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Heat Reactivation of the α-Hemolytic, Dermonecrotic, and Lethal Activities of Crude and Purified Staphylococcal α-Toxin

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ABSTRACT

MANOHAR, M. (University of Minnesota, St. Paul), S. KUMAR, AND R. K. LINDORFER. Heat reactivation of the α-hemolytic, dermonecrotic, and lethal activities of crude and purified staphylococcal α-toxin. J. Bacteriol. 91:1681-1685. 1966.—Crude staphylococcal toxin loses its α-hemolytic activity more rapidly at 60 than at 100 C. This paradoxical behavior has been postulated to be due to the presence of a thermolabile inhibitor in crude toxin. This work provides experimental evidence for the presence of a thermolabile "protective inhibitor." This substance(s) protects the α-toxin against destruction at 60 C, yet simultaneously inhibits the hemolytic activity of α-toxin under the same conditions. Of greater importance, this work also demonstrates that the dermonecrotic and lethal activities of crude toxin are inactivated and reactivated in parallel with the α-hemolytic activity. Crude staphylococcal toxin possessing a high α-hemolytic titer when heated to 60 C for 30 min lost its α-hemolytic, dermonecrotic, and lethal activity. However, when this same toxin was immediately exposed to 100 C, a remarkable simultaneous reactivation of all three of these activities occurred. Contrariwise, electrophoretically purified α-hemolysin, which also possessed dermonecrotic and lethal activity, showed no reactivation under these conditions, thus demonstrating that reactivation is due to a substance(s) distinct from the α-toxin. The fact that α-hemolytic, dermonecrotic, and lethal activities were inactivated at 60 C and simultaneously reactivated at 100 C provides additional proof that these activities are all associated with one toxic component. The probability is remote that three separate entities would exhibit the same rate of inactivation and the same strange reactivation.

As early as 1907, Arrhenius (1) reported on the heat reactivation of staphylococcal hemolysin. He demonstrated that exposure of staphylococcal hemolysin to 60 C for 30 min resulted in the complete inactivation of hemolytic activity. Of greater interest was the fact that additional heating of these samples at 100 C resulted in a remarkable reactivation of hemolytic activity. Landsteiner and von Rauchenbichler (9) concluded from their dilution studies that the "Arrhenius effect" was due to the conjugation of an inhibitor(s) with the hemolysin at 60 C, thereby masking its hemolytic activity. They also postulated that the union between hemolysin and inhibitor was disrupted at higher temperatures. Tager (14) in an intensive study obtained the inhibitor(s) in concentrated form and showed that the activity of the inhibitor itself was impaired by heating to 80 C.

These studies suggested that a relatively thermolabile substance combines with the hemolysin at 60 C and inhibits its activity. At higher temperatures, the inhibitor itself is inactivated, thus releasing active hemolysin, which apparently has a greater stability to heat than the inhibitor.

In the work reported herein, this heat reactivation phenomenon is used to provide additional evidence that the α-hemolytic, dermonecrotic, and lethal activities are three different expressions of one toxic entity.

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If the α-hemolytic, dermonecrotic, and lethal activities of crude staphylococcal toxin are all expressions of one molecule (2, 4–8, 11), it should be possible to demonstrate the heat reactivation of the dermonecrotic and lethal effects in addition to the reactivation of the α-hemolytic activity. The reactivation of these activities would be most apt to occur if all three effects were associated with the same active sites. In addition, the availability of purified α-toxin (7, 8) makes possible a more exact study of the mechanisms involved in the heat reactivation of α-toxin. If the "Arrhenius effect" is due to the presence of an inhibitor(s) which is distinct from α-toxin, purified α-toxin should not show reactivation, since the purification procedure would have removed the inhibitor. This work also reports the presence of a factor in crude toxin which protects α-toxin from destruction by heat at 60 C.

MATERIALS AND METHODS

Toxin preparation. The Wood 46 strain of *Staphylococcus aureus* was used for toxin production. This strain has never produced detectable amounts of β- or Δ-hemolysin. The methods of Leonard and Holm (10) for toxin production were modified in that the agar concentration of the medium was increased to 0.5%, and the cultures were incubated without shaking in an atmosphere containing 50% CO_2 and 50% O_2. After incubation, the crude toxin was extracted from the medium by the saline extraction method of Tasman and van der Slot (15). The cells were removed from the extracted toxin by high-speed centrifugation at 4 C.

Preparation of crude α-antitoxin. Adult New Zealand white rabbits were used to produce crude antitoxin. Each rabbit received a 0.3-ml intradermal injection of crude toxin every 5th day for a total of five injections. Two weeks after the last injection, the animals were bled and the sera were frozen for subsequent use in immunoelectrophoretic studies.

Preparation of crude β-hemolysin. Crude β-hemolysin was prepared in the same way as α-toxin, with two exceptions. *S. aureus* T-19 was used for the production of β-hemolysin, and cultures were incubated at 41 C instead of 37 C.

Purification of α-toxin. A Spinco (model CP) continuous-flow curtain electrophoretic apparatus was used for the purification of α-toxin. An initial fractionation was carried out at *p*H 5.6 by use of lactate buffer, ionic strength of 0.02, 98 ma. The eluate showing peak α-hemolytic activity was dialyzed against distilled water, concentrated by perevaporation, and refractionated by use of Veronal buffer (*p*H 8.6), ionic strength 0.02, and 98 ma. The eluate showing peak α-hemolytic activity was tested for purity with immunoelectrophoretic methods. Previous work (7, 8) has established that this procedure results in an α-toxin preparation with an extremely high degree of purity. After purification, the toxin was dialyzed against saline at 4 C.

Immunoelectrophoresis. Schiedeger's (13) method was used with modification. Noble agar (Difco) was used in a Veronal buffer (*p*H 8.6), ionic strength 0.02. The antigen was allowed to migrate for 2 to 5 hr at 4 ma (six standard microscope slides per experiment). After migration of the antigen, the antitoxin was added and the slides were incubated for 72 hr at room temperature in a humidified chamber. After washing in Veronal buffer (*p*H 8.6), the slides were stained with amido black (10B—color index, 246).

Measurement of α-hemolysin. The activity of α-hemolysin was measured by incubating 0.5 ml of a 2% suspension of washed rabbit red blood cells (washed and suspended in Veronal buffer, *p*H 7.4, containing 0.0015 M Mg^{++}) with 0.5 ml of serial twofold dilutions of each eluate. The tests were incubated for 1 hr at 37 C, followed by 1 hr at room temperature. The highest dilution of toxin showing complete hemolysis was considered to be the end point of the titration.

Measurement of Δ-hemolysin. This activity was measured in the same manner as the α-hemolysin, except that horse erythrocytes were used instead of rabbit erythrocytes.

Measurement of β-hemolysin. The procedure used for α-hemolysin was used, with two exceptions. A 1% suspension of sheep red blood cells was substituted for the 2% suspension of rabbit red blood cells, and the tests were refrigerated at 4 C during the second hour instead of incubating at room temperature.

Measurement of α-antitoxin. An α-hemolysin titration was carried out in the manner outlined above to establish the hemolytic unit. The hemolytic unit was taken as the smallest amount of α-hemolysin causing complete hemolysis. Veronal buffer was used to prepare serial twofold dilutions of α-antitoxin in 0.25-ml volumes. To each of these dilutions 2 hemolytic units of toxin was added in a volume of 0.25 ml. After a 10-min incubation period at 37 C, 0.5 ml of a 2% suspension of washed rabbit erythrocytes was added to each tube. The tests were incubated for 1 hr at 37 C, followed by 1 hr at room temperature. Rabbits whose serum showed no inhibition of α-hemolysis were used in testing for dermonecrotic activity.

Measurement of β-antitoxin. β-Antitoxin was measured in the same manner as α-antitoxin, except that conditions suitable for the measurement of β-hemolysin were used.

Measurement of dermonecrotic activity. Adult New Zealand white rabbits were used for these measurements. The rabbits were pretested, and only animals free from α- and β-antitoxin were used. The degree of dermonecrosis was determined by injecting 0.1 ml of each sample intradermally into each of five rabbits. Two days after injection, the diameter of the lesions was measured, and the average area of dermonecrosis due to each sample was calculated from the measurement.

Lethal activity. White Swiss mice (6 weeks old) were used in these titrations. Four mice were injected intraperitoneally with 0.1 ml of each of the decimal dilutions. Death within 12 hr was considered to be

due to toxin. The LD_{50} was calculated from these results by the method of Reed and Muench (12).

Exposure to heat. Crude or purified α-toxin was divided into several 1-ml samples in rubber-stoppered tubes of uniform size (100 by 12 mm). These samples were placed in a water bath for the indicated times and temperatures. After heat exposure, the tubes were removed and placed in an ice bath until the various activities were measured.

RESULTS

Heat inactivation and reactivation of crude α-toxin. In the first experiment, crude α-toxin with an initial titer of 1:2,048 was used. Eight samples of toxin were placed in a water bath at 60 C. One sample was removed at 10, one at 20, and one at 30 min. The remaining five samples were immediately placed in a water bath at 100 C for the following additional periods of time: 5, 10, 15, 30, and 45 min. After the indicated heat exposures, the samples were immediately placed in an ice bath. The α-hemolytic, dermonecrotic, and lethal activity of each sample was then measured, with the results shown in Table 1 and Fig. 1. An examination of Table 1 shows clearly that, after 30 min of exposure at 60 C, none of the three activities could be detected. However, it may also be seen that, after an additional 5 min of exposure at 100 C, all three of the activities reappeared. The three activities continued to increase until 10 min of exposure and then gradually dropped away until they disappeared after 45 min of exposure at 100 C. Figure 1 shows pictorially the inactivation and reactivation of the dermonecrotic effect of crude toxin.

These results show clearly that these three activities are inactivated and reactivated in parallel.

TABLE 1. *Heat reactivation of the hemolytic, dermonecrotic, and lethal activities of crude staphylococcal α-toxin*

Temp	Time	α-Hemolysin titer*	Avg dermonecrosis in the rabbit	Lethal activity in mice (LD_{50})/ml
C	*min*		*cm²*	
21	0	2,048	7.5	$10^{3.3}$
60	10	32	—†	Negative
60	20	16	—†	Negative
60	30	0	0	Negative
100	5	256	2.3	$10^{1.3}$
100	10	512	3.8	$10^{2.2}$
100	15	256	1.3	$10^{1.0}$
100	30	32	0.01	Negative
100	45	0	—†	Negative

* Reciprocal of titer.
† Not tested.

Heat inactivation of purified α-toxin. The purity of the α-toxin used in these studies is demonstrated by the immunoelectrophoretic results shown in Fig. 2. The crude α-toxin shows five or six lines of precipitate, whereas the purified α-toxin shows only one line. The purity of α-toxin prepared by these methods has previously been demonstrated (7, 8) by ultracentrifugation and double agar diffusion methods.

Five samples of purified α-toxin with an initial titer of 1:512 were placed in a water bath at 60 C. At intervals of 10, 20, and 30 min, a sample was removed. The remaining two samples were immediately transferred to a water bath at 100 C for additional periods of 10 and 20 min. After the heat exposures indicated above, the samples were placed in an ice bath until the three activities were measured. The results of this experiment are shown in Table 2. After 30 min of exposure at 60 C, the three activities were either not detectable or were present in a very low titer. No reactivation of these activities occurred when these samples were placed at 100 C.

Demonstration of a "protective inhibitor" in crude α-toxin. A re-examination of the results presented in Table 1 shows that the crude toxin

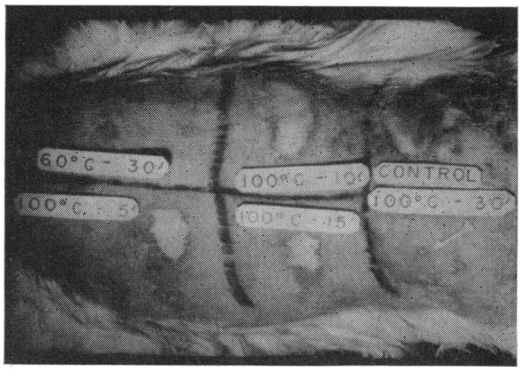

FIG. 1. *Heat inactivation and reactivation of the dermonecrotic effect of crude α-toxin.*

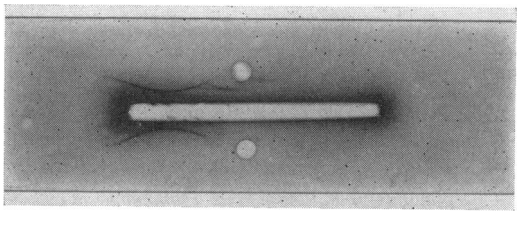

FIG. 2. *Demonstration of purity of α-toxin. The top well contained crude α-toxin; the bottom well, purified α-toxin; the center moat, antitoxin to crude α-toxin.*

used had an original α-hemolytic titer of 1:2048. When crude toxin was heated to 60 C for 30 min and for an additional 10 min at 100 C, the titer was 1:512. This represented a four fold overall drop in titer. However, when purified toxin (Table 2) was subjected to the same heat exposure, the α-hemolytic titer dropped from 1:512 to 1:4, or a 128-fold drop. This suggested that something in crude toxin was protecting α-hemolysin from destruction by heat.

To substantiate these results, a third study was conducted in which the hemolytic titer of the crude and purified toxins was carefully adjusted to the same level. These preparations were then subjected to heat under identical conditions, with the results shown in Table 3. The α-hemolytic activity of crude toxin underwent a total drop from 1:800 to 1:256 (approximately threefold), whereas the α-hemolytic activity of purified toxin dropped from 1:800 to 0 after the same heat exposure. In addition, it is evident that a comparable protective effect was also exerted on the dermonecrotic and lethal activities.

It is also evident from these results that some substance in crude toxin is inhibiting the activities of α-toxin, since the three activities in crude toxin are all lower than the comparable activities in purified toxin after heating for 30 min at 60 C.

DISCUSSION

In this investigation, the heat reactivation of α-toxin was used as a tool to provide additional evidence that the α-hemolytic, dermonecrotic, and lethal activities of crude staphylococcal toxin are all effects of one toxin. The probability that three distinct substances would be inactivated in parallel is low. Further, the probability that these three substances would all simultaneously show the same strange heat reactivation is even more remote.

The validity of the postulations of earlier workers (9, 14) regarding the mechanism of heat inactivation and reactivation of α-hemolysin are substantiated by this work. Their results suggested that a thermolabile conjugating inhibitor was responsible for the paradoxical heat reactivation of α-hemolysin. The results presented in this paper show clearly that the activities of purified α-toxin are not reactivated by heat. It follows, therefore, that heat reactivation is not a function of the α-toxin molecule itself, but is brought about by some substance(s) which was removed by the purification procedure.

This work also demonstrates that the "Arrhenius effect" is due to the presence of a substance(s) which inhibits the activities of α-toxin at 60 C and at the same time protects the α-hemolysin molecule from destruction by heat. The inhibitor accounts for the fact that the activities of crude α-toxin after heating at 60 C for 30 min are consistently lower than those of purified α-toxin under the same conditions. The protective effect is evidenced by the fact that crude α-toxin heated at 100 C for an additional 10 min always has higher activity than purified α-toxin treated in the same way.

These results would indicate that the present explanation of reactivation should be altered to

TABLE 2. *Heat inactivation of the α-hemolytic, dermonecrotic, and lethal activities of purified staphylococcal α-toxin*

Temp	Time	α-Hemolysin titer*	Avg dermonecrosis in the rabbit	Lethal activity in mice (LD_{50}/ml)
C	*min*		*cm²*	
21	0	512	2.7	$10^{1.5}$
60	10	64	0.3	Negative†
60	20	32	0.1	Negative
60	30	8	0	Negative
100	10	4	0	Negative
100	20	0	0	Negative

* Reciprocal of titer.
† Mice ill, no deaths.

TABLE 3. *Effect of "protective inhibitor" on the heat inactivation and reactivation of staphylococcal α-toxin*

Exposure		α-Hemolysin titer (reciprocal)		Dermonecrosis in the rabbit* (cm^2)		Lethal activity in mice (LD_{50}/ml)	
Temp	Time	Crude	Purified	Crude	Purified	Crude	Purified
C	*min*						
21	0	800	800	5.0	4.8	$10^{2.5}$	$10^{2.3}$
60	30	4	64	0	0.8	Negative	$10^{0.6}$
100	10	256	0	2.5	0	$10^{1.3}$	Negative

* Average of three rabbits.

include the presence of a protective factor which apparently is as important as the inhibitory factor in explaining the "Arrhenius effect."

From these results, a working hypothesis has been formulated. This hypothesis holds that a relatively thermolabile substance in crude α-toxin preparations conjugates with α-toxin at 60 C. This substance(s) inhibits the three activities of α-toxin, but at the same time protects the active sites of the α-toxin molecule from destruction by heat. At 100 C, the "protective inhibitor" is itself progressively inactivated, accounting for the gradual return of the activities of the relatively heat-stable α-toxin. The released α-toxin is then gradually inactivated by the higher temperature.

Studies are in progress to determine whether the protective and the inhibitory effects are due to one, or more than one, substance.

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