Complement: Substitution of the Terminal Component in Immune Hemolysis by 1,10-Phenanthroline

Abstract. *The participation of metal ions in the terminal step of immune cytolysis was investigated with chelating agents. 1,10-Phenanthroline induced lysis of sensitized sheep erythrocytes which had reacted with the first eight components of human complement. Hemolysis caused by 1,10-phenanthroline resembled lysis produced by the ninth component of complement in dependence on cell-bound eighth component and on temperature and in inhibition by 0.09*M *ethylenediaminetetraacetic acid. Bivalent metal ions reduced the hemolytic capacity of 1,10-phenanthroline, and* Fe^{++} *inhibited the activity of the ninth component. Since trivalent iron had no such effects, it is postulated that the hemolytic activity of 1,10-phenanthroline and the ninth component of complement is a function of their affinity for* Fe^{++}.

Immune cytolysis by antibody and complement requires participation of all nine components of the serum complement system (C'1 to C'9) (*1*). The reaction of complement with antibody-coated cells proceeds in well-defined steps, yielding characteristic intermediate reaction products (*1*, *2*). In the final reaction step the ninth component (C'9) acts on a surface site on the cells which has been generated by the action of the preceding eight components of complement and which is referred to as a C'8 site. Erythrocytes (E) which have reacted with antibody (A) and which carry C'8 sites are designated systematically EAC'1a,4,2a,3,5,6,7,8 (*3*) and are characterized by their ability to undergo slow, low-grade lysis in the absence of C'9 (*4*) and rapid and extensive lysis upon addition of C'9 (*5*).

The aim of this study was to explore the possible participation of metal ions in the terminal step of the complement reaction. It was found that the chelating agent dipyridinobenzene [1,10-phenanthroline hydrate (*6*), hereafter termed phenanthroline] can mimic C'9 action in that it causes rapid, temperature-dependent lysis of cells which carry C'8 sites. The various intermediate products which occur in the reaction of sensitized sheep erythrocytes and complement were prepared according to previously published methods, with purified human complement components (*7*, *2*). The components C'8 and C'9 were purified from human serum according to methods developed in this laboratory (*8*). A combination of chromatography on cellulose ion exchangers, filtration through Sephadex gels, and electrophoresis on polyvinyl powder blocks was used. Usually 10^8 cells of a given intermediate complex were incubated for 20 minutes at 37°C with recrystallized phenanthroline in isotonic veronal-NaCl buffer, *p*H 7.3 (*9*). The total reaction volume was 2 ml. The degree of lysis was determined by spectrophotometric assay of extracellular hemoglobin.

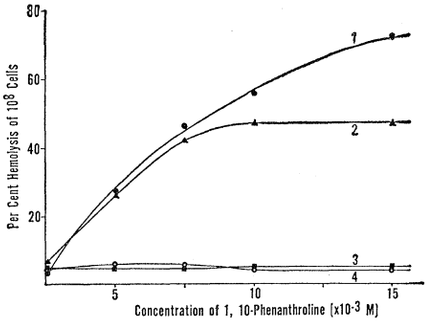

Fig. 1. Relationship between concentration of 1,10-phenanthroline and hemolysis of EAC'1a,4,2a,3,5,6,7,8. Curve 1 (●———●), lysis resulting after removal of EDTA and phenanthroline from the cells shown in curve 4. Curve 2 (▲———▲), lysis in the absence of EDTA. Curve 3 (■———■), lysis of EAC'1a,4,2a,3,5,6,7 in the absence of EDTA, included as a control. Curve 4 (○———○), lysis in the presence of 0.09*M* EDTA.

The specificity of the phenanthroline effect is shown in Table 1. Of seven different intermediate complexes tested, only that containing C'8 sites was susceptible to lysis by phenanthroline. The extent of lysis was found to be approximately proportional to the amount of C'8 employed in preparing the C'8 containing intermediate complex. It was also proportional to the concentration of phenanthroline in the reaction mixture, which was varied between 0.5 and $1.5 \times 10^{-2}M$ (Fig. 1). The extent of lysis was minimal at 0°C, but increased with temperature up to 40°C.

Frank *et al.* (*10*) reported that lysis of complement-treated cells can be inhibited by 0.09*M* ethylenediaminetetraacetic acid (EDTA). That C'9 action is not inhibited under these conditions was recently demonstrated (*5*). To explore further the analogy of phenanthroline-induced lysis to lysis produced by C'9, the effect of 0.09*M* EDTA was investigated. As shown in Fig. 1, 0.09*M* EDTA completely inhibited lysis by phenanthroline (curve 4). However, when both EDTA and phenanthroline were removed from the reaction mixture by centrifugation and resuspension of the cells in veronal buffer, rapid lysis occurred (curve 1), indicating that phenanthroline had acted on the cells and that lysis was prevented by the high concentration of EDTA. Figure 1 also shows that phenanthroline-induced lysis is apparently inhibited by phenanthroline concentrations greater than $1 \times 10^{-2}M$ (curve 2). Removal of phenan-

Table 1. Lysis of various erythrocyte-antibody-complement complexes by 0.01*M* 1,10-phenanthroline.

Intermediate complex	% Lysis of 10^8 cells* (20 min at 37°C)
EA	0
EAC'1a	0
EAC'1a,4	0
EAC'1a,4,2a	0
EAC'1a,4,2a,3	1.6
EAC'1a,4,2a,3,5,6,7	0.9
EAC'1a,4,2a,3,5,6,7,8 (C'8 × 1)	14.6
EAC'1a,4,2a,3,5,6,7,8 (C'8 × 2)	24.5
EAC'1a,4,2a,3,5,6,7,8 (C'8 × 4)	61.6

* Values are corrected for lysis of the controls not containing phenanthroline, which in no instance exceeded 2 percent.

Table 2. Percent inhibition, by various metal ions (at various concentrations), of hemolysis induced by C'9 or phenanthroline.

Metal ion	C'9 (5 μg)		Phenanthroline ($1 \times 10^{-2}M$)
	$1 \times 10^{-4}M$*	$5 \times 10^{-5}M$*	$4 \times 10^{-3}M$*
$ZnCl_2$	0	8.0	34.9
$CuSO_4$	4.9	4.4	7.8
$CdCl_2$	1.7	1.5	6.8
$CoCl_2$	0	0	28.1
$NiSO_4$	0	0	84.5
$FeCl_3$	6.4	0	0
$FeCl_2$	81.8	100.0	86.4
$FeSO_4$	100.0	100.0	88.3

* These values are the concentrations of the salts.

throline by centrifugation and resuspension of the cells in veronal buffer was followed by an increase in total hemolysis to a level similar to that described by curve 1.

These results indicate that lysis of sensitized sheep erythrocytes by phenanthroline resembles lysis produced by C'9 in requirement for C'8 sites, dependence on number of C'8 sites, dependence on temperature, and in inhibition by 0.09*M* EDTA. Like C'9, phenanthroline can exert its effect on the cell in the presence of 0.09*M* EDTA which inhibits hemolysis. It may be concluded that the two chelating compounds act at different steps of the terminal phase of the hemolytic reaction. Phenanthroline acts, as C'9, following C'8; EDTA acts following C'9 or phenanthroline. Even at a molar concentration which was six times greater than the highest phenanthroline concentration employed, EDTA showed no lytic effect, as evidenced by lack of lysis following removal of EDTA from cells bearing C'8 sites (not shown).

The possible relationship between the hemolytic and the metal-binding capacity of phenanthroline was investigated next. Before addition to C'8 cells, phenanthroline was mixed with solutions of various metal ions; the extent of inhibition of lysis is shown in Table 2. A definite reduction of the hemolytic activity of phenanthroline is apparent, indicating that the ability to bind metals may be essential for its hemolytic function.

As phenanthroline-induced lysis of C'8 cells appeared to be dependent upon the chelating activity of the compound, it was considered possible that C'9 functions similarly, and, hence, the effect of metal ions on C'9 activity was tested. As shown in Table 2, incubation of C'9 for 15 minutes at 37°C with 1×10^{-4} or $5 \times 10^{-5}M$ Fe^{++} ($FeCl_2$ or $FeSO_4$) led to complete inhibition of activity. None of the other ions tested exhibited a comparable effect. In particular, Fe^{+++} was virtually ineffective. Dialysis of C'9 treated with Fe^{++} did not restore its activity.

Two other ion-binding substances, desferrioxamine (*11*) (0.01*M*) and iron-free transferrin (*12*) (1mg/ml), were found to lack the ability to induce lysis of cells containing C'8 sites. These two substances are known to bind iron only in its trivalent form (*13*), whereas phenanthroline is capable of binding iron only in its bivalent form (*14*). Since C'9 could be inhibited by Fe^{++}, but not by Fe^{+++}, it appears that C'9 shares with phenanthroline an affinity for Fe^{++} and a lack of reactivity with Fe^{+++}. It may be postulated that the capacity of C'9 and of phenanthroline to lyse C'8 cells is due to their reactivity with Fe^{++}. Work is needed to determine whether activation of C'8 sites by C'9 is brought about by withdrawal of Fe^{++}.

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15. This is publication No. 226 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California. This work was supported by PHS grant 7007-01 and by American Heart Association grant 65-G-166.

24 May 1967

Tribolium castaneum: Morphology of "Aureate" Revealed by the Scanning Electron Microscope

Abstract. *Counts of setae in "aureate"* (au), *spontaneous autosomal recessive mutation of good penetrance and viability, show that the* au *gene causes a threefold increase in setation over the normal in the visible abdominal sternites but not in the membranous wings of the tenebrionid flour beetle* Tribolium castaneum. *Micrographs taken with the scanning electron microscope demonstrate that the* au *gene increases setation throughout the body.*

All living stages (eggs, larvae, pupae, and adults) of *Tribolium confusum* can survive being placed in the chamber of the scanning electron microscope (*1*) and exposed to a pressure of about 10^{-4} torr and to an electron beam (whose current and electron energy are, respectively, 2×10^{-11} to 2×10^{-10} amp and 25 kev) for as long as half an hour (*2*). In the scanning electron microscope, the radiation leaving the specimen is not focused in the optical sense, and therefore a great increase of usable radiation is possible.

Secondary electrons, visible light, characteristic x-rays, or specimen current could theoretically be used to build up the image, and the information carried by these various radiations can be resolved at dimensions comparable to those used in electron optical design. The scanning electron microscope separates localization from information content, and we report further uses of this instrument.

We exposed the flour beetles to low pressure (10^{-4} torr) in the chamber of the scanning electron microscope for a few minutes to anesthetize them. One beetle was then selected and mounted in an aluminum "boat" attached to a specimen holder and reintroduced in the scanning electron microscope. Beetles from two strains were used: (i) an unselected wild-type strain derived from Texas, kept in the laboratory by mass matings since 1958, and (ii) a mutant of spontaneous occurrence designated "aureate" (*au*).

This mutant is autosomally recessive, having good penetrance and viability; it is characterized by an increase in the number of setae over the whole body, with the result