Two-Dimensional Separation of Embryonic and Adult Colony Forming Units. A Study of Differentiation in Hemopoiesis[1] (35832)

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The earliest detectable embryonic spleen colony forming units (CFU-S) in fetal liver can be characterized by density and volume as being distinct from those which develop in later embryonic life or are present in the adult animal (1–3). Embryonic CFU-S are known to be more sensitive to drugs such as tritiated thymidine (4), to possess a lower seeding efficiency to the spleen (2, 5), to have different self-renewal properties (6), and have been reported to have a lower sensitivity to suppression in a polycythemic environment (7). It is the purpose of this communication to describe a two-dimensional separation of embryonic and adult CFU-S accomplished by sequential density and velocity separations. Each of the populations which it has been possible to separate have been characterized in terms of volume, sensitivity to tritiated thymidine suicide, sensitivity to suppression in polycythemic recipients, self-renewal properties, and seeding efficiencies to the spleen. The results represent a developmental sequence and have been summarized in a fingerprint of the adult and embryonic CFU-S.

Materials and Methods. Mice. C57Bl/6 male (Jackson Laboratories), at 8 weeks of age, were used as the recipients for bone marrow and liver. Animals were irradiated to 850 rad using an Atomic Energy of Canada γ cell 20 at a dose rate of 100 rads/min. At this irradiation dose, background level of colony formation was less than 0.2 CFU/spleen. Timed pregnancies obtained from Jackson Laboratory were used as the source of the embryonic liver CFU-S. Spleens were fixed in Bouin's fixative to facilitate counting of surface nodules.

Plethorization. Animals were rendered polycythemic by injections of 0.5 ml of freshly washed packed red cells on days 0 and 2. Recipients were randomly checked for effective plethorization by hematocrit measurement. In all cases, the plethorized recipients had hematocrits greater than 0.5 on day of sacrifice.

Cell separations. The equilibrium density gradient centrifugation method was carried out as described elsewhere (8). The velocity separation method was that employed by Miller and Phillips (9) using fetal calf serum (Armour Pharmaceutical Co.) and Eagle's minimal essential medium (Grand Island Biochemical Co.) buffered with Tris to pH 7.4 as the gradient material (3). Because the density separation conveniently separates a much larger number of cells ($1–10 \times 10^8$), this method was always used as the first separation stage.

Cell volume determinations. Volume measurements of cells were carried out using the Tris buffered Eagle's MEM solution and a Nuclear Data pulse height analyzer which had been matched to a Coulter transducer with a 100-μ aperture (9). The aperture and pulse height analyzer were standardized using fresh human erythrocytes.

Self-renewal. Experiments designed to study the ability of either embryonic or adult CFU/S to self-renew in irradiated recipients, that is to produce progeny of identical physical parameters, were carried out by transplanting approximately 2×10^6 embryonic liver or bone marrow cells, either from the unfractionated material or from density separated populations, into 850 R irradiated

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recipients for 7 days. Spleens from these animals were removed, teased, and subjected to a second separation on the basis of velocity. The fractions were than assessed for their content of CFU-S in the usual manner.

Tritiated thymidine suicide. Liver or bone marrow cells (1×10^6) were cultured for 30 min in the presence or absence of 100 μCi/ml of ^{3}H TdR (Amersham 6.3 mCi/mm), in thymidine-free Eagle's MEM medium, or 1 μg/ml of vinblastine (Velb, Eli Lilly and Co.). Cells were washed twice in medium containing thymidine before injection.

Results. Embryonic liver, at 19 days of gestation, contains three major CFU-S populations (2), of which one of these, the light density component, is the only one detectable at 10 days of gestation (Fig. 1). Early and late embryonic populations were compared in their ability to be suppressed in polycythemic recipients. The results shown are the means of three to four experiments on each population and clearly indicate that the earliest embryonic population is the least suppressed in polycythemic recipients with regard to surface colony counts.

Preliminary histological evidence suggested that the 7–8-day surface colonies present in plethorized irradiated recipients which had received embryonic liver cells were not erythroid as reported by Feldman and Bleiberg (7) but appeared to be granulocyte and megakarocyte in nature. Therefore, we did ^{59}Fe incorporation studies to determine the level of erythropoiesis in these plethorized recipients. These unpublished data showed quite clearly that neither embryonic or adult cells were capable of inducing an erythroid response in heavily plethorized recipients.

The sensitivity to tritiated thymidine suicide in the three populations in 19-day embryonic liver and the five major regions in bone marrow were tested *in vitro*. In addition to this, the sensitivity of embryonic CFU-S to the mitotic inhibitor vinblastine was also compared in one experiment. The results indicate that the earliest embryonic population is exquisitely sensitive to the killing effect of tritiated thymidine (23% survival). The next oldest population present at density 1.064 is approximately as sensitive as

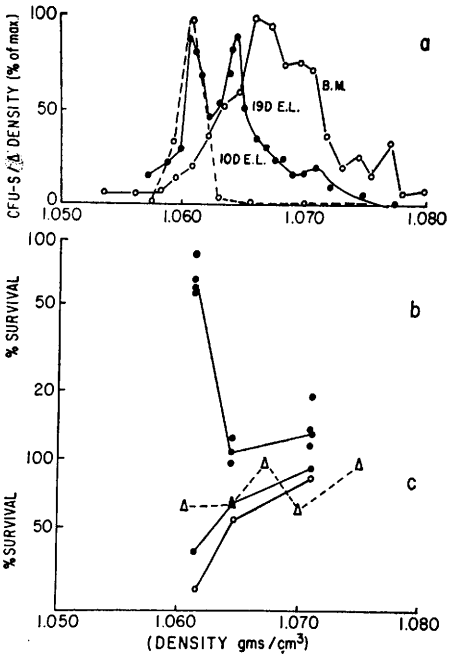

FIG. 1a. Equilibrium density gradient distribution profiles for 10- and 19-day embryonic liver and 8-week bone marrow CFU-S: Results are similar to those previously published (2). (b) Effect of plethorization on 8-day surface colony counts derived from the 19-day embryonic liver CFU-S populations. Each point represents a separate experiment with 5–7 recipients/point. (c) Effect of tritiated thymidine suicide and vinblastine on the initial survival of embryonic liver and bone marrow CFU-S populations. Each point is the mean of 5–7 animals. The values for thymidine survival in the embryonic liver are an average of two experiments: (O - -) ^{3}HTdR embryonic liver; (● —) vinblastine embryonic liver; (△ - -) ^{3}HTdR bone marrow.

are the most sensitive populations present in the bone marrow. The most dense populations in both embryonic liver and bone marrow are least sensitive, in fact, showing no significant killing by either thymidine or vinblastine. Although some regions of the bone marrow profile show significant killings by the thymidine, the average value calculated from all fractions is 82%, in reasonable agreement with the results of Becker *et al.* (4).

The density similarity between embryonic and adult populations suggested that these CFU-S might be identical. However, this has previously been shown not to be true for at

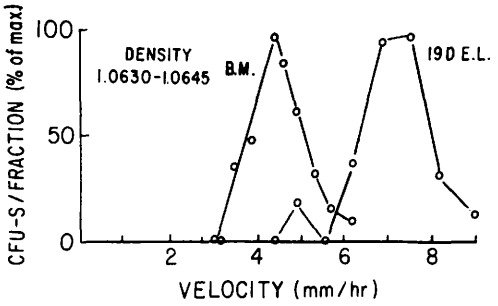

FIG. 2. Demonstration of differences in velocity and therefore volume between adult and embryonic CFU-S of equivalent density: Density purified cells were subjected to a 3-hr velocity separation before being transferred to lethally irradiated recipients.

least the earliest embryonic population (3). This point was further investigated by analyzing each of the density populations in Fig. 1 by a second separation stage based on velocity. Velocity separations in theory should be determined by the volume and the density of the cell. Therefore, a velocity separation following a density separation results in separation which should be dependent only on the basis of volume. An example of this approach to separating different populations of similar density is given in a velocity separation of the embryonic and adult populations of density 1.0635 (Fig. 2). Since the embryonic population sediments considerably faster than the adult type it must be larger in size.

The question of how much larger these two cell types were, had to be answered by obtaining relationships between the density of a cell, the velocity v of that cell type, and the volume as determined on a Coulter counter.

From the equation for a sphere of density ρ_1 and radius r falling under a gravitational force g in a medium of viscosity η and density ρ_0

$$v = \frac{2}{9}\frac{\rho - \rho_0}{\eta} g r^2. \qquad (1)$$

Therefore, the following equation relating volume v, density, and velocity can be obtained

$$\log V = \frac{3}{2}\log v - \frac{3}{2}\log \rho - \rho_0 + \text{const}, \qquad (2)$$

from which, for cells of equal volume, but different in densities

$$\frac{v_1}{v_2} = \frac{\rho_1 - \rho_0}{\rho_2 - \rho_0}. \qquad (3)$$

The necessary information to obtain standard curves for the relationships in Eqs. (2) and (3) could not be obtained from the embryonic cell material because of the presence of a high proportion of erythroid elements which show a definite pH dependency of both volume and density (10) (BSA separation, pH 5.1; FCS–Tris, pH 7.4). Therefore, normal spleen cells were used as a source of cells heterogeneous in size and density. Populations representing the density extremes observed for the function of cells so far tested (ρ 1.057 $\pm$ 0.002, ρ 1.074 $\pm$ 0.002) were subsequently separated by velocity separation. Each velocity fraction was then analyzed on the Coulter counter to measure its modal volume. From this data a plot of velocity and volume for each of the two density populations of cells was obtained (Fig. 3). Also shown in Fig. 3 are the data for spleen

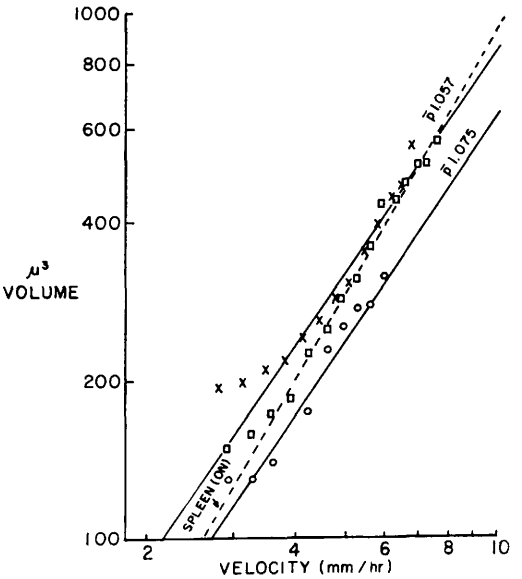

FIG. 3. Demonstration of the density and volume dependency of velocity separations: Spleen cells were subjected to a density separation and two distinct density regions were simultaneously separated by velocity in separate chambers. The velocity fractions were analyzed for volume by means of a pulse height analyzer-Coulter transducer. The data for the original (ON) spleen cells is also included for reference.

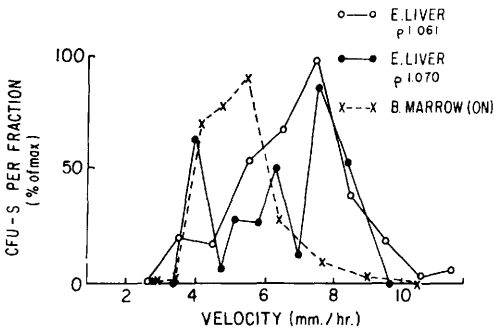

FIG. 4. A comparison of the self-renewal capabilities of early and late embryonic liver CFU-S as compared to adult bone marrow: Irradiated recipients were injected with 2×10^6 fractionated cells or unfractionated bone marrow for 7 days; and the pooled recipient spleens were teased and subjected to velocity separation.

cells not previously subjected to a density separation.

The best fit for the volume–velocity data on unfractionated spleen is a line of slope greater than the theoretical 1.5 suggested by Eq. (2) and Miller and Phillips (9) for a density homogeneous population of spleen cells. However, the data for the density 1.057 cells and the density 1.074 cells plot with a best fit line of slope 1.5. The two parallel lines are displaced in velocity by a factor of 1.25 which is in close agreement with the density difference between these populations (1.33) predicted from Eq. (3). It therefore appears that differences in density of spleen cells can play a significant role in a separation by sedimentation velocity.

Velocity separations on each of the various density populations of CFU-S was then carried out and the results calculated on the basis of volume by interpolation of the data in Fig. 3.

The self-renewal capabilities of these early embryonic populations and those present in mature bone marrow were tested by transplanting lethally irradiated recipients with these cells and determining the sedimentation velocity profile of the CFU-S present in the irradiated recipients 7 days after transplant. The data shown in Fig. 4 indicate that the earliest embryonic CFU-S give rise to the late embryonic populations, as well as those classified as being adult on the basis of their sedimentation velocity (see Fig. 5 for velocity values of embryonic and adult CFU-S). This is true also of the later embryonic population. However, the adult bone marrow population appears capable of self-renewing only adult type CFU-S.

A two-dimensional map or fingerprint graphically representing all of the experimental data was prepared. The squares shown in Fig. 5 represent the density widths of each peak as employed in the second dimension velocity separation and the height of each of the squares represents the width at half-peak height on the velocity separation (see Fig. 2, for example). The data shown for both 10-, 19-day and adult 8-week-old CFU-S includes values for volume, seeding efficiency (1, 2), sensitivities of each population to tritiated thymidine suicide (Fig. 1), and the self-renewal properties of the embryonic populations (Fig. 4). The resulting fingerprint describes, on a physical basis, interrelationships between the embryonic and adult cells and the differentiation changes within these populations.

Discussion. The data summarized by the fingerprint of adult and embryonic CFU-S outline a novel approach to an understanding of the interrelationships between populations of cells differing in such biological parameters as cell-cycle state, size, self-renewal parameters and susceptibility to hematological stress. The results indicate that the earliest embryonic CFU-S are large cells which are exquisitely sensitive to tritiated thymidine and possess an unusual degree of insensitivity to suppression in polycythemic recipients. Under the test conditions, they are incapable of self-renewal but they do give rise to embryonic progeny which are both smaller in size and higher in density. These smaller embryonic populations are separated into CFU-S which are in cell cycle and which are either in an extended G_1 phase or a true G_0 state. In addition, both of these populations have lost the resistance to the polycythemic environment possessed by the earliest population of cells. They are capable of self-renewal into themselves, as well as giving rise to the adult-type colony forming units. Experiments

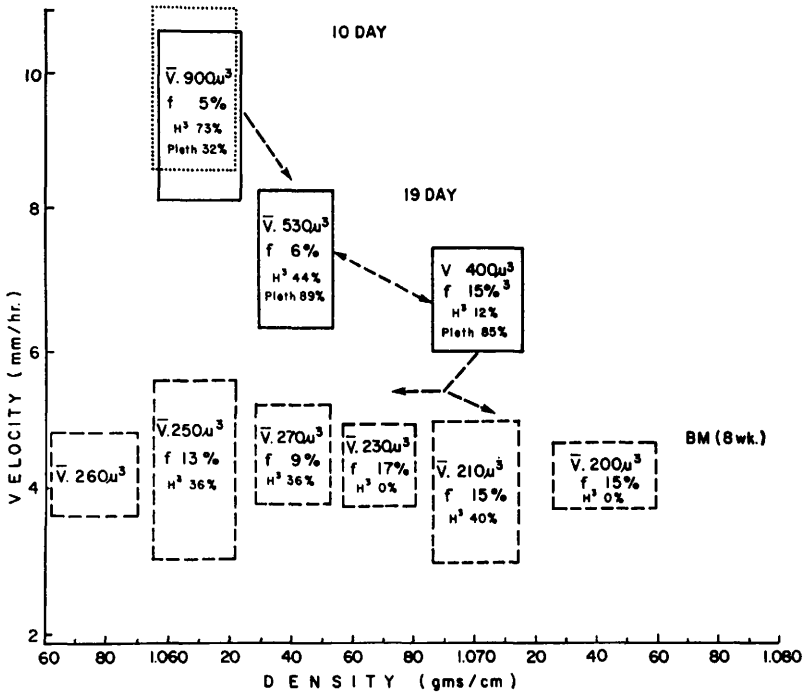

FIG. 5. Volume and density fingerprint of embryonic and adult CFU-S: (rectangles) represent the density range of each population and the half-height widths of each velocity profile subsequently obtained (see Figs. 1 and 2). The values for seeding efficiency were taken from Refs. (1, 2). The values for suppression by tritiated thymidine suicide or plethorization were taken from Fig. 1. The arrows demonstrating the path of self-renewal are taken from Fig. 4.

were not carried out to determine if an altered recruitment or a change in differentiation were responsible for the observed insensitivity of early embryonic CFU-S to polycythemic suppression.

The adult CFU-S possess a narrower spectra of seeding efficiencies, sizes, and two of the five populations tested are insensitive to thymidine suicide. It is impossible, at the present stage, to explain the heterogeneity of the adult colony forming units, except in terms of resting and cycling populations of cells. The possibility that some of these populations are artifacts seems unlikely, as similar heterogeneity has been observed in the immune system and significant immunological differences have been ascribed to each population (11). Studies on the self-renewal properties of these various density fractionated populations of bone marrow have previously indicated that there is no significant difference in the ability to produce normal numbers of progeny per colony in irradiated recipients, nor are there any differences in either the embryonic or adult populations in their ability to give rise to a normal ratio of erythroid, granulocyte, and megakarocyte colonies (1).

The data suggests that general properties such as seeding efficiency, thymidine sensitivity, sensitivity to polycythemic suppression, and self-renewal properties may be best described, not in terms of the overall populations of cells present in the particular organ, either under normal conditions or under circumstances of stress, but in terms of selected populations of cells which either have survived the treatment or have been induced as a result of it. For example, the decreased seeding efficiency observed in bone marrow colony forming units following sublethal doses of irradiation are easiest explained in terms of an increase in the proportion of the cycling population of colony forming units (12). Similarly, the changes in seeding efficiency after endotoxin stimulation are reflect-

ed in selection for particular populations rather than a change in all CFU-S (1, 12).

It is also of some interest to note that the techniques employed have been capable of separating cells involved in a differentiation sequence within the hemopoietic system; that is, the most primitive embryonic colony forming unit in liver at about day 12 of gestation appears to give rise to a CFU-S which possesses significantly different cell-cycle parameters, size, density and, apparently, some other property associated with the ability of the cell to preferentially seed in the spleen and give rise to granulocyte and megakarocyte colonies under conditions of polycythemia.

Summary. This study was undertaken to determine interrelationships between early and late embryonic and adult CFU-S. Values for density, volume, seeding efficiency, self-renewal, sensitivity to tritiated thymidine suicide and response in plethorized recipients were determined and graphically displayed as a fingerprint of embryonic and adult CFU-S. The data demonstrate the usefulness of density and velocity separations in characterizing heterogeneous cell populations.

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