Effect of Zinc on the Viability and Phagocytic Capacity of Peritoneal Macrophages[1] (37190)

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The finding of the stabilization of the integrity of liver lysosomes by zinc (1, 2) suggested that possibly some other biomembranes or biostructures might be stabilized by this metal. This has relevance since it is known that zinc is a normal constituent of various cells (3, 4). Furthermore, one of the methods to preserve intact plasma cell membranes employs zinc treatment with the assumption that this metal interacts with thiol groups of macromolecules by the formation of zinc mercaptides, thus increasing the integrity of the membrane (5). Cox (6) postulates that the well-known stabilization of membranes by glucocorticoids could be related to the increased accumulation of zinc within some cells treated with these steroidal hormones.

When discussing the possible mechanism(s) of the stabilizing effect of zinc on lysosomal particles, three principle reactions are proposed: (a) interaction with some functional groups of the intrinsic macromolecules of the membrane, (b) effect on enzymes controlling the integrity of the membrane (*i.e.*, phospholipase A_2), and (c) the effect on the degree of the oxidation of the polyunsaturated fatty acid constituents of the membrane by inhibiting lipid peroxidation. The last mechanism was proved in our recent studies (7). There are indications, however, that the other two mechanisms are also involved (8).

Our interest in this study was focused on plasma membranes, mainly of macrophages and lymphocytes. We demonstrated that accumulation of zinc in various cells is dependent on pH. It was found that the increased viability of macrophages treated with zinc and exposed to the cytotoxic effect of silica parallels a decrease in phagocytic activity.

Methods. Isolation of macrophages and other cells. Peritoneal macrophages were obtained from white male mice, CD 1 strain. The mice were injected with 2 ml of thioglycolate 3 days before sacrifice by decapitation. Ten milliliters of buffered saline solution was injected. The abdomen was lightly massaged for 90 sec. Then the macrophage rich solution was withdrawn. The macrophages were washed twice with saline before the *in vitro* experimental manipulations were performed. Differential counts on the peritoneal wash showed that more than 70% of the cells were macrophages.

Platelets were isolated from 100 ml of heparinized sheep blood and lymphocytes from 100 ml defibrinated human blood by standard procedures (9).

Lysosomes were isolated from rat liver as described elsewhere (1). This fraction in fact represents mitochondria particles and lysosomes, as no further purification was carried on. One milliliter of the suspension was treated with 2 vol of saline or with $ZnCl_2$ (1 m*M* final concentration) for 15 min at room temperature. The cells were washed 6 times with saline, hydrolyzed, and assayed for zinc by atomic absorption spectrophotometry. Protein was determined in unhydrolyzed aliquots at 280 and 260 nm.

Zinc treatments. Four days before the terminal procedure, the mice were divided into three groups; one getting injections of 0.5 ml Hepes buffer daily, the second getting 0.5 ml daily ip injections of 0.1 mg/ml $ZnCl_2$ in Hepes buffer, and the third group getting 0.5 ml daily injections of 0.5 mg/ml $ZnCl_2$ in Hepes buffer.

In vitro *experiments.* Viability index was determined on the washed macrophages before and after their exposure to 0.5–0.7

[1] Supported in part by N.I.H. grants ES 00790 and AM 14047.

mg/ml silica (99% pure crystalline SiO_2, monodispersive 1 μ particle size) in saline at 37° for 30 min. The viability of the cells was ascertained by making smears and staining with Trypan blue (dye exclusion). Dead cells picked up the stain; viable ones did not. When possible, ten counts of 100 cells each were made.

Phagocytosis. Measurement of macrophage phagocytosis followed the method of Bercovici (10). The washed macrophages were suspended in minimal essential medium supplemented with a 5% fetal calf serum and glutamine. The suspension was placed in a well containing a coverslip (Falcon Microtest Plates). *Staphylococcus albus* was added to the suspension. The final macrophage concentration was approximately 10^5 cells/ml; the final *Staphylococcus* concentration was approximately 10^8 bacteria/ml. All *Staphylococcus* and macrophage concentrations were constant among all groups being compared on a single day and were incubated together under gentle rotary shaking for 1 hr at 37°. The coverslips to which macrophages had attached were then removed and rinsed in a dilute saline solution. The cells were fixed in methyl alcohol for 3 min; the coverslips were attached to slides and stained with Giemsa.

Two criteria of phagocytosis were evaluated: the phagocytosis index given as an average number of bacteria per cell of 200 cells counted per coverslip, and the rate of phagocytosis, referring to the percentage of cells examined which showed any phagocytic activity after 60 min incubation time.

Results and Discussion. Content and accumulation of zinc in some cells. Platelets, lymphocytes, macrophages, and mitochondrial-lysosomal particles isolated as described under Methods were incubated at pH 7.4 or 6.0 in 0.01 *M* phosphate or phosphate-acetate buffer containing 1 m*M* $ZnCl_2$ for 15 min at 22° under slight shaking. The cells were washed in an excess of saline six times, hydrolyzed, and assayed for zinc. There was an appreciable amount of zinc present in all intact cells studied (Table I). Platelets contain, however, five times more zinc than macrophages or lymphocytes. Isolated liver mitochondrial fraction contains only one-third the zinc present in lymphocytes. Cells incubated with zinc at physiological pH increase the content of zinc by a factor of 5–10; the content of zinc in the cells incubated at pH 6.0 was, with all three species of cells studied, significantly lower than at pH 7.4. This indicates that the functional state of the cell is important for its capacity to absorb and bind zinc, as reported also by Cox (6, 11) for various epithelial-like cell lines grown in tissue culture and by Saito *et al.* (12) for spermatozoa. It is worth mentioning that liver mitochondrial-lysosomal fraction incubated with zinc under the same conditions accumulated the same amount of zinc at both pH's studied.

Zinc and viability of macrophages exposed to silica (Table II). The viability of macrophages from mice treated for four days with high doses of zinc was decreased. Low doses of zinc alone either did not affect or rather increased the viability of macrophages. The protecting effect of low doses of zinc becomes quite obvious in macrophages exposed to silica. In cells not treated with zinc, silica is quite toxic. The average viability of macro-

TABLE I. Zinc Accumulation in Macrophages, Platelets, Lymphocytes and Liver Lysosomal-Mitochondrial Fraction.

	Zinc (μg/mg protein)			
	Macrophages	Platelets	Lymphocytes	Lysosomes-mitochondria
Control	1.98 ± 0.17[a]	11.59 ± 3.77	1.63 ± 0.15	0.47 ± 0.07
$ZnCl_2$, pH 6	8.50 ± 2.2	30.10 ± 6.35	7.60 ± 2.01	3.32 ± 0.402
$ZnCl_2$, pH 7.4	21.0 ± 1.9	68.13 ± 1.63	15.26 ± 0.20	3.31 ± 0.070

[a] Each value is presented as mean ± SE and is the average of 3 separate incubations for 15 min each, followed by six thorough washings in the buffer solution. Final concentration of zinc in 0.01 *M* Tris buffer was 1 m*M*.

TABLE II. Effect of the Dose of Zinc on the Viability of Mouse Peritoneal Macrophages Exposed to Silica.[a]

Treatment of macrophages	Group of animals		
	Control	Low zinc	High zinc
No silica exposure			
Viability index	85.2 ± 4.5	91.1 ± 1.5	67.1 ± 6.5*
In percent of control	100.0 ± 5.3	107.1 ± 1.7	79.0 ± 7.9*
Exposed to silica			
Viability index	55.9 ± 2.8***	79.1 ± 2.8	37.6 ± 13.7
In percent of control	100.0 ± 5.1***	142.3 ± 5.1	67.2 ± 24.0

[a] There were four mice in each group. Viability index is given as percent of viable cells in a sample, consisting of ten counts of 100 cells each. Variability is given by mean ± SE. The last entry in each group presents the data in percent of controls. Asterisks (*, **, ***) refer to $p < 0.05$, 0.01, or 0.001, respectively, comparing control with low or high zinc group.

phages isolated from eight control animals and further incubated for 30 min in the basic medium at 37° amounted to 86.7 ± 2.64%. Cells exposed for 30 min to silica particles in the same incubation medium showed significantly lower viability, 58.6 ± 2.0% ($p < 0.001$). The deleterious effect of silica is even more pronounced in cells from mice treated with high doses of zinc. Pretreatment of animals with a low dose of zinc reduced significantly ($p < 0.001$) the death rate of macrophages exposed to silica. This striking effect is summarized in the lower line of Table II presenting the viability in percent of control macrophages not treated with zinc.

It has been shown that cytotoxic effect of silica particles relates to the formation of hydrogen bonds between the surface of the crystalline silica and macromolecular constituents of the lysosomal membrane. By this mechanism, a lysis of lysosomes occurs resulting in the death of the cell (13, 14). As shown by Gabor *et al.* (15), silica induces lipid peroxidation in the lung. This mechanism is known to distintegrate various biomembranes containing polyunsaturated fatty acids (16). It is also known that substances masking the surface of the silica crystal inhibit its cytotoxicity (17). We tested, therefore, the possibility that the protective effect of zinc against the cytotoxic effect of silica particles is mediated by masking the reactive groups on the silica surface with zinc.

Macrophages were isolated from the peritoneal cavity by the standard thioglycolate technique from mice not treated with zinc. An aliquot of the suspension of silica was incubated with 1 m*M* $ZnCl_2$ solution and then repeatedly washed with the first buffer used for incubation of macrophages.

The results (Table III) showed that the cytotoxicity of silica was not changed by pretreatment of particles with zinc. The mechanism for the protective effect of zinc lies,

TABLE III. Cytotoxic Effect of Silica on Macrophage is not Affected by Pretreatment of Silica with Zinc.[a]

	Treatment of macrophages			
	Control	Exposed to silica		Exposed to silica treated with Zn^{2+}
Viability index	88.3 ± 3.2***	61.3 ± 2.3	N.S.	62.1 ± 2.8***
In percent of control	100.0 ± 3.6***	69.4 ± 2.6	N.S.	70.3 ± 3.2***

[a] There were four mice in each group. Suspension of uniform size silica particles was incubated in 1 m*M* $ZnCl_2$ for 15 min, then exhaustively washed with the buffer medium used for incubation of macrophages. All data on viability of macrophages exposed to silica alone or zinc treated silica are statistically significantly lower ($p < 0.01$) than controls. There is no statistical difference between the last two columns. Asterisks at the last column refer to significance between this group and control group. See legend to Table II.

therefore, in the modification of some properties of the cell as such.

Zinc and viability of lymphocytes. Another evidence of the increased viability of cells by zinc was obtained with lymphocytes. During the isolation and purification procedure, consisting of six consecutive washings and centrifugations in buffered saline (pH 7.4, 0.146 *M*), the viability of lymphocytes decreased significantly from 92.0 ± 1.3% to 80.2 ± 1.3% ($p < 0.001$). When 0.01 m*M* $ZnCl_2$ was added to the washing medium, the viability changed only insignificantly from 92.0 ± 1.3 to 90.0 ± 0.85 in the last wash. This indicates that zinc treatment increases the resistance of another type of cell against a different form of stress.

Zinc and phagocytosis. It may be assumed that zinc interacting with some components of the cell, specifically of membrane constituents, will change both the structural as well as functional characteristics of the membrane. To evaluate this, we studied how zinc treatment affects the total capacity to phagocytose *Staphylococcus albus* and the rate of phagocytosis by peritoneal macrophages.

The results summarized in Table IV show significantly inhibited phagocytic capacity of macrophages isolated from the mice treated with either low (0.05 mg $ZnCl_2$/mouse) or high doses of zinc (0.25 mg $ZnCl_2$/mouse). Also, the rate at which macrophages were phagocytosing the bacteria was significantly slower after zinc treatment.

In light of these findings, it seems that the increased viability of macrophages pretreated *in vivo* with zinc and then exposed *in vitro* to silica particles is related to the inhibition of phagocytic activity of macrophages. The reasons for such a modification of the function of a cell by zinc may be multifold: an inhibitory effect of zinc on both Mg^{2+}-dependent and Na^+- and K^+-stimulated ATPases of pulmonary alveolar macrophages was demonstrated by Mustafa *et al.* (18) and Donaldson (8). Considering the possible functional implication of this finding, a modification of phagocytosis could be expected. Zn^{2+} was shown to be a particularly effective inhibitor of phospholipase A_2, which cleaves many lipoprotein-bound phospholipid components of biological membranes (19, 20). Still, a close resemblance of the effect of zinc on stabilization of membranes and inhibition of phagocytosis with that of cortisone is quite intriguing.

Summary. Peritoneal macrophages ioslated from mice treated for four days with low doses of zinc showed higher viability when exposed to the toxic effect of silica particles (1 μ). By pretreatment with high doses of zinc, the cytotoxic effect of silica was increased. The protective effect of low doses of zinc was not found to be related to the interaction of zinc with silica particles and is thought to lie within the cell.

Some functions of macrophages, screened by determination of phagocytosis of *Staphylo-*

TABLE IV. Effect of Zinc Administration to the Mice on the Phagocytic Activity and Rate of Phagocytosis of *Staphylococcus albus* by Peritoneal Macrophages.[a]

Group of animals	Phagocytosis index			Rate of phagocytosis		
	Control	Low zinc	High zinc	Control	Low zinc	High zinc
M	11.53 ± 0.54	6.89 ± 0.40	6.48 ± 0.39	81.50 ± 1.79	66.00 ± 1.14	59.20 ± 0.86
N	6.12 ± 0.28	3.98 ± 0.22	3.74 ± 0.20	64	43	64
O	10.53 ± 0.77	4.85 ± 0.27	7.53 ± 0.60	80.20 ± 0.96	47.20 ± 2.05	65.00 ± 1.78
P	13.13 ± 0.80	10.21 ± 0.76	4.96 ± 0.28	81.20 ± 1.52	67.80 ± 2.39	52.60 ± 1.88
Q	10.78 ± 0.60	6.96 ± 0.48	—	86.60 ± 1.40	54.80 ± 3.18	—
In % of control	100 ± 5.7**	62.80± 5.20	48.7 ± 7.6**	100 ± 1.8***	73.6 ± 4.0	79.70 ± 7.6*

[a] Phagocytosis index refers to average number of bacteria per macrophage of 200 cells counted. Rate of phagocytosis refers to percent of cells showing phagocytosis after 60 min incubation with *Staphylococcus albus*. Number of asterisks refer to the significancy ($p < 0.05$, 0.01, or 0.001) between control group and zinc treated animals. Letters M–Q refer to the pair-matched mice tested under identical conditions the same day. See also legend to Table II.

coccus albus and rate of phagocytosis, were inhibited in mice treated with both low as well as high doses of zinc.

The concentration of zinc in macrophages, and also in platelets and lymphocytes, increased by a factor of 5–10 when the cells were incubated in medium containing zinc. Zinc accumulation in these cells was pH dependent, but was not pH dependent in the mitochondrial-lysosomal fraction.

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Received Sept. 18, 1972. P.S.E.B.M., 1973, Vol. 142.