Virchows Arch. A Path. Anat. and Histol. 375, 129–146 (1977)

Virchows Archiv A

Chronic Megakaryocytic-Granulocytic Myelosis – An Electron Microscopic Study Including Freeze-Fracture*

II. Granulocytes, Erythrocytes, Plasma Cells and Myeloid Stroma

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Summary. In 5 patients with chronic megakaryocytic-granulocytic myelosis (CMGM) bone marrow specimens were studied by electron microscopy to investigate possible abnormalities of the granulocytic cell lineage. Thin sections were compared with freeze-fracture replicas to elucidate further aspects of leucocyte cytology. The atypia exhibited in these cells (eosinophils, basophils and neutrophil granulocytes) consisted mostly of a disorganization of granulopoiesis with hyper- and hypogranulation, a pathological increase in the number of nuclear blebs and a maturation asynchrony sometimes leading to Pelger-like cell forms. Moreover, a presumptive stem cell was demonstrated in the erythopoietic and granulocytic cell lines resembling CFU cells. In conclusion, granulopoiesis in CMGM exhibited abnormalities as generally observed in chronic myelogenous leukaemia. When considered with our previous finding of malignancy in megakaryopoiesis, CMGM has to be classified as a myelosis of mixed cellularity.

Key words: Myeloproliferative disorder – Megakaryocytic-granulocytic myelosis – Granulocyte lineage – Bone marrow biopsy – Freeze-fracture – Electron microscopy.

Introduction

Chronic megakaryocytic-granulocytic myelosis (CMGM) is considered to be a myelosis of mixed cellularity with neoplastic proliferation of cells of megakaryocytic and granulocytic lineage (Georgii and Vykoupil, 1976). Part one of this paper concentrated on the atypia, suggestive of malignancy, found in the

Abbreviations: Chronic megakaryocytic-granulocytic myelosis: CMGM; Chronic granulocytic leukaemia: CGL; Osteomyelofibrosis: MF; Rough surfaced endoplasmic reticulum: RER

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* Supported by the Deutsche Forschungsgemeinschaft, grant Ge 121/15

megakaryocytic-thrombocytic series (Thiele et al., 1977). This report is devoted to the other cellular elements of the bone marrow including the myeloid stroma. Attention is focused on the granulocytic series, to determine whether there are abnormalities similar to those in chronic granulocytic leukaemia (CGL). Thus, this study sets out to confirm or negate the possibility of malignancy involving two different cell lines in certain myeloproliferative disorders. To provide a three-dimensional impression of cellular membranes and a generally more advanced morphological interpretation, freeze-fracture is employed.

Materials and Methods

The history and clinical data of the five patients investigated are discussed in our previous report (Thiele et al., 1977) and methods of bone marrow biopsy and specimen preparation for thin section electron microscopy follow procedures already published (Thiele et al., 1977).

For freeze-fracture aldehyde fixed marrow samples were transferred to 30% glycerol in Ringer's solution for 60 min, placed on specimen-holders and quickly frozen in liquid Freon 22 (monochlorodifluoromethane) at −150°C. Fracture at −120°C and shadowing with platinum-carbon were performed in a Balzers BA 360 M unit (Balzers AG, Liechtenstein). The carbon-platinum replicas were cleaned in hypochlorite bleach and chromic acid, repeatedly rinsed in distilled water and collected on formvar-carbon membranes suspended in platinum rings (Thiele and Wermbter, 1974).

Results

A. Granulocytes

1. Maturation. Low magnification survey pictures demonstrate overt granulocytic proliferation and, as commonly observed in CGL, immature forms are in the majority. Eosinophils, including primitive forms, are conspicuous, located between clusters of neutrophils, a very few basophils and some precursor cells. Further description of granulocyte maturation is limited to points essential for the understanding of the morphological aspects revealed by freeze-fracture.

In thin sections the most primitive cell of the granulocytic series is identified as a large blastic cell generally lying at the centre of a cluster of maturing granulocytes (Fig. 1a). This cell (promyeloblast) exhibits a high nuclear-cytoplasmic ration and contains an ovoid nucleus with some indentations and many pores in the nuclear envelope. Thin sections of the cytoplasm show an abundance of free polysomes, a Golgi apparatus, a small amount of rough surfaced endoplasmic reticulum (RER) and a few mitochondria, vesicles and dense bodies (precursors of primary granules?). Further maturation is characterized by appearance of primary granules of an immature type. The content of these granules is of low electron density and has a flocculent nature, very characteristic of an early myeloblast (Fig. 1b). In freeze-fracture replicas those granules and the cisternae of the RER are easily recognized (Fig. 1c). The cell surface undulates slightly and includes small pinocytic vesicles scattered at random (Figs. 1b and d).

The neutrophil promyelocyte exhibits a more elongated nucleus with a slight decrease in total number of nuclear pores (Fig. 2b). The nuclear-cytoplasmic

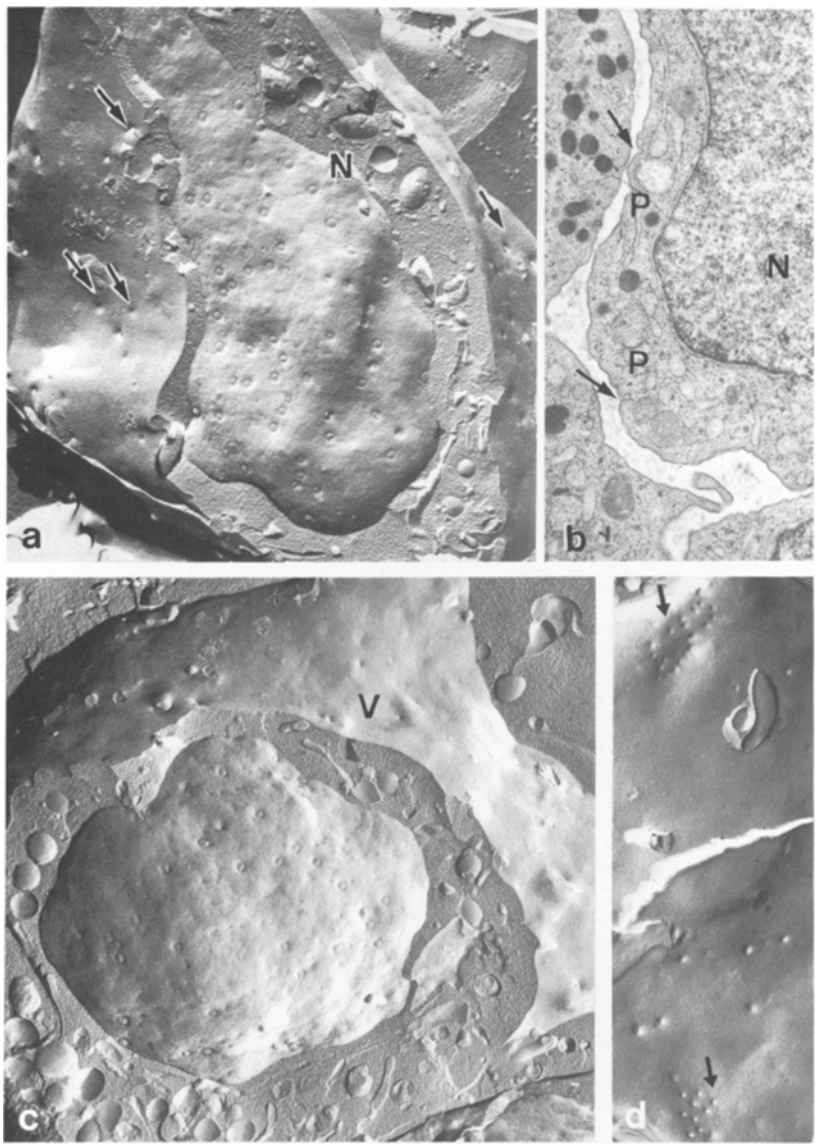

Fig. 1. a Freeze-fracture replica of primitive myeloid precursor cell (promyeloblast). Large nucleus (*N*) with many pores, few cytoplasmic organelles and several pinocytic vesicles of the plasma membrane (*arrows*). **b** Thin section of early myeloblast with a few immature primary granules with flocculent content (*P*). Several pinocytic vesicles (*arrows*). Nucleus (*N*) with dispersed chromatin. **c** and **d** Freeze-fracture replica of myoblast. **c** Cytoplasm containing many spherical bodies (primary granules) and large cisternae of the rough surfaced endoplasmic reticulum. Nucleus with slight decrease of pores, some pinocytic vesicles (*V*). **d** En-face-view of myeloblast plasma membrane with openings of many pinocytic vesicles (arrows). All freeze-fracture replicas are mounted that shadowing is directed from below. **a–d** × 12,800

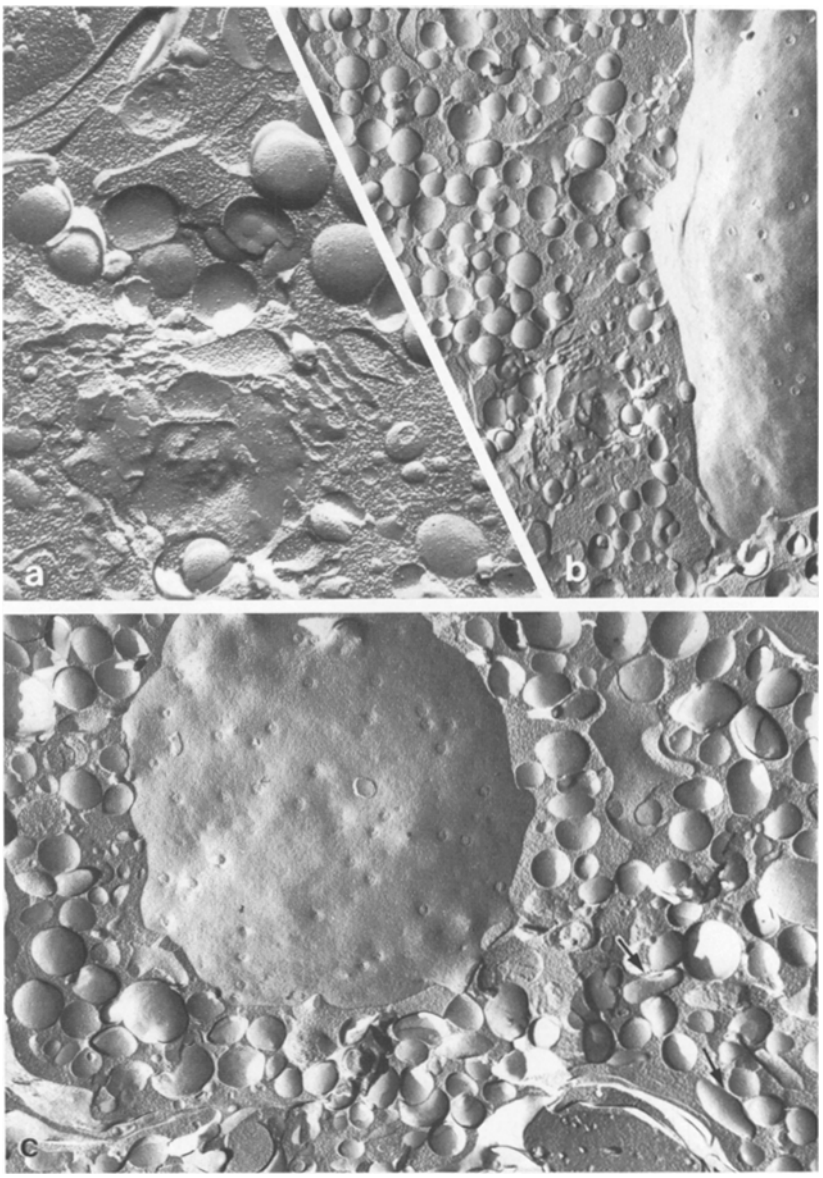

Fig. 2. **a** Promyelocyte with large Golgi apparatus consisting of stacks of perforated flattened saccules surrounded by many spherical bodies (primary granules). **b** Survey with part of elongated nucleus and cytoplasm filled by primary granules. **c** Myelocyte with many granules (mostly primary) and a few elongated roll-like secondary specific granules (*arrows*). **a** ×32,000; **b** and **c** ×15,800

ratio diminishes gradually, the cytoplasm develops an extensive Golgi apparatus and there is conspicuous granulogenesis. These granules may differentiate into three types as described in thin sections by Cawley and Hayhoe (1973). Freeze-fracture shows the Golgi apparatus to consist of a stack of flattened saccules with pores of different sizes and distribution (Figs. 2a and 3a). In thin sections this stack often pursues a twisted undulated course enclosing the centrioles. Therefore in different section planes the maturing surface with the most electron dense and smallest saccule may by chance occupy the outer aspect of the stacks (Fig. 6a). At the neutrophil myelocyte stage the nucleus starts to lobulate and the cytoplasm displays two kinds of granules distinguishable by their different size and shape even in freeze-fracture replicas (Fig. 2c). The nuclear chromatin starts to condense whilst the RER and free polysomes decrease in amount. The appearance of tertiary granules in the metamyelocyte is accompanied by nuclear lobulation and heavy chromatin condensation.

The cytoplasm at this stage contains bodies with a wide variation in shape: the largest spherical ones display primary granules (azurophilic granules), more elliptical ones are secondary neutrophilic granules and the smallest dumbbell-shaped ones are tertiary (specific) granules (Fig. 3b). When almost mature the neutrophil granulocyte contains predominantly elongated tertiary granules and only a few primary and secondary ones (Fig. 3c). In spite of favorable fracture planes running through the interior of each of these different granules no special inner structure is revealed.

2. Atypia. Abnormalities of the granulocytic cell lines are not as conspicuous as those described for the megakaryocytic cells (Thiele et al., 1977). Most of the leucocytes mature normally, the most striking atypicality being due to nuclear-cytoplasmic asynchrony causing maturation anarchy and abnormal granulopoiesis. Disfiguration of the nucleus at all stages of granulopoiesis, consisting of nuclear blebs and loops embracing cytoplasm and organelles, are most remarkable (Figs. 4a–c). These blebs consist of three layers: two layers of fibrous lamina and a very small central portion of chromatin (Fig. 4b). Rarely, membrane-bound and whorl-like nuclear inclusions can be seen. Moreover, there are ringshaped extensively developed nucleoli and sometimes a lack of nuclear segmentation in mature metamyelocytes. Disorganization of maturation is documented by the persistence of stack-like arranged cisternae of the RER even in the more mature cells of granulopoiesis, consistent with the so called Döhle bodies (Döhle, 1911). Some cells exhibit hypogranulation (Fig. 5a), others hypergranulation (Fig. 4c) at a stage when the nucleus is at the metamyelocyte phase of maturation. Granule production is also qualitatively disorganized. This is shown for example, by the frequent occurrence of predominantly secondary granules at the late metamyelocyte stage. On a few occasions, myeloblasts are observed with granules that had coalesced producing small, abortive Auer rod-like structures (Fig. 4d). However, the eosinophil pro-and metamyelocyte also exhibits an anarchy in granulogenesis (Figs. 5b and c). The cytoplasm of these cells is often filled with abnormal, immature granules with atypical inner structures (Fig. 5c). Abnormalities also occur in the basophil series, generally associated with hypogranulation.

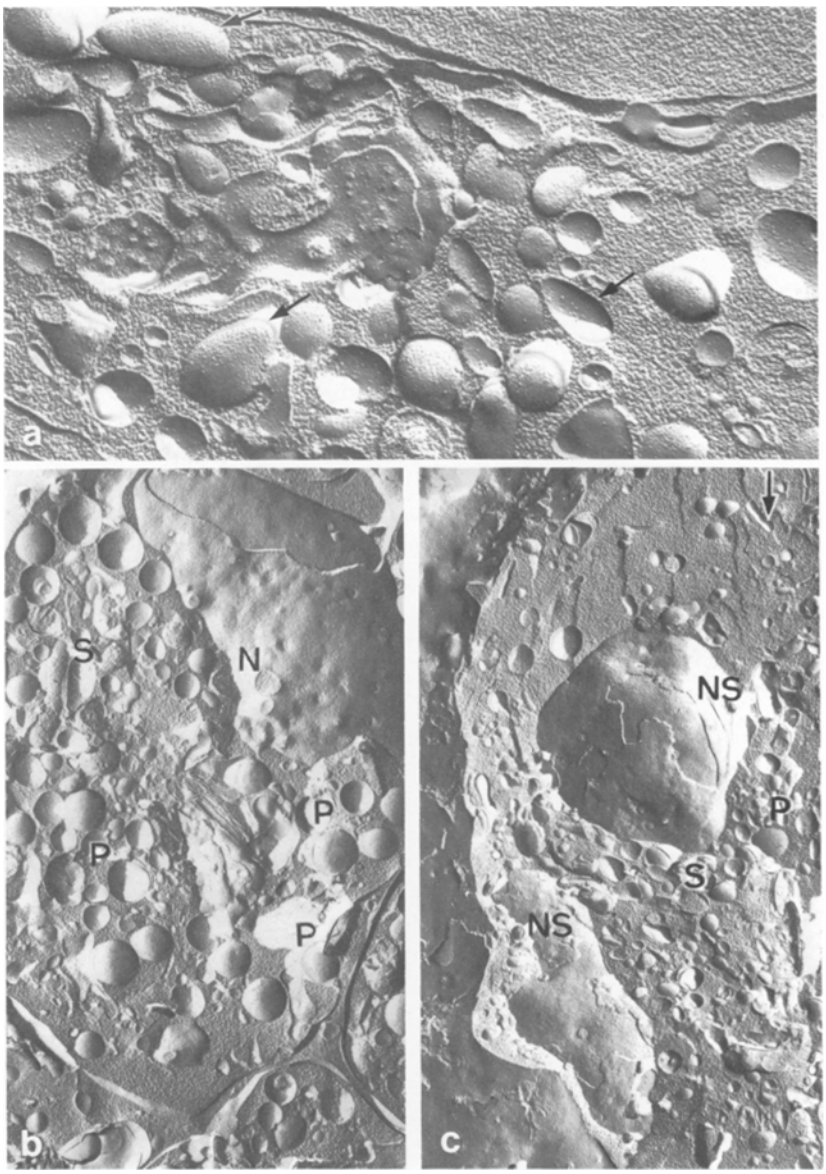

Fig. 3. a Golgi apparatus of myelocyte with perforated saccules surrounded by spherical primary and elliptical and roll-like secondary specific granules (arrows). **b** Metamyelocyte with part of the segmented nucleus (*N*) a large Golgi apparatus and cytoplasm containing primary (*P*), secondary (*S*) and a very few tertiary specific granules (*arrow*). **c** Almost mature neutrophil granulocyte with two nuclear segments (*NS*) and dumbbell shaped tertiary specific granules (*arrow*). There are still some primary (*P*) and secondary granules (*S*). **a** ×32,000, **b** and **c** ×12,800

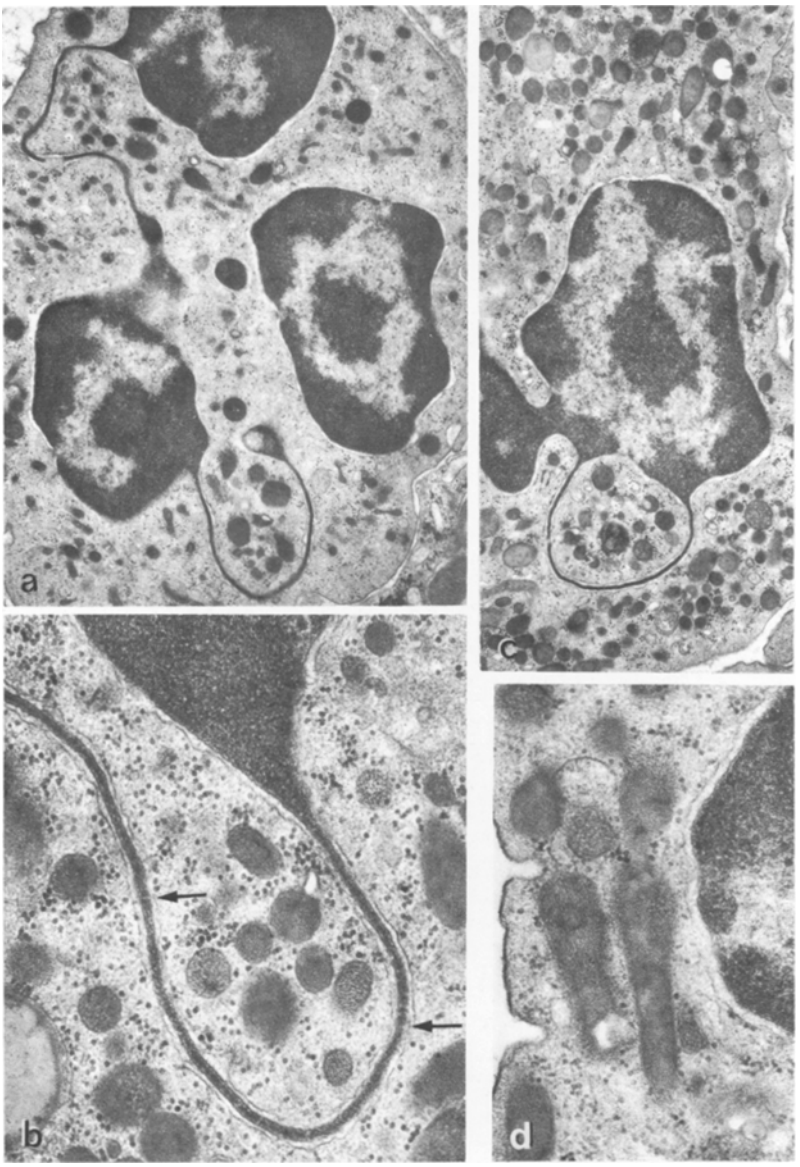

Fig. 4a–d. Atypia of granulopoiesis. **a** Mature neutrophil granulocyte with nuclear bleb and loop. Cytoplasm containing relatively few granules including several primary and scanty tertiary specific granules. **b** Nuclear bleb embracing large portion of cytoplasm (?) with several primary granules in a mature granulocyte. Small chromatin layer between fibrous lamina with periodical banding in some places (*arrows*). **c** Mature neutrophil metamyelocyte with hypergranulation and large nuclear loop embracing granule filled cytoplasm. The granules predominantly of the primary and secondary type. **d** Late neutrophil myeloblast with large partially coalescing primary granules (?) probably forming precursors of Auer rods. **a** ×12,800; **b** ×32,000; **c** ×11,600 – **d** ×32,000

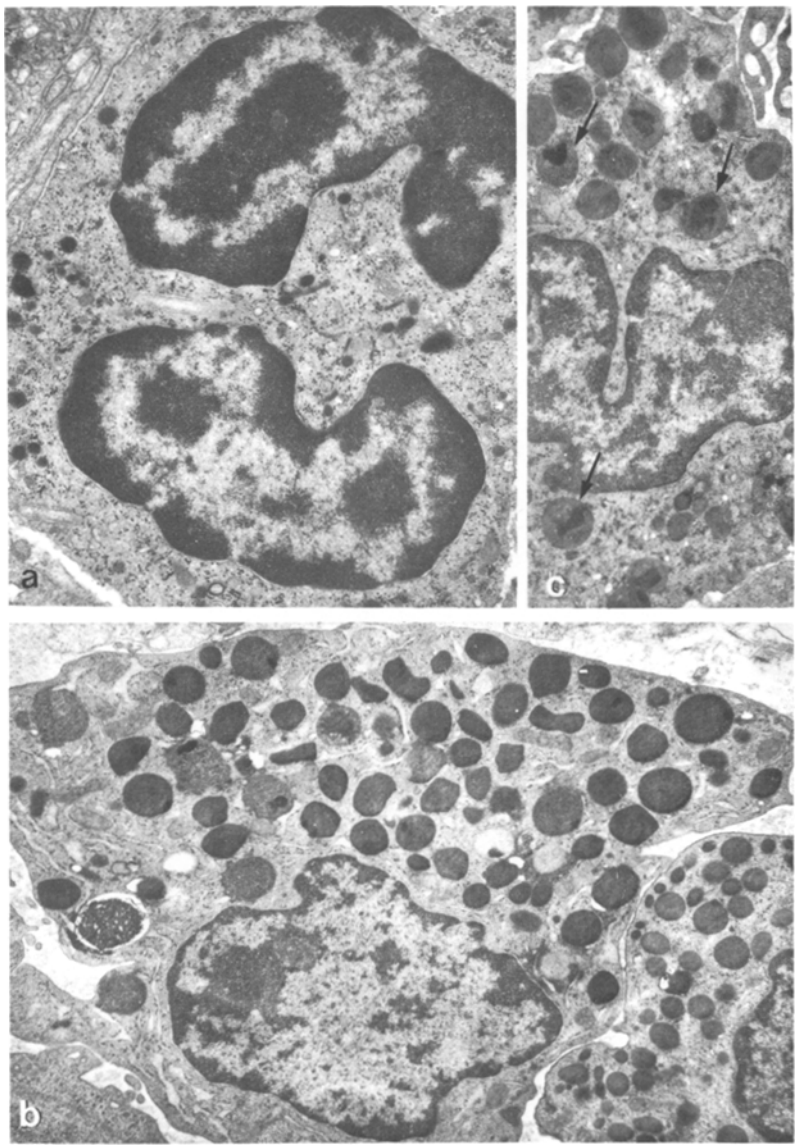

Fig. 5. Late neutrophil metamyelocyte with hypogranulation and segmented nucleus with condensed chromatin. Cytoplasm with few secondary, primary and tertiary specific granules. **b** Late eosinophil promyelocyte with immature primary granules showing different sometimes bizarre inner structures. **c** Late eosinophil metamyelocyte with starting segmentation of nucleus and some mature specific granules displaying abnormal inner structures (*arrows*). **a** ×12,800 – **b** ×8000 – **c** ×8800

B. Erythrocytes

Normal erythropoiesis is observed in the many erythroblastic islands investigated. Freeze-fracture reveals a decrease in the number of nuclear pores occuring during maturation from proerythroblasts (Fig. 6b) to the so called basophilic late erythroblasts (Fig. 6c).

C. Stem Cells

The combination of normal erythropoiesis with highly proliferating granulopoiesis encouraged a search for a presumptive stem cell. This stem cell should be the most primitive blastic cell in erythropoietic islands and should be identical to the most primitive cell at the centre of clusters of maturing granulocytes. This primitive cell is identified as having a diameter of 5–6 μm, and an extremely high nuclear to cytoplasmic ratio (Fig. 7a). The nucleus contains finely dispersed chromatin or minimal marginal chromatin condensation and one or two nucleoli. The rim of cytoplasm displays only free polysomes. Gradual maturation with differentiation of cytoplasm and decrease in the nuclear to cytoplasmic ratio can be traced in cells progressing from the centre to the periphery of clusters of erythropoiesis or granulopoiesis. Enlargement of the cytoplasmic compartment occurs together with the appearance of mitochondria and later on small cisternae of the RER; polysomes become abundant. Next the Golgi apparatus gradually develops and the first dense bodies can be observed. The maturation towards the granulocytic lineage proceeds with formation of the first primitive primary granules (promyeloblast, Fig. 7b) and towards the erythrocytic lineage with further enlargement of the cell to finally produce the proerythroblast (Fig. 7c).

D. Plasma Cells

The plasma cell series (Figs. 8a–c) shows no striking abnormalities except for lamellated crystal-like inclusions in dilated cisternae of the RER (Fig. 8c). Freeze-fracture replicas demonstrate the cisternae of the RER to contain pores and channels of about 70 nm diameter in random distribution (Fig. 8b).

The monocyte and lymphocyte series are normal in their ultrastructure throughout the different stages of maturation.

E. Myeloid Stroma

1. Reticulum Cells. The phagocytic reticulum cell (histiocytic cell) is spiderlike in outline. The cytoplasm is characterized by much RER and large membrane bound dense bodies of various shapes and inner structure. These inclusions are easily recognized in freeze-fracture replicas (Fig. 9a and b) and it is seen that frequently the large peculiarly shaped, dense bodies contain laminated

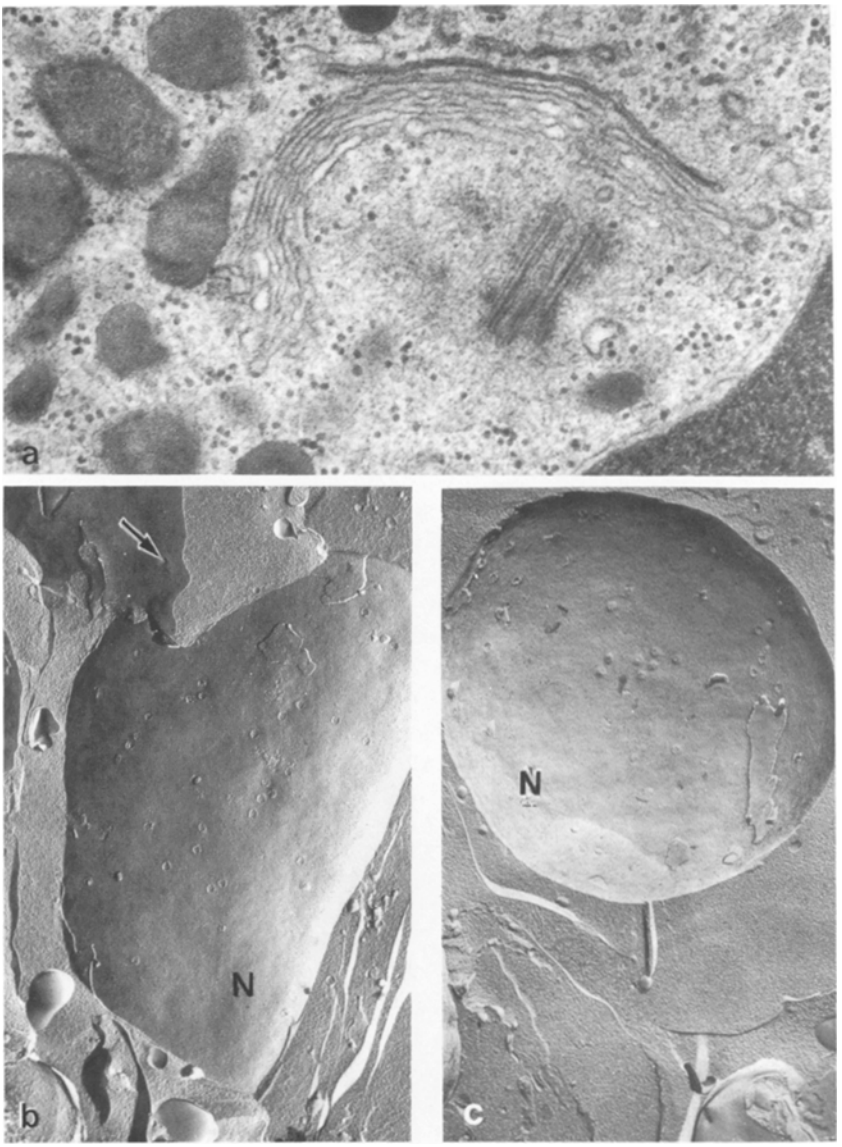

Fig. 6. **a** Golgi apparatus of a myelocyte (compare with Figure 3a) with secondary granules and twisting of the saccules with innermost dense saccule apparently turned to the outer aspect of the stack. In the centre one centriol. **b** and **c** Freeze-fracture replicas of erythrocyte series. **b** Late proerythroblast with large elongated nucleus (*N*) and cytoplasm with a few dense bodies and pinocytic vesicle of plasma membrane (*arrow*). **c** Late erythroblast with spherical nucleus (*N*), decrease of nuclear pores and almost no cytoplasmic organelles. **a** ×41,500 — **b** and **c** ×12,800

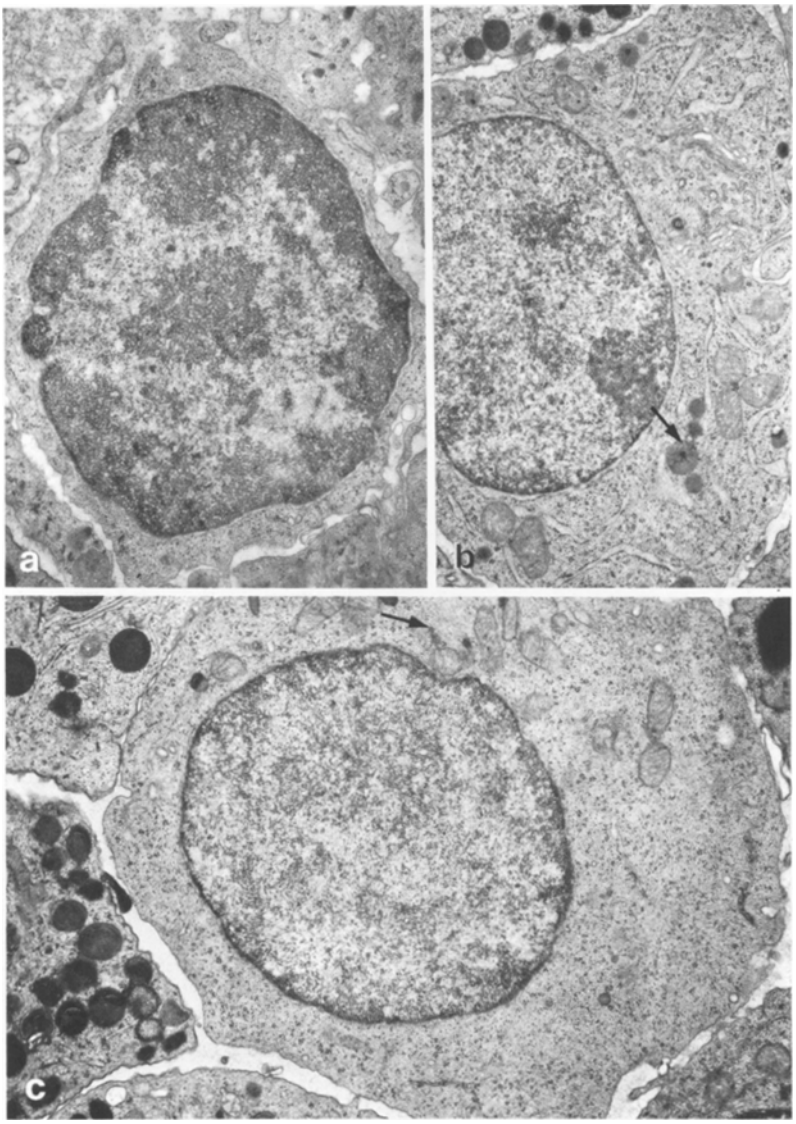

Fig. 7. a Stem cell with large nucleus surrounded by small portion of cytoplasm containing numerous polysomes. **b** Myeloid precursor cell (promyeloblast?) with large Golgi complex, few mitochondria and some dense bodies (*arrow*). **c** Primitive blastic well (early proerythroblast?) with polysome rich cytoplasm, few mitochondria and bundles of microtubules (*arrow*). **a** ×12,800; **b** and **c** ×9200

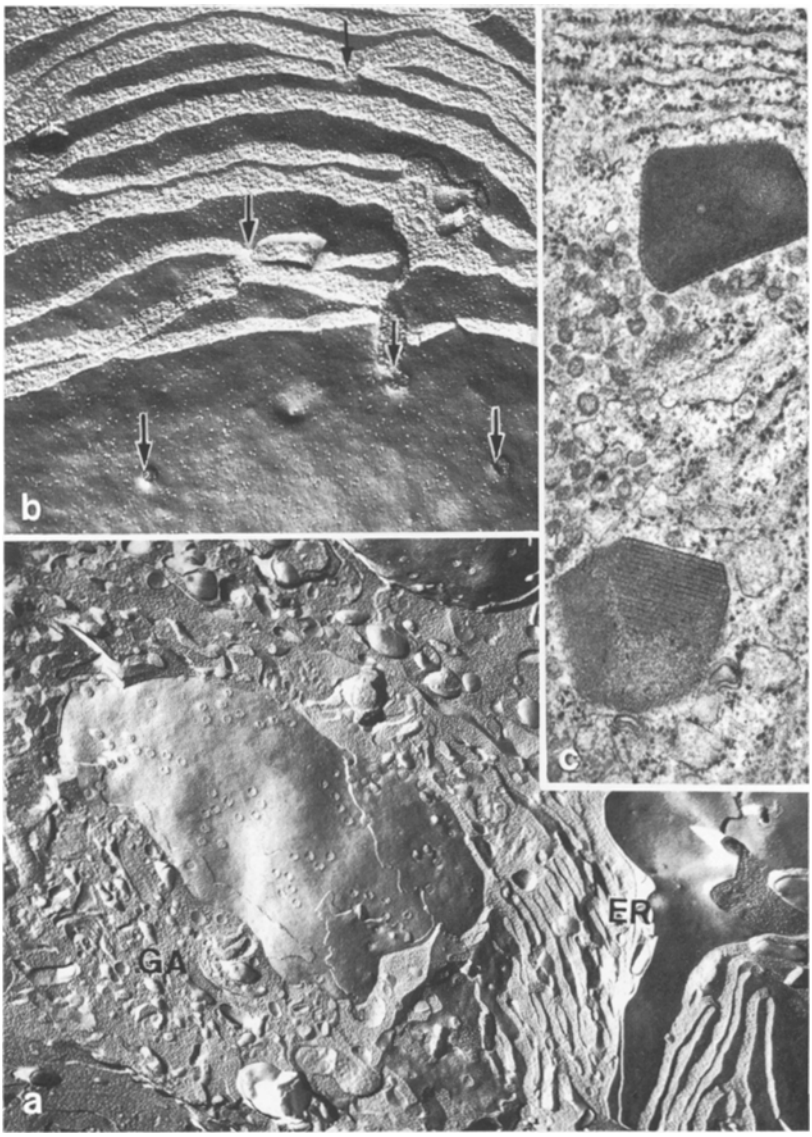

Fig. 8a–c. Plasma cell series. **a** Survey with immature plasma cell (plasmacytoblast) containing large nucleus with wavy outline and many pores. Cytoplasm with large Golgi apparatus (*GA*) and cisternae of the endoplasmic reticulum (*ER*). **b** Cisternae of the rough endoplasmic reticulum in longitudinal and transverse cleavage planes with several pores (*arrows*). **c** Crystalloid inclusions of plasma cytoblast apparently lying in dilated cisternae of the endoplasmic reticulum. **a** ×12,800; **b** and **c** ×32,000

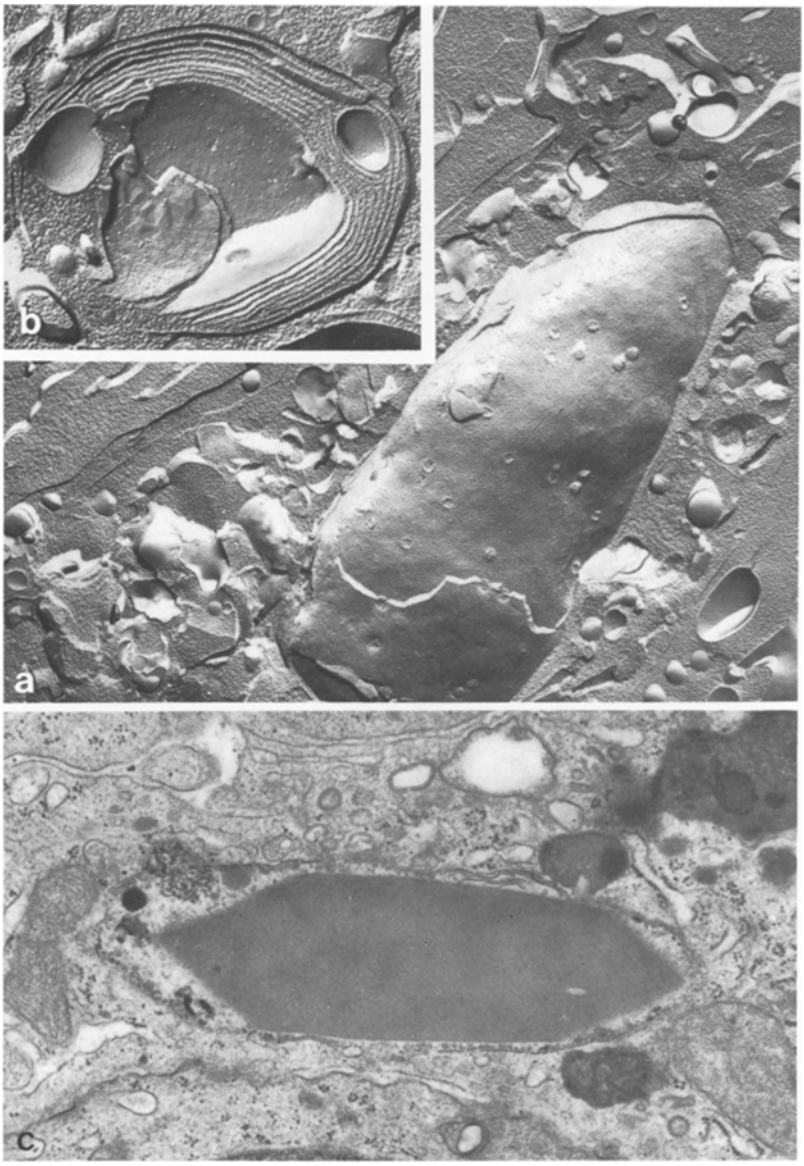

Fig. 9a–c. Phagocytic reticulum cell of the bone marrow. **a** Survey of reticulum cell with large roll-like nucleus and cytoplasm containing many fractured bodies of variable shape and cisternae of the endoplasmic reticulum. **b** Large cytoplasmic inclusion with laminated inner structure and small spherical bodies in between the lamellae. **c** Membrane bound inclusion with prismatic homogenous body surrounded by several dense bodies of variable inner structure. **a** ×16,000; **b** ×32,000; **c** ×24,000

cleavage planes (Fig. 9b). At the centre of large cytoplasmic inclusions, plump, prismatic dense bodies of a homogenous structure are conspicuous. They are probably proteinaceous in nature (Fig. 9c). Frequently ghosts of erythro-, granulo- und most often thrombocytes can be observed.

2. Vessels. Freeze-fracture confirms the presence of endothelial cells as an uninterrupted layer along the sinus walls. The endothelial cells have no fenestrations but their cellular processes overlap to a remarkable extent thus forming a vascular channel which is interrupted by the passage of various haemic cells entering the lumen. The close relationship of megakaryopoiesis to the vascular bed, which is surrounded and invaded by this process, is conspicuous, as already described (Thiele et al., 1977). The sinusendothelial cells contain many pinocytic vesicles and sometimes dense membrane bound bodies probably formed as the result of phagocytosis. In the small capillaries, however, the endothelium does show fenestrations and multiple Weibel-Palade granules (Weibel and Palade, 1964) are also seen in the endothelial cells of arterioles. These "granules" are elongated, often being present in high concentrations and having a fibrillar inner structure.

Discussion

A. Thin Sections

1. Granulocytes. The atypia found in the granulocyte series can be compared with those generally encountered in the leukocytes of CGL (Bessis, 1968, 1973; Ross and Harnden, 1969; Cawley and Hayhoe, 1973). This is particularly true of hypo- and hypergranulation, the maturation asynchrony and the pathological increase in the number of nuclear blebs.

Disorganization of granulopoiesis is conspicuous not only in the neutrophil but also in the basophil and eosinophil lineages. In the latter, the atypia is comparable with the findings of Wulfhekel et al. (1975) in eosinophilic-myelomonocytic leukaemia. Thus, all three types of granulocytes are involved in this neoplastic process indicating a malignant transformation at a very early stage of cellular differentiation. The very low levels of alkaline phosphatase activity commonly found in patients with this myeloproliferative disorder can be attributed either to an enzymal defect of the ultrastructurally normal-appearing specific granules or to the observed hypogranulation of mature neutrophils (Ullyot and Bainton, 1974).

Maturation anarchy is documented by the persistence of Döhle bodies (Döhle, 1911) and the failure of complete segmentation in mature metamyelocytes resulting in a Pelger-like anomaly as sometimes observed in CGL (Darte et al., 1954). The nucleoli frequently exhibit formations as described in human leukaemic myeloblasts by Smetana et al. (1969) and the nuclear bodies resemble those found in tumour cells (Bouteille et al., 1967, Krishan et al., 1967).

The frequent formation of veil-like projections of the nucleus (nuclear blebs) is also described by several authors as a property of malignant cells (Epstein

and Achong, 1965; Achong and Epstein, 1966; McDuffie, 1967; Mollo and Stramignoni, 1967; Smith and O'Hara, 1968). Sometimes, unit-like structures of the nuclear envelope-limited central chromatin sheet can be observed in the nuclear blebs, identical to those described by Davies and Small (1968) in leucocytes. As nuclear blebs are shown to occur with drugs which inhibit DNA synthesis (Ahearn et al., 1967; Stalzer et al., 1965) or following radiation (Duplan et al., 1969); their presence is probably a sign of disturbance in DNA-synthesis. Moreover, recent investigations show a close relation between these structures and aneuploidy in human acute leukaemias (Ahearn et al., 1974). From all these findings, therefore it appears that our granulocytic cell line exhibits many morphological features attributable to malignancy. Further support for the neoplastic nature of many forms of myelofibrosis (MF) as proposed by Georgii and Vykoupil (1976), Schäfer et al. (1975) and Hill and Schäfer (1976), is provided by Tavassoli and Weiss (1973) in their investigations of haemotopoiesis of the spleen in agnogenic MF associated with myeloid metaplasia. They found abnormalities in haemopoietic cells, particularly of the granulocytic series, similar to those seen by us in the bone marrow and relate their findings to neoplastic haematopoiesis in an otherwise unaltered spleen. It should be noted however, that only two of our five patients exhibit any degree of MF and clinical features pointing to myeloid metaplasia.

Still, it is tempting to consider the myeloid metaplasia of MF as some kind of metaplastic/neoplastic growth and not as a compensatory one (Nathan and Berlin, 1959; Szur and Smith, 1961; Nakai et al., 1962).

Thus our electron microscopic observations confirm the clinical and morphological bone biopsy findings of a myelosis of mixed cellularity involving the megakaryocytes and granulocytes.

2. Stem Cells. There is a good chance of discovering the stem cell (haemocytoblast) among the precursor cells of the atypical proliferating leukocytes in CGL, by comparing it with the most primitive cell of normally maturing erythrocytes. In this situation we have the opportunity of investigating extensive areas, with large numbers of proliferating cells in contrast to the normal bone marrow. The cell kinetics of CGL also help us, particularly regarding persistence of a regulatory mechanism even in myelopoiesis (Morley et al., 1967; Vodopick et al., 1972; Gatti et al., 1973). Finally in patients with MF there is an increase in the circulating stem cell population (Chervenick, 1973). Our stem cell candidate closely resembles the haemopoietic stem cell derived by Dicke et al. (1973) and Rubinstein and Trobaugh (1973) from colony-forming unit clutures. These so called CFU-cells are slightly larger (8–10 μm diameter) than our presumptive stem cell (about 5–6 μm) but do show the same fine structure that readily distinguish them from lymphocytic cells.

By Freeze-Fracture Replicas

Generally, freeze-fracture confirms the morphological findings derived from thin sections but also extends knowledge of some cytological aspects. This

is particularly true of *nuclear pores, RER, Golgi apparatus, granules* and the *inclusions* of phagocytic reticulum cells.

The number of *nuclear pores* gradually diminshes during maturation in granulo- and erythropoiesis. This may be related to a decrease in the rate of protein synthesis as found experimentally in other cells (Maul et al., 1971) and in actively secreting human endocrine cells (Thiele and Wermbter, 1974).

In plasma cells, the highly developed *RER* exhibits circular pores similar to those described by Orci et al. (1972) in pancreatic exocrine cells.

Freeze-fracture contributes to our knowledge of the fine structure of the *Golgi apparatus* specially regarding the appearance of the individual saccules. Our findings in human haemopoietic cells are in complete agreement with those found in plant cells (Fineran, 1973).

At least three different types of *granules* are distinguishable in replicas of the neutrophil series, primary (azurophilic), secondary and tertiary (specific) neutrophil granules. This confirms earlier findings of Kriz (1969). Even in mature, normal neutrophil granulocytes, a very small number of large, rounded primary granules as well as the smaller, more elongated specific ones are to be observed as previously reported by Bainton et al. (1971).

The large *inclusions* of the reticulocytes show laminated fracture planes. This is a characteristic of lipids and according to the experimental findings of Ruska et al. (1972) these inclusions should consist mainly of a mixture of neutral fat and cholesterol compounds.

Comparison of thin sections with freeze-fracture replicas of the sinuses of the bone marrow generally confirms earlier observations mostly made in animals (Zamboni and Pease, 1961; Huhn and Steidle, 1967; Hudson and Yoffey, 1968; Huhn and Stich, 1969). In addition the capillaries and small arterioles show a fine structure similar to other regions of the body with many pinocytic vesicles and Weibel-Palade granules (Weibel and Palade, 1964).

In conclusion, the use of the electron microscope in comparing thin sections and freeze-fracture replicas, confirms that granulopoiesis in CMGM is neoplastic. This, together with our previous finding of malignancy in megakaryopoiesis of CMGM, classifies CMGM as a myelosis of a mixed cellularity leading gradually to MF.

Acknowledgements. We are greatly indebted to Professor E. Reale, Hannover for providing the freeze-fracture preparations and for his critical review of the manuscript.

The excellent technical assistance of Mrs. M. Rißmann is appreciated.

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Received March 22, 1977