Copper and immunity\textsuperscript{1,2}

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ABSTRACT The immune system requires copper to perform several functions, of which little is known about the direct mechanism of action. Animal models and cells in culture have been used to assess copper’s role in the immune response. Some of the recent research showed that interleukin 2 is reduced in copper deficiency and is likely the mechanism by which T cell proliferation is reduced. These results were extended to show that even in marginal deficiency, when common indexes of copper are not affected by the diet, the proliferative response and interleukin concentrations are reduced. The number of neutrophils in human peripheral blood is reduced in cases of severe copper deficiency. Not only are they reduced in number, but their ability to generate superoxide anion and kill ingested microorganisms is also reduced in both overt and marginal copper deficiency. This mechanism is not yet understood. Neutrophil-like HL-60 cells accumulate copper as they differentiate into a more mature cell population and this accumulation is not reflected by increases in Cu/Zn superoxide dismutase or cytochrome-c oxidase activities. The identity of copper-binding proteins in this cell type may be useful in learning new functions of copper or assessing copper status. Neutrophils, because they are short-lived and homogeneous cell populations, are predicted to be an effective and valuable tool for assessing nutrient status in human populations.

KEY WORDS Copper, immunity, mitogen proliferation, phagocytes, HL-60 cells, neutrophils, Cu/Zn superoxide dismutase, copper binding proteins

MODELS OF COPPER DEFICIENCY

Copper is known to play an important role in development and maintenance of the immune system but its exact mechanism of action is not yet known. The objective of this review is to examine some of the recent research that has contributed new knowledge to this field. The animal model most commonly studied is the rodent. Immunity has been examined in rodents using two different paradigms of copper deficiency. One paradigm places animals on a deficient diet when they are weaned (at \textasciitilde 21 d old). The observations, then, are made on an immune system that is fairly well established and, as a result, conclusions are drawn about how copper deficiency influences the animal’s ability to maintain its immunity. The other model places pregnant animals on a deficient diet shortly before or on the day of parturition. Pups are weaned to the deficient diet and fed for another 2–6 wk before they are killed. This perinatal model reflects the effect of copper deficiency on the neonatal development of the immune system. We cannot necessarily apply the findings of one paradigm to the other.

Cell culture is another model in which to study immunity. There are several established cell lines as well as transformed cell lines that are used to study growth and development of the immune system; in addition, cell cultures can be used to study activation or a particular function of an immune cell apart from the host’s hormonal influence.

A model that we used in our studies on neutrophil differentiation is the HL-60 cell model. This continuous cell line can terminally differentiate into neutrophil-like cells by incubating them with retinoic acid. Although the cell itself is leukemic, the process of differentiation is thought to be normal (1). We have characterized copper homeostasis during this process. In addition, we have attempted to influence this process by supplementing the cultures with copper, ceruloplasmin, or a copper chelator, tetraethylenepentamine (TEPA).

APPROACHES TO STUDYING THE IMMUNE SYSTEM

Studies examining copper’s role in immunity can be categorized into three strategies. First, one could quantitate the components of the immune system, such as the leukocytes, antibodies, and effector molecules, eg, cytokines. This would provide a picture of the immune system at a given time point. Second, one could perform a functional analysis of the immune system. Common functional measures of leukocytes are their ability to migrate to the site of action, phagocytose particles or microorganisms, and generate superoxide anion or other chemicals in the host’s arsenal designed to kill invading microorganisms. Physiologically, these are relevant to the purpose of the immune system. Third, one could measure a general immune response to a challenge. For example, edema and delayed hypersensitivity are common measures that require a challenge to the immune system.

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system. Quantitating the proliferation of T and B cells is a functional analysis that requires the cells to respond to a stimulus. Researchers are still defining and characterizing the effect of copper deficiency using a variety of these strategies.

**EFFECTS OF COPPER DEFICIENCY ON IMMUNE FUNCTION**

The immune system is conventionally divided into two systems, innate and acquired. In 1992 an extensive review on copper and these branches of immunity was published by Prohaska and Failla (2). These authors discussed clinical and experimental evidence that underscored the importance of copper for immunity. This article will cover research that has been published since that review.

**Specific acquired response**

The specific acquired immune system is the portion of the immune system that comes into action when a foreign invasion occurs, hence the name *acquired*. The host must devise specific responses to individual organisms. Lymphocytes are components of the acquired response that include the T and B cells. When blood cells are isolated by density-gradient centrifugation, both these cell types sediment as the mononuclear cell layer. These two cell types are responsible for synthesizing and secreting antibodies, providing memory, activating the classical complement cascade, and killing invading microorganisms. Proliferation of these cells is an important step in fighting invasion.

Copper deficiency, in general, reduces the effectiveness of the acquired response (3–5). Early studies of copper deficiency, in which ceruloplasmin was almost nondetectable, determined that the copper-deficient animals were anemic, their thymus weights were significantly lower, and their spleen weights were significantly higher than in control animals (4). Antibody production by spleen cells was significantly reduced in copper-deficient animals (4, 5). Splenocytes in copper-deficient animals had a reduction in the incorporation of tritiated thymidine into cellular DNA in a standard mitogenic proliferation assay. Recently, a mechanism for this impaired proliferation response was proposed that involved cytokines.

Cytokines are the components of the immune system responsible for communication between different cells of immunity. Lukasewycz and Prohaska (4) observed that interleukin 1 was significantly greater in copper-deficient animals whereas interleukin 2 was significantly reduced. Bala and Failla (6) extended these observations in the rat weanling model of copper deficiency. A copper-adequate diet (6 ppm Cu) and a copper-deficient diet (0.6 ppm Cu) were fed to weanling rats for 5 wk. Splenocytes were isolated and the proliferative response to mitogens was determined. The proliferative capacity of splenocytes in copper-deficient rats was significantly lower than in copper-sufficient control rats. Adding interleukin 2 back to the cultures brought the response back to normal. Adding copper to the cultures also restored the response although it did not reach control concentrations. The authors concluded that the mitogen-induced synthesis of DNA was impaired by copper deficiency and that this impairment stemmed from the reduction in the concentrations of interleukin 2. Exactly why interleukin 2 concentrations are reduced in copper-deficient animals is not yet known.

Alterations in the mitogenic response to interleukin 2 were also found in marginally copper-deficient rats (7). Pregnant rats were placed on a marginally copper-deficient diet (2.7 ppm) on the 11th day of gestation. This amount of copper was chosen to reflect the estimated amount consumed by the American public relative to the estimated safe and adequate daily dietary intake (1.5–3.0 mg Cu/d); the 1987–1988 Nationwide Food Consumption Survey estimated copper intakes of 0.86–0.95 mg/d in adult women and 1.18–1.23 mg/d in adult men (8). In the study in rats, common indexes of copper status such as organ tissue copper, serum copper, and ceruloplasmin activity were not reduced by this protocol (7). The bioactivity of interleukin 2 was 10–25% of control values in males but not significantly different in the females. Splenocytes from the males were unable to proliferate in response to mitogens. These rats were marginally copper deficient in utero for half of their gestation, therefore, the lack of copper exerted its effects on the development, as well as the maintenance, of the immune system. These findings underscore that immunity is impaired even when the standard indexes of copper status are normal.

**Innate system**

The innate system is the functional arm of the immune system that is considered the nonspecific branch. The skin, along with mucus layers in the nasal passages and the intestine that serve as barriers to microorganisms, are considered part of this system; research on the effect of copper deficiency on this portion of the innate system has not been done.

Another component of the innate system is a vast array of serum proteins known as the complement system. There are some 20 different proteins that are converted and activated to cause microbial cell lysis, some of which are antibody dependent. Research that has examined the effect of copper deficiency on the complement proteins or the cascade of events that occurs when a foreign invader is present has not been done.

Phagocytic cells are also components of the innate system. Phagocytes include the neutrophils, monocytes, eosinophils, and basophils. Neutrophils circulate in the blood whereas monocytes travel into tissues where they further differentiate into macrophages. It is known that copper deficiency has a profound effect on neutrophils and macrophages, but little is known about the effects on eosinophils and basophils. These latter two cell types are difficult to study because they are found in such small numbers. Basophils mature into mast cells in tissues. Schuschkke et al (9) found an increase in the mast cell population in the cremaster muscle of copper-deficient animals, suggesting that copper deficiency might alter the distribution of blood cells into tissues or the maturation patterns of the leukocyte population.

Neutropenia is a clinical sign of copper deficiency in humans (10, 11). It was first observed in the 1960s (12). Bone marrow aspirates from copper-deficient humans showed an increased number of promyelocytes and a decreased number of metamyelocytes and banded cells. This was interpreted as an arrest of maturation of granulocytes due to copper deficiency (13–15). Other potential mechanisms that could result in neutropenia include impaired secretion from the marrow, an early death of progenitor cells in the marrow, reduced life span of the circulating peripheral cells, and redistribution into tissues or organs (16). Higuchi et al (17) detected anti-neutrophil antibodies in the serum of copper-deficient patients, which might indicate a mechanism of neutrophil loss. In addition to a reduced number of circulating neutrophils in copper deficiency, the function of those neutrophils was impaired. Reduced superoxide anion production and reduced candidacidal activities without alterations in phago-
cytosis were found to result from copper deficiency (18, 19).
Similar changes were observed in macrophages (20). Marginal copper
deficiency also resulted in impaired neutrophil function.
In the perinatal model that was described previously, neutrophils
elicted from the peritoneal cavities of rats with marginal copper
deficiency generated 60% less superoxide anion than did those
of control rats (7). This again illustrates that immunity is
impaired even when indexes of copper status are normal. The
effect of marginal copper status on human health may be signifi-
cant, but our efforts to determine whether the general population
is marginally deficient have not been productive. A recent study
using low-copper diets in young men suggested that the require-
ment for copper fell somewhere in the range of 0.4–0.8 mg/d
(21); however, the optimal amount of copper one should con-
sume has not been established.

If copper-deficient neutropenia is an arrest of maturation in
the bone marrow, then this process can be investigated in the HL-
60 cell line that acquires mature neutrophil characteristics when
incubated with retinoic acid (1). We approached this concept in
two ways: 1) by evaluating changes in copper homeostasis during
differentiation, and 2) by varying medium copper concentra-
tion and evaluating the effect on the cells’ ability to differentiate.

Bae and Percival (22) found that on differentiation, cells incu-
bated with retinoic acid accumulate twofold more copper. The
rate of copper uptake was increased twofold after 24 h of retinoic
acid induction. The ability of the cells with retinoic acid to accu-
cumulate copper was greater than that of the HL-60 cells (22, 23).
Cu/Zn superoxide dismutase (SOD) activity was reduced in cells
with retinoic acid, but was recovered by copper supplementation
of the medium (24). The concentration of Cu/Zn SOD protein
was reduced by retinoic acid differentiation. Radioactive copper
was distributed into two peaks after fractionating the cytosol by
molecular weight chromatography. Cells with retinoic acid have
a greater proportion of copper in the high-molecular-weight frac-
tion than do HL-60 cells (23). Endogenous copper measured by
graphite furnace atomic absorption is also higher in these frac-
tions. We concluded that neutrophils accumulate copper that is
not accounted for by changes in Cu/Zn SOD or cytochrome-c
oxidase. The function of this additional copper is not known, but
it is found in the same concentrations as in the peripheral neu-
traphils isolated from human volunteers (22). Moreover, the copper
accumulation in retinoic acid–induced cells is greater than in
cells induced to differentiate along monocytic cells lines with
cholecalciferol (vitamin D3) (25). The identity of these proteins
needs to be determined if we are to understand the role of copper
in neutrophils and how it affects the ability of these cells to
differentiate and function.

A second approach we used to examine the influence of copper
on differentiation was to measure changes in the differentia-
tion process resulting from alterations in the copper status of
the cells. Cell culture media is relatively low in copper, \( \approx 0.5 \mu \text{mol/L} \)
(24). If copper is critical for granulopoiesis, then providing cop-
per to these cells during differentiation should enhance expres-
sion of mature characteristics. We first assessed differentiation as
the ability to produce the superoxide anion, also known as the res-
piratory burst. The components of the enzyme responsible for
generating superoxide anion are synthesized during retinoic
acid–induced differentiation of HL-60 cells (26). Incubating the
cells with 12 \( \mu \text{mol} \) copper sulfate/L or 2 \( \mu \text{mol} \) ceruloplasmin/L
during differentiation enhanced the cells’ ability to produce
superoxide anion (22). We then had to ask whether this increase
was due to an increase in the number of differentiated cells or a
greater activation of the burst itself. We assessed the morphology
of the cells by light microscopy and found that there was a greater
proportion of differentiated cells in the copper-treated cultures
(22). Therefore, we concluded that copper supplementation
enhanced retinoic acid-induced differentiation.

The opposing question was asked: If copper is removed from
the cell, is differentiation impaired or prevented? We hypothe-
sized that if copper is essential for differentiation, then chelation
of copper with TEPA should prevent the cells from differentiat-
ing. HL-60 cells were incubated with this chelator for 4–21 d
before adding the retinoic acid. The amount of copper was
reduced by 40–70% and the activity of Cu/Zn SOD was reduced by
60–80% (27). Differentiation was again assessed as the produ-
don of superoxide anion. Cells incubated with TEPA and
retinoic acid produced the same amount of superoxide anion as
did the cells with retinoic acid, indicating that differentiation had
occurred (27). These results were extended by Sergeant and John-
son (28) in HL-60 cells grown in media that had much lower cop-
per concentrations than did ours. In their study, the HL-60 cells
were cultured in serum-free medium that contained \( < 5 \mu \text{mol}
\text{Cu/L} \). The cells differentiated to the same degree as cells grown
in fetal bovine serum— or copper-supplemented serum-free
medium. Sergeant and Johnson interpreted their results to mean
that either the remaining copper in the cells was sufficient for dif-
ferentiation or the cells were already sufficiently differentiated
such that removal of copper did not affect subsequent differenti-
tion. So whereas our TEPA model is useful in some studies
related to manipulating copper concentrations and Cu/Zn SOD
activity, it does not prevent the HL-60 cells from differentiating.

The lack of effect of TEPA on HL-60 cell differentiation
prompted us to develop a mouse model to continue our investiga-
tion of copper’s role in granulopoiesis. We chose the perinatal
model in which the dams are started on a deficient diet on the day
of parturition. The diets were formulated as the AIN93G diet (29)
and contained 6.0 and 0.6 ppm of copper as determined by
graphite furnace atomic absorption spectrophotometry. The pups
were weaned to the same diet as their respective dam and were fed
the diet for 4 wk. The deficient pups had 10-fold less serum copper
and 100-fold less ceruloplasmin oxidase activity (Table 1). Cu/Zn SOD activity was reduced by 70% in the periph-
ernal leukocytes and 40% in the bone marrow cells. Peripheral neu-
traphils were assessed in whole blood by flow cytometry using
CD11b, a cell adhesion molecule found on peripheral leukocytes,
to characterize the neutrophils. CD11b is the molecule responsi-
able for adherence of granulocytes on the endothelial cell before dia-
pedesis into the tissue. The expression of CD11b in the copper-
deficient group was 50% of that found in the copper-adequate
group (Figure 1). It is not yet known whether the reduced expres-
sion of CD11b results in any functional impairment in neutrophil
adherence to the endothelium. Expression of CD11b is upregu-
lated in inflammation. Our future investigations will focus on neu-
traphil functions in inflammation in copper-deficient animals. We
will determine whether the amount of CD11b is reduced or
whether the expression on the cell surface is reduced.

COPPER AND IMMUNITY IN HUMAN STUDIES

In 1985, Heresi et al (30) studied immunoglobulins and phago-
cytosis in copper-deficient marasmic children. Eleven infants in
the study had \( < 800 \mu \text{g} \) plasma Cu/L and \( < 200 \text{mg} \) serum cerulo-
plasmin/L, whereas another eight infants had subnormal plasma copper but normal ceruloplasmin. These children were neither anemic nor neutropenic. The results of the analyses were obtained before and after 1 mo of copper supplementation. Concentrations of serum immunoglobulins G, M, and A, as well as salivary immunoglobulin A were not statistically different after copper supplementation. The phagocytic index, however, was significantly improved after supplementation, as measured by the number of bacteria ingested per isolated neutrophil. The impairment in phagocytosis due to copper deficiency was found regardless of serum factors because they used both autologous and normal homologous serum. Although these infants were copper deficient and their phagocytic index improved with copper supplementation, the authors also state that the interpretation of this study is complicated by the fact that the infants were malnourished.

The most complete examination of the role of copper in human immunity was published in 1995. Kelly et al (31) examined the effect of a low-copper diet on several indexes of immunity in 11 young men in a metabolically controlled trial. The study was designed to provide a diet of 0.66 mg Cu/d for 24 d, then deplete further with a diet of 0.38 mg Cu/d for another 40 d, and then replete with 2.49 mg Cu/d for the remaining 30 d. Peripheral white blood cell numbers, phagocytosis, mononuclear cell proliferation, and interleukin 2 receptor concentrations were determined. The ability of the peripheral blood mononuclear cells to proliferate was significantly reduced at the end of the 0.38-mg/d diet relative to the start of the study as well as relative to the end of the 0.66 mg Cu/d-diet. The diet containing 2.49 mg Cu/d prevented any further decline, but did not restore the proliferative response to what it was at the beginning of the study.

These authors also measured interleukin 2 receptor concentrations in the serum and in the culture medium of cells that had been cultured in vitro. Interleukin 2 receptors are shed by the peripheral blood mononuclear cells and indicate early T cell activation, that is, the change from G0 to G1 in the phases of the cell cycle. Concentrations in the culture medium, but not in the subject’s serum, were significantly reduced at the end of the 0.38-mg Cu/d diet, and did not return to prestudy concentrations at the end of the 2.49 mg Cu/d diet.

The B cells in the periphery were significantly increased at the end of the 0.38-mg Cu/d diet and also did not return to prestudy concentrations at the end of the 2.49-mg Cu/d diet. This observation was also reported in animal studies on copper-deficient diets (32).

Phagocytosis was measured by histologic examination and light microscopy in whole blood samples. Neither the percentage of neutrophils involved in phagocytosis nor the number of bacteria engulfed was altered. Another report in young men on the same low-copper diets showed that the copper concentrations of the neutrophils reflected the amount of copper in the diet (21).

In addition, Kelley et al (31) reported what did not change as a result of the diet. These diets did not result in any alteration in the peripheral blood cell numbers of leukocytes, monocytes, neutrophils, lymphocytes, or natural killer cells.

The authors believe that the changes that they did observe were due to the low amount of dietary copper, however they did not rule out other dietary or nondietary factors that may have changed the immune response. The fact that the 2.49-mg Cu/d diet prevented further decreases in the response substantiates the belief that it was indeed the effect of low copper. A longer time of repletion (> 24 d) or a higher concentration of copper may be needed to restore and clarify copper’s role in human immunity. Adults appear to be resistant to change, both in depletion and repletion.

### SUMMARY AND FUTURE DIRECTIONS

We have opportunities to find new functions for copper. In the retinoic acid–differentiated cells, there is a potential to discover new copper-binding proteins. This research will perhaps help us define nutrient requirements for cell differentiation. New functions for copper will open the door for new status measures.

Immune cells may be a sensitive means by which to measure nutrient status. Cu/Zn SOD can be readily manipulated in cells in culture by copper supplementation and by TEPA chelation. Furthermore, Cu/Zn SOD is readily measured in neutrophils and mononuclear cells from humans. It will become important to know whether or not this index is affected by other conditions unrelated to copper nutriture, such as inflammation or infection.

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**TABLE 1**

<table>
<thead>
<tr>
<th>Indicators of copper status in copper-adequate and copper-deficient mice</th>
<th>Copper-adequate mice</th>
<th>Copper-deficient mice</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma copper (μmol/L)</td>
<td>12.0 ± 4.2</td>
<td>1.22 ± 0.71</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Ceruloplasmin activity (u/L)</td>
<td>17.08 ± 4.52</td>
<td>0.16 ± 0.31</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Bone marrow Cu/Zn SOD</td>
<td>1533.4 ± 384.1</td>
<td>934.7 ± 271.5</td>
<td>0.022</td>
</tr>
<tr>
<td>Leukocyte Cu/Zn SOD</td>
<td>122.7 ± 29.8</td>
<td>44.5 ± 17.7</td>
<td>0.005</td>
</tr>
</tbody>
</table>

1 Cu/Zn superoxide dismutase (SOD) activity is expressed in u/mg cell protein where 1 unit = 50% inhibition of the oxidation rate of pyrogallol.

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**FIGURE 1.** Percentage of peripheral leukocytes expressing the cell adhesion molecule designated as CD11b. The determination was made by flow cytometry using the fluorescein isothiocyanate–labeled antibody against CD11b. $\bar{x} \pm$ SD.
before its utility as a status measure can be established.

Neutrophils are, in theory, good cells to use as a status indicator. The cells have a rapid turnover of \( \approx 3-5 \) days and are terminally differentiated in the peripheral blood. They are homogeneous in comparison with the mononuclear cells, which are made up of several T cell subsets as well as various populations of memory cells. These cells may have life spans on the order of years, thus making repletion difficult to study. Eventually, one of our goals is to be able to measure status using a copper-dependent enzyme or a copper-specific cellular function in the neutrophil population. Much remains to be done in this area.

**REFERENCES**


