

Alterations in Lymphocyte Subpopulations in Copper-Deficient Mice

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Analyses of cell surface determinants of splenocytes from copper-deficient C58 mice indicate alterations in lymphocyte subpopulation characteristics. Both the absolute number and the relative percentage of surface immunoglobulin-bearing (B) cells from copper-deficient mice were significantly greater than those from copper-supplemented controls. The relative percentage of Thy 1.2-positive (T) cells was decreased, and the decrease was most prominent within the Lyt 1-positive (helper) T-cell subset. The functional responsiveness of both B cells and T cells was decreased in copper deficiency.

Nutrition can influence immunological function. It is now firmly established that deficiencies of protein, calories, vitamins, or certain trace elements can lead to impairment of immunity (6, 9).

Copper, a trace metal, is essential for many biological processes, including the immune response (2, 5). Infants with Menkes' disease, a congenital X-linked copper deficiency, usually succumb to bronchopneumonia at a very early age (7). Even the milder allelic forms of Menkes' disease are associated with frequent infection (10). Nutritional copper deficiency in humans, although rare, is also characterized by recurrent infections that can lead to pulmonary sepsis (1, 14). Copper supplementation is necessary to reduce infections in infants recovering from marasmus (4). Domestic animals with insufficient copper intake show decreased bactericidal activity and impaired neutrophil function (3, 13). Laboratory animals fed a low copper diet exhibit an increased susceptibility to bacterial infections (12, 17) and decreased resistance to tumor challenge (15).

A mouse model of copper deficiency to investigate the role of copper in immune function recently was established in our laboratory (15, 16, 19, 20). Previous results suggested that both humoral (20) and cell-mediated (15, 16) immunity were compromised in copper-deficient (Cu^-) mice. It was in this context that we undertook an analysis of the subpopulation characteristics of lymphoid cells from Cu^- mice.

MATERIALS AND METHODS

Mice. Inbred C58 mice were maintained at the animal facilities of the School of Medicine of the University of Minnesota, Duluth.

Pregnant dams were housed in polycarbonate cages with stainless-steel covers and wood shavings for bedding. Offspring were weaned at 3 weeks of age and were transferred to stainless-steel cages with wire-mesh floors at 4 weeks of age. Mice were used for experiments at 8 weeks of age.

Diet. On the day of parturition, C58 dams were switched from a nonpurified diet (Purina Mouse Chow; Ralston Purina Co., St. Louis, Mo.) containing 12 to 14 mg of copper per kg to a purified diet formulated to omit copper from the salt mix (modified AIN 76A; Teklad Laboratories, Inc., Madison, Wis.) which contained 0.6 mg of copper per kg. This diet has previously been used to establish copper deficiency in mice

(18). Half of the dams were given supplemental copper (20 $\mu\text{g}/\text{ml}$ as CuSO_4) in their drinking water, whereas the remaining dams were given deionized water to drink. Pups were maintained on the treatment of their respective dams until the experiments began. For any given experiment, copper-supplemented, control (Cu^+) and Cu^- animals were matched as to age, sex, and weight. Each experiment consisted of a minimum of five Cu^+ and five Cu^- mice. Several additional mice from each experiment were kept for biochemical analysis (19).

Copper status. Plasma from individual microhematocrit tubes was assayed for ceruloplasmin (EC 1.16.3.1) activity with *o*-dianisidine as the substrate as described by Prohaska (18). Ceruloplasmin, a cuproenzyme, was routinely used to monitor the copper status of the mice. It previously was shown (15) that ceruloplasmin levels in this mouse model fall to undetectable levels before weaning.

Lymphocyte enumeration. Single-cell suspensions of splenocytes from Cu^+ and Cu^- mice were evaluated by flow cytometry as described by Marder et al. (P. Marder, A. Hinson, C. Russo, S. Ferrone, and E. Ades, Immunobiology, in press). In brief, individual groups of cells were treated with fluorescein-tagged goat anti-mouse immunoglobulin M (Cappel Laboratories, Cochranville, Pa.) or monoclonal fluorescein-conjugated anti-Thy 1.2, anti-Lyt 1, or anti-Lyt 2 (Becton Dickinson and Co., Mountain View, Calif.) to enumerate total B cells, total T cells, helper T cells, and cytotoxic-suppressor T cells, respectively. Flow cytometry was performed on an EPICS V cell sorter (Coulter Electronics, Inc., Hialeah, Fla.) equipped with an argon ion laser emitting 800 mW of power at 488 nm. For each sample the log of fluorescence intensity from 10,000 light scattered cells was collected. The resultant histograms were integrated above channel 20 to determine the cells that were positively fluorescent.

Mitogen reactivity. For the mitogen studies, cells (5×10^5) were incubated with or without the designated mitogen in a volume of 0.2 ml of Click medium supplemented with 0.5% normal mouse serum for a total of 72 h. At 18 h before harvest, 1 μCi of tritiated thymidine (15 Ci/mmol) was added in 50 μl of complete medium. Cultures were collected by precipitation onto glass-fiber filters with a 12-place mechanical harvester. Filters were placed in plastic scintillation vials containing standard scintillation fluid and counted for ^3H activity in a Beckman LS 3100 scintillation counter. The

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TABLE 1. Lymphocyte cell surface antigens of Cu⁻ and Cu⁺ mice^a

Expt.	Status	Total no. of splenocytes (10 ⁶)	SIG ^b		Thy 1.2		Lyt 1		Lyt 2	
			Total no. of cells (10 ⁶)	Relative %	Total no. of cells (10 ⁶)	Relative %	Total no. of cells (10 ⁶)	Relative %	Total no. of cells (10 ⁶)	Relative %
1	Cu ⁺	42 ± 3	16 ± 1	39.4 ± 0.7	20.0 ± 1.3	48.6 ± 0.6	15.0 ± 1.0	34.8 ± 0.7	5.3 ± 0.4	13.3 ± 0.4
	Cu ⁻	64 ± 9 ^c	33 ± 6 ^c	51.2 ± 2.0 ^c	22.0 ± 2.0	34.4 ± 0.8 ^c	15.0 ± 1.6	23.5 ± 1.3 ^c	6.5 ± 0.8	10.3 ± 0.6 ^c
2	Cu ⁺	74 ± 9	41 ± 8	52.0 ± 1.9	29.0 ± 3.6	43.0 ± 1.1	22.0 ± 3.0	29.8 ± 0.9	7.0 ± 0.8	10.0 ± 1.2
	Cu ⁻	171 ± 9 ^c	117 ± 16 ^c	68.0 ± 2.7 ^c	32.0 ± 3.2	19.0 ± 1.1 ^c	18.0 ± 1.7	10.6 ± 1.4 ^c	14.0 ± 2.1 ^c	8.0 ± 0.6
3	Cu ⁺	61 ± 7	26 ± 3	42.0 ± 1.3	39.0 ± 4.7	63.0 ± 1.6	24.0 ± 3.2	40.1 ± 1.1	8.7 ± 1.9	14.3 ± 1.7
	Cu ⁻	43 ± 6 ^c	24 ± 4	56.0 ± 2.4 ^c	15.0 ± 1.6 ^c	36.0 ± 2.9 ^c	10.0 ± 1.4 ^c	24.0 ± 1.3 ^c	5.3 ± 0.9	12.3 ± 0.8

^a Single-cell suspensions of spleen cells from Cu⁺ and Cu⁻ mice were enumerated by flow cytometry as described in the text. Values are the means ± the standard error for five animals per group.

^b SIG, Surface immunoglobulin.

^c Mean comparisons by Student's *t* test indicated significant differences (*P* < 0.05).

incorporation of [³H]thymidine into DNA was expressed in counts per minute. Phytohemagglutinin (Difco Laboratories, Detroit, Mich.), concanavalin A (Sigma Chemical Co., St. Louis, Mo.), and lipopolysaccharide B (Difco Laboratories) were used at concentrations of 0.5, 0.1, and 50 µg per microtiter well, respectively.

Results of triplicate samples were expressed as mean counts per minute ± the standard deviation. A stimulation index (SI) for mitogen reactivity analyses was determined for some experiments by the formula: SI = counts per minute of cell culture at the optimal dose of mitogen/counts per minute of cell culture without mitogen.

RESULTS

Mice fed the copper-deficient diet without supplementation (Cu⁻) developed visible signs of copper deficiency including hypopigmentation. Verification of the Cu⁻ state was confirmed by measuring ceruloplasmin activity in plasma. Ceruloplasmin values were negligible in the Cu⁻ group (less than 0.4 U/liter) compared with a mean value of 22.2 U/liter for mice given the copper supplement in their drinking water (Cu⁺). Chronic dietary copper deficiency often, but not always, led to enlarged spleens based on fresh weight compared to Cu⁺ mice. The Cu⁻ mice used for lymphocyte enumeration in these studies showed this variable response (Table 1).

Splenomegaly, as indicated by an increase in spleen size and in total splenocyte number, was observed in two of the experiments depicted here (Table 1). In all experiments there was a statistically significant increase in the number of surface-immunoglobulin-bearing (B) cells associated with copper deficiency, in terms of relative number of cells per spleen. The absolute number of splenic B cells in Cu⁻ mice was usually (see experiments 1 and 2 in Table 1) at a level two to three times that seen in Cu⁺ controls. The absolute number of cells bearing the Thy 1.2 marker was not significantly changed by copper deficiency when splenomegaly was observed, although the relative percentage of these T cells was below control values in all experiments (Table 1). The enumeration of Lyt 1-positive T cells indicated that the relative decrease in the total T-cell population was mainly within this subset (predominantly T helper cells). The data also showed that the absolute number and relative percentage of the Lyt 2-positive T cells (cytotoxic-suppressor cells) were not reproducibly changed by copper deficiency.

Fluorescence profiles of representative mice indicated that the density of surface antigen labeling was altered by

dietary copper deficiency (Fig. 1). Copper deficiency appears to diminish those T-cell subsets that would normally possess the higher fluorescence intensity. Interestingly, the B-cell profile, except for the numerical differences, appears similar for both Cu⁻ and Cu⁺ mice.

Another intriguing observation noted with the Cu⁻ animals was that a great number of large (presumably blastoid) cells were routinely observed with chronic copper deficiency. The typical cytofluorometric analysis was gated to count all cells that were approximately 7 µm in diameter. The fluorescence of many cells in spleens of Cu⁻ animals was not counted because they exceeded the gated size. Microscopically, these cells appeared to be blastoid in nature and may represent immature types. Of the total splenocytes in a typical Cu⁻ spleen, 30 to 40% would be such cells, whereas a typical Cu⁺ spleen would have no more than 15%.

The lymphocyte reactivity of the cells used for fluorescence analysis was measured in terms of reactivity to the T-cell mitogens phytohemagglutinin and concanavalin A and to the B-cell mitogen lipopolysaccharide (Table 2). Interpretation of mitogen reactivity was complicated by the reproducible observation that [³H]thymidine incorporation by Cu⁻ splenocytes in the absence of mitogen was significantly elevated (1.7- to 4.3-fold) compared with incorporation by Cu⁺ splenocytes. Nevertheless, in most cases decreased mitogen reactivity was observed both in terms of total counts per minute and in terms of SIs for all three mitogens (statistically significant in six of the nine cases presented in Table 2).

DISCUSSION

These results clearly show that chronic dietary deficiency of copper can influence the lymphocyte population of the spleen. When splenomegaly was observed, the predominant finding was a large increase in B cells, whereas total T cells remained at levels equivalent to those of the control. Consequently, a major shift in the ratio of B cells to T cells was evident in spleens of Cu⁻ mice. Furthermore, even when spleen size was small in Cu⁻ mice, the B-cell compartment, relative to that of T cells, was high, suggesting some fundamental alteration in lymphocyte differentiation associated with copper deficiency. The variability in splenomegaly reported in these studies confirms other work published earlier on C58 mice (19, 20) and C57 black and Swiss white mice (11, 12).

The mechanisms leading to an increase in the total number

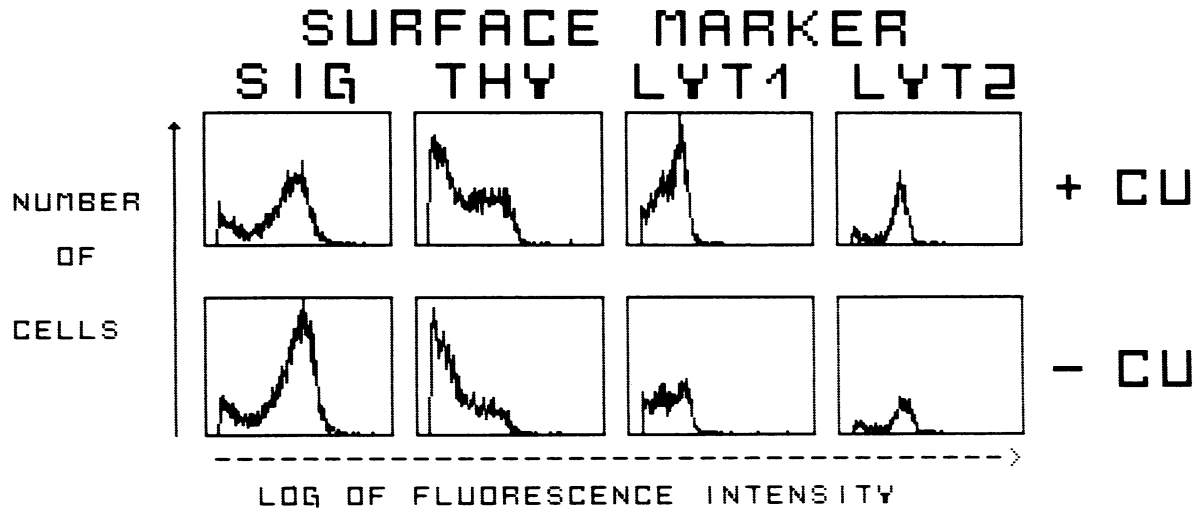


FIG. 1. Surface antigens on splenocytes from Cu^- (-CU) and Cu^+ (+CU) C58 mice. Single-cell suspensions of 6- to 8-week-old Cu^- spleens were compared with age-, sex-, and weight-matched Cu^+ controls. Samples (10^6) of splenocytes were treated with fluoresceinated goat anti-mouse immunoglobulin M (Cappel Laboratories) for detection of surface immunoglobulin (SIG)-bearing cells or with fluoresceinated, monoclonal anti-Thy 1.2 (THY), anti-Lyt 1 (LYT1), or anti-Lyt 2 (LYT2) (Becton Dickinson and Co.) to determine total T cells, helper cells, and cytotoxic-suppressor cells, respectively. Each histogram represents the fluorescent analysis of 10,000 viable lymphocytes as selected by low-angle forward-light-scatter gating. The log of fluorescence intensity is shown in 256 channels along each abscissa versus the number of cells (125 = full scale) on the ordinate. Histograms were integrated above fluorescence channel 20 to determine positive percentages in Table 1. The figures shown are representative data from one experiment.

of B cells and the frequent splenomegaly associated with the Cu^- state are yet to be determined. In this context, two possibilities based on recent observations may be considered. Smith et al. (22) recently have shown that the thymus regulates B-cell number and function in BXSB mice, a strain manifesting a lupus-like autoimmune disease. Neonatal thymectomy in these mice can lead to excessive B-cell number. Dietary copper deficiency in C58 mice leads to thymic atrophy (19) and depressed thymocyte mitogenic reactivity (O. A. Lukasewycz and J. R. Prohaska, unpublished data). If copper deficiency leads to a "functional thymectomy" as suggested by these previous results, the B-cell proliferation could be attributed to lack of thymic regulation. Vyas and Chandra recently have shown decreased serum thymic factor activity in copper-deficient rats (23). Another possibility relates to the finding by Roth and Koshland (21) which indicates that a copper-dependent enzyme is necessary for the formation of the J chain in the production of immunoglobulin M. Insufficiency of copper might lead to B-cell proliferation due to lack of a sufficient feedback signal by the formed immunoglobulin M.

The results of the mitogen studies in these experiments are in accord with previous findings which indicated a decreased mitogenic responsiveness and showed the level of anergy to concanavalin A to be related to the degree of copper deficiency (16). Of particular interest was the observation that increased numbers of B cells did not engender a greater mitogenic response to lipopolysaccharide B. It is possible that the relative decrease in helper T cells may reduce interleukin 2-like proliferative lymphokines in these cultures. Flynn et al. recently showed that interleukin production is decreased in lymphocytes cultured in vitro under copper-deficient conditions (8).

Previous observations from our laboratory and from others show that dietary copper deficiency causes not only a depressed humoral immune response but an impaired cell-mediated immune response as well. Furthermore, the suppression of immunity appears to be directly proportional to the severity of copper deficiency (16, 20). Chronic copper deficiency alters the size, biochemistry, and morphology of both primary and secondary lymphoid tissue (19). Copper-deficient mice have small thymus glands and enlarged spleens

TABLE 2. Immunological reactivity of lymphocytes from Cu^- and Cu^+ mice^a

Expt	Status	Immunological reactivity (means \pm SD) when stimulated by ^b :						
		No mitogen (cpm)	PHA		ConA		LPS	
			cpm	SI	cpm	SI	cpm	SI
1	Cu^+	4,485 \pm 579	114,104 \pm 5,374	25.4	177,101 \pm 7,698	39.5	35,422 \pm 3,064	7.9
	Cu^-	14,966 \pm 3,058 ^c	60,411 \pm 30,833 ^c	4.0	48,756 \pm 41,068 ^c	3.3	28,772 \pm 8,294	1.9
2	Cu^+	4,910 \pm 408	92,420 \pm 4,479	18.6	148,450 \pm 4,037	30.2	31,730 \pm 1,190	6.5
	Cu^-	8,190 \pm 1,075 ^c	26,290 \pm 3,760 ^c	3.2	66,210 \pm 14,615 ^c	8.0	23,410 \pm 1,490 ^c	2.9
3	Cu^+	1,920 \pm 443	92,279 \pm 13,464	48.1	36,115 \pm 8,686	18.8	22,942 \pm 9,005	11.9
	Cu^-	8,213 \pm 1,093 ^c	23,017 \pm 11,986 ^c	2.8	24,855 \pm 13,694	3.0	17,846 \pm 4,891	2.2

^a Single suspensions of spleen cells from Cu^+ and Cu^- mice were stimulated by the indicated mitogens as described in the text. Values represent triplicate counts of five animals.

^b PHA, Phytohemagglutinin; ConA, concanavalin A; LPS, lipopolysaccharide B; SI, stimulation index.

^c Mean comparison by Student's *t* test indicated significant differences ($P < 0.05$).

relative to controls. These tissues show low activities of two cuproenzymes, cytochrome oxidase and superoxide dismutase, suggesting a functional copper deficiency. Abnormal mitochondria and misshapen nuclei are also associated with copper deficiency (19).

It appears that copper plays an important role in the maturation sequence of lymphoid tissues and that deficiency of copper early in the developmental continuum leads to impairment of the immune system. This impairment is manifested by lymphocyte subpopulation shifts and a diminished immunoreactivity. This may help explain the high level of infection seen in humans and animals with copper insufficiency.

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