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Review article

The immune system as a physiological indicator of marginal copper status?

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Cu appears to have many important functional roles in the body that apparently relate, among others, to the maintenance of immune function, bone health and haemostasis. Some have suggested a role for long-term marginal Cu deficiency in the aetiology of a number of degenerative diseases. Accurate diagnosis of marginal Cu deficiency, however, has remained elusive despite an increased understanding of the biochemistry of Cu and its physiological roles in the body. Traditional markers of Cu status, such as serum Cu and caeruloplasmin protein concentrations are insensitive to subtle changes in Cu status. Cu-containing enzymes, such as Cu–Zn-superoxide dismutase, cytochrome *c* oxidase and diamine oxidase, may be more reliable but evidence to date is not conclusive. Development of markers sensitive to marginal Cu status is essential before conclusions can be drawn concerning the risks of long-term intake of suboptimal dietary Cu. As Cu appears to be essential for maintenance of immune function, activities of specific immunological markers, altered in Cu deficiency, offer alternatives. This review evaluates a selection of immunological markers that could be considered potentially sensitive markers of marginal Cu status. The indices of immune function reviewed are neutrophil function, interleukin 2 production, blastogenic response to mitogens and lymphocyte subset phenotyping.

Copper: Copper status: Immune system

Essentiality of copper

The potential essentiality of Cu for man was not recognized until 1928 when Hart *et al.* (1928) showed Cu to be essential for erythropoiesis in rats fed a milk-based diet. The actual essentiality of Cu for man was not confirmed until the 1960s by a study on malnourished Peruvian children (Cordano *et al.* 1964). These children had an anaemia refractory to Fe therapy, neutropaenia, and bone abnormalities, which were responsive to Cu supplements. An immense amount of research has since been undertaken to try to determine the functions of Cu in the human body. Current research suggests that most of the Cu in living organisms, including man, plays the role of cofactor for specific enzymes and electron transport proteins involved in energy or antioxidant metabolism (Linder & Hazegh-Azam, 1996). Two of the principal cuproenzymes are cytochrome *c* oxidase and superoxide dismutase (SOD), which are involved in electron transport and antioxidant defences respectively. Other cuproenzymes and proteins that bind Cu are presented in Table 1.

Copper deficiency

The effects of severe Cu deficiency are now well established and have been observed in man under a variety of different clinical conditions, including patients on long-term parenteral nutrition (Dunlap *et al.* 1974), coeliac disease, short bowel syndrome, tropical and non-tropical sprue, intakes of megadoses of Zn and Fe (Allen & Solomons, 1984), premature infants, neonates, and previously malnourished children (Manser *et al.* 1980; Paterson & Burns, 1988). The most frequent signs of Cu deficiency in human subjects are anaemia, neutropaenia

Abbreviations: Con A, concanavalin A; IL, interleukin; MHC, major histocompatibility complex; MNC, mononuclear cells; NK, natural killer cells; PHA, phytohaemagglutinin; SOD, superoxide dismutase.

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Table 1. Functions of established cuproenzymes and copper-binding proteins

Copper enzyme/binding protein	Function
Cuproenzymes	
Amine oxidases (monoamine oxidase, tryamine oxidase and histamine oxidase)	Deamination of primary amines
Caeruloplasmin	Oxidation of iron, copper transport, antioxidant
Copper–zinc-superoxide dismutase	Free radical detoxification
Cytochrome *c* oxidase	Electron transport
Diamine oxidase	Oxidative deamination of amines
Dopamine β-hydroxylase	Catecholamine production
Extracellular superoxide dismutase	Free radical detoxification
Ferroxidase II	Oxidation of iron
Hephaestin	Copper oxidase, export of iron from intestine
Lysyl oxidase	Cross-linking of collagen and elastin
Peptidylglycine α-amidating monooxygenase	α-Amidation of peptides
Thiol oxidase	Disulfide bond formation
Tyrosinase	Melanin production
Intracellular copper transporters	
ATP7A (Menke's disease protein)	Copper transporter
ATP7B (Wilson's disease protein)	Copper transporter
Cox17	Copper chaperone for cytochrome *c* oxidase
CCS	Copper chaperone for superoxide dismutase
ATOX-1 (HAH1)	Copper homeostasis and antioxidant defence
Extracellular copper transporters	
Albumin	Copper transporter
Transcuprein	Copper transporter
Copper proteins with no known catalytic/transport function	
Clotting factor V	Blood clotting
Clotting factor VIII	Blood clotting
Metallothionein	Copper storage

(Williams, 1983) and bone abnormalities (Danks, 1988), while less frequent signs are hypopigmentation (Danks, 1988), impaired growth (Castillo-Duran & Uauy, 1988), increased incidence of infections (Castillo-Duran *et al.* 1983), alterations of phagocytic capacity of neutrophils (Heresi *et al.* 1985) and abnormalities of glucose (Klevay *et al.* 1986) and cholesterol metabolism (Reiser *et al.* 1987). Severe Cu deficiency in both animals and human subjects can be identified by low serum or plasma Cu or caeruloplasmin, low red blood cell SOD activity and neutropaenia.

Clinically defined Cu deficiency is rare, but it has been suggested that long-term intakes of suboptimal dietary Cu may precipitate a number of conditions. Possible manifestations, in addition to the aforementioned features of severe Cu deficiency, are conditions such as arthritis, arterial disease, myocardial disease and neurological effects (Danks, 1988). Adult human dietary recommendations have been variously estimated between 1·2 and 2·0 mg Cu/d (Milne, 1998). Many diets, however, fail to provide this amount. Calculations based on a survey of 849 individual diets from the USA and Europe indicated that >30 % of diets provided <1·0 mg Cu/d (Klevay *et al.* 1993), the approximate amount of dietary Cu found to be insufficient in some short-term Cu depletion experiments in human subjects (Milne, 1998). A recent Cu supplementation trial in Northern Ireland, UK, estimated average dietary intakes of Cu to be 1·43 mg/d for males and 1·03 mg/d for females (Turley *et al.* 2000). Cu intakes for the females were below the UK reference nutrient intake of 1·2 mg/d (Department of Health, 1991). New recommendations for dietary Cu intakes were published in the USA earlier this year (Institute of Medicine, 2001) and the recommended daily dietary allowance for Cu is now 0·9 mg/d. This recommendation proceeds, and is lower than, the estimated safe and adequate daily dietary intake proposed in 1989 by the US National Academy of Sciences (National Research Council, 1989) of 1·5–3·0 mg Cu/d for adults.

With increasing experimental and epidemiological evidence indicating a role for suboptimal Cu status in disease progression, accurate assessment of Cu status in human subjects is becoming more and more important. However, there are two main barriers to the assessment of Cu status in human subjects. First, controversy remains about the precise human dietary requirements for Cu and second, there is currently no known index of Cu status that responds reliably and accurately to small changes in body Cu status or can determine a state of marginal Cu status.

Assessment of copper status

Traditional markers of Cu status, such as caeruloplasmin protein levels and Cu–Zn-SOD activity, known to be reduced in severe Cu deficiency, have been shown to be less sensitive to marginal Cu deficiency (Turnlund *et al.* 1990). These markers have also been shown to be unresponsive to Cu supplementation (Kehoe *et al.* 2000). Recent studies have shown that cytochrome *c* oxidase activity of leucocytes and platelets is reduced in Cu deficiency (Milne & Johnson, 1993) and diamine oxidase activity is increased in Cu supplementation (Kehoe *et al.* 2000). However, these results have not been confirmed by others and warrant further investigation.

Research to find a marker of Cu status may need to move away from traditional markers of Cu status, such

as caeruloplasmin and SOD. Instead, focusing on indices of biochemical and physiological systems, which are most likely to become limiting during periods of suboptimal Cu status, offer an alternative. To be a functional index of Cu status, the chosen marker must respond sensitively, specifically and predictably to changes in the concentration and/or supply of dietary Cu or Cu stores, be accessible for measurement and measurable, and impact directly on health (Strain, 2000). It has been shown recently that a biochemical index of bone turnover (urinary pyridinium cross-link excretion) was increased with a low-Cu diet relative to a medium-Cu diet. This increase, if sustained, would result in a measurable loss in bone mass. Moreover, this increase in bone turnover occurred with no change in serum Cu or caeruloplasmin (Baker *et al.* 1998). These results indicate that measures of bone health might be sensitive indicators of Cu status and support the results of a long-term Cu supplementation study that demonstrated decreased loss of vertebral bone mineral density in perimenopausal women taking Cu compared with controls (Eaton-Evans *et al.* 1996). Conversely, no effects of Cu supplementation (3 mg/d and 6 mg/d) were observed on biochemical markers of bone formation or bone resorption over 6-week periods in twenty-four healthy males and females (Baker *et al.* 1999).

For a comprehensive list of functional indicators that may be useful in the diagnosis of marginal Cu deficiency see Table 2. As alterations in a number of these markers, e.g. caeruloplasmin and fibrinogen, are also predictive of an inflammatory state, the inclusion in Table 2 of markers of inflammation and/or infection, not affected by Cu status, to allow differentiation between Cu deficiency and inflammation is considered important.

Table 2. Functional indices of copper status*

Molecular indices of copper status
Changes in activity/concentrations of cuproenzymes/copper binding proteins
Caeruloplasmin oxidase
Caeruloplasmin protein
Superoxide dismutase
Cytochrome *c* oxidase
Lysyl oxidase
Diamine oxidase
Dopamine β-hydroxylase
Peptidylglycine α-amidating monooxygenase
Tyrosinase
Factor V
Factor VIII
Transcuprein
Biochemical indices of copper status
Pyridinium cross-links of collagen
Various measures of oxidative stress (thiobarbituric-acid reactive substances)
Catecholamines
Encephalins
Polyamines
Physiological indices of copper status
Immune function
Haemostasis
Cholesterol metabolism
Glucose tolerance
Blood pressure
Arterial compliance
Arterial plaque
DNA damage and repair
Bone density
Markers of low level inflammation/infection
C-reactive protein
Slow-reacting substance of anaphylaxis

* Adapted from Strain (2000).

Another physiological system with potentially huge scope for establishing functional marker(s) of Cu status, and one that is compromised by Cu deficiency, is the immune system. Cu deficiency has many varied and pronounced effects on the immune system leading to an overall reduction in immune system integrity. This present review outlines some of the major effects of Cu deficiency on the immune system and discusses the feasibility of using markers of immune status to indicate responses to dietary Cu status.

The immune system and its response to copper deficiency

The immune system comprises two main components, the innate or non-adaptive immune system and the acquired or specific immune system (Fig. 1). Innate immunity is present from birth and comprises mainly non-specific defences, which include structural barriers such as the skin and mucous membranes, and physiological barriers, such as pH and O_2 levels. In addition, blood proteins, including members of the complement system, and phagocytic cells (neutrophils and macrophages) and other leucocytes such as natural killer (NK) cells are involved through their roles in phagocytosis, pinocytosis and the inflammatory response. These processes are not influenced by prior contact with an infectious agent and form the first line of defence for the body and retard the establishment of overt infection.

Acquired (specific) immunity includes the T cell system of cell-mediated immunity and the B cell system (humoral immunity) of antibody production. These mechanisms are adaptive and acquired and are stimulated by exposure to infectious agents. The cells of the acquired immune system are responsible for synthesizing antibodies, providing memory, and killing invading micro-organisms. T cells are subdivided into functionally distinct populations: helper T cells (CD4+), which recognize their specific antigens in association with major histocompatibility complex (MHC) class II molecules and cytotoxic T cells (CD8+), which recognize antigens in association with MHC class I molecules. B cells are defined by the presence of surface immunoglobulin and the majority of B cells carry MHC class II antigens. Both arms of the immune system act in concert to provide an integrated system of host defence.

Innate immunity

Very little research has been concerned with Cu deficiency and the innate immune system. Indeed, no research, to our knowledge, has investigated the effect of Cu deficiency on structural barriers such as the skin, mucus layers in the nasal passage or the intestine. All of these structures function as barriers to micro-organisms. The complement system, which contains an array of plasma proteins that

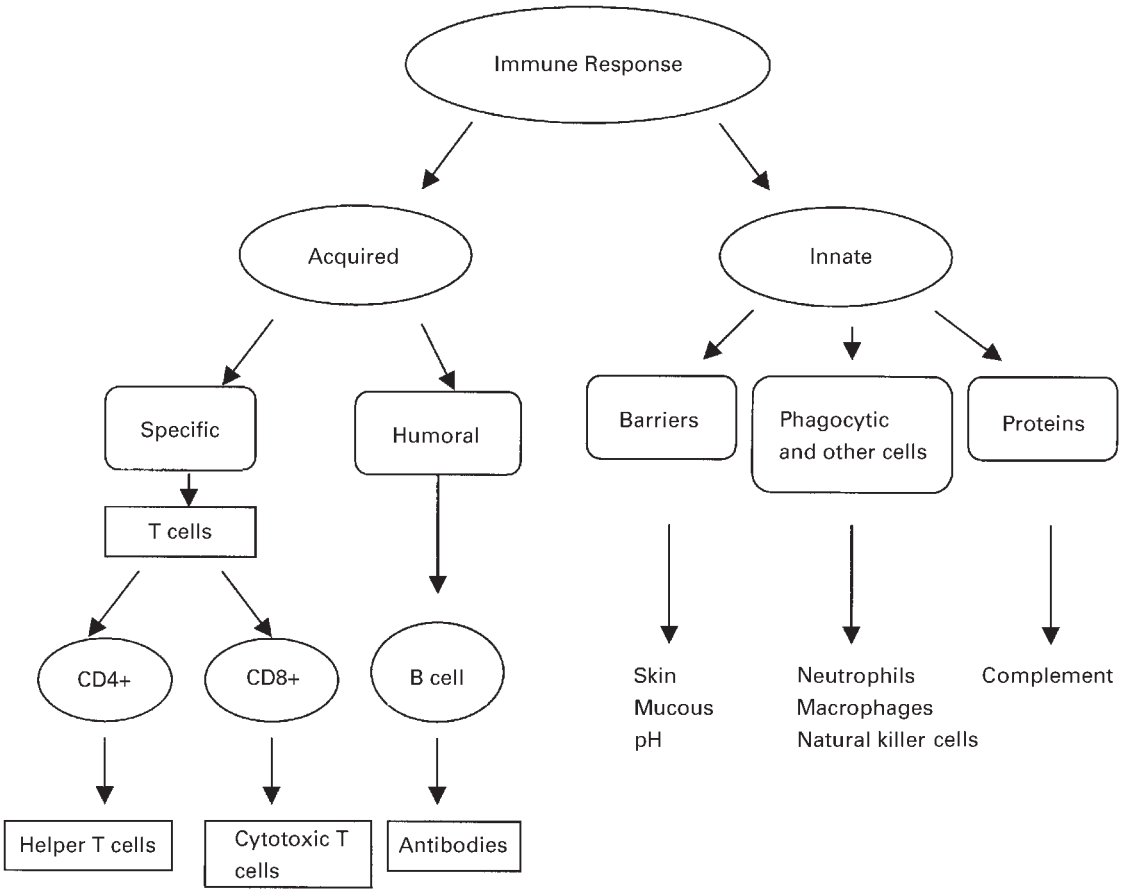

Fig. 1. Overview of the immune system.

are converted and activated to cause microbial cell lysis, has not been examined in detail; albeit it has been reported that Cu may be closely involved with the function of complement (Montgomery *et al.* 1974). Work by Sullivan & Ochs (1978) demonstrated no effect of Cu deficiency on circulating levels of C3 and C4 in a patient with Menke's disease (an inherited defect of Cu metabolism resulting in a Cu deficiency-like syndrome).

The effect of Cu deficiency on phagocytic cells, particularly neutrophils and macrophages, is well detailed in the literature. Functions of neutrophils include travelling to the site of infection, adhering to the endothelium and transmigration across the endothelium, where they are involved in phagocytosis and killing of foreign invaders by activation of the respiratory burst (Karimbakas *et al.* 1998). Cu deficiency causes a decrease in the number of circulating neutrophils, a condition termed neutropaenia. This condition is observed in Cu-deficient animals (Koller *et al.* 1987) and human subjects (Heresi *et al.* 1985; Danks, 1988). Boyne & Arthur (1981,1986) reported that the respiratory burst and microbicidal activity of bovine peripheral blood neutrophils were decreased by Cu deficiency. Cellular Cu status, respiratory burst, and candidacidal (yeast-killing ability) activity of peritoneal macrophages have been shown to decrease in severely Cu deficient rats (Babu & Failla, 1990*b*). In addition, the reduction in candidacidal activity of peritoneal macrophages has been seen in marginally Cu-depleted rats when compared with peritoneal macrophages from Cu-adequate rats. The diminished activities of these phagocytic cells compromises the innate immune defence system and contributes to greater susceptibility to infections (Percival, 1995). Newberne *et al.* (1968) demonstrated the importance of mononuclear phagocytes in Cu-deficient rats infected with *Salmonella typhimurium*. The Cu-deficient rats exhibited higher mortality rates and shorter survival than control animals.

Another type of leucocyte altered in Cu deficiency is NK cells, a population of granular lymphocytes that normally serve to kill target cells that display decreased expression of self class I MHC molecule–peptide complexes or cells coated with specific immunoglobulin G molecules (Abbas *et al.* 1997). A study by Koller *et al.* (1987) compared the cytotoxic activity of NK cells isolated from spleens of Cu-adequate and Cu-deficient rats and controls. The NK cells from the Cu-adequate control rats were five- to sevenfold more effective than NK cells from the Cu-deficient rats in releasing ^{51}Cr from yeast artificial chromosome-1 target cells. In innate immunity, NK cells form part of the first line of defence.

Acquired immunity

Humoral immunity. The effect of Cu deficiency on the

humoral immune system can be demonstrated by the response of Cu-deficient animals to challenge with sheep red blood cells. Production of antibodies to sheep red blood cells requires antigen processing and T lymphocyte cooperation and help. The plaque-forming-cell response to sheep red blood cells has shown to be decreased in both Cu-deficient mice (Mulhern *et al.* 1985; Blakley & Hamilton, 1997) and Cu-deficient rats (Vyas & Chandra, 1983), as a result of an impairment in antibody production in the spleen of these animals. Furthermore, serum antibody titres to sheep red blood cell challenge have been shown to be lower in Cu-deficient rats (Failla *et al.* 1988) and serum immunoglobulin G production to another T-dependent antigen (keyhole limpet haemocyanin) was lowered by Cu deficiency (Koller *et al.* 1987).

Cell-mediated immunity. Phenotypic profiles of lymphoid and myeloid cells from mice (Lukasewycz *et al.* 1985; Mulhern & Koller, 1988), and mononuclear cells (MNC) from rat spleen (Bala *et al.* 1991*b*) and peripheral blood (Bala *et al.* 1990) are altered by Cu deficiency. More specifically, there is a general reduction in T lymphocytes, sometimes elevation of B lymphocytes but never a reduction, and in one study an increase in macrophages was seen (Bala *et al.* 1991*b*). The reduction in T lymphocyte numbers seems to be predominantly within the T-helper subpopulation (CD4+). Reductions in the T cytotoxic subpopulation (CD8+) are also observed, but not to as great an extent as the CD4+ subpopulation. It appears, therefore, that Cu plays an important role in the maturation sequence of lymphoid tissue. Cu deficiency leads to an impairment that is manifested by lymphocyte subpopulation shifts (Lukasewycz *et al.* 1985). Following on from these findings are results that show suppression of [^{3}H]thymidine incorporation into DNA in splenic MNC from Cu-deficient rats (Bala *et al.* 1991*a*,*b*). The authors of these studies point out that this suppression greatly exceeds any reduction that could be attributed to the reduction in T lymphocyte numbers. This suggests that other functions necessary for T lymphocyte activity, e.g. antigen processing and presentation, may be impaired by Cu deficiency.

Effects of Cu deficiency on the acquired immune system can be further demonstrated by examining the response of splenic lymphocytes to T cell mitogens (blastogenesis). Decreased responsiveness of splenic MNC to T cell mitogens is well documented in the literature in a number of species and human cell lines. Bala *et al.* (1992) showed that MNC isolated from the blood of Cu-deficient pigs exhibited decreased reactivity to the T cell mitogens, phytohaemagglutinin (PHA) and concanavalin A (Con A). In hypocupraemic lambs (Suttle & Jones, 1986), decreased DNA synthesis in cultures of PHA, ConA and pokeweed were observed in treated lymphocytes. Results from two studies in which blood and splenic MNC from Cu-deficient rats were examined (Bala *et al.* 1990, 1991*b*) support these findings. Further, these results suggest that the reduced responsiveness to T cell mitogens is a function of Cu deficiency and not secondary Fe deficiency, given that packed cell volume was similar in all animals. Cu deficiency, therefore, may impair mitogen-induced DNA synthesis (Failla & Bala, 1992).

It has previously been reported that the suppressed mitogenic responsiveness of splenic MNC isolated from Cu-deficient rats was highly correlated with interleukin (IL) 2 activity in these cultures. (Bala & Failla, 1992). IL-2 is a cytokine secreted by activated T lymphocytes and has a central role in the regulation of host responses to pathogenic challenge. Cu deficiency decreases the secretion of IL-2 by activated rodent splenocytes (Bala & Failla, 1992), human peripheral blood MNC and the human T lymphocyte cell line Jurkat (Hopkins & Failla, 1997*a*). Progression of the competent T cell to the S phase of the cell cycle is impaired in Cu deficiency (Bala *et al.* 1991*a*) and this is owing to the impaired secretion of IL-2 (Bala & Failla, 1992). Failure to provide T cells with an adequate supply of Cu limits the ability of activated T cells to produce sufficient levels of the cytokine IL-2, which is required for T cell proliferation.

The delayed type hypersensitivity response is a further means of assessing the impact of Cu deficiency on the acquired immune system. Cu-deficient rats exhibited impaired delayed type hypersensitivity to oxazolone, a T cell-dependent contact sensitizing agent, and to a T cell-independent antigen *Pneumococcal polysaccharide*, compared with controls (Kishore *et al.* 1984). In contrast, normal responses were seen to oxazolone in Cu-deficient rats (Koller *et al.* 1987). Lukasewycz & Prohaska (1982), using a C58 mouse-line Ib syngeneic leukaemia tumour system, observed that male Cu-deficient mice were unable to withstand even an immunizing dose of Ib cells (15 % survival) compared with Cu-supplemented controls (100 % survival).

Cu deficiency also has pronounced effects on organs of the immune system. Biochemical and morphological studies have shown that, in mice, severe Cu deficiency is characterized by an enlarged spleen and small thymus (Prohaska & Lukasewycz, 1990). These findings are also seen in Cu-deficient rats (Failla *et al.* 1988).

Some of the major effects of Cu deficiency on the immune system have been outlined earlier. A brief summary is presented in Table 3. The summary incorporates the aforementioned work and additional insults that Cu deficiency has on the immune system. Effects of sex, species, duration of Cu deficiency, time of implementation of Cu deficiency and severity of Cu deficiency must be taken into account and not all of these symtoms will be seen at any one time, if at all.

Biomarkers of copper status

The detrimental impact of Cu deficiency on the immune system is similar to the immunosuppressive influence of marginal and moderate deficiencies of other essential trace elements e.g. Zn and Fe. Other factors such as age and gender of the individual will also impact on the functioning of the immune system, possibly resulting in similar manifestations to those seen in Cu deficiency. These are important issues to consider when trying to target a sensitive biomarker of Cu status and it is unlikely that a biomarker can be found that is totally specific to alterations in body Cu status alone. Indeed, the more sensitive a biomarker is to alteration, for example in Cu status, the less

Table 3. Effects of severe copper deficiency on the immune system

Effect of Cu deficiency	Species	Authors
Neutropaenia and anaemia	Man, cattle	Danks, 1988; Cordano *et al.* 1964; Boyne & Arthur, 1981
↓ Number of antibody producing cells in spleens on exposure to sheep eyrthrocytes	Rats, mice	Mulhern *et al.* 1985; Blakley & Hamilton, 1987; Vyas & Chandra, 1983
↓ Respiratory burst and microbicidal activities of neutrophils and peritoneal macrophages	Rats, cattle	Babu & Failla, 1990*a*,*b*; Boyne & Arthur, 1981
↓ Cytolytic activity of natural killer cells	Rats	Koller *et al.* 1987
↓ Delayed type hypersensitivity response	Rats	Kishore *et al.* 1984
↓ *in vitro* responsiveness to T cell mitogens in splenic peripheral blood MNC	Mice, rats, man	Lukasewycz *et al.* 1985; Bala *et al.* 1990; Kelley *et al.* 1995
↓ T lymphocyte (relative % and absolute values)	Rats, mice	Bala *et al.* 1990; Lukasewycz *et al.* 1985
↑ Levels of B cells (relative %)	Rats, mice	Bala *et al.* 1990; Lukasewycz *et al.* 1985
↓ T cell subsets (CD4 and CD8) (relative and absolute values)	Rats, mice	Bala *et al.* 1990; Lukasewycz *et al.* 1985; Mulhern & Koller, 1988
↓ Suppression of [^{3}H]thymidine incorporation into DNA in splenic MNC	Rats	Bala *et al.* 1990, 1991*a*
↓ Medium IL-2 levels in splenic MNC and peripheral blood MNC	Human T-cell line, man	Bala & Failla, 1992; Kelley *et al.* 1995
↓ IL-2 mRNA in Jurkat cells	Human T-cell line	Hopkins & Failla, 1997*a*
Alterations in NF-κB expression	Human T-cell line	Hopkins & Failla, 1997*b*
↓ Ability of neutrophils–macrophages to kill *Candida albicans*	Rats, sheep, cattle	Bala & Failla, 1990*a*,*b*; Jones & Suttle, 1981
↑ Monocyte population	Rats	Bala & Failla, 1993
↓ Serum thymulin	Rats	Vyas & Chandra, 1983
↑ Dopamine production	Rats, mice	Prohaska *et al.* 1990
↓ Noradrenaline production	Mice	Prohaska *et al.* 1990
↑ Morbidity to infections	Man	Castillo-Duran *et al.* 1983

MNC, mononuclear cells; IL, interleukin.

specific it tends to be. However, as already mentioned, traditional measurements of Cu status tend to be unresponsive to changes in body Cu status and influenced by factors such as age, gender and inflammation. Therefore, it is imperative to consider alternatives.

From the experimental work available, the following variables can be considered as potential biomarkers of Cu status: IL-2 secretion from lymphocytes, neutrophil function, phenotypic profiles of lymphocyte subsets and response of lymphocytes to T cell mitogens (blastogenesis). Neutrophil function, as already mentioned, is a feature of the innate immune system. The assessment of IL-2 production, phenotypic profiling of lymphocyte subsets and the blastogenic response of cell cultures to T cell mitogens, however, are all aspects of the acquired immune system, specifically the cell-mediated response.

Interleukin 2

IL-2 is the principal cytokine responsible for the progression of T lymphocytes from the G1 phase to the S phase of the cell cycle. The action of IL-2 on cells is mediated by binding to the IL-2 receptor. Failure to synthesize adequate quantities of IL-2 has been described as a cause of antigen specific T cell anergy (Abbas *et al.* 1997). Secretion of IL-2 as a marker of Cu status is plausible for a number of reasons. First, reduced production of IL-2 in response to Cu deficiency appears to be common to a number of species. Therefore, observations from animal models may be considered to be applicable to human models. Decreased IL-2 secretion has been seen in activated rodent splenocytes (Bala & Failla, 1992), human peripheral MNC (Hopkins & Failla, 1997*a*), the Jurkat T-cell line (Hopkins & Failla, 1997*a*, 1999) and in splenic non-adherent cells from Cu-deficient mice (Prohaska & Lukasewycz, 1990). Perhaps more important is the finding that not only is IL-2 decreased in severe Cu deficiency, it is also decreased in marginal Cu deficiency. Chronic ingestion of a diet marginally low in Cu (2·7 mg Cu/kg) by adult male rats resulted in reduced production of IL-2 (Hopkins & Failla, 1995). Furthermore, IL-2 production was decreased with no significant alterations of conventional indicators of Cu status, e.g. tissue Cu, tissue Cu–Zn-SOD and serum caeruloplasmin activity. In a study on Jurkat cells (Hopkins & Failla, 1997*a*), the reduction of IL-2 production exceeded the extent of the decline in cellular Cu–Zn-SOD. Moreover, a decline in IL-2 bioactivity in cultures of human peripheral blood MNC exposed to a high affinity Cu chelator, 2,3,2-tetramine, was observed in the absence of any significant changes in Cu–Zn-SOD activity. Specificity of the decrease in IL-2 to Cu deficiency was tested by adding physiological concentrations of Cu to mitogen-treated cultures of splenic MNC from Cu-deficient rats. Cu repletion enhanced IL-2 activity and normalized DNA synthesis (Bala & Failla, 1992).

It is well known that Zn and Fe interact with Cu. Therefore, in order to determine that the decrease in IL-2 was purely a result of Cu deficiency, Bala & Failla (1992) examined Fe and Zn status in the Cu-deficient rats. Both

haemoglobin and concentrations of Zn in several tissues were found to be normal, indicating little effect of marginal Cu status on Fe and Zn status. A study by Prohaska & Lukasewycz (1989) reported a reduction of 50 % in IL-2 production in culture supernatant fractions of mitogen-treated murine splenocytes. As seen in the study of Bala & Failla (1992), Cu supplementation restored DNA synthesis, but only at concentrations greater than 24 μM. Adding Zn and Mn to the medium did not alter mitogenic reactivity, again indicating specificity of Cu in restoring IL-2 production. Hopkins & Failla (1999) demonstrated that in the Jurkat T-cell line, Cu deficiency (induced by the Cu chelator 2,3,2-tetramine) decreased IL-2 synthesis in T lymphocytes by inhibiting transcription of the IL-2 gene. As use of chelating agents can reduce cellular content of other trace elements, additional experiments confirmed it was indeed Cu deficiency that adversely affected transcriptional efficiency of IL-2 and not a secondary Fe deficiency. IL-2 production has not been examined in human trials, although a study by Kelley *et al.* (1995) on Cu-deficient men reported decreased secretion of the IL-2 receptor in peripheral blood MNC cultured with mitogens.

There is obviously a need for more human trials before IL-2 can be considered a potential marker of Cu status, but the evidence suggests that the effect of Cu deficiency on IL-2 appears to be reversible, sensitive to marginal Cu intakes, reproducible in several species and easily measurable in human peripheral blood MNC. Furthermore, the decrease in IL-2 secretion is probably owing to decreased IL-2 mRNA expression (Hopkins & Failla, 1997*a*). This allows sensitive methods, such as real time polymerase chain reaction, to evaluate gene expression as a back-up to measurement of IL-2 production.

Neutrophil function

Neutrophils represent the predominant type of leucocytic cell in the blood of man and many domestic animals (Wolford *et al.* 1986) and, therefore represent a readily accessible population for the possible assessment of Cu status, specifically measurement of neutrophil function. In Cu deficiency, neutrophil numbers, respiratory burst, microbicidal activity, and Cu–Zn-SOD activity can all be decreased significantly relative to controls (Babu & Failla, 1990*a*).

Similar to the production of IL-2, reduced functioning of neutrophils has been observed in a number of species, including man (Heresi *et al.* 1985). Boyne & Arthur (1981) reported that the respiratory burst and microbicidal activity of bovine peripheral neutrophils were reduced in Cu deficiency. Similarly, neutrophils from hypocupraemic lambs, depleted of Cu, had reduced killing ability and peripheral blood granulocytes isolated from ewes fed a Cu-deficient diet had a reduced ability to kill ingested *Candida albicans* (Jones & Suttle, 1981). Furthermore, hypocupraemic lambs exhibited reduced killing capacity of their neutrophils when compared with controls (Suttle & Jones, 1986). These results have also been repeated in rodents (Babu & Failla, 1990*a*) and the work extended to examine marginal Cu deficiency (Babu & Failla, 1990*a*). Results from this work indicated impaired functioning of peritoneal neutrophils in marginal Cu deficiency, as illustrated by decreased activity of neutrophil Cu–Zn-SOD, decreased phorbol myristate acetate induced superoxide production by neutrophils and decreased candidacidal activity of neutrophils. Moreover, these changes occurred without significant alteration in the activity of red blood cell SOD and tissue Cu. A further study (Hopkins & Failla, 1995) showed decreased superoxide production from thioglycollate- (stimulates migration of neutrophils into the peritoneal cavity) elicited neutrophils of adult male rats fed marginally low-Cu diets. Torre *et al.* (1996) observed that mild dietary Cu insufficiency in dairy cattle impaired neutrophil ability to kill ingested bacteria compared with Cu-supplemented controls. In conjunction with evidence highlighting reduced responsiveness of neutrophils to marginal Cu deficiency are results that indicate that Cu repletion reversibly and readily restores functioning of neutrophils in several species, including man.

In a study by Jones & Suttle (1981), three out of five Cu-deficient calves were given oral doses of cupric oxide. Neutrophil killing ability increased by 100 % in these repleted calves. Babu & Failla (1990*a*) observed that Cu repletion for 7 d in rodents restored Cu status and function of neutrophils and in the aforementioned study of Torre *et al.* (1996), Cu repletion improved neutrophil superoxide production and bactericidal capacity in dairy cattle. Cu supplementation has also been shown to increase numbers of neutrophils in peripheral blood and to reduce the incidence of respiratory tract infections in infants recovering from marasmus who were neither anaemic nor neutropaenic (Castillo-Duran *et al.* 1983). Neutrophil phagocytic activity was more than doubled when hypocupraemic infants were supplemented with Cu (Heresi *et al.* 1985).

The results collectively indicate that neutrophil function is reversibly impaired by marginal and severe Cu deficiency and suggest that Cu status and function of these cells may provide a sensitive indicator for assessment of Cu status. Higuchi *et al.* (1995) reported that severely Cu-deficient subjects have high serum titres of anti-neutrophil antibodies. Supplementing with Cu increased serum concentrations of Cu and neutrophils and decreased or eliminated anti-neutrophil titres. This work indicates that it might be useful also to examine levels of anti-neutrophil antibodies in marginally Cu-deficient human subjects as a possible measure of suboptimal Cu status.

Phenotypic profiling of lymphocyte subsets

In severe Cu deficiency, phenotypic profiles of lymphoid and myeloid cells from mice (Lucasewycz *et al.* 1985; Mulhern & Koller, 1988) and MNC from rat spleen (Bala *et al.* 1991*b*) and peripheral blood (Bala *et al.* 1990) are altered. More specifically, in Cu-deficient mice (Lukasewycz *et al.* 1985), there was a statistically significant increase in the number of surface immunoglobulin-bearing cells, in terms of relative number of cells per spleen. The absolute number of splenic cells was at a level two- to threefold higher than controls. Relative percentage of cells expressing the T cell marker, Thy-1·2, was below control levels. The relative decrease in T cell population was found to be owing to altered expression

of a marker for the CD4+ T cell subset, (Lyt-1+) and not the CD8+ T cell subset, as the CD8+ marker (Lyt-2+) was unaltered. A study on Cu-deficient rats (Bala *et al.* 1991*b*) demonstrated decreases in relative percentages and absolute numbers of T lymphocytes. This reduction was manifested in a reduction in CD4+ cells and a reduction in CD8+ cells, albeit the latter decrease was only in the most Cu-deficient animals. In parallel with the reduction in T cell numbers was a relative increase in percentage of splenic B cells.

From this, it seems plausible to consider lymphocyte subset phenotyping as possible marker(s) of Cu status. In particular, the overall reduction in total T lymphocyte numbers seems to be particularly within the CD4+ subset. Furthermore, this reduction is independent of several complications associated with Cu deficiency, e.g. anaemia and altered mass of lymphoid tissue (Bala *et al.* 1991*b*). The greater decrease in CD4+ cells, compared with CD8+ cells, has also been observed in the peripheral blood of Cu-deficient male rats (Bala *et al.* 1991*a*,*b*). These results indicate that T helper cells (CD4+) are more susceptible to Cu deficiency than cytotoxic T cells (CD8+). It has also been demonstrated (Bala & Failla, 1993) that feeding Cu-deficient rats a Cu-adequate diet increased the percentage of splenic CD4+ cells back to control levels, whereas relative percentage and total numbers of splenic CD8+ cells were unaffected by depletion and repletion. A study by Mulhern & Koller (1988) demonstrated that in marginal and severely Cu-deficient rats there was a dose-dependent reduction in Thy-1·2 (T cell marker) and Lyt-1 (CD4+).

It appears, therefore, that absolute numbers and relative percentages of T lymphocytes, specifically the CD4+ subset, could be used as one assessment of marginal Cu status. These T-cell markers appear to be decreased, in a dose-dependent manner, in more than one species, and then increased on Cu repletion. Although the evidence is not as well substantiated (particularly in human subjects) as that for IL-2 production and neutrophil function, lymphocyte phenotyping by flow cytometry offers a simple, accurate and viable method indicative of Cu status in human subjects.

Examination of the pattern of expression of a panel of surface markers might be more indicative of Cu status. For example, current thinking is that most alterations in immune responsiveness to Cu deficiency can be accounted for by impaired T cell function. The efficiency of T cell activation is proportional to the number of class II MHC molecules expressed on the surface of antigen presenting cells, such as monocytes. This interaction is strengthened by complementary pairs of molecules, termed adhesion molecules, on adjacent T cells and monocytes. Intracellular adhesion molecule 1 (ICAM-1 or CD 54) and leucocyte function-associated antigen 1 (LFA-1 or CD11a) are two such molecules that can be easily measured in peripheral blood and give an indication of the integrity of T cell function. T cell activation can also be assessed easily by the presence of intermediate (CD25) and/or late (HLA-DR) activation markers. Indeed, work by Kelley *et al.* (1995) reported that feeding men a Cu-deficient diet for 42 d decreased the percentages of activated circulating peripheral T (HLA-DR) cells. An ongoing Cu depletion–repletion trial in healthy men at our laboratory is examining the effect of Cu deficiency on the expression of a plethora of surface markers including T and B cells, intermediate (CD25) and late (HLA-DR) activation markers and adhesion molecules (CD11a and CD54).

Blastogenic response to T cell mitogens

The last potential candidate for a biomarker of Cu status involves evaluation of leucocyte function by measuring the blastogenic response of cell cultures on exposure to specific mitogens. Lukasewycz & Prohaska (1983) demonstrated a reduced response of splenic lymphocytes from Cu-deficient mice to Con A, PHA and pokeweed. Similar results have also been established in Cu-deficient rodents, although the effect appears to be more severe. Bala *et al.* (1991*b*) showed a suppressed proliferative response to T cell mitogens in Cu-deficient female rats with normal Fe status. This observation indicates that the defect was not dependent on secondary Fe deficiency. Mitogen reactivity of peripheral blood MNC to the T cell mitogens Con A and PHA was suppressed in Cu-deficient rats (Bala *et al.* 1990) and a recent study on Cu-deficient swine reported a modest suppression of peripheral blood MNC to PHA stimulation, but not Con A or pokeweed (Bala *et al.* 1992). Other work by Bala *et al.* (1991*b*) demonstrated that *in vitro* blastogenesis of splenic MNC from male rats exposed to PHA and Con A was decreased by 75–80 % compared with controls and PHA-induced blastogenesis of MNC was only 29 % of that found in controls.

Studies in laboratory and domestic animals are further supported by cellular models. Exposing differentiating U937 promonocytic cells to the Cu chelator 2,3,2-tetramine to induce Cu deficiency resulted in suppression of the respiratory burst activity of the cells. This 2,3,2-tetramine-induced impairment of respiratory burst was blocked in cultures supplemented with Cu but not Zn or Fe (Huang & Failla, 2000).

Most of these studies have concentrated on severe Cu deficiency. However, Windhauser *et al.* (1991) demonstrated a decreased response of MNC to PHA in cultures prepared from young, mildly Cu-deficient rats. Furthermore, this suppression of blastogenic activity occurred in the presence of slightly decreased serum Cu and normal serum caeruloplasmin and liver Cu, an indication of the high sensitivity of blastogenesis to small changes in Cu status.

Cu repletion appears to restore the suppressed blastogenic activity. For example, Soderberg *et al.* (1987) examined the recovery of splenic lymphocytes following irradiation with and without subcutaneous Cu treatment. It was concluded that Cu supplementation, compared with control, enhanced splenic populations and accelerated the reappearance of responsiveness to Con A. Failla & Bala (1992) showed that impaired lymphocyte response of Cu-deficient rats to PHA could be reversed *in vitro* by supplementation with $CuSO_4$. Moreover, the restoration of the normal response was specific for Cu, because *in vitro* addition of Zn and Mn failed to elevate activity of lymphocyte cultures prepared from Cu-deficient rats. A further study (Bala & Failla, 1993) showed that cultures of

PHA-treated splenic MNC from Cu-deficient rats exhibited only 48 % of the activity of the control cultures. Feeding Cu to the Cu-deficient rats partially alleviated the suppressed response to PHA after 7 d.

It seems clear therefore, that Cu deficiency can suppress blastogenic response of lymphocytes to T cell mitogens. There have been exceptions to this finding (Blakley & Hamilton, 1987; Prohaska & Lukasewycz, 1989; Arthington *et al.* 1996) indicating that the response to T cell mitogens is tissue specific and may be different in various animal species. For example, Mo-induced Cu deficiency in heifers resulted in an increased lymphocyte proliferative response to PHA compared with controls (Arthington *et al.* 1996).

However, a study in Cu-depleted men (Kelley *et al.* 1995) showed that proliferation of peripheral blood MNC, isolated and then cultured with PHA and Con A, was lowered at the end of a Cu-deficient diet (0·38 mg Cu/d) compared with baseline values. Cu supplementation prevented further suppression of blastogenic activity.

Blastogenic activity, therefore, appears to be a sensitive marker of marginal Cu status, and one that responds to Cu repletion (Windhauser *et al.* 1991). Furthermore, as blastogenic activity of cell cultures of peripheral blood in both rats (Bala *et al.* 1991*a*), and more recently human subjects (Kelley *et al.* 1995), has been shown to be affected by Cu deficiency, applying this methodology to human trials is already feasible and successful.

Other immune biomarkers

There are a number of other biomarkers that merit consideration in the assessment of Cu status. Although experimental evidence is limited, one example is IL-1. IL-1 is a cytokine involved predominantly with the stimulation of T helper cells to induce secretion of, among other things, IL-2. In a study by Lukasewycz & Prohaska (1989) IL-2 production of non-adherent splenocytes from Cu-deficient mice was decreased compared with controls. Conversely, IL-1 production by macrophages from the same Cu-deficient mice was double that of control levels. It appears, therefore, that Cu deficiency may have a differential effect on cytokine production and analysis of IL-1 and perhaps other cytokines such as IL-4 and IL-6 may warrant investigation.

Gene expression of the transcription factor NF-κB appears to be affected by Cu deficiency but work by Hopkins & Failla (1997*b*) has shown conflicting results. An increased expression was seen in chelator-treated Jurkat cells after exposure to PHA relative to controls, whereas in livers and spleens of Cu-deficient rats NF-κB expression was reduced relative to controls. Interestingly, NF-κB is one of the four transcription factors that regulates IL-2 gene expression which, as previously mentioned, is decreased by Cu deficiency.

Expression of a number of other genes for both Cu metalloenzymes and non-Cu metalloenzymes have recently been shown to be altered by Cu deficiency: Cu–Zn-SOD (Lai *et al.* 1994), Mn-SOD (Lai *et al.* 1996), catalase (Lai *et al.* 1996) and glutathione peroxidase (Prohaska *et al.* 1992), to name a few. With recent advances in molecular biology, such as real time polymerase chain reaction, alterations in gene expression, owing to Cu deficiency, for example, can be determined far more reproducibly and accurately than ever before. This methodology, unfortunately, relies on the user knowing in advance the genes of interest to be examined. However, the recent development of gene array systems allows the expression of hundreds of genes to be examined at any one time. This methodology can identify the expression of genes influenced by Cu deficiency. More detailed analysis on specific genes (once potential genes have been highlighted) can then be elucidated from real time polymerase chain reaction.

Summary

The basis for considering IL-2 production, neutrophil function, phenotypic profiling of lymphocyte subsets and the blastogenic response to T cell mitogens as markers of suboptimal Cu status is from the experimental findings discussed in the present review. What strengthens their case as putative biomarkers of Cu status are that they appear to be altered by marginal Cu deficiency and Cu repletion generally restores function back to control levels (although Cu repletion is not possible on most occasions when assessing Cu status). Furthermore, similar findings are seen in several species of laboratory and domestic animals. How these markers respond to marginal Cu deficiency in man is less clear. Only one trial, in healthy adult men, has examined the effect of Cu deficiency on immune function (Kelley *et al.* 1995) and blastogenic activity to T cell mitogens was the only real indicator of depressed immune function. Effects of acute and chronic marginal Cu deficiency in rats and mice has severe consequences. It is, therefore, important to examine the effects of long-term suboptimal Cu status on immune function in human subjects, if indeed certain sub-populations of man does consume marginal dietary intakes of Cu. As traditional indices of Cu status are unresponsive to marginal Cu status, it is important to identify and examine new candidate markers. The markers suggested in the present review can be measured easily in any trial (not necessarily trials related to immune function specifically) examining Cu status of human populations. One positive 'take-home message' is that several characteristics of immune depression, owing to Cu deficiency, are reversible. So if marginal Cu status can be identified in man, supplementation may go some way toward improving immune functioning.

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