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Research Article

STUDIES OF STREPTOCOCCAL FIBRINOLYSIS. II. THE INHIBITION OF STREPTOCOCCAL FIBRINOLYSIS BY ANTIFIBRINOLYSIN AND ANTIPROTEASE[1]

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The resistance of human plasma clots to dissolution by fibrinolysin is generally considered an immunological response to infection by β-hemolytic streptococci (1, 2). Consequently, the development of resistant clots during convalescence has been used as a diagnostic test for streptococcal disease. However, clot resistance has also been observed in individuals where no evidence of streptococcal infection could be recognized. Positive antifibrinolysin tests, for example, have been reported in the following instances: 15 per cent of normal individuals (3, 4); in a number of unrelated disease states, as lobar pneumonia, gonococcal arthritis, typhoid fever, and acute nephritis (5 to 7); in the newborn (8, 6); and in various animal species (9, 1). These findings, in turn, have placed some doubt on the specificity of the antifibrinolysin response.

Recent advances in the study of the mechanism of streptococcal fibrinolysis, however, have made it necessary to review these non-specific cases of antifibrinolysis in the light of the new findings. It has been shown (10 to 12) that the *lytic factor* (13) which participates in streptococcal fibrinolysis is the precursor of a proteolytic enzyme in plasma, which is activated by streptococcal fibrinolysin. Fibrinolysis results from the proteolytic action of this activated enzyme. Thus two stages are involved in the process:

(*1*) Conversion of the lytic factor by fibrinolysin into an active protease.

(*2*) Dissolution of the fibrin clot by the protease.

As was indicated in a preliminary report (12), the inhibition of either of these two stages would result in the apparent resistance of the clot to fibrinolysin. Accordingly, the resistance of a plasma clot to lysis might result from: (a) the presence in plasma of antifibrinolysin; (b) the presence of antiprotease; or (c) the deficiency of an effective lytic factor.

Deficiency of a lytic factor has been reported in the resistant plasmas of the newborn (14) and of the rabbit (13). The present study is primarily concerned with the rôle and properties of antifibrinolysin and antiprotease.

MATERIALS AND METHODS

1. Fibrinolysin. Fibrinolysin was prepared by alcoholic precipitation according to the procedure of Garner and Tillett (15), and stored in the dried or frozen state as previously described (16). The source of the fibrinolysin was a highly active strain of group A β-hemolytic streptococcus (no. 98) isolated from the blood of a patient with erysipelas.

2. Fibrinogen. The product employed was Fraction I (17) of the plasma proteins.[2] Clots formed from this fibrinogen preparation were readily susceptible to fibrinolysis, indicating that the lytic factor was present in adequate amounts. The fibrinogen solution used contained 0.60 grams of Fraction I in 100 ml. of buffered saline.

3. Serum protease. Preparation of serum protease was accomplished by chloroform treatment of the component of serum which is precipitated by dilution and acidification to pH 5.5. This method has been previously described (18). In addition, a component of Fraction III-2 (17) of the plasma proteins [2] was found to be a highly active

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[2] The products of plasma fractionation employed in this work were obtained through the courtesy of Drs. E. J. Cohn, J. T. Edsall, and S. Howard Armstrong, Jr. They were developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, Mass., under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

source of the protease, and was also employed. Both preparations were equally satisfactory.

4. Thrombin. The thrombin preparation was a 1:10 dilution of a commercial product [3] prepared from rabbit blood.

5. Buffered saline. The buffered saline employed was a solution containing 0.01M phosphate in 0.85 per cent sodium chloride at pH 7.4.

6. Standard fibrin clot. The standard clot was formed from 0.5 ml. of fibrinogen and 0.2 ml. of thrombin made up to a final volume of 2.2 ml.

7. Assay of serum antiprotease. The titer of serum antiprotease was measured by the highest dilution of serum which neutralized the clot-lysing activity of a standard solution of serum protease. The standard solution of serum protease adopted was that dilution which permitted 0.5 ml. to lyse completely the standard fibrin clot in 30 minutes at 37° C. The procedure for the estimation of serum antiprotease was as follows:

One ml. amounts of twofold dilutions of serum beginning with 1/5 were incubated with 0.5 ml. of standard protease solution for 30 minutes at 37° C. The enzyme-serum mixtures were then incorporated in fibrin clots by the further addition of 0.5 ml. of fibrinogen and 0.2 ml. of thrombin. Incubation at 37° C. was carried out for 60 minutes. The reciprocal of the highest serum dilution which completely prevented lysis was taken as the titer of antiprotease.

8. Assay of serum antifibrinolysin. The procedure was similar to that described for the estimation of antiprotease. The titer of serum antifibrinolysin was taken as the highest dilution of serum which neutralized the fibrinolytic activity of a standard solution of fibrinolysin. The standard fibrinolysin solution was that concentration which permitted 0.5 ml. to lyse the standard fibrin clot in 30 minutes at 37° C. One ml. amounts of twofold dilutions of serum beginning with 1/5 were incubated for 30 minutes at 37° C. with 0.5 ml. of fibrinolysin. To each tube was added 0.5 ml. of fibrinogen and 0.2 ml. of thrombin. After incubation for 60 minutes, the tubes were read for the highest serum dilution completely preventing lysis.

It will be observed that in the methods employed for the estimation of antiprotease and antifibrinolysin, the unit of lytic activity was the same.

9. Antifibrinolytic sera were obtained from convalescent cases of acute streptococcal pharyngitis or tonsillitis. The diagnosis was confirmed by the presence of β-hemolytic streptococcus on throat culture, and by the development of another streptococcal antibody, antistreptolysin "O", during convalescence (19).

A. The fibrinolysin-antifibrinolysin reaction

Although the antifibrinolysin test has been found occasionally to be non-specific, it is generally accepted that the clot resistance developed in individuals convalescent from streptococcal disease is a specific immunological response to the infection (2). While it has not been possible to elicit antifibrinolysin by animal inoculation (20), the immunological nature of the antifibrinolytic response in man is supported by the observation that the development of fibrinolytic resistance is correlated with the formation of other streptococcal antibodies, such as antistreptolysin "O" (21, 22, 8).

It has recently been observed that the level of antifibrinolysin in serum was also correlated with the capacity of the serum to inhibit trypsin (23). This observation seemed to contradict the biphasic nature of the fibrinolytic reaction, which suggests that antifibrinolysin and antiprotease represent separate activities. The relationship of antifibrinolysin to the fibrinolytic mechanism was therefore investigated. In addition, observations were extended to the quantitative aspects of the reaction between fibrinolysin and antifibrolysin.

1. The relationship of antifibrinolysin to the fibrinolytic mechanism

Since streptococcal fibrinolysis has been shown to be a two-stage process, it was necessary to determine first whether antifibrinolysin acted by neutralizing fibrinolysin or by blocking the protease system. This problem was approached by comparing the antifibrinolytic effects observed when a given convalescent serum was incubated with lytic factor and when the same serum was incubated with fibrinolysin. If the serum acted on fibrinolysin, it seemed reasonable that incubating the serum with the lytic factor in the fibrinogen preparation would have no appreciable effect on the resistance of the clot; whereas, incubating the serum with fibrinolysin would result in an increase in resistance. The respective capacities of the serum to inhibit the lytic factor and fibrinolysin were quantitated by testing the serum in progressive twofold dilutions. The inhibitory effect in each case was measured by the highest dilution of the serum which completely prevented dissolution.

The procedure employed was as follows: One ml. amounts of twofold dilutions of serum were set up in triplicate. To the first set of dilutions were quickly added 0.5 ml. amounts of fibrinolysin, 0.5 ml. of fibrinogen, and 0.2 ml. of thrombin, without prior incubation.

[3] "Hemostatic Globulin," Lederle Laboratories, Inc.

To the second set of serum dilutions, 0.5 ml. amounts of fibrinogen solution (lytic factor) were added and incubation carried out for 15 minutes at 37° C. To these mixtures were next added 0.5 ml. of fibrinolysin and 0.2 ml. of thrombin. To each tube of the third series of serum dilutions, 0.5 ml. amounts of fibrinolysin were added and incubation of these mixtures carried out for 15 minutes at 37° C. To each mixture was then added 0.5 ml. of fibrinogen and 0.2 ml. of thrombin. The tube of highest serum dilution in which no lysis occurred after one hour at 37° C. was taken as the titer of the serum.

It may be observed from Table I that incubating the serum with the lytic factor did not result in a significant increase in antifibrinolytic titer. However, when incubation was carried out with fibrinolysin, the titer of the serum was increased from 8 to 256. This marked increase in the antifibrinolytic capacity of the serum which resulted from incubating previously with fibrinolysin indicated that the serum acted specifically on fibrinolysin.

TABLE I

Effect of incubating serum with fibrinolysin and with lytic factor on the antifibrinolytic titer

Titer of serum* without incubation	Titer of serum after incubation with lytic factor	Titer of serum after incubation with fibrinolysin
8	16	256

* Reciprocal of highest dilution of serum which completely prevented lysis.

Subsequent comparative studies of the levels of antifibrinolysin and antiprotease in the sera from a large number of individuals, to be presented in the following sections, have similarly indicated that antifibrinolysin has no effect on the protease system. The evidence thus indicated that the factor responsible for the resistance of plasma clots to lysis in cases of hemolytic streptococcal infection does not affect the serum protease system, but reacts specifically with fibrinolysin.

2. *Quantitative aspects of the fibrinolysin-antifibrinolysin reaction*

The observation that an increase in antifibrinolytic titer resulted when a prior incubation of the serum with fibrinolysin was carried out suggested that neutralization did not occur instantaneously. It had been observed previously that plasma clots frequently became more resistant when the plasma sample was first incubated with fibrinolysin (24). While this increased resistance was pointed out as a factor which conditioned the results obtained with the plasma antifibrinolysin test, further investigation of this effect was not carried out. In the present experiment an attempt was made to measure the rate of reaction between fibrinolysin and antifibrinolysin.

The effect of incubation was first studied by measuring the lytic activity of mixtures of fibrinolysin and serum which had been incubated previously at 37° C. for various intervals of time. Lytic activity was measured by the time of dissolution of a standard fibrin clot in which the reaction mixtures were incorporated.

The procedure was as follows: Mixtures of 1.0 ml. of a serum diluted to 1/200 and 0.5 ml. of fibrinolysin were incubated for 0, 15, 30, and 60 minutes at 37° C. At the end of each period, 0.5 ml. of fibrinogen and 0.2 ml. of thrombin were added. The lysis time of each clot was then measured at 37° C. Lysis time was taken as the period between clotting and complete dissolution of the fibrin clot.

The progressive prolongation of the lysis time resulting from such incubation is demonstrated in Table II. Without prior incubation, the lytic activity of the fibrinolysin-serum mixtures was identical with that of the fibrinolysin-saline control, indicating that no union of fibrinolysin and antifibrinolysin occurred. As the period of incubation was lengthened, however, the proportion of fibrinolysin neutralized appeared correspondingly increased.

TABLE II

Prolongation of the lysis time produced by incubating for varying periods constant amounts of fibrinolysin and antifibrinolysin

Fibrinolysin	Antifibrinolysin*	Saline	Lysis time produced after incubating for:			
			0 min.	15 min.	30 min.	60 min.
ml.	*ml.*	*ml.*	*min.*	*min.*	*min.*	*min.*
0.5	0	1.0	10	10	10	11
0.5	0.25	0.75	10	15	17	18
0.5	0.5	0.5	10	23	30	41
0.5	1.0	0	10	64	90	>120

* The antifibrinolysin solution was a 1/200 dilution of a convalescent serum.

In order to subject the reaction to study in quantitative terms, it was necessary first to devise

an accurate method of measuring fibrinolysin concentration. It was found that fibrinolysin concentration could be satisfactorily estimated by means of lysis time if a standard fibrin clot of constant and adequate lytic factor content were employed, and if such measurements were made within a range in which the relationship between lysis time and fibrinolysin concentration most nearly approached linearity (25). A suitable range of proportionality was observed with lysis times between 10 and 60 minutes.

Accordingly, by means of a series of precise titrations, a reference curve was plotted which related lysis time to fibrinolysin concentration (Figure 1). It was found convenient to express

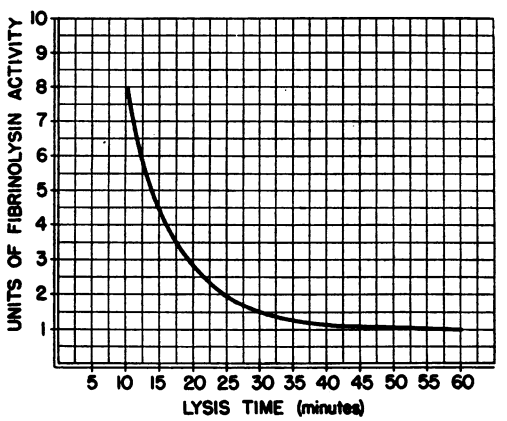

FIG. 1. STANDARD FIBRINOLYSIN CURVE

fibrinolysin concentration in arbitrary units of activity. One unit of fibrinolysin was that amount effecting lysis of the standard fibrin clot in 60 minutes. By means of the reference curve, the lysis time effected by a mixture of fibrinolysin and antifibrinolysin could be converted into terms of residual units of fibrinolysin.

The rate of neutralization was then studied by the following procedure:

To constant amounts of a 1/200 dilution of convalescent serum contained in 1.0 ml. was added 0.5 ml. of fibrinolysin equivalent to 8.0 units of activity. Incubation was carried out for varying periods from 5 minutes to 90 minutes in a water bath at 37° C. At the end of a given period of incubation, 0.5 ml. of fibrinogen and 0.2 ml. of thrombin were added, the clotting time noted, and the time required for dissolution of the clot accurately determined. The lysis time was then a measure of the residual activity of the fibrinolysin-antifibrinolysin mixture after the given period of incubation. Lysis time was converted into terms of relative fibrinolysin concentration in units, by reading directly from the reference fibrinolysin curve. Deducting the residual units of activity from the 8.0 units of fibrinolysin originally present yielded the amount of fibrinolysin bound.

In Figure 2 are given the curves for the rate of reaction between fibrinolysin and antifibrinolysin. The rate of combination is exceedingly rapid during the initial phase of the reaction, particularly during the first 10-minute interval. Subsequently, the reaction proceeds at a progressively diminishing rate, and is essentially complete at the end of 30 minutes. Equilibrium is reached in approximately 60 minutes.

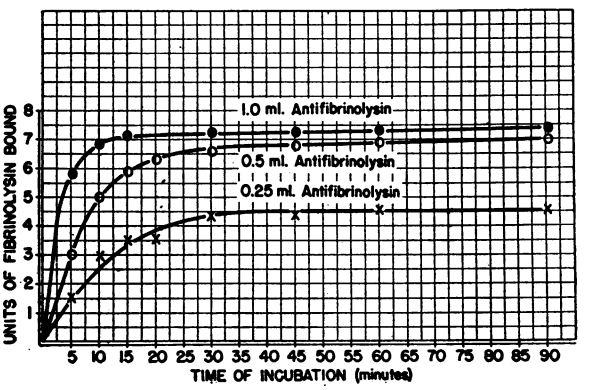

FIG. 2. RATE OF COMBINATION OF FIBRINOLYSIN AND ANTIFIBRINOLYSIN

It will be noted from Figure 2 that the amount of fibrinolysin finally bound was not related stoichiometrically to the quantity of antifibrinolysin added, and consequently could not be reduced to terms of equivalence with a given amount of serum. For example, while 0.5 ml. of antifibrinolysin neutralized 7.0 units of fibrinolysin, the addition of twice as much, or 1.0 ml., neutralized only 7.3 units. These observations then indicated that the amount of fibrinolysin bound was dependent upon the relative proportion of fibrinolysin and antifibrinolysin in the reaction mixture.

This fact is clearly demonstrated in Figure 3, in which is given the amount of fibrinolysin finally bound after the addition of varying quantities of antibody to 8.0 units of fibrinolysin. As the antifibrinolysin concentration was increased, each successive increment combined with progressively smaller amounts of fibrinolysin. It was therefore concluded that fibrinolysin and antifibrinolysin

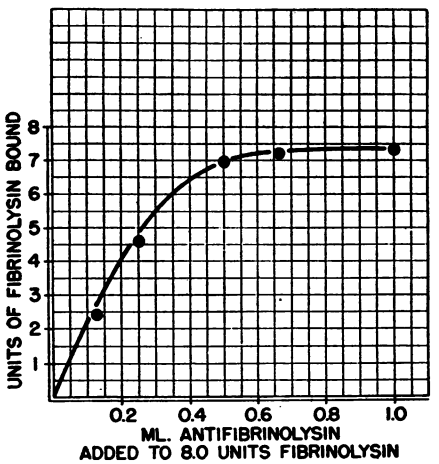

FIG. 3. AMOUNT OF FIBRINOLYSIN BOUND AFTER ADDING VARYING AMOUNTS OF ANTIFIBRINOLYSIN TO 8.0 UNITS OF FIBRINOLYSIN

combine in varying proportions which are dependent upon the ratio of the concentrations of the two substances. The reaction is thus similar to toxin-antitoxin reactions *in vitro* (26).

When the amount of fibrinolysin bound at the end of 90 minutes was taken as the end-point of the reaction, it was found that after 15 minutes incubation, the reaction was 83 to 96 per cent complete, depending upon the amount of antifibrinolysin present; and at the end of 30 minutes, the reaction was 95 to 98 per cent complete.

B. The inhibition of streptococcal fibrinolysis by serum antiprotease

It has been demonstrated that the resistance of plasma clots to lysis in patients recovering from hemolytic streptococcal infections is due to the presence of antifibrinolysin, an antibody specifically neutralizing the action of fibrinolysin. From theoretical considerations of the fibrinolytic mechanism, it has been suggested that the inhibition of fibrinolysis may also result from the presence in plasma of antiprotease.

The nature and physiological significance of serum antiprotease, sometimes termed serum antitrypsin, has been reviewed recently (27). Antiproteolytic activity appears to be a constant property of human and animal sera; however the substance or substances in serum responsible for this property have as yet not been identified. The most widely held hypothesis is that antiprotease is a product of protein hydrolysis. It has been pointed out, for example, that the antitrypsin of normal serum is similar in properties to the trypsin-inhibitor produced during the tryptic digestion of proteins (28). Moreover, the antiproteolytic activity of serum appears to be causally related to the products of protein hydrolysis formed both in the intestine and parenterally after the oral and intramuscular administration of trypsin (27). These findings are also supported by evidence from pathological studies. Thus the serum antiprotease level is increased after meals, in patients with malignant tumors, in tuberculosis and hyperthyroidism, in anaphylaxis, and in many cases of acute infection, especially if accompanied by high fever (27).

In the present work, investigation was made of the relationship of serum antiprotease to the inhibition of fibrinolysin. The association of an increased antiprotease level with acute infections suggested that this factor might be related in some cases to the non-specific antifibrinolytic tests reported. Consequently, comparative studies were made of the antiprotease and antifibrinolysin levels of normal human sera, of sera from patients with hemolytic streptococcal infections and pneumococcal pneumonia, and of sera from various animal species.

1. The relationship of antiprotease to the fibrinolytic mechanism

Previous studies have shown that fibrinolysin may be inhibited by animal and plant antitrypsins (23). These observations were regarded as indicating that fibrinolysin and trypsin were similar in properties. However, since streptococcal fibrinolysis has been shown to be a two-stage reaction, it seemed more likely that the inhibition observed was due to the blocking of the protease system. This assumption was verified in the following experiment.

Ten sera of varied antiprotease level but of low antifibrinolysin content were selected, and each serum tested for its relative capacity to inhibit fibrinolysin and serum protease. Antifibrinolytic and antiproteolytic effect was measured by the prolongation of the lysis time of a standard fibrin clot to which the serum was added. It was necessary that the sera contain little or no antifibrinoly-

sin, since possible inhibition of fibrinolysin by the antiprotease would be masked by the more pronounced effect of the antifibrinolysin. The sera were not incubated with either fibrinolysin or protease prior to clot formation; reagents were added in rapid succession and clots immediately formed.

The procedure was as follows:

To 1.0 ml. of a 1/10 dilution of each serum was added 0.5 ml. of fibrinolysin, and then quickly, 0.5 ml. of fibrinogen and 0.2 ml. of thrombin. To a duplicate series of sera were added 0.5 ml. amounts of serum protease, and clots formed in the same manner. Incubation was carried out at 37° C. and the lysis times of the clots noted. The relative capacity of each serum for inhibiting fibrinolysin was thus directly compared with its relative ability to inhibit the activity of the serum protease.

TABLE III

Relationship of the antifibrinolytic capacities of sera to their antiprotease content

Serum no.	Lysis time with fibrinolysin	Lysis time with protease	Anti-fibrinolysin titer	Anti-protease titer
	min.	*min.*		
26 C	20	36	<5	5
19 B	21	41	<5	10
25 B	20	43	5	10
16 A	26	46	5	40
19 A	49	41	<5	20
19 C	51	48	<5	20
25 C	56	51	5	40
26 B	56	53	5	40
25 A	56	51	5	40
21 A	81	48	<5	40

Table III demonstrates that these properties were directly correlated. Sera possessing elevated levels of antiprotease were those maximally resistant to the action of fibrinolysin. Since the true antifibrinolysin levels of these sera were all negligibly low, this resistance could have been due only to antiprotease. It was concluded that serum antiprotease is an important factor in the resistance of plasma clots to streptococcal fibrinolysis.

2. The antiprotease levels of normal human sera

Sera from 21 normal individuals known to have had no recent illness or infection were assayed for their antiprotease and antifibrinolysin titers by the methods described. As shown in Table IV, variation of the titer of antiprotease in the normal subjects was slight; none of the sera possessed an antiprotease titer greater than 5. In contrast, the antifibrinolysin titers ranged from < 5 to 80. Approximately 70 per cent of the sera possessed an antifibrinolysin titer of less than 10.

TABLE IV

The antiprotease and antifibrinolysin titers of normal human sera

Serum no.	Antiprotease titer	Antifibrinolysin titer
618	5	80
619	<5	40
620	<5	20
621	<5	<5
623	<5	10
624	<5	10
625	5	10
626	5	20
627	<5	5
628	<5	<5
629	5	<5
630	<5	<5
634	<5	<5
637	5	<5
640	<5	5
643	5	10
644	5	5
645	<5	<5
647	<5	<5
649	<5	<5
650	<5	<5

No correlation was observed between the antifibrinolysin and antiprotease content of these normal sera. However, the considerable variation of the antifibrinolysin titer was significant. This observation provided suggestive evidence that the resistant plasma clots reported previously (3, 4) with normal individuals might have been due to the presence in these cases of significant levels of antifibrinolysin.

3. Comparison of the antifibrinolysin and antiprotease titers from patients with hemolytic streptococcal infection

In a recent study of the antifibrinolysin test, it was reported that sera having high antifibrinolysin titers also possessed high antitrypsin titers (23). However, studies of the mechanism of the fibrinolytic reaction and its inhibition have thus far indicated that antifibrinolysin is a specific antibody whose rôle in the antifibrinolytic phenomenon is separate and distinct from that of antiprotease. Observations were further extended to sera from known streptococcal infections.

The antifibrinolysin titers and antiprotease titers of 65 acute and convalescent sera from 21 cases of hemolytic streptococcal pharyngitis or tonsillitis were compared. Hemolytic streptococcal infection was confirmed by bacteriological and

clinical evidence and by the development of streptococcal antibodies (antistreptolysin "O") during convalescence (19).

TABLE V

Comparison of the antiprotease and antifibrinolysin titers of sera from cases of hemolytic streptococcal infection (pharyngitis or tonsillitis)

Case no.	Date of serum	Anti-streptolysin "O" titer	Anti-protease titer	Anti-fibrinolysin titer
E-185	3–22	400	10	1000
	4–13	500	10	1000
	4–28	500	10	1000
E-284	3–27	83.3	<10	200
	5–11	250	10	500
	6–15	250	20	600
E-361	4–2	125	5	150
	4–23	500	10	600
	5–11	500	10	600
T-262	11–21	159	20	150
	12–10	159	20	150
	12–27	250	10	300
	12–31	833	10	1000
T-270	11–21	159	10	50
	11–28	200	5	100
	12–9	2000	10	250
	12–31	1585	5	250
T-202	11–19	159	<5	400
	11–28	317	<5	600
	12–10	500	5	1000
	1–3	400	10	600
D-184	12–18	83.3	<5	300
	1–5	159	<5	800
	1–25	200	<5	1000
E-425	4–10	200	10	400
	5–4	1250	20	1400
	5–18	1250	20	1400
D-226	12–30	200	5	150
	1–20	625	5	400
	2–10	625	<5	400
T-229	11–19	62.5	<5	50
	11–28	159	10	100
	12–9	400	5	500
	12–31	400	20	500

The results are given in Table V. The antistreptolysin titers are also given for the purpose of establishing the validity of the streptococcal infection. The antiprotease titers of these sera ranged from < 5 to 20, while the antifibrinolysin titers extended from < 50 to 1400. No correlation of antifibrinolysin with serum antiprotease was observed, either in terms of relative or of absolute titers. Moreover, in no patient was a rise in antiprotease found which was in the least commensurate with the rise in antifibrinolysin.

These observations confirm the hypothesis that antifibrinolysin and antiprotease are distinct substances, each of which, by a different mode of action, is capable of effecting resistance to fibrinolysin. In cases of hemolytic streptococcal infection, this increased resistance is apparently due to a specific immunological response whereby antifibrinolysin is elaborated. No corresponding changes in the serum antiprotease level were found to occur at the same time.

4. The antifibrinolytic properties of sera from cases of pneumococcal pneumonia

There is some evidence that the changes which occur in the blood during the acute phase of infection may be responsible for the non-specific positive antifibrinolytic tests reported. Such positive tests (6, 8) have been observed in the acute febrile phase of influenza, poliomyelitis, typhoid fever, bacillary dysentery, and lobar pneumonia. Although these studies have been largely confined to patients in the pediatric age group, similar observations have been made in some adults, particularly those with gonococcal arthritis (5), infections due to streptococcus viridans (4, 5), and lobar pneumonia (3, 7). The resistance produced in these cases did not appear to be an immunological response to the infection, since maximal resistance occurred in the acute phase of the disease and then rapidly fell during convalescence. That these effects might be due to a rise of antiprotease is suggested by the fact that antiproteases are known to be elaborated in the blood during acute infection (29, 27).

Consequently, a study was made of the antiprotease levels of acute phase and convalescent sera from 25 cases of lobar pneumonia. The cases available for study were soldiers with illnesses of moderate severity.

Eleven of the 25 cases of lobar pneumonia studied demonstrated antiprotease titers of 20 or greater in either the acute or convalescent sera. The antiprotease levels of these 11 patients are given in Table VI. The level of antiprotease in these cases may be considered appreciably elevated as compared with the titer of 5 obtained with normal sera. Four patients showed a fourfold or greater fall in titer from the acute to the convalescent phase. The evidence suggests that antiprotease may be the factor responsible for the

TABLE VI

Comparison of the antiprotease and antifibrinolysin titers of selected sera from patients with pneumococcal pneumonia

Serum no.	Antiprotease titer		Antifibrinolysin titer	
	Acute	Convalescent	Acute	Convalescent
10	20	20	>640	>640
12	20	5	40	40
16	40	10	5	<5
17	40	20	320	320
18	20	10	80	80
19	20	10	<5	<5
21	40	10	<5	5
22	80	40	40	80
24	80	80	40	20
25	40	40	5	5
26	40	5	5	<5

resistant clots of some patients with pneumococcal pneumonia.

Those sera containing an elevated antiprotease level were also tested for their antifibrinolysin titers. As indicated by Table VI, no correlation between the titer of antiprotease and the titer of antifibrinolysin was obtained. It might be expected from the nature of the fibrinolytic process that the sera which contained appreciable antiprotease titers would show an increased degree of resistance to fibrinolysin. This was not found to be the case. The acute serum of No. 21, for example, which possessed an antiprotease level of 40, had no higher antifibrinolysin titer than the convalescent serum, the antiprotease level of which was 10. It would appear that the present method of antifibrinolysin titration was not readily affected by the levels of antiprotease in the sera studied.

A possible explanation for this is that the serological method does not permit ready union of the antiprotease with lytic factor before activation is effected; nor does it readily permit union of the inhibitor with the activated protease before the fibrin clot is attacked. Since the end-point in the test is based on *lysis-prevention,* it will be seen that antiprotease may not be able to prevent fibrinolysis completely unless present in high concentration.

5. The inhibition of fibrinolysin by the sera of various species

The resistance of plasma clots of various animal species to the action of fibrinolysin was first accounted for by the hypothesis that fibrinolytic streptococci were species-specific; that is, that a given streptococcus was able to lyse only the fibrin of the species from which it was isolated (9, 2). Subsequently it was found that the resistance of rabbit plasma clots could be attributed to the deficiency of an effective lytic factor (13), and perhaps also to the presence of an inhibitor (10). Other studies indicated that human fibrin of all the species tested was most susceptible to fibrinolysis, regardless of the source of strain of streptococcus (30).

These observations suggested that the difference in susceptibility between human and animal plasma clots was, in large measure, due to factors in the plasma itself rather than to the fibrin specificity of the streptococcus. In man it has been demonstrated that resistance may result from at least three plasma factors: antifibrinolysin, antiprotease, or a deficient lytic factor. It seemed likely that these same factors, with the possible exception of antifibrinolysin, were responsible for the observed resistance of animal plasma clots. In the present study the rôle of antiprotease was examined.

The sera of various animal species including the rabbit, guinea pig, horse, cow, and swine were examined for their capacity to inhibit the action of fibrinolysin on human fibrin clots. This property was compared with the capacity of the same sera to inhibit human serum protease. Inhibition of fibrinolysin was measured by the lysis time of a fibrin clot to which was added 0.2 ml. of serum and 0.5 ml. of fibrinolysin. This amount of fibrinolysin regularly lysed the fibrin clot in 15 minutes. Antiprotease was determined by serum titration according to the method previously described.

As shown in Table VII, addition of the animal sera rendered the fibrin clot completely resistant to the action of fibrinolysin. In contrast, the addition of normal human serum to the fibrin clot affected the fibrinolytic rate only slightly. On examination of their antiprotease content, the animal sera all possessed titers of 80 or greater, in striking contrast to the level of 5 obtained with normal human serum. Since these sera were incorporated in fibrin clots which contained adequate amounts of lytic factor, the resistance could be attributed only to inhibiting substances in the added sera. It was concluded that antiprotease is an important factor in the resistance of animal clots to fibrinolysin.

TABLE VII

The inhibition of fibrinolysin and serum protease by the sera of various species

Species	Lysis time with fibrinolysin	Antiprotease titer
Rabbit	>24 hrs.	80
Guinea Pig	>24 hrs.	160
Horse	>24 hrs.	160
Cow	>24 hrs.	>160
Swine	>24 hrs.	>160
Human	20 min.	5
Control (Saline)	15 min.	0

DISCUSSION

From theoretical considerations of the mechanism of streptococcal fibrinolysis, it has been suggested that the resistance of plasma clots to the action of fibrinolysin may result:

(*1*) From the inhibition of fibrinolysin.

(*2*) From the inhibition of the serum protease system.

(*3*) From a quantitative deficiency of the lytic factor.

The results of the various studies made on fibrinolysin inhibition in streptococcal infections, in pneumococcal pneumonia, and in the animal species have furthered this hypothesis.

In streptococcal infection, the inhibition of fibrinolysin is due to antifibrinolysin, a specific antibody presumably elaborated as an immunological response to the infection. Antifibrinolysin does not enter into any other phase of the fibrinolytic mechanism. The true antifibrinolysin level of a given serum is completely independent of its content of antiprotease.

These observations were further confirmed in studies of the antifibrinolytic properties of sera from patients with pneumococcal pneumonia. The absence in cultures of pneumococcus of a possible antigenic constituent similar in action to fibrinolysin (2), as well as the non-immunological character of the antifibrinolytic response during the disease, indicated that here the basis for the inhibition of fibrinolysin was completely different from that in streptococcal infection. It was of marked significance, therefore, that in some of the cases of pneumococcal pneumonia studied, the antiprotease level tended to be elevated particularly during the acute stage of the disease.

In a recent review of the relationship of the serum antiprotease level to various disease states (27), it was reported that antiproteases are liberated during the febrile phase of acute infection. In several cases of lobar pneumonia (31), the antiprotease titer rose markedly just before crisis and then decreased to normal limits within a period of a week. The present data suggest that the positive antifibrinolysin tests observed in cases of pneumococcal pneumonia and other acute febrile diseases may be due in part to the elaboration of serum antiprotease during the disease processes. The rapid loss of antifibrinolytic properties observed in such patients would result from a corresponding decrease in antiprotease. Further data relating the antiprotease level in acute infections with the plasma clot antifibrinolysin test are desirable.

Since the variation of the antiprotease level of normal sera is slight, the positive antifibrinolysin tests reported with normal individuals are probably not due to this factor. On the other hand, the wide variation of the antifibrinolysin level in normal sera is suggestive evidence that such positive tests may have been due to the presence of residual antifibrinolysin elaborated as a result of streptococcal infection in the past. A critical analysis of the occurrence of antifibrinolysin in normal individuals, as well as in the various disease states, has been made possible by the application of a quantitative method for the estimation of serum antifibrinolysin. The results of this investigation will be reported in a succeeding paper.

SUMMARY AND CONCLUSIONS

The inhibition of streptococcal fibrinolysis by human serum may result from the presence of either antifibrinolysin or antiprotease.

Antifibrinolysin combines rapidly and specifically with fibrinolysin, and does not affect the activity of the lytic factor or the serum protease. The reaction between fibrinolysin and antifibrinolysin is 95 to 98 per cent complete at the end of 30 minutes at 37° C. and equilibrium is reached in approximately 60 minutes. Since the two substances combine in varying multiple proportions, the reaction resembles *in vitro* toxin-antitoxin reactions.

Antiprotease prevents streptococcal fibrinolysis by inhibiting the protease system. The antiprotease content of serum is independent of the level of antifibrinolysin. No correlation was observed

either in absolute or relative levels of antiprotease and antifibrinolysin.

In patients with pneumococcal pneumonia, the antiprotease levels tend to be elevated during the acute phase of the disease. It is suggested that the antifibrinolytic response frequently observed in the acute phase of febrile disease is due to the presence of plasma antiprotease.

The sera of various animal species were found to possess exceedingly high levels of antiprotease. It is possible that the resistance of animal plasma clots to the action of fibrinolysin may similarly be due to inhibition of the protease system.

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