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# Copper-thionein in leucocytes

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**Summary.** Upon incubation of peripheral leucocytes with copper sulphate a dramatic cellular copper uptake reaching levels of 25–50-fold compared to that of the natural copper content was measured. The orange-red fluorescence of the copper-treated white blood cells was assigned to the formation of Cu(I)-thiolate clusters in Cu(I)-thionein. A protein of 6–8 kDa was isolated from homogenized bovine leucocytes and characterized by its electronic absorption and amino acid composition to be identical to the above Cu(I)-thionein. More than 70% of the intracellular copper was attributed to this protein in its monomeric and polymeric form. Cu-thionein formation was more pronounced in monocytes than in granulocytes. As most intriguing phenomenon, the release of this Cu-thionein from leucocytes, was also noticed. The occurrence of Cu-thionein in leucocytes and the excretion of the intact Cu(I)-thiolate protein is of considerable interest with respect to the observed elevated copper levels in white blood cells and plasma during tumor malignancies and inflammatory processes.

**Key words:** Copper — Cu-thionein — Metallothionein — Leucocytes

## Introduction

The molecular principles of absorption, transport and storage of copper are amongst the most important tasks in elucidating the biochemical role of this prominent transition metal. There are some intriguing physiological phenomena regarding the levels of biogenic copper, including a dramatic rise of copper in the blood plasma during the course of systemic inflammation and rheumatoid arthritis. Changes in the hormonal status give rise to transient imbalances in copper metabolism (Deuschle and Weser 1985; Weser and Hartmann 1984). The observed rise of copper concentration in the plasma is paralleled by a marked increase in the total number of leucocytes. The plasma copper is essentially bound to caeruloplasmin which is known to be an important antioxidant in vivo (Gutteridge 1985). Inflammatory processes may also be affected by the binding of this serum protein to specific receptors located on leucocyte membranes (Kataoka and Tavassoli 1985). Furthermore, the inhibition of leucocyte-induced degradation of hyaluronic acid by caeruloplasmin (Hartmann et al. 1985) confirms its pronounced ability to control the activity of free radicals and excited oxygen species generated by these cells. Activated neutrophils are able to oxidize Cu(I)-thionein yielding Cu(II) of considerable uncontrolled reactivity (Hartmann et al. 1985). At the same time, and in the presence of apo-caeruloplasmin, the formation of the holoprotein was noticed (Schechinger et al. 1986). This explains the occurrence of 10–15% of caeruloplasmin in its metal-free form in blood plasma (Holtzman and Gaumnitz 1970).

Compared to healthy subjects, a significantly higher copper concentration, sometimes reaching 300% above normal, was observed in serum and lymphocytes from children suffering from acute lymphocytic leukemia (Carpentieri et al. 1986). In general, Cu, Zn and Fe have been associated with normal lymphocyte maturation and regulation of immune function and serum concentrations of these metals are modified in most types of cancer (Carpentieri et al. 1986).

These phenomena concerning leucocytes and copper, together with the fact that metallothion-

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ein synthesis could be induced by Cd salts in human peripheral leucocytes (Peavy and Fairchild 1987; Koizumi et al. 1987) prompted us to investigate the pronounced reactivity of white blood cells with copper ions in more detail. It was of interest to search for the nature of the copper-binding compound.

The thiolate-rich metallothioneins are suggested to be the most likely candidates for the successful binding and control of the cellular copper level. In these proteins, copper is sequestered in the form of stable oligonuclear Cu(I)-thiolates. Thus, the many undesirable reactions of freely diffusing low-$M_r$ Cu complexes are put under control (Felix et al. 1989).

Examination of the cell homogenates of blood leucocytes previously incubated in the presence of copper ions should allow a decision on whether or not metallothionein is involved in leucocyte copper metabolism.

## Materials and methods

*Chemicals.* RPMI 1640 synthetic medium, Trypan blue, xanthine and nitroblue tetrazolium chloride (NBT) were from Serva, Heidelberg. Xanthine oxidase, cytochrome *c* and phorbol myristate acetate (PMA) were purchased from Sigma, Heidelberg.

*Leucocytes.* Bovine and human leucocytes were prepared from fresh citrated blood according to published methods (Hartmann et al. 1985; Dioguardi et al. 1963). For the separate isolation of bovine mononuclear and polynuclear cells, whole blood was centrifuged for 30 min at 2000 *g*. The mononuclear cells concentrated as a thin layer located in between the red cells and plasma. After removing the plasma this layer was decanted from the red cells. Contamination with granulocytes was negligible. The bulk of granulocytes were associated together with the erythrocytes. Repeated treatment of both fractions with 10 vol. 0.83% $NH_4Cl$ was performed until no red cells were present in either preparation. The cells were washed twice with 50 vol. phosphate-buffered saline (PBS). Human peripheral leucocytes were obtained from 40 ml citrated blood without separating the different cell types. Leucocytes ($2 \times 10^8$ cells) were incubated in a PBS-containing RPMI 1640 medium (3 ml PBS, 2 ml RPMI 1640) at 25° C in the presence of 20–100 μM $CuSO_4$. After incubation, the cells were washed twice with PBS and homogenized in 2 vol. water by repeated freezing and thawing prior to sonification for 10 min at 30° C. The supernatant was chromatographed by means of an FPLC system using a Pharmacia Superose 12 gel filtration column. Cell viability was controlled by Trypan blue exclusion.

*Analytical methods.* For metal determination, washed white blood cells were ashed 12 h in the presence of 1.5 vol. hydrochloric acid and 0.15 vol. $H_2O_2$. Copper and zinc were quantified by flameless atomic absorption spectrometry on a Perkin-Elmer 3030 unit. Electronic absorption spectra were run on a Beckman DU 40 spectrophotometer. Fluorescence measurements were carried out on a Spex fluorolog 222 double-beam spectrometer. Amino acid analyses were performed using the hydrolyzed protein as previously described (Hartmann and Weser 1977). Cysteine residues were converted into cysteic acid by treatment of the protein with performic acid prior to hydrolysis.

## Results and discussion

When bovine blood leucocytes ($2 \times 10^8$ cells) were incubated in 5 ml PBS/RPMI medium in the presence of 100 μM $CuSO_4$, a time-dependent copper uptake was observed reaching saturation between 6–8 h at values of nearly 25 μg Cu/$2 \times 10^8$ cells (Fig. 1). It should be emphasized that the copper concentration of untreated cells was less than 1 μg Cu/$2 \times 10^8$ cells.

Concomitant with the metal incorporation, an orange-red luminescence was noticed when washed cells were irradiated with ultraviolet light in the 300-nm region. This fluorescence was of intriguing similarity to that of fetal bovine Cu-thionein, yeast Cu-thionein and reconstituted Cu-thionein (Beltramini and Lerch 1981; Hartmann et al. 1987; Richter and Weser 1988). In this context credit should be given to an earlier observation of Anglin et al. (1971) who treated white guinea-pig epidermal homogenate with copper salts. Unfortunately, they were unable to assign the exact nature of the fluroescent compound. They suggested that this orange-red fluorescence might have originated from transiently formed copper-cysteine complexes. In the present experiments where bovine leucocytes were used, mononuclear cells exhibited this luminescence already within 15 min in the presence of approximately 100 μM copper. In contrast, 2 h were required until polynuclear cells showed the same fluorescence at an intensity reaching only 60–70% of that of the lymphocytes.

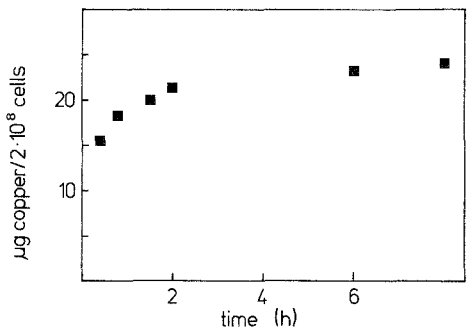


**Fig. 1.** Time-dependent copper uptake by bovine leucocytes. Samples of $2 \times 10^8$ cells were incubated in 5 ml PBS in the presence of 100 μM $CuSO_4$ under shaking at 25° C. Copper was measured in washed cells after ashing. Three independent experiments were carried out. The reproducibiltiy was ± 5%

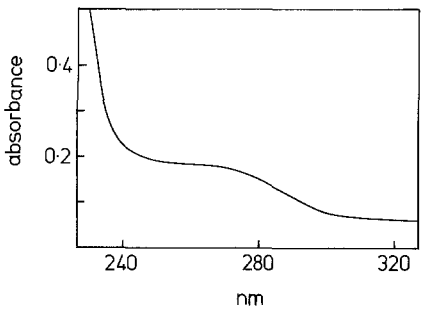


**Fig. 2.** Ultraviolet electronic absorption of bovine leucocyte Cu-thionein after FPLC Superose 12 chromatography. The copper concentration was 3.6 μM and zinc 1.4 μM. Light path: 1 cm

The copper-dependent fluorescence was also seen in human leucocytes. It was attractive to conclude that this phenomenon must be associated with Cu-metallothionein. An induction of metallothionein by $CdCl_2$ in monocyte and lymphocyte cultures but not in granulocytes has been proposed (Peavy and Fairchild 1987). However, no chemical analyses characteristic for metallothionein were shown. Similar results were obtained from Cd-treated monocytes and lymphocytes (Koizumi et al. 1987).

In order to characterize the copper-binding compound the copper-treated white blood cells were homogenized. After centrifugation at 48000 *g*, the supernatant was chromatographed on an FPLC Superose 12 gel filtration column equilibrated with 10 mM Tris/HCl, pH 8 in the presence of 0.1% 2-mercaptoethanol. Under these conditions the main copper-containing fraction co-eluted with the bulk of high-$M_r$ compounds. However, cell homogenization with 1% SDS and 0.5% mercaptoethanol revealed a three fold higher copper solubilisation. A copper-binding polypeptide in the 6–8-kDa region was obtained. The ultraviolet electronic absorption of this fraction was similar to that of earlier preparations of hepatic bovine Cu-thionein (Hartmann and Weser 1977) indicating the absence of aromatic amino acids (Fig. 2).

The most specific proof for metallothionein was expected from the amino acid analysis of this fraction. In fact, the amino acid composition was typical for bovine metallothionein (Table 1).

Because of many uncontrolled oxidation processes, the exact stoichiometry of copper and zinc to the protein portion cannot be given. However, attention should be placed on the intriguing fact that more than 70% of total copper is associated with these monomeric and polymeric metallothioneins. The behaviour of the intracellular, sometimes high-$M_r$ and insoluble, copper-binding proteins is related to the early observation of Porter who successfully demonstrated the occurrence of a polymeric Cu-protein in the liver of newborn species (Porter 1974). These polymeric proteins from pre- and neonatal hepatic tissues were thought to represent a copper-rich form of metallothionein. The mode of the polymerization was shown in our laboratory (Rupp and Weser 1974). A similar situation can be deduced in the leucocytes examined here.

The fast formation of Cu-thionein upon copper incubation of leucocytes may be due to the presence of metal-free and/or zinc-containing thionein which is converted into the copper form. The more pronounced formation of Cu-thionein in monocytes compared to that in granulocytes could be explained by the oxidative action of the latter cells as mentioned in the Introduction.

A copper-binding protein of molecular mass approximately 7 kDa from copper-supplemented lymphoblast and fibroblast cultures was found previously (Riordan and Madapallimattam 1979). However, binding of 1 mol Cu/mol protein, a low cysteine content of 5–6% and an amino acid composition distinctly different from metallothionein were noted. They concluded that their protein was not related to metallothionein. However, the above-mentioned phenomena do not fully exclude a relationship to metallothionein. In earlier work, the oxidative cleavage of the Cu(I)-thiolate bonding has been repeatedly shown (Weser and Hartmann 1984).

**Table 1.** Amino acid composition of bovine leucocyte and fetal liver copper-thionein. Values are expressed as a percentage of total number of residues in the molecule

| Amino acid | Bovine copper-thionein | |
|---|---|---|
| | Leucocytes | Liver [17] |
| Cysteine[a] | 28.1 | 27.8 |
| Aspartate | 5.8 | 6.6 |
| Glutamate | 4.1 | 5.0 |
| Threonine | 4.6 | 4.4 |
| Serine | 10.8 | 12.2 |
| Proline | 4.9 | 6.6 |
| Glycine | 9.2 | 9.1 |
| Alanine | 8.3 | 8.7 |
| Valine | 4.6 | 4.2 |
| Lysine | 9.2 | 12.3 |
| Arginine | 2.3 | 0.7 |
| Leucine | 4.2 | 0.9 |
| Isoleucine | 2.8 | 1.2 |
| Methionine | 0.6 | 0 |

[a] Determined as cysteic acid

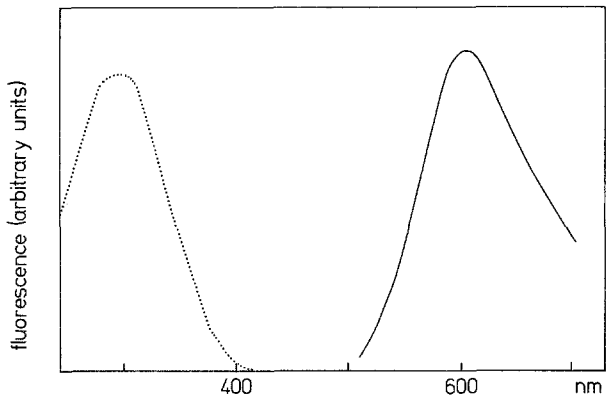


**Fig. 3.** Corrected fluroescence spectrum of the cell-free supernatant of leucocytes after 8-h incubations with copper(II) salt

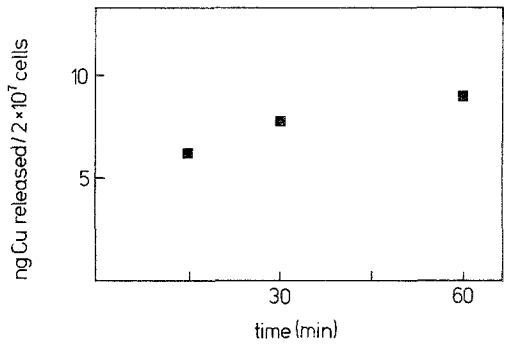


**Fig. 4.** Release of copper from activated bovine granulocytes. Samples of $2 \times 10^7$ granulocytes in 1 ml PBS were activated with 10 µl PMA (1 mM). The copper concentration was measured in the centrifuged cell-free supernatant. Unactivated cells served as control. All measurements were performed in triplicate. The reproducibility was ±7%

A most intriguing phenomenon was observed when the copper incubation of the leucocytes was performed in PBS supplemented with 1 mM $Ca^{2+}$, 1 mM $Mg^{2+}$ and 5.5 mM glucose. Ultraviolet irradiation of the buffer solution produced the same luminescence emission as found in intact Cu-treated leucocytes (Fig. 3).

The release of intact Cu-thionein can be deduced to be similar to that observed with copper-supplemented yeast cells (Felix et al. 1989). This species from leucocytes, although soluble, appeared to be a polymer form of Cu-thionein. Uncontrolled oxidation processes during the course of the incubation may have led to this polymerization. Nevertheless, according to the observed fluorescence, there were sufficient Cu(I)-thiolate chromophores present. An amino acid composition virtually identical to metallothionein was found. 15–20% of the total intracellular copper concentration was measured in this portion of the soluble copper-protein.

In order to examine the degree of cell damage, leucocyte viability was measured before and after copper treatment. No marked damage was noticed. The Trypan blue exclusion assay revealed only 5–8% of stained cells after an 8-h copper incubation and was similar to untreated cells. Furthermore, no superoxide dismutase activity at all was seen in the cell-free supernatant, indicating that no cytosolic enzymes are released due to cell damage. In addition, PMA-activated granulocytes were essentially unaffected in their ability to reduce cytochrome *c*.

The occurrence of Cu-metallothionein in leucocytes and the excretion of the intact copper protein is of utmost importance. Our knowledge of the intracellular catabolism of Cu-thionein is limited. It is assumed to provide the cell with essential copper. The release of Cu-thionein into the blood plasma would help to reconstitute many copper proteins, including the 10–15% of apo-caeruloplasmin always present in blood. Oxidative labilization of Cu(I)-thiolate clusters may be assigned to the action of granulocytes, as shown earlier (Hartmann et al. 1985). Thus, the antioxidizing function of caeruloplasmin is expected to be considerably enhanced. Indeed, copper release from PMA-activated, non-copper-treated granulocytes was measured. Approximately 30% of the natural intracellular copper concentration was excreted within 1 h (Fig. 4).

It is suggested that the released copper derives from metallothionein possibly excreted by the cells. Unfortunately, due to both the low copper concentrations and the neutrophile activity mentioned above, no intact Cu-thionein could be detected. In unactivated granulocytes no detectable copper concentration was noticed in the cell-free supernatant.

At present we do not know how the copper of leucocytes is transported into the cell. A copper uptake from leucocytes into caeruloplasmin has been suggested. However, an inverted process could also have taken place. Due to both its copper-transport function and the interaction of caeruloplasmin with leucocytes (Kataoka and Tavassoli 1985), a copper transfer from this protein into the white blood cells has to be considered.

Nevertheless, the occurrence of Cu-thionein in white blood cells and the release of the metal-loaded protein into the plasma contributes to our understanding of the elevated copper levels in both leucocytes and plasma during some pathological processes including inflammation and tumor malignancies.

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