COLICINE K AND ENDOTOXINS: EFFECT OF HEMOGLOBIN AND ITS SUBUNITS ON THEIR ANTIBIOTIC AND SEROLOGICAL PROPERTIES*

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When the antibiotic, colicine K, is incubated with serum, its lethal action toward Escherichia coli B is enhanced manyfold (Amano et al., 1958). This colicine enhancing factor (CEF) was found to be a heat stable, α_2 globulin associated with Cohn Fraction IV-1 (Van Vunakis et al., 1958). When homogenates of different tissues were assayed in order to find a possible tissue source of CEF, the factor was found to be ubiquitously distributed. In general, the tissues having high specific activity were noted to contain considerable amounts of blood. Hemolyzed blood was also more potent than serum or plasma in enhancing the activity of colicine K.

We have examined the role of hemoglobin and its subunits for their ability to enhance the antibiotic activity of colicine K. Since Amano et al. (1958) had shown that the colicine K complex is probably identical with the O antigen of the bacteria which produced it, we have also studied the ability of hemoglobin components to alter the serological activities of other O antigens.

Materials and Methods. Colicine K was the generous gift of Dr. W. F. Goebel of the Rockefeller Institute. E. coli endotoxin (from strain O111:B_4) and its antisera were given to us by Dr. J. Fine, Beth Israel Hospital, Boston. Shigella flexneri and Salmonella typhimurium

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endotoxins and antisera were given to us by Dr. S. B. Formal, Walter Reed Army Institute of Research. The twice-crystallized human hemoglobin was obtained from Pentex Corp.; the α and β chains of human hemoglobin, purchased from Gallard-Schlesinger Chem. Mfg. Co., had been prepared by countercurrent distribution (Hill et al., 1961). Amino acid analysis of the α chain agreed with the published values; analysis of the β chain revealed the presence of 0.15 residue of isoleucine which may indicate contamination by fetal hemoglobin or a non-heme protein (Hill et al., 1961). Immunochemical analyses were carried out by micro C' fixation (Wasserman and Levine, 1961). Enhancement of the activity of colicine K was tested by the zone inhibition assay on plates seeded with E. coli B (Goebel et al., 1956). Twofold dilutions of the assay sample (0.2 ml) were incubated at 37° for 60 min. with 0.2 ml of colicine K (2.5 µg/ml of 0.05 M NaCl, 0.005 M phosphate buffer, pH 6.8. The final dilution of the sample which prevented growth of E. coli B, i.e., gave a zone of inhibition, was considered the end point. Under our conditions, 0.01 ml of colicine K control mixture did not give a zone of inhibition when applied to the seeded plates.

Results and Discussion. When sera or plasma of various species were analyzed for their ability to enhance the activity of colicine K, it was found that the maximum dilution required to obtain a complete zone of inhibition on E. coli B varied from 1/40 to 1/640. Hemolyzed blood, on the other hand, was active at dilutions between 1/20,000 and 1/80,000.

In order to test the effect of the hemolytic products, fresh sheep blood, which had been collected in an equal volume of Alsever's solution, was centrifuged to separate the cells from the fluid phase. The cells were washed 3 times with isotonic saline and hemolyzed by addition of water. Aliquots of each sample were assayed for colicine

TABLE I

Assay of Blood Components and Proteins for Colicine Enhancement and Alteration of Serological Activity of E. coli Endotoxin and Colicine K

Blood Fraction or Protein	Colicine Enhancement Final dilution or μg required to obtain a complete zone of inhibition	Serological Alteration[3] Dilution or μg required to decrease C' fixation 50%
Sheep blood[2]	1/200	1/40
" plasma	1/200	1/30
" washed R.B.C.[2]	—	1/35
" lysed R.B.C.	1/30,000[4]	1/10,000
" lysed R.B.C.-stromata	1/20,000	1/10,000
Human hemoglobin	1.6	1.5
α-chain	1.6	1.6
β-chain	1.6	4.1
Egg white lysozyme	25	8.0
Bovine ribonuclease	> 250	67
Ovalbumin	> 250	> 200
Horse heart cytochrome	> 250	> 200
Rabbit serum albumin	> 250	> 200
Bovine serum albumin	> 250	> 200
α-Amylase	> 250	> 200

1. All dilutions in blood fractions refer to concentrations of whole blood.

2. After incubation with colicine K or E. coli endotoxin, the red cells were removed by centrifugation before assay by zone inhibition or C' fixation.

3. For blood fractions, 5 μg/ml of E. coli endotoxin were incubated at 37° for 30 min. with various dilutions of the assay sample. The diluent was 0.005 M phosphate buffer, pH 6.8, 0.05 M NaCl. For purified proteins, 1.65 μg/ml of colicine K were used. Other conditions were the same. All reaction mixtures were in a final volume of 3.0 ml.

4. There are 11 gms of hemoglobin in 100 ml of sheep blood (Altman, 1961). As a typical calculation, this dilution represents 3.7 μg Hgb assuming quantitative recovery from the lysed red blood cells. Twice crystallized sheep hemoglobin was active at a concentration of 1.6 μg.

enhancing activity (Table I). While whole blood and plasma were active at a 1/200 dilution, the lysed cells were active at a dilution of 1/30,000. Compared to plasma, the hemolysates were, therefore, more than 100 times more potent in enhancing the activity of colicine K. Removal of the stromata from the lysate did not alter the acti-

vity to an appreciable extent.

Human hemoglobin, the α and β chains of human hemoglobin, and hemin were tested for their ability to enhance the activity of colicine K (Table I). Hemin was inactive; hemoglobin and its chains were active at 1.6 μg. Several other proteins were assayed for colicine enhancement and, with the exception of lysozyme, were found to be essentially inert. On a weight basis, lysozyme was 5% as active as hemoglobin or the α chain. Myoglobin was active at low concentrations, but in view of its possible contamination by hemoglobin, additional purification is required before assigning it a quantitative value.

E. coli endotoxin was also incubated with various blood fractions and assayed for serological alteration as measured by C' fixation with its homologous antibody. The dilution of each fraction required to give 50% decrease in C' fixation (Fig. 1) is shown in Table I. In agreement with the zone inhibition assay, the hemolysate was more than two orders of magnitude more active than either plasma

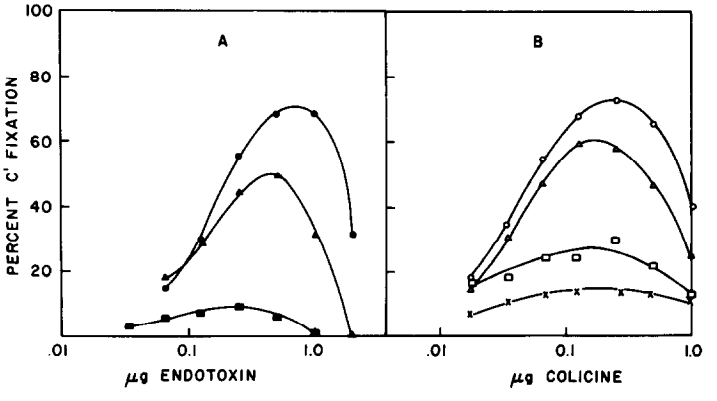

Fig. 1. C' fixation of (A) E. coli endotoxin (5 μg/ml), and (B) colicine K (1.65 μg/ml) after incubation with various amounts of α chain (●) none; (▲) 1.5 μg; (■) 3.0 μg; (○) none; (△) 1.0 μg; (□) 2.0 μg; (×) 4.0 μg. The conditions for incubation are the same as those given in Table I.

or whole blood. Similar results were obtained when colicine K was assayed with its homologous antiserum. Those proteins that had been tested for ability to enhance colicine K activity were also tested for ability to alter the serological activity of colicine K. A similar pattern of results were found. Hemoglobin and the α chain were active at 1.5 μg, the β chain at 4.1 μg, lysozyme at 8 μg, and ribonuclease at 67 μg. The other proteins tested were essentially inert.

C' fixation after incubating colicine K and the O antigen with varying amounts of α chain is shown in Fig. 1. A progressive loss in the ability to fix C' was found with increasing amounts of α chains. Similar decreases in C' fixation were also found using the lipopolysaccharide immune systems from S. typhimurium and Sh. flexneri. However, incubation with the α chain was without effect on the following immune systems: Pneumococcus, types II, VIII, or XIV and T_4 DNA. A small but reproducible alteration was noted with sialic acid containing α_1 glycoprotein, fetuin.

Two interpretations of the loss of serological activity are possible. The antigens either have been fragmented or the hemoglobin components have complexed with them to mask some of the antigenic determinants. Colicine K is a lipocarbohydrate protein complex in which the protein portion contains essentially all of the antibiotic activity (Amano et al., 1958). It is so closely associated with the lipocarbohydrate, however, that is can only be separated using degradation procedures which bring about dissociation of the complex. We have found that incubation of colicine K with the α chain of hemoglobin or with serum, followed by gel filtration on Sephadex 200, resulted in a retardation of the antibiotic activity in the eluting pattern. These results suggest either that the complex has been degraded to yield active fragments of smaller molecular weight or that the active protein has been displaced from the complex which is other-

wise left intact. Additional experiments are required to distinguish between these two possibilities.

There are numerous references in the literature to humoral and tissue factors which can alter the endotoxic properties of O antigens from gram negative bacteria (Cf. Keene et al., 1961; Smith et al., 1963). A loss in immunological properties has, in several cases, been correlated with changes in the biological activities commonly associated with endotoxins (Cluff, 1956; Landy et al., 1959; Rudbach and Johnson, 1962). It could, therefore, be predicted that hemoglobin and its components might alter the activities of endotoxins and indeed, may be one of the factors in serum which alters the physiological effects of endotoxins. We have isolated at least three components from Fraction IV-1 by chromatography on DEAE cellulose that are capable of enhancing the activity of colicine. While they do not contain heme, they may represent globin or its subunits complexed, perhaps, with the haptoglobins which are present in Fraction IV-1 (Laurell and Gronvall, 1962).

Other characteristics of the reaction between hemoglobin components and O antigens, and the effect on the physiological properties of the endotoxins are currently under investigation. In addition, modification of the α chain is under way to determine the amino acid sequence responsible for the interaction.

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