Inhibition of Staphylococcal α -Toxin

THE EFFECT OF AROMATIC POLYSULPHONIC ACIDS ON THE LETHAL EFFECT OF α -TOXIN IN MICE

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1. Certain aromatic polysulphonic acids, previously tested for inhibition of the haemolytic activity of staphylococcal α -toxin, together with some additional related compounds, were tested as possible inhibitors of α -toxin in mice. 2. Compounds that inhibited the haemolytic activity of α -toxin at concentrations of 0.16 mM or less [compounds (I), (II), (IV), (V), (VII) and (VIII)] were found to inhibit the lethal effect of α -toxin. 3. With the exception of compound (VIII), amounts of 1 mg. were required to inhibit 4 LD₅₀ of toxin when the test compounds were premixed with α -toxin before injection; comparable inhibition with 0.3 mg. of compound (VIII) was achieved without prolonged premixing. 4. Mixtures of α -toxin and compounds (I) and (II) containing an excess of test compound showed markedly diminished inhibitory activities. 5. The 'half-molecule' analogues of group 1 [compounds (III) and (XVIII)] were non-inhibitory. 6. Compounds (I)-(V), when administered separately from α -toxin by the same route (intraperitoneal), were active only when injected almost simultaneously with toxin, whereas compounds (VII) and (VIII) were strikingly inhibitory when injected 15 min. before or after the toxin. 7. Compound (VIII) failed to inhibit the lethal effect of α -toxin when injected by a different route (intravenous).

As reported in the preceding paper (Robson Wright, Arbuthnott & Lominski, 1968), certain aromatic polysulphonic acids were potent inhibitors of the haemolytic activity of crude preparations of staphylococcal α -toxin. Since most of these compounds had low toxicity for experimental animals, it was decided to test them as inhibitors of the lethal action of staphylococcal α -toxin in mice. The main aim of this study was to determine whether those compounds that inhibited α -toxin *in vitro* also inhibited the toxin *in vivo*, and to compare the relationship between chemical structure and inhibitory activity *in vitro* and *in vivo*. Also, a compound having good inhibitory activity in both assay systems may have some practical value, as well as be of use in studying the action of staphylococcal α -toxin.

Inhibition of the lethal effect was first tested by incubating mixtures of test compound and α -toxin before injection. Thereafter inhibitory activity was assessed further by injecting the test compounds at different times either before, or after, α -toxin.

MATERIALS AND METHODS

Crude staphylococcal α -toxin. α -Toxin from *Staphylococcus aureus*, strain Wood 46, was prepared and assayed for haemolytic activity as described by Lominski & Arbuthnott (1962). Such preparations contained 16000 minimal haemolytic doses (M.H.D.)/ml. The LD₅₀ in mice was 64 M.H.D.

Animals. Mice (Porton strain) of both sexes, weighing 25-35 g., were usually used.

Tests for inhibition of the lethal effect of α -toxin. The following methods were used to assess inhibitory activity.

(a) Test compounds dissolved in iso-osmotic phosphate-buffered saline (0.6% NaCl in 50 mM-sodium phosphate buffer), pH 7.0, were allowed to react with an equal volume of a dilution of α -toxin at 37° for up to 60 min. before injection of 0.5 ml. of the mixture intraperitoneally into mice. Since each injection consisted of 0.5 ml., the amounts of α -toxin and of test compound were expressed as the final concentration in 0.5 ml.

(b) Test compounds were injected in 0.25 ml. amounts at different time-intervals before, or after, the injection of the same volume of a dilution of α -toxin. Normally both test compound and α -toxin were injected by the

intraperitoneal route, but in a few experiments the test compound was administered intravenously and α -toxin intraperitoneally.

In both types of experiment groups of six or ten mice were used; for each group the number of survivors was

recorded at regular intervals over a period of 1-5 days. To facilitate the comparison of different groups within a single experiment, mice from the same batch were weighed and distributed so as to make the scatter of weights within any group comparable as far as possible. All groups within

Evans blue

Chlorazol sky blue.

Scheme 1. Compounds tested as possible inhibitors of staphylococcal α -toxin

a single experiment were injected on the same day with the same preparation of toxin. Most experiments were repeated several times. The survival data were analysed statistically by using the fourfold table χ^2 test (Hill, 1942).

Test compounds. Most of the compounds used in the present study were described in the preceding paper (Robson Wright *et al.* 1968) and are referred to by the same compound numbers and chemical group numbers. The structures of the compounds tested are shown in Scheme 1. As examples of group 1, compounds (I), (II), (III), (IV) and (V) were tested for inhibition of the lethal effect. Two additional compounds belonging to group 1 were included [compounds (XVIII) and (XIX)] that could not be tested

in vitro because they formed turbid solutions and thus interfered with extinction measurements. Compound (XVIII), supplied as the insoluble acid, was suspended in 0.15M-sodium phosphate buffer, pH 7.2, and solubilized by the addition of a small volume of 5N-NaOH, the final pH being 7.

Within group 2 most experiments were carried out with compound (VIII), which was available in relatively large amounts; compounds (VII) and (X) were also tested.

The diazo sulphonie acid blue dyes Evans blue (compound XX) and Chlorazol sky blue (compound XXI), structurally related to compounds of groups 1 and 2, were also used.

All of the additional compounds described were kindly supplied by Dr F. L. Rose, Imperial Chemical Industries Ltd., Pharmaceutical Division, Alderley Park, Macclesfield, Cheshire.

RESULTS

Compounds that *in vitro* were good inhibitors of the haemolytic activity of α -toxin (causing a 50% decrease in the maximum rate of haemolysis, $R_{\max.}$, at 0.16 mm or less) also inhibited the lethal effect of the toxin in mice. Though lethality proved to be a less sensitive index of α -toxin activity than haemolysis, certain similarities were found between inhibition of haemolysis and inhibition of the lethal effect.

Premixing of test compound with α -toxin

Results obtained in the haemolysis test indicated that the inhibition of α -toxin by compound (I) increased with increasing time of interaction. The

same was true for the inhibition of the lethal effect (Table 1); incubation of mixtures of 250 minimal haemolytic doses (M.H.D.) of α -toxin (4 LD₅₀) and compound (I) (1 mg./0.5 ml.) for 60 min. at 37° before injection resulted in almost complete inhibition of the lethal effect. The degree of inhibition was less marked when the incubation time was 30 min., and little or no inhibitory activity was found when the incubation time was decreased to 5 min. In subsequent experiments of this type mixtures were therefore incubated for 30–60 min. before injection.

The influence of concentration of test compound on inhibition of the lethal effect is shown for compounds (I) and (II) in Table 2. The injection mixtures, containing 1–5 mg. of test compound and 500 M.H.D. (8 LD₅₀) of α -toxin/0.5 ml., were incubated for 30 min. at 37°. Both compounds showed markedly less activity at high concentrations. Compound (I) gave almost complete inhibition at 1 mg./0.5 ml. and little or no inhibition

Table 1. *Influence of time of incubation on the inhibition of the lethal effect of staphylococcal α -toxin by compound (I)*

The injection mixture contained 1 mg. of compound (I) and 250 M.H.D. (4 LD₅₀) of α -toxin/0.5 ml. The mixture was incubated at 37° for various times before injection of 0.5 ml. intraperitoneally into mice. The control group received an injection of 0.5 ml. of α -toxin without test compound.

Injection mixture	Incubation time (min.) Time after injection	No. of survivors/total no. of mice injected				
		... 4 hr.	6 hr.	8 hr.	24 hr.	5 days
α -Toxin + compound (I)	5	10/10	8/10	5/10	0/10	0/10
α -Toxin + compound (I)	30	10/10	9/10	9/10	7/10	5/10*
α -Toxin + compound (I)	60	10/10	10/10	10/10	10/10	9/10†
α -Toxin alone	60	8/10	2/10	2/10	0/10	0/10

* $P \leq 0.05$.

† $P \leq 0.01$.

Table 2. *Effect of increasing amounts of compounds (I) and (II) on the inhibition of the lethal effect of staphylococcal α -toxin*

Mixtures were incubated for 30 min. at 37° before intraperitoneal injection. The concentration of α -toxin was 500 M.H.D. (8 LD₅₀)/0.5 ml.

Incubation mixture	Concn. of test compound (mg./0.5 ml.) Time after injection ...	No. of survivors/total no. of mice injected	
		7 hr.	4 days
α -Toxin + compound (I)	5	9/10	0/10
α -Toxin + compound (I)	2.5	9/10	5/10*
α -Toxin + compound (I)	1	10/10	9/10†
α -Toxin + compound (II)	5	10/10	1/10
α -Toxin + compound (II)	2.5	8/10	5/10*
α -Toxin + compound (II)	1	7/10	5/10*
α -Toxin alone		0/10	0/10

* $P \leq 0.05$.

† $P \leq 0.01$.

Table 3. *Comparison of analogues of group 1 as inhibitors of the lethal effect of staphylococcal α -toxin*

Mixtures were incubated for 60 min. at 37° before injection of 0.5 ml. intraperitoneally. All compounds were tested at a concentration of 1 mg./0.5 ml.; the concentration of α -toxin was 250 M.H.D. (4 LD₅₀)/0.5 ml.

Injection mixture	No. of survivors/ total no. of mice injected
Time after injection ...	4 days
α -Toxin + compound (II)	10/10†
α -Toxin + compound (III)	0/10
α -Toxin + compound (IV)	8/10†
α -Toxin + compound (V)	7/10†
α -Toxin + compound (XVIII)	0/10
α -Toxin + compound (XIX)	10/10†
α -Toxin alone	0/10

† $P \leq 0.01$.

at a concentration of 5 mg./0.5 ml. Compound (II), though less active, showed a similar pattern of results. To determine whether this effect was due to toxicity of the test compounds themselves, doses of 5, 10 and 15 mg. of compound (I), in the absence of α -toxin, were injected intraperitoneally into mice after incubation of the compounds under the same conditions as used in the inhibition test. No deaths were recorded after 7 days. Thus the unexpected lethal activity of toxin-test compound mixtures containing 5 mg. amounts of test compound cannot be explained in terms of toxicity of the test compound alone. A similar paradoxical effect has been described for mixtures of streptolysin S and Congo red (Rosendal & Bernheimer, 1952).

Several analogues belonging to group 1 were tested for inhibitory activity *in vivo* (Table 3); the concentration of test compound was 1 mg./0.5 ml. and the concentration of α -toxin 250 M.H.D. (4 LD₅₀)/0.5 ml. Compounds (II), (IV), (V) and (XIX) were found to be good inhibitors; by contrast compounds (III) and (XVIII), which can be considered as 'half-molecule' analogues, were inactive. The finding is in reasonably good agreement with the results of inhibition of haemolysis, and supports the suggestion that two aromatic sulphonic acid chains may be necessary for good inhibitory activity of group 1 compounds.

Compounds (XX) and (XXI), structurally related to both groups 1 and 2, were tested for inhibition of the lethal effect of 500 M.H.D. (8 LD₅₀) of α -toxin, at a concentration of 1 mg./0.5 ml. (Table 4). Although differing only slightly in structure, they differed strikingly in activity: compound (XX) caused only a slight increase in time to death whereas compound (XXI) completely prevented death.

Table 4. *Inhibition of the lethal effect of staphylococcal α -toxin by compounds (XX) and (XXI)*

Mixtures were incubated for 60 min. at 37° before intraperitoneal injection. The concentration of test compounds was 1 mg./0.5 ml. and that of α -toxin 500 M.H.D. (8 LD₅₀)/0.5 ml.

Incubation mixture	No. of survivors/ total no. of mice injected
Time after injection ...	3 days
α -Toxin + compound (XX)	0/6
α -Toxin + compound (XXI)	6/6†
α -Toxin alone	0/6

† $P \leq 0.01$.

Table 5. *Inhibition of the lethal effect of staphylococcal α -toxin by compound (VIII)*

Mixtures were incubated for 0 and 60 min. at 37° before intraperitoneal injection. The concentration of compound (VIII) was 0.3 mg./0.5 ml. and that of α -toxin 500 M.H.D. (8 LD₅₀)/0.5 ml.

Time of incubation (min.)	No. of survivors/ total no. of mice injected
Time after injection ...	22 hr.
0	6/6†
60	6/6†
α -Toxin alone	0/6

† $P \leq 0.01$.

The results *in vitro* have indicated (Robson Wright *et al.* 1968) that within group 2 compound (VIII) was the most potent inhibitor of haemolysis. This compound, when premixed with α -toxin *in vivo* was found to be a powerful inhibitor of the lethal effect (Table 5). Even when injected immediately after being mixed with α -toxin it completely inhibited the lethal effect of 500 M.H.D. (8 LD₅₀) at a concentration of 0.3 mg./0.5 ml.

Injection of test compounds at different times before or after α -toxin

To investigate further the inhibitory activity of certain compounds, experiments were carried out in which test compound was injected intraperitoneally at different time-intervals before or after an injection of α -toxin by the same route.

The results of a typical experiment with compound (I) are shown in Table 6; the dose of compound (I) was 1 mg. and the challenging dose of α -toxin was 128 M.H.D. (2 LD₅₀). When injected separately from α -toxin, compound (I) only inhibited the lethal effect when introduced into the peritoneal cavity a few minutes before the toxin.

Table 6. *Effect of injecting compound (I) at various times before or after staphylococcal α -toxin*

The dose of compound (I) was 1.0 mg. injected in 0.25 ml. before or after a dose of 128 M.H.D. (2 LD₅₀) of α -toxin, also in 0.25 ml. Both injections were administered intraperitoneally.

Injection time	No. of survivors/ total no. of mice injected
Time after injection of α -toxin ...	5 days
30 min. before toxin	1/10
4 min. before toxin	8/10†
15 min. after toxin	0/10
30 min. after toxin	0/10
α -Toxin alone	0/10

† $P \leq 0.01$.

Table 7. *Effect of injecting compounds (II), (VIII) and (XXI) 15 min. before α -toxin*

The dose of compounds (II) and (XXI) was 3 mg. and the dose of compound (VIII) was 1 mg.; the dose of α -toxin was 128 M.H.D. (2 LD₅₀); both injections were administered intraperitoneally.

Compound	No. of survivors/ total no. of mice injected
Time after injection of α -toxin.....	2 days
(II)	3/10‡
(VIII)	9/10†
(XXI)	2/10‡
α -Toxin alone	0/6

† $P \leq 0.01$.

‡ Not statistically significant.

Injection either 30 min. before or 15 min. after the challenging dose of α -toxin resulted in no inhibition. With the exception of compounds (II), (VIII) and (XXI) all the compounds tested behaved similarly.

The injection of 1 mg. of compound (VIII) 15 min. before 128 M.H.D. (2 LD₅₀) of α -toxin resulted in almost complete inhibition of the lethal effect (Table 7). Compounds (II) and (XXI) showed a similar trend, but were less active; compound (VIII) also proved to be a good inhibitor of toxic activity when injected after a challenging dose of α -toxin. Indeed, compound (VIII) appeared to be more active when injected 15 min. after than when injected 15 min. before the toxin (Tables 7 and 8). For instance, at a dose of 0.3 mg. administered 15 min. after 250 M.H.D. (4 LD₅₀) compound (VIII) completely inhibited the lethal effect (Table 8); similarly this dose when injected before the toxin was not inhibitory. Compound (VII), a closely related analogue of compound (VIII) and

Table 8. *Effect of injecting compounds (VII) and (VIII) of group 2 15 min. and 30 min. after a dose of staphylococcal α -toxin*

The dose of each test compound was 1 mg. and the dose of α -toxin was 250 M.H.D. (4 LD₅₀); both test compound and toxin were injected intraperitoneally.

Compound	Time injected after α -toxin (min.)	No. of survivors/ total no. of mice injected
	Time after injection of α -toxin ...	22 hr.
(VII)	15	6/6†
	30	3/6‡
(VIII)	15	6/6†
	30	2/6‡
α -Toxin alone		0/8

† $P \leq 0.01$.

‡ Not statistically significant.

a potent inhibitor of α -toxin *in vitro*, tested at a dose of 1 mg., was effective when injected 15 min. after the toxin. Neither compound, however, was as effective when injected 30 min. after the toxin.

These results indicate that compound (VIII) was the most inhibitory compound tested *in vivo*. Attempts were therefore made to test it by injecting the toxin intraperitoneally and the test compound intravenously. Thus far, such experiments have proved negative, and animals so treated died if anything more rapidly than those injected with toxin alone. It may therefore be concluded that even *in vivo* α -toxin and test compound must interact before absorption into the bloodstream, and that such an interaction occurs in the peritoneal cavity when the test compound is introduced at short intervals of time either before, or after, α -toxin.

DISCUSSION

Inhibition of the lethal effect of staphylococcal α -toxin by aromatic polysulphonic acids resembled the inhibition of haemolysis *in vitro* in that in both assay systems the degree of inhibition was time-dependent and increased with increasing time of premixing. This supports the view (Robson Wright *et al.* 1968) that inhibition of α -toxin involves the action of the compound on the toxin itself, rather than on the substrate or receptor of the toxin. The finding that the 'half-molecule' analogues of group 1 [compounds (III) and (XVIII)] were not active *in vivo* suggests that both aromatic sulphonic acid chains are necessary for maximum inhibition. On the other hand, the number of sulphonic acid groups alone does not determine the degree of inhibitory activity, since compound (XIX), having only two

sulphonic acid groups, is as inhibitory as those compounds of group 1 that have six sulphonic acid groups.

Rosendal & Bernheimer (1952) have shown that the diazo sulphonic acid dye Congo red inhibits the haemolytic and lethal effects of streptolysin S, a cytolytic toxin of similar general properties to staphylococcal α -toxin. The effect of compounds (I) and (II) on staphylococcal α -toxin also resembles the action of Congo red on streptolysin S in that injection of mixtures of toxin and drug containing an excess of drug was unexpectedly lethal. Unlike the compounds tested in the present study, Congo red inhibited streptolysin S only when premixed with toxin before injection; separate injection of streptolysin S, even at a 1 min. interval, failed to inhibit the toxin *in vivo*. By contrast, compounds of group 1 inhibited staphylococcal α -toxin when injected 4 min. before toxin, and compounds (VII) and (VIII) of group 2 were effective when the time-interval was increased to 15 min. This apparent difference between the inhibition of streptolysin S and staphylococcal α -toxin *in vivo* may be explained partly by the fact that Congo red and streptolysin S were injected intravenously, whereas in the present study the intraperitoneal route was employed.

It is known that compound (I) (suramin) binds to plasma proteins (Spinks, 1948; Town, Wills, Wilson & Wormall, 1950). Thus it appears likely that group 1 compounds when injected separately from α -toxin, even by the same route, become rapidly bound to body protein and rendered unavailable for interaction with the toxin protein. Unlike group 1 compounds, compounds (VII) and

(VIII) of group 2 inhibited the lethal effect of α -toxin when administered by separate injection 15 min. before or after 4 LD₅₀ of α -toxin; extension of the time-interval to 30 min., however, resulted in no statistically significant inhibition. The relatively greater ability of compounds (VII) and (VIII) to abolish the lethal action of α -toxin without prior interaction *in vitro* suggests that they may be less readily bound or otherwise made unavailable *in vivo* than group 1 compounds. However, attempts to prevent death by injecting the toxin and compound (VIII) by different routes have so far failed. It is nevertheless hoped that the present study may provide a basis for the design of compounds of higher affinity for the toxin protein rather than for the proteins of the host.

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