Reaction of Bovine Conglutinin in Human *in Vitro* Phagocytic Systems[1] (34573)

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Considerable information has now been accumulated concerning the reactivity and specificity of bovine conglutinin (K) and human immunoconglutinin (IK) (1–3). These factors appear to interact with the intermediate complement complex EAC'1, 4b, 2a, 3b. In the case of bovine conglutinin reacting exclusively with fixed C'3, the presence of an enzyme–conglutinogen-activating factor (KAF) is required to modify or supply the C'3 receptor site (4). Bovine conglutinin also differs from immunoconglutinin in that calcium ions apparently participate in the reaction with complement, furthermore, this reaction can be inhibited by *N*-acetyl-D-glucosamine (5).

Studies of the biological significance of conglutinin have been mainly concerned with *in vivo* effects in laboratory animals. Like immunoconglutinin, it was shown to enhance resistance of mice to experimental bacterial infections (6, 7). The effect of conglutinin on immune adherence, a C'3-dependent phenomenon, has also been studied. Sell (8), cited by Lachmann (3), showed that large amounts of conglutinin (1000 times that necessary for conglutination) inhibited immune adherence.

Because of the recent demonstration that anti-γ-globulin factors may block *in vitro* phagocytosis (9), we judged it important to examine the reactivity of naturally occurring anticomplement factors such as conglutinin in similar phagocytic systems. This held special interest because it had been demonstrated that heat labile complement components and human 19S anti-γ-globulin factors may compete in phagocytic systems for similar sites on antigen–antibody complexes (9). If human anti-γ-globulin factors could block phagocytosis, it appeared possible that the bovine anti-C'3 factor, conglutinin (K) might also impede phagocytic mechanisms in a parallel fashion. A quantitative method for *in vitro* phagocytosis and killing by measuring the total bacterial count after incubation of test bacteria with human polymorponuclear leukocytes seemed particularly well suited for directly examining the effects of bovine conglutinin. The presence of *in vitro* antiopsonic effects of bovine conglutinin in several phagocytic systems was clearly established.

Material and Methods. Phagocytosis system. The general method of Hirsch and Strauss (10) as modified from Maaløe (11) was used for all phagocytosis studies as described previously (9, 12).

Bacteria. Staphylococcus aureus 502A of phage type 7 and *Escherichia coli* K-12 were the standard strains used for all studies. Bacteria were grown overnight at 37° in Penassay broth (Difco) and washed three times before use.

Opsonins. Human serum derived from normal blood donors and stored at —70° was used as a source of opsonin. In some instances γG opsonins isolated by DEAE-cellulose chromatography (13, 14) from sera of patients with septicemia or subacute bacterial endocarditis were used. Opsonic 7S or 19S serum fractions were obtained by sucrose density gradient separation of serum previ-

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ously inactivated at 56° for 30 min (12). Human umbilical cord serum was utilized as a source of complement. Prior to use, the cord serum was absorbed three times at 0° with killed *E. coli*. For absorption of staphylococcal antibodies, EDTA was added to the serum at a final concentration of 0.01 *M*, followed by absorption three times at 22° with heat-killed staphylococci. Calcium and magnesium ions were then restored by dialysis of absorbed serum against Hanks' balanced salt solution. Such absorbed sera have previously been shown (12, 15) to contain complement activity but are devoid of demonstrable opsonic antibody.

Preparations of conglutinin. Bovine conglutinin was prepared from bovine serum using the zymosan absorption method (16). This procedure resulted in a 200–500-fold purification giving preparations of bovine conglutinin with activity/protein values of 15,000 to 34,000. Another highly purified preparation of bovine conglutinin was generously provided by Dr. P. J. Lachmann. This material had an activity/protein ratio of 56,000 and exhibited the same behavior in phagocytic systems as did our preparations.

Conglutination titration. Conglutinating activity was measured as described by Coombs *et al.* (1) using sheep red cells, bovine serum as the source of antibody, and fresh horse serum as the complement source. One percent suspensions of alexinated cells or control cells were incubated at room temperature with equal volumes of dilutions of conglutinin in plastic titration plates (Cooke Engineering Co., Alexandria, Va.). The resuspension pattern was read after 1–2 hr. Specificity of these reactions was established by the blocking effect of 0.001 *M* *N*-acetyl-D-glucosamine (3).

Results. When fresh normal human sera were used as opsonins for *E. coli*, it was apparent that the major rate-limiting opsonic factor was heat labile since all opsonic activity was abolished by inactivating the serum at 56° for 30 min. In keeping with previous studies (12), it was felt most likely that such opsonization by heat-labile factors was largely complement dependent. When bovine

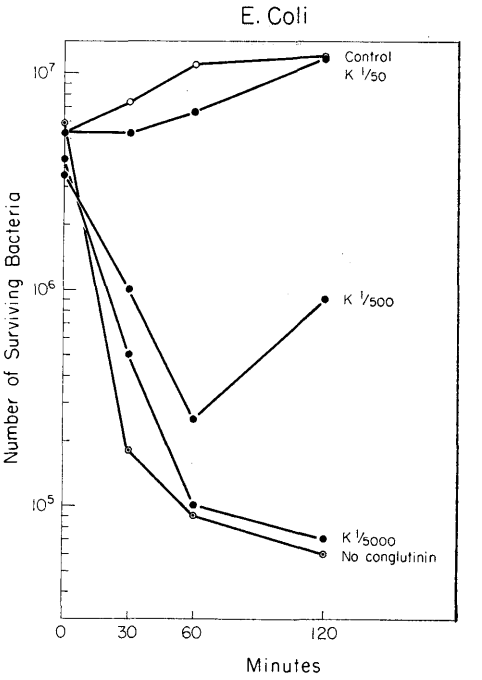

FIG. 1. Effect of conglutinin (K) on the phagocytosis of *E. coli* opsonized with fresh normal human serum. Final dilutions of a purified conglutinin preparation added are indicated in the figure. K 1/50 almost completely abolished the killing of bacteria, whereas less antiopsonic effect was noted with further dilution of K (1/500 and 1/5000). Controls included conglutinin, bacteria and white blood cells, bacteria and opsonin alone, and bacteria with white blood cells. None of these controls produced any killing effects.

conglutinin preparations were added to these opsonic systems, almost complete antiopsonic activity was noted. Representative examples of the blocking of phagocytosis and killing of bacteria by bovine conglutinin are shown in Fig. 1. Complete absence of opsonic, bactericidal or leukotoxic effects of bovin conglutinin alone was noted in the test systems employed. Moreover, when further dilutions of conglutinin were added to these opsonic systems, a corresponding decrease of antiopsonic effect was noted (Fig. 1). Control experiments without white blood cells were also included to check possible agglutination of bacteria. Direct microscopic smears, as well as viable counts, did not indicate any significant clumping.

In addition to heat-labile opsonic systems

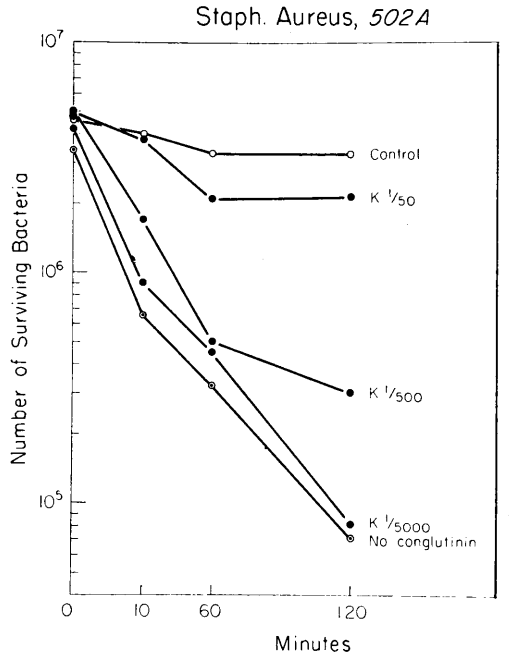

FIG. 2. Effect of conglutinin (K) on the phagocytosis of *S. aureus* strain 502A opsonized with fresh normal human serum. Final dilutions of a purified conglutinin preparation added are indicated in the figure. Similar to the gram-negative system (Fig. 1), this phagocytic system was also inhibited almost entirely by conglutinin (K 1/50). Controls were similar to those in Fig. 1.

using *E. coli* and fresh normal human serum, similar phagocytic systems with *S. aureus* were tested. Heating the serum did not entirely abolish the opsonization in these systems. This indicated that in addition to a major heat-labile opsonic component, some heat-stable opsonization took place. When increasing amounts of conglutinin were added to these systems, an increasing antiopsonic effect was recorded. The residual killing noted was of the same magnitude as the heat-stable opsonization by the inactivated serum. Antiopsonic effect of bovine conglutinin in *S. aureus* phagocytosis is shown in Fig. 2. The inhibition of phagocytosis by conglutinin was thus clearly established in several different test systems using heat-labile opsonins.

Patients with bacterial endocarditis (SBE) develop high titers of heat-stable opsonins (9, 17, 18). Isolated γG globulins from such patients do not require additional heat-labile factors for phagocytosis and killing of the test bacteria. When bovine conglutinin was added to phagocytic test systems using such isolated heat-stable γG opsonins, no blocking of the uptake and killing of test bacteria was seen.

Attempts to reverse conglutinin antiopsonic effects on heat-labile systems by the addition of *N*-acetyl-D-glucosamine (NADG) were also made to test the specificity of the conglutinin reactions. It was calculated that on a molar ratio basis in the test phagocytic systems under study, it would be necessary to use 1.5 *M* NADG to block the amount of conglutinin present. Controls using the NADG-reagent alone showed distinct inhibiting effect on phagocytosis above concentrations of 0.2 *M*. Therefore, the specificity of reaction could not be ascertained using this reagent.

Preincubation studies were also performed to provide an insight in the sequence of complement activation and conglutinin interaction. Test bacteria were preincubated with fresh normal human serum as a source of heat-labile opsonins, washed and then added to phagocytic cells and conglutinin. Only slight antiopsonic conglutinin effect could be demonstrated in these preincubation experiments (Fig. 3). It appeared, therefore, that the reactivity of C'3 with conglutinin either was only temporary or that additional serum factors were essential for the conglutinin blocking of heat-labile phagocytosis.

Experiments were also conducted to study the interaction of bovine conglutinin with separated 7S or 19S serum fractions potentiated by absorbed cord serum as a complement source. No antiopsonic effect was found with 7S opsonins using both gram-positive and gram-negative bacteria. Using *E. coli* and 19S serum fractions, a slight antiopsonic effect was noted when K was added to the 19S fractions.

Discussion. The present report indicates that conglutinin, the naturally occurring anti-C'3 factor of bovine serum, is capable of impeding phagocytosis *in vitro*. This was established using test systems felt to be primarily complement dependent on the basis of

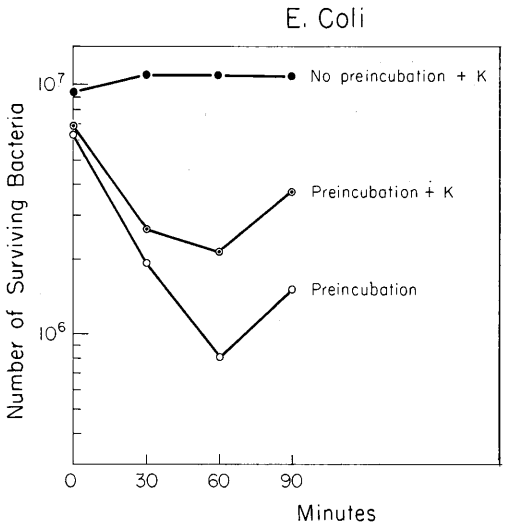

FIG. 3. Effect of preincubation of bacteria with fresh normal human serum on the inhibiting capacity of conglutinin (K). Preincubation reduced the inhibiting capacity of conglutinin markedly (curve labeled preincubation +K) as compared to the same amount of conglutinin added to serum, white cells and bacteria without preincubation (curve labeled No preincubation +K).

their loss of opsonic activity after heating and is in accordance with the known specificity of bovine conglutinin for C'3. Moreover, the absence of an antiopsonic conglutinin effect in phagocytic systems primarily dependent on hyperimmune heat-stable γG opsonins indicated that conglutinin could not block in such completely γ-globulin dependent systems. Tests for specificity of conglutinin reactive sites using *N*-acetyl-D-glucosamine could not be done because of interference of the latter reagent with the phagocytic test system employed.

A blocking of phagocytosis by conglutinin is somewhat surprising in view of the enhancement of resistance to infections noted in experiments with mice (6, 7). However, the concentration used in our *in vitro* experiments are above the normal titer of bovine blood, showing that inhibition is not a natural phenomenon. On the other hand, we have not noted any enhanced killing of bacteria when conglutinin is added in lower amounts. Also, in similar experiments in our laboratory using bovine cells, antibodies and complement, only a blocking effect was seen. The possible physiological role of bovine conglutinin thus has to be sought for in some other biological defense system.

It has previously been shown by Sell (8) as cited by Lachmann (3) that immune adherence can be inhibited by conglutinin. In both immune adherence and phagocytosis, a large excess of conglutinin was necessary for blocking as compared to the amount required for conglutination of alexinated cells. Immune adherence as well as erythrophagocytosis are two phenomena dependent on C'3 (19). Also, receptor sites for C'3 have been found on polymorphonuclear leukocytes—the cell responsible for phagocytosis in our test system (20). Bovine conglutinin, with specific reactivity for C'3, can thus be utilized as a reagent to detect the role of activated C'3 in various biological phenomena.

Results obtained in preincubation experiments, as well as tests utilizing isolated 7S and 19S antibody with fresh absorbed cord serum added as a complement source, did not show any significant blocking by conglutinin. The blocking could not be restored by adding fractions of serum containing conglutinogen activating factor, KAF, in our system (4). As conglutinin is capable of inhibiting only when added simultaneously with complement but not if complement is allowed to react first in these preincubation studies, the mechanism involved might not be a mere steric blocking of a reactive site. The mode of action by conglutinin could be either a blocking of further conformational changes of C'3 or a blocking of activation of a later reacting component. This may indicate that later-reacting complement factors are essential for optimal phagocytosis. Support for this was given recently by the finding that full phagocytic activity in a plasma-associated disorder of phagocytosis and chemotaxis could be restored by adding purified normal C'5 (21, 22).

Summary. Bovine conglutinin, a naturally occurring anti-C'3 factor, was studied in quantitative human *in vitro* phagocytosis systems. Striking antiopsonic effect was noted when conglutinin was added to phagocytic

systems with gram-negative and gram-positive organisms dependent on heat-labile opsonic factors. In noncomplement-dependent test systems using isolated hyper-immune γG opsonins, no blocking by conglutinin was noted.

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