

## The effects of copper deficiency on human lymphoid and myeloid cells: an *in vitro* model

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(Received 9 January 1995 – Revised 6 April 1995 – Accepted 2 May 1995)

Cu has long been known to influence immune responses. An *in vitro* model system was established in which human myeloid (HL-60), B-lymphoid (Raji) and T-lymphoid (Molt-3) cell lines could be grown in culture media of varying Cu levels. Initially Cu was removed from the medium by dialysis of fetal calf serum against a metal-ion chelator, minor depletion of other trace metals being obviated by repletion with appropriate metal salts. The growth rate of HL-60 was significantly ( $P < 0.05$ ) inhibited by 72 h Cu depletion. Molt-3 cells required a longer period, up to 144 h, in Cu-depleted medium before growth was impaired. Raji-cell growth was not affected. These results confirmed clinical observations that T-cell functions were more sensitive to Cu deprivation than B cells. Analysis of intracellular metal levels in Molt-3 cells showed that Cu levels had been significantly lowered ( $P < 0.05$ ) although  $Ca^{2+}$  levels were raised. Intracellular activity of the antioxidant enzyme superoxide dismutase (EC 1.15.1.1) was significantly impaired ( $P < 0.05$ ) in Molt-3 cells grown in Cu-depleted medium. Activity of the mitochondrial enzyme cytochrome c oxidase (EC 1.9.3.1) was also significantly impaired ( $P < 0.05$ ) by Cu depletion. Each of these findings indicates an increase in the potential for cellular damage by reduced antioxidant activity, impairment of normal mitochondrial activity and excessive  $Ca^{2+}$  influx. A major consequence of the type of damage occurring under these circumstances is membrane disruption. This was confirmed by scanning electron microscopy of Molt-3 cells grown under varying Cu levels.

**Copper: Immune response: Antioxidants: Cytochrome c oxidase**

Cu is an essential trace element which has an important role in many physiological functions, including the immune response. Both T- and B-lymphocyte functions are impaired by Cu deficiency (Stabel & Spears, 1989) although T cells are rather more sensitive to Cu deficiency than are B cells (Lukasewycz *et al.* 1985). Neutrophil function is also impaired in Cu deficiency (Babu & Failla, 1990). Jones & Suttle (1981) have observed significantly diminished activity of the antioxidant enzyme superoxide dismutase (SOD; EC 1.15.1.1) and raised levels of extracellular superoxide anion ( $O_2^{\cdot-}$ ) in neutrophil preparations isolated from Cu-deficient ewes. Reduced neutrophil cytotoxic activity in these ewes was suggested to be associated with altered activity of SOD, limiting the formation of  $H_2O_2$  from SOD-mediated reduction of  $O_2^{\cdot-}$ . Nonetheless the precise mechanism(s) by which Cu deficiency alters host immunity is still largely uncertain. Thymic atrophy associated with Cu deficiency has been postulated to result from either accelerated tissue damage through diminished SOD activity (Prohaska *et al.* 1983) or aberrant energy production because of decreased cytochrome c oxidase (CCO; EC 1.9.3.1) activity. Dietary Cu deficiency accompanied by low tissue Cu concentration can reduce Cu,Zn-

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SOD, ceruloplasmin (Cp), catalase (CAT; EC 1.11.1.6) and glutathione peroxidase (GSHPx; EC 1.11.1.9) activity (Strain, 1994). Metabolic and immunological disturbances could be promoted through the accumulation of reactive O species (ROS) which would be facilitated by such a diminution of antioxidant defences. Observable changes in the cell surface of immunocompetent cells could also be caused by high ROS fluxes.

The study reported here was carried out to establish a system for studying Cu deficiency in a lymphoid cell culture model. We examined the effects of Cu depletion on the human T-lymphoid cell line Molt-3 by monitoring cell viability, cell growth, intracellular metal element content, intracellular antioxidant enzyme status, mitochondrial function (CCO activity) and cell morphology. Two other cell types, the human B-lymphoid cell line Raji and the human myeloid line HL-60, were also included to compare the relative sensitivities of cell proliferative capacity in Cu deficiency. These data will facilitate further studies on those lymphoid cell activities affected by Cu deficiency and the connection between these modified functions and the development of immune hyporesponsiveness.

## EXPERIMENTAL

### *Cell lines*

Molt-3 human T-lymphoblastoid cells (Minowada *et al.* 1972) were maintained in routine suspension culture. Cells were subcultured at a density of  $1 \times 10^5$  cells/ml in normal, Cu-deficient (CuD) or Cu-repleted (CuR) media for 72 h at 37° in a humidified CO<sub>2</sub>-air (5:95 v/v) incubator. In some experiments cells were preincubated in the CuD medium for 72 h to reduce the intracellular Cu content before re-culture. Cell growth was monitored by electronic counting (Coulter Electronics Ltd., Luton, Beds) and viability was assessed by acridine orange/ethidium bromide staining, visualized under u.v. microscopy. In investigations on cell growth and viability, Raji human Burkitts lymphoma (B-lymphoid cells; Epstein & Barr, 1964) and HL-60 human myeloid cells (Collins *et al.* 1977) were included for comparison.

### *Cell culture media*

The normal cell culture medium was RPMI 1640 containing fetal calf serum (FCS; 100 g/l), 100 IU penicillin/ml and 100 µg streptomycin/ml. The CuD medium contained RPMI-1640 as before but FCS had been depleted of Cu by dialysis against a specific Cu chelator.

### *Serum copper depletion*

FCS was dialysed at 4° for 8 h against two changes of triethylenetetramine (30 mM, TRIEN) in phosphate-buffered saline (PBS), pH 7.4. The serum was then further dialysed for 12 h against three changes of PBS to eliminate the chelator. Serum was membrane-sterilized. The protein content of FCS was estimated before and after dialysis using the Bio-Rad protein assay (Bio-Rad Laboratories Ltd., Watford, Herts, Cat. no. 500-0006).

### *Determination of the mineral content of fetal calf serum and media*

For Cu, Zn, Fe and Mn analysis serum samples were diluted 1:1 with TCA (200 g/l) and heated at 80° for 15 min. After cooling the samples were centrifuged at 10000 rev./min for 45 min. The supernatant fraction was used for metal analysis by atomic absorption spectrophotometry (AAS; Unicam, Sp9; Pye Unicam Ltd., Cambridge, Cambs). Cu, Zn, Fe and Mn standards were prepared in TCA (100 g/l) which was also used as a zero reference. Culture-medium samples were analysed undiluted and distilled-deionized water (DDW) was used as a zero reference. For Ca and Mg analysis both serum and culture-medium

samples were diluted 1 + 99 with  $\text{LaCl}_3$  solution (1 g/l), standards were prepared similarly and 1 g/l  $\text{LaCl}_3$  solution was the blank.

#### *Preparation of repleted media*

As slight decreases in the levels of a range of metals occurred following dialysis, 0.1 ml of sterile solutions of  $\text{ZnCl}_2$  (0.954 mM),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (39 mM) and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (14.2 mM) were added to the CuD medium to achieve pre-dialysis levels. CuR media were prepared at three different Cu levels by adding 0.1 ml of increasing concentrations of  $\text{CuCl}_2$  into the CuD medium. These were: CuR, 51.89  $\mu\text{M}$ ; CuRI, 0.345 mM and CuRII, 0.689 mM.

#### *Determination of intracellular metal element concentration*

Cells ( $6 \times 10^7$  cells for each element determined) were harvested, washed once in 10 mM-EDTA-PBS and twice in saline (9 g NaCl/l) to remove any non-specifically bound Cu from cell surfaces. The cells were lysed by resuspension in 1.5 ml DDW (or  $\text{LaCl}_3$  (1 g/l) for Ca and Mg analysis) and freezing-thawing (liquid  $\text{N}_2$  to  $37^\circ$ , three times). Following centrifugation of lysates the supernatant fractions were assayed for Cu, Zn, Fe, Mn, Ca and Mg using AAS.

#### *Preparation of Molt-3 cells for scanning electron microscopy (SEM)*

A 0.15 M solution of poly-L-lysine was poured onto sterile glass coverslips in individual Petri dishes. Cells ( $8 \times 10^5$  cells/ml) were poured onto washed and dried poly-L-lysine-coated coverslips and allowed to attach at  $37^\circ$  for 3 h in a humidified  $\text{CO}_2$ -air (5:95 v/v) incubator. Each coverslip was then washed twice in saline, fixed in buffered formaldehyde-glutaraldehyde fixative solution for 4 h, washed twice in 0.1 M-cacodylate buffer and post-fixed in  $\text{OsO}_4$  (10 g/l) at  $4^\circ$  overnight. Coverslips were then washed three times in 0.1 M-cacodylate buffer, dehydrated in acetone and dried (critical point dryer). Finally the coverslips were attached to electron microscopy stubs with conductive C cement and gold-sputtered. The specimens were examined in a Jeol JSM-840 SEM (Jeol Ltd., Tokyo, Japan). Micrographs were taken to assess morphological changes in cell surfaces.

#### *Enzyme assays*

*Superoxide dismutase.* Total SOD activity was measured by a modification of the method of Podcasy & Wei (1988) using the debris-free supernatant fraction from  $2 \times 10^7$  cells. The assay involves the ability of SOD to inhibit the  $\text{O}_2$ -mediated reduction of iodionitro-tetrazolium (INTV) to a water-soluble product with an absorption maximum at 505 nm. A unit of SOD enzyme activity is defined as the amount of enzyme which inhibits the reaction by 50%. Mn-SOD activity was measured by addition of 5 mM-NaCN to the reaction mixture and Cu,Zn-SOD was calculated by subtraction of the Mn-SOD value from the total. Cellular SOD activity was expressed as U/ $10^7$  cells.

*Catalase.* The intracellular activity of CAT was determined at room temperature by following the decomposition of  $\text{H}_2\text{O}_2$  at 240 nm according to the method of Aebi (1983). Activity is expressed as U/mg cellular protein.

*Glutathione peroxidase.* GSHPx activity was quantified by a coupled enzyme procedure at  $37^\circ$ , monitoring loss of NADPH at 340 nm (Paglia & Valentine, 1967). Assays were carried out using the commercially available Ransel GSHPx kit on a Cobas Fara analyser (Roche Products Ltd., Welwyn Garden City, Herts). Results are expressed as U/mg protein.

*Cytochrome c oxidase.* CCO activity was measured by the method of Cooperstein & Lazarow (1951). Results are expressed as U/mg cellular protein.

*Protein.* All protein assays were performed using a Bio-Rad protein assay.

*Statistical analysis*

One-way ANOVA was used for data analysis. A *P* value of < 0.05 was taken to indicate statistical significance.

**RESULTS***Copper depletion from fetal calf serum*

Dialysis against 30 mM-TRIEN reduced the Cu content of FCS to less than 28% of the non-dialysed control (Table 1). Although significant depletion of Zn, Ca and Mg also occurred, the efficacy and specificity of TRIEN were greater than for any other of six chelators tested in preliminary experiments (results not shown). The total protein content of the serum was not modified by dialysis.

*Metal levels of cell culture media*

Cell culture media were produced by adding normal or dialysed FCS (100 g/l) to RPMI. After repletion of Zn, Ca and Mg, levels were measured again by AAS (Table 2). The only significant difference between normal and CuD media was the Cu level; the CuD level was less than 53.6% of the normal value. The concentrations of all six metal elements in CuR medium were similar to those of the control. CuRI and CuRII media were higher in Cu than normal medium, but the level did not exceed the physiological range of 1.00–1.20 ppm found in normal human serum (Underwood, 1977).

*The effect of copper depletion on cell growth*

Depletion of Cu caused a significant decline in the growth rates of Molt-3 and HL-60 cells. The effect on HL-60 cells was apparent within the initial 72 h of culture in CuD medium (Fig. 1) whereas prolonged culture was necessary to impair Molt-3 proliferation (Figs. 2 and 3). Repletion of Cu restored the ability of the medium to support growth of Molt-3 but not of HL-60 cells, the degree of restoration being proportional to the amount of Cu added. A further study showed that Cu repletion was equally effective irrespective of the Cu source added; Cu(histidine)<sub>2</sub>, Cu albumin, and Cu(I)glutathione (K. K. Tong, J. J. Strain and B. M. Hannigan, unpublished results). The growth rate of Raji cells was unaffected by Cu depletion and the mean cell viabilities of all cultures ranged from 94 to 100%. Thus cells could be maintained to permit *in vitro* studies of Cu-deficiency.

*Intracellular metal levels (Molt-3)*

After 72 h culture in CuD medium cellular Cu was reduced by 47.6% relative to control values, cellular Ca was significantly increased ( $\times 97\%$ ) and other metal elements remained unchanged (Table 3). Cells grown in CuR medium had a lower Cu level than those in normal medium. No significant changes in intracellular metal levels occurred in Molt-3 cells cultured in CuRI or CuRII media, relative to the normal control (results for CuRII not shown). Following the initial 72 h culture period in CuD medium, cells were re-cultured for 72 h in media of varying Cu content. In fresh CuD medium cellular Cu continued to decline, to 33% of the normal level, and cellular Ca was further increased, to 100.8% of the control level (Table 4).

*Enzyme activities in Molt-3 cells*

Results of the intracellular Cu,Zn-SOD activity measurements are shown in Fig. 4. Addition of cyanide to the reaction mixture abolished all SOD activity, thus indicating that Molt-3 cells do not contain measurable Mn-SOD. Cellular Cu,Zn-SOD activity was significantly reduced, to 31.5% of the control value, following 72 h incubation in CuD medium. With Cu repletion, SOD activity was restored in a dose-dependent manner to a

Table 1. *The levels of trace metals (ppm) in fetal calf serum before and after dialysis against a specific copper chelator triethylenetetramine (TRIE)*

(Mean values and standard deviations for three determinations)

	Before dialysis		After dialysis v. 30 mM-TRIE	
	Mean	SD	Mean	SD
Cu	0.256	0.001	0.070***	0.002
Zn	4.566	0.006	0.400***	0.002
Fe	2.686	0.015	2.660	0.010
Mn	0.106	0.005	0.101	0.007
Ca	125.0	6.8	9.7***	0.4
Mg	32.6	1.2	3.5***	0.2
Protein (mg/ml)	29.6	3.2	29.5***	1.0

\*\*\* Mean values were significantly different from those before dialysis,  $P < 0.0001$ .Table 2. *The levels of trace metals (ppm) in cell culture media*

(Mean values and standard deviations for three determinations)

Medium		Cu	Zn	Fe	Mn	Ca	Mg
Normal	Mean	0.061	0.413	0.448	0.042	22.8	10.7
	SD	0.002	0.002	0.013	0.001	0.7	0.1
Metal-depleted	Mean	0.033*	0.135*	0.430	0.039	14.2*	8.6*
	SD	0.002	0.005	0.006	0.001	0.3	0.1
CuD	Mean	0.033*	0.432	0.432	0.040	22.6	10.8
	SD	0.002	0.018	0.008	0.002	0.3	0.1
CuR	Mean	0.058	0.438	0.433	0.040	22.2	10.7
	SD	0.002	0.018	0.002	0.001	0.4	0.1
CuRI	Mean	0.187*	0.438	0.428	0.040	22.1	10.7
	SD	0.002	0.019	0.007	0.002	0.2	0.1
CuRII	Mean	0.034*	0.433	0.427	0.039	22.1	10.7
	SD	0.001	0.020	0.008	0.000	0.2	0.1

CuD, copper-depleted; CuR, copper-repleted (0.1 ml 51.89  $\mu\text{M}$ -CuCl<sub>2</sub>); CuRI, copper-repleted (0.1 ml 0.345 mM-CuCl<sub>2</sub>); CuRII, copper-repleted (0.1 ml 0.689 mM-CuCl<sub>2</sub>).\* Mean values were significantly different from those for the normal medium,  $P < 0.05$ .

maximum of 90.7% of the control in CuRII medium. Incubation of cells in CuD medium for longer than 72 h produced no further decline in SOD activity. Neither CAT nor GSHPx activity was altered by Cu depletion (results not shown). Molt-3 levels of the mitochondrial enzyme CCO are shown in Fig. 5.

Incubation of cells in CuD medium for 72 h caused a significant reduction in Molt-3 CCO activity, to 32.5% of the normal value. This reduction in CCO was further increased, to less than 10.3% of the control value, following a second 72 h culture period in CuD medium. Restoration of CCO activity was effected by Cu repletion, in a dose-dependent manner.

#### *Morphological features of Molt-3 cells*

The morphological features of Molt-3 cells, visible under SEM, are presented in Plate 1, (a) to (f). In general, cells cultured in normal medium were relatively round and uniform in shape, panel (b), as were cells following 72 h culture in CuRII, or CuD media (panels (a)

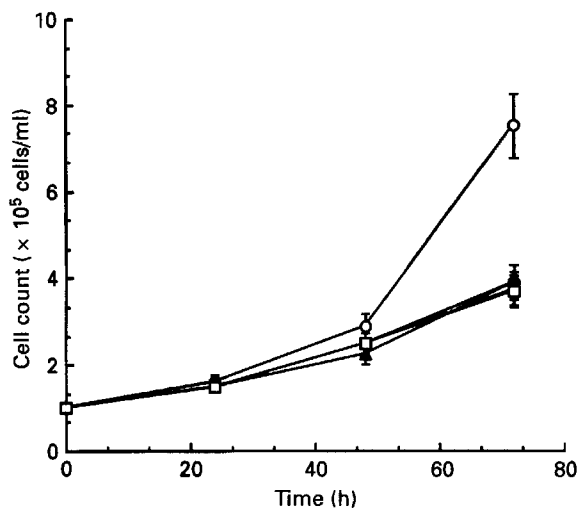


Fig. 1. Growth of HL-60 cells in culture media of varying copper content. (○), Normal medium; (●), copper-depleted medium; (△), copper-repleted medium (0.1 ml 51.89  $\mu\text{M}$ -CuCl<sub>2</sub>); (▲), copper-repleted (0.1 ml 0.345 mM-CuCl<sub>2</sub>); (□), copper-repleted (0.1 ml 0.689 mM-CuCl<sub>2</sub>). Values are means for six determinations, with their standard deviations represented by vertical bars.

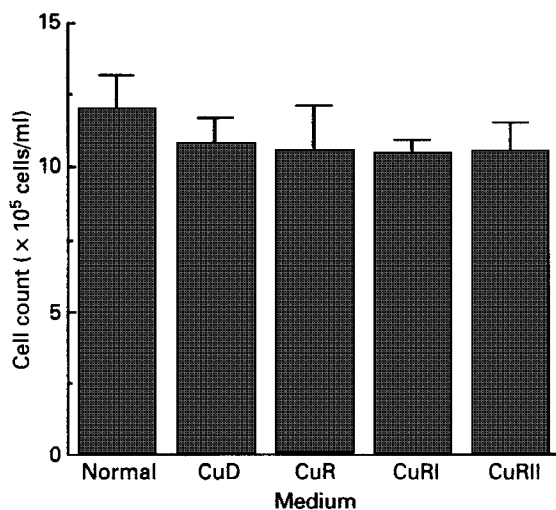


Fig. 2. Growth of Molt-3 cells following 72 h culture in media of varying copper content. Values are means for four determinations with their standard deviations represented by vertical bars. CuD, copper-depleted; CuR, copper-repleted (0.1 ml 51.89  $\mu\text{M}$ -CuCl<sub>2</sub>); CuRI, copper-repleted (0.1 ml 0.345 mM-CuCl<sub>2</sub>); CuRII, copper-repleted (0.1 ml 0.689 mM-CuCl<sub>2</sub>).

and (c) respectively). All cells examined had a generally spherical appearance with a variable number of microvilli. Conversely, a significant number of changes in cell surface topography were observed following a further 72 h culture in CuD medium (panel (f)). Cells showed a pock-marked surface indicative of membrane damage (Roath *et al.* 1978). No surface disruption was noted when cells previously cultured (72 h) in CuD medium were re-cultured (72 h) in CuRII medium (panel (d)). Surprisingly, cells first cultured in CuD medium and then re-cultured in normal medium continued to show significant morphological change (panel (e)).

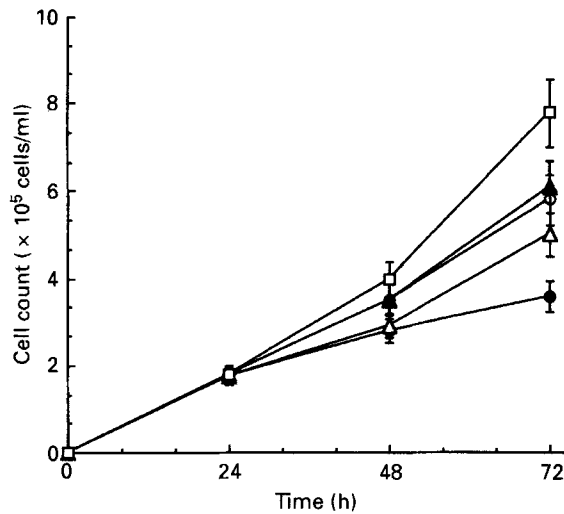


Fig. 3. Growth of Molt-3 cells following 72 h culture in copper-depleted medium and 72 h culture in media of varying copper content. (○), Normal medium; (●), copper-deficient; (△), copper-repleted (0.1 ml 51.89  $\mu\text{M}$ -CuCl<sub>2</sub>); (▲), copper-repleted (0.1 ml 0.345 mM-CuCl<sub>2</sub>); (□), copper-repleted (0.1 ml 0.689 mM-CuCl<sub>2</sub>). Values are means for four determinations, and standard deviations represented by vertical bars.

Table 3. Intracellular levels of trace metals ( $\mu\text{g}/4 \times 10^7$  cells) in Molt-3 cells following 72 h culture in media with varying copper levels

(Mean values and standard deviations for three separate cultures)

Medium		Cu	Zn	Fe	Mn	Ca	Mg
Normal	Mean	0.042	0.404	0.066	0.014	0.266	4.600
	SD	0.002	0.008	0.006	0.004	0.008	0.130
CuD	Mean	0.022*	0.401	0.069	0.018	0.518*	4.545
	SD	0.003	0.021	0.007	0.003	0.021	0.069
CuR	Mean	0.023*	0.418	0.065	0.012	0.345	4.656
	SD	0.006	0.015	0.001	0.002	0.006	0.008
CuRI	Mean	0.035	0.416	0.062	0.013	0.266	4.808
	SD	0.005	0.015	0.001	0.002	0.006	0.008

CuD, copper-depleted; CuR, copper-repleted (0.1 ml 51.89  $\mu\text{M}$ -CuCl<sub>2</sub>); CuRI, copper-repleted (0.1 ml 0.345 mM-CuCl<sub>2</sub>).

\* Mean values were significantly different from those for normal medium,  $P < 0.05$ .

#### DISCUSSION

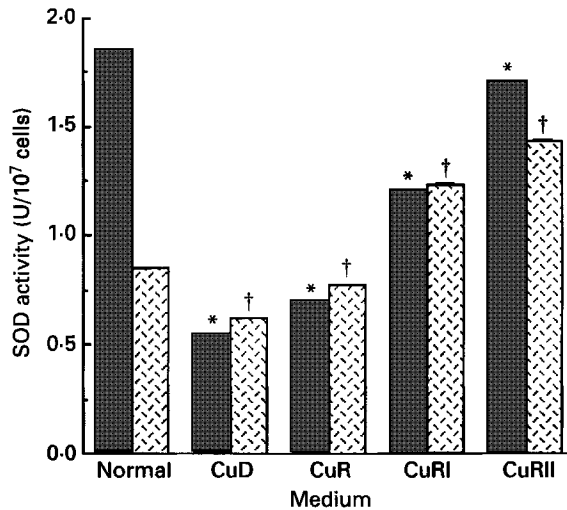
In the present study the influence of varying Cu levels on lymphoid cells *in vitro* was examined. Depletion of Cu from FCS was achieved by dialysis against the chelator TRIEN. Manipulation of culture medium Cu level by FCS depletion and repletion was possible as RPMI alone did not contribute significantly to the Cu content. The restricted growth of Molt-3 cells in CuD medium indicated that Cu can influence lymphoid cell growth. The magnitude of this effect was different in different cell types; Raji (B-lymphoid) cell growth was unaffected by Cu depletion while the proliferative rate of HL-60 cells was significantly impaired, even after only 72 h in culture. A similar pattern of varying sensitivity to trace metal levels was previously noted in experiments using media depleted and subsequently repleted with Zn (Martin *et al.* 1991). The differential effects of Cu depletion on T- and B-

**Table 4.** Intracellular levels of trace metals in Molt-3 cells ( $\mu\text{g}/4 \times 10^7$  cells) after 72 h culture in copper-depleted (CuD) media followed by 72 h culture in media with varying copper levels (Mean values and standard deviations for three separate cultures)

Medium		Cu	Zn	Fe	Mn	Ca	Mg
Normal	Mean	0.024	0.422	0.068	0.015	0.300	4.550
	SD	0.004	0.017	0.001	0.002	0.021	0.055
CuD	Mean	0.014*	0.413	0.070	0.016	0.534*	4.331
	SD	0.001	0.005	0.003	0.002	0.015	0.008
CuR	Mean	0.025	0.410	0.070	0.015	0.322	4.545
	SD	0.004	0.014	0.001	0.002	0.015	0.108
CuRI	Mean	0.036*	0.434	0.067	0.012	0.348	4.401
	SD	0.002	0.013	0.004	0.001	0.030	0.170

CuD, copper-depleted; CuR, copper-repleted (0.1 ml 51.89  $\mu\text{M}$ -CuCl<sub>2</sub>); CuRI, copper-repleted (0.1 ml 0.345 mM-CuCl<sub>2</sub>).

\* Mean values were significantly different from those for the normal medium,  $P < 0.05$ .



**Fig. 4.** Cellular copper-zinc-superoxide dismutase (SOD; EC 1.15.1.1) activity in Molt-3 cells cultured in media of varying copper content for 72 h (■), or for 72 h in copper-deficient medium followed by 72 h in media of varying copper content (▨). CuD, copper-depleted; CuR, copper-repleted (0.1 ml 51.89  $\mu\text{M}$ -CuCl<sub>2</sub>); CuRI, copper-repleted (0.1 ml 0.345 mM-CuCl<sub>2</sub>); CuRII, copper-repleted (0.1 ml 0.689 mM-CuCl<sub>2</sub>). Values are means for three determinations with standard deviations represented by vertical bars. \* Mean values were significantly different from those for the normal medium (72 h),  $P < 0.05$ ; † mean values were significantly different from those for the normal medium (72 h + 72 h),  $P < 0.05$ .

lymphoid cells and myeloid cells would support previous reports of a range of immunological abnormalities in Cu-deficient animals, with T-cell functions being more severely impaired than B cells (Lukasewycz *et al.* 1985). While none of the activities of the known cuproenzymes provides a ready link between Cu levels and cell proliferation, it has recently been suggested that an increase in intracellular Cu levels, particularly within the nucleus, is necessary for cell proliferation (Wlostowski, 1993). It is possible that Cu may help to maintain Fe in its reduced state and so available as a metal co-factor for ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis (Cory, 1983; Harris,



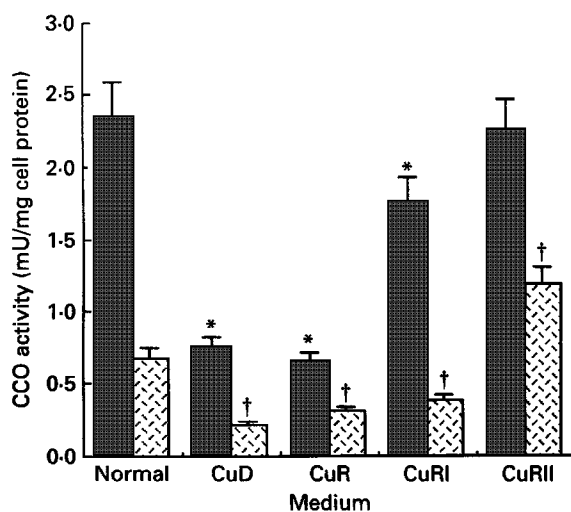


Fig. 5. Cellular cytochrome c oxidase (CCO; *EC* 1.9.3.1) activity in Molt-3 cells cultured in media of varying copper content for 72 h (■) or for 72 h in copper-deficient medium followed by 72 h in media of varying copper content (▨). CuD, copper-depleted; CuR, copper-repleted (0.1 ml 51.89  $\mu\text{M}$ -CuCl<sub>2</sub>); CuRI, copper-repleted (0.1 ml 0.345 mM-CuCl<sub>2</sub>); CuRII, copper-repleted (0.1 ml 0.689 mM-CuCl<sub>2</sub>). Values are means for three determinations, with standard deviations represented by vertical bars. \*Mean values were significantly different from those for the normal medium (72 h),  $P < 0.05$ ; † mean values were significantly different from those for the normal medium (72 h + 72 h),  $P < 0.05$ .

1983). The maintenance of normal cell viability, despite a reduced growth rate, contributes greatly to the usefulness of this cell model, allowing investigation of a range of functions under conditions of varying Cu levels in media.

Evidence that individual cells were rendered Cu-deficient is provided by the fact that intracellular Cu levels were markedly reduced through culture in CuD medium, the extent of the reduction depending on the duration of cell culture. A necessary threshold level of Cu may be indicated because Molt-3 cell proliferation was not affected by the Cu levels pertaining after 72 h culture, the lower levels at 144 h coinciding with a significant impairment of growth rate. The measured cellular levels of Cu, Zn and Fe are similar to values previously published for the murine leukaemic cell line L1210. The depression in intracellular Cu,Zn-SOD activity reported here indicates that the activity of this enzyme is directly influenced by cellular Cu levels. The importance of biologically available Cu, as opposed to the simple presence of CuCl<sub>2</sub> in the medium, may warrant further investigation because Cu when repleted to pre-dialysis levels (CuR medium) did not restore the normal activity level of SOD. Even with excess CuCl<sub>2</sub> in CuRII medium the enzyme level had attained only 90.3% of its normal value. The function of SOD is to prevent cellular damage as a consequence of O<sub>2</sub><sup>·-</sup> build-up. CAT and GSHPx function cooperatively through the removal of H<sub>2</sub>O<sub>2</sub> and hydroperoxides synthesized by SOD action. Neither CAT nor GSHPx activity was changed in the current study. Thus the observed changes in SOD activity, without any compensatory increase in other antioxidant enzymes, can lead to a wide range of profound disturbances in cellular structure and function. The morphological damage observed in SEM studies on Molt-3 cells is a highly likely consequence of O<sub>2</sub><sup>·-</sup> build-up. Interestingly, after 72 h culture in CuD medium, normal cell morphology was restored by CuRII medium but not by normal medium. This finding is very strong evidence for the essential role of adequate Cu levels in maintaining cell membrane integrity. Alterations in cell surface membranes may lead to altered permeability to Ca<sup>2+</sup>. The

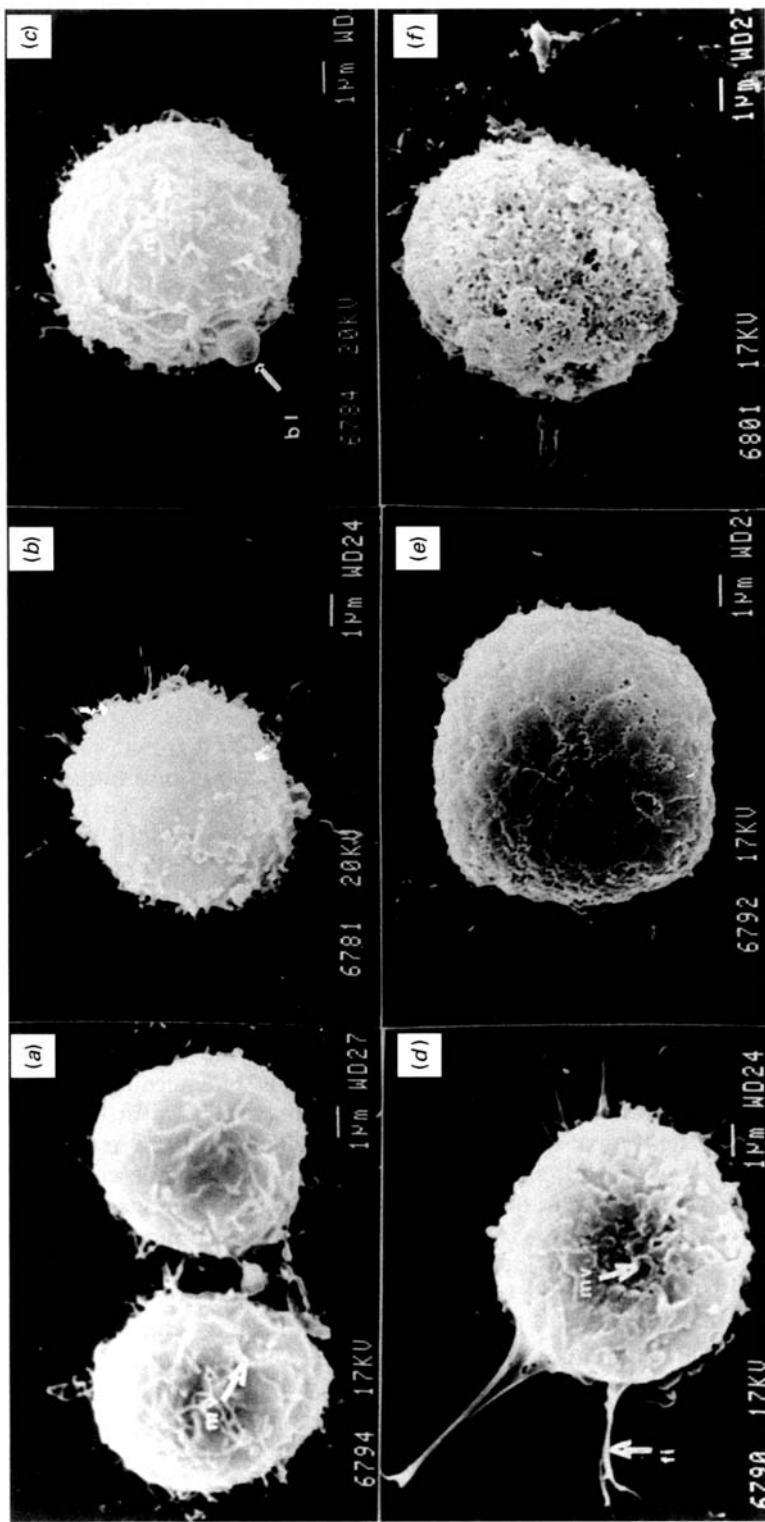


Plate 1. Morphological features of Molt-3 cells cultured in media of varying copper content, visible under scanning electron microscopy. (a), Copper-repleted medium (0.1 ml 0.689 mM-CuCl<sub>2</sub>) (CuRII), 72 h; (b) normal medium, 72 h; (c) copper-deficient (CuD) medium, 72 h; (d) CuD, 72 h + CuRII, 72 h; (e) CuD, 72 h + normal medium, 72 h; (f) CuD, 72 h + CuD, 72 h.

observation of increased accumulation of intracellular  $\text{Ca}^{2+}$  in cells from CuD medium may be a direct result of this damage. Apart from increased membrane permeability, one cellular change which can contribute to  $\text{Ca}^{2+}$  accumulation is the disruption of a number of ATP-dependent enzymes, normally responsible for maintaining the  $\text{Ca}^{2+}$  gradient (Schanne *et al.* 1979). The observed reduction in CCO levels in CuD cells may affect intracellular ATP levels, a consequent rise in intracellular  $\text{Ca}^{2+}$  adding to disruption of the cellular cytoskeleton and plasma membrane (Boobis *et al.* 1989; Orrenius *et al.* 1989). Low CCO activities have been measured in spleen, thymus, heart and liver tissues from CuD mice (Prohaska *et al.* 1983; Bode *et al.* 1992).

#### CONCLUSIONS

Depletion of Cu in culture medium induced measurable changes in the structure and function of the human T-lymphoid cell line Molt-3, the severity of the changes depending on the period of culture in the deficient media. These changes were reversible, in whole or in part, by repletion of Cu in the form of  $\text{CuCl}_2$  added to the culture medium. From the results presented it appears that Cu deficiency can exert an effect on immunocompetence by altering the structure of immune cell membranes and the activity of enzymes which mediate antioxidant defences, ATP production and mitosis. This cellular model allows each possible effect of Cu deficiency to be analysed independently of changes in any other component of the cells' normal growth environment. The many possible sites through which Cu can influence cellular integrity highlight the complex interaction of Cu with immune systems. Progress in understanding this interaction will require not only dietary intervention studies in animals but also *in vitro* model systems such as that described here.

This work was supported by a grant from the Department of Health and Social Security (N. Ireland) Clinical Research Award. K.K.T. was a recipient of a University of Ulster postgraduate studentship.

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