Sedimentation Analysis of the Cells in Mice Required to Initiate an *in Vivo* Immune Response to Sheep Erythrocytes[1] (34988)

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(Introduced by E. A. McCulloch)
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A mouse spleen cell suspension, when mixed with sheep erythrocytes (SRBC) and transplanted into a lethally irradiated mouse, will mount an immune response against the SRBC (1). An interaction between at least two functionally different cells in the transplanted suspension is required to initiate this immune response (2). One cell, the progenitor of hemolysin-producing cells, is found in bone marrow (3) and another cell, whose function is unknown, is present in thymus. Thus, while neither transplanted bone marrow or thymus cells alone can induce an immune response in irradiated mice, mixtures of these two tissues give rise to large numbers of antibody-producing cells. Presumably, organs such as spleen or lymph node that are competent by themselves contain all of the necessary types of cells.

Studies of this complex system should be facilitated by separating the various components of the system so that each can be studied independently. To this end, we used a technique of velocity sedimentation cell separation. Under the conditions of the separation, nucleated mouse cells sediment at a rate determined primarily by their size, although density also makes a minor contribution (4). Separation on the basis of size often yields a separation on the basis of function so that separations of functionally different cells can often be achieved. Thus, different cell types from mouse spleen have sedimentation profiles usually consisting of a single peak and have characteristic sedimentation velocities (s values): 19S antibody-producing cells (4.7 mm/hr), 7S antibody-producing cells (4.4 mm/hr), erythrocytes (2.0 mm/hr), and granulocytes (two peaks at 4.3 mm/hr and 5.9 mm/hr) (5).

This paper describes experiments which determine the sedimentation profiles of two cell types required for the initiation of the immune response. One of these cell types, the direct precursor of the antibody-producing cell, is tentatively identified as also being capable of binding antigen.

Materials and Methods. F1 hybrid mice of genotype C3H/HeOci × C57BL/60ci have been used throughout. They were bred in the animal colony at the Ontario Cancer Institute. Irradiated mice were kept three to a cage and allowed free access to food and water. Sheep erythrocytes (SRBC) have been used as antigen throughout. These were washed three times in phosphate buffered saline (PBS) before use.

Spleen cell suspensions were prepared by putting the spleens in PBS, cutting them into small pieces with scissors, and aspirating the pieces through a 1-ml syringe. After allowing chunks to settle out, the resulting suspension was washed by centrifugation at 250g. The pellet was resuspended and filtered through a 60-mesh stainless-steel screen or, more recently, a 37-μ capillary array filter (Mosaic Fabrications, Sturbridge, Mass.). Bone marrow suspensions were prepared by cutting the ends off femurs and aspirating PBS through them. After allowing chunks to settle out, the suspension was washed and filtered as above. Thymus cell suspensions were prepared by forcing thymus tissue through a fine screen (80 mesh).

The velocity sedimentation cell-separation

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technique is described in detail elsewhere (4); only a brief description is given here. First, the cells suspended in 0.2% bovine serum albumin (BSA) in PBS, are loaded through the bottom of the sedimentation chamber. This is a cylindrical tank, either 12 or 24 cm in diameter, with a conical bottom having a hole at the apex of the cone. Next, a nonlinear gradient of 0.3–2% BSA in PBS is introduced slowly under the cell suspension. This lifts the cells to form a thin band near the top of the chamber. The cells are allowed to sediment under the action of gravity for 3–4 hr at 4°, and fractions, usually about 30 in number, are collected through the bottom.

The ability of various tissues or tissue fractions to initiate an immune response has been assayed by transplantation. The cells to be tested were injected intravenously, along with 10^8 SRBC, into lethally irradiated (950 rads) mice (1). Eight days later the spleens of the recipient mice (five or more per point) were assayed for 19S antibody-producing cells (plaque-forming cells or PFC) using the Jerne plaque assay (6). The geometric average of the number of PFC/spleen was taken as a measure of the ability of the injected cells to initiate a primary immune response.

When assaying the fractions from a cell-separation experiment, fixed volumes from each fraction, the same for all fractions, were tested. The reason for assaying a fixed volume of each fraction rather than a fixed total number of cells was to determine directly the actual shape of the sedimentation profile of the active cells (*i.e.*, the number of active cells per sedimentation velocity interval) rather than the frequency of active cells relative to other cells present.

Rosette-forming cells (RFC) were assayed by taking 2×10^6 nucleated cells from the unfractionated or fractionated tissue to be tested, spinning out the cells at 250*g*, resuspending the pellet in 1 ml of 1% BSA in PBS containing 2×10^7 SRBC, and incubating overnight at 4°. Rosettes were counted using a special hemocytometer with a capacity 100 times that of a clinical hemocytometer. Any cell with four or more SRBC on its surface was scored as a rosette.

Results. The initial experiments were to determine those fractions from the spleens of unimmunized mice that could initiate an immune response upon injection into lethally irradiated recipients. A typical experiment, from numerous replicates, is shown in Fig. 1A. In this experiment a total of 3.2×10^8

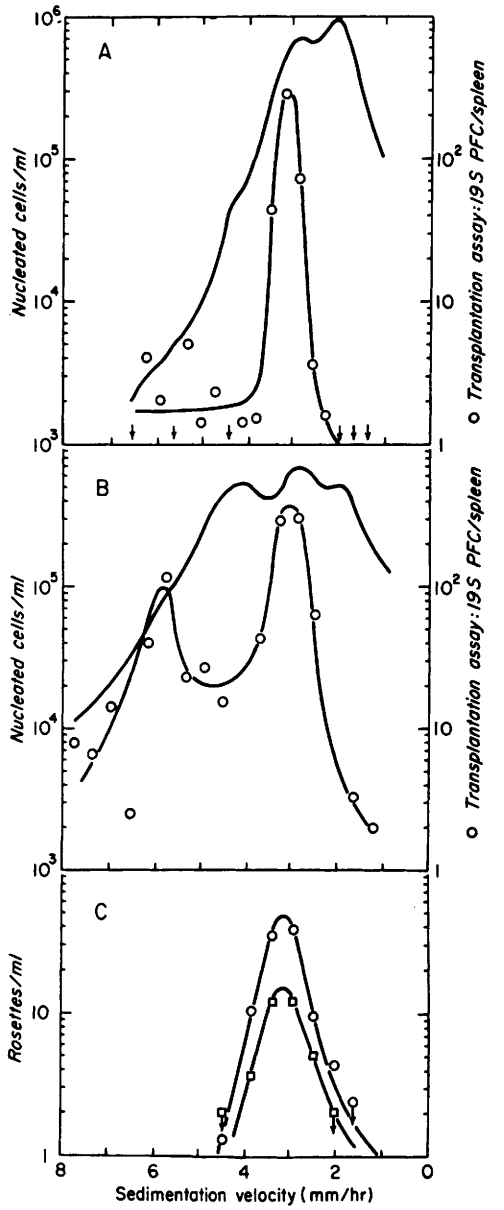

FIG. 1. Sedimentation profiles comparing the position of immunocompetent cells in spleen (A) and bone marrow (B) with the position of rosette-forming cells (C) in spleen (circles) and bone marrow (squares). All donor mice were unimmunized.

nucleated cells was sedimented, and the fractions were assayed for immunocompetence by transplantation. Each recipient mouse received 1/6 of the cells in one of the fractions. The solid line indicates the nucleated cell distribution. The peak at 3 mm/hr includes small lymphocytes and the shoulder at about 4.5 mm/hr is due to granulocytes. The peak at 2 mm/hr represents damaged cells as none of the cells in this region will concentrate fluorescein diacetate (7).

The solid line for which the data points are shown gives the activity obtained in the transplantation assay. Unexpectedly, most of the activity (80% of the unfractionated control) was recovered in a single, symmetric peak at a sedimentation velocity (s value) of 3 mm/hr. Our working hypothesis was that spleen contained two different cells necessary to initiate an immune response to SRBC, and we had hoped to separate these two cells by velocity sedimentation. A complete separation of the two cells would have made all of the fractions inactive in the transplantation assay. The finding that most of the activity can be recovered in a few fractions indicates either that only one type of spleen cell is required to initiate the immune response or that the two cells have the same s value. The available evidence (2, 3) made us believe the latter.

Bone marrow and thymus are each thought to supply one of the cell types required for initiation of a primary immune response (2). If the two cell types in spleen have the same s value, the active cells in bone marrow and thymus might also be expected to have similar s values. Thus, if bone marrow is sedimented and each of the fractions mixed with thymus cells and SRBC, only those fractions in the $s = 3$ mm/hr region should have activity in the transplantation assay. Here, the thymus is being used as a probe to find the active cells in bone marrow. Figure 1B shows an experiment testing this possibility; 3.7×10^8 nucleated bone marrow cells were sedimented, and each of the fractions was mixed with thymus cells and SRBC. The mixtures were injected into lethally irradiated mice. Each recipient mouse received 5×10^7 thymus cells, 10^8 SRBC, and 1/6 of the cells in one of the fractions. The solid line again represents the nucleated cell distribution. The distribution is similar to spleen, the major difference being that the granulocyte peak ($s = 4.5$ mm/hr) is now more pronounced. The distribution of activity obtained in the transplantation assay has two peaks. The major peak has both s value and shape quite similar to the activity profile of spleen cells (see Fig. 1A). The activity in this peak is about 85% of that in the unfractionated bone marrow–thymus control. We concluded that the active cell in bone marrow has an s value of 3 mm/hr.

In Fig. 1B there is also a small peak of activity at an s value of 5.5 mm/hr. Its position and shape correspond closely to a doublet cell population observed microscopically. Therefore, we tentatively assume the peak is due to doublet formation between pairs of cells one of which is the active cell in bone marrow that sediments as a singlet at 3 mm/hr.

We next tested whether the active cell in thymus also has an s value of 3 mm/hr. As above, the procedure was to fractionate thymus (a total of 1.5×10^9 nucleated cells) and assay the fractions, mixed with bone marrow and SRBC, for activity in the transplantation assay. Each recipient mouse received 5×10^6 bone marrow cells, 10^8 SRBC, and 1/6 of the cells in one of the fractions. A major peak of activity is seen at 3 mm/hr (see Fig. 2) as expected. The activity corresponds to about 70% of that in the unfractionated bone marrow-thymus control. As in the bone marrow fractionation, there is a secondary peak of activity although here it has a slightly greater s value (about 6 mm/hr) and does not appear to be associated with doublets. We do not know the

The cells started in a narrow band (located at the zero of the sedimentation velocity scale) and sedimented for 3–4 hr at 4° before fractions were collected. The solid lines in A and B indicate nucleated cells/ml in each fraction; the circles are activity obtained on transplantation (see text). The nucleated cell distributions are not shown in C, but were similar to those of A and B. The arrows in A indicate values off scale. The symbols with arrows in C indicate upper limits; no rosettes were seen in these fractions.

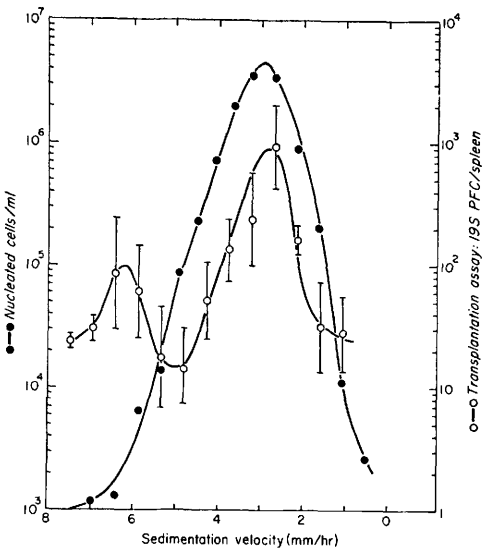

FIG. 2. Sedimentation profile showing the position of immunocompetent cells in thymus. See Fig. 1 caption and text.

origin of this peak but are currently investigating the hypothesis that the cells in it are precursors of the active cells at 3 mm/hr.

We conclude that the initiation of the immune response to SRBC requires two different cells, neither of which can be provided by an irradiated mouse. Both cells occur in spleen but are found separately in bone marrow and thymus. The two cells have the same *s* value (3 mm/hr), quite different than that of 19S antibody-producing cells (4.7 mm/hr).

It would be desirable to have methods for detecting and enumerating the two cell types directly and separately rather than through the synergistic production of antibody-producing cells after transplantation. A possible candidate for one of the cell types is the rosette-forming cell (RFC), a cell which is capable of binding red cells to its surface under various conditions *in vitro*. Although most of the RFC in an immunized animal are probably antibody-producing cells (8, 9), the RFC in unimmunized mice may not be. RFC in unimmunized mice are correlated with tissues that also contain progenitors of antibody-producing cells; they are found in the spleen and bone marrow but only to a very limited extent in the thymus (10). If RFC are one of the two types of cells required to initiate the immune response, the sedimentation profile of RFC should coincide with the activity profiles shown in Figs. 1A and 1B. The data in Fig. 1C confirm this prediction. This figure gives the results of two separations using tissue from unimmunized mice, one with bone marrow (squares) and one with spleen (circles). In each experiment fractions were mixed with SRBC to form rosettes. The distribution of cells able to form rosettes is the same for both tissues and also corresponds to the distribution of active cells in each tissue.

In other experiments with RFC we observed that there are two morphologically distinct types of rosettes. In unimmunized mice, RFC bind large numbers of SRBC, completely obscuring the central nucleated cell; we call this type a "raspberry" RFC. In immunized mice, however, most of the RFC bind only a few SRBC under our conditions, and only a few RFC are of the raspberry type. Sedimentation analysis of RFC from immunized mice shows that the nonraspberry types of RFC have a sedimentation profile identical to that of 19S-PFC (peak *s* value of 4.7 mm/hr).

Discussion. It appears clear that spleen contains two radiation-sensitive cells, both of which are required for an immune response. Either or both of these cells has specificity (11, 12). The data of this paper suggest that the same two cells are found separately in bone marrow and thymus and we shall assume that this is so. It has been proposed that small lymphocytes are the presursors of antibody-producing cells (13). Both functional cells sediment with an *s* value that is consistent with this interpretation.

For ease of discussion, we call the functional cell common to bone marrow and spleen an R cell, and the functional cell common to thymus and spleen a T cell. With the data in this paper and the data of others it is possible to state several properties of R cells. In bone marrow-thymus synergism, bone marrow is known to provide the progenitor of the antibody-producing cell (3). Thus, by its definition, the R cell is the progenitor of antibody-producing cells. Several observations suggest that the R cell and the RFC

found in the spleen and bone marrow of unimmunized mice are the same cell. The sedimentation profiles of R cells (as measured by transplantation activity) and of these RFC are very similar. This suggests that they may be the same cell and implies that the R cell is specific since RFC are known to be specific (10). Observations with other antigens (14–16) are also consistent with the hypothesis that progenitors of antibody-producing cells specifically bind antigen.

Summary. The cells involved in the initiation of the immune response in mice to sheep erythrocytes have been investigated by velocity sedimentation cell separation at unit gravity. The fractions obtained were assayed for immunocompetence by transplantation with sheep erythrocytes into lethally irradiated mice. Immunocompetent cells in mouse spleen were found to sediment as a single band with a sedimentation velocity of 3 mm/hr. Bone marrow and thymus are individually inactive in the transplantation assay but when mixed together produce a large immune response. It was shown that when bone marrow is sedimented, mixed with thymus, and assayed by transplantation, the major peak of activity is at 3 mm/hr. Thymus fractions, when mixed with bone marrow, also show a peak of activity at 3 mm/hr. From this it was concluded that the activity in spleen is probably due to two overlapping populations of cells at 3 mm/hr. It was shown that rosette-forming cells in both the bone marrow and spleen of unimmunized mice also have a sedimentation velocity of 3 mm/hr and it is inferred that these cells may be the direct progenitors of antibody-producing cells.

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