernatant, removed with a wide-bore pipette, was diluted, and the cells were recovered by centrifugation. Some immature non-nucleated red cells remained with the nucleated cells, but the red cell : nucleated cell ratio was always less than the original spleen suspension. This procedure would also markedly reduce the level of nucleated erythroid cells in the small lymphocyte preparation (25).

**Adherence Column Separation.**—This procedure selects cells on the basis of active adherence to the glass bead surface. The technique has been modified from the Rabinowitz (6) procedure to give more efficient separation with various mouse lymphoid organs. Full details will be presented elsewhere (Shortman et al. manuscript in preparation). Briefly, cells in 50% mouse serum were passed as a band over a period of 10 min through a pre-equilibrated column of ~450 µ diameter siliconed beads at 37°C. The column filtrate represented the “nonadherent” fraction. It contained a ratio of erythrocytes to viable nucleated cells not appreciably different from the starting material. Cells trapped in the column were recovered by first washing them with a salt solution containing the chelating agent ethylenediaminetetraacetate and then by gently disrupting the bead of beads and washing again. Both washes were combined to give the “adherent” fraction.

**Total Nucleated Cell Counts.**—Nucleated cell counts were usually performed in a hemocytometer. Cells from density gradient fractions were counted with a Model B Coulter cell counter, the lower threshold set at 100 µ to eliminate debris and nonnucleated cells.

**Viable Cell Counts.**—The exclusion of eosin was used as a rough measure of cell viability. Cells in a balanced salt solution containing 16% fetal calf serum were mixed with an equal volume of 1% eosin in saline, and the proportion of cells excluding the dye were counted under phase-contrast microscopy after 3 min.

**Differential Counts for Morphological Classification.**—Small samples of cells from each fraction were layered above fetal calf serum in a small tube, and the cells were sedimented. The supernatant was carefully removed from the upper zone first, and the cells were resuspended in a small volume of residual serum. Smears narrower than the width of the slide were prepared, dried, fixed in methanol, and stained with Giemsa. Counts were made, including all cells encountered by proceeding directly across the smear from one edge completely to the other, at positions corresponding to 1/4, 1/2, and 3/4 of the length of the smear. Each count included a minimum of 1000 cells. Conventional morphological criteria were used.

**Counts of Cells Showing Phagocytic Activity.**—In all cases phagocytosis was assessed on cells harvested after 3-5 hr incubation with the appropriate antigen under conditions and antigen concentrations identical with those used for tissue culture. For POL antigen, 125I-labeled polymerized flagellin was used, and phagocytosis was assessed by counting labeled cells after radioautography of smeared preparations. The method could not distinguish between true phagocytosis and surface bound antigen. For SRC antigen, phagocytosis was assessed by counting smeared and stained preparations for cells showing complete SRC within their cytoplasm. Further details are given elsewhere (Shortman et al. manuscript in preparation).

**Antibody-Forming Cell Counts.**—The immune response in culture was assayed by counting the number of individual cells forming antibody against the particular antigen. For SRC antigen, the assay was the formation, in the presence of complement, of plaques of lysis in a monolayer of SRC, using the modification of the Jerne technique introduced by Cunningham and Szenberg (15). This technique should detect only hemolytic antibody, predominantly IgM, released by the cells. For POL antigen, the assay was adherence of motile bacteria to antibody
forming cells using the technique of Diener (16). The test strain was Salmonella derby (strain SW721, H antigen fg; O antigen 1, 4, 12) which shares the H but not the O antigen with the Salmonella strain used as the source of POL. This technique should detect all classes of antibody on the surface of cells.

Treatment of Antibody-Forming Cells with Anti-H2-Isoantisera.—C57 anti-CBA and CBA-anti C57 antisera, together with control C57 and CBA sera, were kindly provided by Dr. G. Mitchell and Dr. Miller and were prepared as described by Miller and Mitchell (28). After harvesting and washing, samples of 8-50 X 10⁶ cells from tissue culture or intact spleen were mixed with 0.15 ml balanced salt solution containing 10% fetal calf serum, 0.05 ml of guinea pig serum as a complement source, and 0.05 ml of test antiserum or control serum. The mixture was incubated at 37°C for 30 min with occasional shaking. After incubation the samples were diluted, and the intact cells were recovered by centrifugation and washed once. This final preparation was then used for the hemolytic antibody-forming cell assay.

Antigens.—Polymerized bacterial flagellin (POL) was prepared from flagellin of S. addaide (strain 1338, H antigen fg, O antigen 35) according to the method described by Ada et al. (17). Sheep erythrocytes (SRC) were stored in Alsever's solution for 1 wk and then washed 4 times in saline before use.

In Vivo Assay for Initiation of Immune Responses.—This assay depended in each case on the formation of foci of antibody forming cells in the spleen of irradiated animals after the injection of cell fractions to be tested together with the appropriate antigen. The response to POL antigen was enumerated using the method of Armstrong and Diener (18). The response to SRC antigen was assayed by the hemolytic focus assay of Kennedy et al. and Playfair et al., carried out as described by Mitchell and Miller (19). The mice used as irradiated recipients ranged from 54-250 days of age but were usually in the range 70-90 days. They were irradiated using a Phillips RT 250 X-ray machine operating at 250 kv, 15 ma, and a HVL of 0.8 mm Cu. The dose was 800 rads at the mid-point of the animal, given at 127 rads/min with full backscatter conditions. The test cells were injected intravenously into the lateral tail vein approximately 4 hr after irradiation. Normally each animal received 3 or 4 X 10⁶ viable cells. In the case of density gradient fractions for the SRC response, a constant proportion of each fraction was injected, the dose corresponding to 3 or 4 X 10⁶ cells if it was assumed that antigen sensitive cells were equally distributed in all fractions. The antigen, 25 µg POL or 0.1 ml of a 20% suspension of SRC, was usually injected with the cells, but was occasionally injected 24 hr later to reduce the background focus count. The number of antibody-forming cell foci per spleen was determined 63/4-7½ days after antigen injection. About 10 irradiated recipients were used for each point. Each experiment included controls for background response in the absence of transferred cells, and this background was subtracted from all experimental values.

Tissue Culture.—The assay for the development of an immune response in vitro to both antigens was the technique of Marbrook (2) and Diener and Armstrong (3) as given in detail by Diener and Armstrong (20). Cultures of the original suspension or various fractions normally contained 12 X 10⁶ viable nucleated cells. This corresponded to ~ 16 X 10⁶ total (viable and nonviable) nucleated spleen cells in the original spleen suspension. The volume of medium in the inner chamber was 2 ml. POL antigen was added at the level of 0.02 µg/ml, or 0.04 µg per culture, SRC at the level of 2 X 10⁶ SRC per ml, or 4 X 10⁶ per culture. Both antigens were normally added to the same culture vessel. A series of control experiments failed to demonstrate any inhibition of the response to one antigen by the presence of the other. All basic findings obtained with both antigens in the same culture have been verified by experiments with the antigens in separate cultures. Cultures were harvested at 3½ days, near the peak of the response, and separate samples were taken for counts of cells forming antibody against the two antigens. Results were expressed either as antibody-forming cells per culture or as antibody-forming cells per 10⁶ viable cells placed into culture. Expression of data per 10⁶ viable cells
harvested at the time of assay gave results which were comparable but about two-fold higher. All cultures were in triplicate or quadruplicate. Variation between cultures in the one experiment ranged from 25 to 34% SD for both assays, leading to an error of ~15% (SEM) for the mean. Thus differences of more than 30% between fractions could be considered significant. The absolute response between one experiment and another varied over a 3-fold range, mainly due to differences between batches of fetal calf serum. Because of a limited supply, some batches of fetal calf serum giving a relatively low response were used in this work. The results remained consistent and independent of the serum used, despite this variation in the absolute response. Background responses in the absence of added antigen were exceptionally low with the batches of fetal calf serum used, being 0.0-0.3 antibody-forming cells per 10⁶ viable cell input in the SRC system, 0.1 antibody-forming cells per 10⁶ viable cell input in the POL system.

RESULTS

Density Distribution of Spleen Cells.—The first method used to obtain a degree of separation of different types of mouse spleen cells was equilibrium density gradient centrifugation in isotonic gradients of albumin. The degree of separation attained on the basis of density differences is depicted in Fig. 1. In general small lymphocytes were found in the central regions of the gradient, the larger dividing lymphocytes in the upper regions. Macrophages were found in the upper regions of the gradient, most polymorphs in the lower regions. There was considerable overlap in the density distribution of these classes of cells. In addition, each given morphological class of cell could be resolved into a number of density peaks. This could be seen more clearly with narrow range gradients giving better resolution and was in accordance with previous observations (5). There is evidence that various density peaks of a given cell class represent different stages in the differentiation of that lineage (21, 22). There was morphological evidence for this in the case of the granulocyte series, the small proportion of cells in the lighter regions being predominantly the immature elements, and the dense major peak being predominantly mature polymorphs.

Density Distribution of Phagocytic Cells.—The data of Fig. 1 include the density distribution of two classes of phagocytic cells, "macrophages" and "polymorphs." This classification was based purely on morphological appearance, and it was important to determine by direct functional testing the cells which would actually phagocytose the two different antigens under the conditions of tissue culture. Accordingly, tests for phagocytosis were performed after 3–5 hr of culture.

There was a marked difference in the types of cells phagocytosing the two antigens in tissue culture. In the case of SRC, all the phagocytic cells could be classed as macrophages or monocytes, with only a small proportion of these representing a doubtful morphological classification. By contrast, 91% of the

1 Shortman, K. 1970. The density distribution of thymic and thoracic duct lymphocytes. Manuscript in preparation.
Fig. 1. The density distribution of CBA mouse spleen cells. The albumin gradient technique is described in Materials and Methods. Total nucleated cell counts were performed on each fraction. Smear and stained preparations were used to assess the proportion of cells of each class in the fraction on the basis of conventional morphological criteria. From this data the total number of cells of each class in each fraction was calculated, and the density distribution was computed. Each curve is normalized so the peak value is the same, regardless of absolute numbers. The relative amounts of each type in spleen suspensions was small lymphocytes, 87%; large-medium lymphocytes, 8.5%; macrophages, 1.8%; polymorphs, 2.8%. No attempt was made to differentiate erythroid from lymphoid cells, the small proportion of erythroid cells being included in the lymphocyte count. The macrophage count included cells with the appearance of blood monocytes. The polymorph count included all cells identified as members of this granulocyte series, mature and immature. A single, typical experiment is presented.
cells scored as phagocytic for POL were clearly polymorphs (mature and immature), 5% were macrophages, and 4% were lymphocytes. It should be noted that this assay could not distinguish surface binding of POL from true phagocytosis. The level of lymphocytes scored as phagocytic was in fact similar to the level of lymphocytes showing specific binding of POL to the external membrane under conditions where phagocytosis was suppressed (13). It is therefore unlikely that any lymphocytes were phagocytosing POL.

![Diagram of density distribution of phagocytic cells](image)

**Fig. 2.** The density distribution of CBA mouse spleen cells showing phagocytic activity in vitro. The broken line gives total nucleated cell distribution, the solid line gives the distribution of active cells. The absolute level of active cells is given in Table I. The assays for phagocytosis are given in Materials and Methods, and other details are as for Fig. 1. Single but typical experiments are presented.

The density distribution of the phagocytic cells reflected the difference in antigen handling seen at the morphological level. Cells phagocytosing SRC (Fig. 2) were found in the upper regions of the density gradient and corresponded to three of the four zones where macrophages in general were found (Fig. 1). Macrophages less dense than 1.053 were inactive. These may have been a different class of cells, or alternatively may have simply represented a region of concentration of damaged macrophages (5). In contrast to results with SRC, cells phagocytosing POL concentrated in the lower regions of the gradient with a spread towards the lighter regions (Fig. 2). In agreement with the morphological data, the density profile of activity followed that of granulocytic cells in general (Fig. 1).
Immune Responses by Different Density Fractions.—The preceding results indicated a separation, with varying degrees of overlap, of different classes of cells into different regions of the density gradient. To determine the effect of this degree of separation on the initiation of immune responses, fractions were assayed.

Fig. 3. The response of density fractions of CBA mouse spleen cells to SRC and POL antigens in vivo and in vitro. Samples of each fraction were assayed in vivo by transfer to irradiated recipients, or in vitro by the tissue culture technique, as described in Materials and Methods. The total activity present in each fraction was then calculated to give the density distribution. Other details are as in Fig. 1. The in vitro assays are individual experiments, typical of many runs. The dominant peak of in vitro response to SRC was always the 1.063 g/cm³ density region. The dominant peak of in vitro response to POL varied from one experiment to another, but all fractions in the density range 1.063–1.075 gave a substantial response in all experiments. The in vivo assays represent single experiments performed with the focus assay. The general outline of both profiles has been confirmed in other experiments using an assay based on counts of antibody-forming cells in the recipient spleens.
in vivo (by transfer to an irradiated recipient) or in vitro (by the tissue culture assay system). A typical series of results is given in Fig. 3, which shows the relative activity in different density regions.

The density distribution of cells initiating immune responses to both antigens in vivo was similar in general outline to the results of Haskill (21, 22). Activity was found over a wide density range, corresponding to lymphocyte-rich regions, but did not coincide with the total lymphocyte profile. A series of peaks was observed, perhaps reflecting different metabolic states of antigen-sensitive cells, as in the rat (21, 22). There was evidence for differences between the antigen-sensitive cell profiles for the two antigens.

The in vitro response to POL likewise fell in the lymphocyte-rich zones and showed a broad density distribution with evidence for heterogeneity. There was no convincing evidence, at this degree of resolution, of a fundamental difference between cells reacting in vivo and in vitro.

The response to SRC in vitro showed a very marked contrast, activity being restricted to the upper regions of the gradient. The activity profile parallels that obtained independently by Haskill, Byrt, and Marbrook (23). This contrast between the in vitro responses to the two antigens persisted even when the same cell samples were assayed with both antigens in the same culture vessel; dense fractions responded to POL but not to SRC.

Two explanations may be offered for the restriction of in vitro response to SRC to the upper regions of the gradient (23). It was possible that after density fractionation only "primed" cells could proliferate in culture, and the correspondence between the regions of large and medium lymphocytes and mitotic figures (Fig. 1) with regions of activity would support this view. However the fact that the more dense small lymphocyte-rich regions responded to POL seemed to contradict this theory. An alternative explanation is that, whereas lymphocytes alone can respond to POL, both lymphocytes and phagocytic macrophages are required for a response to SRC in vitro. This view was supported by the fact that activity was indeed found in regions of overlap between lymphocytes and phagocytic macrophages (Figs. 1 and 2). This hypothesis required that the irradiated host served as a sufficient source of phagocytic cells, so only lymphocytes were required to initiate the response in vivo.

Separation of Lymphocytes and Phagocytes on Adherence Columns.—The density separation technique in one sense provided too detailed a system for testing the above hypothesis by back-mixing fractions, since this could reflect interaction between different density classes of lymphocytes, as well as between lymphocytes and nonlymphoid "accessory cells." To obtain separation of lymphocytes as a class from phagocytes, the principle of active adherence of phagocytic elements to solid surfaces was used. Elsewhere (Shortman et al. Manuscript in preparation.) we have given full details of the separation of mouse spleen cells on large glass bead columns at 37°C. Large, medium, and small lymphocytes all pass through such a column, although there may be some
selective retention of the larger elements. Lymphocytes showing nonphagocytic surface binding of antigen also penetrate the column. The separation of phagocytic cells from the lymphocytes in the column filtrate fraction is shown in Table I. On a morphological basis, the level of macrophages was reduced 14-fold, the level of polymorphs 47-fold. Since retention on the column depended on active adherence, even this low level may have represented inactive phagocytes. In support of this, out of a total of approximately 10^8 scanned, no cells capable of phagocytosing SRC were detected in the column filtrate. A low level of cells in the filtrate did show POL uptake. However, none of these were macrophages. 47% of these were lymphocytes and probably represented cells showing nonphagocytic cell surface binding of the antigen as discussed earlier. The remainder were polymorphs and probably represented true “leakage” of active phagocytes.

### TABLE I

|                     | Total viable nucleated cells | Cells per 1000 nucleated cells in fraction |
|---------------------|-----------------------------|------------------------------------------|
|                     | All lymphocytes  | Macrophages | Polymorphs | Phagocytosis in vitro SRC | POL |
| Original spleen suspension | 100 | 951 | 18 | 28 | 1.19 | 14.1 |
| Filtrate fraction     | 46  | 997 | 1.3 | 0.6 | 0.00 | 0.5  |
| Adherent fraction     | 30  | 876 | 52 | 68 | 1.22 | 15.5 |

Results are the means of three experiments in the case of phagocytic activity, eight experiments in the case of morphological classification.

One limitation of the procedure is also seen from Table I. Although the filtrate fraction was efficiently depleted of phagocytes, lymphocytes still remained the dominant cell type in the adherent fraction, possibly as a result of non-specific trapping. Phagocytic elements were however enriched from three- to fourfold in this fraction.

**In Vivo Responses after Adherence Column Separation.**—The two fractions from adherence column fractionation were compared to the original spleen suspension in their ability to initiate immune responses on transfer to irradiated recipients. The results (Table II) showed that the filtrate fraction contained lymphocytes capable of initiating responses to both antigens and that phagocytic cells were not required in the inoculum for assay in vivo. The partial drop in response to POL after passage through the column might be correlated with some selective loss of larger lymphocytes. In both cases the adherent fraction showed activity, as would be expected from its content of lymphoid cells.

**In Vitro Responses after Adherence Column Separation.**—The two fractions from adherence column fractionation were then compared to the original spleen
suspension in their ability to initiate immune responses in tissue culture. The results (Table III) gave a marked contrast to the in vivo situation and showed pronounced differences between the two antigens. Although the absolute response in culture varied from one experiment to another over the range shown, the following effects were obtained within each experiment, where the response showed little variation (see Materials and Methods).

The filtrate fraction, devoid of detectable phagocytic cells, failed to respond to SRC significantly above background, although it responded fully in vivo. The adherent fraction, containing both classes of cells, responded in culture.

| TABLE II | Initiation of Immune Responses In Vivo after Active Adherence Column Separation |
|----------|-----------------------------------------------------------------------------|
|          | Spleen colonies per 10^6 cells injected                                      |
|          | SRC antigen                  | POL antigen                  |
| Original spleen suspension | 0.60 ± 0.13                  | 0.74 ± 0.14                  |
| Filtrate fraction       | 0.81 ± 0.19                  | 0.40 ± 0.17                  |
| Adherent fraction       | 0.56 ± 0.06                  | 0.52 ± 0.19                  |

Results are the means of three experiments ± SEM.

| TABLE III | Initiation of Immune Responses In Vitro after Active Adherence Column Separation |
|-----------|--------------------------------------------------------------------------------|
|          | Antibody-forming cells per 10^6 viable nucleated cells cultured                 |
|          | SRC antigen                  | POL antigen                  |
| Original spleen suspension | 65 (44-121)                  | 131 (48-286)                |
| Filtrate fraction       | 0.8 (0.0-1.2)                 | 108 (36-285)                |
| Adherent fraction       | 51 (27-155)                  | 71 (18-195)                 |

Results are the means of four experiments. The range of variation from one experiment to another is presented. Within each experiment, results were typical of these means but only varied ± 15% SEM. Background responses in absence of added antigen have not been subtracted. Further details are in Materials and Methods.

In contrast, the filtrate fraction responded well in culture to POL, despite the absence of phagocytic macrophages and the very low level of phagocytic polymorphs. Even the small drop in activity compared with the starting material was no greater than the drop in in vivo activity (Table II), and hence it could not be correlated with the reduction in phagocytes.

This contrast between the cellular requirements for the two antigens, originally obtained when the antigens were tested on separate samples of cells in separate culture vessels, persisted when the antigens were present together with the same cells in the same culture vessel.

Reconstitution of Anti-SRC Activity by Mixing Fractions.—These results sup-
ported the original hypothesis that phagocytic elements were needed as well as lymphocytes for a response to SRC, but not to POL. To check that loss of activity in the filtrate fraction was not due to some form of specific damage to the SRC system, reduced levels of the adherent fraction, enriched for phagocytic elements, were mixed with the filtrate lymphocyte fraction, as shown in Table IV.

Mixing the two fractions gave a marked synergistic effect, restoring activity to near the original level. The nonlinearity of the cell dose-response curve makes estimation of the extent of enhancement difficult. However, even if the contribution of the adherent fraction alone was calculated on the basis of its activity at higher cell levels (as in Table III), a synergistic effect remained.

TABLE IV

| No. of viable nucleated cells into culture | Antibody-forming cells per culture at 3.8 days |
|-------------------------------------------|-----------------------------------------------|
| **Original spleen**                       |                                               |
| 12 $\times$ 10^6                          | 313 (121-529)                                 |
| **Filtrate fraction**                     |                                               |
| 12 $\times$ 10^6                          | 8 (0-18)                                      |
| **Adherent fraction**                     |                                               |
| 4 $\times$ 10^8                           | 4 (0-12)                                      |
| **Mixture:**                              |                                               |
| **Filtrate fraction**                     | 12 $\times$ 10^6                             |
| **Adherent fraction**                     | 4 $\times$ 10^8                              |
|                                           | 303 (72-495)                                 |

Results are the means of three experiments. Other details are as for Table III, but note that results are expressed on a per culture, rather than a per cell basis.

The Origin of Antibody-Forming Cells in Reconstitution Experiments.—The previous mixing experiments were most simply explained by assuming that phagocytic cells in the adherent fraction were "priming" the lymphoid elements in the filtrate fraction to develop into cells forming hemolytic antibody. The response of the filtrate fraction alone to POL antigen and the response to both antigens in vivo supported the view that the filtrate fraction contributed the progenitors of the final antibody-forming cells. However, it remained entirely possible that the actual progenitors of the hemolytic antibody-forming cells were contributed by the lymphocytes in the adherent fraction (or the irradiated host for the in vivo assay), lymphoid cells in the filtrate fraction serving some essential accessory role such as antigen recognition. Such an interaction between different classes of lymphocytes has been described for the SRC response (19, 28). To test this possibility, the adherent fraction from C57 mice was mixed with the filtrate fraction from CBA mice, and the origin of antibody-forming cells resulting from stimulation in culture with SRC was tested with the aid of strain-specific anti-H2 isoantisera. There was no
evidence that the genetic incompatibility of the mixed cells had any effect on
the response in culture. The experiment offered the additional advantage of
negligible response by the C57 phagocytic fraction alone; even unfractionated
C57 spleen cells responded poorly in tissue culture.

The results of a typical experiment are presented in Table V. Control ex-
periments with cells from intact animals demonstrated that the antisera dis-
tinguished clearly between antibody-forming cells of CBA or C57 origin. In
tissue culture, both the CBA filtrate fraction and C57 adherent fraction were
devoid of detectable response. On mixing, a response equivalent to unfraction-
ated CBA spleen cells was obtained. Testing the antibody-forming cells after
harvesting showed they had the characteristics of CBA cells, not those of C57.

It was clear that in this particular mixture the C57 adherent fraction was
truly "accessory," in the sense that all the antibody forming cells originated in
the CBA filtrate fraction. In the previous experiments using CBA adherent
fraction, it was likely that a small proportion of the progenitor cells derived
from the lymphocytes in this fraction, since it alone did give some response in
culture. However this would not alter the essential conclusion that progenitor
cells were present in the filtrate fraction and that they required some accessory
cell, completely removed by the adherence column, in order to respond to SRC
in culture.

Irradiated Spleen as a Source of Accessory Cells.—The fact that lymphocytes

| TABLE V |
| The Source of Antibody-Forming Cells in Mixed Fractions from Active Adherence Columns |

| Test serum | Normal CBA control | Normal C57 control | CBA anti-C57 | C57 anti-CBA |
|-----------|--------------------|--------------------|--------------|--------------|
| Antibody-forming cells/spleen × 10³, assayed | 200 | 187 | 4 | 175 |
| 5 days after SRC stimulation of normal, non-irradiated mice of strain: C57 | CBA | 57 | 78 | 61 | 4 |

Tests on tissue culture cells

| Antibody-forming cells/culture, assayed after 3.8 days of culturing with SRC antigen. |
|------------------------------------------|
| A: Unfractionated CBA spleen cells, 12 × 10⁶ | 290 | 175 | 155 | 0 |
| B: CBA filtrate fraction, 12 × 10⁶ | 0 | 0 | 0 | 0 |
| C: C57 adherent fraction, 4 × 10⁶ | 1 | 0 | 0 | 0 |
| D: Mixture of B + C | 180 | 270 | 290 | 5 |

Cells were treated with specific antisera or control sera after harvesting but prior to antibody-forming cell assay. The results are means of three to four cultures of a single experiment. A second experiment gave identical results, but the tests for antiserum specificity on intact animals were omitted.
alone initiated a response to SRC when transferred to an irradiated recipient and that the same cells required some accessory cell to respond in culture could have two explanations. The additional requirement could be in some sense a tissue culture artifact. Alternatively, the irradiated host could act as a sufficient source of such accessory cells in the transfer situation. To test the latter possibility, intact CBA mice were irradiated exactly as for the in vivo assay, but the spleens were immediately removed and tested for their ability to restore an anti-SRC immune response to a filtrate fraction from nonirradiated animals. The results are shown in Table VI.

Both the filtrate fraction from normal animals and the unfractionated cells from irradiated animals gave no response to SRC alone. On mixing, an immune

| Cell source                        | Antibody-forming cells per culture |
|------------------------------------|-----------------------------------|
| Unfractionated, normal CBA spleen cells | 300                               |
| CBA lymphocytes                    | 0.3                               |
| Irradiated CBA spleen cells        | 0.3                               |
| Mixture:                           |                                   |
| CBA lymphocytes                    |                                   |
| Irradiated CBA spleen cells        | 80                                |

The lymphocyte fraction was the filtrate fraction from active adherence column separation of normal CBA spleen suspensions. Irradiated animals were killed immediately after 800 rads X-irradiation to provide the irradiated spleen cells. 12 × 10⁶ viable nucleated cells were used per culture, or 12 × 10⁶ total nucleated cells in the case of irradiated spleen. The antigen was SRC. The results represent a single experiment.

response was obtained, indicating that the irradiated spleen indeed acted as a source of accessory cells. The activity was not fully restored to that of normal, unfractionated spleen. This might indicate that the accessory cells were partially inactivated by irradiation, a point that would not negate their role as a sufficient source of this activity in the in vivo assay. Alternatively, the large excess of irradiation damaged lymphocytes in the mixture could have caused partial inhibition of the tissue culture response.

In Vivo and In Vitro Responses of Size Filtration Separated Small Lymphocytes.—The adherence column separation allowed testing of the immune responses of all classes of lymphocytes, large, medium, and small, with and without phagocytes. It was of interest to see if the small, normally nondividing lymphocytes would behave in a manner similar to lymphocytes in general, especially in view of the suggestion that under certain conditions most of the response in tissue culture could be due to “primed” cells (23). To obtain a sample of spleen small lymphocytes, the cold, small glass bead size filtration
columns were employed (4, 24). This technique selects by size, not by active adherence. Applied to spleen suspensions, it normally provided a column filtrate consisting of 5-10% of all viable small lymphocytes, with large lymphocytes reduced 30-fold and medium lymphocytes fivefold. The level of macrophages was reduced 10-fold, less than with adherence columns, and the level of polymorphs was not significantly changed.

The immune responses of several such samples of small lymphocytes, compared to the original spleen suspension, are summarized in Table VII. On a cell for cell basis, the response to both antigens in vivo was reduced about one-fifth that of the starting material, or of the wider spectrum of lymphocytes obtained by adherence columns (Table II). A similar drop in response to SRC after column filtration was seen with rat spleen. This result stands in contrast to the unaltered activity of mouse or rat thoracic duct lymphocytes to SRC antigen after size filtration (26, 27). However the spectrum of antigen sensitive cells to SRC in spleen is known to be markedly different from those in thoracic duct (22).

The in vitro response to POL was reduced by size filtration about the same extent as the in vivo response. However, the in vitro response to SRC showed a more drastic drop to at most 4% of the starting material, and less if the background was subtracted. In experiments where size filtration was continued to further deplete the preparation of large cells, this response was entirely eliminated (e.g., Table VIII).

One possible cause for this drop could be the enhanced levels of erythrocytes, and possibly also nucleated erythroid cells, in the column filtrate. Direct addition of similar levels of mouse erythrocytes caused partial inhibition of the response of unfractionated mouse spleen cells to SRC, but not to POL. However, elimination of erythrocytes by a density separation failed to restore the re-

| Antigen                     | In vivo response | In vitro response |
|-----------------------------|------------------|-------------------|
|                             | Spleen colonies per 10^6 viable nucleated cells injected | Antibody-forming cells per 10^6 viable nucleated cells into culture |
|                             | SRC              | POL              | SRC | POL |
| Unfractionated CBA spleen cells | 0.46            | 0.65             | 16  | 86  |
| Filtrate (small lymphocyte fraction) | 0.10            | 0.11             | 0.6 | 11  |
| Relative activity of small lymphocytes | 22%             | 17%              | 4%  | 13% |

Results are means of three experiments in the case of in vivo responses, seven experiments in vitro. Background responses in absence of added antigen have not been subtracted from the in vitro response.
Nonlymphoid Accessory Cells in Immune Response

Such an erythrocyte depleted fraction was used in the experiment of Table VIII.

Restoration of Small Lymphocyte Response by Adherent Cells.—The failure of purified small lymphocytes to respond to SRC in vitro could have been due to the loss of some accessory cell such as phagocytic macrophages, or could indicate that these cells were inherently incapable of a tissue culture reaction. To distinguish between these possibilities, a sample of small lymphocytes was prepared by extensive column size-filtration, and it was depleted of erythrocytes and any nucleated erythroid cells. This fraction gave no response to SRC in culture (Table VIII). The actively adherent fraction from C57 spleen was used as the cleanest source of accessory cells, since it was devoid of any response alone (Tables V and VIII). However, on mixing, an active preparation was obtained (Table VIII). The reconstituted activity was in fact higher than the original unfractionated CBA spleen, an observation possibly related to the removal of inhibitory mouse erythroid cells. In the presence of accessory cells, it appeared that small lymphocytes could respond well in tissue culture.

The Density Distribution of Accessory Cells for In Vitro SRC Response.—The results of two quite different column separation procedures appeared to give good support to the original postulate based on density separation; for in vitro responses lymphocytes alone are sufficient for POL antigen, but there is an additional nonlymphoid cell required for SRC response. However, it remained to be demonstrated that the accessory cell separated by column procedures was in fact the light density cell postulated from density separation. To investigate this aspect, density fractions were tested for their ability to reconstitute a SRC response in vitro to CBA lymphocytes depleted of accessory cells by ad-

| Cell source               | Viable nucleated cells per culture | Antibody-forming cells per culture |
|---------------------------|-----------------------------------|-----------------------------------|
| Unfractionated CBA spleen cells | $10 \times 10^6$                  | 280                               |
| CBA small lymphocytes     | $14 \times 10^6$                  | 0                                 |
| C57 adherent fraction     | $4 \times 10^6$                   | 0                                 |
| Mixture                   |                                   |                                   |
| CBA small lymphocytes     | $10 \times 10^6$                  | 830                               |
| C57 adherent fraction     | $4 \times 10^6$                   |                                   |

Small lymphocytes were purified from CBA mouse spleen by extensive size filtration and were then depleted of erythroid elements by density separation. The adherent fraction from C57 spleen cells was separated in active adherence columns. The results represent a single experiment.
herence column filtration. The spleens from C57 mice were used as a source of accessory cells (see Table V). The spleen cells were not fractionated prior to density separation, since adherence column separation might have altered their density. To ensure that only the accessory cell activity was assayed, all harvested cultures were treated with anti-C57 antisera before assay. The density distribution of the ability to restore activity to CBA lymphocytes is given in Fig. 4.

The results show that the accessory cell activity was separate from the bulk of the nucleated cells and was found in the upper regions of the gradient. Since

![Figure 4](image)

**Fig. 4.** The ability of density fractions of C57 mouse spleen to restore an in vitro anti-SRC immune response to CBA lymphocytes depleted of phagocytic cells. The broken line represents the total nucleated cell distribution, the solid line activity. A constant proportion of each fraction was mixed with a fixed amount of purified CBA lymphocytes; the mixture was cultured and assayed for numbers of hemolysin-forming CBA cells. The total potential number of CBA antibody-forming cells induced per fraction was calculated and used to compute the density distribution profile. Further details are given in the text. The result represents a single fractionation experiment assayed with replicate cultures.

the profile was obtained with C57, not CBA spleen, the results may not be directly comparable with Figs. 1–3. However two general points could be made. First, activity was found in the same general regions as macrophages phagocytosing SRC. Second, an overlap between such a profile and the regions of responsiveness in irradiated hosts to SRC could conceivably generate the density profile of the in vitro response to this antigen.

**DISCUSSION**

In this paper we have attempted to collate those results from the three separation procedures that bear on the question of the role of antigen-processing cells in the immune response. The question of the heterogeneity of the reacting lymphocytes has not been emphasized, although evidence for such heterogeneity is provided by both the density and the size based separation methods. Further study of this aspect, along the lines pioneered by Haskell (22) for the antigen-sensitive cells of the rat, is underway. A related aspect is the possible interaction
between different lymphoid cells. Evidence for such an interaction in the response to SRC has been provided by in vivo studies (e.g., 19, 28) and to some extent from in vitro work (23, 30). Again this aspect will be investigated using the density separation technique, but care will be needed to experimentally separate lymphocyte-lymphocyte interaction from the lymphocyte-accessory cell interaction considered in the present report.

The in vivo studies presented in this report all suggest that only lymphoid elements need to be transferred to obtain an adoptive immune response in the irradiated animals. If accessory, nonlymphoid cells are involved in the response, it appears that these are adequately provided by the irradiated host. The tissue culture system is therefore the clearest way of demonstrating the role of such radiation-resistant elements.

The evidence that some form of accessory cell is required for a response to SRC seems very strong. Our results are in line with those of Mosier (29) who separated adherent and nonadherent components from mouse spleen by successive cultures in dishes and found these components did not respond in culture unless mixed together. Our results also agree with those of Haskill, Byrt, and Marbrook (23) who found a radiation-resistant, light-density cell involved in the response to SRC in culture, but the detailed density profile of these two components does not agree well. Our data show that the cell behaves in several ways (active adherence, size, and density distribution) like phagocytic macrophages. The simplest hypothesis, and the one we set out to investigate, is that the accessory cell is a macrophage involved in antigen processing. However, even if the cell is a macrophage, other roles are possible. For example, Mosier (9) has suggested the need for cell aggregate formation to obtain a response to SRC, and the accessory cell could have a "structural" role here. Other more trivial roles for the cell, such as production of some nutrient not present in the culture medium, seems less likely in view of the good response of lymphocytes to another antigen in the absence of this accessory element.

The results with POL antigen suggest that in contrast to the situation with SRC, no such accessory cell, and in fact no phagocytic components, are required to obtain a response with this system. Elsewhere these findings with POL have been extended to show that macrophages can in fact be inhibitory and that they do not appear to be involved in either immunity or tolerance in vitro. However, it is technically difficult to establish such a negative conclusion with the same precision as a positive dependence, and we would hesitate to advance this view were it not for the almost absolute difference between the two antigens under identical conditions. It still remains possible that some extremely low level of phagocytic activity is adequate but still essential for the

\footnote{Diener, E., K. Shortman, and P. Russell. 1970. Immunity and tolerance in vitro in the absence of phagocytic cells. Manuscript in preparation.}
response to POL. All fractions contained some phagocytic polymorphs, often in minute amounts only, and this cell may serve to process antigen. Alternatively, cells not detected as phagocytic in the first 5 hr of culture may become active at later times. It is also possible that some of the cells tentatively identified as lymphocytes showing binding of POL to their surface membranes actually processed the antigen. However, the absence of any requirement for antigen processing in a response to POL would fit well with the recent results of Ada and Byrt (8), which suggest the fundamental role of direct interaction of antigen with the lymphocyte surface.

Assuming the differences in the cellular requirements for the response to the two antigens are as absolute as the data suggests, it is of interest to speculate on the basis for this radical difference. Three reasons for the difference may be suggested.

(a) Differences in antigen size: The fact that SRC is of cellular, and POL of molecular dimensions might explain the difference. Thus interaction of a recognition unit on the lymphocyte surface with SRC antigenic determinants may be diffusion limited, and the accessory cell may simply serve to reduce SRC to an effective size.

(b) Different immune systems: It may be relevant that the response to SRC in the CBA mouse is thymus dependent, whereas the response to POL is not (31). We may have fortuitously chosen antigens which initiate responses by two radically different systems in the one animal. In the SRC response there may be a requirement for antigen-RNA complexes, or accessory cells may be needed to promote interaction between thymic-derived and other lymphocytes.

(c) Different assay procedures: The plaque assays used for antibody-forming cells differed in several respects. The assay for the SRC response probably measured only \( \gamma M \)-type antibody released from the cell. The assay for POL response would probably have measured all classes of antibody, on the surface of cells. The accessory cell could therefore influence the type or the quantity of antibody produced.

All these possibilities are open to experimental investigation. Further work combining cell separation procedures with tissue culture studies should permit a much more precise and detailed understanding of cell interactions, now considered an integral part of cellular immunology.

**SUMMARY**

Tissue culture techniques were combined with cell separation procedures to investigate the cellular requirements for a response to antigen leading to the production of antibody-forming cells. Mouse spleen was dissociated, and the

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3 Martin, W. J., and J. F. A. P. Miller. 1970. Influence of thymectomy and antilymphocyte globulin on the antibody response to *Salmonella adelaide* flagellin in mice. Manuscript in preparation.
cells were separated into various groups on the basis of density, size, and active adherence. The ability of fractions to initiate a response in vivo, on transfer to an irradiated recipient, was compared to the response in vitro; and this ability was correlated with the presence or absence of phagocytic cells. Two different antigens were studied, sheep erythrocytes (SRC) and polymerized bacterial flagellin (POL).

Density distribution analysis of spleen showed a wide density range of cells responding to both antigens in vivo. The same fractions responded to POL in vitro as in vivo. By contrast, only the light density regions responded in vitro to SRC. Response occurred in regions of overlap between lymphocytes and phagocytic macrophages.

Separation by active adherence on columns of large glass beads gave a preparation containing large, medium, and small lymphocytes but no detectable phagocytic macrophages and very low levels of phagocytic polymorphs. This lymphocyte preparation responded to both antigens in vivo. In vitro it gave a full response to POL, but no response to SRC. Addition of a small quantity of the adherent fraction, enriched for phagocytic cells, restored response to SRC. The use of strain-specific antisera in a mixed culture containing a C57 phagocytic fraction and CBA lymphocytes showed that the lymphocyte fraction contributed the precursors of the final antibody-forming cells. The accessory cells from C57 spleen banded in the light regions of the density gradient where phagocytic macrophages were found. Irradiated spleen cells also activated the lymphocyte preparation, suggesting that the irradiated host provided the accessory cells for the in vivo response to SRC.

Small lymphocytes were purified from spleen by the small glass bead size filtration technique. This sample of small lymphocytes responded less well to POL than the total lymphocyte population, but it responded as well in vitro as in vivo. The small lymphocyte preparation responded in vivo to SRC but not in vitro. Addition of a small quantity of the phagocyte-rich fraction from adherence columns restored the in vitro response to SRC.

The results indicated that phagocytic cells are not required in the initiation of an immune response to POL. By contrast some accessory cell, possibly a phagocytic macrophage, is required for a response to SRC. The basis for this marked difference is discussed.

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