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SURFACE ULTRASTRUCTURE OF HUMAN MEGAKARYOCYTES SORTED ON THE BASIS OF DNA CONTENT

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Abstract

The relationship of polyploidization (DNA content) to differentiation is not well defined. We have developed centrifugal elutriation and Percoll density gradient centrifugation to obtain large numbers of highly-purified megakaryocytes which subsequently were stained for DNA content with Hoechst 33342 and sorted by FACS into 8C, 16C and 32C ploidy classes for correlated analysis of cell surface structures by scanning electron microscopy. Each ploidy class revealed unique surface characteristics that reflect differentiation occurring in megakaryocytes independent of their DNA content.

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KEY WORDS: Scanning electron microscopy, megakaryocytes, ploidy class, differentiation, flow cytometry.

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Introduction

Megakaryocytes undergo a unique form of differentiation during which they exhibit nuclear endoreplication without cell division. In addition to the resultant extensive polyploidization, these cells exhibit concomitant changes in cytoplasmic development that lead ultimately to platelet formation.

Despite much study and speculation, the relationship of polyploidization to cell differentiation is not well understood. As reviewed recently [14], several techniques including scanning microdensitometry have been used to quantitate DNA content in Feulgen-stained megakaryocytes of several species, and have confirmed that these cells contain DNA levels of 8C, 16C, and 32C [5-7,9,17]. Previous attempts to relate ploidy to cytoplasmic maturation have involved light microscopic analysis of megakaryocytes from each ploidy class [15,16], as well as ultrastructural analysis evaluated by transmission electron microscopy (TEM) [18-20]. Such studies have offered a qualitative analysis of megakaryocyte differentiation within ploidy classes based primarily on morphologic considerations. However, as a result of the complexity and tediousness of Feulgen microdensitometric measurements, this data base is rather limited.

The surface ultrastructure of megakaryocytes has also been examined by scanning electron microscopy (SEM) in several species [1-3,8,10,11], including man [4,7,23,25]. These latter studies have focused on the mechanism of platelet biogenesis and no data are available on the surface ultrastructure of cells within specific polyploid classes.

In this study, we have used centrifugal elutriation [26] and discontinuous Percoll density gradient centrifugation to isolate large numbers of megakaryocytes from human bone marrow. After staining these cells with Hoechst 33342, they were sorted on the basis of DNA fluorescence according to ploidy class, using flow cytometry [13,27]. We were able to examine in excess of 500 intact megakaryocytes per ploidy class by SEM. We conclude from this analysis that cell surface characteristics may be classified into specific categories that reflect differentiation occurring within each ploidy class that is independent of DNA content.

Materials and Methods

Bone marrow preparation

Megakaryocytes were isolated from human ribs removed from patients during routine thoracotomy. Hematologic parameters of each donor studied were normal, with no patient having undergone radiation or chemotherapy. Immediately after surgical removal, ribs were placed in CATCH medium [21]. The bone segment (usually 12-18 cm long) was cut into 3 cm pieces using a bone ronguer and each piece opened to expose the marrow cavity. The marrow was flushed from the split bone fragments by gentle pipetting with CATCH + 0.5% albumin. As many subsequent procedures as possible were performed at 12°C [26]. Cells in the pooled marrow were monodispersed by gentle pipetting with a polypropylene pipette and then filtered by gravity through a 100 micron nylon mesh (TETKO, Elmsford, NY). Total nucleated cell (TNC) and megakaryocyte counts were performed using a Neubauer hemocytometer and light microscopy (American Optical, Buffalo, NY).

Megakaryocyte isolation

Centrifugal elutriation of the bone marrow suspension was performed as described by Worthington and Nakeff [26]. Briefly, 5 x 10^6 TNC/ml in 40 ml of CATCH medium were introduced into a centrifugal elutriator (Beckman, Palo Alto, CA) at a flow rate of 20 ml/min and speed of 2200 RPM at 12°C, using a Sanderson separation chamber and the JE-6 rotor. After introduction of the load sample, 400 ml of CATCH medium was used as a wash leaving megakaryocytes suspended in the chamber volume (approximately 5 ml). This sample was removed, layered on 5 ml of 30% Percoll (density 1.035 g/ml) diluted in CATCH medium pH 7.2 and 300 mOsm, (Pharmacia, Piscataway, NJ) and centrifuged at 700g for 20 min at 12°C. The interface which contained the megakaryocytes was washed with CATCH medium without albumin. Cells were fixed immediately in 1% glutaraldehyde (Polyscience) in 0.1M cacodylate buffer, pH 7.2, at 22°C for 1 h and stored at 4°C.

Flow cytometry

DNA staining. Megakaryocytes were stained with Hoechst 33342 (H342) (Calbiochem, San Diego, CA) which is presumed to be a DNA-specific stain with preference for adenine-thymine rich regions of DNA. 1-2 x 10^5 megakaryocytes/ml were stained with H342 at a final concentration of 10 micromolar for 60 min at 4°C.

Analysis and sorting. Megakaryocytes were analyzed and sorted according to ploidy class as described by Nakeff et al. [13] and Worthington et al. [27] on the basis of H342 DNA related fluorescence using a Becton-Dickinson FACS 440 flow cytometer (Mountainview, CA). The instrument was equipped with a 5 watt Argon ion laser (Spectra-Physics Model 164-05) operating at 30 mW output power in the UV (351 and 364 nm). For analysis, cells were flowed one at a time through a 70 micron nozzle tip and past the laser beam at rates of about 500/sec. The H342 signal was then detected using a 470 nm bandbass (458-482 nm) filter (Omega, Brattleboro, VT) combined with a 1.0 nominal density filter (Oriel, Stanford, CT) to reduce signal intensity. For sorting, cells were jetted one at a time into air, analyzed for DNA content as described and the liquid jet then broken into droplets at resonant frequencies (approximately 28 kHz). The droplets containing megakaryocytes to be sorted were then electrically charged and separated from the others during passage through a high-voltage electrical field and collected onto slides (for SEM) at rates that varied from 5-50 megakaryocytes/sec, depending on the particular ploidy class being sorted. Specific ploidy classes (8C, 16C and 32C) were defined by electronic windows on the resulting frequency distribution histograms (256 channels) of megakaryocyte DNA content. For sorting, ploidy classes were defined relative to the modal intensity of the diploid DNA region. Boundaries for each ploidy class were then designated as the point half-way between the modal intensity of adjacent peaks. A 5 channel buffer zone was included on each side of the minimum channel between adjacent DNA distributions to ensure minimal overlap.

SEM preparation

Megakaryocytes of each ploidy class were sorted onto poly-L-lysine coated coverslips into 0.1 ml of 0.09% phosphate buffered saline. The cells were allowed to adhere for 30 min at 22°C with precautions taken to prevent complete evaporation. The coverslips then were immersed in 1% glutaraldehyde and stored at 4°C. They were washed with distilled water and freeze-dried (Edwards High Vacuum, Sussex, England) under vacuum at -65°C. Coverslips were gold-coated (Edwards High Vacuum) and viewed on an ETEC autoscanning electron microscope (Haywood, CA) at 20 kV with tilt of 25°.

Results

Megakaryocytes were studied from 8 human rib specimens. After isolation, a total of 1-3 x 10^6 megakaryocytes were obtained from each rib sample which represented an average recovery of about 70% of those present in the starting material. Freeze drying of the SEM specimen resulted in minimal cell loss. Using the unique morphology of cells with a DNA content greater than 8C, it was possible to examine 500 megakaryocytes from each ploidy class without difficulty.

Following SEM examination, the most striking observation was that megakaryocytes displayed a marked heterogeneity of surface ultrastructure within each of the major ploidy classes. Nevertheless, they could be classified into distinct categories of which some were common to all three classes, as summarized in Table 1.

Megakaryocytes were observed that had a relatively smooth surface, with occasional minor projections (Figure 1). This type of surface structure was noted in all the ploidy classes and was characteristic of megakaryocytes that exhibited the smallest cell diameters. In

Table 1

MEGAKARYOCYTE SURFACE CHARACTERISTICS
BY PLOIDY CLASS

DNA CONTENT	SEM-TYPE				
	Smooth	micro-villi	nodules	blebs	other
8C	30*	40	10	<10	<10
16C	30	30	20	<10	<10
32C	30	10	30	0	30

*Percent of all megakaryocytes within a specific ploidy class.

addition, it was clear that in this category, cell size increased with increasing DNA content such that smooth 32C megakaryocytes generally were larger than 16C and those in turn larger than 8C of the same type. Megakaryocytes with a smooth surface constituted approximately 30% of each ploidy class.

The next most frequent category was composed of cells with surface villi. Microvilli found on 8C cells (Figure 2) were shorter and broader than those found on 16C and 32C megakaryocytes. On the latter cells, they became longer and more filamentous (Figure 3). The diameter of villous cells increased with DNA content in a fashion similar to that observed for smooth cells. In contrast to the relatively constant proportion of cells with a smooth surface, the fraction of villous cells in each

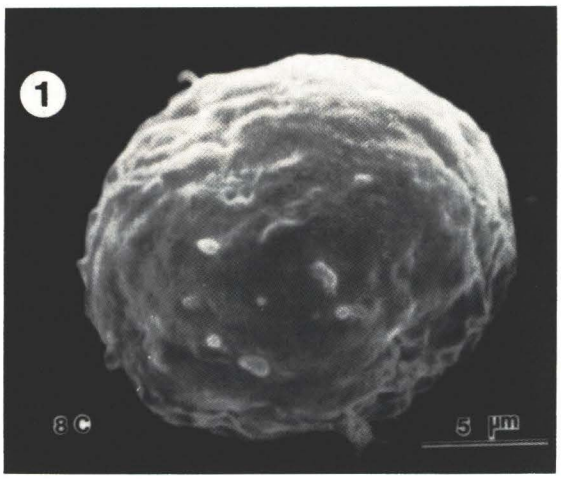

Figure 1. 8C megakaryocyte having a smooth surface with a few small projections.

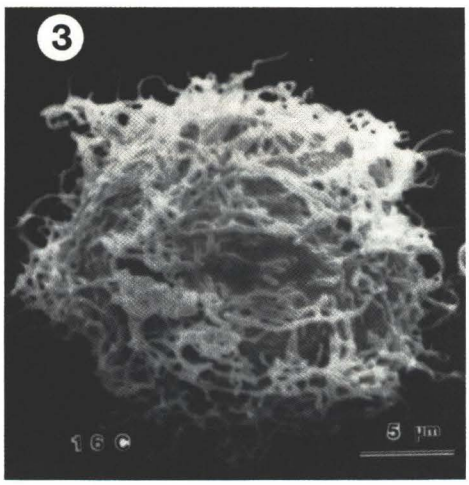

Figure 3. 16C megakaryocyte with longer, filamentous microvilli which are characteristic of this ploidy class.

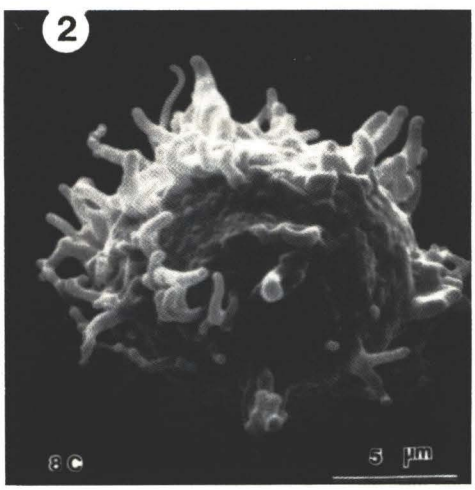

Figure 2. 8C megakaryocyte displaying short, broad microvilli spread randomly over the entire surface.

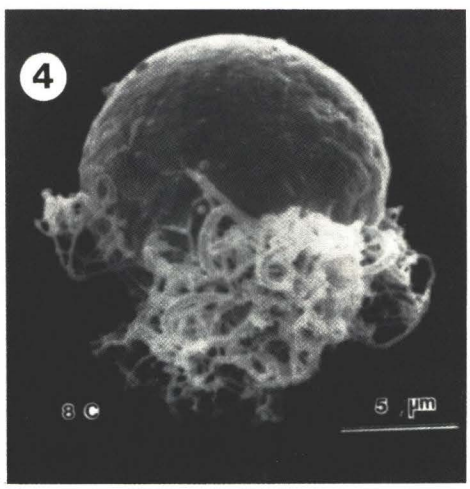

Figure 4: 8C megakaryocyte with polarized microvilli.

ploidy class decreased with increasing DNA content. At least 40% of 8C megakaryocytes displayed microvilli, while 30% of 16C, and only 10% of 32C cells belonged to this category.

Although the number of villi varied, cell surfaces were randomly covered by them. However, among 8C and 16C cells, we did observe a peculiar microvillous formation. As shown in Figure 4, microvilli were polarized at one end of the cell, with the remainder of its surface smooth. This type was rather rare and accounted for less than 10% of all megakaryocytes in each of these two classes.

All ploidy classes contained megakaryocytes covered with small nodules which had diameters of about 0.2-0.5 microns (Figure 5). Many of these cells also had smooth areas where the nodules were not apparent. As seen at higher magnification in Figure 6, the cell surface was intricate and was also covered with numerous fenestrations which revealed a complicated internal network. Although there was a large variation in size within ploidy classes for cells exhibiting this particular surface, cell diameters increased with increasing ploidy. This surface was found on 10% of 8C, 20% of 16C, and 30% of 32C megakaryocytes (Table 1).

Although smooth, microvillous and nodular were the surface characteristics that predominated in megakaryocytes of all ploidy classes, a minority (approx. 5%) of megakaryocytes exhibited surface ridges. Less than 5% of 8C and 16C megakaryocytes displayed surface blebs (approximately 2 microns in diameter) or, as shown in Figure 7, a combination of blebs and slender filaments.

A novel, though by no means rare, megakaryocyte was noted among the 32C ploidy class (Figure 8). This cell had a round, central mass (presumably the nucleus) surrounded by a veil of cytoplasm. The uniqueness of this topography among 32C megakaryocytes must be a property intrinsic to these cells since their fixation prior to sorting would be expected to prevent any "active spreading" on the poly-L-lysine substrate. The perimeter of this cell was round, and its surface smooth with an absence of projections. These cells were consistently among the largest in diameter within this ploidy class and represented about 30% of all 32C megakaryocytes.

Discussion

To our knowledge, the SEM evaluation of the surface features of megakaryocytes that have been sorted into distinct ploidy classes has never before been reported. This work represents a complex combination of techniques which permitted the analysis of relatively large numbers of isolated, intact megakaryocytes.

Centrifugal elutriation and discontinuous Percoll density gradient centrifugation are gentle yet quantitative methods for enriching megakaryocytes while the use of CATCH medium prevented cellular activation [12] which may have altered surface characteristics prior to fixation. Osmolarity (300 mOsm), pH (7.2) and temperature (12°C) were optimized for maximal yield and preservation of megakaryocytes. In addition, flow cytometry permitted their rapid separation into ploidy classes. We believe that this combination of techniques represents a unique approach to elucidate the relationship between polyploidization and differentiation. Multivectorial analysis [27] provides a means to correlate several parameters, including DNA content, cell size (by light scatter and microscopy) and ultrastructural characteristics defined at the cell surface (SEM).

From previous work [18,20] combining Feulgen microspectrophotometry and TEM, it has been reported that the principal ploidy classes in murine marrow contain megakaryocytes in several stages of differentiation, in some cases including cells undergoing "platelet formation". Although we make no attempt, using SEM alone, to propose that surface structure reflects differentiation, we do feel, in agreement with others [7], that megakaryocytes exhibiting similar surface features may be cells at the same stage of differentiation. The fact that we found similarities in terms of surface structure type among ploidy classes may indicate that each class contains cells at the same stage of differentiation. Early studies [18,20] suggested that there was no simple correspondence between ploidy level and differentiation. Also, cell diameter alone may not be a reasonable parameter of differentiation. The latter conclusion was based on the observation [18] that some megakaryocytes in the 8C class which were "producing" platelets by TEM examination were smaller than some of the 32C cells which exhibited a characteristically immature cytoplasm.

The complexity of this interpretation is supported by the studies reported here as it appears that the size of the megakaryocyte generally increases with ploidy while maturation may be independent of ploidy. Megakaryocyte size among the predominant surface types did indeed increase with increasing DNA content although there was substantial overlap among ploidy classes which most probably reflects the finding that mature cells of a lower ploidy class may be similar in size to immature cells of a higher ploidy class. Within each ploidy class, smooth megakaryocytes were among the smallest in cell diameter, with microvillous cells being intermediate, and those covered with nodules constituting the largest. For example, some 32C smooth megakaryocytes were smaller than 8C megakaryocytes that were covered with nodules.

The presence of megakaryocytes with microvilli present solely at one pole of the cell (Figure 4) may be the *in vitro* correlate of such cells reported *in vivo* by SEM [22]. These cells were reported to be located next to the abluminal surface of the bone marrow endothelium with their microvilli serving to secure the megakaryocyte in position counter to the blood flow. This has also been demonstrated in TEM preparations by Tavassoli and Aoki [24]. The presence of these rare cells may indicate that the isolation and analysis methods we have applied are gentle and quantitative.

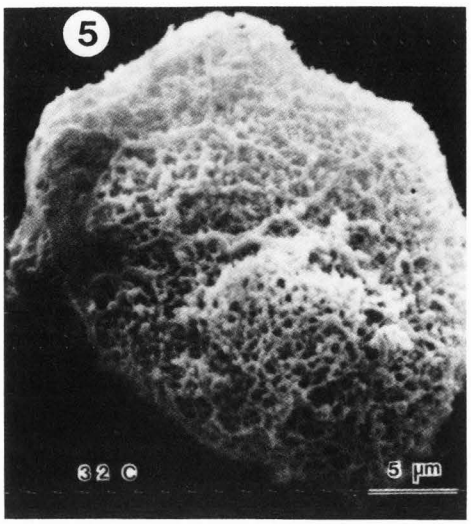

Figure 5: 32C megakaryocyte displaying nodules (0.2-0.5 microns in diameter) over most of its surface.

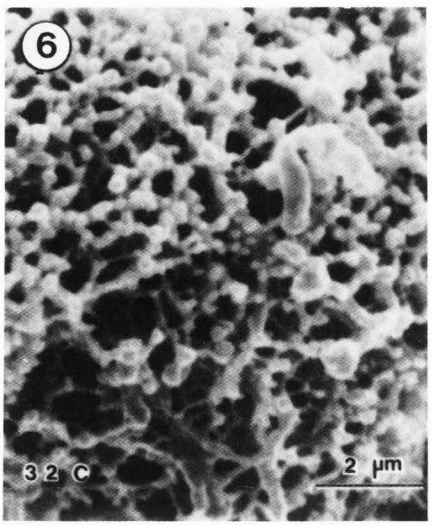

Figure 6: A higher magnification of the same 32C megakaryocyte (Figure 5) revealing numerous fenestrations and a complex internal network.

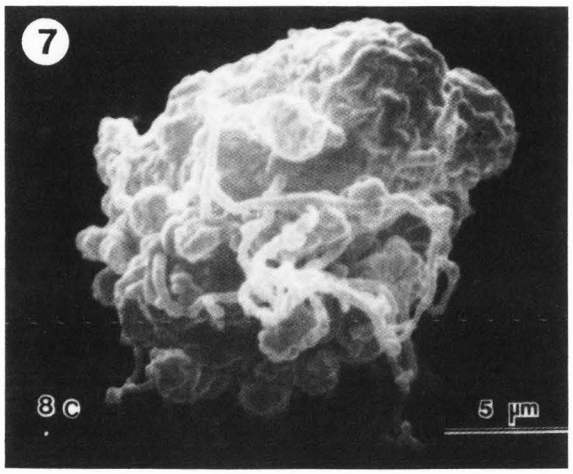

Figure 7: 8C megakaryocyte covered with round blebs (2 microns in diameter) and long filaments.

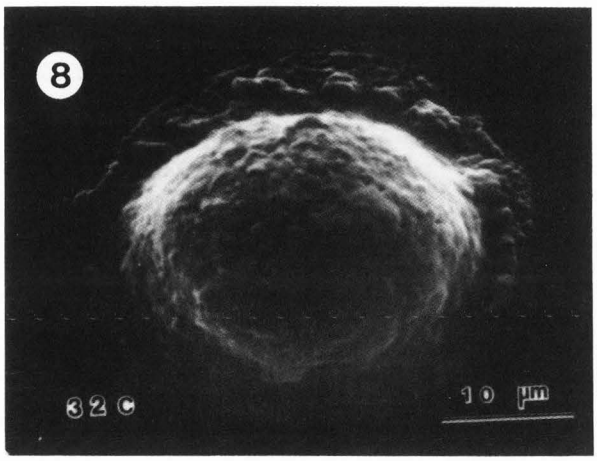

Figure 8: 32C megakaryocyte of a type unique to this class. The round central mass (presumed to be the nucleus) is surrounded by a thin veil of cytoplasm.

We believe that these studies have provided a fascinating look at the complexities that may be inherent in the interpretation of polyploidization and differentiation in megakaryocytes. Future studies being carried out in this laboratory should permit us to examine the surface and internal ultrastructure of individual megakaryocytes from discrete ploidy classes in an attempt to begin to unravel these complexities.

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This manuscript is dedicated to the memory of Dr. Marion I. Barnhart whose tragic death resulted in the deeply-felt loss of a mentor and colleague.

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Discussion with Reviewers

M. Tavassoli: How much do the elutriation and flow cytometry interfere with the cell viability and is it possible to obtain viable cells through this technique for in vitro culture? Have the authors attempted such cultures?
Authors: Following elutriation and Percoll density gradient centrifugation, viabilities of human megakaryocytes were always > 80% by Trypan Blue exclusion. We have never attempted cultures of these cells. However, Worthington and Nakeff [26] have used rat marrow in this same system and have demonstrated thromboxane production by elutriated megakaryocytes attesting to the preservation of their metabolic capabilities.

R.P. Becker: Have you seen megakaryocytes fixed in the process of producing platelets? If so what was the ploidy of these cells?

Authors: As stated in the Discussion, we cannot discern which megakaryocytes, if any, are producing platelets. This is, however, of great interest and we are currently involved in single cell analysis by SEM and subsequent TEM examination. This will permit correlation of surface characteristics with internal ultrastructure.

D. Zucker-Franklin: Why do you perform the experiments at 12°C?
Authors: We have found 12°C to give the optimum recovery of megakaryocytes during elutriation [Ref. 26].

D. Zucker-Franklin: Please explain the column designated "other" in Table 1.
Authors: For 8C and 16C megakaryocytes, "other" refers to megakaryocytes with polarized microvilli (similar to Fig. 4) or those with a combination of surface features (Fig. 7). For 32C megakaryocytes, "other" refers to cells similar to Fig. 8.

D. Zucker-Franklin: Figs. 1, 2, 4, and 7 all have a ploidy of 8N, but what features do they share?
Authors: These figures clearly demonstrate the degree of heterogeneity in cell surface ultrastructure that was surprising to us for cells that had the same DNA content, namely 8C. Whether this heterogeneity reflects cells at different physiological stages remains to be determined. We also wish to emphasize that since our measurement of DNA was the total H342 nuclear fluorescence per megakaryocyte, relative to the DNA content of diploid cells, it is most reasonable to express the value for this ploidy class as 8C rather than 8N.

J.C. Mattson: Which morphologic features do the authors believe represent platelet formation? Are there any correlative TEM studies that might shed light on this?
Authors: The surface detail in these SEM photomicrographs raises several questions concerning the physiologic events occurring within each morphologic category. As previously stated, we cannot discern which surface represents platelet formation. The fine microvillous projections (Figs. 2,3,4) are approximately 0.2-0.4 μm in diameter which is much too small for platelets. The surface blebs (Fig. 7) are about the proper size (2μm) but we cannot say, based on size alone, that the blebs represent platelets or "proplatelet formation". It is further interesting to speculate that the fenistrations seen in Fig. 6 could represent the formation of demarcation membranes. These questions should be answered by correlative TEM studies which are ongoing in our laboratory.

J.C. Mattson: It has been suggested that platelet size is related to the ploidy class of the parent megakaryocyte. Do the authors have any data to support this; i.e., could they observe formation of different sized platelets from megakaryocytes of different ploidy classes?
Authors: Observations of this type could not be made on the basis of surface structure. The correlative TEM work should, however, provide the means to obtain this data.