Dietary Copper Deficiency Increases the Mast Cell Population of the Rat (43816)

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Abstract. **Mast cell-released histamine has been implicated in the enhanced acute inflammatory response of copper-deficient rats. The present study examined possible changes in the copper-deficient mast cell which may account for increased macromolecular leakage and edema formation. Mast cell populations were determined in the cremaster muscle of copper-adequate and copper-deficient rats. Total histamine content, unstimulated histamine release and concentration-dependent histamine release with the mast cell secretagogue compound 48/80 were also determined in isolated peritoneal mast cells. A significantly higher number of mast cells were found in the cremaster muscle of the copper-deficient rats (78 ± 7 cells/5 μm section) than in the copper-adequate controls (51 ± 4). Total histamine content per cell as well as unstimulated and stimulated release of the inflammatory mediator per cell were not different between the groups. The results suggest that dietary copper deficiency increases the mast cell population but does not alter the mast cell histamine content or sensitivity to degranulation in the rat. This increase in the number of mast cells may be a mechanism by which acute inflammation is enhanced in copper deficiency.**

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Mast cells have a physiological role as an effector of host defense mechanisms, and their degranulation is an important step in acute hypersensitivity reactions. The release of inflammatory mediators such as histamine and serotonin from the mast cell cause an increase in vascular permeability of post-capillary venules and associated edema formation.

Dietary copper deficiency is known to increase the acute inflammatory response in experimental animals (1–7). In rats, carrageenan challenge resulting in greater paw edema formation (2, 5) is significantly increased by copper deficiency. *In vivo* microscopy studies showed that the mast cell degranulating agent compound 48/80 significantly increases macromolecular leakage from post-capillary venules in the cremaster muscle of copper-deficient rats (6, 7).

In our previous work on acute inflammation in copper-deficient rats (6, 7), we demonstrated that there was not a generalized change in receptor sensitivity to inflammatory mediators. In addition, endothelial barrier mechanisms which normally restrict macromolecular leakage were intact. We concluded that increased macromolecular leakage in copper deficiency was associated with mast cell degranulation by a primary effect on the mast cell rather than on the endothelium (7).

In the present study, we have examined mast cells from copper-deficient rats to determine what changes may have occurred to account for the enhanced acute inflammatory response (6, 7). The *in vivo* rat cremaster muscle preparation and *in vitro* isolated peritoneal mast cells were used to examine mast cell numbers, mediator content, and sensitivity to secretagogues all of which could contribute to altered mast cell functions (8, 9).

Materials and Methods

Approval for this project was obtained from the University of Louisville Animal Care and Use Com-

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mittee. Male, weanling Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed in stainless steel cages in a temperature- and humidity-controlled room with a 12:12-hr light:dark cycle. They were given distilled water to drink and were fed *ad libitum* a purified diet (7) that was made copper adequate by addition of 6 μg of copper per gram of diet (CuA diet) or was left deficient by not adding copper (CuD diet). The animals were maintained on their respective diets for 4 weeks.

The CuA diet corresponded closely to the AIN-76 diet recommended for rats with the copper content equal to that recommended by the National Research Council (10). Growth and reproduction for rats fed the AIN-76 diet are similar to that for rats fed commercial nonpurified diet. Diet analysis by atomic absorption spectrophotometry indicated that the CuA diet contained 6.3 μg copper/g diet and the CuD diet contained 0.6 μg copper/g diet. Parallel assays of National Institute of Standards and Technology (NIST) reference samples (citrus leaves, #1572) yielded values within the specified range, which validated our mineral assays.

In the first protocol, mast cells were counted in the cremaster muscle preparations. The preparation was identical to that previously used to identify an enhanced macromolecular leakage response to the mast cell secretagogue, compound 48/80 (6, 7). Rats were anesthetized with pentobarbital (50 mg/kg ip) and had a tracheal cannula inserted to maintain a patent airway. The skin of the right scrotum was opened and the cremaster incised longitudinally, keeping the principal nerves and blood vessels to the muscle intact. The cremaster was spread with sutures over a cover slip in the bottom of a specially designed Plexiglas bath containing modified Krebs solution (in m*M:* NaCl, 113; $NaHCO_3$, 25; glucose, 11.6; KCl, 4.7; $CaCl_2 \cdot H_20$, 2.6; $MgSO_4 \cdot 7H_20$, 1.2; KH_2PO_4, 1.2). The Krebs solution was replaced every 15 min and was maintained at pH 7.4 ± 0.05 by bubbling nitrogen and carbon dioxide into the bath. An indwelling heater coil with a negative feedback system was used to maintain the bath at 35° ± 0.5°C. Animals were placed on a heating pad to maintain rectal temperature at 37°C. After a period of 60 min, the Krebs solution was replaced with 10% neutral buffered formalin, and the cremaster was fixed in the bath for 20 min. The cremaster muscle was then removed, further fixed in additional neutral buffered formalin overnight, and then embedded in paraffin. The cremasters were serial sectioned in 5-micron cross-sections through the center of the muscle perpendicular to the long axis of the muscle. Mast cells were stained with toluidine blue. All of the stained mast cells in each cross-section of muscle were counted by light microscopy. Two or three sections were counted per animal in six CuA rats and seven CuD rats. The values presented in Table II are the mean number of mast cells per 5-micron section per animal.

In the second protocol the histamine content of peritoneal mast cells and the percent histamine released from these cells by degranulation with compound 48/80 were determined. Eight CuA and eight CuD rats were used for this protocol. Rats were anesthetized with ketamine/xylazine (37.5/5 mg/kg im) and exsanguinated by cardiac puncture. Crude peritoneal cell suspensions were obtained by lavage with about 20 ml HEPES-buffered salt solution with 0.1% bovine albumin (HBSS-BA) having the composition (m*M*): NaCl, 137; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 0.5; NaH_2PO_4, 0.4; glucose, 5.6; HEPES, 10.0. The cells were washed twice with HBSS-BA and isolated by centrifugation at 125*g* for 15 min in Percoll (11). The cells were then washed with HBSS-BA and resuspended in HBSS-BA to a concentration of 10^5 cells/ml. To stimulate histamine release, the mast cell degranulator 48/80 (Sigma Chemical Co., St. Louis) was added to cell suspensions to give a final concentration of 0 (control), 0.01, 0.03, 0.1, and 1.0 μg/ml. Ten microliters of the releasing agents in HBSS-BA were added to 1-ml aliquots of cells and secretion was allowed to proceed for 30 min at room temperature; nonstimulated cells received an equal volume of HBSS-BA. The secretions were then terminated by placing the tubes on ice and adding 3 ml of ice-cold HBSS-BA. Cells and supernatants were recovered by centrifugation at 4°C for 2 min at 125*g*. The cell pellets were resuspended in 4 ml HBSS-BA and both cell suspensions and supernatants were allowed to stand in a boiling water bath to release residual histamine (12). Histamine was determined fluorimetrically in the supernatants and cell suspensions and in histamine standards (0.03–3 μg/ml) by the method of Shore *et al.* (13) but without the extraction steps. The amount of histamine was determined from the histamine standard curve and was expressed as the amount of histamine released in $\mu g/10^6$ cells. Stimulated secretion was expressed as the percentage of histamine in the supernatant as part of the total histamine (supernatant histamine + residual cell histamine).

Copper status of the rats was determined from liver samples. The median lobe of the liver was collected from each rat and frozen for subsequent analysis. The livers then were lyophilized and digested in nitric acid and hydrogen peroxide (14). Copper content was determined by inductively coupled plasma emission spectrometry. Parallel assays of reference sample #1577a (bovine liver) from the NIST yielded mineral contents within the specified ranges.

Statistical analysis compared groups by using Student's *t* test for unpaired data. Differences were considered significant at $P < 0.05$. Values are means ± SEM.

Results

Rats fed the CuD diet for 4 weeks became copper-deficient as indicated by significantly lower hepatic copper concentrations and hematocrit (Table I). However, there was no difference in growth rate of the animals as demonstrated by a comparison of body weight between the two groups.

The results of the count of cremaster mast cells is shown in Table II. The values represent the number of mast cells per 5-micron cross-section of the cremaster muscle. The results demonstrate a significantly greater number of mast cells in the cremaster of CuD rats as compared with the CuA rats after 4 weeks on their respective diets.

Assay of histamine content in mast cells isolated from the rat peritoneum did not demonstrate a difference between dietary groups (Table II).

Spontaneous release of histamine from the isolated mast cells was not different between the CuA and CuD groups (Fig. 1, control values). Likewise, there was no difference in histamine release to compound 48/80 at any concentration of the degranulating agent used (Fig. 1).

Discussion

The present study demonstrates that 4 weeks of dietary copper deficiency caused a significant increase in the number of mast cells in the rat cremaster muscle compared to age-matched copper-adequate animals (Table II). This larger mast cell population occurred in the same tissue where an enhanced acute inflammatory response to mast cell degranulation has been reported in CuD animals (6, 7). The increased number of mast cells is coincident with an increase in number of platelets (15) which also come from precursor stem cells. Conversely, anemia, as identified by a depressed hematocrit (Table I) and hemoglobin, has been shown to occur in copper-deficient rats compared with copper-adequate controls (16). An increase in the number of mast cells and platelets and a decrease in red blood cells suggests a role for copper in hematopoiesis and differentiation of stem cells. Others have shown that mast cell populations can demonstrate heterogeneity

Table I. Characteristics of Rats Fed Copper-Adequate (CuA) and Copper-Deficient (CuD) Diets for 4 Weeks

Parameter	CuA (n = 6)	CuD (n = 7)
Body wt (g)	225 ± 6	224 ± 15
Liver copper (μg/g dry wt)	10.14 ± 0.28	1.20 ± 0.23*
Hematocrit, %	39.3 ± 1.4	28.9 ± 2.9*

Note. All values are mean ± SEM.
* $P < 0.05$ compared with copper adequate animals.

TABLE II. Cremaster Mast Cell Concentration and Peritoneal Mast Cell Histamine Content in Copper-Adequate (CuA) and Copper-Deficient (CuD) Rats

Parameter	CuA	CuD
Cremaster mast cells (Protocol 1) (number/5-μm cross-section)	51 ± 4 (6)	78 ± 7 (7)*
Histamine content (Protocol 2) (μg/10^6 peritoneal cells)	45 ± 2 (8)	51 ± 8 (8)

Note. All values are mean ± SEM (n).
* $P < 0.05$ compared with copper-adequate animals.

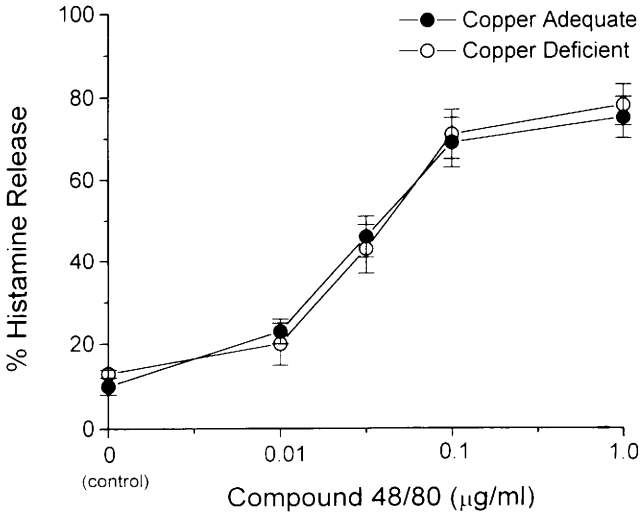

Figure 1. Comparison of histamine released from mast cells of copper-adequate and copper-deficient rats. Mast cells were stimulated by vehicle (control) only or by increasing concentrations of the mast cell degranulator compound 48/80. Values are means ± SEM; n = 8 CuA, 8 CuD.

to several phenotypic characteristics, including mediator content, sensitivity to agents that induce activations, and mediator release (8, 9, 17). These, as well as other mast cell characteristics such as stage of maturation, differentiation, and proliferation, are regulated by many factors and are subject to alterations in the cell's microenvironment (8, 9, 17).

Previous work in the rat cremaster muscle microcirculation of copper-deficient rats demonstrated that topical application of the histamine H_1-receptor blocker diphenhydramine in the bath significantly decreased the macromolecular leakage response to compound 48/80 in both CuA and CuD rats (7). Diphenhydramine also eliminated the significantly higher macromolecular leakage in CuD animals compared with CuA animals. These results lead us to conclude that histamine was the primary mediator of the enhanced inflammatory response resulting from mast cell degranulation (7). In the present study, we determined that the histamine content of isolated peritoneal mast cells from CuD animals was not different than the his-

tamine in mast cells from CuA rats (Table II). There was also no difference between groups in the release of histamine from unstimulated mast cells (Fig. 1, control). This would correspond with previous *in vivo* work which showed that there was no spontaneous macromolecular leakage in either CuD or CuA animals (6, 7). The amount of histamine released by compound 48/80 from rat peritoneal mast cells is similar to that previously reported using this technique (12).

The sensitivity of mast cells to degranulation by the secretagogue compound 48/80 was also tested as a possible source of the enhanced acute inflammatory response associated with dietary copper deficiency. Concentration-response curves for a range of 48/80 concentrations demonstrated that there is no difference in histamine release from peritoneal mast cells at any concentration of 48/80 tested. This data also concurs with results from cremaster studies where there was no difference in the threshold dose of 48/80 that induced macromolecular leakage (6, 7). Extrapolation to other pharmacological and immunological stimuli of mast cell degranulation can be made as a wide variety of stimuli are believed to act by the common pathway of increased free calcium in the cell cytosol (12). Thus, it is unlikely that histamine release from CuD mast cells to other stimuli would be different from that presented here.

The data in the present study demonstrate that dietary copper deficiency causes an increase in the number of mast cells in the cremaster muscle of rats. There is, however, no change in histamine content of the mast cell nor in the amount of histamine released, either stimulated or unstimulated. Based on this data, we propose that copper deficiency preferentially increases the number of mast cells and that it is the increased mast cell population that accounts for the enhanced acute inflammatory response seen in the cremaster muscle microcirculation of copper-deficient rats.

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