Arrested Maturation of Granulocytes in Copper Deficient Mice[1,2,3]

Joanne Karimbakas, Bobbi Langkamp-Henken and Susan S. Percival[4]

Food Science and Human Nutrition Department, University of Florida, Gainesville, FL 32611

ABSTRACT The objective of this study was to examine the role of copper in neutrophil development and function. Mice were made copper deficient by feeding dams a diet containing 1.05 μg copper starting at parturition. Control mice were fed the same diet containing 6 μg copper. The pups were weaned to the diet and killed when they were 5–6 wk old. Peripheral blood cell counts, margination and cell maturity were measured. The response to an intraperitoneal injection of lipopolysaccharide (LPS) was also determined. Copper deficiency resulted in twice as many neutrophils and fewer than half the number of lymphocytes. Half as many cells in copper-deficient mice expressed Ly-6G, a granulocytic marker of cell maturity. In addition, copper-deficient cells expressed only half the amount of Ly-6G per cell than was expressed by copper-adequate cells. This suggested that the cells were younger, or arrested in their maturation as a result of copper deficiency. An arrest of maturation has been proposed as the cause of neutropenia in human copper deficiency. Injection of LPS in copper-adequate mice resulted in twice as many Ly-6G-expressing cells in the periphery. LPS injection into copper-deficient mice resulted in a severe leukopenia but did not influence Ly-6G expression any more than did copper deficiency alone. LPS treatment caused an increase in myeloperoxidase activity associated with the lungs of copper-deficient mice. The results suggest that although the neutrophils of copper-deficient mice are immature, they can be sequestered by the lung when stimulated to do so. J. Nutr. 128: 1855–1860, 1998.

KEY WORDS: • *copper deficiency* • *mice* • *neutrophil* • *differentiation* • *immunity*

A lack of copper results in neutropenia in humans receiving parenteral nutrition lacking copper. Individuals described in these case studies received long-term parenteral nutrition for months to years before being diagnosed as copper deficient. Neutropenia was reversed upon reintroduction of copper (Percival 1995). Studies in these copper-deficient humans suggested that copper is essential for normal granulopoiesis. It has been shown in many species that resistance to infection is impaired in copper deficiency (Prohaska and Failla 1993), and ineffective granulopoiesis could be a potential cause of this impairment. Neutrophils elicited from copper-deficient mice produce less oxidative burst, although their ability to migrate (chemotaxis) and engulf (phagocytosis) microorganisms is not affected (Babu and Failla 1990, Boyne and Arthur 1981). Impaired oxidative burst may also be explained by ineffective granulopoiesis. Nearly all immune cells are affected by copper deficiency. It is our goal to understand the influence of copper on neutrophils and on granulopoiesis.

Neutrophils, apart from acting as circulating phagocytes, play an important role in the inflammatory response. After injury, the bone marrow releases more neutrophils into the circulation. A few of these newly released neutrophils are immature banded cells. Neutrophils migrate to the site of damage, penetrate the tissues and kill invading microorganisms. How copper deficiency affects neutrophil responses to inflammatory stimuli is not well understood and is also a part of this research.

To determine the relationship between dietary copper intake and peripheral blood neutrophils, a murine model was studied. The objectives were to characterize the peripheral blood neutrophil response to copper deficiency and, subsequently, to determine the response of neutrophils to an inflammatory stimulus.

MATERIALS AND METHODS

Mice. Procedures were approved by the Institutional Animal Care and Use Committee of the University of Florida. Timed pregnant outbred ICR strain mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) at 8 wk of age; upon arrival, mice were acclimated to a standard nonpurified diet (Harlan Teklad 4% mouse/rat diet, 7001, Madison, WI) for at least 5 d. The mice had free access to the diet and to distilled, deionized water. On the day of parturition, the dams were fed either a copper-deficient (CuD)[5] diet, 1.05 ± 0.13 μg/g or a copper-adequate (CuA) diet, 6.4 ± 0.32 μg/g; diets were prepared by Dyets (Bethlehem, PA) using the AIN 93G formulation (Reeves et al. 1993). At weaning (3 wk old), genders were separated and the mice were fed the same diets as their dams for an additional

[1] This is Florida Agricultural Experiment Station Journal Series number R06326.

[2] Supported by DDK R29–43518.

[3] The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

[4] To whom correspondence and reprint requests should be addressed.

[5] Abbreviations used: Cp, ceruloplasmin; CuA, copper adequate; CuD, copper deficient; CuZn-SOD, copper zinc superoxide dismutase; DPTA, diethylenetriaminepentaacetic acid; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; HETAH, hexadecyltrimethyl ammonium hydroxide; LPS, lipopolysaccharide; MPO, myeloperoxidase; TMB, 3,3′,5,5′-tetramethylbenzidine.

Manuscript received 6 October 1997. Initial review completed 4 December 1997. Revision accepted 17 June 1998.

2–3 wk, or until they were 5–6 wk old. Only male mice were studied. Mice were housed in polycarbonate cages in an environmentally controlled room (12-h light:dark cycle, 25°C). Water bottles were fitted with polyethylene stoppers and stainless steel sipper tubes; distilled, deionized water was used throughout the study. Before the mice were killed, they were transferred to wire-bottomed cages and food was withheld overnight. Copper-deficient mice did not exhibit outward signs of infection, and spot checks for systemic bacteria by culturing whole blood onto blood agar plates and into brain-heart infusion broth were negative. When the mice reached 5–6 wk of age, they were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg) and blood collected via the inferior vena cava. After exsanguination, the mice were killed by cervical dislocation.

Lipopolysaccharide treatment. Pups were randomly assigned to treatment groups. One half of each group of mice was injected intraperitoneally with lipopolysaccharide (LPS) in isotonic saline 17 h before killing. A preliminary study showed that neutrophilia resulted 17 h after injection and occurred before the peak increase in ceruloplasmin activity or serum copper levels. The mice were injected with 50 μg of LPS from *Escherichia coli* 026:B6 (cell cultured, tested and chromatographically purified by gel filtration) (Sigma Chemical, St. Louis, MO). Control mice were injected intraperitoneally with saline.

Blood collection. Blood was collected via the inferior vena cava after opening the body cavity. A 1-mL syringe containing EDTA was used to bleed the mice. Whole blood (60 μL) was set aside for flow cytometry analysis. A 300-μL blood sample was placed in lithium heparin microtainer tubes (Becton Dikenson, San Jose, CA) for complete blood count analyses, including hemoglobin, hematocrit and fibrinogen, by the University of Florida Veterinary Medical Center Hematology Department. The remaining blood was centrifuged at 350 × *g* for 20 min at room temperature. Plasma was immediately frozen at −20°C to be used later for ceruloplasmin (Cp) activity and copper analysis.

Flow cytometry analysis. A direct two-color immunofluorescence staining technique using whole blood was employed to quantitate expression of the cell-surface marker Ly-6G in peripheral blood (McCarthy and Macey 1993). Whole blood was used to avoid density gradient separation of the leukocytes. Separation techniques have been shown to increase the expression of some markers on the cell surface. Whole blood in EDTA (15 μL) was mixed with 250 μL of LDS-751 (Exciton, Dayton, OH). The working solution of LDS-751 was prepared by diluting the stock solution (0.2 g/L, wt/v, in methanol) 1:100 in Hanks' balanced salt solution (HBSS). LDS-751 binds to nuclear DNA; therefore it does not label erythrocytes or platelets and they can be gated out during data acquisition. Samples were placed on ice and incubated with 5 μL of antibody conjugated with fluorescein isothiocyanate (FITC) at 4° C. Samples were adjusted to 300 μL with 1% fetal bovine serum in HBSS. Monoclonal antibodies to Ly-6G (isotype Rat IgG2κ) and the corresponding IgG2κ isotype control were obtained from Pharmingen, San Diego, CA. Sample analysis was conducted within 5 h. Analyses of labeled cell samples were done with a FACScan Flow cytometer with an argon laser (FACScan, Becton-Dickinson, San Jose, CA). Nonnucleated cells were excluded from data acquisition by gating at ~100 units of fluorescence, based on the LDS-751 fluorescence. Ten thousand intact cells that fluoresced above 100 units were acquired for each measurement. Graphic plots and statistics for flow cytometry analyses were produced using WinMDI software, Version 2.5, build #6 (developed by Joe Trotter of The Scripps Research Institute, La Jolla, CA). "Mean fluorescence" reflects the average fluorescence of the subpopulation of cells having fluorescence greater than the background fluorescence of the isotype controls. "Percent gated" refers to the percentage of cells of the total 10,000 nucleated cells acquired during FACS analysis that was in the region of specifically bound Ly-6G antibody.

Superoxide dismutase assay. The activity of Cu/Zn-superoxide dismutase (Cu/Zn-SOD, EC 1.15.11) was measured in erythrocytes and lung tissue as reported previously (Percival 1993). Lung tissue preparation was slightly modified (De Haan et al. 1994). After thawing on ice, lung tissue (~0.05 g) was homogenized in 300 μL of Tris buffer [50 mmol/L Tris, 1 mmol/L diethylenetriaminepentaacetic acid (DPTA), pH 8.2]. Samples were then sonicated on ice 10 times, at 1 cycle/s, 70% duty cycle, and then centrifuged at 100,000 × *g* for 1 h at 4°C (TLA 100.4 fixed angle rotor, Optima Ultra Centrifuge, Beckman, Fullerton, CA). Protein was determined by the Bradford Protein Assay (Bio-Rad Laboratories, Richmond, CA).

After the protein concentration of the supernatant was adjusted to 0.1 g/L, the samples were extracted with chloroform/methanol (15:25, v/v). Aliquots (50 μL) of extracted supernatant were serially diluted four times in triplicate with Tris buffer (pH 8.2, 50 mmol/L Tris/1 mmol/L DPTA) in flexible microtiter plates. Another 50 μL of Tris buffer was added to each well. The reaction was initiated by the addition of 50 μL of 0.2 mmol/L pyrogallol containing 1 mmol/L DPTA to all wells. The change in absorbance was monitored every 10 s for 3 min in a microplate reader (UVMax, Molecular Devices, Menlo Park, CA) at an absorbance of 340 nm.

The program software (Softmax, Molecular Devices, Menlo Park, CA) calculated the change in absorbance as mOD/min. One unit of activity was defined as the amount of Cu/Zn superoxide dismutase that inhibits the autoxidation of pyrogallol by 50%. Results were expressed as units/mg lung protein. Lung protein content per gram of tissue was not different among the experimental groups.

Myeloperoxidase activity in lung. Lung myeloperoxidase (MPO, EC 1.11.1.7) activity was determined by an adaptation of the method used by Grisham et al. (1990). Briefly, the tissue was minced and homogenized at a 10% (wt/v) in 20 mmol/L KH_2PO_4 with 1 mmol/L EDTA at pH 7.4. Samples were diluted to 1.5 times their initial volume in the same buffer and centrifuged at 10,000 × *g* at 4°C for 20 min. The pellet was reconstituted to a final volume of 1 mL with 50 mmol/L acetic acid containing 5 g/L hexadecyltrimethyl ammonium hydroxide (HETAH) at pH 6.0. The sample was then rehomogenized, sonicated, frozen and thawed twice on dry ice. The sample was centrifuged at 10,000 × *g* at 4°C for 20 min. The supernatant was assayed by measuring the H_2O_2 -dependent oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB). This yielded a blue chromogen possessing a wavelength maximum of 655 nm. Samples (100 μL) were added to a reaction buffer containing 315 μL of 0.8 mol/L KH_2PO_4, pH 5.4, to which 25 μL of 10% HETAH and 50 μL of 16 mmol/L TMB in dimethylformamide were added. The sample was incubated in a shaking water bath at 37°C for 5 min. The reaction was initiated by adding 10 μL of 30 mmol/L H_2O_2 and incubated for 3 min at 37°C. Catalase (10 μL) was added, followed by the sequential addition of 2 mL of 0.2 mol/L sodium acetate. Units of myeloperoxidase activity were calculated from a standard curve that used peroxidase enzyme EC 1.11.1.7 (P-8375, Sigma Chemical) as the standard enzyme.

Ceruloplasmin oxidase activity assay. Cp (EC 1.16.3.1) activity was determined by the method of Schosinsky et al. (1974) using *o*-dianisidine. Cp oxidase activity was reported in IU, and determined by calculating the molar absorptivity after subtracting the 5-min reading from the 15-min reading. Copper in the plasma was determined by graphite furnace atomic absorption spectrophotometry as previously reported (Bae and Percival 1994).

Statistical methods. All comparisons between groups were carried out using SigmaStat (version 1.01), produced by Jandel Scientific (San Rafael, CA). Significantly different means after one-way ANOVA were detected by a Student-Newman-Keuls multiple comparison test. When unequal variances were found, data were log transformed. When normality failed, a one-way ANOVA was performed on ranks. All data are expressed as means ± SD. The probability level at which differences were considered significant was $P < 0.05$.

RESULTS

Indices of copper status of the mice are presented in **Table 1**. All measures were significantly different in the copper-deficient compared with the copper-adequate mice. The mice sustained signs that are commonly found in copper deficiency, such as enlarged heart, lower copper levels, reduced copper-dependent enzymes and anemia.

Complete blood cell counts of the dietary groups and LPS

TABLE 1

Copper status in mice fed a copper-deficient (CuD) or copper-adequate (CuA) diet from birth[1]

	Cu adequate	Cu deficient[2]
Body weight, *g*	25.7 ± 2.54	20.8 ± 2.6
Heart weight, *g*	0.161 ± 0.02	0.216 ± 0.03
mg/g body wt	6.23 ± 0.8	11.0 ± 0.9
Spleen, *mg*	84.9 ± 19.0	106.6 ± 35.1
mg/g body wt	3.3 ± 0.5	5.2 ± 0.9
Thymus, *mg*	81.2 ± 12.0	42.0 ± 14.1
mg/g body wt	3.16 ± 0.5	1.99 ± 0.6
Ceruloplasmin activity, *units/L*	17.1 ± 4.5	0.63 ± 0.3
Plasma copper, *umol/L*	12.0 ± 4.2	1.22 ± 0.71
Liver copper, *nmol/g dry liver*	261.8 ± 4.56	170.0 ± 25.2
Hemoglobin, *g/L*	124 ± 5	30 ± 16
Hematocrit	0.33 ± 0.05	0.09 ± 0.04
RBC, cell number × 10^6	6.82 ± 1.01	1.37 ± 0.38
RBC CuZn-SOD activity, *units/mg protein*	3999 ± 1189	436.5 ± 143

[1] Values reported are means ± SD, n = 6–8 mice.
[2] All means of CuD mice are significantly different from those of CuA controls, $P < 0.05$.

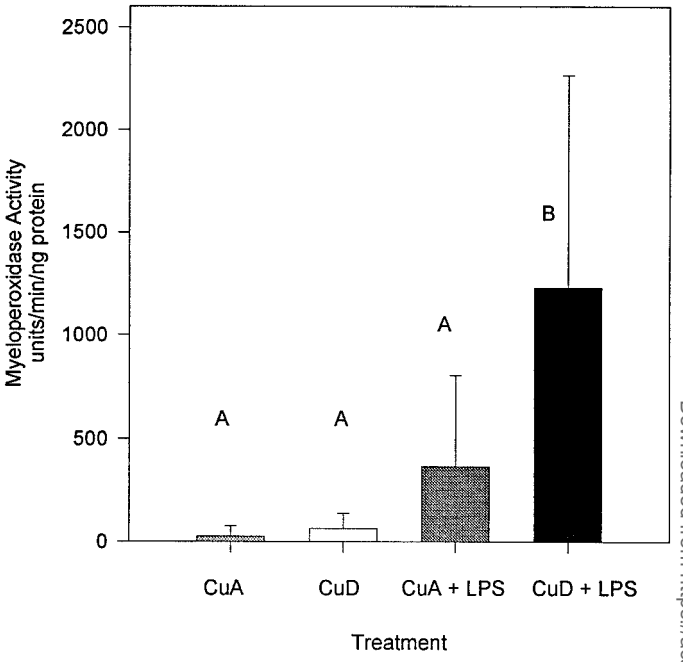

FIGURE 1 Myeloperoxidase activity in the lung tissue of copper-adequate (CuA) and copper-deficient (CuD) mice stimulated or not stimulated with an injection of lipopolysaccharide. Results are expressed means ± SD, n = 6. Different letters (A, B) indicate significantly different means, $P < 0.05$.

groups are shown in **Table 2**. Overall, the CuD group was leukopenic, with 60% fewer lymphocytes but with twice as many neutrophils as the CuA controls. Banded neutrophils were detected in the peripheral blood of the CuD mice.

When mice were challenged with LPS, the number of peripheral lymphocytes decreased by 92% and the number of neutrophils increased more than threefold in CuA mice. Exposure of the CuD group to LPS resulted in an 84–91% reduction in total leukocytes compared with CuD and CuA, respectively. However, instead of the greater number of neutrophils that are expected during inflammation, there were 85% fewer neutrophils in CuD + LPS mice than in CuD mice without LPS injection. Banded cells were observed in both CuA and CuD mice stimulated with LPS.

The MPO activity measured in the lungs of CuD mice was not significantly different from that of the control mice (P = 0.33), although the activity was twice that of the control mice (**Fig. 1**). LPS injection resulted in a 15-fold greater MPO activity in the lungs of the CuA mice, but the difference was not significant because of the variability among the mice (P = 0.09). LPS caused a 50-fold increase in MPO activity in the lungs of the CuD mice (P < 0.05). No MPO activity was detected in the kidney or intestinal tissues in any group (data not shown).

As expected, copper deficiency resulted in significantly lower plasma copper concentration and Cp activity (**Table 3**). Plasma fibrinogen concentration and lung Cu/Zn SOD activity were significantly reduced in CuD mice compared with CuA mice.

Exposure of CuA mice to LPS would be expected to result in significantly higher levels of acute phase proteins such as fibrinogen, Cp, and therefore, copper. The CuA mice had more than twofold greater fibrinogen concentrations after LPS challenge; however, in this study, the mean Cp activity and plasma copper concentrations were not different 17 h after LPS injection. Challenging the CuD group with LPS did not affect Cp activity, plasma copper or fibrinogen concentrations, or lung CuZn-SOD activity.

Results of Ly-6G expression from representative mice are

TABLE 2

Peripheral blood cell differentials in copper-adequate (CuA) and copper-deficient (CuD) mice with and without lipopolysacchanide (LPS) stimulation[1]

	Cells, *n*	Bands	Segmented neutrophils	Lymphocytes	Monocytes
			cells/uL		
CuA	2850 ± 840^A	ND[2]	428 ± 116^C	2163 ± 730^A	82 ± 37
CuD	1600 ± 697^B	144 ± 168	868 ± 490^B	792 ± 155^B	73 ± 33
CuA + LPS	2300 ± 490^A	188 ± 235	1854 ± 341^A	174 ± 92^C	116.8 ± 52
CuD + LPS	255 ± 80^C	33 ± 32	124 ± 55^C	91 ± 25^C	8.2 ± 4

[1] Values are means ± SD, n = 5 except CuD + LPS, where n = 4. Values within a column with different superscript letters are significantly different at $P < 0.05$.
[2] ND, banded cells were not detected in 4 out of 5 CuA mice; a blood sample from one mouse contained 20 bands, or 1% of the total cells.

TABLE 3

Measures of copper status and inflammation in copper-adequate (CuA) and copper-deficient (CuD) mice with and without lipopolysaccharide (LPS) stimulation[1]

	Plasma			Lung
Treatment	Copper	Ceruloplasmin	Fibrinogen	Cu/Zn -SOD[2]
	μmol/L	*mU/L*	*g/L*	*U/mg protein*
CuA	$12.0^{A} \pm 4.2$	$11.0^{A} \pm 4.9$	$1.60^{B} \pm 0.55$	$483.1^{A} \pm 64.5$
CuD	$1.2^{B} \pm 0.7$	$0.6^{B} \pm 0.8$	$1.00^{C} \pm 1.23$	$252.7^{B} \pm 29.0$
CuA + LPS	$11.9^{A} \pm 2.7$	$14.8^{A} \pm 5.2$	$5.20^{A} \pm 1.79$	$413.3^{A} \pm 58.9$
CuD + LPS	$0.2^{B} \pm 0.1$	$0.2^{B} \pm 0.4$	$2.25^{C} \pm 1.26$	$249.2^{B} \pm 34.1$

[1] Values are means ± SD, $n = 6–8$ mice. Values within a column with different superscript letters are significantly different, $P < 0.05$.
[2] Copper-zinc superoxide dismutase.

shown in **Figure 2**. The results were analyzed by single-parameter histograms based on the fluorescence of the specific cell marker and the corresponding isotype control. Markers were used to exclude 95% of nonspecific binding in the analyses.

A 50% lower mean fluorescence ($P < 0.05$) and a 50% reduction in the percent gated ($P = 0.21$) were found in the CuD group compared with the CuA group (**Table 4**).The difference in the percent gated is biologically important but failed to reach significance because of the great variability within groups. This suggests not only fewer cells in the Ly-6G region, but also lower expression of this marker per cell. Exposure to LPS caused the CuA mice to have a significantly greater percent gated, but significantly less mean fluorescence, indicating a greater number of Ly-6G–expressing cells in the periphery, but lower expression of Ly-6G per cell. LPS challenge to the CuD mice did not affect Ly-6G expression compared with the unstimulated CuD group.

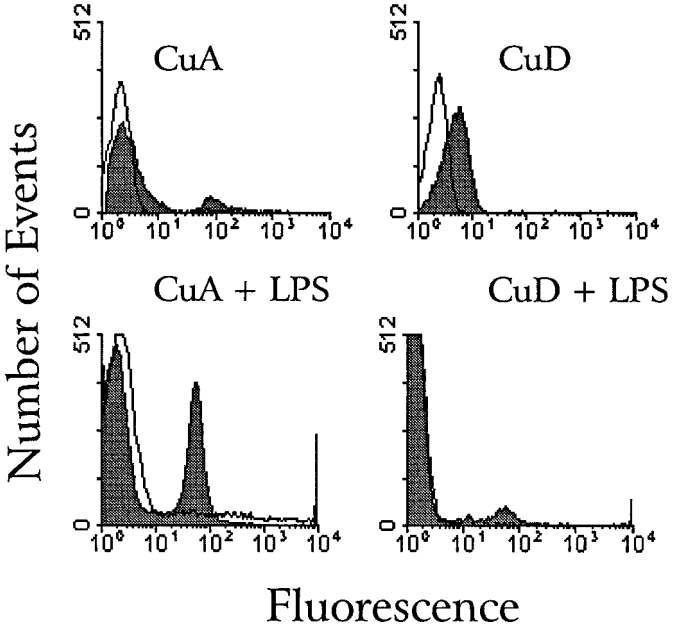

FIGURE 2 Expression of Ly-6G on the surface of peripheral blood cells in copper-adequate and copper-deficient mice stimulated or not stimulated with an injection of lipopolysaccharide. These are representative histogram plots of Ly-6G antibody staining of peripheral leukocytes from whole-blood preparations. The shaded histogram represents the Ly-6G antibody, whereas the open histogram represents the nonspecific bound isotype control antibody. Values for mean fluorescence number of cells were obtained from specifically bound Ly-6G antibody after excluding 95% of the nonspecifically bound control.

DISCUSSION

The mice nursed by dams fed the copper-deficient diet and then weaned to the copper-deficient diet were very copper deficient. The values reported in Table 1 are in good agreement with values reported by Prohaska's group (Lukasewycz and Prohaska, 1990, Prohaska et al. 1983, Prohaska and Lukasewycz 1990) and with those of Mulhern and Koller (Koller et al. 1987, Mulhern and Koller 1988). These two groups used the perinatal mouse model, whereas this study used an immediately postnatal model.

Anemia complicates the understanding of immune changes that are specifically due to copper. In this study, the mice were anemic. Which of the changes in immunity are due to a secondary iron deficiency? Iron deficiency impairs the function of neutrophils and T cells (Scrimshaw and SanGiovanni 1997), similar to the functional impairment observed in copper deficiency (Prohaska and Failla1993). However, the phenotypic changes in leukocyte differentials do not occur in iron deficiency as they do in copper deficiency. Iron deficiency may result in a reduction in neutrophils and lymphocytes, but the proportions of cells remain normal (Kriete et al. 1995 and cited in Fairbanks 1994). Others report no change in leukocyte numbers in iron deficiency (Hrycek et al. 1991). Copper deficiency results in a reversal in the proportion of leukocytes

TABLE 4

Flow cytometric analysis of cell surface Ly-6G molecule on leukocytes of copper-adequate (CuA) and copper-deficient (CuD) mice with and without lipopolysaccharide (LPS) stimulation[1]

Treatment	% Cells in Ly-6G region	Mean fluorescence of Ly-6G Region
CuA	$13.77^{B} \pm 8.00$	$74.6^{A} \pm 14.4$
CuD	$6.17^{B} \pm 6.92$	$35.6^{C} \pm 14.4$
CuA + LPS	$31.65^{A} \pm 7.41$	$55.6^{B} \pm 13.7$
CuD + LPS	$6.08^{B} \pm 5.93$	$25.9^{C} \pm 14.3$

[1] Values are means ± SD, $n = 4$ CuA and CuD, $n = 7$ mice + LPS. Values within a column with different superscript letters are significantly different, $P < 0.05$.

from 16% neutrophils and 75% lymphocytes to 51% neutrophils and 44% lymphocytes (Table 2). Data from Rickard et al. (1975) indicated that iron was not a major requirement for granulopoiesis, whereas our preliminary data in bone marrow (unpublished) indicate an important role for copper. Furthermore, changes in immunity due to copper deficiency are detected whether or not anemia is present. In humans (Dunlap et al. 1974) and Hayton et al. (1995) and in rodents (Babu and Failla 1990, Lukasewycz and Prohaska 1990, Prohaska et al. 1983) changes in immune cell function have been demonstrated when hemoglobin and/or hematocrit was low. On the other hand, similar changes in immunity without an accompanying anemia have been shown in humans (Goyens et al. 1985, Higuchi et al. 1988, Sriram et al. 1986), mice (Mulhern and Koller 1988) and cattle (Arthington et al. 1996). We conclude that those changes in immunity due to copper deficiency occur regardless of the hemoglobin levels and, moreover, iron deficiency does not result in changes in the differential profile of leukocytes as does copper deficiency.

Neutropenia has been reported as a sign of copper deficiency in humans. The copper-deficient mice in this study, however, exhibited neutrophilia, with more than twice as many peripheral blood neutrophils as the copper adequate mice. Neutrophilia was also observed in Hereford heifers consuming a molybdenum-supplemented, copper-deficient diet (Arthington et al. 1996). Species difference may be a reason that the mice and cattle become neutrophilic consuming a copper-deficient diet and humans become neutropenic. Another explanation for the differences could be the duration of the copper deficiency. Our study initiated copper deficiency in mice at birth, and it continued until the mice were young adults. Humans who became neutropenic did so as adults, after several months and, in some cases, years of parenteral nutrition before presenting symptoms required attention. It is not known whether humans were neutrophilic at early stages of copper deficiency or whether the mice will eventually become neutropenic. Finally, as one last alternative explanation, we showed that mice become neutropenic after an injection of LPS. It is possible that the neutropenia of copper-deficient humans may be the result of an inflammation or infection, which was the stimulus that initially brought the individual to the clinician who then diagnosed neutropenia. A review of 13 case studies citing copper-deficient neutropenia in 17 people described 13 individuals with some complication involving infection or inflammation (Botash et al. 1992, Dunlap et al. 1974, Fujita et al. 1989, Goyens et al. 1985, Hayton et al. 1995, Karpel and Peden 1973, Phillip et al. 1990, Prasad et al. 1978, Sriram et al. 1986, Summerfield et al. 1992). No complications were mentioned or apparent in four individuals exhibiting neutropenia (Dunlap et al. 1974, Higuchi et al. 1988, Naveh et al. 1981, Zidar et al. 1977).

The maturity of the murine cells in the periphery was determined by using the antibody against the Ly-6G cell surface marker and by histologic examination. Fleming et al. (1993) showed that granulocytes are the predominate population that stained with this antibody. The function of this marker is not known, but Hestdal et al. (1991) clearly showed the correlation between this marker and the maturity of the myeloid lineage. More Ly-6G is expressed on the cell surface of mature cells. In these studies, copper deficiency resulted in twice as many peripheral blood neutrophils, but the expression of Ly-6G was half that of the copper-adequate cells. Not only were there a lower number of cells expressing Ly-6G, but the expression per cell was also lower. This suggests that the peripheral blood neutrophils from the CuD group were less mature compared with controls. It also concurs with the evidence of banded cells in the periphery and supports the idea that copper deficiency resulted in younger, less differentiated cells in the periphery. We could interpret this as an arrest of maturation, the commonly stated postulate for human neutropenia (Dunlap et al. 1974, Hirase et al. 1992, Zidar et al. 1977).

Banded cells are not normally found in the periphery, and their presence usually indicates inflammatory activity. As expected, banded cells were not detected in the periphery of copper-adequate mice. Copper-deficient mice, however, had the same number of banded cells as the copper-adequate, LPS-stimulated mice. Inflammation in copper-adequate control mice resulted in more cells in the periphery that expressed Ly-6G, although the expression per cell was less. Similarly, neutrophils from copper-deficient mice displayed a reduction of Ly-6G expression. Neutrophilia, the presence of bands and reduction of Ly-6G expression in copper deficiency are signs similar to those found in the copper-adequate mice stimulated with LPS. Therefore, these characteristics of copper deficiency are reminiscent of the peripheral distribution of leukocytes in inflammation.

As expected, plasma copper and Cp levels were significantly reduced by copper deficiency as was lung CuZn-SOD activity. Plasma fibrinogen levels were also significantly reduced by copper deficiency, in agreement with Lominadze et al. (1996). CuA mice had greater fibrinogen levels when challenged with LPS, but the CuD mice did not mount a significant increase in fibrinogen in response to LPS.

The functions of neutrophils include traveling to the site of infection, adhering to the endothelium and subsequently undergoing diapedesis, or transmigration, across the endothelium. These steps are followed by engulfment (phagocytosis) and killing of the foreign invader by activation of the oxidative burst. Migration and phagocytosis are not affected by copper deficiency, but the killing ability of the oxidative burst is significantly lower (Babu and Failla, 1990, Boyne and Arthur, 1981). We estimated the adhesion ability of neutrophils from copper-deficient mice by measuring a neutrophil-specific enzyme in lung homogenates after LPS stimulation. Because of the significant increase in MPO activity in the lungs of the copper-deficient mice after LPS stimulation, it is suggested that adhesion of neutrophils to the endothelium is not impaired. This is somewhat surprising because the cells are less mature and are therefore expected to express fewer cell adhesion molecules. However, the ability of these copper-deficient cells to up-regulate surface expression of the adhesion molecules is unknown. We also do not know the stage of maturity achieved by the copper-deficient neutrophils and therefore cannot predict the expression of adhesion molecules that may or may not be expressed due to the age of the cell.

Additional evidence that the copper-deficient mice could respond to an inflammatory stimulus was provided by the remarkable leukopenia after LPS injection. About 85% of the total cells were gone from the periphery. These data plus the MPO data support the hypothesis that the cells marginated and were sequestered by the lung. We cannot determine from this experiment if they migrated into the tissue. Nonetheless, adherence is an important first step in diapedesis; future research will also include histologic and immunohistochemical examination of the lung to localize the neutrophils.

The leukopenia observed in copper-deficient mice after LPS stimulation was severe. Although our data suggest that the cells marginated, it also suggests that the bone marrow may have been defective in supplying new cells to the periphery. We have examined bone marrow cells from copper-deficient

mice and have preliminary evidence that shows immaturity of bone marrow cells as well.

In summary, copper-deficient mice, when not inflamed by an injection of LPS, exhibit neutrophilia, not neutropenia. The leukocyte population profile is altered in copper deficiency to resemble one that is reminiscent of an inflammatory situation. The peripheral cells in copper deficiency are more immature, supporting the observation in humans that copper deficiency leads to an arrest of maturation. The CuD neutrophils, although immature, appear able to respond to an inflammatory stimulus by margination from the periphery and sequestration by the lung. The resulting neutropenia after LPS injection is severe and suggests an inadequate replenishment of cells from the bone marrow. Future work will examine the bone marrow and potential mechanisms (e.g., relationship with cytokines) that result in changes in the process of cellular differentiation.

ACKNOWLEDGMENTS

The authors thank Richard A. Satcher and Kelli Herlinger-Garcia for technical assistance and Neal Benson at the UF-ICBR Flow Cytometry Core Laboratory for assistance and helpful discussions.

LITERATURE CITED

Arthington, J. D., Spell, A. R., Corah, L. R. & Blecha, F. (1996) Effect of molybdenum-induced copper deficiency on in vivo and in vitro measures of neutrophil chemotaxis both before and following an inflammatory stressor. J. Anim. Sci. 74: 2759–2764.

Babu, U. & Failla, M. L. (1990) Copper status and function of neutrophils are reversibly depressed in marginally and severely copper-deficient rats. J. Nutr. 120: 1700–1709.

Bae, B. & Percival, S. S. (1994) Copper uptake and intracellular distribution during retinoic acid-induced differentiation of HL-60 cells. J. Nutr. Biochem. 5: 457–461.

Botash, A. S., Nasca, J., Dubowy, R., Weinberger, H. L. & Oliphant, M. (1992) Zinc-induced copper deficiency in an infant. Am. J. Dis. Child. 146: 709–711.

Boyne, R. & Arthur, J. R. (1981) Effects of selenium and copper deficiency on neutrophil functions in cattle. J. Comp. Pathol. 91: 271–276.

De Haan, J. B., Tymms, M. J., Cristiano, F. & Kola, I. (1994) Expression of copper/zinc superoxide dismutase and glutathione peroxidase in organs of developing mouse embryos, fetuses, and neonates. Pediatr. Res. 35: 188–196.

Dunlap, W. M., James, G. W. & Hume, D. M. (1974) Anemia and neutropenia caused by copper deficiency. Ann. Intern. Med. 80: 470–476.

Fairbanks, V. F. (1994) Iron in medicine and nutrition. In: Modern Nutrition in Health and Disease. (Shils, M. E., Olson, J. A. & Shike, M. eds.), pp. 185–213. Lea and Febiger, Philadelphia, PA.

Fleming, T. J., Fleming, M. L. & Malek, T. R. (1993) Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. J. Immunol. 151: 2399–2408.

Fujita, M., Itakura, T., Takagi, Y. & Okada, A. (1989) Copper deficiency during total parenteral nutrition: clinical analysis of three cases. J. Parent. Enteral Nutr. 13: 421–425.

Goyens, P., Brasseur, D. & Cadranel, S. (1985) Copper deficiency in infants with active celiac disease. J. Pediatr. Gastroenterol. Nutr. 4: 677–680.

Grisham, M. B., Benoit, J. N. & Granger, D. N. (1990) Assessment of leukocyte involvement during ischemia and reperfusion of intestine. Methods Enzymol. 188: 729–733.

Hayton, B. A., Broome, H. E. & Lilenbaum, R. C. (1995) Copper deficiency-induced anemia and neutropenia secondary to intestinal malabsorption. Am. J. Hematol. 48: 45–47.

Hestdal, K., Ruscetti, F. W., Ihle, J. N., Jacobsen, S.E.W., Dubois, C. M., Kopp, W. C., Longo, D. L. & Keller, J. R. (1991) Characterization and regulation of RB6–8C5 antigen expression on murine bone marrow cells. J. Immunol. 147: 22–28.

Higuchi, S., Higashi, A., Nakamura, T. & Matsuda, I. (1988) Nutritional copper deficiency in severely handicapped patients on a low copper enteral diet for a prolonged period: estimation of the required dose of dietary copper. J. Pediatr. Gastroenterol. Nutr. 7: 583–587.

Hirase, N., Abe, Y., Sadamura, S., Yufu, Y., Muta, K., Umemura, T., Nishimura, J., Nawata, H. & Ideguchi, H. (1992) Anemia and neutropenia in a case of copper deficiency: role of copper in normal hematopoiesis. Acta Haematol. 87: 195–197.

Hrycek, A., Kalina, Z., Badowski, R. & Wartalska, G. (1991) Spontaneous migration and adherence of leukocytes and the NBT test of peripheral blood neutrophils in patients with iron deficincy anemia. Acta Hematol. Pol. 22: 261–266.

Karpel, J. T. & Peden, V. H. (1973) Copper deficiency in long-term parenteral nutrition. J. Pediatr. 52: 525–533.

Koller, L. D., Mulhern, S. A., Frankel, N. C., Steven, M. G. & Williams, J. R. (1987) Immune dysfunction in rats fed a diet deficient in copper. Am. J. Clin. Nutr. 45: 997–1006.

Kriete, M. F., Champoux, M. & Suomi, S. J. (1995) Development of iron deficiency anemia in infant rhesus macaques. Lab. Anim. Sci. 45: 15–21.

Lominadze, D. G., Saari, J. T., Miller, F. N., Catalfamo, J. L., Justus, D. E. & Schuschke, D. A. (1996) Platelet aggregation and adhesion during dietary copper deficiency in rats. Thromb. Haemostasis 75: 630–634.

Lukasewycz, O. A. & Prohaska, J. R. (1990) The immune response in copper deficiency. Ann. N.Y. Acad. Sci. 587: 147–159.

McCarthy, D. A. & Macey, M. G. (1993) A simple flow cytometric procedure for the determination of surface antigens on unfixed leucocytes in whole blood. J. Immunol. Methods 163: 155–160.

Mulhern, S. A. & Koller, L. D. (1988) Severe or marginal copper deficiency results in graded reduction in immune status in mice. J. Nutr. 118: 1041–1047.

Naveh, Y., Hazani, A. & Berant, M. (1981) Copper deficiency with cow's milk diet. Pediatrics 68: 397–400.

Percival, S. S. (1993) Cu/Zn superoxide dismutase activity does not parallel copper levels in copper supplemented HL-60 cells. Biol. Trace Elem. Res. 38: 63–72.

Percival, S. S. (1995) Neutropenia caused by copper deficiency: possible mechanism of action. Nutr. Rev. 53: 59–66.

Phillip, M., Singer, A. & Tal, A. (1990) Copper deficiency in an infant with giardiasis fed with cow's milk. Isr. J. Med. Sci. 26: 289–290.

Prasad, A. S., Brewer, G. J., Schoomaker, E. B. & Rabbani, P. (1978) Hypocupremia induced by zinc therapy in adults. J. Am. Med. Assoc. 240: 2166–2168.

Prohaska, J. R., Downing, S. W. & Lukasewycz, O. A. (1983) Chronic dietary copper deficiency alters biochemical and morphological properties of mouse lymphoid tissues. J. Nutr. 113: 1583–1590.

Prohaska, J. R. & Failla, M. L. (1993) Copper and immunity. In: Human Nutrition—A Comprehensive Treatise, Vol. 8: Nutrition and Immunology (Klurfield, D. M., ed.), pp. 309–332. Plenum Press, New York, NY.

Prohaska, J. R. & Lukasewycz, O. A. (1990) Effects of copper deficiency on the immune system. Adv. Exp. Med. Biol. 262: 123–144.

Reeves, P. G., Nielsen, F. H. & Fahey, G. C., Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 123: 1939–1951.

Rickard, K. A., Brown, R. D., Dunleavy, L., Robin, H. & Kronenberg, H. (1975) The bone marrow colony forming cell in megaloblastic anemia and iron deficiency anemia. Aust. N.Z. J. Med 5: 424–430.

Schosinsky, K. H., Lehmann, H. P. & Beeler, M. F. (1974) Measurement of ceruloplasmin from its oxidase activity in serum by use of *o*-dianisidine dihydrochloride. Clin. Chem. 20: 1556–1563.

Scrimshaw, N. S. & SanGiovanni, J. P. (1997) Synergism of nutrition, infection and immunity: an overview. Am. J. Clin. Nutr. 66: 464S–477S.

Sriram, K., O'Gara, J. A., Strunk, J. R. & Peterson, J. K. (1986) Neutropenia due to copper deficiency in total parenteral nutrition. J. Parent. Enteral Nutr. 10: 530–532.

Summerfield, A. L., Steinberg, F. U. & Gonzalez, J. G. (1992) Morphologic findings in bone marrow precursor cells in zinc-induced copper deficiency anemia. Am. J. Clin. Pathol. 97: 665–668.

Zidar, B. L., Shadduck, R. K., Zeigler, Z. & Winkelstein, A. (1977) Observations on the anemia and neutropenia of human copper deficiency. Am. J. Hematol. 37: 177–185.