Bovine Monocyte-derived Macrophage Function in Induced Copper Deficiency

S. Cerone, A. Sansinanea, S. Streitenberger, C. García and N. Auza

Departamento de Fisiopatología, Facultad de Cs. Veterinarias, UNCPBA, Tandil, Buenos Aires, Argentina

Abstract. The effect of molybdenum-induced copper deficiency on monocyte-derived macrophage function was examined. Five female calves were given molybdenum (30 ppm) and sulphate (225 ppm) to induce experimental secondary copper deficiency. Oxidant production by bovine macrophages was measured after stimulation with phorbol myristate acetate (PMA) and opsonized zymosan (OpZ). Lipoperoxidative effects inside of macrophage, superoxide dismutase activity, superoxide anion and hydrogen peroxide formation were determined. Copper deficiency was confirmed from decreased serum copper levels, and animals with values less than 5.9 µmol/l were considered deficient. The content of intracellular copper decreased about 40% in deficient cells compared with the controls. The respiratory burst activity determined by nitroblue tetrazolium reduction was significantly imppaired with both stimulants used. Superoxide anion formation was less affected than hydrogen peroxide generation. In addition, increased lipid peroxidation was observed. It could be concluded that the effect of these changes may impair the monocyte-derived macrophage function in the immune system.

Key words: Copper — Bovine — Macrophage — Superoxide anion — Peroxide hydrogen

Introduction

The concept that the nutritional status influences the susceptibility of a host to infectious disease is well established. Copper is an essential trace element which has an important role in many physiological functions, including the immune response either humoral or cellular. Lymphocyte function is impaired in Cu deficiency (Lukasewycz et al. 1985; Stabel and Spears 1989). Neutrophil function is also impaired in Cu deficiency (Babu and Failla 1990; Cerone et al. 1998) with significantly diminished activity of the antioxidant enzyme superoxide dismutase (SOD). Nonetheless the precise mechanism by which Cu deficiency alters host immunity is still uncertain.

Correspondence to: Dra. Silvia Cerone, Fac. Cs. Veterinarias, UNCPBA, Pinto 399, 7000 Tandil, Bs.As., Argentina. E-mail: scerone@vet.unicen.edu.ar
Macrophages, which are a heterogeneous population existing in various tissues and organs, are responsible for numerous metabolic, immunological and inflammatory processes. Cells of the monocyte/macrophage lineage have been shown to participate in immune response by phagocytosis and killing of foreign agents, by processing and presenting antigens to lymphocytes, by modulating of proliferative responses.

There are several steps in the process of phagocytosis: attachment to the phagocytic cell via ligands on the particle surface to receptors on the cell surface, ingestion of the agent, production of oxygen metabolites, release of lysosomal contents, and eventual killing.

Macrophages possess on their surfaces numerous molecules that function as specific receptors for various ligands (Wright and Silverstein 1986). Several of these receptors are important for phagocytosis and cell to cell interactions (Kaufmann and Reddehase 1989), and are often utilized by invading microorganisms as a mechanism of entry into host phagocytes.

Compromise of macrophage Fc receptors by particle binding IgG generally leads to particle ingestion and oxidant species formation: superoxide anion (O$_2^-$), hydroxyl radicals (OH$^-$) and hydrogen peroxide (H$_2$O$_2$) known as reactive oxygen intermediates (ROIs) (Ehlenberger and Nussenzweig 1977).

Likewise, the occupation of macrophage complement receptors by particle bound C3b in common mediates particle ingestion and may promote respiratory burst and oxidant production (Griffen 1980; Shaw and Griffen 1981).

Both soluble and particulate agents stimulate phagocytic cells to produce the respiratory burst. Phorbol myristate acetate is a potent soluble agent, the stimulation is independent of cell surface receptors, and it directly activates protein kinase C both in vivo and in vitro. Protein kinase C activity has been shown to be important for the oxidative burst of macrophages (Johnston and Kitagawa 1985).

The respiratory burst is a marked increase in metabolic activity which takes place when the phagocytic cells are exposed to stimuli. It consists in an increase of oxygen consumption, ROIs formation and hexose monophosphate shunt activity (Henricks et al. 1986). The NADPH-oxidase, a membrane associated enzyme system, is activated and catalyzes the one-electron reduction of oxygen to O$_2^-$, which can either lose or accept an electron and can act either as reductant or as oxidant. When two O$_2^-$ molecules interact one of them is oxidized and the other one is reduced. In the presence of hydrogen hydrogen peroxide is formed. This reduction is catalyzed by Cu,Zn-superoxide dismutase (Cu,Zn-SOD, EC 1.15.1.1) which is located in the cytosol as an integral part of the body’s defense mechanism against the consequences of superoxide metabolism. OH$^-$ and H$_2$O$_2$ provide microbicidal oxidative activity both within the phagosome and in the extracellular environment.

The objective of this study was to examine differences in the ability of normal and induced copper deficient bovine monocyte-derived macrophages to phagocytize and produce oxidant species upon different stimuli and the lipid peroxidative effects, as a manner to determine the metabolic functionality of the macrophage.
Macrophage Function in Copper Deficiency

Materials and Methods

Animals

Eight female Aberdeen Angus calves, were assigned into control \((n = 3, \text{ group 1})\) or Cu-deficient \((n = 5, \text{ group 2})\) groups. All animals were fed basal diet containing alfalfa hay and concentrate ration with 6.8 and 3.6 mg of Cu and Mo per g dry matter, respectively, in a small pen at the experimental farm of our University. The animals had free access to water. Additionally, the heifers in the Cu-deficient group were daily supplemented orally with 30 ppm of Mo as ammonium molybdate and 225 ppm sulphate as sodium sulphate, five days a week. This supplementation was provided during 120 days to induce a low Cu status (Allen and Gawthorne 1987) and the copper status was checked every 30 days. The decrease of serum copper level was used as the indicator of the body copper status.

Blood samples were taken from the jugular vein. Peripheral blood leucocyte cells were isolated from animals with low copper status as described below.

Serum copper levels

Serum copper levels were determined both in serum and in monocyte-derived macrophages by atomic absorption spectrophotometry (AAS) with a Perkin Elmer spectrophotometer (Norwalk, Ct). Sera used for the determination were diluted 1:1 with TCA (200 g/l). Cells were harvested, washed three times in saline solution (9g NaCl/l) to remove Cu from cell surfaces, resuspended in deionized water at \(6 \times 10^7\) cells/ml, and diluted 1:1 with TCA.

Cell culture

Blood from each animal was drawn into acid citrate dextrose anticoagulant at a ratio 10:1 (Roth and Kaeberle 1981). The anticoagulated blood was centrifuged at 20°C at 1000 \(\times\) g for 30 min. The buffy coat was removed and diluted 1:1 with Ca,Mg-free phosphate- buffered saline, pH 7.2 (PBS-CMF), layered over Ficoll-Hypaque 1.083 and centrifuged at 20°C at 1000 \(\times\) g for 45 min to separate neutrophils and erythrocytes from mononuclear cells (Barta et al. 1984). The mononuclear cell layer was harvested and washed six times with PBS-CMF by centrifuging for 10 min. at 200 \(\times\) g. The cells were then placed into tissue culture flasks with RPMI 1640 medium supplemented with 10% autologous serum. After incubation at 37°C with 5% CO₂ for 60 min, non-adherent cells and medium were decanted and fresh medium was added to the flasks. The cells were incubated for additional 36 h in order to obtain a more uniform and homogenous population of cells. Then, the medium was decanted, and the adherent cells were gently scraped from the bottom of the flasks using a plastic cell scraper (Bounous et al. 1992). The scraped cells were washed in PBS-CMF and resuspended to \(5 \times 10^7\) cells/ml in serum free RPMI 1640 medium. The recovered cells were identified as macrophages based on morphological features, adherence to plastic, and nonspecific esterase staining; 85–90% were viable as determined by exclusion of 0.2% trypan blue.
**Determination of Cu,Zn-superoxide dismutase (Cu,Zn-SOD) activity**

The cells harvested were washed twice in saline solution and lysed by freezing-thawing. Following centrifugation of the lysate, the supernatant fraction was used to measure SOD activity by a modification of the method of Beauchamp and Fridovich (1971). The assay involves the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide, which is generated by the reaction of photoreduced riboflavin and oxygen.

For each sample, six tubes were set up each containing 0.1 mol/l EDTA buffer, 0.15 mol/l phosphate buffer, 1.5 mmol/l NBT, 0.12 mmol/l riboflavin and variable quantities of cell extract. The tubes were placed in a light box containing an 18 W fluorescent tube, and received uniform illumination for 12 min. Optical densities were measured at 560 nm. Mn-SOD activity was measured by addition of 5 mmol/l NaCN to the reaction mixture and Cu,Zn-SOD was calculated by subtracting the Mn-SOD value from the total. A unit of SOD enzyme activity is defined as the amount of enzyme which inhibits the reaction by 50%. Cellular SOD activity was expressed as U/10^7 cells.

**Preparation of opsonized zymosan (OpZ) particles**

Plasma was collected from heparinized blood. Zymosan (25 mg) was added to 10 ml of plasma, the mixture was sonicated for 5 min and incubated for 30 min at 37°C. The solution was centrifuged at 900 × g for 15 min to obtain the zymosan pellet. The pellet was resuspended in 10 ml of RPMI 1640 medium (Carey et al. 1995).

**Macrophage function test**

Nitroblue tetrazolium (NBT) reduction test. The NBT is a yellow dye that, when incorporated into the phagolysosome, is biochemically transformed into a blue formazan crystal. The more metabolically active the cell is the more NBT is incorporated (Humbert et al. 1971). Two hundred µl NBT solution (0.11% in HBSS), 200 µl macrophages (4 × 10^5 cells) were mixed with 40 µl opsonized zymosan or 100 µl of 10 µg/ml PMA. Duplicate tubes were prepared without the stimulant. Some tubes were incubated at 37°C for 15 min, others for 30 min, the reaction was stopped by adding 3 ml of 0.5N HCl. Then, the tubes were centrifuged at 1000 × g for 10 min, the cells washed twice with 3 ml of 0.5N HCl for 5 min, the sediment resuspended in 3 ml of dimethylformamide (DMF) and heated in a boiling water bath for 10 min, subsequently 2 ml of 10N KOH was added and mixed thoroughly, centrifuged, and the upper DMF layer was read in OD_{710}. The results are expressed as Δ OD/2 × 10^6 macrophages/15 and 30 min. (Nagahata et al. 1986).

**Measurement of superoxide anion production**

O_2^- was measured by the SOD-inhibitable reduction of ferricytochrome c. Briefly, macrophages (2 × 10^6/ml) in HBSS were kept in several wells at 37°C, with 160 µmol/l cytochrome c (Sigma) in the presence or absence of 1 µg/ml PMA. Reference samples also contained 75 µg/ml of SOD (Sigma). At 15, 30 and 60 min
of incubation in different wells, reduction of cytochrome c was measured at 550 nm in a MSE-spectrophotometer. The amount of SOD-inhibitable $O_2^-$ production was calculated on the basis of the extinction coefficient of cytochrome c $E_{550} = 21 \text{ mmol/l}^{-1} \text{ cm}^{-1}$ and is expressed as nmol/2 $\times 10^6$ cells (Johnston 1984).

Production of hydrogen peroxide ($H_2O_2$)

The production of $H_2O_2$ was measured by a modification of the method described by Pick and Mizel (1981). The cells ($1 \times 10^6$) were incubated in 1 ml of PBS, in the presence of 5 mmol/l glucose, under 5% CO$_2$ : 95% air atmosphere at 37°C and in the presence of 0.125 mg/ml OpZ or 1 µg/ml PMA. After 1 h of incubation, solution of phenol red and 0.2 µmol/l horseradish-peroxidase were added to the medium to quantify the hydrogen peroxide content. After 10 min the reaction was stopped with 100 µl of 1 N NaOH and the amount of hydrogen peroxide formed was measured spectrophotometrically at 620 nm. Results are expressed as nmol/10$^6$ cells.

Determination of thiobarbituric acid-reacting substances (TBARS)

TBARS were determined as an indication of lipid peroxidation using a modification of the method of Balla et al. (1990). For this purpose, $1 \times 10^7$ macrophages resuspended in HBSS were incubated with OpZ (0.125 mg/ml) for 1 h; then, 50 µl of trichloroacetic acid (300 g/l) was added and the suspension was placed on ice for 10 min, and the precipitate was subsequently removed by centrifugation. The supernatant (500 µl) was added to 250 µl of a 0.75% solution of thiobarbituric acid in 0.5 N HCl, incubated at 90°C for 15 min and the optical density was read at 535 nm. Standards were prepared with malondialdehyde tetrabutylammonium salt. Lipid peroxide levels were expressed as nmol of malondialdehyde (MDA)/$\times 10^7$ cells.

Statistics

All results are the average of measurements on three separate monocyte-derived macrophage cultures and were analyzed by Student’s $t$ test, $p \leq 0.05$ was considered statistically significant. Data are expressed as mean ± standard deviation.

Results

Animals supplemented with molybdate and sulphate had significantly lower serum levels of Cu than those not supplemented, as determined by atomic absorption spectrophotometry, decreasing from 10.8 µmol/l to 5.6 µmol/l at 120 days after starting the trial; the control group maintained their initial values ($11 \pm 0.5 \mu$mol/l). As soon as the animals from group 2 reached the above values their blood samples were processed and the cells cultivated as mentioned above.

The quantity of intracellular copper was 35–40% less in monocyte-derived macrophages from deficient bovines than that in control cells, being 0.028 ± 0.005 µg/6 $\times 10^7$ cell and 0.045 ± 0.004 µg/6 $\times 10^7$ cell, respectively.
Table 1. Oxidant production by monocyte-derived macrophages from control and Cu-deficient group

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<thead>
<tr>
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<th>Control group</th>
<th>Deficient group</th>
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<tr>
<td>NBT reduction</td>
<td></td>
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</tr>
<tr>
<td>with PMA</td>
<td>0.75 ± 0.05ₐ</td>
<td>0.73 ± 0.05</td>
</tr>
<tr>
<td>with OpZ</td>
<td>0.61 ± 0.06ₐ</td>
<td>0.62 ± 0.06ₐ</td>
</tr>
<tr>
<td>without stimulant</td>
<td>0.23 ± 0.04</td>
<td>0.23 ± 0.02ₐ</td>
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NBT reduction is expressed as ∆ OD/2 × 10⁶ cells. Control group n = 3, Deficient group n = 5. Letters (ₐ,ₐ) indicate significant difference (p ≤ 0.05).

Table 2. SOD activity, H₂O₂ and TBAR production by monocyte-derived macrophages from control and Cu-deficient group

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Deficient group</th>
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<tbody>
<tr>
<td>SOD activity</td>
<td>1.80 ± 0.25ₐ</td>
<td>0.70 ± 0.20ₐ</td>
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<tr>
<td>H₂O₂ nmoles</td>
<td></td>
<td></td>
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<tr>
<td>with PMA</td>
<td>15.0 ± 2.3ₐ</td>
<td>11.0 ± 2</td>
</tr>
<tr>
<td>with OpZ</td>
<td>11.3 ± 1.8ₐ</td>
<td>7.1 ± 2</td>
</tr>
<tr>
<td>TBAR</td>
<td>1.45 ± 0.12ₐ</td>
<td>3.07 ± 0.38</td>
</tr>
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</table>

SOD activity is expressed as U/10⁷ cells, H₂O₂ in nmoles/1.5 × 10⁶ cells, and TBAR as nmol MDA/10⁷ cells. Control group n = 3, Deficient group n = 5. Letters (ₐ,ₐ,ₐ,ₐ) indicate significant difference (p ≤ 0.05)

Mean oxidant production was higher following PMA stimulation than that produced by OpZ, although the oxidant production was lower in deficient monocytes-derived macrophages as compared to control cells. After PMA stimulation, NBT reduction, as a means of assaying the metabolic integrity of phagocytosing cells, was approximately 50% lower in deficient macrophages as compared to control cells, and 39% after OpZ challenge. The results are expressed as ∆ DO/2 × 10⁶ macrophages/15 and 30 min (Table 1).

The results of the intracellular Cu,Zn-SOD activity measurements are shown in Table 2. Addition of cyanide to the reaction mixture abolished the Cu,Zn-SOD activity and allowed to determine its activity by subtracting from the total value. Cellular Cu,Zn-SOD activity was significantly reduced, to 30% of the value for control cells (expressed as U/10⁷ cells).

The concentrations of the superoxide anion produced in response to the potent stimulant PMA were not significantly different in deficient Cu monocyte-derived macrophages compared to those induced in normal copper-nondeficient cells, mea-
Figure 1. Anion superoxide generation after phorbol myristate acetate stimulation. Control group \( n = 3 \); Deficient group \( n = 5 \). There were no significant differences between the values obtained (\( p \leq 0.05 \)).

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Discussion

The basal metabolism of macrophages can be significantly affected by receptor-ligand interactions, which often result in a respiratory burst with the metabolism of large quantities of glucose by way of the hexose monophosphate shunt, and an increased oxygen consumption (Babior 1984).

Monocyte-derived macrophages from copper deficient bovines were less active than those from control animals. The oxidant production demonstrated by the NBT reduction as a means to determine the metabolic integrity of this kind of cells, was lower in macrophages from Cu-deficient animals. PMA caused a greater quantity of oxidant production than that produced by OpZ. This soluble agent may allow faster
activation of the NADP-oxidase than do receptor-mediated events. The mechanism is different: PMA avoids several steps in membrane-stimulated activation of the respiratory burst, while OpZ stimulates oxidant production by surface phenomena.

In particular, the $O_2^-$ production after the stimulation with soluble agents was not statistically significant between the groups, but the $H_2O_2$ generation was smaller in monocyte-derived macrophages from copper deficient bovines with both soluble and particulate stimulants. The $O_2^-$ thus generated should be rapidly converted to hydrogen peroxide ($H_2O_2$) and hydroxyl radicals. The transformation to $H_2O_2$ proceeds under the Cu,Zn-SOD action which is one of the free radical-metabolizing enzymes eliminating peroxide radicals. The lower Cu,Zn-SOD activity, determined in monocytes-derived macrophages from copper deficient animals may be the cause of $O_2^-$ permanency in the macrophage and its inability to be transformed into $H_2O_2$, as was demonstrated in this assay where its level reached only 62% of the value in normal challenged cells. The lower $H_2O_2$ concentrations in cells from Cu-deficient animals might perturb the myeloperoxidase activity since this compound is the substrate for the generation of bactericidal hypochlorites.

A second consequence of the receptor-ligand interaction is the release of arachidonic acid from cellular stores of phospholipids by phospholipase A2, and its conversion to a series of leukotrienes or prostaglandins. The release of these compounds constitutes an important aspect of the macrophage function (Bonney and Davies 1984).

Assuming that macrophages and other active phagocytes generate reactive oxygen intermediates for microbicidal purposes, sometimes this potent defense mechanism may constitute a risk to the host itself. High concentrations of oxidants might modify important residues in regulatory proteins and excessively alter the receptor function. (Wolff et al. 1986). The sulfhydryl (SH) groups are the most susceptible of the nucleophilic centers in proteins that are potential targets for oxidants. ROIs can also initiate a destructive peroxidative cascade that consumes a large percentage of plasma membrane lipids, leading to peroxidation of unsaturated fatty acids, as was demonstrated by the increase in TBARS in Cu-deficient cells compared to the controls. Lipid peroxidation may reduce the arachidonic acid concentration, and thus impair the regulatory signals to responding cells. To confirm this possibility, further assays are required.

Although the present model cannot distinguish whether the alterations are due to copper deficiency or molybdenum excess, thiomolybdates or derivatives have been shown to reduce the absorption of dietary copper from the gut (Dick et al. 1975; Allen and Gawthorne 1987); in addition, Mo-induced Cu deficiency is widely recognized in ruminants. We can conclude that the decreased levels of Cu,Zn-SOD activity, $O_2^-$ and $H_2O_2$ production and the increase in lipid peroxidation products found in vitro in deficient macrophages may be associated with an impaired macrophage respiratory burst capacity and a lower prostaglandins and leukotrienes formation, causing a greater risk for infections, as observed in animals with secondary copper deficiency in our country.
Acknowledgements. This study was supported by a grant from Secretaría de Ciencia y Tecnología of UNCPBA and Comisión de Investigaciones Científicas de la Pcia. de Buenos Aires.

References


Final version accepted December 28, 1999