Thrombocytopoiesis—Analysis by Membrane Tracer and Freeze-Fracture Studies on Fresh Human and Cultured Mouse Megakaryocytes

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ABSTRACT The origin of platelets (Pt) from megakaryocytes (MK) is beyond question, but the mechanism whereby Pts are released from the precursor cell is still debated. A widely-held theory claims that the MK plasma membrane invaginates to form demarcation membranes (DMS), which delineate Pt territories. Accordingly, Pts would be derived mostly from the periphery of the MK, and the MK and Pt plasma membranes would have to be virtually identical. Since, on morphologic grounds, this theory is untenable, several aspects of thrombocytopoiesis were reexamined with the help of membrane tracer and freeze-fracture analyses of freshly-collected human and cultured mouse MK. To our surprise, freeze-cleavage of the MK plasma membrane revealed that the vast majority of intramembranous particles (IMP) remained associated with the protoplasmic leaflet (P-face), whereas the partition coefficient of IMPSs of the platelet membrane was the reverse. This is the first time that any difference between MK and Pt membranes has been determined. Replicas of freeze-fractured MK that were in the process of thrombocytopoiesis revealed an additional novel phenomenon, i.e., numerous areas of membrane discontinuity that appeared to be related to Pt discharge. When such areas were small, the IMP were lined up along the margin of the crevice. At a later phase, a labyrinth of fenestrations was observed. Thin sections of MK at various stages of differentiation showed that Pt territories were fully demarcated before connections of the DMS with the surface could be found. Therefore, the Pt envelope is probably not derived from invaginations of the MK plasma membrane. When living, MK were incubated with cationic ferritin or peroxidase at 37°C, the tracers entered into the DMS but did not delineate all membranes with which the DMS was in continuity, suggesting the existence of distinctive membrane domains. Interiorization of tracer was not energy-dependent, but arrested at low temperatures. At 4°C the DMS remained empty, unless there was evidence that Pts had been released. In such instances, the tracers outlined infoldings of peripheral cytoplasm that was devoid of organelles. Thus, the majority of Pts seem to originate from the interior of the MK, and the surface membranes of the two cells differ in origin and structure. The observations do not only throw new light on the process of thrombocytopoiesis, but also strengthen the possibility that MKs and Pts may be subject to different stimuli.

Ever since the recognition by Heidenhain in 1894 (1) that the cytoplasm of megakaryocytes (MK) is organized into distinct territories, the discovery by Wright in 1906 (2) that megakaryocytes give rise to circulating platelets, and the electron microscopic description of the demarcation membrane system (DMS) by Yamada in 1957 (3), the mechanism whereby platelets are released from their precursor cells has been debated. The majority of investigators have adduced...
evidence in support of Wright’s original theory that the periphery of the MK forms ameboid processes that fragment into platelets (Pt) (4-6). Phase-contrast cinematography and scanning electron microscopy have been employed to show that pseudopod-like processes of MKs extend from the marrow compartment into the sinusoidal space (7-9). These pseudopods are believed to dissociate into Pts. On the other hand, there is incontrovertible evidence derived from studies on numerous mammals including man, that intact, mature MKs can also enter the peripheral circulation and that their nuclei may become denuded of cytoplasm in the pulmonary vascular bed (10-13). In addition, it has been established that the DMS, which delineates the Pt territories within the confines of the MK cytoplasm, is in continuity with the extracellular space (14). This alone has led to the conclusion that the DMS is formed by invagination of the MK plasma membrane and has necessitated models explaining how the tubules resulting from invaginations of the MK surface membrane could give rise to the plasma membrane of individual Pts (15). Indeed, it is generally assumed that the Pt membrane is identical to the MK membrane. There is extensive antigenic cross-reactivity between the two cells (16, 17) and some monoclonal antibodies raised against specific Pt glycoproteins react with identical components of MKs (18). In addition, there is histochemical evidence supporting the view that the Pt surface ‘coat’ resembles the coat associated with the DMS (19). All these observations provide no proof that the membranes of the DMS constitute the plasma membranes of future Pts. In fact, with recognition by Paulus et al. (20) of the log normality of the Pt volume distribution, it became unlikely that each Pt territory would give rise to an individual Pt, particularly in conditions when thrombocytopoiesis is accelerated or depressed. On the basis of purely mathematical considerations leaning heavily on the observed log normal distribution of Pt volume, Trowbridge et al. (21) have also concluded that a physical fragmentation process of the MK cytoplasm is the most likely explanation for Pt release. This would imply that the plasma membrane of individual Pts may not derive from the MK plasma membrane, since most potential Pt territories may never have been located near the surface of the precursor cells. Therefore, MK and Pt plasma membranes could have properties that are distinct from each other. We wished to explore this possibility by previously-used methods to elucidate some unique characteristics of the Pt membrane (22). Our observations suggest that, as a rule, Pts do not arise from the surface of MKs and that their plasma membranes differ from those of the mother cells.

MATERIALS AND METHODS

Cells: Human MKs were prepared from heparinized bone marrow obtained from patients whose marrow was sampled for diagnostic purposes, and which subsequently proved to be normal. The specimens were washed several times with Hanks’ saline, after which they were centrifuged at 250g for 10 min to sediment most of the erythrocytes. The ‘buoy coat’ was obtained with a Pasteur pipette and placed into 3% phosphate-buffered glutaraldehyde. Pts were collected from anticoagulated peripheral blood as described repeatedly (22). However, the vast majority of studies were conducted on cultured mouse MKs. To this end, marrow was flushed from the femurs of 8-10-wk-old male BDF, mice (Jackson Laboratories, Bar Harbor, ME) into Fischer’s medium. Contin-

FIGURE 1 Cultured mouse MKs prepared by elutriation, cytocentrifuged, and stained with Wright’s and Giemsa. Various stages of Pt development are illustrated: (A) MKs with a smooth periphery. The cells have a very large lucent region that appears granulated but without evidence of Pt territories. The nucleus is mature. Neutrophils (N) help to appreciate the large size of the MKs. (B) Three MKs showing a smooth contour. In contrast to the cells depicted in A here the development of Pt territories is seen clearly (arrow). (C) The MK at the top appears to have been fixed in the process of cytoplasmic fragmentation. Some of the Pts are still attached to the main body of the cell. Others, at the periphery, have completely separated. On the bottom, a denuded MK nucleus is shown closely associated with the mass of platelets which are probably derived from its cytoplasm. × 600.
uous liquid cultures were initiated by pooling the marrow cells in 25-cm² plastic flasks (Falcon Labware, Oxnard, CA) at concentrations of 2 x 10⁶ cells/ml, essentially as described by others (23, 24). Each flask contained 10 ml of Fischer's medium supplemented with 20% horse serum (Flow Laboratories, McLean, VA), penicillin and streptomycin (Grand Island Biological Co., Grand Island, NY), and hydrocortisone sodium succinate (Solu Cortef, Upjohn, MI).

**Figure 2**
Electron micrographs of cultured mouse MKs illustrating the process of thrombocytopoiesis. (a) Elutriated; (b–d) not elutriated. (a) Detail of the peripheral cytoplasm of a cell comparable to the one illustrated in Fig. 1b. Note that the marginal zone is devoid of organelles and cytoplasmic invaginations, whereas the intermediate zone is beginning to show Pt territories. x 8,500. (b) A mature MK with a highly convoluted 'staghorn' nucleus shows fully-developed platelet territories. The intervening cytoplasm is replete with membranes forming vesicles and tubules. The surface membrane of this cell is folded into numerous processes but there is still a narrow peripheral zone without cytoplasmic invaginations. P, Pt territory. x 3,700. (c) A fully mature MK showing dissociation of the cytoplasm into Pts. Note that the peripheral zone (PZ) appears to have fragmented into cytoplasmic segments with relatively few organelles. It cannot be deduced from this illustration whether the peripheral zone will remain attached to the nucleus of the cell. x 300. (d) Section through the pole of a MK illustrates opening through the peripheral zone. It is possible that unattached Pts (arrow) derived from the interior of the MK may be discharged via such an opening. Note that the peripheral zone is not demarcated into platelet territories. L, lymphocyte. x 2,800.
at a final concentration of $10^{-4}$ M. Incubation took place at 33°C in a 5% CO₂ atmosphere. At weekly intervals, half of the medium and half of the freely suspended cells were removed from the culture flasks, pooled, and assayed for total cell count, Mks, CFU-M, CFU-GM, and differentiated cells. The removed medium was replaced with an equal volume of fresh medium. The cultures were not recharged with fresh cells at 3 wk as has been described in other reports (23, 24). The concentration of Mks in the flasks was determined by the technique of Ebbe et al. (25). Briefly, 2 ml of the cell suspensions were mixed with 0.5% new methylene blue (vol/vol). The Mks were counted directly in a hemocytometer at 40x. The total number per flask was calculated from the hemocytometer readings after making corrections for the 10% dilution with new methylene blue. The specimens used in the experiments described here were harvested between 2-20 wk of culture and always 1 wk after half of the cells had been removed and half of the medium had been replaced with fresh medium. Most of the studies were carried out on samples enriched for Mks by subsequent centrifugal elutriation as follows: 30-100 ml of supernatant culture fluid containing cells in suspension were placed into a Beckman JE-6 elutriator rotor held in a J2-6B Beckman centrifuge (Beckman Instruments, Palo Alto, CA). A strobe light permitted visualization of the cells in the chamber during separation and a digital tachometer was used to measure the rate. The rates of flow of the cell suspension passing through the rotor were controlled by a Masterflex pump (Coll Financial Instrument Corp., Chicago, IL) with an external potentiometer. As recommended by Worthington and Nakoff (26) cells and elutriation fluid were drawn rather than pushed through the system by the Masterflex pump attached to the efflux side of the rotor. This spared Mks from the mechanical action of the pump. Dulbecco's phosphate-buffered saline containing 20% horse serum was used as the elutriation fluid. Elutriation proceeded at room temperature at 750 rpm. The samples were loaded into the chamber at a flow rate of 10 ml/min, but they were withdrawn at a slower rate of 25 ml/min. A total of 250 ml of elutriation fluid was used to remove most of the granulocytes and macrophages. The head of the elutriator was then dismantled and the separation chamber containing the enriched Mks was removed.

**Tracer Studies:** After light microscopy of cytocentrifuged and stained samples had established that Mks were well-preserved, aliquots of the cell suspensions were incubated with horseradish peroxidase (Sigma Chemical Co., St Louis, MO) 5 mg/ml or cationized ferritin (Miles Biochemicals, Elkhart, IN) 2 mg/ml final concentration. The incubation periods ranged from 10 min to 2 h and were conducted at 4°C, 20°C, and 37°C. The interiorized peroxidase was localized with the substrates H₂O₂ and 3,3'-diaminobenzidine as described in detail elsewhere (22, 27). Metabolic inhibition was brought about by addition to the cell suspension of $10^{-3}$ M NaN₃, $10^{-2}$ M 2-deoxyglucose, and $10^{-3}$ M iodoacetic acid. The inhibitors were added to the MK/Pt suspensions 30 min before the tracer substances. For experiments conducted at 4°C, the MK suspensions were cooled in ice for 30 min before addition of the tracers.

**Freeze-Fracture and Electron Microscopy:** The incubation or culture periods were terminated by addition of 1% phosphate-buffered glutaraldehyde for 2 h and processed for transmission electron microscopy by postfixation with osmium tetroxide for 2 h followed by staining en bloc with 0.5% uranyl acetate in saline for 1 h. Dehydration and embedding were carried out as described elsewhere (22). For freeze-fracture studies, aliquots of the glutaraldehyde fixed specimens were washed with distilled water and resuspended in 25% glycerol for 2 h at room temperature to effect cryoprotection. The glycinated cells were sedimented in a Beckman microfuge (Beckman Instruments, Inc.), quick-frozen with Freon 22 and further cooled with liquid N₂. Membrane cleavage was executed in a Balzers high vacuum freeze etch unit BAF (Hudson, NH) in a vacuum of $10^{-4}$ and temperature of −100°C. The cleaved surfaces were shadow-cast with platinum and carbon at angles of 45° and 90°, respectively. After thawing the replicas were cleared of proteinaceous debris by soaking overnight with Chlorox, rinsed in 50% Chlorox/50% distilled water for 1 h, and then subjected in sequence to three changes of distilled water, 100% acetone for 2 h, three changes of distilled H₂O₂, 2% acetic acid for 2 h, and five rinses with distilled H₂O. Intramembranous particles (IMP) were counted on prints having a final magnification of 90,000. The surface areas were measured with a compensating polar planimeter (Keuffel and Esser Co., Morristown, NJ). The specimens to be used for transmission electron microscopy were dehydrated and embedded in Poly/Bed 812 and sectioned with an LKB ultratome (LKB Instruments, Inc., Gaithersburg, MD). Thin sections were contrasted with uranyl acetate and lead citrate. A Siemens Elmiskop 1 electron microscope was the instrument used for all studies.

**RESULTS**

It should be mentioned at the outset that the observations to be described here were made on fresh mouse and human bone marrow as well as on cultured mouse Mks. The illustrations were selected from cultured mouse marrow because this material was more abundantly available. Moreover, although elutriation resulted in a 10-fold enrichment of Mks, when specimens were designed for embedding it was often deemed convenient to eliminate this step in favor of a larger pellet. No morphologic differences were noted whether the specimens were elutriated or not.

Since the morphology of Mks maintained in liquid culture has been described (28), and since it did not differ much from that of fresh cells illustrated in detail elsewhere (29), only features relevant to the subject under investigation, i.e., thrombocytopoiesis will be analyzed. The cultures provided every stage of MK maturation and numerous free Pts were seen even in 3-mo-old cultures. Light microscopy of cytocen-

**FIGURE 3** Examples of P-face (a) and an E-face (b) of Pt plasma membranes obtained from mouse MK cultures. The preparations were not elutriated. The IMPS associated with the P-face are smaller and not as numerous as those on the E face. Also note that the P-face reveals pits (arrow), whereas the E-face has complementary protrusions (arrow). A more detailed description of the freeze-fracture properties characteristic for platelets can be found in reference 22. × 62,000.
trifuged, stained preparations permitted clear delineation of platelet territories, even though the DMS could not be resolved by this technique (Fig. 1, A–C). The majority of cells with mature multilobed nuclei had a dense, smooth peripheral zone which was devoid of organelles and free of Pt 'budding' (Fig. 1A). In some cells, the light-appearing area of the cytoplasm ('intermediate zone' of Yamada [3]) was well-granulated, but the organelles had not aggregated into recognizable territories. In other cells with a smooth peripheral zone, Pt territories could be distinguished readily (Fig. 1B). The apparent disintegration of the cytoplasm into individual Pts, a process which extended from the vicinity of the nucleus to the periphery of the cell (Fig. 1C), was observed more commonly in some specimens than in others, but was never exhibited by >10% of cells. There was considerable variability in the percentage of MKs engaged in Pt release from one culture to another. This did not depend on the age of the culture or subsequent manipulations. However, the final outcome appeared to be exactly what is believed to take place in vivo, namely complete fragmentation of the cytoplasm and separation from an all but denuded nucleus (Fig. 1C, bottom). It should be noted that ameboid processes believed to consist of long chains of unseparated Pts, as suggested originally by Wright (4) and illustrated by others (5) were not seen in the cultured specimens.

On electron microscopy, the process of MK fragmentation was even more striking (Fig. 2, a–d). A detail of a cell that would probably have appeared 'smooth' in light microscopy is seen in Fig. 2a. Although the Pt territories are formed, the DMS does not extend into the peripheral zone, which is devoid of organelles. On the other hand, the MK illustrated in Fig. 2b presents a much narrower marginal zone and an almost 'villous' periphery. Pt territories have fully formed and, with the exception of the perinuclear area, the mitochondria and granules are confined to these regions. The intervening cytoplasm is replete with profiles of smooth endoplasmic reticulum that make up the vesicles and channels of the DMS. In many cells this membrane system seemed hypertrophied. Such hypertrophy of the DMS is often seen in MKs of pathologic marrow (30–33), but only rarely in cells obtained from healthy individuals. The cell shown in Fig. 2c is an example of one in which the cytoplasm is beginning to disintegrate into individual Pts of various sizes, while Fig. 2d illustrates a section through the pole of a MK that was fixed fortuitously at a stage when fragmentation was almost complete. The last image shows particularly well that individual Pts may originate from the interior of the MK (see arrow in Fig. 2d). Such Pts are unlikely to be enveloped by the plasma membrane of the mother cell. It also should be noted that Pt territories are not as well delineated at the periphery as at the center of the cell shown in Fig. 2d.

**Freeze-fracture**

To appreciate the structure of MK membranes in the

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**Figure 4** The inset shows a replica of a freeze-fractured cultured mouse MK. In contrast to the cells shown in Fig. 3, this preparation was not elutriated. The area demarcated by the rectangle is shown at higher resolution. Note that the E-face of the plasma membrane exhibits very few intramembranous particles. Occasional protrusions are evident (arrow). × 60,000.
FIGURE 5  Freeze-fracture replicas illustrating portions of P-faces of mouse MK plasma membranes (specimen not elutriated). Area demarcated by the rectangle in a is shown at higher resolution in b. Arrows indicate an area over which 'pits' seem to be concentrated. In b, continuity of the plasma membrane and the DMS via a pit is suggested. Arrow indicates a small area where the membrane has disrupted. c shows continuity between the DMS and MK surface. The number of IMPs associated with the DMS appears to be about the same as on the P-face of the MK plasma membrane. Compare with a and b. (a) $\times 13,000$. (b and c) $\times 60,000$. 
context of the subject under study, it is necessary to recall some aspect of the Pt membrane. The Pt is unusual in that freeze-cleavage of its plasma membrane causes a much larger number of IMP to remain associated with the outer leaflet of the bilayer (E-face) than its protoplasmic aspect (P-face) (22, 34, 35). The reverse is true of most other mammalian cells. Moreover, the IMPs of the E face are heterogeneous in size and shape as has been reported in detail elsewhere (22). In addition, the P-face of the Pt surface membrane reveals a variable number of pits or pores that measure ~25 nm in diameter and that have been shown to be in continuity with the open canalicular system. The E-face shows complementary protrusions. These observations held equally true for Pts that had formed in the MK cultures (Fig. 3, a and b). If Pts dissociated from the periphery of the MK, it seemed likely that the plasma membrane of the MK would have a similar partition coefficient. Much to our surprise, the freeze-cleaved MK membrane had an IMP distribution characteristic for other mammalian cells and unlike that of the Pt. Fig. 4 shows the dearth and small size of the IMPs on the E-face of the MK plasma membrane while Fig. 5 illustrates the density of the IMPs on the P-face. However, like the Pt plasma membrane, the P-face of the MK surface membrane revealed pits (with complementary protrusions on the E face) that were in continuity with the DMS. Because it has been suggested by others (14, 15) that the DMS is formed by invagination of the surface membrane, particular attention was focused on the distribution of IMPs associated with the membranes making up this system. In general, the impression was gained, that in areas where continuity existed between the DMS and the plasma membrane, the partition coefficient was the same, i.e., P-face > E-face (Fig. 5 c). Casual inspection of freeze-fracture images of DMS membranes located proximal to the peripheral zone did not permit a similar conclusion. In fact, as illustrated in Fig. 6, DMS membranes with a convex curvature and protrusions seem to have about the same number of IMPs as membranes presenting a concave curvature exhibiting pits. To substantiate this impression the IMPs were counted. To this end, it was assumed that P-faces are identifiable by pits and E-faces by protrusions independent of membrane curvature. Only DMS replicas presenting such structures were evaluated.

When IMPs of eight P-faces and nine E-faces were counted, the P-faces proved to have 672.98 ± 135 and the E-faces 778.18 ± 190 IMPs per μm² (P 0.3 > p > 0.2). Thus, there was no significant difference in the number of IMP that remained associated with either membrane half of the DMS following cleavage. Therefore, the partition coefficient of the internal DMS membranes appears to be unlike that of the Pt or MK envelope.

Freeze-fracture replicas of MKs engaged in thrombopoiesis presented an entirely novel phenomenon, which to our knowledge, has not been reported before. The plasma membranes appeared to be discontinuous. This was manifested by irregularly-shaped crevices that interrupted membrane continuity over variable distances (Figs. 7 and 8). Perhaps the earliest indication of imminent membrane dissociation was clearing of IMPs. When membrane continuity was broken over even short distances, the IMP were seen lined up at the edge of the crevice. At times, a fractured area of cytoplasm believed to represent a Pt territory abutted a crevice in the plasma membrane (Fig. 7). In such areas, the discontinuity of the MK surface membrane and the reorganization of IMPs was easy to appreciate. Although membrane

![Figure 6](https://example.com/figure6.png) - Detail of a replica of a freeze-fractured mouse MK from an elutriated specimen showing various aspects of the demarcation membranes. It is assumed that profiles exhibiting pits represent P-faces (arrows), and those showing protrusions are E-faces (arrowheads). Note that the size and distribution of IMPs is similar on both membrane halves. × 40,000.
dissociation was first recognized in freeze-fractured, cultured MKs, this phenomenon was subsequently also observed in freshly collected human bone marrow specimens.

**Tracer Studies**

The continuity of the extracellular space and the channels of the DMS has been elegantly traced by others. However, the present studies led to some new observations. Incubation of the MK with peroxidase or cationic ferritin at 37°C delineated the plasma membrane and the DMS as expected, but it became also clear that these tracers remained excluded from a large portion of the vesicles and tubules that occupied the cytoplasm separating platelet territories (Figs. 9–11).

Whether these channels became filled with the tracer did

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**FIGURE 7** Detail of a P-face of a mouse MK plasma membrane. Note that IMPs are lined up along crevices where membrane continuity is interrupted (arrows). Clearing of IMPs is believed to occur before rupture. When cleared areas are small, they cannot be etched. This specimen was elutriated. × 63,000.
FIGURE 8. Details of the P-face of cultured mouse MK plasma membranes. The cells were probably engaged in thrombocytopoiesis: a was prepared from a culture which had been elutriated, whereas b was obtained from a culture which had been fixed without prior elutriation. a shows a labyrinth of surface crevices from which IMPs have cleared (arrows) but, which could probably not have been 'etched.' In b, numerous points of membrane disorganization are apparent. Similar areas occurred also elsewhere on the surface of the same cell. The inset shows what is probably the beginning of membrane disruption. It has also been obtained from the same cell. In thin sections, these plasma membrane interruptions would probably appear as those illustrated in Fig. 1C. Crevices were only seen over some areas of the plasma membrane. In other areas, the peripheral zone (see Figs. 1 and 2) remained intact. (a) × 58,000. (b) × 63,000. (Inset) × 63,000.

FIGURE 9. Detail of a mouse MK from an unelutriated specimen that had been incubated with cationized ferritin at 37°C for 2 h before fixation. There are relatively few entry channels through the peripheral zone (arrow). Marked ramification occurs towards the interior of the cell. Large areas of cytoplasm (X, Y, and Z) have excluded the tracer to a large extent. × 39,000.
FIGURE 10  Overview of MK cytoplasm representing portion of a cell taken from the same specimen as the cell illustrated in Fig. 9, i.e., the specimen had been incubated with cationized ferritin at 37°C for 2 h before fixation. The arrows indicate membrane domains devoid of ferritin in continuity with profiles coated with the tracer. Note that the size of the channel does not seem to dictate entry of the tracer. Of particular interest is the fusion of an 'empty' coated vesicle with the 'smooth' membrane of a tracer-delineated channel (asterisk). × 70,500.
Figures 11 and 12  Fig. 11: Detail of a mouse MK taken from a culture that had been incubated with cationic ferritin at 4°C for 2 h before fixation. Elutriation was omitted. Note that the tracer has coated the plasma membrane (arrow), but that the channels of the DMS have remained uncoated. X 64,000. Fig. 12: MK derived from the same specimen as the one illustrated in Fig. 11. The cell appears to have released most of its Pts. Much of the peripheral zone (PZ) devoid of organelles has remained associated with the nucleus. The surface is thrown into many folds which are coated with ferritin. They are probably in continuity with the extracellular milieu. No tracer is seen in vesicles of the cytoplasm which make up the nuclear zone, an area not believed to give rise to Pts. X 9,500.
not seem to depend on their size nor on the location within the cell. Tubules close to the nucleus filled as readily as those located at the periphery, i.e., within a few minutes of incubation. Ramification of the tracer-filled channels was much more marked towards the interior of the MK than at the surface. Indeed in some cells the marginal zone exhibited only a few straight channels that began to curve and branch on entering the intermediate zone (Fig. 10). Perhaps of greatest interest was the observation that some profiles of smooth ER devoid of ferritin were in continuity with profiles that were coated with the cationic tracer (Fig. 10). As had been the case for the canalicular system of Pts, metabolic inhibitors did not impair the uptake of the tracers by the DMS. A more complicated picture emerged when the cells were incubated with cationic ferritin at 4°C. Under these conditions the tracer entered a few large channels at the periphery of the cell where they outlined Pt territories if these chances were located in the marginal zone, or if the Pts had already separated from each other, but were still located within the confines of the cell (i.e., at the stage illustrated in Fig. 2C). When Pt territories had not completely formed, even though there was evidence of considerable DMS development, ferritin remained entirely limited to the coat of the plasma membrane and was not interiorized at 4°C (Fig. 11). Thus the channels of the DMS were not ‘open’ to the extracellular environment at 4°C. On the other hand in cells that appeared to have released most of their platelets and that had numerous in-foldings of the peripheral zone, the tracer was found along the invaginated membranes (Fig. 12) even at 4°C. In most sections obvious continuity between such in-foldings and the extracellular environment could be demonstrated.

DISCUSSION

The availability of cultured MKs that are actively engaged in thrombocytopoiesis has made it possible, for the first time, to subject an almost unlimited number of the cells to structural analysis. Our studies have led to the inescapable conclusion that the entire MK cytoplasm may fragment into Pts and that it is a rare event for Pts to be derived from the peripheral zone of the mother cell (Figs. 1 and 2). These observations do not only lend credence to the theoretical models of thrombocytopoiesis conceived by others (20, 21), but also explain our own difficulty in finding ‘budding’ Pts on ultrastructural analysis of human MK (36). The concept of Pt ‘budding’ was probably based on light microscopic studies of non-anticoagulated bone marrow specimens that had been allowed to dry between cover slips, or on routinely prepared smears of bone marrow aspirates. Such ‘buds’ may have represented cellular processes commonly displayed by living MKs, which are cells known to have ameboid properties (5). This new information is by no means in conflict with ultrastructural observations showing chains of unseparated Pts in marrow sinusoids or even in the peripheral circulation (37, 38). Such MK fragments could have originated from the interior of the cell. Two considerations make this most likely: First, the fact that Pt territories and the DMS can be seen fully developed without involving the peripheral zone (Figs. 1 and 2). Therefore, it is difficult to conceive how the membranes of future Pts could originate from the surface of such cells. Second, the fact that the freeze-fracture morphology of the Pt membrane differs from that of the MK (Figs. 3–5). To our knowledge, this is the first time that a structural difference between MK and Pt membranes has been demonstrated. Except for the finding by Rabellino et al. (39) that the la antigen is present on 15% of MKs while it appears to be absent from the Pt surface, the literature abounds with reports attempting to prove the virtual identity of Pt and MK membranes. On physiological grounds however, there is reason to believe that MK and Pts might also have distinctive surface properties that would permit them to respond differently to some stimuli. For instance, the introduction of Pt aggregating substances which will induce thrombosis and subsequent thrombocytopenia enhances thrombocytopoiesis, i.e., a process requiring disintegration of MKs. There is also indirect evidence from studies by Kalmaz and McDonald (40) that some specific antisera prepared to destroy Pts may not injure MKs. Animals treated with these antisera were able to raise new Pts within hours, a time period too short to attribute their generation to the differentiation of new MKs from stem cells. None of these considerations detract from the significance of the experiments by Behnke (14) which established that there is continuity of the extracellular space with the channels of the DMS. These studies did not prove however, that the DMS is formed by invagination of the MK plasma membrane, nor that such membranes actually become the envelope of newly-formed Pts. The freeze-fracture analyses have failed to help in this regard. Although the IMP partition coefficient (IMP-PC of the platelet plasma membrane) differs from that of the MK, the IMP-PC of the internal DMS is at variance with both. On the other hand, if the vesicles and cisterns of the DMS would arise de novo and establish contact with the surface membrane at a later stage, there would be a time when tracers like cationic ferritin or peroxidase would not fill the entire system. This hypothesis proved to be correct, particularly in MK with a hypertrophied DMS (Fig. 9). Moreover, as has been demonstrated for Pts (22) the tracers appeared to be interiorized by membrane flow rather than by membrane invagination. The process was not energy-dependent, but inhibited at 4°C. It should be reiterated that at 37°C the tracer-filled channels of the DMS remained relatively straight while traversing the peripheral zone. They appeared to reach the surface at only a few points. A more circuitous route was evident towards the interior of the cell, where fusion with ‘empty’ as well as tracer-filled compartments appeared to have occurred. The fact that, even after prolonged incubation some profiles of the system did not become delineated with the tracer, although they were in continuity with membranes bearing ferritin (Fig. 10), may be taken as additional support for the view that the DMS is not entirely derived from the surface membrane. Since the degree of filling was unrelated to the size or location of the channels it is possible that the cationic tracers react only with some and not with other membrane domains. Images showing continuity between channels delineated by the tracer and those devoid of ferritin were not uncommon (Fig. 10). It is conceivable that various segments of the DMS subserve different functions as has been shown in other cells whose membranes are involved in intracellular transport or

2 As has been observed in Pts (22), some freeze-fracture replicas of MK showed areas with innumerable ‘pits’ (Fig. 5). Comparable areas showing numerous channels of the DMS reaching the surface close to one another were also seen. This observation has invited the suggestion that MK membrane interruption may commence in a specialized area (15). Although this is a viable hypothesis, we tend to believe that occasional aggregation of ‘pits,’ i.e., DMS ‘openings’ may be related to chance movement or deformation of the cell at the time of fixation.
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