ISOLATION OF INTACT MEGAKARYOCYTES FROM GUINEA PIG FEMORAL MARROW

Successful Harvest Made Possible with Inhibitors of Platelet Aggregation; Enrichment

Achieved with a Two-Step Separation Technique

RICHARD F. LEVINE and MARTHA E. FEDORKO

From The Rockefeller University, New York 10021

ABSTRACT

Methods have been devised to harvest megakaryocytes from guinea pig femoral marrow and to isolate them in high yield. When marrow tissue was disaggregated the megakaryocytes underwent degenerative changes characterized by the loss of cytoplasmic granules and alterations in membrane topography, similar to the changes seen in aggregating platelets. These morphologic changes were interpreted to mean that megakaryocytes possessed functional attributes of platelets. The use of agents which inhibit platelet aggregation (0.38% sodium citrate, 10^{-9} M adenosine, and 2 \times 10^{-8} M theophylline) in a medium free of bivalent cations prevented these changes. This solution resulted in both an excellent morphologic preservation and a significantly increased recovery of megakaryocytes from marrow tissue. A two-step purification of the intact megakaryocytes was carried out on the basis of their low density and large size, with equilibrium density gradient centrifugation followed by velocity sedimentation. This sequence gave approximately a 100-fold enrichment of megakaryocytes, significantly better than that achieved with either method alone. These techniques for harvesting and concentrating megakaryocytes make it possible for the first time to study megakaryocytes in vitro.

Circulating platelets are produced in mammals by megakaryocytes. The relationship between these two types of cells was deduced from their tinctorial similarities on light microscopy by Wright in 1906 (37). Megakaryocytes are unique cells: they are large and polyploid (7, 13), and each one gives rise to numerous progeny by a process appearing to be an organized fragmentation (3, 38). Their 2- or 3-day lifespan (10, 11) from the onset of differentiation to platelet shedding encompasses a great deal of activity, but the functions and cellular physiology of megakaryocytes remain little understood because there has been no satisfactory method to study them in vitro.

Until now, megakaryocyte morphology and physiology have been examined only in sections of marrow tissue or in marrow smears. In past studies the number of observations on megakaryocytes has been limited by their relative infrequency in the marrow, where they comprise a fraction of 1%
of all cells. Recent advances in cell separation procedures (16, 23) suggested the possibility of obtaining relatively pure populations of megakaryocytes for study in vitro.

We now report the first method for isolating large numbers of intact megakaryocytes. We also report evidence supporting the concept of behavioral kinship between megakaryocytes and platelets.

MATERIALS AND METHODS

Removal of Bone Marrow from the Animal

Male guinea pigs, 350–400 g in weight, of the Chase-Lemoen Rockefeller University strain, were killed by exsanguination and injection of air into the thorax. The femurs were promptly removed, cleaned of adherent tissue, and gently cracked open with a bone rongeur. The marrow tissue was scooped out in large pieces with a small curved spatula.

Preparation of Bone Marrow Suspensions

Bone marrow tissue from one femur was immersed in 5 ml of one of the solutions described below, in a 3.5-cm plastic petri dish (polystyrene, no. 3001, Falcon Plastics, Oxnard, Calif.). The entire sample was cut up with small scissors and forceps into pieces no longer than 2 mm. The dish was swirled a few times and the supernate aspirated of lacton, Dickinson & Co., Parsippany, N. J.). The entire sample was cut up with small scissors and forceps into pieces no longer than 2 mm. The dish was swirled a few times and the supernate aspirated of lacton, Dickinson & Co., Parsippany, N. J.).

The solution in which the marrow tissue was immersed was transferred to a 17 x 100-mm plastic tube (all tubes used were polypropylene, Falcon Plastics), using a Pasteur pipette with its narrow part broken off to leave an orifice of about 3 mm diam. All glassware was routinely treated with Siliclad (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.). The marrow particle suspension was vigorously pipetted 8–10 times with the wide-bore Pasteur pipette to break up the cell clumps into single cells.

Suspension Media

The solution in which the marrow tissue was immersed and then resuspended was either Hanks’ Balanced Salt Solution, containing calcium and magnesium (HBSS), or calcium- and magnesium-free Hanks’ balanced salt solution (CMFH; Grand Island Biological Co., Grand Island, N. Y.). Citrated solutions were made by adding one-tenth vol of 3.8% sodium citrate. Other additives included prostaglandin E1 (PGE1, courtesy of Dr. John Pike, Upjohn Co., Kalamazoo, Mich.), adenosine, theophylline, apyrase, epsilon-aminocaproic acid, N-ethylmaleimide, dibutyryl cyclic adenosine monophosphate, and ouabain (all from Sigma Chemical Co., St. Louis, Mo.). The pH of each solution was adjusted by addition of isotonic HCl or NaOH after all other additions.

Quantitation of Megakaryocytes in Marrow Cell Suspensions

Freshly prepared marrow cell suspension was added to Turk’s solution (Hellige, Inc., Garden City, N. Y.) and the nucleated cells were enumerated in hemocytometers (improved Neubauer type, American Optical Corp., Southbridge, Mass.). Megakaryocytes and other cells were counted at 160 magnification with phase-contrast optics. The megakaryocytes were identified by their size (＞16 μm), nuclear morphology (bilobed or multilobed), and prominent light refraction. One pipette sample was used to fill both chambers of each hemocytometer and four samples were taken from each marrow cell suspension. From these eight determinations a mean megakaryocyte count and a total nucleated cell count were calculated. The results were expressed as the number of megakaryocytes per 1,000 nucleated marrow cells. The result of suspending marrow cells from one femur in a particular medium was compared to the result obtained with the cells of the other femur of the same animal in a different medium. The same mechanical procedure was employed for all specimens. Each medium tested differed from its control in a single parameter. The effects of different media on the same population sample were analyzed for statistical significance, using a paired one-tailed Student’s t test (12).

Concentration of Megakaryocytes from Marrow Cell Suspensions

The harvested marrow cells were poured through a stainless steel sieve (100 mesh, hole diameter 149 μm) into petri dishes. The cells were washed twice by centrifuging (250 g for 10 min) and replacing the medium with CMFH containing 10^-4 M adenosine, 2 x 10^-8 M theophylline and 3.5% bovine serum albumin (BSA, Fraction V, Armour Pharmaceutical Co., Chicago, Ill.), pH adjusted to 7.4. For all experiments the albumin was obtained from a single batch and used without prior dialysis. The cells were then resuspended in this protein-containing solution at a concentration of approximately 10^6 marrow cells/ml. This suspension was layered (1.8 ml per tube) over four discontinuous density layers (1.8 ml each) of solutions of BSA in CMFH, pH 7.4, with specific gravities ranging from 1.035 to 1.050, as shown in Fig. 1. A 23.5% (wt/vol) stock BSA solution in
Washed bone marrow cell suspension in albumin-Hanks' solution

Equilibrium density gradient centrifugation

Mega-enriched cell suspension in protein-free medium

Velocity sedimentation

FIGURE 1 Procedure to concentrate megakaryocytes from guinea pig marrow cell suspensions.

CMFH was prepared by adding 29 g BSA to 100 ml of glass-distilled water and 7.9 ml of 10x strength CMFH; the pH of the solution was then adjusted by slow addition of approximately 7 ml of 1 N NaOH and then 2-3 ml of 0.15 ml N NaOH as necessary. The total volume was approximately 123 ml. The solutions of different specific gravities were derived from stock solutions of 22% BSA by comparisons of refractive indices (AO T/C Refractometer, American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.) with a standard curve. Four cellulose nitrate tubes (4 x 3 in, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) containing the density gradients were spun in a swinging bucket rotor at 10,000 g for 30 min (Lourdes model LRA centrifuge, maintained at 4°C). The low-density population of cells suspended at the density interfaces was collected and pooled. After one wash in CMFH, the cells were resuspended in 4.2 ml of CMFH (approximately 3 x 10⁶ cells/ml); 1.0 ml was layered over each of four glass tubes (17 x 75 mm) containing a very shallow discontinuous density gradient of BSA in CMFH (cf. Fig. 1), with specific gravities of 1.0022-1.0069 (1.8 ml of each layer). The tubes were kept upright on the table top while the cells were allowed to settle at room temperature. After 30 min the uppermost ~3 ml of solution in each of the four tubes was withdrawn and discarded; the cloudy bands of cells at the first two interfaces were thus excluded. The remaining cells were centrifuged into a pellet and washed once with 3.5% BSA in CMFH and then used for morphologic and quantitative studies. In preliminary experiments, the concentrations, the specific gravities of the solutions, and the times employed for these two techniques were systematically varied in order to maximize the megakaryocyte enrichment. The conditions specified above resulted in the best enrichment.

Quantitation of Megakaryocytes before and after Each of the Concentrating Procedures

At this step, suspensions of cells were counted directly, i.e. not in Turk's solution. Four determinations were made for each cell suspension. The number of megakaryocytes per 1,000 cells in suspension was calculated; this method included erythrocytes as well as nucleated cells.

Determination of Viability of Megakaryocytes

Cell integrity was determined by counting the proportion of megakaryocytes which excluded trypan blue. Cells were suspended in CMFH with 3.5% BSA (wt/vol).
100-μl samples (Yankee Micropets, Clay Adams, Parsippany, N. J.) of the cell suspensions were added to 100 μl of 2% trypan blue (Allied Chemical Corp., New York) and 50 μl of 1.8% NaCl. After mixing, duplicate hemocytometer counts of 100-200 megakaryocytes each were evaluated at magnification 160 with phase-contrast optics.

Morphologic Observations of Megakaryocytes in Suspension

Phase-contrast microscopy: Living cells were examined by phase-contrast microscopy at magnification 160 in hemocytometers. For observations at greater magnification, cover slip preparations were made of cells fixed in 2.5% glutaraldehyde in cacodylate buffer, pH 7.4, for 15 min, then resuspended in saline. Phase-contrast (×1,000) and interference phase-contrast (Normarski, ×400) observations were carried out on this type of preparation. Sizes of individual megakaryocytes were determined with the use of an eyepiece micrometer disk (Bausch & Lomb Inc., Rochester, N. Y.) which was standardized with the engraved hemocytometer chamber; for megakaryocytes not completely spherical, the average of the longer and shorter axes was calculated. Human erythrocytes from a healthy subject were similarly fixed and sized as a control for possible osmotic changes induced in the cells by the fixation medium.

Bright-field microscopy: After centrifugation of a cell suspension onto cover slips in flat-bottomed vials (1 × 10⁶-2 × 10⁷ cells/cover slip), the medium was aspirated and the cells were rapidly air dried. The cover slips were stained with Wright's (Hartman-Leddon Co., Philadelphia, Pa) and Giemsa's (Grady-wohl Laboratories, St. Louis, Mo.) stains. On the basis of this staining, maturation stages of megakaryocytes could be classified easily by the following scheme: stage I, the youngest megakaryocytes, with a high nuclear/cytoplasmic ratio and basophilic cytoplasm but no granulation present; stage II, maturing cells, with granule formation but with some basophilia remaining in the cytoplasm; stage III, mature megakaryocytes, with eosinophilic cytoplasm full of granules and without residual basophilia. Occasional stage I cells had a pale, slightly eosinophilic area in the center of the cell where, as shown by electron microscopy, the Golgi zone was located; nonetheless, since these cells had no definite granulation they were assigned to stage I. Essentially the maturational distinctions were among all basophilic cells, partially eosinophilic and basophilic cells, or wholly eosinophilic cells.

Electron microscopy: Cell suspensions were prepared for electron microscopy by centrifugation (250 g for 10 min). Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 10 min, then chilled on ice for 10 min. The cells were centrifuged once more (250 g for 2 min) and the fixative was replaced with an ice-cold mixture of one part 2.5% glutaraldehyde and two parts 1% osmium tetroxide, both in cacodylate buffer for 15 min (11). The cells were washed three times with ice-cold saline by centrifugation at 250 g for 2 min. The cells were then placed in cold 0.25% uranyl acetate (0.1 M acetate buffer, pH 6.3) for 1–16 h. After two more washes in cold saline, the cells were centrifuged in small glass conical tubes (250 g for 2 min); the supernate was removed and the cells were suspended in molten 2% Noble agar at 140°C (Difco Laboratories, Detroit, Mich.) and immediately centrifuged (18). The cell pellet in agar was dehydrated in a graded series of alcohol concentrations, embedded in Epon (39), and thin sections were obtained with a Porter-Blum MT-2 ultramicrotome (Dupont Instruments, Sorvall Operations, Newtown, Conn.). Sections were stained with lead citrate and uranyl acetate (33) and photographed with a Siemens IA electron microscope. Marrow tissue blocks were prepared in a similar manner as controls for morphologic changes produced by the various procedures employed.

Megakaryocyte surface membrane staining was enhanced by fixation of cells in suspension with 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 8% tannic acid and 0.015 M CaCl₂, as outlined by Fedorko and Levine. Thin sections were not stained.

RESULTS

The Effect of Harvesting Megakaryocytes in Different Media

When bone marrow tissue was suspended in HBSS, the megakaryocytes were found usually to have extensive vacuolization (Fig. 2, inset). Compared to their ultrastructural appearance in marrow fixed en bloc, the apparent alterations induced in megakaryocytes during marrow disaggregation were loss of granules and the appearance of membrane-enclosed spaces or vacuoles (Fig. 2). In these cells, although granules were greatly diminished in number, other organelles, including mitochondria, endoplasmic reticulum, Golgi apparatus, and nuclei, showed excellent preservation. These apparent vacuoles were investigated using tannic acid during fixation to enhance the staining of the plasma membranes (Fig. 3). Because the tannic acid as used does not penetrate intact cells (31), the spaces designated V in Fig. 3 are continuous with the extracellular space. The membranes enclosing these spaces are stained with electron-dense deposits. They are continuous with the outer surface membrane and thereby identified as platelet membranes.
Vacuolization (V) extends throughout the cell but does not affect the Golgi zones (Go), nucleus (N), mitochondria (m), or rough endoplasmic reticulum. × 18,800. Inset, This megakaryocyte shows extensive cytoplasmic vacuolization. Cell air dried and stained with Wright's-Giemsa's × 530.

let-demarcating membranes. Thus, these apparent vacuoles are derived from the demarcation channels apparently by separation of the invaginated surface membranes which were formerly closely apposed (3, 20), as discussed below.

In an attempt to block these alterations we examined the condition of megakaryocytes after suspending marrow in different media. Substantial differences in the preservation of megakaryocytes were observed with phase-contrast microscopy. It was evident that often one medium was much more successful than another in preventing vacuolization. It also became apparent in these comparisons that the use of certain substances resulted in an increase in the number of megakaryocytes obtained from marrow tissue. This observation led us to undertake a systematic evaluation of the effects of different media on the content as well as on the preservation of megakaryocytes in marrow suspensions.

In Table I are the results of paired assays evaluating the quantitative benefits of a single agent or condition of the harvest of megakaryocytes from equivalent marrow tissue. The exclusion of bivalent cations (comparison a) and stepwise inclusions of citrate, adenosine, and theophylline in CMFH (comparisons b, d-f) gave statistically significant improvements in content of identifiable megakaryocytes over those obtained with simpler solutions. A slightly low pH did not enhance the content (comparison c). In a direct comparison, adenosine was equal to theophylline in increasing the content of megakaryocytes (Table I, g); however, when the two drugs were combined, an additive effect on the content was produced (comparison j). The addition of albumin, aspirin, dipyridamole, imipramine, chlorpromazine, heparin, or procaine did not improve the content.

Marked improvements in the degree of preservation of megakaryocytes generally paralleled their enhanced content. Sometimes the morphological differences between cells in two different media were noted to be more substantial than
FIGURE 3 The demarcating membranes (small arrow) and the surface membrane (double arrows) have increased electron density due to the use of tannic acid in the initial fixative. Also shown are the extracellular space (E), vacuoles (V), and cytoplasmic granules (g). x 23,300.

FIGURE 4 Megakaryocytes harvested in CATCH medium and concentrated by density equilibrium centrifugation followed by velocity sedimentation. At low magnification the megakaryocytes, which are large, are readily distinguished from the contaminating cells. x 2,810.

the differences in content. The use of PGE\(_1\) (10\(^{-5}\) M), for example, had a more impressive effect on inhibiting vacuolization than it did in enhancing the content (comparison h). Some other substances added to facilitate the harvest of megakaryocytes gave morphologic and quantitative results which were similar to those obtained with adenosine or theophylline but were not additive to those obtained with the combined CATCH medium; examples included N-ethyl maleimide (10\(^{-3}\) M), dibutyryl cyclic adenosine monophosphate (5 x 10\(^{-4}\) M), and ouabain (10\(^{-3}\) M). The combined medium, citrate + adenosine + theophylline in CMFH (CATCH), gave a 67% mean enhancement of content, a highly significant improvement compared to the harvest with HBSS (Table I, f). The quantitative data indicated that the CATCH medium gave the greatest enhancement of megakaryocyte content of all the substances and combinations tested. Though cell preservation in different media was not so thoroughly documented, no other agents added to CATCH medium gave further improvement in the appearance of megakaryocytes. Light and electron microscope evaluation of megakaryocytes harvested in this medium showed that, though loss of granules and dilation of the platelet-demarcating channels were not totally abolished, the extent of these alterations was much less in most megakaryocytes and only small numbers of them had the extensive alterations usual in cells harvested in HBSS. Otherwise the megakaryocyte morphology was identical to that seen in tissue blocks. Consequently, CATCH was used as the standard medium for making marrow cell suspensions.

Concentration of Megakaryocytes from the Harvested Marrow Cell Suspensions

The most efficient way to enrich the megakaryocytes was to use equilibrium density centrifugation followed by velocity sedimentation (Table II). The
density gradient centrifugation resulted in a substantial concentration of megakaryocytes, 15.5-fold (Table II), but the recovery was not high, 27% (middle line of Table II). The velocity sedimentation step (bottom line in Table II) was able to conserve most of the megakaryocytes while its effect on concentration was not so great (sixfold) as with the first part of the procedure. The complete concentration procedure gave a 93-fold enrichment of megakaryocytes from the marrow cell suspension harvested in CATCH medium. Overall megakaryocyte recovery was 21%. Most

### Table I

Comparison of Megakaryocyte Content of Paired Femurs with Different Harvesting Media

| Comparison | Composition of medium | Number of observations | Nucleated cell counts (megakaryocytes/10^6 cells) mean ± SD | Statistical significance |
|------------|-----------------------|------------------------|-----------------------------------------------------------|--------------------------|
| (a) HBSS | CMFH                  | 4                      | 1.77 ± 0.15                                               | <0.005                   |
| (b) CMFH  | CMFH + citrate 0.38% (C) | 12                     | 2.48 ± 0.42                                               | <0.005                   |
| (c) CMFH, pH 6.8-7.0 |                      | 6                      | 3.14 ± 0.49                                               | NS                       |
| (d) CMFH + C | CMFH + C + adenosine 10^-2 M (A) | 8                      | 2.96 ± 0.17                                               | <0.01                    |
| (e) CMFH + C | CMFH + C + theophylline 2 × 10^-3 M (T) | 6                      | 2.96 ± 0.44                                               | <0.005                   |
| (f) CMFH + C + A | CMFH + C + A + T | 8                      | 2.95 ± 0.78                                               | <0.005                   |
| (g) CMFH + C + A | CMFH + C + T | 8                      | 3.41 ± 0.63                                               | NS                       |
| (h) CMFH + C | CMFH + C + PGE, 10^-3 M | 8                      | 3.66 ± 0.79                                               | <0.05                    |
| (i) HBSS | CMFH + C + A + T | 6                      | 2.15 ± 0.46                                               | <0.0005                  |

### Table II

Quantitative Results of Concentrating Megakaryocytes from Femurs of Two Guinea Pigs*

| Cell suspension                | Yield (total megakaryocytes ± SD x 10^-6) | Recovery from prior step | Parity (megakaryocytes/10^6 marrow cells ± SD) | Concentration over prior step | Viability |
|-------------------------------|-------------------------------------------|--------------------------|-----------------------------------------------|--------------------------------|-----------|
| Starting marrow suspension    | 2.76 ± 0.72                               | 3.55 ± 1.17              |                                               |                                | 73        |
| After density centrifugation  | 0.758 ± 0.503                             | 27                       | 55.1 ± 22.9                                  | 15.5                           | 81        |
| After velocity sedimentation  | 0.574 ± 0.178                             | 76                       | 329 ± 102                                    | 6                              | 92        |

*Results of seven experiments; viability determined on three of these.

**LEVINE AND FEDORKO Isolation of Intact Megakaryocytes from Femoral Marrow**
of the megakaryocytes not recovered in these two procedures were found in the pellets and supernates, respectively.

Trypan blue exclusion was used to determine how the two-part procedure affected the megakaryocytes. In three of these experiments (data in Table II), 73% of the megakaryocytes in the freshly harvested cell suspensions were viable, 81% after the density centrifugation steps, and 92% after the velocity sedimentations. Therefore, both of the concentration techniques favored the enrichment of viable megakaryocytes, so that after the enrichment procedure the cell integrity was excellent. If the data in Table II were recalculated on the basis of only dye-excluding megakaryocytes, there was a final concentration of 117-fold and a recovery of 25.7%.

Attempts to concentrate megakaryocytes by reversing the sequence of the procedure, i.e. velocity sedimentation followed by density gradient centrifugation, gave lesser yields. When the velocity sedimentation was carried out first, the high degree of cell loading (approximately $2 \times 10^8$ cells/ml) might have impaired the efficiency of the separation procedure; however, the use of a discontinuous gradient resulted in no visible streaming or clumping below the second interface (i.e. 1.0022:1.0045), presumably because of the much lower cell load at that interface. A velocity sedimentation without a preceding density gradient centrifugation generally gave an average 10-fold concentration with good recovery. Two successive velocity sedimentations never surpassed a 44-fold concentration.

The megakaryocytes were concentrated by the two-part procedure to 33% purity. Fig. 4 shows the appearance of the enriched population. Other types of cells present included nucleated erythrocyte precursors, promyelocytic and myelocytic neutrophils and eosinophils, and small numbers of erythrocytes, mononuclears, and undifferentiated cells. Lymphocytes, macrophages, mature granulocytes, fat cells, osteoblasts, and osteoclasts were absent or rare.

The mean yield with the two-part procedure was about $6 \times 10^6$ megakaryocytes from four guinea pig femurs. Cell pellets of adequate size for easy and convenient ultrastructural study could be prepared from the yield from two femurs. The electron micrograph in Fig. 5 shows that many megakaryocytes could be easily studied in a single specimen. In contrast, marrow blocks contain about one megakaryocyte per section.

**Maturity and Sizes of Megakaryocytes before and after Concentration**

Observations were made to determine whether the megakaryocytes enriched by the two-step procedure were representative of the harvested pool of megakaryocytes or were a selected subpopulation. Maturation stages of megakaryocytes before and after concentration were compared. The appearance of megakaryocytes on stained smears was used to classify the maturational stages (see Materials and Methods). In three experiments, 200 cells were evaluated from the cell suspensions before and after concentration. The harvested marrow cell suspensions contained an average of 25% immature, 56% intermediate, and 20% mature megakaryocytes. The concentrated populations consisted of 21% immature, 62% intermediate, and 17% mature megakaryocytes.

When the diameters of megakaryocytes from a marrow cell suspension and of megakaryocytes in a concentrated population derived from it were compared (Fig. 6), there was a negligible difference in the mean sizes of the two groups. The similarity of the histograms suggests that on the basis of size as well as maturation the megakaryocytes isolated by this procedure were not a subpopulation of the original marrow cell suspension.

**Morphologic Observations on Concentrated Megakaryocyte Populations**

Megakaryocytes harvested and concentrated by the above method typically showed densely granulated cytoplasm (Figs. 4 and 5), in contrast to the foamy cytoplasmic appearance of megakaryocytes harvested in HBSS (Fig. 2 and inset).

When fixed in suspension, the megakaryocytes usually were spherical and occasionally ellipsoidal, with a mean diameter of 27.5 μm but occasionally as small as 10 μm.

When the megakaryocytes were kept in protein-containing solutions during the concentration procedure the artifacts were further minimized or reversed. There were minimal or no differences between most of the isolated megakaryocytes (Figs. 5 and 7) and the megakaryocytes fixed in marrow blocks. There was excellent preservation of most megakaryocytes; the only artifact was an occasional area of separation of the apposed membranes of the platelet demarcation system (Fig. 5). Especially impressive examples of the preservation achieved were cells such as those seen in Figs. 5 (center-right cell) and 7, in which platelet
FIGURE 5  Agar-embedded cell pellet from concentrated megakaryocyte population (see Materials and Methods). Representative electron micrograph shows the large number of megakaryocytes that can be seen in one specimen. Compared to Fig. 1, artifactual vacuolization is minimal and seen mostly in the periphery of some cells. Maturation stage varies from one cell to another and is most advanced in the center-right cell. × 3,400.
sheding seemed immanent at the time of fixation. Although no exact quantitation was attempted, it was estimated that such fully mature megakaryocytes comprise approximately 1% of the megakaryocytes both in situ and in the enriched populations.

DISCUSSION

Intact megakaryocytes have been enriched approximately 100-fold from guinea pig femoral marrow. The excellent preservation and the good yields of megakaryocytes were made possible by the use of certain agents not required for the harvest of other cell types. The purification procedure involved two different techniques—equilibrium density centrifugation followed by velocity sedimentation. The methods used to harvest and concentrate megakaryocytes were short and simple and required no unusual equipment.

Guinea pigs were chosen in preference to other species for this study because an adequate mass of marrow tissue could be obtained simply and quickly, the gelatinous tissue was easily handled, little fat was present in the femoral marrow of young guinea pigs, and not least, because guinea pigs appeared to us and others to have a greater concentration of megakaryocytes in the marrow. We obtained 2–10 times more megakaryocytes from guinea pigs than were previously found in marrow cell suspensions from man (27, 34), rats (30), and mice (9, 26).

Successful isolation of megakaryocytes involved two separate problems: harvesting the marrow and concentrating the megakaryocytes. In previous reports on megakaryocytic counts in marrow cell suspensions by other authors (9, 26, 27, 30, 34), there were no comments on the condition of the megakaryocytes. Other workers have observed, however, that disaggregation of marrow tissue generally affects megakaryocyte morphology adversely.

Our analysis of electron micrographs has made apparent the selective nature of the changes in megakaryocytes: the most obvious alteration was dilation of the spaces separating apposing membranes of the platelet demarcation system. This system consists of a small number of invaginations of the surface membrane (20) which proliferate throughout the megakaryocyte and eventually divide the cytoplasm into individual platelets (3, 38). The narrow space between the apposed membranes is continuous with and accessible to the space outside the cell. These membranes are similar histochemically and structurally to (and are probably identical with) the megakaryocyte

* Paulus, J.-M, personal communication.

* White, J. G., personal communication.

* Pennington, D. G., personal communication.
FIGURE 7  Mature megakaryocyte with cytoplasm well demarcated into prospective platelet areas (P). No significant dilation of demarcating channels is seen. Cytoplasmic granules are abundant. × 6,950. Inset, Portion of the same cell, showing that these platelet areas (P) are similar to mature platelets. × 18,500.

LEVINE AND FEDORKO  Isolation of Intact Megakaryocytes from Femoral Marrow  169
and platelet surface membranes (3). In the "vacuolated" megakaryocytes seen in HBSS, the spaces of the demarcation system could conceivably have enlarged because of fusion of the granules with the demarcating membranes and/or a retraction of the membrane-enclosed cytoplasm.

The other major change in the megakaryocytes harvested in HBSS was the large-scale loss of cytoplasmic granules. These granules in platelets contain adenosine diphosphate, serotonin, calcium, and several other substances (25). Platelet shape changes and degranulation are physiologic responses preceding aggregation and occurring in response to collagen, subendothelial microfibrils, adenosine diphosphate, epinephrine, and many other substances (4, 8, 36). In megakaryocytes the internal topographical alterations and the loss of granules are reminiscent of and may be comparable to platelet reactions.

It seemed likely that the changes seen in harvested megakaryocytes represent a platelet-like response to the same chemical and surface stimuli that affect platelets. Some of the agents that cause platelet aggregation are normally found in all tissues. The alterations observed in megakaryocytes, then, might be due primarily to chemical, not mechanical, trauma. Furthermore, it seemed that those agents and conditions that inhibit platelet aggregation might also decrease the extent of the megakaryocyte alterations. This latter proposition was tested by evaluating the effects on morphology and yield of a number of known inhibitors of platelet function. Some of the substances tested had a noticeable effect on the harvest of marrow tissue so that the megakaryocytes were morphologically more like those that we and others (3, 20, 38) observed in situ. Moreover, a quantitative improvement was found in the number of harvested megakaryocytes. The data demonstrated that megakaryocyte alterations could be blocked by inhibitors of platelet function. The idea of protecting the integrity of the megakaryocytes as they are harvested and concentrated is conceptually related to the work of Ardlie (1), who developed complex solutions to preserve the function of platelets during their isolation from blood.

The benefits of CATCH medium in harvesting megakaryocytes are probably due to several factors. The disaggregation of marrow tissue is possibly more complete, as is the case with many other tissues, when bivalent cations are not available. In addition, calcium ions are required for platelet degranulation and aggregation (14, 32). Citrate might contribute by chelating endogenous calcium ions. Adenosine stimulates adenylate cyclase (24). Thymophylline is a known antagonist of phosphodiesterase and thereby inhibits platelet changes by preventing the breakdown of cyclic adenosine monophosphate (2, 29). The data in the current study are consistent with the concept that the latter two substances affect megakaryocytes by synergistic mechanisms (5). The amount of adenosine found effective is approximately 10-fold greater than the concentration necessary to inhibit human platelet aggregation in vitro; that of thymophylline was no different (39). Since the specific stimuli that affect the megakaryocytes during marrow tissue disruption are not known, it is not possible to compare the sensitivity of megakaryocytes to that of platelets.

Without the protection afforded by the inhibiting agents, up to 40% or more of marrow megakaryocytes appeared to have been lost during the harvesting procedure. Their fate is not known. It might be that marked vacuolization and degranulation made the cells much more susceptible to physical trauma such as tissue mincing and pipetting, so that some of the cells were disrupted and were no longer identifiable. The other cell types present are known not to be drastically affected by these agents, conditions, and mechanical procedures, as used. Since the inhibiting agents clearly preserved the megakaryocyte morphology, we believe that the improved content of megakaryocytes in marrow cell suspensions is a quantitative expression of this preservation and represents a better recovery or yield from marrow tissue.

In comparing the effects of different media, an assumption was made a priori that marrow tissue from opposite femurs of the same animal contained equivalent cell populations. A comparison of the megakaryocyte content in identical media gave results that were consistent with this hypothesis. The differences in content between an arbitrarily chosen first femur and the other femur processed in the same medium, or between media of insignificantly different pH, were never more than a few per cent. The quantitation of nucleated cells for these comparisons obviated the unpredictable contributions of variable amounts of circulating blood and/or marrow tissue included in the specimens.

The theory underlying cell sedimentation states that the rate is largely a function of cell size (16, 23). Therefore, it seemed logical that velocity sedimentation should be an appropriate method to
concentrate the intact megakaryocytes. Other authors have reported some degree of success in other species with the use of velocity sedimentation to achieve megakaryocyte concentrations of 1–3% (26, 30). These reports give no data on the intactness or total yield of megakaryocytes. Mel reported that the STA-FLOW velocity sedimentation technique was suitable for megakaryocyte enrichment (22), but quantitative data are lacking. In our experience, velocity sedimentation was not so effective a concentrating procedure for megakaryocytes as expected. The large difference in specific gravity between megakaryocytes and most other marrow cells prevented a good separation of megakaryocytes solely on the basis of size. The density equilibrium centrifugation was a more effective single technique for concentrating guinea pig megakaryocytes. The combination of the two techniques gave significantly increased enrichment compared to that obtainable by a single technique. Other workers (17, 28) have used velocity sedimentation to "characterize" further cells separated by a prior density gradient centrifugation, but this combination has not had much use for the primary purpose of purifying a single cell type.

The recovery of megakaryocytes at the end of the velocity sedimentation step could be varied to give greater purity or greater yield, depending on the objective of the study. There was an inverse relationship between these two parameters. If a greater volume of the gradient was discarded, relatively more of the contaminating cells were removed, resulting in megakaryocyte purity up to 42% with a total yield of $4 \times 10^8$ megakaryocytes per experiment. If a lesser volume was discarded, the yield was often higher, up to $9 \times 10^8$ megakaryocytes from four femurs, but purity fell as low as 24%.

The data on size and maturation profiles showed that megakaryocytes in concentrated populations were quite similar to megakaryocytes from marrow cell suspensions. Exactly how representative the latter cells are of the in vivo pool cannot be answered directly. Previous estimates of mean size of megakaryocytes were based on data from histologic sections. If one accepts Harker's estimate that the latter measurements should be corrected to reflect a 25% shrinkage during fixation (15), the results of several authors agree closely with our own, despite the species differences (6, 15, 21). Erythrocytes that were fixed in the same way as our megakaryocyte preparations had the same mean size as unfixed erythrocytes, as reported by Westerman et al. (35), implying that our size data are accurate and need no correction.

We conclude that the pool of concentrated megakaryocytes is probably representative of the in vivo population.

The previous handicap of having to study megakaryocytes in situ and in limited numbers has been overcome. Extensive morphologic, physiologic, and chemical studies may now be carried out with the large numbers of megakaryocytes obtained.

We are indebted to Ms. Jessica Pash for excellent technical assistance. We thank Drs. James Hirsch, Zanvil Cohn, Ralph Nachman, and Eric Jaffe for helpful criticism and encouragement.

Portions of this material were presented at the 16th Annual Meeting of the American Society of Hematology, December, 1974. The work was supported by United States Public Health Service grant HL16271 and by a Leukemia Society of America, Inc. Fellowship to Richard F. Levine and a Leukemia Society of America, Inc. Scholarship to Martha E. Fedorko.

Received for publication 18 July 1975, and in revised form 16 December 1975.

REFERENCES

1. ARDLIE, N. G. 1971. Studies on the mechanism of adenosine diphosphate-induced platelet aggregation. Ph.D. Thesis. McMaster University, Hamilton, Ont. xx:1–192.
2. ARDLIE, N. G., G. GLEN, B. SCHULTZ, AND C. SCHWARTZ. 1967. Inhibition and reversal of platelet aggregation by methyl xanthines. Thromb. Diath. Haemorrh. 18:670–673.
3. BENNEKE, O. 1968. An electron microscopic study of the megakaryocyte of the rat bone marrow. I. The development of the demarcating membrane system and the platelet surface coat. J. Ultrastruct. Res. 24:412–433.
4. BORN, G. V. R. 1970. Observations on the change in shape of blood platelets brought about by adenosine diphosphate. J. Physiol. (Lond.). 209:487–511.
5. BORN, G. V. R., AND D. C. B. MILLS. 1969. Potentiation of the inhibitory effect of adenosine on platelet aggregation by drugs that prevent its uptake. J. Physiol. (Lond.). 202:41–42.
6. COWAN, D. H. 1973. Thrombokinetic studies in alcohol-related thrombocytopenia. J. Lab. Clin. Med. 81:64–76.
7. DE LEVAL, M. 1964. Dosages cytophotometriques d'ADN dans des megacaryocytes normaux de cobaye. C. R. Séances Soc. Biol. Fil. 158:2198–2201.
8. DROLLER, M. J. 1973. Ultrastructure of the platelet release reaction in response to various aggregating agents and their inhibitors. Lab. Invest. 29:595–606.
9. EBBE, S., E. PHALEN, and F. STOHLMAN, Jr. 1973. Abnormalities of megakaryocytes in W/Wv mice. Blood. 42:857-864.

10. EBBE, S., and F. STOHLMAN, Jr. 1965. Megakaryocytosis in the rat. Blood. 26:20-35.

11. FEINENDEGEN, L. E., N. ODARTCHENKO, H. COTTER, and V. P. BOND. 1962. Kinetics of megakaryocyte proliferation. Proc. Soc. Exp. Biol. Med. 111:177-182.

12. FERGUSON, G. A. 1966. Statistical Analysis in Psychology and Education. 2nd edition. McGraw-Hill Book Co., New York.

13. GARCIA, A. M. 1964. Feulgen DNA values in megakaryocytes. J. Cell Biol. 20:342-345.

14. HARKER, L. A. 1968. Megakaryocyte quantitation. J. Clin. Invest. 47:452-457.

15. HARKER, L. A. 1968. Megakaryocyte quantitation. J. Clin. Invest. 47:452-457.

16. HARWOOD, R. 1974. Cell separation by gradient centrifugation. Int. Rev. Cytol. 38:369-403.

17. HASKILL, J. S., and M. A. S. MOORE. 1970. Two dimensional cell separation. Comparison of embryonic and adult haematopoietic cells. Nature (Lond.). 226:853-854.

18. HIRSCH, J. G., and M. E. FEDORKO. 1968. Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and "postfixation" in uranyl acetate. J. Cell Biol. 38:615-627.

19. LUTF, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409-414.

20. MACPHERSON, G. G. 1972. Origin and development of the demarcation system in megakaryocytes of rat bone marrow. J. Ultrastruct. Res. 40:167-177.

21. MATTER, M., J. R. HARTMANN, J. KANTZ, Q. B. DEMARSH, and C. A. FINCH. 1960. A study of thrombopoiesis in induced acute thrombocytopenia. Blood. 15:174-185.

22. MEI, H. C. 1970. Stable-flow free boundary cell fractionation as an approach to the study of hematopoietic disorders. In Myeloproliferative Diseases in Animals. W. J. Clarke, E. B. Howard, and P. L. Hackett, editors. Atomic Energy Commission Series. National Technical Information Service, U. S. Dept. of Commerce, Springfield, Va. 665-686.

23. MILLER, R. G., and R. A. PHILLIPS. 1969. Separation of cells by velocity sedimentation. J. Cell. Physiol. 73:191-201.

24. MILLS, D. C. B., and J. B. SMITH. 1971. The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3',5'-cyclic monophosphate in platelets. Biochem. J. 121:185-196.

25. MUSTARD, J. F., and M. A. PACKHAM. 1970. Factors influencing platelet function: adhesion, release, and aggregation. Pharmacol. Rev. 22:97-187.

26. NAKIEN. A., and B. MAAT. 1974. Separation of megakaryocytes from mouse bone marrow by velocity sedimentation. Blood. 43:591-595.

27. PIZZOTATO, P. 1948. Sternal marrow megakaryocytes in health and disease. Am. J. Clin. Pathol. 18:891-897.

28. PRETLOW, T. G., and I. M. CASSADY. 1970. Separation of mast cells in successive stages of differentiation using programmed gradient sedimentation. Am. J. Pathol. 61:323-339.

29. SAZMAN, E. W., and L. L. NERI. 1969. Cyclic 3',5'-adenosine monophosphate in human blood platelets. Nature (Lond.). 224:609-610.

30. STINSON, A. J., and T. G. PRETLOW II. 1975. Separation of megakaryocytes from marrow cells by velocity sedimentation. Fed. Proc. 34:873.

31. TILNEY, L. G., J. BRYAN, D. J. BUSH, K. FUJIIWARA, M. S. MOOSEKER, D. B. MURPHY, and D. H. SNYDER. 1973. Microtubules: evidence for 13 protofilaments. J. Cell Biol. 59:267-275.

32. VALDORF-HANSEN, J. F., and M. B. ZUCKER. 1971. Effect of temperature and inhibitors on serotonin-11C release from human platelets. Am. J. Physiol. 220:105-111.

33. VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25:407-408.

34. VOGEL, P., and F. A. BASSEN. 1939. Sternal marrow and other variables in the release reaction of human platelets. Am. J. Physiol. 121:819-824.

35. WEATON, A. J., and R. C. NELSON. 1961. A direct method for the quantitative measurement of red cell dimensions. J. Lab. Clin. Med. 57:819-824.

36. WHITE, J. G. 1971. Platelet morphology. In The Circulating Platelet. S. A. Johnson, editor. Academic Press, Inc., New York. 45-121.

37. WRIGHT, J. H. 1906. The origin and nature of the blood plates. Boston Med. Surg. J. 154:643-645.

38. YAMADA, E. 1957. The fine structure of the megakaryocyte in the mouse spleen. Acta Anat. 29:267-290.