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Surface morphology and ultrastructure of normal and cyclic hematopoietic canine bone marrow in long-term liquid cultures

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Summary. Long-term liquid cultures of normal and cyclic hematopoietic (CH) dog bone marrow produce committed granulocyte-macrophage progenitor cells (CFU-GM) and differentiated granulocytes for several weeks. Analysis of in situ fixed cultures or of cells harvested from the culture supernatants revealed that the cells had ultrastructure and surface morphology characteristic of immature and mature myeloid cells. The surface morphologies of adherent cells from both normal and CH dogs were similar. The characteristic abnormalities previously reported in neutrophils obtained from CH dogs were not observed in neutrophils obtained from long-term marrow cultures of CH dogs. These results indicate that the cellular abnormalities in the neutrophils of CH dogs may be secondary manifestations of the disease and are not inherent to the pathogenesis of the hematopoietic cells.

Key words: Bone marrow culture, Cyclic hematopoiesis, Ultrastructure

Introduction

Continuous bone marrow cultures have been used to study normal marrow hematopoiesis in vitro and the complex interaction of hematopoietic stroma cells, regulatory factors, and developing blood cells (Dexter et al., 1973, 1977; Allen and Dexter, 1976a). Dexter et al. developed a method for the long-term culture of mouse bone marrow which sustained hematopoietic activity for several months (Dexter and Lajtha, 1974; Dexter et al., 1977). This long-term bone marrow culture system is composed of two distinct cell populations: 1) cells which adhere to the culture dish are made up of fibroblasts, endothelial-like cells, and fat-containing cells; and 2) a non-adherent cell population made up mainly of committed hematopoietic progenitor cells, and mature granulocytes, monocytes and macrophages (Dexter et al., 1978). The adherent cells apparently function as the regulatory microenvironment for the hematopoietic stem cells (Dexter et al., 1973; Dexter et al., 1977). Initial establishment of an adherent cellular microenvironment is a prerequisite for the subsequent long-term proliferation and differentiation of a second inoculum of bone marrow cells. Long-term bone marrow cultures from several species have been developed, based on modifications of the murine system (Greenberg, 1979; Moore et al., 1979; Gartner and Kaplan, 1980; Al-Lebban et al., 1987).

Cyclic hematopoiesis is a genetic disease of the bone marrow which affects both humans and grey collie dogs. The hallmark of this disease is the periodic production of hematopoietic cells by the bone marrow, which may be due to a regulatory defect of the pluripotent stem cells (Jones and Lange, 1983). Ultrastructural studies of neutrophils separated from bone marrow aspirates of grey collies and humans with cyclic neutropenia have been carried out (Scott et al., 1973; Machado et al., 1981; Parmley et al., 1984). In grey collies, a synchronous differentiation of stem cells to neutrophils occurs following severe neutropenia (Scott et al., 1973; Machado et al., 1981). On days 6 to 9 of the cycle, associated with the formation and condensation of secondary granules, the neutrophils had evidence of cellular injury as demonstrated by the appearance of large aggregates of particulate glycogen in the cytoplasm. In some cells there was also evidence of autophagy. In human patients, during the early neutropenic period of the cycle, increased autophagy and granule abnormalities were present in promyelocytes, myelocytes, and the few segmented neutrophils (Parmley et al., 1984). In the late neutropenic period, most promyelocytes appeared morphologically normal, while the few segmented neutrophils present frequently contained an increased number of autophagic vacuoles and a decreased number of cyto-

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plasmic granules. In the non-neutropenic period, the majority of blood and marrow segmented neutrophils appeared morphologically normal, whereas marrow promyelocytes demonstrated increased autophagy with decreased condensation of primary granules. It is not known if the observed defects in the CH neutrophils are inherent to the CH trait or are secondary changes due to an altered in vivo microenvironment. We previously found that the in vivo neutrophil and monocyte cell cycles were abolished in long-term marrow culture, even though bone marrow aspirates from different days of the cycle were used to establish the cultures (Al-Lebban et al., 1987).

The purpose of the present study was to determine whether distinct ultrastructural abnormalities existed in the adherent and/or nonadherent cells derived from long-term bone marrow cultures of CH dogs by scanning and transmission electron microscopy.

Materials and methods

Animals

Two hematologically normal adult dogs and two cyclic hematopoietic dogs were used in this study. The dogs had been previously vaccinated and dewormed, and they were in apparent good health at the time of the study. The CH dogs were born in the colony maintained at this institution (Jones et al., 1975). All dogs were housed in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care. Bone marrow aspirates were obtained from the iliac crest and the long bones by percutaneous aspiration after oxymorphone/xylazine sedation. The marrow was placed in heparinized Iscove's modified Dulbecco's medium (IMDM) supplemented with 100 U/ml penicillin and 100 mcg/ml streptomycin. The mononuclear cells were recovered by Ficoll-paque sedimentation washed three times, and the viable cells were counted as previously reported (Al-Lebban et al., 1987). Slides of fresh marrow specimens and cultured nonadherent cells were also prepared for staining with May-Grunwald and Giemsa.

Bone marrow cultures

To establish the adherent layer, each flask (Corning, 25 cm^2 growing area) and Linbro well (1.67 cm^2 growing area) was inoculated with 8.0 ml and 0.8 ml of bone marrow cell suspension (2×10^6 Ficoll-paque purified mononuclear cells/ml), respectively. The cells were cultured with IMDM supplemented with 100 U penicillin/ml, 100 mcg streptomycin/ml, 12.5% fetal bovine serum (FBS), 12.5% horse serum (HS), 1% dog serum (DS), 1% L-glutamine, and 10^{-6}M hydrocortisone sodium hemisuccinate (Al-Lebban et al., 1987). The cultures were maintained at 33°C, 4% CO_2 in air, in a humidified atmosphere and were fed twice a week. After 3 or 4 weeks each flask and well was reinoculated with 8.0 ml and 0.8 ml, respectively, of autologous cell suspension containing 2×10^6 nucleated cells/ml of growth medium. The cultures were fed twice a week; at each feeding the number of nonadherent cells were counted, and CFU-GM assay was performed. The remainder of the nonadherent cells were used for scanning electron microscope (SEM) and transmission electron microscope (TEM) studies. The adherent and nonadherent cells were studied at the time of inoculation (nonadherent) and at 1 wk, 3 wks, and 4 wks after inoculation. The adherent layers were studied in culture wells only.

Weekly CFU-GM assays were performed using a modified method of Iscove et al. (Iscove et al., 1974; Al-Lebban et al., 1987) with conditioned medium from phytohemagglutinin stimulated dog peripheral blood mononuclear cells as colony stimulating activity. Cells (1.0×10^5 cells/ml) were dispensed into Falcon 24-well flat-bottom plates, in a total volume of 0.5 ml/ml, and the cultures were maintained at 37°C in humidified air with 5% CO_2. Colonies were counted on days 7 and 14 utilizing a Nikon inverted microscope.

Scanning Electron Microscopy

Adherent layer

Cells cultured in Linbro wells were used in the SEM studies. The cultures were fixed for 30 minutes in situ with 3% glutaraldehyde in 0.1M cacodylate buffer, pH 7.3. The fixative was gently decanted, and the adherent layer was washed briefly with three changes of cacodylate buffer alone. It was then postfixed in 1% osmium tetroxide for 60 minutes in 0.1 M cacodylate buffer, pH 7.3. After several washes with cacodylate buffer, the culture dishes were rinsed with distilled water. The cultures were dehydrated through an ascending series of graded alcohol (25%, 50%, 75%, 100%) and critical point dried with liquid CO_2. The cells were then coated with Gold Palladium, sectioned to 100 Å, deposited by sputtering, and examined in an ETEC Autoscan Scanning Electron Microscope at an accelerating voltage of 20 kV.

Nonadherent cells

The nonadherent cells recovered from the cultures (flask or well) were suspended in the growth medium and allowed to settle on glass cover slips for 1 hour at 37°C, 5% CO_2 in a humidified atmosphere. The cells were fixed and dehydrated as described above.

Transmission Electron Microscopy

Adherent layer

The Linbro cultures were fixed and dehydrated as described above and embedded in situ in Epon. Thin sections were cut at right angles to the growing surface and stained with uranyl acetate and lead citrate. The cells were examined with a Philips 201 microscope at 60 kV.

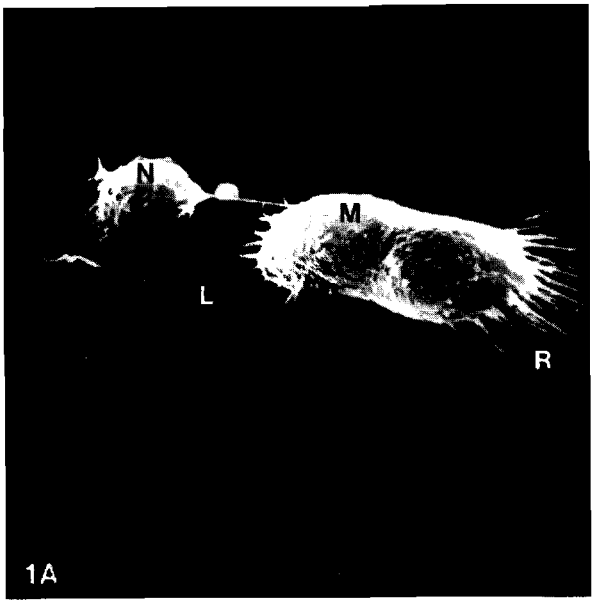
Nonadherent cells

The cells were washed three times with cacodylate buffer, pelleted, and prepared as described above. The cells were examined with a Philips 201 microscope at 60 kV.

Results

For the first 4 weeks normal and CH dog bone marrow cultures produced predominantly differentiating granulocytic cells (Table 1) followed by a progressive decline in cell production with conversion to a monocyte morphology by 8 weeks. Generation of CFU-GM was sustained for at least 4 weeks in both normal and CH marrow cultures.

The adherent layer of long-term bone marrow cultures of both normal and CH dogs contained four morphological cell types: 1) phagocytic mononuclear cells which are a functional component of the adherent layer and have numerous cytoplasmic extensions and an overall tendency to spread along a single axis on glass coverslips (Figs. 1A,B); 2) spindle-shaped fibroblast-like cells (Fig. 2A); 3) flattened polygonal endothelial-like cells that tend to form a confluent monolayer (Fig. 2B);



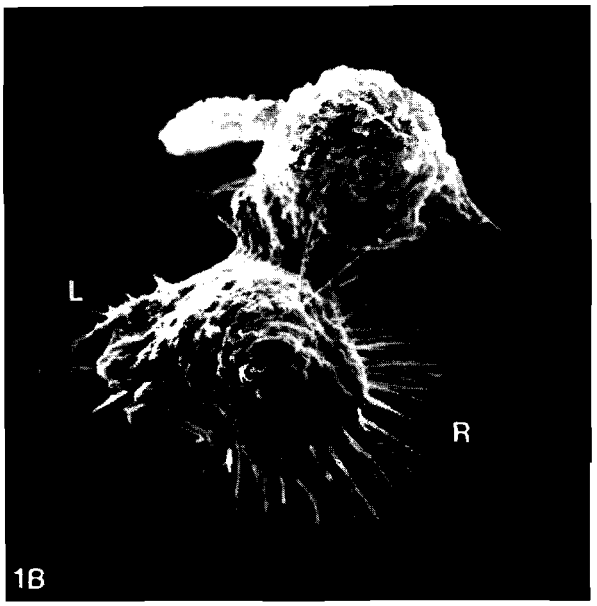


Fig. 1. Scanning micrograph of adherent layer monocytes (M) on the substratum, showing: A) spread morphology mainly along a longitudinal axis. Neutrophil (N) is spreading in a radial manner. × 936, B) a monocyte orientated with a well marked leading edge (L) and trailing reaction fibers (R) × 1,310

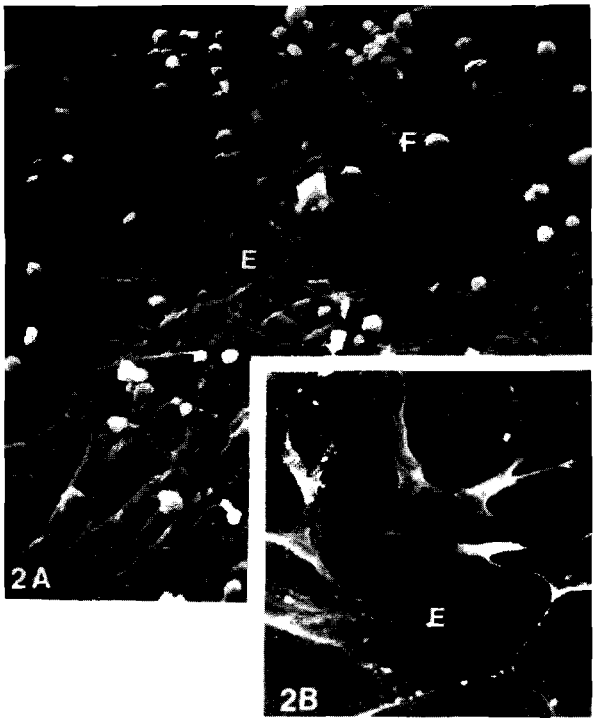
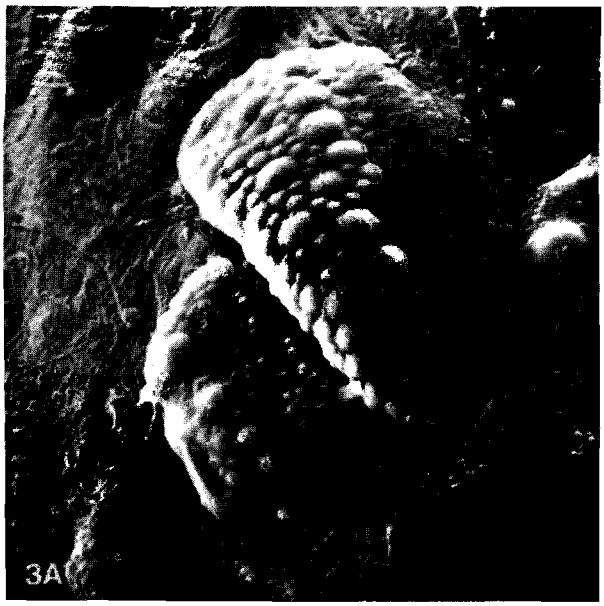
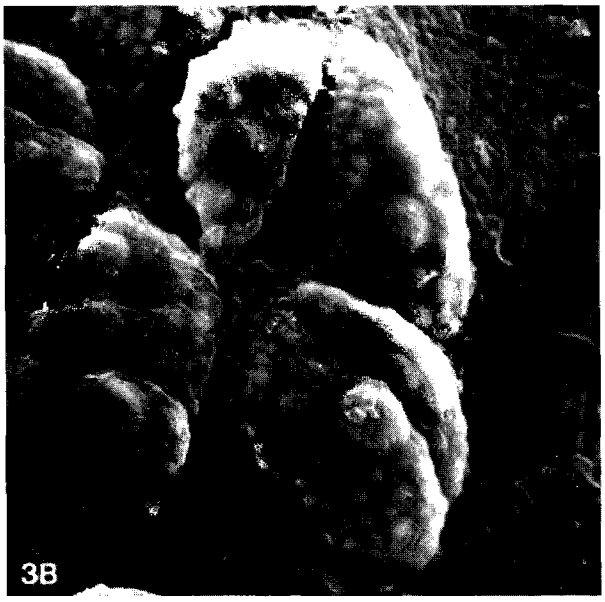


Fig. 2. Scanning electron micrograph of one week old adherent layer cells of a CH dog marrow culture. A) several spindle-shape fibroblast-like cells (F) × 225 B) higher magnification of flattened polygonal shape endothelial-like cells × 351. Several non-adherent cells are also shown.





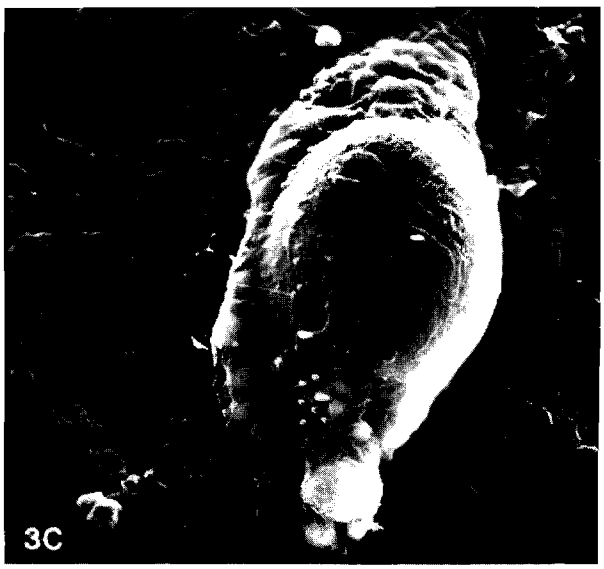
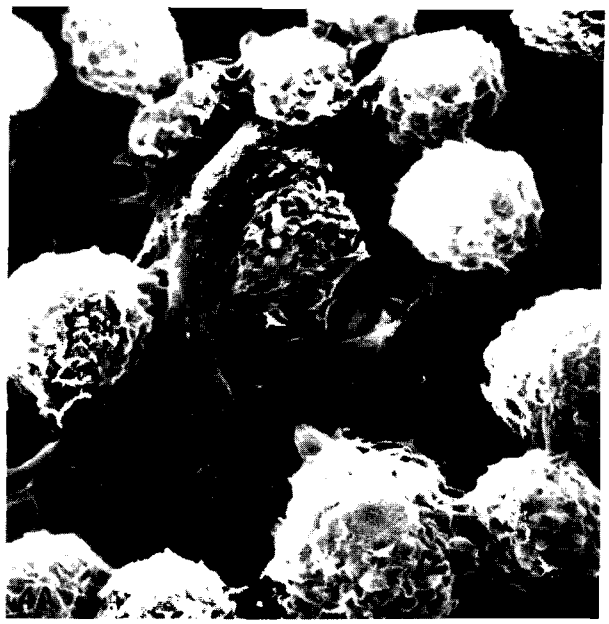


Fig. 3. Scanning electron micrograph of fat accumulation during the establishment of the adherent layer, A) two cells with multiple small fat inclusions are shown. × 1,040 B) Aggregate of fat-containing cells. × 1,170 C) Fat-containing cell with a large fat inclusion surrounded with small fat inclusions. × 1,620



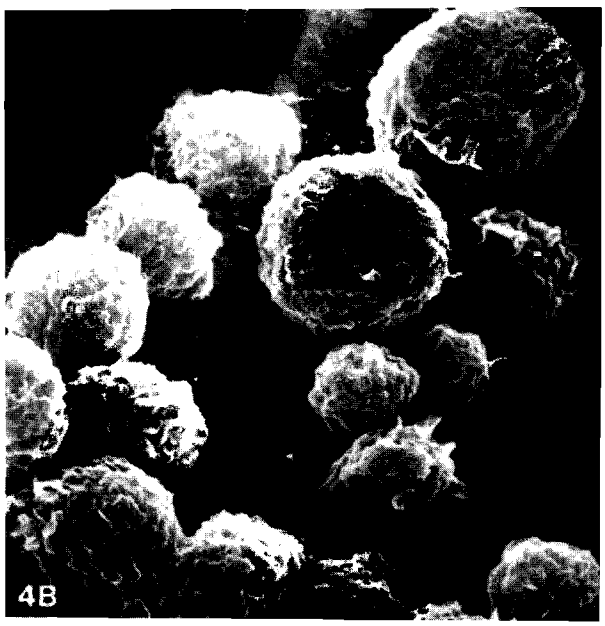


Fig. 4. Scanning electron micrograph of four week old adherent layer of CH dog culture A) shown are several macrophages either settled on the surface of other adherent layer cells or in between them. × 1,217 B) Culture with high proportion of mononuclear cells, showing undulating ruffled membrane with different sizes cells. × 1,123.

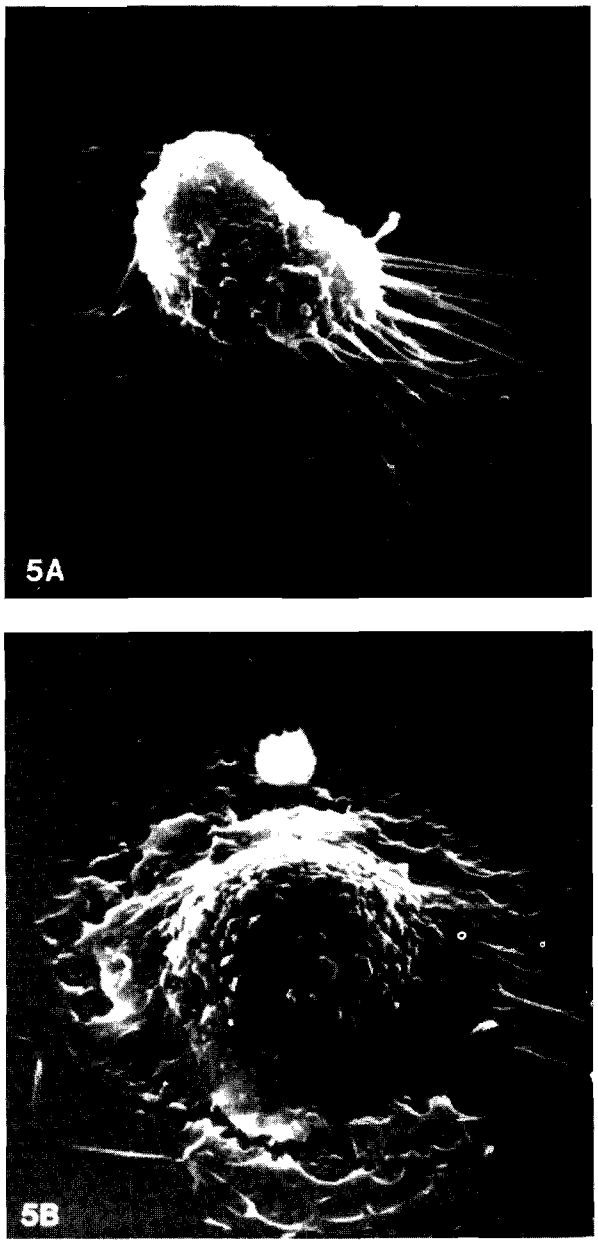


Fig. 5. Scanning electron micrograph of nonadherent mononuclear cells obtained from marrow culture of CH dog with a high proportion of mononuclear cells then settled on glass coverslip A) Cells which extended along a longitudinal axis with numerous cytoplasmic projections. × 1,037 B) Cell spreading in a radial manner. × 2,160

and 4) a fourth cell type, which developed over a period of 10 to 21 days, formed clusters, and was characterized by an accumulation of a lipid type material that stains with oil red 0 as previously described (Al-Lebban et al., 1987) (Figs. 3A-C). Fat started to accumulate in the form of small fat inclusions, which coalesced to form large fat droplets as the cells assumed an overall spherical shape (Fig. 3C). When the cultures lacked these fat-containing cells no CFU-GM recovered from the cultures. There were no morphological differences in the adherent layer

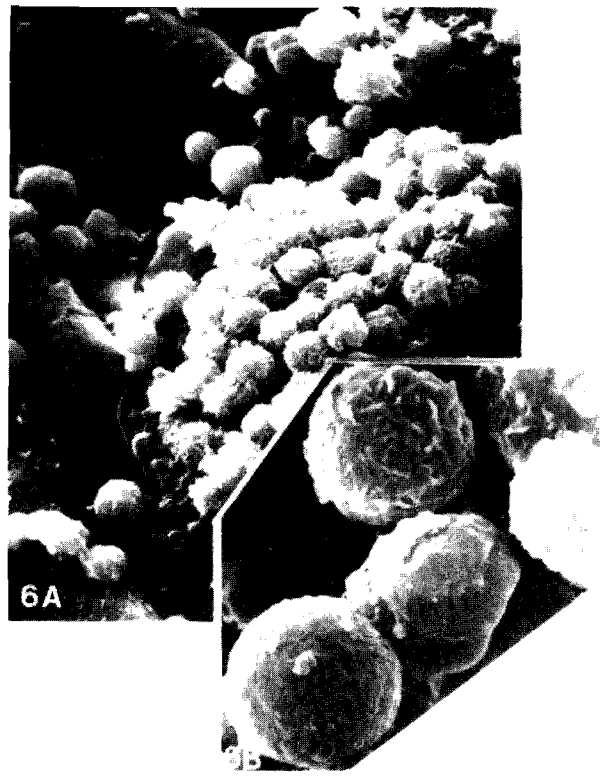


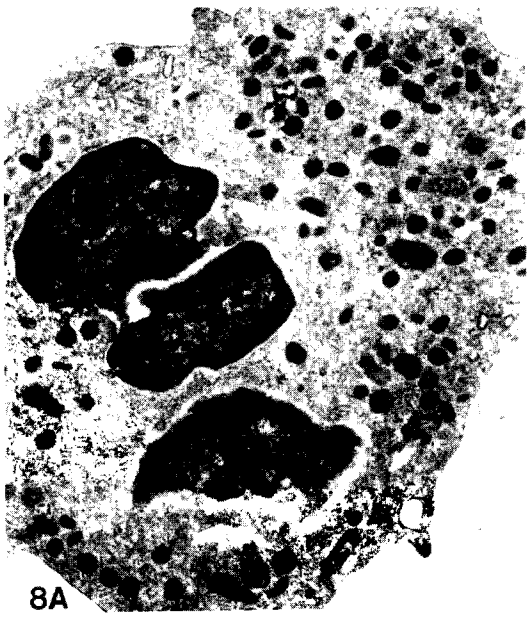
Fig. 6. A) Scanning electron micrograph of aggregates of granulocytes from long-term bone marrow culture of CH dog. The cells are at different stages of maturation as indicated by their varying surface morphology. The majority were mature as indicated by the sophistication of their surface. × 1,123 B) Immature cells with a more smooth surface. × 4,680

cells when bone marrow cultures of normal and CH dogs were compared.

Non-adherent cells

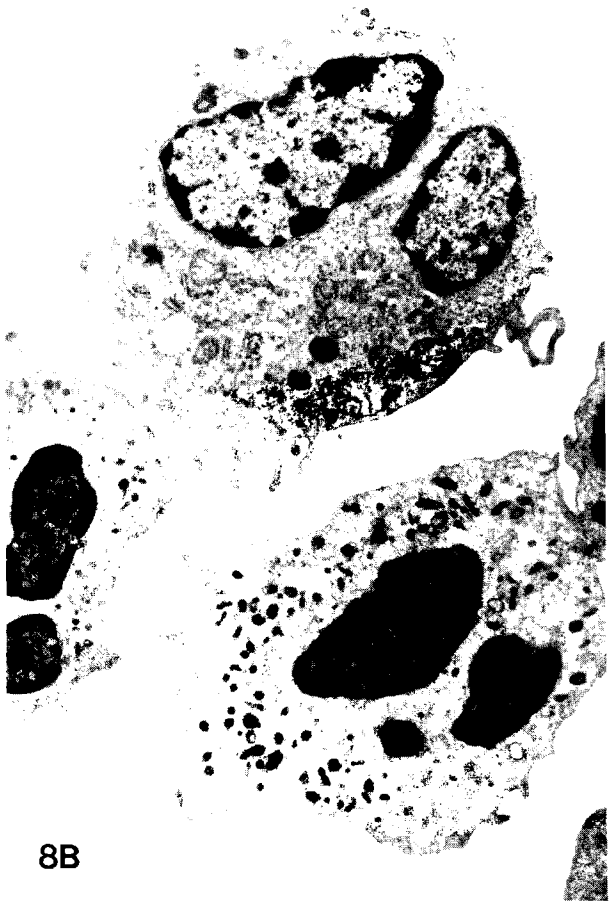
The non-adherent cells were comprised mostly of monocytes, immature and mature granulocytes. Monocytes of both normal and CH dogs settled and spread on the culture dishes (Fig. 4A). They were distributed over the entire culture with only a rare tendency for aggregation. The monocytes occured at varying proportions in different cultures (between 8 and 96%) depending on the culture age. Cells from cultures with a high monocyte proportion were allowed to settle on glass coverslips and prepared for SEM. The cells which attached tended to spread and flatten on the glass surface along a single longitudinal axis with a well marked leading edge and trailing retraction fibers (Fig. 5A), or in radial symmetry (Fig. 5B). However, when monocytes attached to the surface of other adherent layer cells, the monocytes remained spherical (Fig. 5A). Undulating ruffled membranous filopodia and an increase in size of the cells with time were observed (Fig. 4B).

7A



8A

7B



8B

Fig. 7. Transmission electron micrograph of a cell from bone marrow culture of (A) CH and (B) normal dogs to represent the morphology of myeloblast-promyelocyte stage in cultures. The nucleus contains condensed peripheral chromatin which is limited to a narrow margin, and one nucleolus. × 7,350

Fig. 8. Transmission electron micrograph of mature granulocytes. A) The nuclear segmentation which shows three nuclear profiles with largely condensed chromatin. × 15,750 B) The surface shows an elaboration of relatively long projections running in different directions. × 4,725.

Table 1. Morphology and CFU-GM potential of cells obtained from bone marrow cultures of normal and CH dogs.

Dog	Week #	Total cells $\times 10^5$	Total CFU-GM/ $\times 10^{5*}$	Differential %		
				Blast	Granulocytes	Monocytes
N	1	9.5 ± 0.6	46 ± 3.7	8 ± 2.5	72 ± 3.4	8 ± 2.4
	2	6.0 ± 0.3	38 ± 3.3	2 ± 1.2	68 ± 2.5	10 ± 2.5
	3	0.8 ± 0.2	23 ± 2.5	0	71 ± 3.8	18 ± 2.9
	4	0.3 ± 0	18 ± 2.5	0	53 ± 3.6	35 ± 4.4
CH	1	8.4 ± 0.3	43 ± 3.2	12 ± 2.7	75 ± 4.2	11 ± 2.9
	2	7.0 ± 0.3	34 ± 2.6	3 ± 1.1	66 ± 4.3	27 ± 3.1
	3	2.2 ± 0.1	25 ± 2.0	0	61 ± 3.4	31 ± 3.1
	4	0.6 ± 0.1	17 ± 3.8	0	48 ± 2.3	38 ± 2.4

The readings are the mean ± standard deviation of the specified parameters. All cultures were inoculated with 2×10^6 cells/ml growth medium. Cultures were incubated at 33°C, 4% CO_2 and at least five flasks were used per week for both normal and CH dogs.

* Weekly production of CFU-GM in long-term bone marrow cultures of normal and CH dogs.

Granulocytes had a spectrum of surface topography ranging from a relatively smooth surface to a surface covered with ridges and projections (Fig. 6A). When mature polymorphonuclear granulocytes settled on a cellular substratum, they retained their spherical shape and often exhibited a localized region of numerous petal-like projections characteristic of mature granulocytes (Fig. 6B). However, when granulocytes were allowed to settle in growth medium on a glass coverslip for 1 hr. at 37°C, a range of attachment, spreading pattern, and surface morphology were observed. Frequently, the cells attached and spread to an overall hemispherical shape with stubby projections that were more concentrated in the central region of the cell with extensive folds at the margins (Fig. 1A)

Transmission electron micrographs of promyelocytes from CH dog marrow cultures showed a nucleus with condensed peripheral chromatin that was limited to a narrow margin of the nucleus and contained a clear nucleolus (Fig. 7A). Similar morphology was observed with promyelocytes from marrow cultures of normal dogs (Fig. 7B). Mature granulocytes had nuclear segmentation, a granule population characteristic of normal circulating neutrophils, and increased elaboration of cell surface (Figs. 8A,B). Surface morphology and ultrastructural characteristics of culture-derived mature granulocytes were similar in both normal and CH dog marrow cultures.

Discussion

To separate genetic and acquired cellular defects in cyclic hematopoiesis, we compared surface and ultrastructural morphology of normal and CH dog bone marrow cells grown in long-term liquid culture. Adherent and nonadherent cells from both normal and CH dog marrow cultures had similar types and numbers of accessory and progenitor cells. Qualitative abnormalities were not observed in monocytes or granulocytes obtained from the CH marrow cultures, although ultrastructural abnormalities have been reported in studies of freshly collected CH marrow cells at different phases of the cycle. This finding indicates that the observed abnormalities (Scott et al., 1973; Machado et al., 1981; Parmley et al., 1984) are caused by some factors other than the genetic program of the CH neutrophils.

Ultrastructural characteristics of neutrophil granulocytes in grey collie dogs have been reported to vary according to the day of the cycle (Scott et al., 1973; Machado et al., 1981). Autophagy, secondary granule condensation, and abnormal glycogen accumulation are among the abnormalities that were seen in both cyclic hematopoietic dogs and human cyclic neutropenic patients (Scott et al., 1973; Machado et al., 1981 Parmley et al., 1984). These changes were not observed in granulocytes from the bone marrow cultures of CH dogs. It is likely, therefore, that factors which affect the bone marrow microenvironment in vivo are not present in our in vitro culture system. This suggests that the ultrastructural abnormalities previously reported in granulocyte lineage cells of CH dogs are secondarily acquired defects.

In a similar study of long-term cultures of Chediak-Higashi Syndrome (CHS) bone marrow cells (Newburger et al., 1985), it was reported that some of the granulocyte abnormalities that were seen in CHS patients such as increased neutrophil cAMP concentration and abnormal concanavalin- A capping were normal in culture derived CHS granulocytes. Newburger et al. (1985) suggested that some of the granulocyte abnormalities in CHS patients may be secondary manifestations of the disease, and were not an expression of the genetic program of the hematopoietic cell.

In conclusion, the results of the present studies of the ultrastructure and surface morphology of both normal and CH dog adherent and nonadherent cells are similar to murine and human marrow cells in long term cultures (Parakkal et al., 1974; Leake et al., 1975; Allen and Dexter, 1976b; Allen, 1978). The granulocyte abnormalities observed in CH dogs are likely to be a secondary to an altered in vivo microenvironment since the neutrophil abnormalities were not present in the in vitro marrow culture derived granulocytes.

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