JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1979, p. 15-19
0095-1137/79/01-0015/05$02.00/0
Vol. 9, No. 1

Pseudocoagulase Activity of Staphylococci

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Received for publication 19 September 1978

A total of 245 strains of staphylococci isolated from various pathological specimens derived from cases of human infections was tested for staphylocoagulase activity. Test systems employing normal citrated rabbit plasma and the same substrate supplemented with inhibitors of thrombin and proteolytic enzymes (but not influencing the staphylocoagulase activity) were used for testing suspensions of bacteria and cell-free culture supernatants. A total of 237 strains clotted normal rabbit plasma; however, addition of Trasylol and heparin resulted in positive results in 222 strains, whereas plasma supplemented with Trasylol and hirudin was coagulated definitely by only 173 strains. It is postulated that proteolytic enzymes of staphylococci interfere with staphylocoagulase-induced clotting and may simulate coagulase-positive activity of staphylococci. To avoid such false results, a test system for detection of staphylocoagulase should include proteolytic enzyme inhibitors. Possible mechanisms of these findings are discussed.

Staphylocoagulase is a protein produced and excreted extracellularly by human strains of *Staphylococcus aureus*. Therefore, it is generally accepted as a typical feature of these microorganisms, enabling their differentiation from other similar and related bacteria (4, 5, 15, 26). Staphylocoagulase reacts specifically with prothrombin in a stoichiometric process. Both reactants possess no enzymatic activity, but their interaction results in formation of a stable complex of specific proteolytic activity called staphylothrombin (25, 27, 29). It converts fibrinogen, a soluble plasma protein, into insoluble fibrin in a way similar to the action of physiologically formed thrombin. Staphylothrombin differs from thrombin in susceptibility to different inhibitors, being resistant to such natural inhibitors of thrombin as heparin, hirudin, and antithrombin III (16, 25). Staphylothrombin, however, is inhibited by specific antibodies and diisopropylfluorophosphate, an inhibitor of serine proteases (10).

Detection of staphylocoagulase activity in staphylococci is performed routinely by mixing an overnight broth culture of a tested strain with rabbit plasma diluted 1:5 with saline. The plasma serves as a source of prothrombin (coagulase-reacting factor) and fibrinogen. Clotting of plasma is detected after incubation of the mixture at 37°C for 1, 2, 4, 8, and 24 h. This procedure is based on the assumption that coagulation of plasma is caused exclusively by staphylocoagulase (26). However, proenzymes of plasma, such as prothrombin and plasminogen, can be activated in another way. It has been shown that such proteases as trypsin or papain applied in a very low concentration are able to digest prothrombin and/or plasminogen (17, 20, 24). During degradation of plasma proenzymes, they are temporarily converted enzymatically to thrombin or plasmin. This type of activation of the clotting process has been demonstrated recently by us for *Bacteroides melaninogenicus* enzymes (28). It is known that staphylococci produce several enzymes exhibiting proteolytic activity. Several of them have been extensively purified and characterized (1-3, 6, 9, 22). It seemed probable, therefore, that some staphylococcal proteases may simulate, under certain conditions, the clotting effect of staphylocoagulase in the absence of this factor.

The purpose of this investigation was to verify this hypothesis by testing a collection of staphylococcal strains against rabbit plasma in a typical coagulase test versus the same plasma supplemented with specific inhibitors of proteases and thrombin.

MATERIALS AND METHODS

Bacterial strains. A total of 245 staphylococcal strains was isolated from different pathological specimens obtained from patients of Cologne University

Hospitals. The strains were identified by catalase production, anaerobic fermentation of glucose, and other standard methods (13) and stored in a freeze-dried state.

Clotting of plasma. Overnight cultures of staphylococci in heart infusion broth (Difco) were added in parallel in volumes of 0.05 ml to three rows of tubes, each containing 0.5 ml of one of the following: (i) sterile fresh rabbit citrated plasma diluted 1:5 with sterile saline; (ii) plasma containing (in 1 ml) 100 U of aprotinin (Trasylol; Bayer) and 50 U of hirudin (Reanal); and (iii) plasma with 100 U of aprotinin and 20 U of heparin (Polfa). All tubes were incubated at 37°C in a water bath after addition of the inoculum. Clotting of plasma was recorded after 30 min and 1, 2, 4, 6, 8, 18, and 24 h. Results were considered positive when a trace of clot was present. Formation of slight precipitate present at the bottom of the tube was regarded as a weak reaction. To all tubes showing negative results after 24 h of incubation, 0.05 ml of saline containing 6 U of thrombin (Behringwerke) was added. Eventual clotting of plasma was finally recorded after a 30-min incubation at 37°C.

Phage typing. All strains were typed by standard methods and with phages of the international phage set (7).

Antibiotic susceptibility. Sensitivity to 28 antibiotics was investigated as described previously (21).

Fermentation of sugars and acetoin production. Aerobic fermentation of mannitol and maltose was tested by the method of Kloos and Schleifer (15).

Clumping factor. This test was performed exactly as described elsewhere (18).

Bacterial filtrates. A total of 20 selected strains with positive, weak, or negative results of plasma clotting in the presence of inhibitors was cultured in 500-ml portions of heart infusion broth (Difco) at 37°C for 18 h with intensive shaking. The bacteria were sedimented by centrifugation at 10,000 rpm for 30 min at 4°C, and the supernatants were filtered through type 11605 membrane filters of pore size 0.2 nm (Sartorius). They were then stored frozen at −70°C.

Plasma clotting by filtrates. Portions (0.2 ml) of filtrates were added to 0.4-ml samples of the following: (i) sterile fresh rabbit citrated plasma diluted 1:5 with sterile saline; and (ii) plasma containing 6 mM ethylenediaminetetraacetic acid (Serva), 2 mM *N*-ethylmaleimide (Serva), 100 U of heparin (Polfa) per ml, and 100 U of Trasylol (Bayer) per ml. Tubes were inspected continuously for 8 h and then after 17 h of incubation at 37° in a water bath.

Activation of prothrombin. This was performed by incubation of 0.1 ml of filtrates with the same volume of bovine prothrombin solution (20 U) prepared by the method of Malhotra and Carter (19) at 37°C for 30, 90, 150, and 180 min. After this time, 0.4 ml of a 0.2% solution of bovine fibrinogen isolated by the method of Kekwick et al. (14) was added to each tube, and the clotting time was recorded after further incubation at 37°C.

Proteolytic activity. Staphylococcal filtrates were checked for their ability to split casein by the method of Arvidson (2).

Staphylokinase. Detection of this activity was carried out by the method of Brakman and Astrup (8), modified by flooding the plates after 17 h of incubation at 37°C with a 10% solution of perchloric acid (Merck) to detect zones of proteolysis (fibrinolysis).

RESULTS

All except eight tested strains (Table 1) clotted rabbit plasma in 18 h. Addition of Trasylol (inhibitor of plasmin and other proteases) and hirudin (inhibitor of thrombin) markedly decreased the number of clotting-positive strains and increased the number of strains with weak or negative results of the clotting test. Addition to rabbit plasma of the same amount of Trasylol and heparin inhibitory for thrombin action resulted in an increased number of weakly positive, but not negative, results of the clotting test. Thrombin added to all negative samples containing either plasma alone or plasma with inhibitors always produced a strong positive clotting reaction.

Most of the strains producing weak or negative results in the plasma clotting test in the presence of inhibitors required more time to coagulate diluted plasma without added inhibitors than did the strains with coagulating activity unaffected by inhibitors (Table 2). This correlation was highly significant statistically as tested by the chi-square test ($\chi^2 = 33.861$; $P = 0.001$).

All 237 staphylococcal strains coagulating rabbit plasma also produced the clumping factor and acetoin. Most of them anaerobically fermented mannitol and maltose, and only three strains were mannitol or maltose negative. Two strains were simultaneously both mannitol and maltose negative. Two coagulase-negative strains fermented mannitol and maltose. Over 15% of the tested strains belonged to phage group I, 7.8% belonged to group II, 13.2% belonged to group III, and 1.1% belonged to group IV. A total of 22% of the strains were lysed by phages of mixed groups, and 40% of all strains were not typable. They were mostly isolated

TABLE 1. *Clotting of rabbit plasma by 245 staphylococcal strains in the presence of inhibitors of proteolytic enzymes*

Test system	No. of strains clotting plasma in 18 h		
	Positive	Weak	Negative
Rabbit plasma diluted 1:5 with saline	237	0	8
Rabbit plasma diluted 1:5 with saline with Trasylol and hirudin	173	34	38
Rabbit plasma diluted 1:5 with saline with Trasylol and heparin	222	14	9

TABLE 2. *Clotting time of rabbit plasma with inhibitors of proteolytic enzymes added*

Results of clotting test[a]	No. of strains clotting plasma after:							No. of strains with no clot
	1 h	2 h	3 h	6 h	8 h	18 h	24 h	
Negative	0[b]	21	5	4	0	0	0	8
Weak	4	29	1	0	0	0	0	0
Positive	139[b]	34	0	0	0	0	0	0

[a] Rabbit plasma (citrated) was diluted 1:5 with saline plus Trasylol and hirudin.
[b] Difference was statistically significant ($\chi^2 = 33.861$; $P = 0.001$).

from skin lesions (45.5%), but 28.1% were derived from deep purulent lesions, 9.9% were from infected wounds, and the remaining strains were from the respiratory tract. A majority of strains were resistant to penicillin and tetracycline. No correlation between plasma clotting in the presence of inhibitors and sensitivity to antibiotics, origin of strains, or mannitol and/or maltose fermentation was found. The strains with coagulase activity blocked by inhibitors belonged mostly to group II, but not to the mixed phage group. These correlations were also statistically significant ($\chi^2 = 15.956$, $P = 0.001$ and $\chi^3 = 3.832$, $P = 0.05$, respectively).

Filtrates of cultures of 20 staphylococcal strains selected on the basis of their positive, weak, or negative results of clotting in presence of Trasylol and hirudin were also checked for clotting abilities on rabbit plasma alone or plasma supplemented with a mixture of four known inhibitors of different proteolytic enzymes (Table 3). Distinct differences in clotting times between tested filtrates were noted. The 10 most active filtrates clotted plasma in 45 to 90 min (or in the case of strain 51 even in 20 min), whereas four other strains formed a clot after 840 min. As has been demonstrated before with whole staphylococcal cultures, only the fast-coagulating filtrates clotted rabbit plasma containing various inhibitors. Clotting time of plasma with inhibitors increased two or three times compared with results of the test with plasma alone.

All tested filtrates exhibited fibrinolytic activity on fibrin plates. However, they differed in diameters of the lysis zones. Filtrates of strains with coagulating properties unaffected by inhibitors produced smaller zones than those of the strains with completely inhibited coagulase activity. No trace of lysis was found on the plates after heating them for 60 min at 80°C.

Various proteolytic activities were demonstrated on agarose-casein plates. Nearly all filtrates produced zones of proteolysis in three different tests. Only the filtrate of strain 129 was completely inactive. All three proteases (I, II, and III) were found in one filtrate (strain 68), whereas combinations of two of them were detected in seven filtrates. Other filtrates exhibited single proteases.

TABLE 3. *Clotting of rabbit plasma by staphylococcal culture filtrates in the presence of inhibitors of proteolytic enzymes*

Strain no.	Clotting time (min) with:	
	Plasma alone	Plasma + EDTA + NEM + heparin + Trasylol[a]
27	240	—[b]
51	20	75
68	145	—
69	840	—
79	90	—
82	90	130
88	840	—
91	90	—
100	90	160
102	840	—
105	90	130
127	150	—
129	45	160
134	840	—
135	840	—
141	60	130
155	240	—
171	90	130
204	90	—
220	840	—

[a] EDTA, Ethylenediaminetetraacetic acid (6 mM); NEM, *N*-ethylmaleimide (2 mM). The concentration of heparin was 100 U/ml, and that of Trasylol was 100 U/ml.
[b] —, No trace of clotting after 18 h of incubation.

Incubation of the filtrates with bovine prothrombin for various periods of time uniformly caused its activation, resulting in clotting of fibrinogen added thereafter. The clotting time was always inversely related to incubation time, and after 3 h was as short as 2 to 10 min. No distinct differences between filtrates of various strains were found in this type of experiment (Table 4).

DISCUSSION

The present study indicates that the ability to clot plasma can be inhibited in some staphylococcal cultures by substances inactive against

TABLE 4. *Activation of bovine prothrombin by staphylococcal culture filtrates*

Strain no.	Clotting time (min) after addition of bovine fibrinogen to filtrates incubated with prothrombin for:				
	0 min	30 min	90 min	150 min	180 min
27	120	98	29	21	10
51	120	80	30	6	4
68	120	70	21	7	3
69	120	90	20	4	2
79	145	100	25	13	7
82	150	108	20	17	9
88	120	90	22	3	2
91	145	90	35	13	8
100	135	100	25	8	2
102	135	70	28	8	2
105	149	100	25	7	3
127	135	100	28	15	9
129	140	90	35	8	6
134	120	100	28	14	7
135	143	100	35	9	6
141	120	70	25	4	2
155	120	90	25	3	2
171	120	85	20	7	3
204	135	65	32	20	8
220	120	100	35	3	2
Control[a]	>360	>360	270	210	135

[a] Sterile heart infusion broth.

true staphylocoagulase or its substrate but possessing a property of blocking proteolytic enzymes. Moreover, purified bovine prothrombin can be activated by staphylococcal filtrates. This may suggest that some extracellular factors of staphylococci other than staphylocoagulase and which are unable to form staphylothrombin, can exert a clotting effect.

A relatively long plasma clotting time by staphylococcal strains, the staphylocoagulase activity of which is blocked by inhibitors, also indicates the presence of enzymes different from staphylocoagulase, because the dynamics of their production seem to be longer than that of coagulase (which is present in considerable quantities during the first hours of incubation, as demonstrated by Arvidson [2]).

It is possible that diluting the rabbit plasma used for the staphylocoagulase test increases the clotting effect of proteases because of simultaneous dilution of natural protease inhibitors present in fresh plasma.

It seems, therefore, that addition of protease inhibitors to the staphylocoagulase test is a proper way to determine true staphylocoagulase activity and not the clotting produced by proteolytic enzymes of staphylococci.

Arvidson (1, 2), Arvidson et al. (3), Drapeau (9), and Rydén et al. (22) have isolated and characterized several proteases exhibiting different properties from single strains of *S. aureus*. These enzymes possess broader or narrower substrate specificities, and some of them are inhibited by ethylenediaminetetraacetic acid or diisopropylfluorophosphate (1, 22). It is impossible at present to determine which protease plays a role in clotting and fibrinolysis simulating staphylocoagulase and staphylokinase activities. Because it seems very probable that the clotting is an effect of interactions of several staphylococcal enzymes, we have used whole bacterial cultures and a mixture of different protease inhibitors.

The mixture of Trasylol and hirudin exhibited stronger inhibitory properties for nonspecific clotting than did the mixture of Trasylol and heparin. This difference can be explained not only by application of different enzyme concentrations, but also on recent evidence that commercial preparations of hirudin contain, besides their basic enzymatic activity, some inhibitors of various proteases (11). Unfortunately, broad usage of hirudin is limited by its low availability and high price.

It should be stressed that the presence of fibrinogen as a substrate for clotting must be verified in every negative coagulase test by addition of thrombin because, in spite of coagulase activity, fibrinogen partially cleaved by proteases and/or staphylokinase-induced plasmin cannot form a fibrin clot. Application of protease inhibitors in the staphylocoagulase test excludes this danger, as proteolytic enzymes are blocked by the inhibitors and staphylokinase-produced plasmin is inhibited by Trasylol.

The question arises as to whether staphylococcal strains unable to clot rabbit plasma in the presence of inhibitors of proteolytic enzymes can be classified as *S. aureus*. Kloos and Schleifer in their scheme for classifying staphylococci (15) listed a group tentatively named *Staphylococcus* sp. The cocci identified as *Staphylococcus* sp. behave as typical *S. aureus* and clot rabbit plasma, but do not ferment mannitol and maltose or produce acetoin. They have recently been separated from the species *S. aureus* to form a newly proposed species, *Staphylococcus intermedius*, typical for animal coagulase-positive staphylococci (12). Our isolates possessed, in general, all of these three properties, and they are typable by phages of the international phage set. Therefore, they cannot be placed into this group. Also, Schleifer et al. (23) have recently found that coagulase-positive staphylococci form a heterogeneous group. It is possible that human staphylococci lacking true staphylocoagulase activity, although probably as potentially pathogenic as the staphylocoagulase pro-

ducers, form a distinct natural group.

The results of experiments presented in this paper suggest that not just staphylocoagulase can clot rabbit plasma under conditions generally used for detection of this factor. It is very probable that some staphylococcal proteolytic enzymes can, in identical conditions, simulate both staphylocoagulase and staphylokinase by activation of prothrombin and/or plasminogen by limited proteolysis. A more precise test for detection of staphylocoagulase is needed to diminish or eliminate the possibility of obtaining false-positive or false-negative results due to other staphylococcal extracellular products present in cultures of staphylococci.

ACKNOWLEDGMENTS

We thank H. L. Ko for her generous help in performing coagulase tests.

This investigation was supported in part by Public Health Service research grant 05-339-C from the Center for Disease Control. P.B.H. was a Senior Scientist of the Alexander von Humboldt Foundation when these investigations were conducted.

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