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The Effect of Zinc and Other Metals on Complement Hemolysis of Sheep Red Blood Cells *in Vitro*[1] (37789)

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Previous work by our group has concentrated on cellular mechanisms of tissue damage and their mediation by zinc and other metals. We have reported a stabilization of lysosomes by lysosomal membrane-bound zinc (1), inhibition of endogenous and CCl_4-stimulated lipid peroxidation by zinc *in vivo* as well as *in vitro* (2), and a reduced ability of collagen to aggregate platelets in the presence of this metal (3).

Recent reviews (4–7, 8) have pointed out the crucial role played by complement as the principal noncellular effector of cell destruction and as a mediator of acute inflammation. In view of the demonstrated ability of zinc to interfere with some mechanisms of tissue damage, we have extended our investigations to include studies of zinc and other metal interactions with the complement hemolytic system *in vitro*. Our results show that only zinc and copper of the metals tested produced pronounced inhibition of complement hemolysis of sheep red blood cells *in vitro*. This inhibition of cytolysis appears to occur by preventing the formation of an active erythrocyte–antibody–complement complex, rather than interfering with the membrane attack mechanism.

Materials and Methods. Guinea pig complement (C′), rabbit anti-sheep red blood cell hemolysins (A), and Alsever's preserved sheep red blood cells (E) were obtained from the Grand Island Biological Company, Grand Island, NY. Complement and hemolysin titers were measured by the methods of Kabat and Mayer (9) immediately upon receipt. Complement was divided into aliquots and stored at −90° for use as a standard reagent. Hemolysin and sheep red blood cells were stored at 4°.

A commonly used complement hemolytic assay procedure (9) was selected and modified slightly for our purposes. Preserved red cells were washed 3 times in barbital buffer with 0.15 m*M* $CaCl_2$ and 0.50 m*M* $MgCl_2$ (pH 7.2), and resuspended in 15 times the packed cell volume of buffer. The cell suspension was sensitized with an equal volume of 1:100 hemolysin in the same buffer by slow addition during mixing. Aliquots (0.5 ml) of sensitized cells were hemolyzed with 4.0 ml of distilled water and the absorbence of hemoglobin at 541 nm measured with a Gilford 2000 spectrophotometer. Appropriate additions of barbital buffer to the cell suspension were made until the absorbence at 541 nm reached a value of 0.700 ± 2%. This cell concentration was used in all assay procedures.

Complement dilutions were made immediately before use in ice-cold barbital buffer with Mg^{2+} and Ca^{2+} containing 0.1% recrystallized bovine serum albumin (Sigma Chemical Co.) as a stabilizer. Metal solutions were prepared in 0.9% saline, adjusted to pH 6.8 and stored until needed.

When zinc preincubation of individual reaction components was required, they were diluted 1:1 with 1 m*M* $ZnCl_2$ in 0.9% NaCl and incubated for 15 min at room temperature. Cells treated in this manner were washed free of excess zinc by three changes of barbital buffer and centrifugation. The

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zinc in C′ and hemolysin was reduced to less than 14 μM when diluted to the appropriate experimental levels subsequent to incubation. Each zinc preincubated component was used in an assay system in which the remaining reagents were not zinc treated.

The standard reaction mixture contained 0.50 ml of senstitized cell suspension (EA), 0.10 ml of either 0.9% NaCl (control) or metals in 0.9% NaCl and 0.10 ml of guinea pig complement diluted in barbital buffer. Sensitized cells were placed in test tubes prechilled in ice water and saline or metals, then complement, were added to complete the reaction mixture. The tubes were incubated 1 hr at 37°, ± 0.2°. The reaction was stopped by dilution with 3.8 ml of ice-cold barbital buffer and the tubes were immediately centrifuged for 5 min at 500g at 4° to sediment nonhemolyzed cells. The extent of hemolysis was determined by spectrophotometrically measuring the amount of free hemoglobin present in the supernatant liquid at 541 nm. The concentrations of metals and complement used in each experiment are shown in the appropriate table or figure.

Results. Five metals (Zn, Cd, Co, Cu and Mn) were evaluated for their effects on complement hemolysis of sheep red blood cells. These metals were added to the assay mixture in a volume of 0.10 ml and in sufficient strength to raise the total metal concentration to values ranging from 0.05 to 0.70 mM. As shown in Fig. 1, zinc and copper produced marked inhibition of complement cytolysis. Zinc inhibition became noticeable at 0.10 mM, increased sharply from 0.20 to 0.30 mM, then gradually increased until at 0.70 mM only 17% of the control level of hemolysis occurred.

Copper was an effective inhibitor at lower concentrations than zinc, yet did not produce as great an effect. Maximum depression of hemolysis by copper was at 0.25 mM, at which point only 34% of control hemolysis occurred. Interestingly, at copper concentrations above 0.30 mM, a sharp increase in hemolysis occurred. Based on the report by Chvapil, Ryan and Zukoski (1) that copper labilizes lysosomal membranes *in vitro*, we attribute this increased hemolysis to a similar effect on erythrocyte membranes.

Manganese exerted a minor effect as well, lowering hemolysis to 83% of control at 0.60 mM, the highest concentration tested. Neither cadmium nor cobalt affected the hemolytic activity of complement at the concentrations used. If we rank these metals ac-

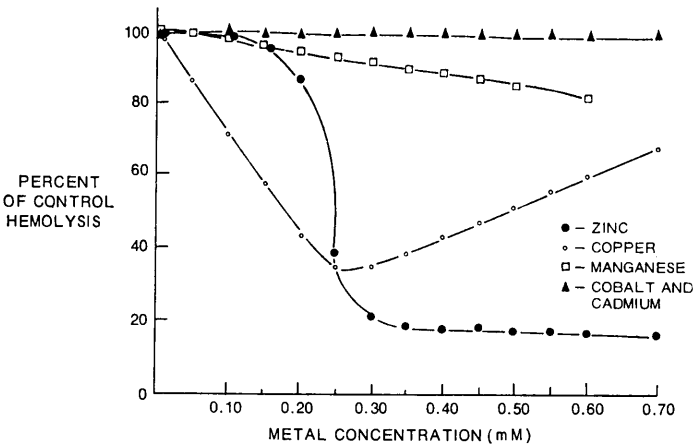

FIG. 1. Effect of different metal concentrations on complement hemolysis of sheep red blood cells *in vitro*. The reaction vessels contained 0.50 ml standard suspension of sensitized red cells, 0.10 ml of metal solution in saline or saline only (control), and 0.10 ml of 1/20 dilution of guinea pig complement. The reaction was allowed to proceed 1 hr at 37° (pH 7.2), then stopped by dilution with 3.8 ml cold barbital buffer, centrifuged, and the supernatant measured spectrophotometrically for the presence of free hemoglobin at 541 nm wave length. Each point represents 6 determinations. The standard error of measurement of this procedure $< \pm$ 1.6%.

cording to the maximum degree of inhibition obtained, we find that Zn > Cu > Mn > Cd and Co.

In order to further quantitate the zinc inhibition of complement activity, a series of assays were run in which the 50% hemolytic endpoint (9) for complement was determined in the presence of 0.10, 0.20, 0.30, 0.40 and 0.50 m*M* zinc chloride. In Fig. 2 the dilution of complement that produced 50% hemolysis in the presence of each zinc concentration is expressed relative to the control endpoint. Our data show that zinc affects the complement requirement in a concentration-dependent manner. It is apparent from the shape of the resulting curve that the magnitude of complement inhibition by zinc is an exponential function of the zinc concentration. While it required 2.92 relative complement units to produce 50% hemolysis at 0.30 m*M* $ZnCl_2$, 29 such units were required at 0.50 m*M* $ZnCl_2$. This accelerating effect of zinc suggests the presence of some critical zinc-complement stoichiometry.

Since our standard assay for metal inhibition was performed with these agents present in the reaction system, any one, or combination of the reactants might be affected. To determine which component(s) was involved, several experiments were run in which each reactant (E, A, C′), and one complex (EA), was separately preincubated in 0.50 m*M* zinc prior to addition to the remaining reactants. The results (Table I) are compared to control reactions using untreated reactants and expressed as percentage of control hemolysis. No decrease in hemolytic activity occurred as a result of any of these treatments, showing that zinc does not directly inactivate any of the components. Rather, it is apparent that zinc must be present in the reaction system and probably affects some interaction between components. That zinc did not alter the sensitization of red cells by hemolysin, nor the susceptibility of EA to hemolysis suggests that C′ itself is the reagent affected.

An experiment was performed in which the reaction system was made 0.50 m*M* with respect to $ZnCl_2$ by addition of the metal at various time intervals (0, 1, 2, 4, 10, 15 min) after the start of the assay. The influence of time of zinc addition on hemolysis at the 1 hr endpoint was compared to the progression of hemolysis in controls at each time interval in an attempt to separate the complement activation steps from the hemolytic phase. Our results (Fig. 3) show that the susceptibility of this reaction to zinc inhibition was greatest when zinc was added at the same time as the other reactants. When added 1

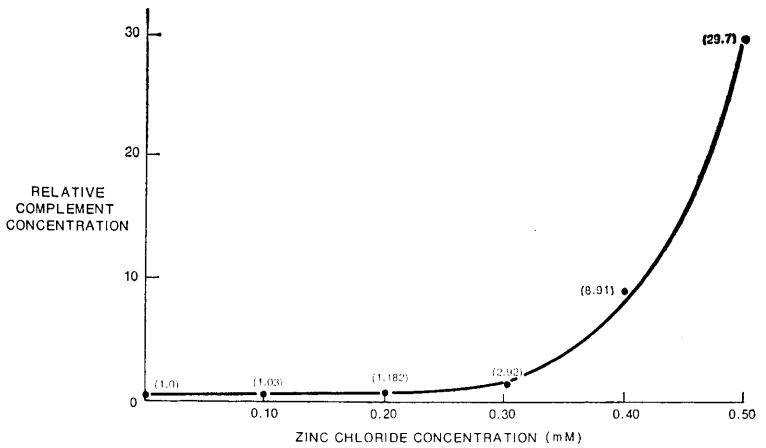

FIG. 2. The relative amount of complement required to produce 50% hemolysis in the presence of different zinc concentrations. Assays were run in triplicate at 8 complement dilutions (1/2–1/100) for each metal concentration, plus a control titration. The complement dilution required to produce 50% hemolysis (supernatant OD 541 nm = 0.350) was determined graphically for each metal concentration and compared to the control dilution achieving 50% hemolysis. Each point represents the product of 24 determinations; SEM ± 1.5% hemolysis.

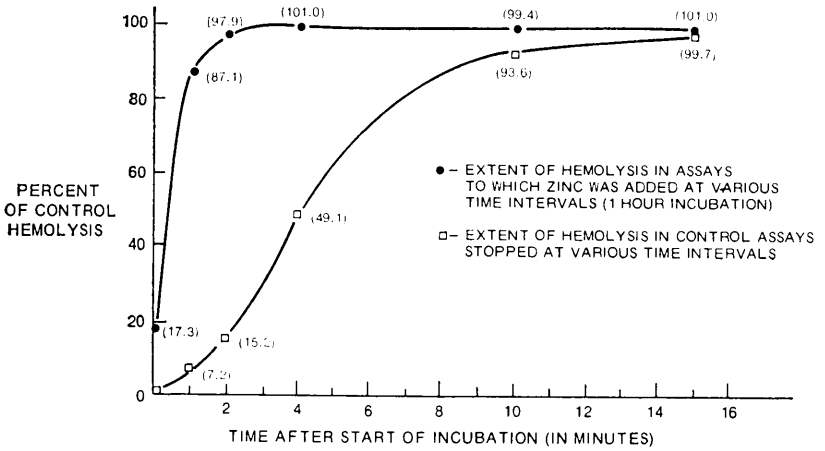

FIG. 3. The influence of time of zinc addition to the assay system on hemolysis after 1 hour incubation at 37° as compared to the progression of hemolysis in controls at each time interval. The reaction vessel contained 0.50 ml sensitized cell suspension, 0.10 ml 1/20 dilution of complement and 0.10 ml 0.9% saline or 0.10 ml zinc of sufficient strength to bring the total concentration to 0.50 m*M*. The reaction was stopped by addition of 3.8 ml ice-cold barbital buffer (pH 7.2), centrifuged, and the amount of free hemoglobin in the supernatant measured at 541 nm. The results are expressed as percentage of control hemolysis after 1 hr of incubation. Each point represents 10 determinations with standard error less than ± 1.1% for all points.

min after the start of incubation only minimal (13%) inhibition occurred. The data in Fig. 3 also show that within this 1 min period only a small degree of hemolysis (10%) had yet occurred in the control reactions, with total hemolysis not achieved until 15 min later. That zinc addition during the actual lysis phase produced no inhibition strongly suggests that zinc acts on the complex formation phase of this reaction.

Discussion. Of the metals tested, zinc and copper produced a marked concentration-dependent inhibition of complement hemolysis of sheep red blood cells *in vitro*; manganese inhibited to a much lesser extent. Despite the close relationship between zinc and cadmium in the IIB group and copper and cobalt in the transition series, neither cobalt nor cadmium inhibited cytolysis. On the basis of maximum inhibitory activity attained, these metals may be ordered in the following manner: Zn > Cu > Mn > Co and Cd.

This order does not correspond to either the Irving–Williams series (10) or to the order of stabilities for metal binding to metallocarboxypeptidases reported by Li (11). On the other hand, Donaldson *et al.* (12) have reported a similar order (Zn > Ca > Fe Mn) of metal efficacy as inhibitors of brain Na^+, K^+ activated ATPase. It has long been recognized that metal activity in biological

TABLE I. The Effect of Preincubating Individual Reaction Components in 0.50 m*M* Zinc Chloride on the Extent of Red Cell Hemolysis after 1 hr of Incubation at 37°.[a]

Complement dilution	Zinc Pretreated component			
	Complement	Hemolysin	Red cells	Sensitized red cells
1/20	103 ± 1.7	99.4 ± 2.1	99.7 ± 1.3	97.4 ± 1.1
1/40	—	—	104 ± 2.4	99.1 ± 1.9
1/60	—	—	101 ± 1.0	102 ± 1.3

[a]The results are presented as mean percentage of control hemolysis ± SE of 10 determinations.

systems seldom conforms to predictions based on their positions in the periodic table (13). Any attempts to rationalize our results on this basis without first knowing the nature of which, if any, biological ligands are involved would undoubtedly be misleading. It is apparent, however, that some functional distinction is made between similar metals by the affected complement fraction or reaction step.

A number of experiments were designed to determine whether zinc exerts its effect on the interactions between red cell, antibody and complement, or acts selectively on one of these reactants. We found that reaction components separately pretreated with zinc yielded a normal level of activity when subsequently used in our assay system (Table I). However, when zinc was added at the same time as complement, pronounced inhibition of cytolysis resulted, suggesting interference by zinc with some interreactional process(es) between reaction components. These data, in conjunction with the failure of zinc to inhibit the enzymatic hemolysis step per se (Fig. 3), lead us to conclude that zinc acts to prevent the activation and/or binding of complement components to form a complete complex. Work is currently under way to identify the affected steps and components and to define the mechanisms by which zinc mediates complement activity.

Summary. Several transition series and IIB group metals were evaluated for effects on complement hemolysis of sheep red blood cells *in vitro*. Zinc and copper (10^{-4} *M*) strongly inhibited complement activity by 83% and 66%, respectively. However, while manganese inhibited slightly (17%), neither cobalt nor cadmium had effect in this concentration range. Further results showed that zinc pretreatment did not inactivate individual reaction components, and that zinc did not affect the membrane-lysis step. Rather, zinc exerted its effect only when added concomitantly with complement, suggesting that it interferes with the binding and/or activation steps necessary to form the complete complement complex.

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