Comparison of Bactericidal and Hemolytic Serum Systems. II. Analysis of Inhibitors in Normal Serum Fractions.* (29588)

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The results of a previous study showed that an increase in concentration of certain normal serum components can interfere with bactericidal and hemolytic serum reactions which are dependent upon antibody and complement(1). In the actual tests each of 32 fractions, obtained from a "normal" human serum pool, was tested singly for its influence on the bactericidal and hemolytic activity of a normal human serum pool. Under these conditions the bactericidal reaction was inhibited most significantly by γ-globulin fractions (added to diluted normal serum in concentrations ranging from 7.5 to 60% of the serum content of the reaction mixture) whereas the hemolytic reaction was inhibited by constituents of the β-globulin and albumin fractions (also added in concentrations of 7.5-60%). No enhancing effect by an excess of any one of the 32 fractions was detected. We now have collected data that elucidate, in part, the mode of action of the inhibitors as well as some of the reasons for differences in their activity in the bactericidal and hemolytic reactions.

Materials and methods. Bactericidal and hemolytic reactions were carried out by previously described methods(1,2) using 0.1 ml of a pool of so-called "normal" human serum (diluted 1/10, 1/20 or 1/40) as a source of specific antibody, complement and other possible accessory factors, and electrophoretic fractions of a normal human serum pool (diluted 1/8, 1/16 or 1/50) as a source of inhibitors. The properties of the inhibitory serum fractions, which were obtained from Dr. B. Björklund, Immunological Research Laboratory, Stockholm, have been described (1).

The extent of inhibition by the serum fractions was determined by adding varying amounts of each of the fractions to a basic bactericidal or hemolytic test system and comparing the percentage of bacterial killing or red cell lysis with that of an unsupplemented system. The preparation and use of reagents for titrating components of hemolytic complement have been detailed elsewhere(3). The lysozyme-free fraction of serum (RL) was obtained by absorption with bentonite. Nitrogen determinations on serum fractions were carried out according to the method of Markham(4).

Results. Previously published data(1) had revealed that serum fractions producing maximal inhibition of the bactericidal system differed in their electrophoretic mobility from those producing maximal inhibition of the hemolytic system. Since the active fractions differed in protein content, it was necessary to determine their specific activities, *i.e.*, the percentage of inhibition per μg of nitrogen. Results of such an analysis, using the immune hemolytic reaction as the test system, showed (Fig. 1) that the percentage of inhibition by each fraction was essentially a linear function of the amount of nitrogen added, but that the slopes differed markedly for the various inhibitory fractions. This may be taken as an indication of either qualitative differences among inhibitory serum components or the presence of variable amounts of inert materials in the inhibitory fractions.

The next question concerned the possible mechanism of action of these distinct inhibitors, particularly whether they reacted primarily with serum constituents or with the cells themselves. Normal human serum, therefore, was pre-incubated with inhibitory serum fractions prior to addition of sensitized sheep red blood cells or *E. coli* organisms. Increased inhibition of both hemolytic and bactericidal activity was obtained (Fig. 2) and this increase was directly proportional to

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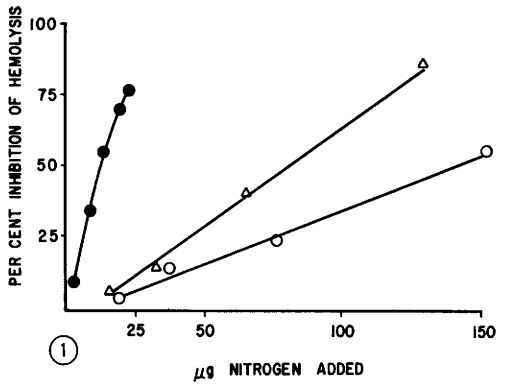

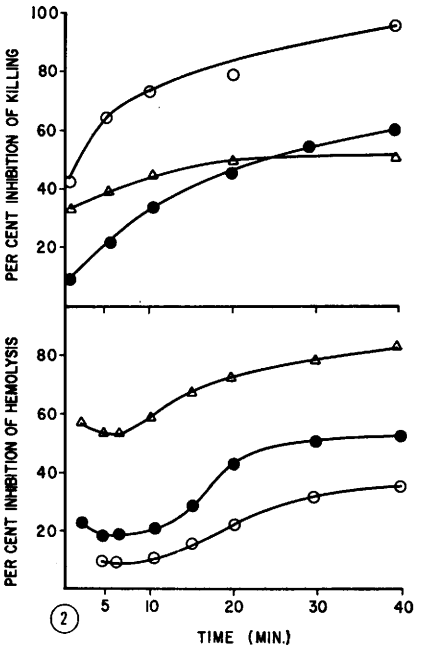

FIG. 1. Inhibition of immune hemolysis by electrophoretic fraction of a pool of normal human serum as a function of weight. ●——●, β-globulin; △——△, albumin; ○——○, γ-globulin.

FIG. 2. Influence of time of incubation at 37°C of normal human serum plus inhibitory serum fractions on subsequent bactericidal (upper curves) and hemolytic (lower curves) reactions. ○——○, γ-globulin; ●——●, β-globulin; △——△, albumin.

the time of pre-incubation. In contrast, pre-incubation of the bacterial or red cells with the inhibitory fractions did not alter the degree of inhibition.

Having thus ascertained that the major effects of the inhibitory serum fractions involved components of serum rather than of the cell, an effort was made to identify the serum components that were inactivated.

TABLE I. Hemolytic Components of C′ Inactivated by Inhibitory Serum Fractions.

Component assayed*	% Inactivation† by serum fraction with mobility of		
	γ-Globulin	β-Globulin	Albumin
C′1	3	0	4
C′2	83	40	0
C′3	56	0	28
C′4	49	78	96

* Normal human serum (diluted 1:10) was incubated with an equal volume of inhibitory serum fraction (diluted 1:10), or with buffer, for 40 min at 37°C and titrated with R1, R2, R3 and R4 to determine the dilution giving 50% hemolysis.

† % Inactivation was based on a 50% end-point.

Representative inhibitory serum fractions were added to normal human serum. The mixture was incubated at 37°C for 30 minutes and then titrated for the known hemolytic components of complement. As shown in Table I, the γ-globulin fraction, which inhibits the bactericidal system more than the hemolytic system, inactivated primarily C′2. The β-globulin and albumin fractions, which contain the principal inhibitors of the hemolytic system, inactivated primarily C′4.

The just cited data furnished evidence for differences between hemolytic and bactericidal systems in their relative requirements for individual components of the complement system. Evidence was also obtained that the bactericidal system requires factors that are without effect on the hemolytic system. Such factors may not represent absolute requirements but at least they enhance bactericidal activity to a marked extent. Lysozyme is one such factor(5,6). In our test system it produced a significant increase in killing when added following the exposure of sensitized bacteria to cation-dependent components of complement (Tables II, III). Similar effects did not occur in the hemolytic system. Finally, it should be noted that exposure to lysozyme, as just described, did not suffice to complete the bactericidal reactions but that additional steps, involving at least one additional component of the C′3 complex was required.

Discussion. This study has shown that the inhibitors for bactericidal and hemolytic serum reactions, present in "normal" human serum and revealed by adding serum frac-

TABLE II. Enhancing Effect of Lysozyme on the Bactericidal Serum Reaction.

Sensitized bacteria incubated with:*	Viable counts/ml after incubation at 37°C with EDTA-RL (lysozyme-free serum) 0 min	40 min	% Killing
RL3 (1:2)	1.8×10^8	6.5×10^7	64
RLP3 (1:2)	1.3×10^8	1.1×10^8	15
RL3 (1:2) + lysozyme	4.3×10^7	$<10^6$	99
RLP3 (1:2) + lysozyme	1.4×10^8	$<10^6$	99
Heated serum + lysozyme	1.8×10^8	1.7×10^8	6

* 0.2 ml of reagents or of heated serum, was added to 0.3 ml of a suspension of *E. coli* B sensitized with antibody, and incubated at 37°C for 10 min prior to transfer of the cells to EDTA-RL. Where lysozyme was included during the first incubation period, 0.1 ml of a 100 μg/ml solution was added.

RL3 = Serum deficient in lysozyme and C'3; RLP3 = *ditto* plus deficiency in properdin.

tions to a basic hemolytic and bactericidal system, are distinct factors. They differ in specific inhibitory activity in the hemolytic test system and they also differ with respect to the hemolytic components of complement that are inactivated by them. It has been confirmed that among the inhibitory serum factors those in the γ-globulin fraction are more inhibitory for the bactericidal than for the hemolytic reaction, whereas those in the β- and albumin fractions, when added to whole serum, inhibit the hemolytic activity of serum more than its bactericidal activity. These inhibitions, according to the data presented here involve effects on specific components of complement rather than effects on the bacteria or erythrocytes. These observations, particularly the fact that different inhibitory serum fractions alter activities of different components of complement and affect hemolytic and bactericidal systems differently, have permitted us to recognize differences between these two systems. If it is assumed that both systems require all of the components of complement, the data would suggest that the 2 systems differ in their quantitative requirements for individual components. Such differences also have become apparent from data recently published by Michael and Braun(7) and from additional data which are currently being collected by us.

The data presented here also indicate that hemolytic and bactericidal systems differ not only in their quantitative requirements, but also, as suggested by the lysozyme data, qualitatively. Such differences are not surprising in view of the known differences in the chemical composition of the target of the hemolytic and bactericidal serum factors, namely the cell envelopes of erythrocytes and bacteria. Also, the two cell systems show major differences in metabolic and reproductive capacities which can greatly influence the outcome of cytotoxic serum reactions(8, 9). However, the resulting quantitative and qualitative differences in requirements for the hemolytic *vs* the bactericidal serum reactions have not been recognized sufficiently in the past.

Finally, based on the known structure of Gram-negative bacterial cell walls the suggestion has been made that lysozyme is re-

TABLE III. Analysis of the Bactericidal Reaction with Respect to the Stage at Which Lysozyme Acts.

Treatment of sensitized bacteria in Step 1*	Step 2†	Viable counts/ml before and after incubation in EDTA-RL (lysozyme-free serum) at 37°C 0 min	40 min	% Killing
R3	Lysozyme	2.5×10^8	5.0×10^7	80
Lysozyme	R3	1.3×10^8	6.5×10^7	48
R3	Buffer	1.5×10^8	8.5×10^7	43
Lysozyme	"	2.7×10^7	2.4×10^7	11

* A suspension of *E. coli* B (3 ml), sensitized with antibody, was incubated for 10 min at 37°C with either 3 ml R3 (diluted 1:4) or 3 ml lysozyme (12.5 μg/ml); the cells were then washed and resuspended in 3 ml cold buffer.

† Bacteria treated in step 1 were exposed to either lysozyme or R3 for 10 min; they were then incubated for 40 min in EDTA-RL (step 3).

quired in a late step of the bactericidal reaction because the susceptible mucopeptide is found in the innermost layer of the cell envelope(10). The results of our studies on sequential steps in bactericidal serum reactions (Table III) now provide experimental evidence that the lysozyme-dependent reaction is indeed a late reaction, the uncovering of the substrate presumably requiring prior action of specific antibody and cation-dependent components of complement.

Summary. The inhibition of bactericidal and hemolytic human serum reactions by addition of certain fractions from a pool of normal human serum has been analyzed. The fractions with major inhibitory effects for bactericidal reactions, namely γ-globulins, proved to be qualitatively different from those which inhibited immune hemolysis, namely β-globulins and albumin. The inhibitory effects of these serum fractions were not on the cells but on different components of the complement system; addition of γ-globulin to whole serum affected C'2, whereas addition of β-globulin and albumin fractions affected principally C'4. Such differences have permitted identification of quantitative differences in requirements for components of complement by the hemolytic and bactericidal reactions. In addition, a qualitative difference in the requirement for lysozyme has been recognized.

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Immunological Reactivity of Thymic Autografts in the Rat.* (29589)

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After antigenic stimulation the lymphatic tissue undergoes several histological changes among which lymphocyte and plasma cell proliferation are the most characteristic. Notwithstanding its lymphatic structure the thymus does not develop the cellular changes that appear in other lymphatic organs after parenteral antigenic stimulation(1).

It has been clearly demonstrated that thymus grafts have the capacity to restore the immunological functions of thymectomized mice(2). This finding would indicate that the immunological functions of the thymus in an heterotopical position are similar to the functions observed when the organ is in its normal location. However, no studies have been reported of the immunological reactivity of the thymic cells in that abnormal localization of the gland. It seemed of interest to determine whether thymic autografts implanted in the subcutaneous tissue would not react to antigenic stimulation with any of the cellular changes associated with the immune response as it usually occurs when the organ is in its thoracic position. In this

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