Virchows Arch [Pathol Anat] (1981) 390:93–108

Virchows Archiv A

Ultrastructural Changes of Bone Marrow in Canine Cyclic Hematopoiesis (CH Dog)

A Sequential Study

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Summary. The pathogenesis of cyclic hematopoiesis (CH) in the grey collie dog is still unknown. It has been proposed that periodic bursts of necrosis of the bone marrow neutrophils would induce cyclic arrests of the stem cell differentiation. In the present study, the sequential changes undergone by the erythroid and neutrophil series of the bone marrow of CH dogs were evaluated by electron microscopy. Erythroid cells presented quantitative periodic oscillations but the morphologic features of both immature and mature cells were normal. On the contrary, nonspecific necrotic changes were observed to occur in the myeloid series. Those abnormalities, which were more marked between days 9 and 11 of the cycle, mainly involved the immature cells and, to a lesser extent, the mature neutrophils. The number of necrotic cells was variable in different cycles, but always represented a small portion of the myeloid cells. In addition, few bone marrow macrophages displayed signs of phagocytic activity containing cell debris. The ultrastructural changes of the myeloid series were accompanied by an abnormal decrease of peroxidase activity and the permanence of large acid phosphatase-positive Golgi complexes in mature neutrophils, as defined by morphologic criteria. Döhle-like arrays of rough endoplasmic reticulum were present in many cells. Our findings suggest that an asynchronic development of myelocytes occurs as a result of regulatory abnormalities related to the congenital defect of the bone marrow which interferes with the differentiation and maturation of the stem cells. Necrosis in some myeloid cells would be a secondary phenomenon rather than a causal factor for the cyclic arrest of cell maturation as has been previously submitted. Furthermore, the small

This study was supported by Grants NIH RR00874, HL15647, and FR5541, from the National Institutes of Health, DHHS

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0340-1227/80/0390/0093/$0 3.20

size of the necrotic cell population could not justify the production of "inhibitors" in sufficient amounts as to block the normal evolution of the bone marrow stem cell pool.

Key words: Cyclic hematopoiesis – Bone marrow – Ultrastructure – Nuclear-cytoplasmic asynchrony – Döhle-like bodies.

Introduction

Cyclic hematopoiesis (CH) in the grey collie dog (Lund et al. 1976) is a genetic disease (Ford 1969; Lund et al. 1970) characterized by periodic cyclic changes, occurring at intervals of approximately 12 days, in the number of circulating and bone marrow hematopoietic cells (Adamson et al. 1974; Dale et al. 1972; Lange et al. 1980; Scott et al. 1973). Erythropoietic and colony-stimulating factor also vary in a cyclic fashion (Adamson et al. 1974; Dale et al. 1971; Yang et al. 1974). The disorder, transmitted genetically as an autosomal recessive trait, can be abrogated by transplantation of normal bone marrow (Dale et al. 1974; Jones et al. 1975; Weiden et al. 1974). Due to the periodic cycles of granulocytopenia, CH neonates and pups are subject to recurrent bacterial infections. Widespread, age-associated amyloidosis of the secondary type (Cheville 1968; Cheville et al. 1970; Gregory et al. 1977; Machado et al. 1978; Machado et al. 1979) consistently occurs in this dog.

Due to the regularity of the periodic cyclic changes, the CH dog is an excellent animal model for studies on hematopoietic differentiation and maturation. The widespread involvement of the bone marrow cell series and the abrogation of the disease resulting from transplantation of normal bone marrow (Dale et al. 1974; Jones et al. 1975) justify the assumption that the genetic defect must influence very early stages of cell differentiation, i.e., the stem cell population (Weiden et al. 1974).

As far as we are aware, only one study has reported attempts to define whether the cyclic oscillations of hematopoietic cells have a characteristic morphologic counterpart (Scott et al. 1973). However, a sequential examination of the ultrastructural characteristics of bone marrow cells of CH dogs throughout the cycle could provide additional parameters for comparison with functional studies. In this paper, we report the results of such an electron microscopy study at intervals on the bone marrow of CH and control dogs. Out attention was particularly focused on the neutrophil and erythroid series because, in the CH dog, these cells display the most marked quantitative cyclic changes, both in the bone marrow and in the peripheral blood.

Material and Methods

Dogs. Our study involved five CH dogs from the colony maintained at the University of Tennessee Department of Medical Biology/Memorial Research Center. The establishment and care of this colony have been described (Jones et al. 1975). Two normal littermates and one dog without the CH background were used as controls.

Blood Counts. Daily blood samples from the jugular vein were collected into EDTA. Total white blood cells were counted with an autocytometer (Fisher Scientific Co., Pittsburgh, PA), and smears were stained with Wright's stain for differential leukocyte counts. Cycle day 1 was designated as the first day the absolute neutrophil count fell to less than 1600/mm^3.

Bone Marrow Aspirations. After appropriate analgesia, marrow was aspirated from the iliac crests or the ends of the long bones of the CH dogs over several cycles. The specimen was immediately smeared for differential cell counts and a portion processed for electron microscopy. The volume of marrow aspirated was not varied from day to day and aspiration sites were systematically rotated so that four days elapsed between taps of any one site. Marrow was aspirated from control dogs at similar intervals and following the same procedures.

Light Microscopy. The bone marrow smears were stained with Wright's stain. At least 2,000 cells were counted in each sample and then classified according to standard morphologic criteria. Granulocytic cells were classified as "early" (myeloblast, promyelocyte, myelocyte) and "late" (metamyelocyte, bands, segmented). Early and late erythroid precursors were tabulated together.

Transmission Electron Microscopy (TEM). The bone marrow aspirates were fixed in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer at pH 7.2 for 2 h, rinsed 3 times in buffer for 10 min each, and then processed for cytochemistry or postfixed for 1 h in 2% osmium tetroxide in 0.1 M sodium cacodylate at pH 7.2. After dehydration, the specimens were embedded in Epon.

Thick (1 μ) sections were stained with toluidine blue 0 solution and periodic-acid-Schiff (PAS), and then studied by light microscopy.

Thin sections were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined on a Zeiss EM 9-S electron microscope.

Cytochemistry. After fixation in glutaraldehyde, portions of the bone marrow were incubated in Novikoff's acid phosphatase medium (Novikoff et al. 1971); Beard and Novikoff's technique was used for demonstrating peroxidase (Beard et al. 1969). The samples were then fixed in osmium tetroxide and processed for electron microscopy examination as described above.

Results

Peripheral Blood and Bone Marrow Differential Counts

Daily peripheral blood cell counts demonstrated that the CH dogs had the expected 12–14 day fluctuations in the number of neutrophils. The absolute neutrophil counts from one dog are shown in Fig. 1. For consistency, cycle day 1 is given as the day in which the neutrophil count falls below 1600/mm^3.

Bone marrow cellularity (as assessed by histologic sections) was fairly constant throughout all phases of the cycle. As shown in Fig. 2, quantitative data demonstrated a reciprocal oscillation of granulopoiesis and erythropoiesis. A clear wave of erythroid elements coincided with the nadir of granulocyte counts in the peripheral blood. The decline in bone marrow erythroblasts was followed by a wave of granulopoiesis as shown by both bone marrow and peripheral blood counts. When the peripheral blood granulocytic counts were normal, the number of bone marrow myeloid precursors once more declined and, conversely, erythropoietic cells began to proliferate again. This rhythmic oscillation was consistently maintained throughout the life span of the CH dog. Control dogs, on the contrary, had a constant, normal pattern of bone marrow cell maturation and peripheral blood cell numbers.

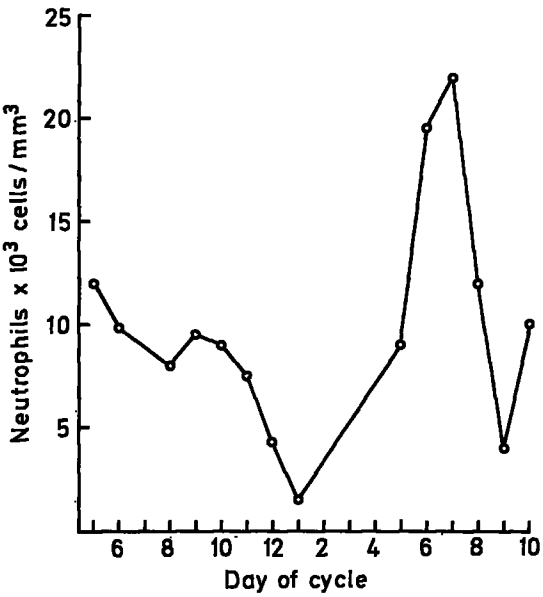


Fig. 1. Cycle of peripheral neutrophils in a CH dog. Cycle day 1 is given as the day of the neutropenic nadir. Counts were made daily during a 12-day period

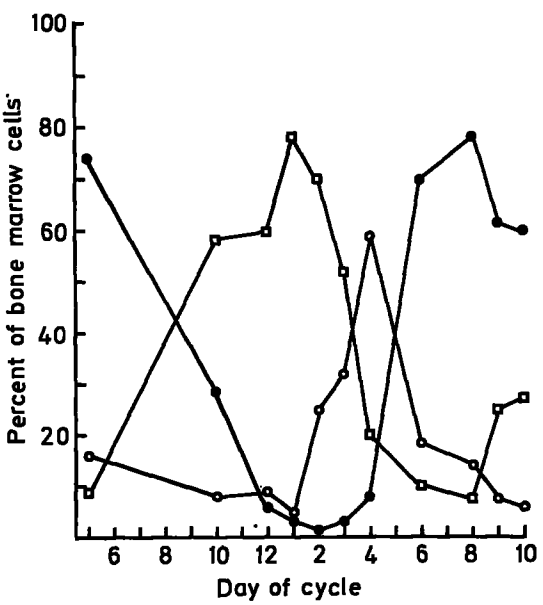


Fig. 2. Cycle of hematopoietic cells in the bone marrow of a CH dog during a 12-day period. □ erythroid precursor cells; ○ early granulocyte precursors (myeloblasts to myelocytes); ● late granylocyte precursors (metamyelocytes to segmented nuclei). The values are given as percentages of the total bone marrow cells

TEM Examinations

The morphologic and cytochemical abnormalities of the myeloid cells were consistently associated with the quantitative oscillation in bone marrow and peripheral blood. Therefore, the results of TEM examination of CH dog bone marrow could be divided into three phases of the cycle: 1) days 1–3, in which cell maturation was absent, corresponding to the peripheral granulocytopenia; 2) days 4–8, showing myelopoietic differentiation and maturation, and initial cellular abnormalities but normal peripheral granulocyte counts; and 3) days 9–11, exhibiting marked changes in myelocytes and granulocytes which preceded the next phase of peripheral neutropenia.

Morphologic Findings

Days 1–3. The cell population of the bone marrow aspirates obtained on day 1 was mainly composed of large undifferentiated cells with round or slightly indented nuclei containing irregular peripheral clusters of chromatin. The cytoplasm of these cells was primitive; multiple free ribosomes were noted as well as a few small mitochondria and sparse cysternae of smooth (SER) and rough (RER) endoplasmic reticulum (Fig. 3).

At days 2 and 3, some of the large primitive cells showed larger mitochondria and more advanced stages of development of the SER, RER, and Golgi complex. Large, dense cytoplasmic bodies, similar to cytosegregosomes, were occasionally found in a few undifferentiated cells (Fig. 4). The absence of minimal specific characteristics made it impossible to determine to which line the primitive cells corresponded. Intermediate forms of maturation of the myeloid line or granulocytes were not observed. Thus, the cell composition of the myeloid series of CH bone marrow was strinkingly different from that of normal dogs in which all stages of neutrophil maturation were present at any given time.

Although the bone marrow erythroblasts and normoblasts of CH dogs showed marked quantitative variations at this as well as other stages of the hematopoietic cycle, the erythroid cells did not display ultrastructural morphologic abnormalities (Fig. 5).

Days 4–8. The granulocyte lineage in the bone marrow from the CH dogs displayed a nearly normal cellular pattern. Undifferentiated cells were scarce, while intermediate forms of maturation (myelocytes, metamyelocytes, and bands) were numerous. Mature neutrophils appeared during this phase.

Signs of cell injury, such as autophagosomes containing mitochondria or fragments of organelles as well as granular material, were frequently found in intermediate myeloid cells (Fig. 6a, b, c). In all specimens examined, the myelocytes and metamyelocytes of CH dogs appeared to contain a smaller number of primary granules with a typical lucent core than did the cells at similar stages of maturation from normal dogs (Fig. 7).

Morphologic signs of cell injury in mature neutrophils were less frequent and marked than in myelocytes and metamyelocytes. However, some neutrophils

showed minimal abnormalities, such as diminished electron density of the cytoplasm and the presence of small vacuoles and autophagosomes (Fig. 8). These changes were not observed in mature neutrophils from normal dogs (Fig. 9).

Early abnormalities in the myeloperoxidase and acid phosphatase reactions of the neutrophil series preceded the appearance of the more marked changes found during the next stage of the cycle.

The erythroid cell line did not show pathologic changes.

Days 9–11. Metamyelocytes, band cells, and mature granulocytes were predominant in bone marrow specimens from the CH dogs. Signs of cell injury were more marked during this phase. Autophagic vacuoles containing cytoplasmic debris, and large lysosomes were observed in some metamyelocytes and band cells (Fig. 10). The Golgi apparatus of metamyelocytes could appear as an ill-defined conglomerate of multiple, tiny vacuoles and irregular cysternae (Fig. 11).

The RER cysternae of immature cells were irregularly dilated and, in some cells, appeared grouped in parallel arrays having morphologic characteristics resembling Döhle bodies (Figs. 10, 11).

The development of cytoplasmic granules in immature myeloid cells of CH dogs did not display a synchronic fashion as was found in the bone marrow of normal dogs. Thus, in a given specimen, it was possible to observe metamyelocytes and bands which contained, almost exclusively, granules of the secondary type. Other cells at similar stages of maturation had partially condensed granules, some exhibiting a lucent core characteristic of primary granules.

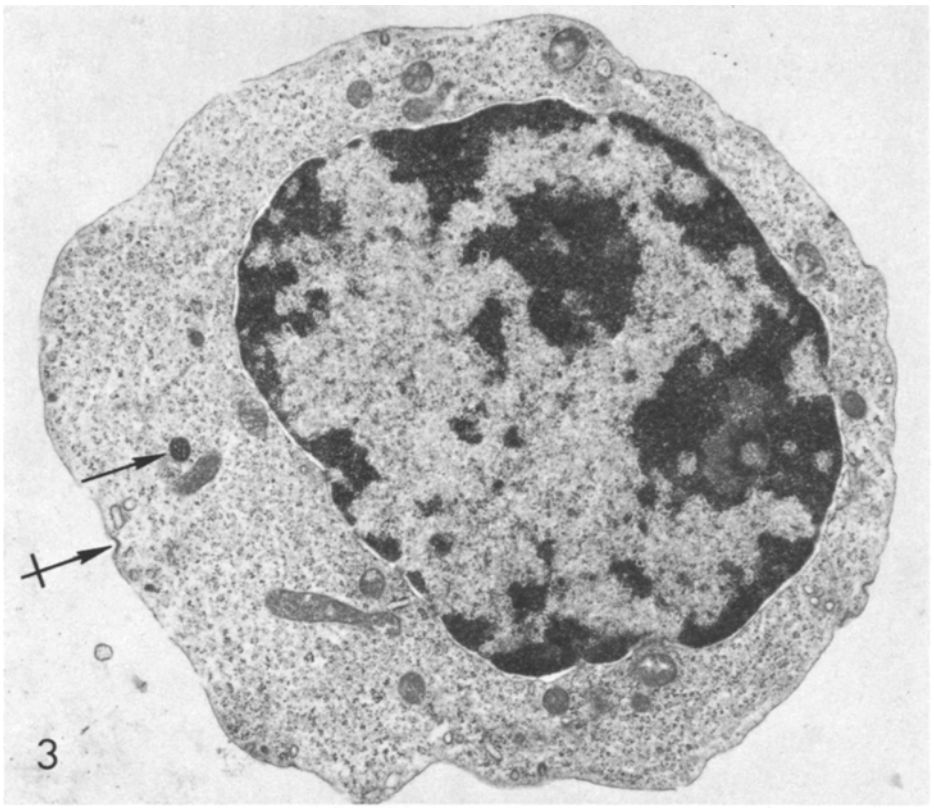
Cell injury in neutrophils was milder than in the immature cells. Although the cytoplasmic granules in most neutrophils had a normal morphology, in some cells the lucent cores of primary granules appeared enlarged and occupied by cytoplasmic ground substance and particulate material. Other elongated granules had a horse-shoe shape (Fig. 12). Such morphologic characteristics were not observed in the granules of neutrophils from normal dogs (Fig. 13).

Between days 7–11, many mature neutrophils exhibited abnormal cytochemical reactions. After incubation for myeloperoxidase, less than one-third of the total population of primary and secondary granules of CH dog neutrophils gave a positive reaction (Fig. 14). This sharp decrease in enzymatic activity

Fig. 3. Day 1 of the cycle. Large undifferentiated cell in the bone marrow of a CH dog. The cell has a slightly indented nucleus. The cytoplasm contains a few mitochondria and numerous ribosomes. Scarce strands of RER and a few granule-like particles (*arrow*) can be noted. Well-defined, small surface invaginations are observed (*crossed-arrow*) (×9,200)

Fig. 4. Day 2 of the cycle. This immature cell of the bone marrow of a CH dog contains more developed RER cysternae and a large cytosegregosome (*arrow*) containing a membranous inclusion (×13,900)

Fig. 5. Group of erythroid cells of the bone marrow of a CH dog at day 2 of the cycle showing normal characteristics (×4,180).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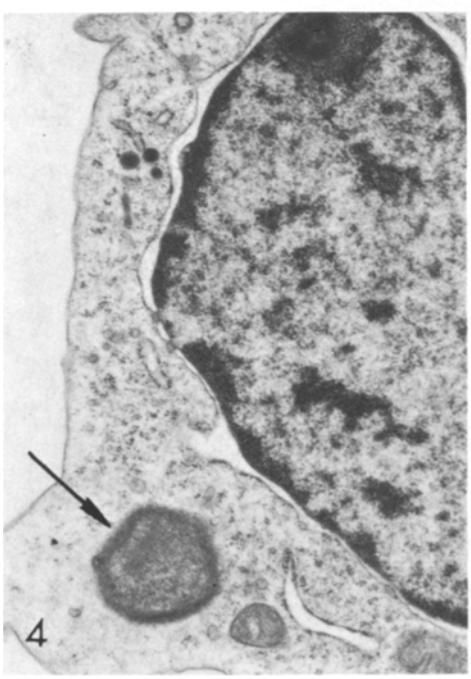
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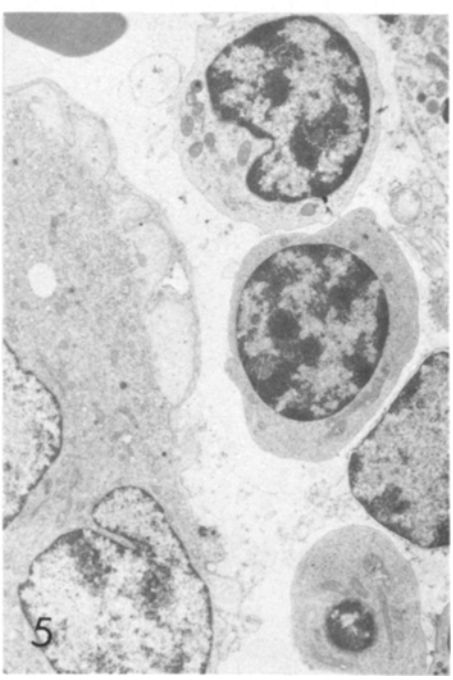
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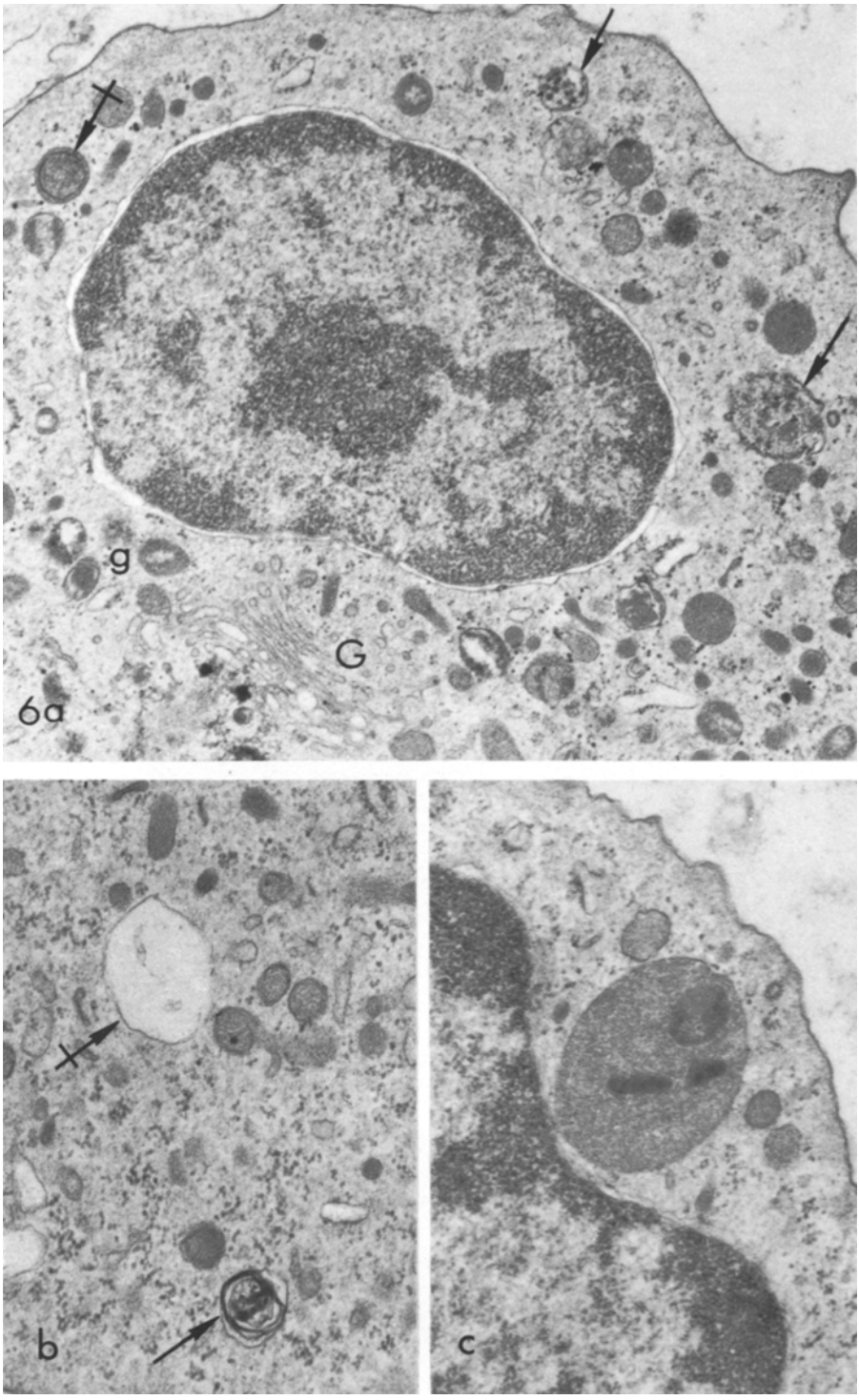
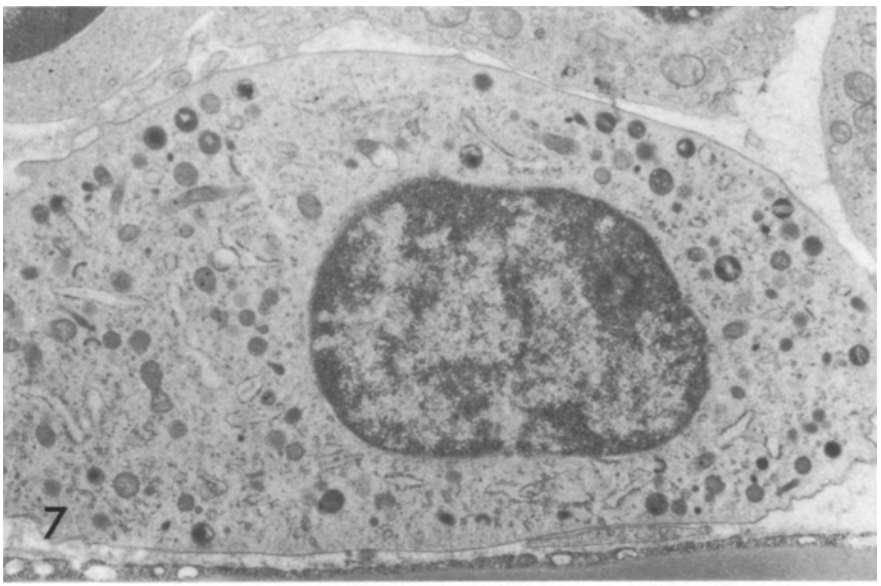


Fig. 6a–c. Days 4–8 of the cycle. **a** High magnification of a myelocyte. The cytoplasm shows a few dilated strands of RER and vacuoles of various sizes (*arrows*). One of them contains a mitochondria (*crossed-arrow*). The Golgi complex (*G*) is well developed. Some of the granules (*g*) display a characteristic shape and lucent core, but most of them have irregular electron density and form (×28,500), **b** High magnification of a metamyelocyte. A myelin figure (*arrow*), vacuoles (*crossed-arrow*), and clumps of glycogen can be noted (×27,730), **c** Large autophagic vacuole containing organelle debris in the cytoplasm of a band cell (×27,910)



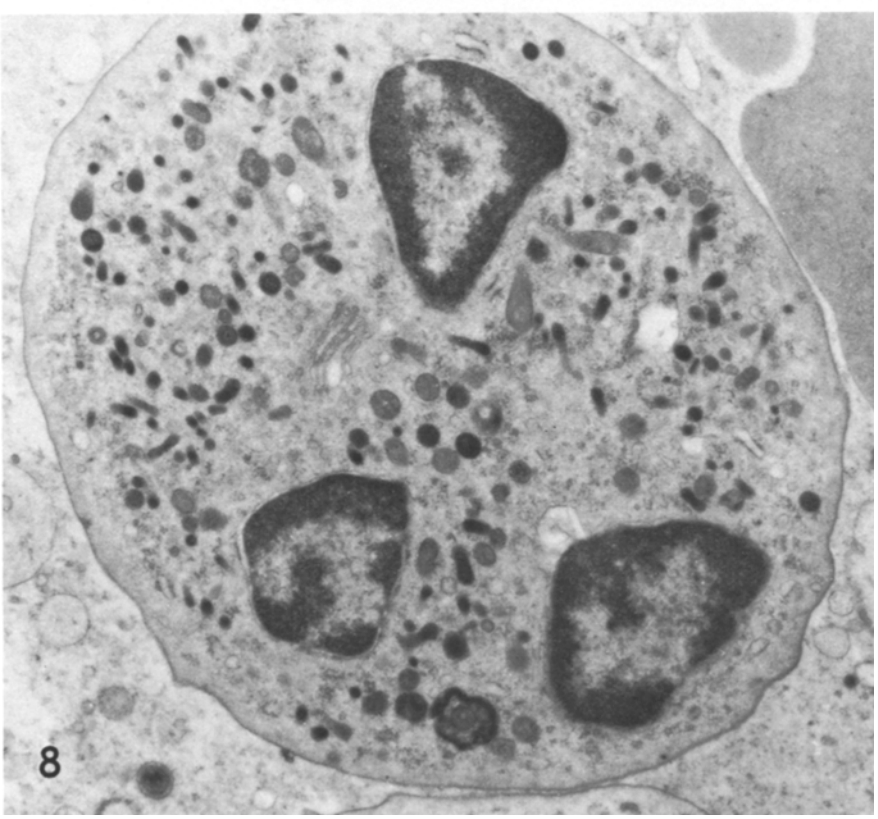
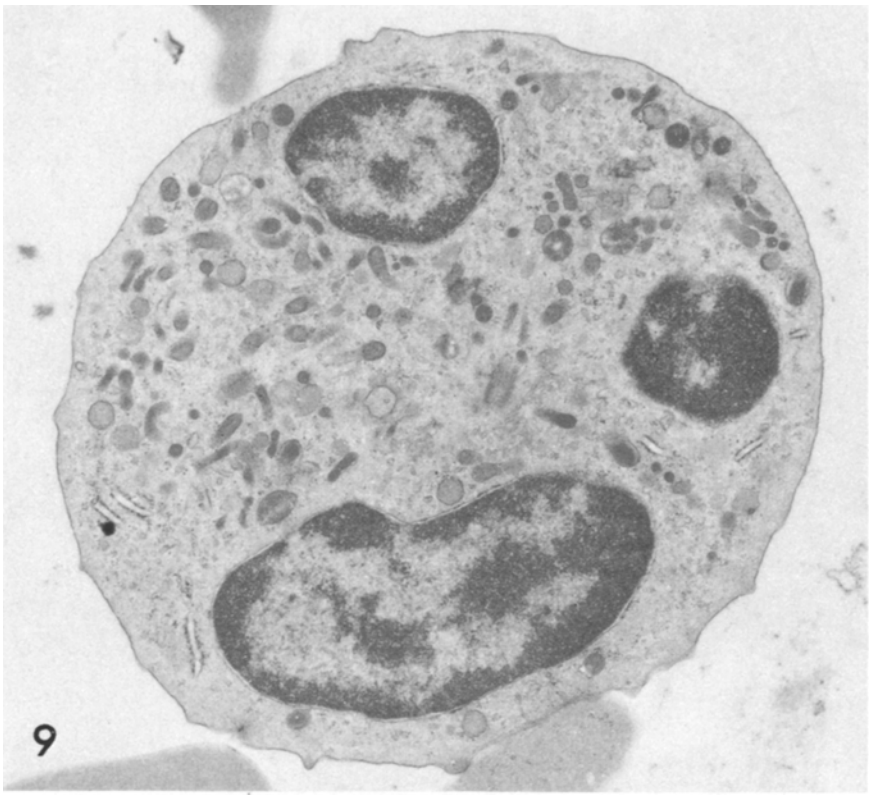


Fig. 7. Metamyelocyte in bone marrow of normal dog. Signs of necrosis as those observed in CH dogs and shown in Fig. 6 are not present. The cytoplasm contains a rich granular population (× 11,750)

Fig. 8. Neutrophil from CH dog displaying small vacuoles and one dense phagosome (× 14,100)



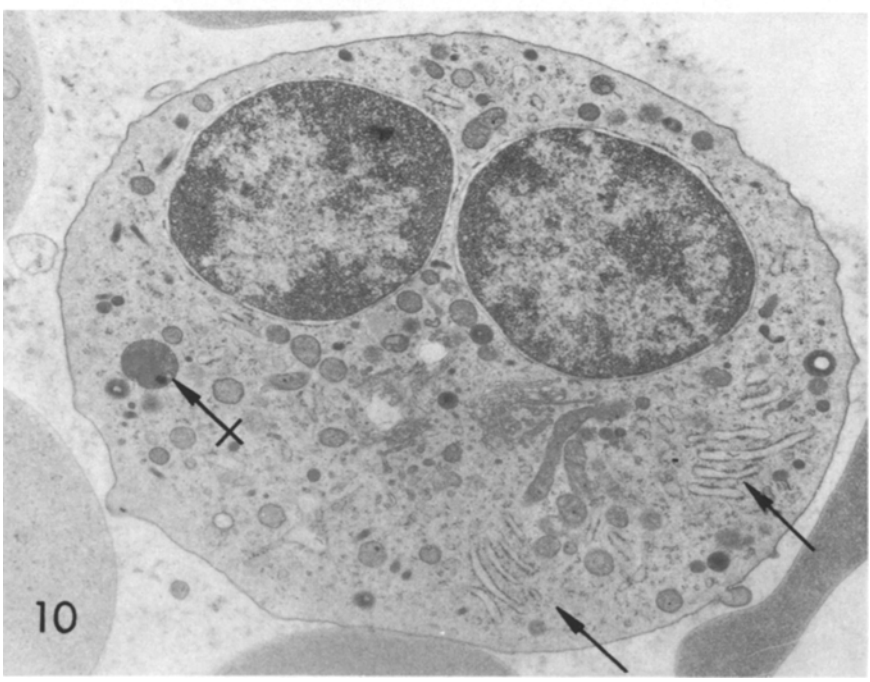
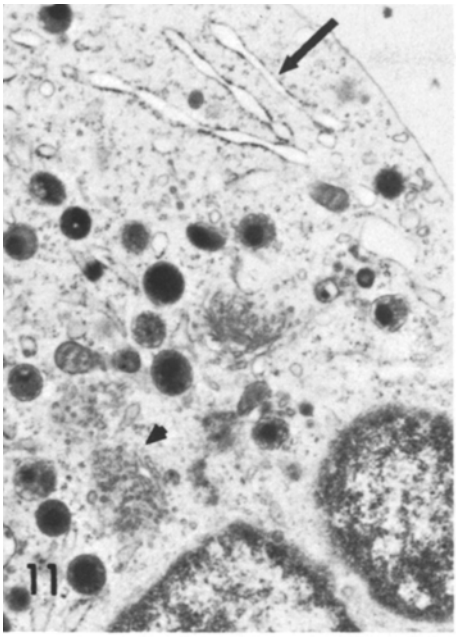
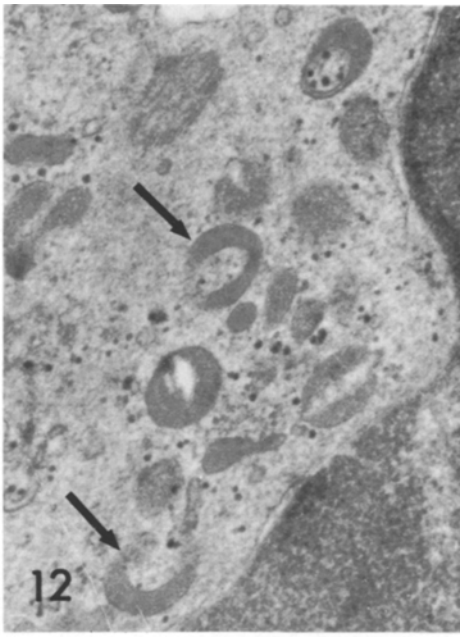


Fig. 9. Early neutrophil from the bone marrow of a control dog. Signs of cytoplasmic necrosis are not observed (×14,100)

Fig. 10. This is very likely a transversally cut band cell. Parallel arrays of RER are noted (*arrows*). Granules are relatively normal. A large, lysosome-like body is present (*crossed-arrow*) (×13,270)





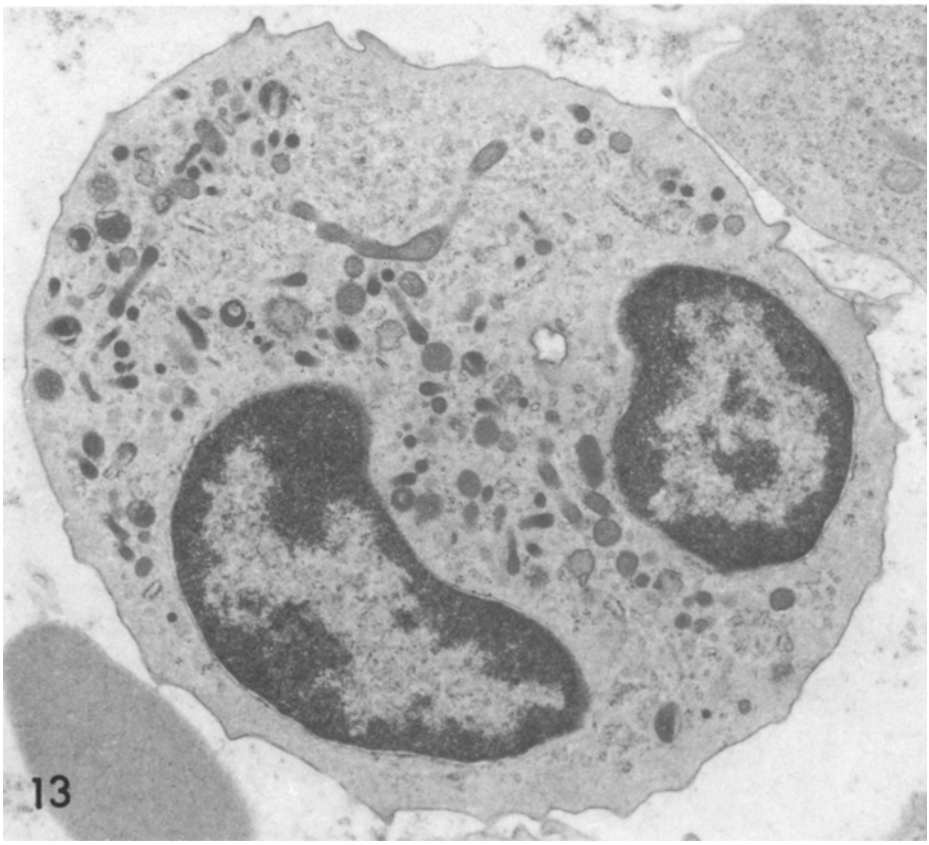


Fig. 11. Parallel arrays of RER (*arrow*) and abnormal Golgi area (*arrowhead*) are noted in this metamyelocyte (× 13,630)

Fig. 12. Large, horse-shoe shaped granules (*arrows*) characteristic of CH neutrophils at day 12 of the cycle (× 19,450)

Fig. 13. Segmented cells of bone marrow from control dog showing normal morphology of granules (× 14,570)

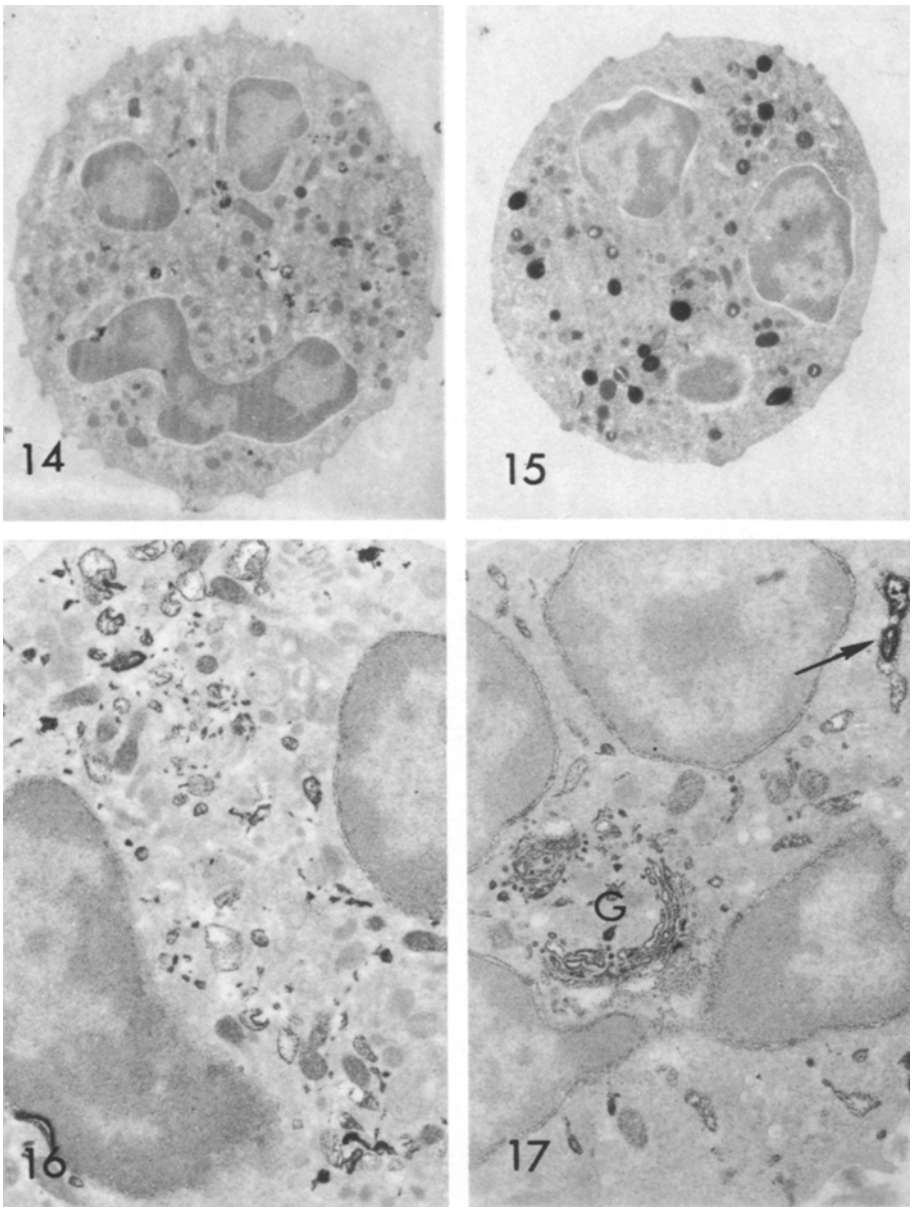


Fig. 14. Extreme reduction of myeloperoxidase-positive granules in mature bone marrow neutrophil of CH dog (×8,460)

Fig. 15. Myeloperoxidase reaction of bone marrow neutrophil from normal dog. Compare with Fig. 14 (×6,650)

Fig. 16. Extensive acid phosphatase reaction in cytoplasmic vacuoles of neutrophil from the bone marrow of a CH dog (×13,630)

Fig. 17. Acid phosphatase stain of a CH dog mature neutrophil. The Golgi complex (*G*) remains highly developed and gives a positive acid phosphatase reaction. Some phagosomes are also stained (*arrow*) (×15,040)

was more marked than occurred in normal bone marrow during the final stages of neutrophil maturation. Approximately half of the granules of neutrophils from control dogs (which had been incubated simultaneously with those from CH dogs) were myeloperoxidase-positive (Fig. 15).

After CH dog bone marrow was incubated for acid phosphatase, multiple precipitates were found in the cytoplasm of neutrophils. The precipitates appeared associated either with vacuoles and phagosomes scattered throughout the cytoplasm (Fig. 16) or with the perinuclear space and Golgi complex (Fig. 17). The persistence of a large, acid phosphatase-positive Golgi complex was observed in CH dog neutrophils which, by standard morphologic criteria, could be considered mature cells. Morphological and enzymatic changes in both immature and mature neutrophils involved only a variable portion of the cell population. Many myelocytic cells did not present abnormalities.

A small number of bone marrow macrophages showed signs of active phagocytosis. The macrophages exhibited numerous large lysosomes, vacuoles, and phagosomes, all of which contained nuclear and cytoplasmic cell debris (Fig. 20).

As in the previous phases, the erythroid cell series did not show morphologic abnormalities.

Light Microscopy of Bone Marrow

Since the subtle cytoplasmic changes depicted by TEM could not be identified in Wright-stained smears, Epon sections (1 μ thick) adjacent to those used for ultrastructural studies were stained with toluidine blue and PAS and examined by light microscopy. Although the sections of the Epon-embedded bone marrow specimens had a small number of myelopoietic cells (30–50 cells per section) than the smears, the structural definition was much more readily seen. The presence of PAS-positive, granular cytoplasmic aggregates was indicative of cellular alteration; in normal myelopoietic cells and, particularly, in neutrophils, the PAS reaction rendered a diffuse pink cytoplasmic color. At days 9–11, when the most marked cellular necrotic changes were observed by TEM, 5–10% of the myelopoeitic cells in the sections had PAS-positive cytoplasmic granular material. Although the different specimens showed slight variations, signs of cell injury were more frequent in immature cells (metamyelocytes, band cells) than in neutrophils. The results of light microscopy examinations agreed with electron microscopy findings of numerous non-necrotic neutrophils in bone marrow specimens at similar intervals.

Discussion

The quantitative studies of bone marrow and circulating blood cells demonstrated the characteristic pattern of canine CH (Dale et al. 1972; Dale et al. 1971; Dale et al. 1972; Lund et al. 1976). The data indicated an orderly appearance of myeloid precursors of increasing maturity associated with the peripheral

blood neutropenia which ends during the period of peripheral blood granulocytosis and is followed by a wave of bone marrow erythropoiesis.

The morphological and cytochemical studies by electron microscopy of the bone marrow demonstrated consistent cellular changes associated with the hematopoietic cycling. Thus, the predominantly undifferentiated cell population found at early stages of the cycle was replaced after the 4th day by intermediate and mature myeloid cells in which autophagia, myelin figures, and abnormalities in the cytoplasmic membranes and granules were apparent. The characteristics of these necrotic signs are not specific to the CH syndrome; similar changes have been found in human granulocytes as a toxic effect of drugs (Fedorko 1967). The ultrastructural evidence of cell injury was consistently more marked in immature myeloid cells than in neutrophils. This finding is at variance with that of Scott et al. (Scott et al. 1973) who reported that cell abnormalities in canine CH are mainly associated with the formation of secondary granules in neutrophils. Repeated examinations of marrow from our CH dogs demonstrated that only a small number of immature and mature neutrophils showed necrotic changes but that a significant proportion of bone marrow myeloid cells displayed a normal morphology or minimal cytoplasmic changes similar to those occasionally observed in normal canine myeloid cells. Only a few bone marrow macrophages evidenced active phagocytosis of cell debris during the period (9–11 days) in which more marked cellular abnormalities occurred. This finding could indirectly signify a low incidence of intramarrow cell necrosis because macrophages in the CH dog are under continual stimulation by infection and amyloid deposition (Cheville 1968; Cheville et al. 1970; Gregory et al. 1977; Machado et al. 1978) and, consequently, a proliferation of macrophages engulfing multiple cell debris should be found if an extensive necrosis of myeloid tissue had taken place.

The results of ultrastructural cytochemistry in bone marrow of CH and normal dogs are open to debate until quantitative analyses can be made. However, the cytochemical studies in a large number of samples have provided consistent information which may be useful as a complement to the morphological findings. A gradual decrease in the number of peroxidase-positive granules is known to occur during the normal maturation of granulocytes in dogs as well as in other species (Ackerman 1968; Cawley et al. 1973; Spicer et al. 1969). The number of positively-reacting granules in the neutrophils may vary according to the particular cytochemical procedure used for detecting the enzyme. When the procedure of Novikoff and Goldfisher (as described by Beard and Novikoff 1969) is followed, peroxidase activity is demonstrated in both primary and secondary granules; approximately half of the granules show a positive reaction in normal neutrophils (Cawley et al. 1973). We found by this technique that peroxidase activity in CH bone marrow neutrophils was markedly reduced compared to that in neutrophils of normal dogs incubated under identical conditions.

Simultaneously, large, acid phosphatase-positive Golgi complexes, similar to those found in immature cells, were demonstrated in morphologically mature CH neutrophils. The extreme reduction in peroxidase (possibly due to either a deficient synthesis during cell differentiation or to increased disappearance in mature cells) and the persistence of well-developed Golgi complexes in many

neutrophils point to the existence of an abnormal development of cells which, by conventional morphologic criteria, display normal mature aspects. A defect of cytoplasmic maturation in tandem with an absence of secondary granules in neutrophils with segmented mature nuclei have been observed in human congenital neutropenia (Parmley et al. 1975).

Necrosis, enzymatic abnormalities, and cytoplasmic inclusions in immature and mature myeloid cells were characteristic signs that apparently were associated with the regular hematopoietic cycles in the CH dog. Since these abnormalities were always found combined, they may have a common causative factor. Thus, the maturation of myelocytes could be impaired by an intrinsic metabolic defect, leading to asynchronic cellular development, an enzymatic defect, or, eventually, to necrotic changes.

The presence of non-necrotic myeloid and granulocytic cells reveals that the severity of the impairment is not identical in all cells. However, the existence of a functional deficiency in the morphologically normal granulocytes entering the circulation during the period of recovery from neutropenia cannot be ruled out. Although the circulating neutrophils in CH dogs have a normal life span (Dale et al. 1972) and the neutropenic episodes are short, the persistence of mild infections along with the extremely early development of amyloidosis (Machado et al. 1978; Machado et al. 1979) strongly suggest the existence of a functional deficiency of granulocytes which may be similar to that of human congenital neutropenia (Parmley et al. 1975).

The erythroid cells in the bone marrow from the CH dogs did not show morphological abnormalities. However, these cells had well defined, quantitative cyclic oscillations, which indicates that they were also affected by the periodic arrests of maturation. The observation of simultaneous quantitative and/or morphologic changes in two different bone marrow cell lines lends support to the hypothesis of a common defect influencing the stem cell population.

The regularity of the quantitative changes along the cycles suggest that the magnitude of hypothetical feedback mechanisms or inhibitory factors for arrest of cell maturation must be fairly constant. Therefore, it is difficult to believe that the necrotic changes in myeloid cells could constitute a pathogenic factor through the liberation of inhibitors because cell injury affects a small and variable number of myeloid cells in each cycle.

According to our results, the abnormalities in the myeloid cells of CH bone marrow must be considered a secondary phenomenon to a still unknown, more complex functional defect involving the very early stages of cell differentiation.

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Accepted August 31, 1980