Retinol Is Sequestered in the Bone Marrow of Vitamin A–Deficient Rats\textsuperscript{1,2}

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ABSTRACT Retinoic acid bound to the nuclear retinoic acid receptor-\textalpha{} is required for the differentiation of promyelocytes to mature neutrophils. However, severely vitamin A–deficient rats have normal numbers of neutrophils in the blood and inflamed tissues. This paradox was explored using four dietary groups of rats: 1) vitamin A–deficient rats; 2) vitamin A–deficient rats subsequently receiving vitamin A; 3) weight-matched pair-fed rats; and 4) nonrestricted, vitamin A–complete diet–fed rats. Plasma and liver retinol concentrations of the vitamin A–deficient rats were \textlt{}1% of those of the other three groups. In contrast, the bone marrow retinol concentrations of the vitamin A–deficient rats were fourfold higher than those in the other three groups. The distribution of myeloid-derived cells in the bone marrow was similar in all four groups of rats with the exception of a significantly greater (\textit{P} < 0.05) occurrence of hypersegmented neutrophils (six or more lobes) in the vitamin A–deficient rats (2.1%) relative to the control groups (0–0.1%). The blood of the vitamin A–deficient rats also contained significantly higher numbers (\textit{P} < 0.01) of hypersegmented neutrophils (67%) relative to those in the control groups (2–7%). The hypersegmentation of the neutrophils in this group of rats was not due to a concurrent deficiency of vitamin B\textsubscript{12} or folate. The importance of bone marrow–derived cells to the survival of the animal is suggested by retinol sequestration in the bone marrow of vitamin A–deficient rats, allowing the differentiation of myeloid cells to neutrophils. J. Nutr. 126: 1618–1626, 1996.

INDEXING KEY WORDS:
\begin{itemize}
  \item vitamin A deficiency
  \item bone marrow
  \item neutrophil hypersegmentation
  \item rats
\end{itemize}

Retinoids are a family of molecules having a broad range of physiological functions. In the liver and some extrahepatic tissues, retinol (vitamin A) is stored in the ester form [Blaner and Olson 1994]. Retinol can be reversibly oxidized to retinaldehyde, which can then be irreversibly oxidized to retinoic acid. Retinol, retinaldehyde and retinoic acid can be metabolized to other retinoid forms. Some of the best-studied functions of retinoids include visual transduction, maintenance of epithelial cells, growth and development, viability of lymphocytes and the differentiation of myeloblastic cells to neutrophils [Buck et al. 1991, Garbe et al. 1992, Gratas et al. 1993, Gudas et al. 1994, Robertson et al. 1992, Saari et al. 1994, Tsai and Collins 1993].

In bone marrow, multipotent stem cells differentiate to either myeloid or lymphoid stem cells [Edwards 1994b]. The myeloid stem cell can then differentiate to four types of cells [megakaryocyte progenitor, erythroid progenitor, monoblast and myeloblast]. These cells can further divide and differentiate to eventually become mature cells [platelets, erythrocytes, monocytes and polymorphonuclear leukocytes [eosinophils, neutrophils or basophils]] that are released into the blood stream. The neutrophil is derived from the myeloblast, which matures sequentially into the promyelocyte, myelocyte, metamyelocyte, band cell and finally to the mature segmented neutrophil [Bainton 1980]. In neutrophils, synthesis of individual proteins occurs at specific times during the maturation of these cells [Borregaard et al. 1993 and 1995].

Neutrophil functions as the first line of defense against infections. Their major function is to find and destroy microorganisms. People and animals with neutropenia or a deficiency of one or more key neutrophil enzymes are more susceptible to infections that can be life threatening. Examples of diseases due to abnormal

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neutrophil function are chronic granulomatous disease in which neutrophils do not mount a respiratory burst, myeloperoxidase deficiency, specific-granule deficiency, myelodysplastic syndrome and CD11/CD18 leukocyte glycoprotein deficiency (Edwards 1994a).

Retinoids are involved in the differentiation of myeloid cells into neutrophils. Myeloid leukemic cell lines such as HL-60 and teratocarcinoma P9 cells differentiate to a neutrophil-like cell upon exposure to retinoic acid (Gratas et al. 1993). In cultures of normal human bone marrow cells, all-trans-retinoic acid, 13-cis-retinoic acid and 4-oxo-all-trans-retinoic acid enhanced the formation of granulocytic colonies and macroclusters while decreasing the number of macrophagic colonies (Gratas et al. 1993). Besides these retinoids, 9-cis-retinoic acid (Kizaki et al. 1993) and retinol (Eppinger et al. 1993) also induce differentiation in HL-60 cells.

A retinoid requirement for myelocytic cell differentiation to neutrophils was demonstrated in experiments in which one of the retinoic acid receptor (RAR)^4 genes or a dominant negative retinoic acid receptor α (RARα) construct was transduced into myeloid lineage cells. Transduction of RARα, RARβ, RARγ or RXXα genes into retinoic acid–resistant HL-60 cells restored retinoic acid sensitivity to these cells (Robertson et al. 1992). These experiments showed that several retinoic acid receptors can mediate the differentiation of myelocytes to neutrophils. However, the major retinoic acid receptor found in hemopoietic cells is RARα. The importance of RARα in neutrophil differentiation was shown by transduction of a dominant negative RARα construct into both normal mouse bone marrow cells and the multipotent interleukin 3–dependent murine hematopoietic cell line FDCP mix A4 (Tsai and Collins 1993). After transduction, the differentiation of both cell types was blocked at the promyelocyte state.

The requirement of retinoids for the differentiation of promyelocytes to neutrophils suggests that vitamin A–deficient humans and animals would be neutropenic. However, neutrophils are present in the blood of vitamin A–deficient animals (Hayashi et al. 1989, Twining et al. 1985) and humans (Sommer et al. 1982), and they are capable of responding to infections. This suggests retinoids are present in the bone marrow of vitamin A–deficient animals and humans. The purpose of this study was to determine whether the bone marrow of vitamin A–deficient rats contained retinoids and to examine the morphology of the neutrophils produced.

MATERIALS AND METHODS

**Materials.** Unless otherwise stated, chemicals and enzymes were obtained from Sigma Chemical (St. Louis, MO).

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4 Abbreviations used: A−, vitamin A–deficient rats; A+, weight-matched pair-fed control rats; N, nonrestricted vitamin A–complete diet rats; R, vitamin A–deficient rats subsequently receiving vitamin A; RAR, retinoic acid receptor; RXR, retinoic acid X receptor.

<table>
<thead>
<tr>
<th>Table 1 Components of autoclavable casein-based vitamin A–deficient and vitamin A–complete diets</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet ingredient</strong></td>
</tr>
<tr>
<td>Vitamin A–deficient</td>
</tr>
<tr>
<td>Vitamin-free test casein</td>
</tr>
<tr>
<td>Cornstarch</td>
</tr>
<tr>
<td>Cottonseed oil</td>
</tr>
<tr>
<td>Cellulose</td>
</tr>
<tr>
<td>AIN-76 mineral mix²</td>
</tr>
<tr>
<td>Vitamin mixture³</td>
</tr>
<tr>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>DL-Methionine</td>
</tr>
<tr>
<td>Ethoxyquin (antioxidant)</td>
</tr>
<tr>
<td>Vitamin A–complete</td>
</tr>
<tr>
<td>All of the above components</td>
</tr>
</tbody>
</table>

1 Supplied by Harlan-Teklad (Madison, WI).
2 The AIN-76 mineral mix contained the following (g/kg diet): calcium phosphate dibasic, 17.5; chromium-potassium sulfate·12H2O, 0.019; cupric carbonate, 0.011; ferric citrate, 0.21; magnesium oxide, 0.84; manganous carbonate, 0.12; potassium citrate monohydrate, 7.7; potassium iodate, 0.00035; potassium sulfate, 1.8; sodium chloride, 2.6; sodium selenite pentahydrate, 0.00035; zinc carbonate, 0.056.
3 The vitamin mixture contained the following (mg/kg diet): p–aminobenzoic acid, 165; ascorbic acid, 1487; biotin, 0.66; cholecalciferol, 0.0825; choline, 2150; folic acid, 10; menadione, 75; niacin, 149; pantothenate, 91; pyridoxine 27; riboflavin, 33; thiamine HCl, 83; all–rac–a–tocopherol, 160; vitamin B12, 45.

**Rats.** WAG/Rij/MCW rats were derived from animals established in 1970 at Yale University using rats supplied by H. R. Reinhold (Radiological Institute TNO, Rijswijk, The Netherlands). These rats were associated with a restricted number of bacterial species (Enterococcus, Enterobacter cloacae, Escherichia coli, Bacillus sp., Providence rettgeri, Staphylococcus epidermis, Staphylococcus aureus, and Streptococcus faecalis) but not to mycoplasma or viruses when the colony was established at the Medical College of Wisconsin in 1978. For the current experiments, representative rats from this colony were tested for bacteria, mycoplasma and viruses by standard microbiological tests. These tests confirmed the presence of the associated bacteria and ruled out the presence of other bacteria, viruses and mycoplasma. An autoclavable casein-based vitamin A–deficient diet (Table 1) was developed for these rats by Ken Rose (Harlan-Teklad, Madison, WI). This diet was formulated to provide a margin of safety for the vitamins due to the autoclaving process and the stress of the vitamin A deficiency. The control diet consisted of the vitamin A–deficient diet supplemented with retinyl palmitate (15 mg/kg diet). Nursing females were fed the autoclavable casein-based vitamin A–deficient diet beginning 16 d postpartum. At 21 d of age, the weaned male pups were divided into four dietary groups and fed one of the following diets for 79
d: 1) 12 rats (A−) were fed the casein-based retinoid-deficient diet. 2) six rats (R) were fed the retinoid-deficient diet until d 63 of the study (16 d before they were killed). On that day, the rats were fed 500 μg of retinyl palmitate in 200 μL of safflower oil and then given free access to the retinyl palmitate–supplemented diet for the next 16 d. 3) 12 rats (A+) were restricted in their intake of the retinyl palmitate–supplemented diet so that their weight gain matched that of the A− rats. 4) 11 nonrestricted rats (N) were given free access to the retinyl palmitate–supplemented diet. All rats were weighed every 3 or 4 d to monitor the progress of the deficiency.

The completeness of the retinyl palmitate–supplemented diet was shown by successful breeding of a separate group of four pairs of WAG/Rij/MCW rats raised on the diet. The rats delivered normal healthy pups as determined by autopsies and microscopic examination of the organs by the staff veterinarian.

On the last day of the experiment (47-99 of dietary treatment), each rat was anesthetized with methoxyflurane and bled by heart puncture into a 10-mL syringe containing 100 U of sodium heparin (Elkins-Sinn, Cherry Hill, NJ) in 100 μL of PBS (4.36 mmol/L Na2PO4, 1.46 mmol/L K2HPO4, 2.68 mmol/L KCl and 1.55 mmol/L NaCl, pH 7.4). The blood was used to prepare blood cell smears. The animals were then killed using an overdose of pentobarbital (Abbott Laboratories, North Chicago, IL).

Bone marrow was harvested from the major femur and tibia. The bones were removed from the rats and dissected free of soft tissue. The epicondyle was removed, and the bone marrow cells were extracted using a syringe containing 10 mL of PBS. These cells were dissociated by flushing up and down through the syringe needle. A hemocytometer was used to count the cells, and trypan blue (Allied Chemical, Morristown, NJ) exclusion was used to determine cell viability.

Livers were removed and stored at −80°C until extracted for retinoids. All procedures were performed under yellow light to preserve the retinoids. All samples were stored in foil or in yellow tubes for protection from light.

The rat protocols used in these experiments were approved by the Animal Care Committee of the Medical College of Wisconsin and complied with the Guide for the Care and Use of Laboratory Animals (NRC 1985).

**Bone marrow, liver and serum retinoid concentrations.** Serum retinoids were extracted using hexane [Sigma-Aldrich Chemical, Milwaukee, WI] in the presence of 5.7 mmol/L ascorbic acid [J. T. Baker, Phillipsburg, NJ] (Driskell et al. 1985). Bone marrow cells were homogenized, and the retinoids in the homogenate were extracted in the same manner as the plasma samples. The entire liver was weighed, homogenized, saponified and extracted using hexane in the presence of 80 mmol/L pyrogallol [Nauss et al. 1985, Thompson et al. 1971]. As an internal standard, retinyl acetate was added to all samples prior to extraction. The samples were solubilized in ethanol [Quantum Chemical, Tuscola, IL] and separated by HPLC [Dionex, Sunnyvale, CA] on a Whatman (Hillsboro, OR) EQC 125A C18 column (4.6 mm i.d. times 108 mm) using a linear gradient of methanol-H2O (90:10, v/v) and ethyl acetate [Fisher Scientific, Pittsburgh, PA] plus isopropanol [Baxter Diagnostic, McGraw Park, IL] (90:10, v/v) at a flow rate of 1.0 mL/min [Rudy et al. 1992]. The chromatography was monitored at 325 nm or 295 nm. A mixture of standards containing retinoic acid [J. T. Baker], retinol [J. T. Baker], retinyl acetate, α-tocopherol and retinyl palmitate was added to some bone marrow extracts to determine coelution of retinoids and α-tocopherol.

**Protein determination.** Proteins extracted from bone marrow were quantified by the method of Lowry et al. (1951). Bovine serum albumin [Intergen Company, Purchase, NY] was used as the protein standard.

**Differential cell counts.** Bone marrow and blood cell smears were prepared by the hanging droplet technique. The cells were stained with LeukoStat [Fisher Diagnostics, Orangeburg, NY]. Because the cytospin technique can induce artificial hypersegmentation, cell smears were used for these experiments to distribute bone marrow and blood cells. During counting, the identity of the animal group was unknown to the experienced cell counter. Bone marrow and blood leucocytes were classified based on shape and staining of the nuclei, cytoplasm and granules.

**Folate and vitamin B-12 quantification.** The Quantaphase II B-12/Folate Radioassay [Bio-Rad Laboratories, Hercules, CA] was used to determine vitamin B-12 (cobalamin) and folate concentrations in plasma samples from the A−, R, A+ and N rats. This is a competitive assay using [57Co]cobalamin and 125I-labeled folate. These labeled molecules compete with those in the samples for sites on their respective binding proteins immobilized on beads. The beads were pelleted, the supernatant fraction was removed, and the beads were counted on a gamma counter [Packard Instrument, Meriden, CT].

**Statistical analysis.** Statistical analysis of the data for overall differences was performed using SigmaStat statistical software [Jandel Scientific Software, San Rafael, CA]. Either the one-way ANOVA or the Kruskal-Wallis ANOVA [Kruskal and Wallis 1952] was used to determine whether an overall difference existed among the A−, R, A+ and N groups. The Kruskal-Wallis ANOVA was used when variances were unequal among groups or when the data did not pass the normality test. Multiple comparisons were performed using the two-tailed Scheffé comparison procedure [Scheffé 1953] except where the data did not pass the normality test. In this case, Dunn’s multiple comparison of ranks [Dunn 1961] was used.
TABLE 2

Plasma and total liver retinol concentrations of vitamin A–deficient and control rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Plasma retinol</th>
<th>Liver retinol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>concentrations</td>
<td></td>
</tr>
<tr>
<td>A−</td>
<td>12</td>
<td>&lt;4a</td>
<td>1.20 ± 0.38a</td>
</tr>
<tr>
<td>R</td>
<td>6</td>
<td>500 ± 88c</td>
<td>367 ± 64b</td>
</tr>
<tr>
<td>A+</td>
<td>12</td>
<td>330 ± 76b</td>
<td>310 ± 89b</td>
</tr>
<tr>
<td>N</td>
<td>11</td>
<td>479 ± 64c</td>
<td>312 ± 60b</td>
</tr>
</tbody>
</table>

1 Values are means ± SD. An overall difference was shown among the groups using the Kruskal-Wallis analysis of variance on ranks [Kruskal and Wallis 1952]. The Scheffé Method [Scheffé 1953] was used to determine individual differences between the groups. P < 0.001 for a vs. b or c. P < 0.01 for b vs. c.

2 Group abbreviations: A−, vitamin A–deficient rats; R, vitamin A–deficient rats then fed the diet containing vitamin A; A+, weight-matched pair-fed control rats; N, nonrestricted, vitamin A–complete diet rats.

RESULTS

Vitamin A–deficient rats (A−) were used in this study in the late weight plateau stage of the deficiency (98 to 105 d of age). For the animals used in these experiments, the onset of weight plateau (failure to gain weight) occurred at 63 ± 3 d after birth. The final weight of the A− rats at 100 d of age (79 d of consuming the diet) was 196 ± 13 g. The final average weights of the R rats, the A+ rats and the N rats at 100 d of age were 226 ± 6, 186 ± 21 and 260 ± 14 g, respectively.

Comparison of the plasma and liver retinol concentrations among the four groups confirmed that the A− rats were severely vitamin A deficient (Table 2). The plasma retinol concentrations of the A− rats were less than 1% of those of the N rats. The liver retinol concentration in the A− rats was approximately 0.4% that for the N rats. The 16-d retinyl palmitate repletion period for the R group of formerly A− rats was sufficient for restoration of normal liver and plasma retinol concentrations. Although the plasma retinol concentrations of the A+ rats were lower than that of the N rats (P < 0.05), they were still much higher than those of the A− rats (P < 0.001). Because the liver retinol concentrations of the A+ and N rats were similar, the lower plasma retinol concentrations in the A+ rats probably reflect either lower amounts of the plasma retinol-binding protein or a lower amount of total retinol from the diet due to the restriction of the diet.

HPLC analysis was used to determine whether the retinoid content of bone marrow cells from the A− rats was also <1% that of the control dietary groups. At 325 nm, a peak was observed in the chromatogram that eluted with the same retention time as retinol (Fig. 1). To confirm that this peak was retinol, the bone marrow samples were spiked with retinol and other standard retinoids (Fig. 1A). The 325 nm peak assigned to retinol coeluted with the retinol standard (Fig. 1A) and its absorbance decreased at 295 nm (Fig. 1B), suggesting the peak was a retinol-like molecule. The retinol concentration in the bone marrow of A− rats was approximately fourfold greater than the amount found in the N rats (Table 3). This may be a result of the protein malnutrition observed in vitamin A deficiency due to lower than normal intake of food. This possibility was eliminated because the retinol concentration in the bone marrow of the weight-matched pair-fed A+ rats, which were also protein malnourished, was not significantly different from that of the N rats. The bone marrow of R rats contained amounts of retinol similar to those observed in the N and A+ rats, suggesting that the elevated concentrations of retinol in the A− rats were reversible.

Several other peaks were noted in the chromatograms at 325 nm [Fig. 1A and B, top]. The first peak, which eluted much earlier than retinol, eluted at the same time as retinoic acid. Unlike retinoic acid, however, this peak at 295 nm was approximately 30% greater in intensity. This indicated that the peak may not contain a retinoid, may be an unsaturated form of retinoic acid, or may be a mixture of retinoic acid plus a compound that absorbs at 295 nm to a greater extent than at 325 nm. The third peak of the chromatograms was the internal standard retinyl acetate. A fourth peak eluted in the retinyl ester portion of the chromatogram but had the properties of α-tocopherol rather than a retinyl ester. The peak coeluted with an α-tocopherol standard and its absorbance was greater at 295 nm than at 325 nm (Fig. 1B) to the same degree as observed for the standard. The α-tocopherol concentrations in the bone marrow of the variously treated rats were not dependant on their retinoid status (Table 3). The N rats had the lowest amounts of α-tocopherol and the R rats the highest amounts, with the A− and A+ rats having intermediate concentrations. These results suggest retinol is specifically deposited or retained in the bone marrow of the A− rats.

The effect of these higher retinol concentrations on bone marrow cell populations was studied. An evaluator who was unaware of treatment groups identified and tabulated the myeloid-type cells in bone marrow. The bone marrow of the four groups of rats contained the same relative number of total neutrophils, eosinophils, basophils, monocytes and mast cells (Table 4). The mature neutrophils, found in bone marrow and blood, were divided into three groups of cells: mobius, normal and hypersegmented (Fig. 2). Mobius neutrophils appeared as band cells having condensed unsegmented nuclear material. Normal neutrophils were defined as cells with three to five lobes and the hypersegmented neutrophils as cells with six or more lobes. The only significant difference (P < 0.05) observed in the neutrophil lineage cell populations from the bone marrow of the four experimental groups was the presence of hypersegmented neutrophils in the A− rats (Table 4). Nine of the 10 samples from A− rats contained hypersegmented neutrophils (range 1 to 27 per 300 my-
eloid lineage cells). In contrast, only two of the 6 bone marrow samples from A+ rats contained one hypersegmented neutrophil out of 300 cells. In the other two control groups, the N and R groups, no hypersegmented neutrophils were observed.

The numbers of the three types of mature neutrophils (mobius, normal and hypersegmented) were determined in blood from the rats (Fig. 3). The total numbers of mature neutrophils were similar in the four groups: 4.86 ± 2.12 times 10^6 for the A− rats, 3.05 ± 1.56 times 10^6 for the R rats, 4.94 ± 1.68 times 10^6 for the A+ rats and 4.38 ± 2.74 times 10^6 for the N rats. However, the number of hypersegmented cells relative to total mature cells was very different in the blood of A− rats compared with the control (N) animals. In the blood of the A− rats, 67% of the neutrophils were hypersegmented compared with 2 to 7% for the R, A+ and N rats (Fig. 2). In addition, only 15% of the mature neutrophils were normal (three to five segments) in the A− rat blood compared with 66−84% in the R, A+ and N rat blood. No differences were noted in the numbers of circulating mobius type cells among the groups.

One possible reason for the hypersegmentation of the A− rat neutrophils would be a concurrent deficiency of folate or vitamin B-12 due to intestinal malabsorption. To explore this possibility, the concentrations of these vitamins were measured in plasma (Fig. 4). No significant differences were observed among groups for the folate concentrations. The plasma vitamin B-12 concentration of A− rats was 36% lower than that found in the plasma of N rats (P < 0.05). No significant differences in the vitamin B-12 plasma concentrations were found between the A− and A+ or R rats. These results indicate the presence of a mild deficiency of vitamin B-12 in the A− rats relative to the N rats but not when compared with the R rats. Because no differences were noted between the plasma vitamin concentrations in these latter rats and the A− rats, a deficiency of vitamin B-12 is not the cause for neutrophil hypersegmentation in the A− rats. This suggests that the excessive hypersegmentation of neutrophils is a direct effect of the differences in retinol concentrations in the A− rats.

### TABLE 3

| Retinol concentrations of bone marrow from vitamin A−deficient and control rats |
|---|---|---|---|
| Group2 | n | Retinol (nmol/g protein) | α-Tocopherol (nmol/g protein) |
| A− | 6 | 9.7 ± 0.9a | 6.4 ± 0.7c |
| R | 4 | 1.6 ± 0.5b | 10.8 ± 0.2de |
| A+ | 4 | 2.3 ± 0.6b | 8.6 ± 1.7e |
| N | 4 | 2.1 ± 0.5b | 3.9 ± 1.2f |

1 Values are means ± SD. An overall difference among the groups was observed using ANOVA. Individual contrasts were performed using the Scheffé method (Scheffé 1953). P < 0.01 for a vs. b. P < 0.01 for c vs. d and for e vs. f.

2 Group abbreviations: A−, vitamin A−deficient rats; R, vitamin A−deficient rats then fed the diet containing vitamin A; A+, weight-matched pair-fed control rats; N, nonrestricted vitamin A−complete diet rats.

**FIGURE 1** Separation of extracted bone marrow retinoids from vitamin A−deficient rats (A−) by HPLC. Retinoids were separated on a C18 column using a linear gradient of methanol-water vs. ethyl acetate-isopropanol. A. Extracted bone marrow retinoid HPLC chromatogram of an A− rat without (top) and with (bottom) added retinoic acid, retinol, α-tocopherol and retinyl palmitate. B. Extracted bone marrow retinoid HPLC chromatogram monitored at 325 nm (top) and 295 nm (bottom).
**TABLE 4**

*Comparison of granulocytic and monocylic cell populations in bone marrow of vitamin A-deficient and control rats*

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Group²</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>A−</td>
<td>R</td>
<td>A+</td>
<td>N</td>
</tr>
<tr>
<td>Total monocyte</td>
<td>1.2 ± 1.0</td>
<td>3.6 ± 2.8</td>
<td>1.5 ± 1.4</td>
<td>1.6 ± 1.6</td>
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<td>Total eosinophil</td>
<td>9.8 ± 3.0</td>
<td>11.8 ± 4.2</td>
<td>10.2 ± 3.7</td>
<td>12.3 ± 2.3</td>
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<tr>
<td>Total basophil</td>
<td>11.6 ± 6.0</td>
<td>7.5 ± 3.0</td>
<td>12.9 ± 2.9</td>
<td>13.5 ± 8.5</td>
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<tr>
<td>Total mast</td>
<td>3.8 ± 2.4</td>
<td>4.0 ± 1.5</td>
<td>2.8 ± 2.2</td>
<td>1.5 ± 1.0</td>
</tr>
<tr>
<td>Total neutrophil</td>
<td>73.5 ± 13.2</td>
<td>72.7 ± 5.8</td>
<td>73.1 ± 14.6</td>
<td>71.0 ± 15.6</td>
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<tr>
<td>Myelocyte</td>
<td>18.5 ± 10.3</td>
<td>13.6 ± 6.9</td>
<td>20.2 ± 10.1</td>
<td>20.7 ± 9.9</td>
</tr>
<tr>
<td>Metamyelocyte</td>
<td>11.1 ± 4.9</td>
<td>11.5 ± 6.7</td>
<td>8.1 ± 6.6</td>
<td>12.0 ± 6.3</td>
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<tr>
<td>Band</td>
<td>13.9 ± 5.2</td>
<td>25.1 ± 5.9</td>
<td>19.8 ± 2.9</td>
<td>17.7 ± 5.8</td>
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<tr>
<td>Mobius³</td>
<td>2.3 ± 1.9</td>
<td>1.8 ± 0.9</td>
<td>1.1 ± 0.9</td>
<td>0.6 ± 0.5</td>
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<tr>
<td>Normal⁴</td>
<td>25.7 ± 7.6</td>
<td>21.1 ± 2.0</td>
<td>23.2 ± 6.2</td>
<td>20.0 ± 6.1</td>
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<tr>
<td>Hypersegmented⁵</td>
<td>2.1 ± 2.2a</td>
<td>0.0b</td>
<td>0.1 ± 0.1b</td>
<td>0.0b</td>
</tr>
</tbody>
</table>

1 Values are means ± SD; n = 10 for A−; n = 5 for R; n = 6 for A+; n = 6 for N. No significant overall differences were found among groups for variables without letters (ANOVA).

2 Group abbreviations: A−, vitamin A-deficient rats; R, vitamin A-deficient rats then fed the diet containing vitamin A; A+, weight-matched pair-fed control rats; N, nonrestricted vitamin A-complete diet rats.

3 The Mobius type is an intermediate between the typical stab cell and the normal 3–4 segmented nuclear cell having condensed nuclear chromatin that has not yet segmented into lobes (See Fig. 2).

4 Normal neutrophils were defined as those with more than six or more nuclear lobes. An overall differences was found among the groups using the Kruskal-Wallis analysis of variance on ranks [Kruskal and Wallis 1952]. Dunn’s test was used to determine individual differences among groups [Dunn 1961]. P < 0.05 for a vs. b.

**DISCUSSION**

Although the plasma and liver retinol concentrations of A− rats were less than 1% of those in the R, A+ and N rats, the retinol concentration in the bone marrow of A− rats was about fourfold higher than those found in the R, A+ and N rats. This indicates retinol is sequestered in the bone marrow of A− rats. In rabbits, retinyl esters and retinoic acid were also detected [Blaner et al. 1993, Skrede et al. 1993], with retinyl esters accounting for 20% [Blaner et al. 1993] to 57% [Skrede et al. 1993] of the retinoids present. The ratio of retinol to retinoic acid was 58:1 [Blaner et al. 1993]. Retinoic acid may be present in rat bone marrow but was not detected in the present study because of the limited amount of bone marrow recovered from the rats and the possible coelution of a molecule with a greater absorbance at 295 nm. Considering these factors, the estimated possible retinoic acid concentration is <0.05 nmol/g bone marrow. Other retinoids may also be present, such as 14-hydroxy-retinoic acid and 14-hydroxy-retinoic acid, which are found in a number of cells including the human granulocyte-like leukemic HL-60 cells [Eppinger et al. 1993]. Again, the expected concentrations of these retinoids would be below our level of detection because of the small amount of bone marrow obtained from the rats and because cells synthesize very low amounts of these metabolites [Eppinger et al. 1993].

In the A− rats, α-tocopherol was detected on the chromatograms at the same general concentration as retinol, but the concentration was not influenced by the retinol status of the animals. Thus, α-tocopherol is probably important in protecting the bone marrow cells from oxidative damage.

**FIGURE 2** Representative forms of mature neutrophils found in the blood of vitamin A-deficient and control rats. Similar cells were observed in the bone marrow of the rats. A: Mobius neutrophil from a weight-matched pair-fed rat. B: Normal neutrophil from a weight-matched pair-fed rat. C: Hypersegmented neutrophil from a vitamin A-deficient rat.
It is not known whether the higher retinol concentration in bone marrow of vitamin A--deficient rats is due to greater uptake or less remobilization of retinol. Under normal conditions, the bone marrow receives a constant supply of retinol from chylomicrons [Hussain et al. 1989]. In rabbits, when chylomicrons containing [3H]retinyl palmitate are administrated by an intravenous route, the chylomicrons are initially taken up at the same rate by liver and bone marrow. Within 4 h, 80% of the retinoid taken up by the bone marrow is remobilized and is probably taken up by the liver for storage (Hussain et al. 1989). Similar results were observed in normal children and those with acute myelocytic leukemia when given an oral dose of retinyl palmitate [Skrede et al. 1994]. Their bone marrow retinyl ester concentrations increased, with peak concentrations occurring at 5 h. Treatment of acute myeloid leukemic children with 52 μmol retinol/m² for 2 y did not significantly alter bone marrow or plasma retinol concentrations when the samples were taken after an overnight fast (Skrede et al. 1994). The high initial uptake of chylomicrons and the ability to regulate retinol concentrations by bone marrow probably reflect the importance of maintaining bone marrow lipid and lipid-soluble vitamin concentrations.

The mechanism by which bone marrow mobilizes retinol and retinyl palmitate is not known. One possible route is the release of retinoids as chylomicron components. Because bone marrow does synthesize plasma retinol-binding protein (Blaner et al. 1993), another route may be the formation and release of plasma retinol-binding protein- retinol complexes.

Bone marrow probably has two sources of retinol: chylomicrons under well-nourished conditions and plasma retinol binding protein-retinol complexes under fasting and starvation conditions. Bone marrow may specifically take up retinol-binding protein-retinol complexes through a receptor-mediated process [Smeal et al. 1995]. The chylomicrons present in the A--rats would contain little, if any, retinyl palmitate; therefore, the retinol found in the bone marrow of these rats is probably from the liver stores. In the A--rats, the bone marrow and liver retinol concentrations were about the same. Assuming 1.0 g of protein per 10 g of bone marrow, the average retinol concentration in bone marrow was approximately 0.97 nmol/g, which was just lower than the average of 1.20 nmol retinol/g liver. Therefore, it is plausible that, in vitamin A deficiency, bone marrow cells sequester retinol released from the liver to ensure the growth of myeloid type cells [Epinger et al. 1993] and lymphoid cells [Buck et al. 1991] and the differentiation of myeloid cells to neutrophils [Tsai et al. 1992, Tsai and Collins 1993].

In the A--rats, 7% of the mature neutrophils in bone marrow and 67% of the mature neutrophils in blood were hypersegmented. In comparison, only 0.3% of the mature neutrophils in A+ rat bone marrow were hypersegmented. Bone marrow of R and N rats did not contain hypersegmented neutrophils, and blood from these groups contained only 2 to 7% hypersegmented neutrophils. The high concentration of circulating hypersegmented neutrophils in the A--rats may be due either
to their preferential release from the bone marrow over the normal neutrophils or to their progressive hypersegmentation once released into circulation where retinol concentrations are very low.

Several routes to neutrophil hypersegmentation may be possible. Neutrophil hypersegmentation can occur because of abnormal DNA synthesis, as observed in vitamin B-12 and folate deficiencies [Nath and Lindenbaum 1979]. Hypersegmentation may be a stage in the neutrophil aging process leading to apoptosis. This possibility is supported by the finding that retinoic acid induces both differentiation and apoptosis of the neutrophil-like HL-60 cells [Martin et al. 1990]. It is also possible that the higher concentrations of retinol in the A- bone marrow accelerate the apoptosis process.

A concurrent deficiency of folate and/or vitamin B-12 was considered a potential cause of neutrophil hypersegmentation in the A- rats because deficiencies of these vitamins are the most common causes of neutrophil hypersegmentation [Nath and Lindenbaum 1979] and because of the possibility of abnormal intestinal absorption of these vitamins due to the vitamin A deficiency associated changes in the intestinal epithelial cells [Holland et al. 1993, Moore 1957]. Although lower concentrations of vitamin B-12 (64% of normal) were found in the plasma of A- rats, these concentrations were not low enough to be considered deficient (<10–20% of normal). Additionally, no significant differences were observed among the plasma concentrations of folate for the various groups of rats or among the plasma concentrations of vitamin B-12 for the A-, R and A+ rats. A higher than normal frequency of neutrophil hypersegmentation was noted in the A- rats but not in the R and A+ rats. These results suggest that the hypersegmentation of the A- rat neutrophils was not due to a concurrent deficiency of folate or vitamin B-12 but may be a direct result of the alteration of retinol concentrations in the A- rats.

In conclusion, retinoids are sequestered as retinol in bone marrow of A- rats, which explains why myeloid cells of vitamin A-deficient animals differentiate to neutrophils. A small percentage (7%) of the mature neutrophils in the bone marrow of the A- rats and 67% of these cells in circulation are abnormally hypersegmented. This is not due to vitamin B-12 and/or folate deficiency but is probably a direct effect of the retinol concentration.

LITERATURE CITED


National Research Council (1985) Guide for the Care and Use of...


