Ultrastructural Morphology and Cytochemistry of Iron-Deficient Polymorphonuclear Leukocytes[1]

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Previous studies have documented decreased activities of certain enzymes and altered function in polymorphonuclear leukocytes (PMN) during iron deficiency. The present study was undertaken to determine if the enzymatic abnormalities could be correlated with morphologic or quantitative change in PMN granules. Ultrastructural examination of primary and secondary granules and assessment of the secondary granule components alkaline phosphatase and vicinal glycol-containing glycoconjugates was performed in rabbit bone marrow, peripheral blood, and peritoneal heterophils. In addition, biochemical quantifications of the secondary granule component alkaline phosphatase and the primary granule marker β-glucuronidase were performed. The results confirmed that a marked, significant decrease in alkaline phosphatase occurs in iron-deficient animals; however, no biochemical decrease in β-glucuronidase activity was observed. Ultrastructurally, PMN secondary granules of iron-deficient rabbits tended to be more numerous than in controls when examined with morphometric and glycoconjugate staining methods, but lacked staining in alkaline phosphatase preparations. These results demonstrate that iron-deficient rabbits produce normal to increased quantities of primary and secondary granules, despite a uniform deficiency of alkaline phosphatase, a secondary granule marker. © 1986 Academic Press, Inc.

INTRODUCTION

The bactericidal capacity of PMN is decreased in iron deficiency (Chandra, 1973; Yetgin et al., 1979). Hexose monophosphate shunt activation and reduction of nitroblue tetrazolium are decreased in iron-deficient rabbits and humans (Celada et al., 1979; Yetgin et al., 1979). Similarly, myeloperoxidase-mediated iodination is decreased in PMN from iron-deficient humans (Prasad, 1979). Myeloperoxidase activity has been reported to be normal (Yetgin et al., 1979) or decreased (Mackler et al., 1984; Sinha and Swarup-Mitra, 1981) in iron deficiency. A decrease in leukocyte alkaline phosphatase (LAP) has been demonstrated in iron-deficient rabbits (Celada et al., 1979). In some humans with iron deficiency, a decrease in PMN and serum lactoferrin has been reported (deVet and Ten Hoopen, 1978). The present study was undertaken to determine if morphological or quantitative decreases in PMN granules could be correlated with a decrease in granule constituents during iron deficiency.

MATERIALS AND METHODS

Preparation of animals. The principles of laboratory animal care as promulgated by the National Research Council were observed. New Zealand White rabbits (Myrtle's Rabbitry, Thompson Station, Tenn.) were designated randomly as either control animals ($n = 4$) or those to be made iron-deficient (FeD, $n = 5$).

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Throughout the experiments, control rabbits were fed a purified agar gel diet (Hunt and Harrington, 1974) and FeD rabbits were given a similar diet from which $FePO_4$ was omitted. These diets thus contained approximately 592 and 3 mg Fe/kg dry wt, respectively; these calculated results were confirmed by analysis of diet samples using atomic absorption spectroscopy (Perkin–Elmer Model 303, Perkin–Elmer Corp., Instrument Div., Norwalk, Conn.). Diets and demineralized deionized water were given *ad libitum*. All rabbits were individually housed in galvanized steel cages in a room with automatically controlled temperature (22°C) and lighting (12 hr). FeD rabbits were bled by cardiac puncture (78.4 ± 26.6 ml/kg of original weight) at intervals thereafter to produce iron deficiency. Neither control nor FeD rabbits were bled within 7 days of the termination of the experiment.

Marrow and blood samples. Initially, 10 ml of blood was removed by cardiac puncture from control ($n = 4$) and FeD ($n = 5$) animals for hematologic, light microscopic, and chemical determinations. Microhematocrits were determined by a capillary tube method. Serum iron concentrations and total serum iron-binding capacities were quantified using an automated bathophenanthroline technique (ACA II: DuPont Automated Clinical Analyzer, DuPont Systems Automatic Clinical Analysis Division, Atlanta, Ga.). Peripheral blood LAP was quantified using a standard method of staining and scoring (Okun and Tanaka, 1978). At the termination of the experiment, similar studies were repeated and, in addition, a heparinized blood sample (5 cc) taken by cardiac puncture was obtained for ultrastructural evaluation as described below. The rabbits were then killed by cervical dislocation and a sample of femoral marrow was obtained for ultrastructural studies.

Peritoneal wash samples. An additional set of control ($n = 2$) and FeD ($n = 4$) rabbits were similarly prepared for the obtainment of peritoneal wash heterophils for ultrastructural and biochemical studies. Iron deficiency was documented using microhematocrit, serum iron concentration, and total serum iron-binding capacity. After light sedation of rabbits with an intramuscular injection of Rompum (2 mg/kg) and Ketamine (20 mg/kg), the peritoneal cavity was injected with 150–200 ml of 0.9% saline containing 0.1% oyster glycogen (Sigma Chemical Co., St. Louis, Mo.). After 24 hr, the rabbits were heavily sedated with Rompum (10 mg/kg) and Ketamine (50 mg/kg) and the peritoneal cavity was injected with 200 ml of 0.9% saline with 1 unit/ml of heparin. A surgical incision was made, a catheter was inserted into the peritoneum, and the peritoneal fluid was collected by gravity. The cells were collected by centrifugation (1500g for 10 min) at 4°C. Some cells were then processed for electron microscopy as described below. Other samples were resuspended for 30 sec in a lysing solution consisting of 0.15 mM NH_4Cl, 0.01 mM $NaHCO_3$, and 1.0 mM ethylenediaminetetraacetic acid. The suspensions were centrifuged, and the cells were collected and rinsed twice in phosphate-buffered saline (PBS). The cells were divided into 1-ml aliquots in PBS containing $10^7–10^8$ cells each and frozen at $-20°C$. The samples were then processed for biochemical determination of β-glucuronidase (Michell *et al.*, 1970) and alkaline phosphatase (Bretz and Baggiolini, 1974).

Electron microscopy. Blood samples were collected in heparinized syringes and centrifuged in 1-ml glass tubes at 1500g for 3 min. The plasma was removed and the buffy coat overlaid with 3% glutaraldehyde/0.1 M cacodylate buffer, pH 7.35, at 4–10°C. After 10 min, the buffy coat was removed and minced in fixative

with a razor blade and allowed to fix an additional 50 min. Femoral marrow tissue was either directly minced in fixative and allowed to fix for 1 hr, or resuspended in PBS, centrifuged, and resuspended in fixative for 1 hr. Peritoneal wash cell pellets were fixed as a tissue pellet and minced in the fixative or resuspended in the fixative. All samples were then rinsed in 0.1 M cacodylate, 7 g/dl sucrose, pH 7.35.

In addition to routine morphologic studies, specimens were processed for alkaline phosphatase (Mayahara et al., 1967); a secondary granule constituent (Wetzel et al., 1967b); peroxidase (Graham and Karnovsky, 1966); a primary granule marker (Dunn et al., 1968); acid phosphatase (Barka and Anderson, 1967); a primary granule constituent (Wetzel et al., 1967b); and vicinal glycol staining after amylase digestion for localization of glycoprotein-rich secondary granules (Fittschen et al., 1983). All specimens except those processed for vicinal glycol staining were postfixed in 1% OsO_4, 0.1 M cacodylate. The specimens were then routinely dehydrated in graded alcohols and propylene oxide and embedded in Spurr low-viscosity medium.

Thin sections (50–70 nm) were obtained with a diamond knife and collected on copper grids for morphologic and cytochemical evaluation of content. Specimens processed for vicinal glycol staining were collected on stainless-steel grids for sequential incubation in periodic acid–thiocarbohydrazide–silver proteinate (PA–TCH–SP) as described previously (Fittschen et al., 1983). Thin sections were counterstained with uranyl acetate and lead citrate, and examined with a Philips 300 electron microscope at an accelerating voltage of 60 kV.

Methanolic uranyl acetate and lead citrate stained marrow late heterophils (containing two or more nuclear lobes with condensed nuclear chromatin) were sequentially photographed ($n = 10$ micrographs) from each of the first three rabbits studied in FeD and control groups. For morphometric studies, the micrographs (7 × 9 in format) were evaluated with a graf/Pen sonic digitizer connected to a Hewlett-Packard 9825 A calculator. The outline of each cell and its nucleus were traced with a sonic pen and the x and y coordinates of successive points were transmitted to a calculator, thus allowing calculation of cytoplasmic and nuclear area (Bishop and Drummond, 1979). In the point counting mode, the number of primary and secondary granules were enumerated. Primary granules were identified by their content of variably extracted osmiophilic material and large size (0.2–0.8 μm) and secondary granules by their homogeneous moderately dense content and generally smaller diameter (0.1–0.5 μm) (Bainton and Farquhar, 1966; Wetzel et al., 1967a). The granule counts were expressed as numbers of granules per square micrometer of cytoplasm. Although only the first three rabbits in each group were evaluated statistically for granule number, qualitative assessment of heterophil granules using cytochemical and morphologic techniques were performed on samples from all rabbits.

RESULTS

Hematologic Parameters

At the initiation of experiments, control rabbits ($n = 4$) weighed 2.49 ± 0.22 kg and those to be made iron-deficient ($n = 5$), 2.75 ± 0.36 kg (mean ± SD). Control and FeD rabbits were fed their designated diets for 30.0 ± 15.0 and 33.8 ± 12.1 days, respectively. Expressed as a percentage of initial weight, control

rabbits gained 9.5 ± 12.5% during the experiments and FeD rabbits lost 3.5 ± 6.7%. Moderately severe iron deficiency was produced in the FeD animals, indicated by decreased hematocrit and serum iron concentration and by elevated serum total iron-binding capacity (Table I). FeD rabbits also exhibited a significantly decreased LAP score and significantly increased reticulocyte percentage (Table I) as previously described in iron-deficient rabbits (Celada *et al.*, 1979).

Electron Microscopy

Morphology. Heterophils from bone marrow, peripheral blood, and peritoneal washes in FeD rabbits had similar or increased numbers of primary and secondary granules (Fig. 1, Table II) despite the observed decrease in alkaline phosphatase in FeD rabbits (Table I). Similarly, no significant difference in either cell or cytoplasmic area could be demonstrated (Table II). Primary granules in both animal groups varied from 0.2 to 0.8 μm in diameter and contained variable amounts of osmiophilic material which either diffusely filled the granule or appeared more prominent in the granule rim. In contrast to primary granules, secondary granules were less dense in unstained specimens, lacked significant osmiophilia, and measured 0.1–0.5 μm in diameter in both control and FeD rabbits. Methanolic uranyl acetate and lead citrate moderately and homogeneously stained secondary granules (Fig. 1). Although some vacuolization was seen in heterophils from FeD rabbits, morphologic differences in secondary granules could not be demonstrated in the FeD and control rabbits. Mitochondria were infrequently observed in control and iron-deficient rabbits. No vesiculation or abnormal membranes were observed in mitochondria from iron-deficient animals when compared to controls.

Cytochemistry. PA–TCH–SP staining of vicinal glycols in secondary granules was similar in all FeD and control specimens (Fig. 2). Immature primary granules in promyelocytes and myelocytes stained moderately whereas staining appeared much weaker in mature primary granules of late heterophils in FeD and control animals, although a tendency toward increased primary granule staining was observed in FeD animals. Alkaline phosphatase was markedly decreased or absent in FeD heterophils, whereas characteristic crescent staining of control heterophil secondary granules was observed (Fig. 3). Acid phosphatase stained immature primary granules in promyelocytes and myelocytes but appeared masked in the mature primary granules of segmented heterophils (Fig. 4). Tertiary granules varied from 0.1 to 0.2 μm in diameter and demonstrated homogeneous acid phosphatase staining. Generally less than three acid phosphatase-positive tertiary granules were observed per cell profile. Peroxidase normally stained most primary granules (Fig. 5).

Biochemical Data

A decrease in alkaline phosphatase was consistently observed in FeD (n = 4) peritoneal wash heterophils (0.061 ± 0.016 μmole/10^6 cells) in contrast to simultaneously prepared (n = 2) and nonsimultaneous controls (n = 10), which had values of 0.420 and 0.155, and 0.170 ± 0.033 $M/10^6$ cells, respectively (P < 0.001, Student's t test). β-Glucuronidase, a primary granule marker, was present in similar quantities in FeD and control animals with values of 0.512 ± 0.246 and 0.656 ± 0.141 OD/ml/10^6 cells, respectively.

TABLE I

Hematologic Parameters of Control and Iron-Deficient Rabbits

	Initial		Final	
	Control (n = 4)	Iron-deficient (n = 5)	Control (n = 4)	Iron-deficient (n = 5)
Microhematocrit (%)	35.5 ± 2.7	40.2 ± 4.1	38.3 ± 3.4	17.6 ± 3.2*
Serum iron concentration (µg/100 ml)	189.8 ± 68.6	193.8 ± 37.3	202.8 ± 75.4	51.2 ± 19.6**
Serum total iron-binding capacity (µg/100 ml)	330.0 ± 20.5	303.8 ± 43.1	394.0 ± 149.6	759.0 ± 81.9**
Saturation, serum total iron-binding capacity (%)	57.5 ± 20.3	65.8 ± 20.7	52.8 ± 17.0	6.7 ± 2.4*
Leukocyte alkaline phosphatase (LAP) score	156.5 ± 10.9	175.8 ± 29.4	182.5 ± 20.5	41.4 ± 14.3*
Reticulocytes (%)	—[a]	—	1.9 ± 0.1	17.2 ± 4.0*

[a] Not performed.

* Value of $P < 0.0005$ by comparison to controls at termination of experiment (Student's t test).

** Value of $P < 0.0025$ by comparison to controls at termination of experiment (Student's t test).
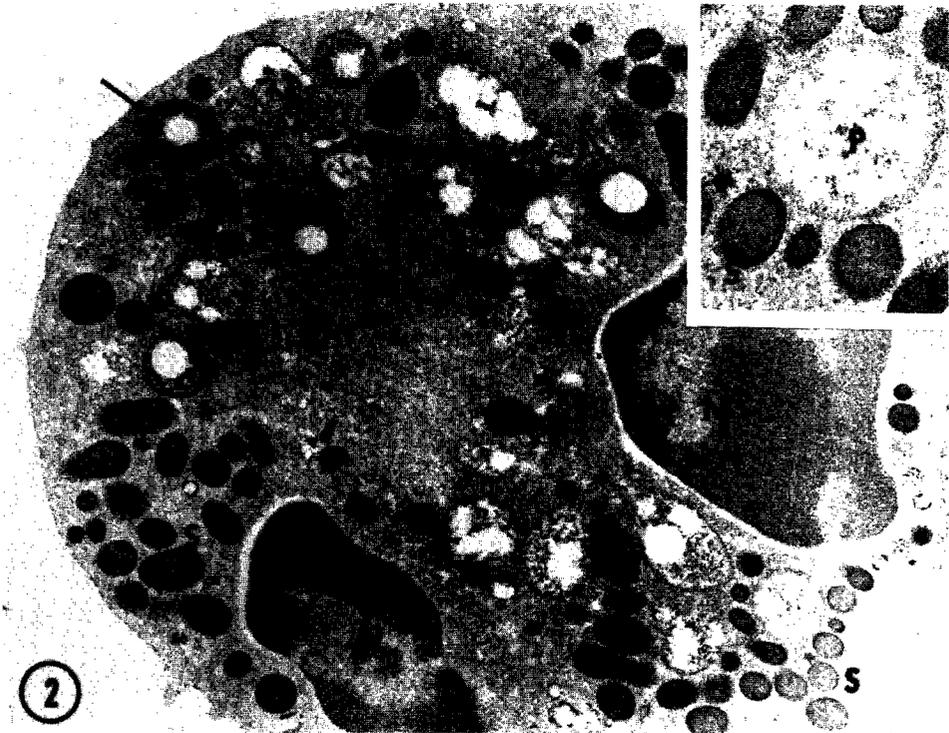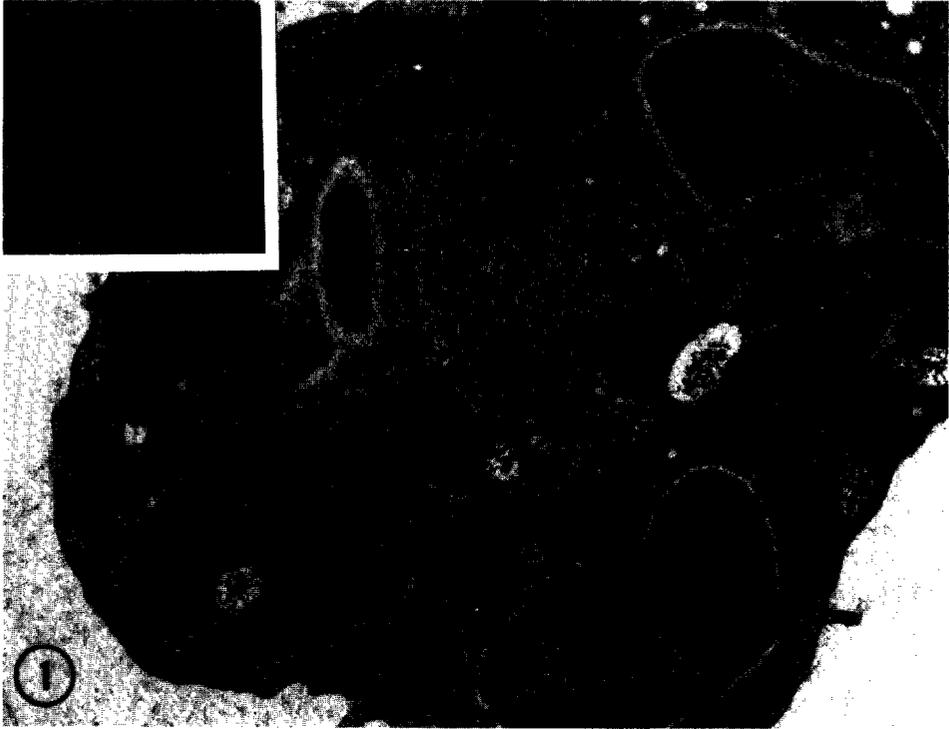
FIG. 1. Primary and secondary granules are abundant in this heterophil from an iron-deficient rabbit. Primary granules (P) are larger and demonstrate increased peripheral staining, whereas secondary granules (S) are smaller and stain homogeneously (enlarged in inset). Some primary granules appear extracted (arrows). Nuclei (N). Bone marrow specimen. Thin section stained with methanolic uranyl acetate and lead citrate. ×20,000; Inset ×50,000.

TABLE II
Heterophil Cytoplasmic Granules

	Primary granules per 10 μm² cytoplasm (Mean ± SD)	Secondary granules per 10 μm² cytoplasm (Mean ± SD)	Cell profile area in μm² (Mean ± SD)	Cytoplasmic area in μm² (Mean ± SD)
Iron-deficient rabbits[a]				
Rabbit 1 (n = 10)	8.2 ± 3.0	22.8 ± 4.9	26.1 ± 6.9	19.5 ± 5.8
Rabbit 2 (n = 10)	8.4 ± 1.8	31.9 ± 6.5	25.1 ± 3.8	17.1 ± 4.0
Rabbit 3 (n = 10)	4.8 ± 2.7	27.7 ± 6.2	27.9 ± 4.6	20.0 ± 4.5
Total (n = 30)	7.2 ± 3.0	27.4 ± 6.84*	26.4 ± 5.2	18.6 ± 5.2
Control rabbits				
Rabbit 1 (n = 10)	6.4 ± 2.7	17.4 ± 4.5	31.0 ± 7.8	21.1 ± 6.8
Rabbit 2 (n = 10)	5.5 ± 2.4	21.0 ± 7.0	28.9 ± 9.3	20.2 ± 5.7
Rabbit 3 (n = 10)	5.5 ± 2.5	17.8 ± 4.4	28.6 ± 5.0	21.7 ± 6.4
Total (n = 30)	5.8 ± 2.5	18.7 ± 5.5*	29.5 ± 7.4	21.0 ± 6.1

[a] Data derived from 10 consecutive and random micrographs of heterophils from each rabbit marrow specimen with $n = 3$ rabbits in FeD and $n = 3$ in control groups. Each heterophil profile contained at least two nuclear profiles with predominantly condensed nuclear chromation.
* P value <0.001 by Student's t test.

DISCUSSION

The present study demonstrates that despite the acquired deficiency of secondary granule enzymes induced by iron deficiency, a decrease in granule number and size does not occur. The decrease in alkaline phosphatase consistently observed in these cells is similar to that previously reported for iron-deficient heterophils (Celada et al., 1979) and extends those results to the ultrastructural level. The decrease in enzyme activity cannot be attributed to a general decrease in lysosomes, but to a selective decrease in specific enzymes. In humans, neutrophil alkaline phosphatase has been localized in a vesicle fraction (Borgers et al., 1978; Wilson et al., 1981), rather than secondary granules as in rabbit heterophils (Bainton and Farquhar, 1968; Wetzel et al., 1967b), but its deficiency is frequently associated with abnormalities or numerical deficiencies of secondary granules (Parmley et al., 1980a; Strauss et al., 1974). The present study demonstrates that a deficiency of alkaline phosphatase is not necessarily associated with a decrease in number of secondary granules. The crescent distribution of alkaline phosphatase in normal control heterophil secondary granules is similar to that in previously reported studies (Bainton and Farquhar, 1968; Wetzel et al., 1967b). The marked decrease in ultrastructural staining of alkaline phosphatase in iron deficiency is consistent with light microscopic and biochemical documentation of decreased akaline phosphatase in these cells from FeD rabbits. This decrease in activity is present in bone marrow, peripheral blood, and tissue compartments.

Vicinal glycol content, assessed using Thiery's PA–TCH–SP method, does not appear altered in iron-deficient secondary granules in the present study. Secondary granules are known to have a large amount of glycoprotein (Noseworthy et al., 1975), which presumably represents the predominant staining with this

FIG. 2. PA–TCH–SP intensely stains vicinal glycol groups in secondary granules (S) of this iron-deficient rabbit (enlarged in inset). Mature primary granules (P) stain weakly whereas stronger irregular staining is evident in immature primary granules (arrows), some of which appear extracted or vacuolated. The staining of FeD primary granules appears slightly increased when compared to control rabbits. Nuclei (N). Bone marrow specimen. ×18,000; inset ×45,000.
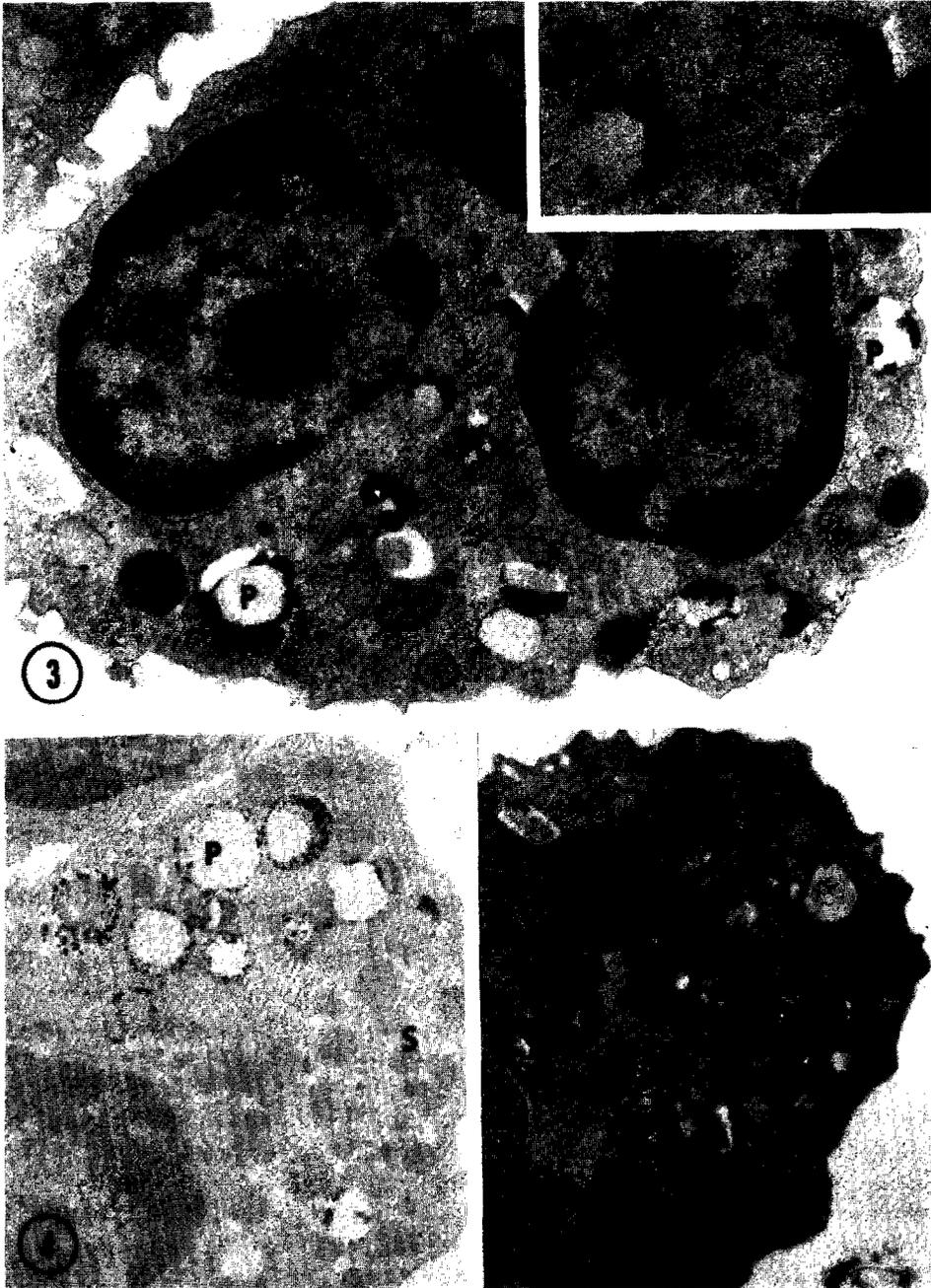
FIG. 3. This metamyelocyte from a control rabbit demonstrates typical, crescent distribution of alkaline phosphatase staining in several secondary granules (S, enlarged in inset). Primary granules (P) lack staining. Staining was markedly decreased or absent in iron-deficient rabbits. Bone marrow specimen, not counterstained. ×20,000; inset ×45,000

FIG. 4. Acid phosphatase reactivity is present in a diffuse or rim pattern in primary granules (P) of a myelocyte from this iron-deficient rabbit (similar to control rabbits). Secondary granules (S) lack staining. Bone marrow specimen. Thin section not counterstained. ×15,000.

FIG. 5. Primary granules (P) from this iron-deficient rabbit demonstrate myeloperoxidase reactivity. Although some granules appear to be less reactive, similar variability was seen in control rabbits. Secondary granules (S) lack staining. Nucleus (N). Bone marrow specimen. Thin section not counterstained. ×13,000.

method. Vicinal glycol staining does appear to be relatively specific for human neutrophil and rabbit heterophil secondary granules (Fittschen *et al.*, 1983; Parmley *et al.*, 1980b). The normal-to-increased quantities of positively stained granules further confirms morphologic observations in iron deficiency.

Primary granules of iron-deficient heterophils also do not appear to be decreased in number in comparison to control cells. This is consistent with the relatively normal amount of β-glucuronidase observed in these cells in this study and the presence of acid phosphatase and peroxidase observed cytochemically. Previous studies have suggested that some deficiency in heme-containing myeloperoxidase may be present (Mackler *et al.*, 1984; Sinha and Swarup-Mitra, 1981). Our failure to document this with ultrastructural benzidine staining may be related to the increased sensitivity of the technique, which results in intense staining even when small amounts of heme are present. The present study demonstrates that if enzymatic deficiency is present in these primary granules, the deficiency is not expressed by a decrease in granule number.

The tendency of increased granules per unit area observed in iron-deficient animals may be comparable to an increase in the hepatocyte mitochondrial compartment (although largely due to an increase in organelle size) in iron-deficient animals despite a decrease in their cytochrome enzymes (Dallman and Goodman, 1971). The finding in heterophils conceivably could be a secondary phenomenon related to either marrow environment or hypercellular marrow activity. Alternatively, an increase in granules may represent a compensatory mechanism to increase granule number when granules are produced with deficient constituents.

Although decreases in granule number and size were not observed, some cytoplasmic vacuoles were observed. However, even this appeared relatively subtle. Previous ultrastructural and biochemical studies of iron-deficient hepatocytes have shown an increase in mitochondrial vesiculation and a decrease of cytochromes A and C (Dallman and Goodman, 1971). The paucity of mitochondria in control and iron-deficient heterophils and the relatively short life span of heterophils may account for the lack of a noticeable involvement of this organelle in heterophils.

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