Two morphologically distinct types of murine megakaryocyte (MK) colonies are present after three to seven days in soft agar culture: (a) "big-cell" colonies composed of ten to 30 large, mature-appearing megakaryocytes and (b) "heterogeneous" colonies consisting of approximately 100 or more cells at various stages of differentiation. Cytoc hemical and immunocytochemical techniques were used to study MK maturation in colonies as well as normal mouse bone marrow. Acetycholinesterase (AChE), a specific marker for murine platelets and MK, was found in the perinuclear cisterna, endoplasmic reticulum, and occasionally, Golgi cisternae of MK in three-day big-cell colonies and immature bone marrow MK. MK in seven-day big-cell colonies and mature bone marrow MK showed additional reaction sites in the demarcation membrane system and occasional granules. In seven-day heterogeneous colonies, small cells resembled immature bone marrow MK with respect to AChE localization, whereas large cells corresponded to mature bone marrow MK. With immunogold procedures at the ultrastructural level, polyclonal antibodies against human platelet membrane glycoprotein IIa and antimouse platelet antiserum labeled bone marrow MK and all MK from colonies grown in soft agar cultures for three to seven days. Granulocytes and macrophages in both bone marrow and soft agar cultures were negative for AChE and these immunocytochemical markers. These data indicate that the pattern of expression of AChE during maturation of MK is similar in vivo and in vitro and demonstrate, when using this marker at the fine-structural level, that a greater range of MK maturational stages is present in heterogeneous colonies than is observed in MK in big-cell colonies. Furthermore, we have confirmed that small cells in heterogeneous colonies are MK and that these colonies are composed solely of MK and their precursors.

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in megakaryocytes at early time points in culture (and hence presumably at various stages of differentiation) and, particularly, the study of small, immature cells that partially constitute the heterogeneous megakaryocyte colonies. In contrast to bone marrow cell suspensions, these megakaryocyte colonies permit the study of purified populations of cells of the megakaryocytic lineage at different stages of maturation, without the requirement for initial physical separation from other cell lineages. We specifically compared at the ultrastructural level the pattern of expression of AChE and the distribution of plasma membrane glycoproteins in heterogeneous megakaryocyte colonies and big-cell megakaryocyte colonies to determine whether this system might be useful to study megakaryocyte differentiation.

MATERIALS AND METHODS

Mice

C57BL/6J mice, obtained from Simonsen Laboratories, Inc (Gilroy, CA), were used for these experiments. These mice were bred under specific pathogen-free conditions and then maintained under conventional conditions.

Preparation of Murine Bone Marrow Samples

Mouse femoral marrow samples were obtained from animals killed by cervical dislocation. A 25-gauge needle was inserted into the proximal end of the femur, and fixative was gently pushed through the length of the bone to remove the marrow core. The marrow cells were dispersed into a single-cell suspension by gentle pipetting. The cells were fixed in either 1.44% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, for two hours at ambient temperature for AChE staining or in 2% paraformaldehyde with 0.05% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for two hours at ambient temperature for immunocytochemical staining.

Preparation of Murine Megakaryocyte Colonies

Spleen cells were obtained as previously described, and these were used rather than bone marrow because spleen cells produce a greater proportion of megakaryocyte colonies. Cultures of spleen cells were maintained in 0.3% agar in Dulbecco’s modified Eagle’s medium as described previously. The final concentration of horse serum was 20%. One milliliter of a splenic cell suspension in agar medium was cultured with 0.1 mL of pokeweed mitogen stimulated, spleen cell-conditioned medium and incubated for three to seven days in a humidified atmosphere of 10% CO2 in air. In this culture system, megakaryocyte colonies achieve their maximum frequency and ploidy (DNA) levels after seven days. At various times, colonies were removed from culture dishes with a finely drawn Pasteur pipette and placed either in a vial containing fixative or a small drop of fixative. In the latter case, an equal volume of warm 3% agar was layered onto the colony-fixative drop, allowed to harden, and removed as a disc into a Petri dish of fixative. Fixatives were as described earlier. Granulocyte and macrophage colonies, which were also present in this culture system, were similarly obtained and studied. After fixation, the samples were rinsed in the appropriate wash buffer.

Cytochemical Procedures for Detection of AChE

AChE cytochemistry was performed on suspensions of bone marrow cells or colonies obtained from soft agar cultures by using the technique of Tranum-Jensen and Behnke as modified by Paulus et al. Control preparations included the omission of substrate or the substitution of butyrylthiocholine iodide for acetylthiocholine iodide (Sigma Chemical Co, St Louis). Other samples were preincubated and stained in the presence of 10⁻³ mol/L eserine (Sigma), an inhibitor of both AChE and nonspecific cholinesterase. The samples were then processed for electron microscopy.

Immunocytochemical Procedures

Immunocytochemical procedures on suspensions of bone marrow cells or colonies obtained from soft agar culture were performed as follows. After fixation, the cells were rinsed several times in 0.1 mol/L phosphate buffer, pH 7.4, with 50 mmol/L NH₄Cl and 2% sucrose. Then they were incubated with rabbit antihuman glycoprotein IIIa serum (a gift of Dr Rodger P. McEver, San Antonio, TX) or rabbit antimonocyte platelet antiserum (PAS) (both of which were diluted 1:500 in phosphate-buffered saline) overnight at 4°C. We have previously demonstrated that antisera directed against human platelet antigens identify murine megakaryocytes. Rabbit antisera PAS was prepared and carefully absorbed with both BRC and WBC as described previously, and an immunoblot assay with solubilized platelet proteins as the target antigens demonstrated that this PAS was active against multiple murine platelet proteins ranging from less than 45 kilodaltons (kD) to more than 300 kD, the resolving limits of the gel system. The most intense labeling occurred against the region of the gel that contained the platelet glycoprotein IIb/IIIa complex. Microscopy with immunoperoxidase-labeled reagents demonstrated no reactivity of the PAS against nonmegakaryocytic bone marrow cells or granulocyte or macrophage colonies, whereas megakaryocytes of all maturation stages were labeled by this polyclonal PAS. Subsequently, bone marrow cells or colonies were rinsed in phosphate-buffered saline several times and then incubated with protein A–gold probes (5-, 10-, or 15-nm-sized particles) (Jansen Pharmaceuticals, Beere, Belgium) diluted 1:20 in 20 mmol/L Tris buffer, pH 8.2, with 0.9% NaCl and 0.1% bovine serum albumin. The samples were rinsed four times with the same Tris buffer and then fixed in 1.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, with 1% sucrose for 45 minutes. They were left overnight at 4°C in 0.1 mol/L cacodylate buffer, pH 7.4, with 1% sucrose and then processed for electron microscopy as will be described. Control procedures included the substitution of irrelevant antibodies, buffer, or preimmune serum for the primary antibodies.

Preparation of Bone Marrow and Colony Samples for Transmission Electron Microscopy

Fixed, stained samples were osmicated (2% OsO₄ in veronal-acetate buffer) and then dehydrated in a graded series of ethanol, infiltrated with propylene oxide, and embedded in Epon. Thin sections (100 nm) were cut, stained with either uranyl acetate and lead citrate or lead citrate alone, and examined with a Philips EM-201 transmission electron microscope.

RESULTS

Classification of the maturation stages of megakaryocytes is complex because cytoplasmic and nuclear development are not synchronous events. The present study is primarily based on the system proposed by Paulus in his ultrastructural studies of guinea pig megakaryocytes and used by Bentfield-Barker and Bainton in their ultrastructural studies of rat and human megakaryocytes. In brief, immature megakaryocytes are small cells measuring 6 to 24 μm in the long axis, containing a large, round, indented, or lobed nucleus with prominent nucleoli, and having a high nuclear:cytoplasmic ratio. These cells contain few cytoplasmic organelles other than polyribosomes and mitochondria.
Maturing megakaryocytes are heterogeneous in size (14 to 30 μm) and in the level of polyploidy (8 to 32N). They contain a large protein synthetic apparatus and variable numbers of demarcation membranes and α granules. Mature megakaryocytes are approximately 20 to 50 μm in diameter with a low nuclear:cytoplasmic ratio and contain an extensive demarcation membrane system and a reduced protein synthetic apparatus. In contrast to murine bone marrow in which immature megakaryocytes constitute only a small proportion of the cells and are difficult to identify without...
the use of specific markers, megakaryocyte colonies from soft agar cultures allow the study of sufficient populations of cells of the megakaryocyte lineage that may be more precisely evaluated (ie, in regard to age) than in the bone marrow.

Megakaryocyte colonies present after seven days in soft agar culture (at which time the maximum number of megakaryocyte colonies has been reached) were composed of megakaryocytes at various stages of maturation, from immature to mature cells. Morphologically, mature cells in big-cell and heterogeneous colonies (Fig 1) resembled bone marrow megakaryocytes with the following exceptions. These cells had greater numbers of multivesicular bodies, which presumably contain agar ingested from the surrounding culture medium, than bone marrow megakaryocytes. Additionally, the number of α granules that were uncondensed was greater in megakaryocytes in colonies than in bone marrow megakaryocytes. The size of mature megakaryocytes in seven-day colonies approximated the size of mature bone marrow megakaryocytes, although no evidence of platelet production was observed. At least two thirds of maturing (Fig 2) and mature megakaryocytes examined from both big-cell and heterogeneous colonies contained demarcation membranes only in one area of the cell cytoplasm. These membranes had fewer connections to the megakaryocyte plasma membrane and compartmentalized less cytoplasm than did membranes in bone marrow megakaryocytes. Areas of these demarcation membranes in cells from colonies did not contain many other organelles such as mitochondria and α granules in contrast to bone marrow megakaryocytes, which exhibited a more uniform distribution of organelles. The appearance of these cells partially resembled that of megakaryocytes described previously by Zucker-Franklin and Petursson.34

**Distribution of AChE in Murine Bone Marrow Megakaryocytes and Megakaryocytes in Soft Agar Culture**

**Bone marrow megakaryocytes.** In immature mouse bone marrow megakaryocytes, reaction product for AChE was confined to the perinuclear cisterna and endoplasmic reticulum (Fig 3A). Few granules and demarcation membrane system profiles were observed in these cells and, if present, were generally unreactive. Occasionally, mouse erythroblasts also demonstrated AChE reaction product in the perinuclear cisterna and endoplasmic reticulum, as reported previously by Paulus et al.28 In mature mouse bone marrow megakaryocytes, reaction product for AChE was observed in the perinuclear cisterna, endoplasmic reticulum, some cisternae of the Golgi complex, some profiles of the demarcation membrane system, and occasional granules (Fig 3B). All other bone marrow cells including eosinophils were completely unreactive for AChE. Control samples in which specific substrate was either omitted or substituted with butyrylthiocholine or in which preincubation and incubation with eserine was performed resulted in megakaryocytes that were negative for AChE (not shown).

**Megakaryocytes in colonies in soft agar culture.** Most megakaryocytes in colonies, after three days of soft agar culture, were immature in appearance with a high nuclear:cytoplasmic ratio and few cytoplasmic organelles.

![Fig 2. A maturing mouse megakaryocyte obtained from a four-day big-cell megakaryocyte colony. The demarcation membrane system (dms) is concentrated in one area of the cell cytoplasm and contains few other organelles. N, nucleus; Gc, Golgi complex; mvb, multivesicular body (original magnification x 6,000).](image-url)
These cells contained reaction product for AChE within the perinuclear cisterna and endoplasmic reticulum (Fig 4A). Occasional megakaryocytes were observed in three-day big-cell colonies, which were considerably more mature and larger in size and contained reaction product in the perinuclear cisterna, many profiles of endoplasmic reticulum, and some cisternae of the Golgi complex (not shown). Megakaryocytes in seven-day big cell colonies resembled mature megakaryocytes in bone marrow with respect to the distribution of AChE. Megakaryocytes in seven-day heterogeneous colonies varied considerably in size and stage of maturation. Small megakaryocytes from these colonies resembled immature megakaryocytes from three day big-cell colonies. All small megakaryocytes examined contained AChE reaction product in the perinuclear cisterna, endoplasmic reticulum (Fig 4B), and some cisternae of the Golgi complex, if this organelle was present. In contrast to data obtained earlier that showed these small cells to be 2N or 4N in ploidy and AChE-negative, we observed positive label in every cell examined at the ultrastructural level. This may be explained by the greater sensitivity of the ultrastructural cytochemical technique as compared with the light microscope level technique. Furthermore, increasing the time of incubation with substrate to 6 hours has demonstrated that all cells in
Fig 4. (A) An immature mouse megakaryocyte from a three-day big-cell megakaryocyte colony reacted for AChE. As in Fig 2A, reaction product is confined to the perinuclear cisterna (pc) and rough endoplasmic reticulum (rer) (original magnification x13,000; current magnification x12,090). (B) An immature mouse megakaryocyte from a seven-day heterogeneous colony reacted for AChE. As in Figs 2A and 3A, reaction product is found in the perinuclear cisterna (pc) and rough endoplasmic reticulum (rer) (original magnification x13,000; current magnification x12,090). (C) A maturing megakaryocyte from a seven-day heterogeneous colony reacted for AChE. Reaction product is present in the perinuclear cisterna (pc), some cisternae of the Golgi complex (Gc), and some profiles of the rough endoplasmic reticulum (rer). Very few demarcation membranes or granules are present in this cell (original magnification x13,000; current magnification x12,090); mvb, multivesicular body. (D) A mature megakaryocyte from a seven-day heterogeneous colony reacted for AChE. Reaction product is distributed similarly to mature bone marrow megakaryocytes: perinuclear cisterna (pc), rough endoplasmic reticulum (rer), Golgi complex (Gc), demarcation membrane system (dms), and some granules (g) are labeled. The dms is thicker than the rer. The marked increase in the size of megakaryocytes as they mature in culture is clearly shown by the increased area occupied by the cells in this figure, each of which is shown at the same magnification (original magnification x13,000; current magnification x12,090). N, nucleus.
heterogeneous colonies are AChE-positive at the light microscopic level (J. Levin and F. Levin, unpublished observations). Maturing and mature megakaryocytes from seven-day heterogeneous colonies resembled maturing and mature bone marrow megakaryocytes with respect to the distribution of AChE (Figs 4C and 4D). Cells from granulocyte or macrophage colonies grown for three to seven days in soft agar culture were negative for AChE (not shown).

**Distribution of Plasma Membrane Glycoproteins in Murine Bone Marrow Megakaryocytes and Megakaryocytes in Soft Agar Culture**

**Bone marrow megakaryocytes.** Mature, recognizable bone marrow megakaryocytes exhibited extensive immunogold label along their plasma membranes when cell suspensions were incubated with either PAS or antiligycoprotein IIIa antibodies and any of the three sizes of protein A-gold probe used, although the greatest amount of label was obtained with PAS followed by protein A-gold (particle size, 5 nm; not shown). All other bone marrow cells were completely negative. Control samples of all bone marrow cells including megakaryocytes in which normal rabbit serum or buffer was substituted for specific primary antibodies also were negative (not shown).

**Megakaryocytes in colonies in soft agar culture.** All megakaryocytes in either big-cell or heterogeneous colonies after three to seven days in soft agar culture showed immunogold label for PAS or glycoprotein IIIa along the extent of the plasma membrane, regardless of their stage of maturation (Fig 5 and Figs 6A and B). Megakaryocytes in colonies demonstrated a lower concentration of gold particles along plasma membranes than mature bone marrow cells. Although these experiments were technically difficult to perform, all of the immunoreagents passed through the thin agar shell surrounding the colonies.

**DISCUSSION**

We have investigated the maturation of murine megakaryocytes in soft agar cultures at the ultrastructural level with respect to their morphological appearance and the distribution of AChE and plasma membrane glycoproteins and determined that both big-cell and heterogeneous colonies contain only megakaryocytes. Immature cells in three-day big-cell colonies, seven-day heterogeneous colonies, and bone marrow demonstrated reaction product for AChE predominantly in the perinuclear cisterna and endoplasmic reticulum and also in Golgi cisternae, if present. In contrast, large megakaryocytes from seven-day big-cell and heterogeneous megakaryocyte colonies and mature bone marrow cells were much more reactive than immature cells, with reaction product present in the perinuclear cisterna, endoplasmic reticulum, Golgi complex, demarcation membrane system, and some granules. All cells in megakaryocyte colonies, regardless of the time in culture or extent of morphological maturity, exhibited immunogold label along the plasma membrane with PAS or anti-GP IIIa serum. Importantly, cells in big-cell colonies achieve their ultimate mean ploidy distribution four days after the initiation of cultures, whereas...
Fig 6. (A) Immature megakaryocyte from a seven-day heterogeneous colony reacted with PAS followed by a protein A–gold (size, 10 nm) probe. At higher magnification (inset) the immunogold label is clearly evident along the plasma membrane (arrows). This cell has a high nuclear:cytoplasmic ratio and only a few dms profiles (original magnification ×5,000; inset ×21,000). (B) Maturing megakaryocyte from a seven-day heterogeneous colony reacted with PAS followed by a protein A–gold (size, 10 nm) probe. Note that this cell is larger, with a lower nuclear:cytoplasmic ratio, and contains more demarcation membranes than the cell in (A). The large, dense granules are multivesicular bodies. At higher magnification (inset) the immunogold label is seen along the plasma membrane at a concentration comparable to that seen in (A) (original magnification ×5,000; inset ×21,000). N, nucleus; dms, demarcation membrane system; Gc, Golgi complex.
the mean ploidy level of heterogeneous colonies continues to increase after four days of culture. Therefore, megakaryocytes express lineage-specific glycoproteins before they attain their final polyploid stage. Pertinently, although heterogeneous colonies are derived from a single precursor cell, they are comprised of megakaryocytes at different stages of maturation (as determined by morphological appearance, size, and AChE localization) in contrast to megakaryocytes in big-cell colonies, which are more uniformly mature in appearance. Because approximately half of the cells in heterogeneous colonies contain 2N or 4N levels of DNA,5,6 our current fine-structural studies further indicate that cells at low ploidy levels express AChE and lineage-specific membrane glycoproteins. Previous studies have localized AChE to the Golgi complex as early as day 2 of human bone marrow cultures (6 to 8 μm in diameter), which they considered to be megakaryocyte precursor cells.22 As previously reported for bone marrow cells,10,28 the Golgi complex, endoplasmic reticulum, and nuclear envelope as well as demarcation membranes and some granules were labeled in more mature megakaryocytes in soft agar culture. Reaction product in the Golgi complex of cells examined in our study was sometimes confined to only one face of the Golgi complex or was present throughout the Golgi complex, as Tranum-Jensen and Behnke also previously reported.10 Therefore, AChE is synthesized and packaged into granules in megakaryocytes maturing in soft agar culture, similarly to megakaryocytes in vivo. This perhaps supports the suggestion of Paulus et al28 that cholinergic regulation may be involved in the control of megakaryocyte growth and therefore operative both in vivo and in vitro. Additional support for this hypothesis is obtained from the report of Burstein et al37 that cholinergic agonists increase the frequency of megakaryocyte colonies in vitro.

Platelet peroxidase is currently the earliest recognized marker of human megakaryocyte maturation because it is present in cells five to six days after the initiation of plasma clot cultures. More recently, Jenkins et al28 have reported that megakaryocytes derived from human committed megakaryocyte progenitor cells in vitro synthesize glycoproteins IIb and IIa. Jackson has demonstrated that small rat marrow cells expressing AChE activity also were labeled by fluorescent anti-PAS.28 We have determined that both AChE and lineage-specific membrane glycoproteins are detectable in immature murine megakaryocytes three days after the culture of Meg-CFC in soft agar, the first day of culture in which murine megakaryocyte colonies can be confidently visually identified under the light microscope. Because it is difficult to reliably retrieve megakaryocyte colonies from soft agar cultures before this time, we were unable to determine which of these markers appears first in our culture system.

It is of interest that during neutrophil differentiation some antigens are lost (as identified by monoclonal antibody MY-10 or those to la antigen) or acquired (as identified by monoclonal antibodies Mo1, MY8, 80H.3, or B34.3) as these cells mature from myeloblasts to polymorphonuclear leukocytes, whereas expression of other antigens (as identified by monoclonal antibodies 82H5, R1B19, and S4-7) occurs throughout the neutrophil maturation sequence.40 Recently, using monoclonal antibodies with light microscopy, Levene et al25 have identified three maturational levels in the human megakaryocyte lineage with distinct immunologic phenotypes; some monoclonal antibodies recognize epitopes expressed only at certain stages of maturation (eg, on immature megakaryocytes or on both immature and mature megakaryocytes) or that, in contrast, are present throughout the entire megakaryocyte/platelet lineage. Utilization of the soft agar culture system in conjunction with similar probes to examine murine or human megakaryocyte maturation at the ultrastructural level will yield important information concerning the time of expression and localization of specific proteins during the developmental sequence of this cell. Future studies will examine the effects of administration to mice of PAS or 5-fluorouracil, agents known to perturb thrombopoiesis, to obtain additional information about megakaryocyte differentiation.

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Ultrastructural analysis of murine megakaryocyte maturation in vitro: comparison of big-cell and heterogeneous megakaryocyte colonies

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