Separation of Immunocompetent Cells From Human and Mouse Hemopoietic Cell Suspensions by Velocity Sedimentation

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Cell suspensions from human and mouse bone marrow, heterogeneous with respect to size, were physically separated by a technique of velocity sedimentation at unit gravity. Fractions were collected, cell counts performed, and a sedimentation "profile" obtained. Fractions were assayed in culture for response to phytohemagglutinin and to allogeneic irradiated leukocytes. The peak activity in these assays occurred in fractions taken from the region of small, slowly sedimenting, nucleated cells, which in humans have an average sedimentation velocity at 4°C of approximately 3.5 mm/hr. The results suggest that velocity sedimentation can be used to separate human stem cells from cells that cause graft-vs.-host disease.

Transplantation of human marrow is frequently complicated by serious graft-vs.-host (GVH) disease. It has been suggested that this complication might be prevented or lessened by physically separating immunocompetent cells from donor marrow before grafting. Separation procedures, however, depend on the availability of suitable functional assays for the cell classes required for successful engraftment and those responsible for GVH disease. Separation of mouse marrow can be monitored by procedures directly associated with these functions; spleen colonies provide a measurement of hemopoietic stem cells, while cells causing GVH disease can be identified by their lethal effects or other parameters of immune responsiveness under genetically controlled conditions in cell transfer experiments. Equivalent validation for separation procedures using human marrow is not available, since only cell culture methods are applicable as assays. However, a comparison of the results obtained using such culture assays applied to separated suspensions of human and mouse cells may provide the needed bridge between the in vivo mouse results and clinically useful separation procedures.

It is the purpose of this paper to present such a comparison. Cells responsive to stimulation by phytohemagglutinin (PHA) or by allogeneic irradiated leukocytes (the one-way mixed leukocyte reaction or MLR-1) were measured in fractions of human and mouse marrow separated by velocity sedimentation using the method of Miller and Phillips. The results are compatible with the view that the human cells detected by PHA stimulation or by the MLR-1 have...
a sedimentation velocity similar to the cells responsible for GVH disease, and consequently these assays may be employed to monitor separation of human marrow to be used for transplantation.

MATERIALS AND METHODS

Preparation of Cells

Human marrow with morphologically normal myeloid and lymphoid cells was obtained from patients undergoing investigation of anemia or from patients with solid tumors (baseline prior to chemotherapy) by aspiration either from sternum or from iliac crest; the volume obtained was usually 3-4 ml. It was placed into a sterile plastic tube (Falcon) containing 1 ml phenol-free heparin (100 U/ml) and centrifuged at 150 \( g \) for 3-5 min at room temperature. The buffy coat was removed and suspended in CMRL 1066 tissue culture medium containing penicillin (100 U/ml) and streptomycin (100 \( \mu g/ml \)). The cells were washed by a further centrifugation and resuspended in 0.3% human serum albumin (HSA) in 1066. Total and nucleated cell counts were performed using a standard hemocytometer.

Mouse marrow was obtained from adult CBA/1 and C57BL/6JOCi animals in the usual manner by washing out the femoral diaphyses with 1066. The cells were washed, resuspended in 3% heat-inactivated fetal calf serum (FCS) in 1066, and counted.

Human peripheral blood from normal subjects was mixed with a small amount of heparin and allowed to sediment at 37\( ^\circ \)C for 60-90 min. The leukocyte-rich plasma was removed, washed, and diluted appropriately in 0.3% HSA/1066.

Mouse spleens were dissected from freshly killed animals, and the cells teased from the stroma and stirred in a flask for 30 min to obtain a suspension of single cells, which was then washed and resuspended in 3% FCS/1066.

Sedimentation of Cells

The procedure has been described previously. Briefly, the cell suspension was loaded into the sedimentation chamber from the bottom, and this was followed by introduction of the gradient (0.5%, 1%, and 2% HSA/1066 for human cells; 5%, 15%, and 30% FCS/1066 for mouse cells) according to the buffered-step method. The entire sedimentation was carried out at 4\( ^\circ \)C for a period of 3-4 hr.

Fractions of equal volume were collected, and small aliquots removed for cell counts. Cell counts and determinations of cell volumes were performed with an electronic cell counter and pulse height analyzer (Technical Measurement Corporation, Model 403). Nucleated cell counts were obtained with 1% eosin or Zap-Isoton (Coulter).

Cell Cultures

PHA: After the sedimentation profile was determined in a given experiment, replicate cultures were set up from individual fractions or, when necessary because of low cell numbers, from pools of several successive fractions. All cultures in a given experiment contained approximately equal cell numbers. For human marrow and peripheral blood this was usually 1 \( \times \) 10^6 nucleated cells in 2 ml of 20% pooled human serum in 1066, while cultures of mouse marrow and spleen contained 5 \( \times \) 10^6 nucleated cells in 2 ml of 20% FCS/1066. (It is customary to express results of PHA and MLR-1 assays in terms of number of mononuclear cells cultured. This usage was deliberately avoided in the present experiments, since fractions obtained from the region of more rapidly sedimenting cells contained predominantly polymorphonuclear cells.) Each PHA tube was inoculated with 0.02 ml PHA (Wellcome). Unsedimented leukocytes were also cultured with and without PHA.

MLR-1: Cultures of human cells from the various fractions were set up in a similar manner, usually containing 1 \( \times \) 10^6 nucleated cells in 2 ml of 20% autologous plasma. Unfractionated allogeneic peripheral blood leukocytes were used as stimulating cells in a
concentration of $1 \times 10^6$ irradiated (2500 R) leukocytes per culture. Unfractionated responder leukocytes were also cultured alone and in the presence of irradiated allogeneic leukocytes.

For mouse marrow or spleen, control cultures contained $5 \times 10^6$ nucleated CBA cells from the various fractions in 2 ml of 20% FCS. MLR-1 cultures contained $2.5 \times 10^6$ fractionated CBA cells as responders and $2.5 \times 10^6$ unfractionated, irradiated (2500 R), nucleated C57BL cells from the corresponding organ as stimulators.

Irradiation of cell suspensions was carried out in a $^{137}\text{Cs}$ apparatus at a dose rate of approximately 100 R/min.

All cell mixtures were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and air.

**Termination of Cultures**

Preliminary experiments indicated that the optimal duration of culture for human cells was 3 and 7 days for the PHA and MLR-1 assays, respectively. In the mouse, these times were 2 and 4 days, although PHA cultures in some experiments were terminated at 4 days. On the day of harvest, 2.5 μCi of $^3\text{H}$-thymidine (Amersham/Searle, specific activity 19.7 Ci/mM) were added to each culture. Labeling time was generally 4 hr; in some of the MLR-1 experiments with human cells it was 20–24 hr. Nonradioactive thymidine, 0.2 mg, was added to each tube at the end of the labeling period. The tubes were centrifuged, the supernatant aspirated, and the cells washed with phosphate-buffered saline (PBS). The contents of each tube were collected on a Whatman glass fiber filter paper (GF/A, 2.4 cm diameter). Each paper was then washed twice with cold PBS, cold 5% trichloroacetic acid, and absolute methanol. The filters were dried, placed in counting vials with 10 ml Omnifluor, and counted for 1 min each in a liquid scintillation spectrometer (Picker).

**Expression of Results**

Although all cultures contained approximately equal numbers of nucleated cells, the single or pooled fractions from which these cultures were set up contained varying numbers of cells. Thus the total activity, in counts per minute (cpm), for an entire fraction was calculated as follows:

$$\frac{(\text{cpm/culture}) - (\text{cpm/blank})}{\text{nucleated cells/culture}} \times \text{nucleated cells in entire fraction}$$

(The assumption has been made that the assay would remain linear if conditions existed for culturing all the cells in a given fraction.)

The arithmetic means of these values for replicate cultures were used to plot the points in the figures showing total activity per fraction.

**RESULTS**

A typical sedimentation profile for human marrow is shown in the upper left panel of Fig. 1. There are two major nucleated-cell peaks, with modal sedimentation velocities of 3.9 and 7.5 mm/hr, respectively. The absolute sedimentation velocities vary from one individual to another and with small changes in the temperature of the cold room, but their relative positions are always the same in normal subjects. The larger, faster sedimenting cells morphologically resemble granulocytes and granulocytic precursors. Erythroblasts and normoblasts reach their highest concentrations in the trough between the two major nucleated-cell peaks.

The slower sedimenting nucleated-cell peak consists almost entirely of small, round, mononuclear cells with little cytoplasm, i.e., small lymphocytes. The erythrocytes sediment at a slightly slower rate, with a modal velocity in
this experiment of 3.6 mm/hr. The fourth major peak consists of cellular debris and platelets; these invariably sediment at a velocity less than 1 mm/hr.

The responses, as measured by $^3$H-thymidine uptake, of fractions taken from this distribution to PHA are shown in the lower left panel of Fig. 1. The width of each interval along the abscissa indicates the number of fractions pooled from the original distribution; thus the largest pool consisted of four fractions (fractions 16–19) from the original distribution. The total activity per fraction was calculated for each culture (as described in Materials and Methods), and the points plotted are the arithmetic means for each pooled fraction.

The peak of activity in this assay occurs in fraction 15, the same fraction in which the peak of small lymphocytes occurs. The modal sedimentation velocity for both peaks is 3.9 mm/hr. While some response to PHA does exist in the region of the profile occupied by larger nucleated cells, this is much lower than that seen at the peak of activity. In addition, the $^3$H-thymidine uptake for control tubes in the region of larger nucleated cells is fairly high, presumably due to the presence in these cultures of relatively primitive hemopoietic cells capable of undergoing several divisions in culture.

Since it had been shown in the mouse that in vivo GVH activity was also localized to the region of slowly sedimenting (3 mm/hr) cells, it was of interest to determine whether the cultural assays, when applied to mouse hemopoietic cells, would yield similar results. Accordingly, mouse marrow cells were subjected to velocity sedimentation, yielding a profile as shown in the upper right panel of Fig. 1. The lower right panel indicates the total $^3$H-thymidine incorporation per fraction for various fractions cultured with PHA (closed triangles) and with allogeneic irradiated marrow cells (crosses). The peak activity in the MLR-i occurs in fraction 15 of the original distribution (corresponding to a velocity of 3.1 mm/hr), while the peak of PHA responsiveness is one fraction to the left of this (sedimentation velocity of 2.5 mm/hr). There is again very little activity in the fractions containing larger cells.

Using these two assays (PHA and MLR-1), 18 experiments were carried out on cell suspensions separated in this manner. The cells used included human marrow, human peripheral blood leukocytes, mouse marrow, and mouse spleen cells. Table 1 summarizes our experience. In most instances, the modal velocity of the cells responding in the assay was the same as the modal velocity of the slowly sedimenting cells. In only one experiment did the modal velocities differ by more than 0.6 mm/hr.

It will be noted that the range of modal sedimentation velocities of lymphocytes is greater among humans than among inbred mice, which is not surprising, but in each case the sedimentation velocity of the large cells changes proportionally, so that the two peaks are in the same relative position. In addition, the PHA- and MLR-responsive cells occur in the same relative region of the sedimentation profile for both species.

DISCUSSION

Velocity sedimentation offers a convenient means of separating relatively large numbers of cells primarily on the basis of size. Thus a functional
Fig. 1. (A) Sedimentation profile of normal human marrow after removal of most erythrocytes. Modal sedimentation velocities of major populations are indicated (arrows). Solid line, nucleated cells; evenly dashed line, total cells in fractions 11-19 (= nucleated cells + erythrocytes); variably dashed line, cellular debris in fractions 20-24. (B) Response of single and pooled fractions along profile to PHA in 3-day culture, as measured by 3H-thymidine incorporation. Widths of intervals correspond to fractions pooled from initial profile. All cultures contained 1 x 10^6 nucleated cells. Crosses, PHA cultures; open circles, control cultures; blank tube, 28 cpm. (C) Sedimentation profile of normal mouse (CBA) bone marrow. Explanation of symbols same as in (A). (D) Response to various (legend continued at bottom of facing page)
Table 1. Comparison of Modal Sedimentation Velocities of Slowly Sedimenting Cells and Cells Reactive in Assays for Immunocompetence

<table>
<thead>
<tr>
<th>Cell Suspension</th>
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<th>Modal V, of Slowly Sedimenting Cell Population</th>
<th>Modal V, of Cell Population Reactive in Assay</th>
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*V, sedimentation velocity (mm/hr).

separation can be obtained between classes of cells that differ in both size and function. Heterogeneous cell populations that have been separated successfully by this method include marrow cells of the guinea pig, rat, mouse, and man; mouse spleen; human peripheral blood; and mouse spermatogonia.

The work of Worton et al. and of Phillips and Miller has shown that this procedure can be used in mice to separate hemopoietic stem cells from immunocompetent cells and thus obtain successful marrow transplants without GVH disease.

The purpose of the experiments reported in this paper was to establish cultural assays that could be used to monitor GVH activity in separations of human and murine cells, thereby providing a bridge between the in vivo results in the mouse and clinical application of the separation procedure for human bone marrow transplantation.

While the exact mechanism of PHA-induced transformation remains to be elucidated, there is general agreement that the in vitro response to this substance usually correlates with thymus-dependent cell-mediated immunocompetence. The MLR-1 has been shown to correlate well with major histocompatibility differences and is felt to represent an in vitro model for the homograft reaction, one type of cell-mediated immunity. The fact that the single and pooled fractions to allogeneic (C57/Bl) irradiated marrow cells (crosses) and to PHA (closed triangles), both in 4-day cultures. Open circles, control cultures. All MLR-1 cultures contained 5 × 10⁶ nucleated cells. Blank tube, 22 cpm.
class of cells responding in these two assays sediments, in both mouse and man, at the same relative velocity as the class of cells responsible for GVH disease in the mouse supports the use of these assays to monitor this function (GVH activity) in separations of human marrow. Dicke and co-workers have previously shown a correlation between the PHA assay and acute GVH disease using density gradient centrifugation. They have also applied this method of separation to human bone marrow transplantation with at least one long-term success to date, although the mildness of the GVH disease seen in this recipient could have been due to HL-A identity with the donor.

The second major functional cell type to be monitored in marrow transplantation is that class of cells required for successful engraftment (i.e., pluripotent stem cells). In both the mouse and the human, assays are now available that detect hemopoietic cells capable of forming granulocytic colonies in culture (colony-forming units in culture, or CFU-C). There is evidence that these cells, while not identical to the pluripotent stem cells (CFU-S) detected by the spleen colony technique, are closely related to the latter and are probably early differentiated descendants of them.

Worton et al. have shown in mouse marrow that CFU-S and CFU-C sediment with the population of larger cells. Others have recently demonstrated that the same is true of human CFU-C. Thus, this cultural assay provides for the indirect monitoring of the cell class required for engraftment.

The fact that, in all of the assays studied (in vivo assays in the mouse, cultural assays in mouse and in man), colony-forming cells are among the more rapidly sedimenting population, while immunocompetent cells are among the more slowly sedimenting population, suggests that velocity sedimentation might provide a useful and practical method of processing human donor marrow to decrease the severity of GVH disease in the recipient. The majority of rapidly sedimenting cells could be infused into the recipient, with the majority of slowly sedimenting cells being discarded. The apparatus has been suitably scaled up and tested and clinical experiments to test this hypothesis are currently under way at our institution.

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