Brit. J. Pharmacol. (1961), 17, 159–166.

A COMPARATIVE STUDY OF STAPHYLOCOCCUS HAEMOLYSINS AND COLISAN

BY

F. BERGMANN, S. LEON, M. CHAIMOVITZ AND F. BENZAKEIN

From the Department of Pharmacology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

(*Received April* 6, 1961)

Staphylococcus β-haemolysin exhibits the same broad spectrum of antagonistic activity against smooth muscle stimulants as described recently for Colisan. The antispasmodic effect develops slowly and can be removed only by repeated washing. The pattern of *haemolytic* activities of Colisan is characteristically different from that of β-haemolysin, but resembles closely α-toxin. Both staphylococcus toxins can destroy paramecium and entamoeba, but the ratio of the titres at which they kill paramecium and entamoeba respectively (P:A value) is different. The P:A value of β-haemolysin is similar to that of Colisan.

In a recent publication, the spasmolytic effect of a bacterial extract (" Colisan "), prepared from a new mutant (RB-103) with antibiotic activities against certain bacteria and protozoa, was described (Bergmann, Reitler, Chaimovitz & Bergmann, 1960). It was then mentioned that the only other bacterial principle with similar pharmacological properties appeared to be staphylococcus β-haemolysin (Anderson, James & Marks, 1954; Kelsey & Hobbs, 1954). A more detailed study of this toxin was therefore undertaken, which revealed antagonistic activities against various smooth muscle stimulants, very similar to those observed with Colisan. This resemblance led us to search also for possible antiprotozoal effects of the β-haemolysin, analogous to those of Colisan.

METHODS

Staphylococcus α- and β-haemolysins. The commercial products of the Wellcome Research Laboratories, Beckenham, containing respectively 4.3 and 0.08 international units (i.u.) per mg, were used. They contained varying amounts of protein, originating from the culture medium. Throughout the terms staphylococcus haemolysins and toxins are used synonymously.

An extract of the mutant RB-103 (Colisan) was obtained through the courtesy of Hillel Remedies Inc., Haifa, Israel.

Haemolytic titre. (a) For β-haemolysin, the short " hot-cold " method of Jackson & Mayman (1958) was used with the following modified buffer: sodium chloride, 8.0 g; potassium chloride, 0.2 g; disodium hydrogen phosphate, 1.15 g; potassium dihydrogen phosphate, 0.2 g, dissolved in 800 ml. of water, were mixed with calcium chloride, 0.1 g in 100 ml., and magnesium chloride hexahydrate, 0.1 g in 100 ml., to give 1 litre of buffer of *p*H 7.0. Sheep erythrocytes, washed thrice with 0.9% sodium chloride solution, adjusted to *p*H 7.0, were suspended in this medium to give a final concentration of 1% by volume. A suitable amount of haemolysin was added and the mixture was incubated first at 37° C for

25 min and then at +4° C for 15 min. The cells were separated by centrifugation at 2,500 rev/min and the supernatant fluid examined with a Klett-Summerson photoelectric colorimeter, using filter no. 54. The same suspension without haemolysin served as blank. Percentage haemolysis was calculated by comparison with a solution, obtained by complete haemolysis of a 1% suspension of sheep erythrocytes in distilled water. Between 20 and 80% haemolysis, a linear dependence on haemolysin concentration was observed. From the appropriate plot, the concentration producing 50% haemolysis ($=H_{50}$) was evaluated as 5.3×10^{-3} i.u./ml. (b) The activity of α-haemolysin was determined on rabbit erythrocytes, according to Jackson & Little (1957). The H_{50} value, defined as above, was equivalent to 0.27 i.u./ml. (see Fig. 4a).

Amoebicidal titre (=A-titre). Strain no. 17 of *Entamoeba histolytica,* obtained through the courtesy of Professor S. Adler, Department of Parasitology, was grown in the following medium:

(a) Bacto-Peptone (Difco), 10 g; Bovril (beef extract), 3 g; sodium chloride, 5 g; disodium hydrogen phosphate, 2.5 g; water to 1,000 ml. The *p*H of this mixture is usually near 7.0. The solution was boiled to dissolve all components, filtered and sterilized at 15 lb. pressure for 30 min.

(b) Fresh beef blood was kept in the refrigerator for 24 hr and then filtered through gauze. The filtrate was centrifuged in a cold room for 15 min at 10,000 rev/min and the supernatant passed through a Seitz filter into a sterile vessel. It was then incubated at 60° C for 30 min and subsequently stored in a refrigerator.

Solutions (a) and (b) were mixed in a ratio of 10:1. To this mixture were added a few grains of sterile rice starch and a suitable size of inoculum, containing trophozoites from a 2 to 4 days' old culture (usually 0.1 to 0.2 ml. of inoculum/5 ml. of culture medium). Drugs were added simultaneously with the inoculum.

Amoebicidal tests give widely varying results if the conditions of the experiment, the composition of the medium or the strain are varied. The figures reported in this paper were obtained under strictly identical conditions; however, activity of an extract is only of value for comparison with other biological effects of extracts from the same organism.

Microscopical examinations were performed daily. When no trophozoites could be detected, a subculture was made after 48 hr. The subculture again underwent daily examination and, if no trophozoites could be found, a second subculture was initiated after an additional 48 hr. Only if the second subculture remained negative during 2 days (that is, after a total observation time of 6 days) was the absence of trophozoites assumed to be established.

Strain 17 of *Entamoeba histolytica,* which was isolated originally from the faeces of a patient, contained a single species of gram-negative rod. Therefore it appeared of importance to ascertain that the amoebicidal action affected the protozoal cells directly and did not act through an antibacterial effect. This was done in two ways: (a) After the trophozoites had been exterminated, the bacteria could easily be subcultured on blood agar. (b) Direct application of amoebicidal concentrations of Colisan to the bacteria, growing in the medium used for the amoebicidal tests, but lacking starch, revealed only slight inhibitory activity.

These results cannot be considered as unambiguous proof of a direct amoebicidal action, as slight interference with bacterial growth may be sufficient to prevent development of the amoebae and thus to cause their extermination. In a future paper we shall give new evidence for the direct amoebicidal activity of Colisan.

Lethal action against Paramecium caudatum (=P-titre). A hay infusion (1 g of hay in 200 ml. of tap water) was sterilized at 15 lb. pressure for 30 min and then inoculated with 5 vol. % of a paramecium culture, about 4 days of age. Microscopical examination at low magnification was performed after 3, 24 and 48 hr. The test substances were added in solid form or dissolved in sterile tap water. As with entamoeba, the minimum lethal dose was used for comparison of activities.

Tests on guinea-pig or rabbit ileum. The method used previously (Bergmann *et al.,* 1960) was again applied. The intestinal loops were incubated with the haemolysin for 7 min before

a smooth muscle stimulant was added, as this is the minimum interval to give the maximal antagonistic effect. The concentration of inhibitor, reducing the height of contraction by 50%, was determined for each stimulant. Between subsequent applications of a stimulant, 3 washings with Locke solution were performed in the course of 3 min.

RESULTS

Antagonism between β-haemolysin and various smooth muscle stimulants. It has been reported by Anderson, James & Marks (1954) and by Kelsey & Hobbs (1954) that staphylococcus β-toxin paralyses the spontaneous peristalsis of rabbit intestine, after an initial contraction. β-Haemolysin likewise blocked the effect of acetylcholine. Our own tests were carried out with guinea-pig ileum. Here again, the preparation of β-haemolysin at our disposal caused first a strong contraction, followed by a gradual relaxation. The initial contraction may be due to the presence of peptone or other components from the culture medium. A decisive answer can only be given when chemically pure β-haemolysin is available. On the other hand, contamination of the β-toxin by α-toxin is improbable, in view of the practically negligible action of the preparation on rabbit erythrocytes (see Table 2).

After 7 to 8 min incubation with β-haemolysin, the effect of smooth muscle stimulants was reduced to various degrees. β-Haemolysin was antagonistic to all stimulants tested (acetylcholine, 5-hydroxytryptamine, histamine, nicotine and barium chloride) (Figs. 1 and 2).

Only after the first washing did the full inhibitory activity of the toxin become apparent. This—partial or complete—block could be removed only slowly by repeated washings, recovery taking about 24 to 40 min. A similar behaviour was observed with rabbit intestine.

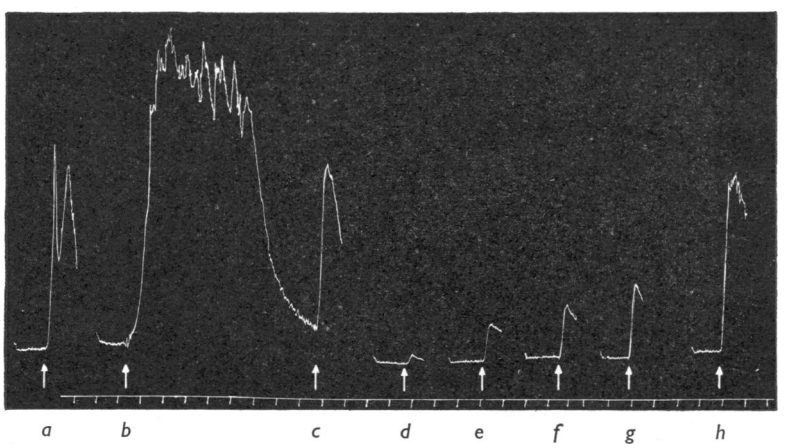

Fig. 1. Antagonism of β-haemolysin and 5-hydroxytryptamine on the isolated guinea-pig ileum. The intestine was stored in the refrigerator for 24 hr prior to use. The standard test dose of 5-hydroxytryptamine, used throughout, was 0.3 μg/ml. At a, 5-hydroxytryptamine; b, β-haemolysin, 0.08 i.u./ml.; c, 8 min later, without washing, 5-hydroxytryptamine was added. The tests with 5-hydroxytryptamine were repeated from d to h. Time intervals: c to d, 3 min; d to e, 4 min; e to f and f to g, 5 min; g to h, 10 min.

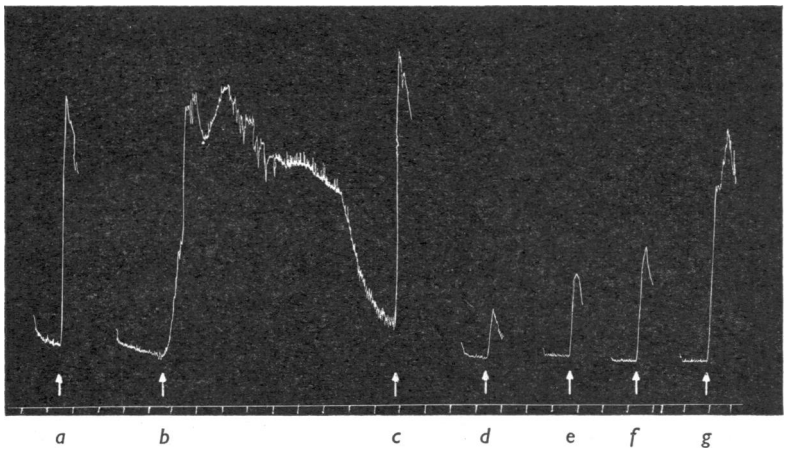

Fig. 2. Suppression of barium-chloride-induced contractions of the guinea-pig ileum by β-haemolysin. The intestine was stored, prior to use, in the refrigerator during 24 hr. The standard dose of barium chloride was 40 μg/ml. At a, barium chloride; b, β-haemolysin, 0.08 i.u./ml.; c, 9 min later, without washing, barium chloride was added; tests with barium chloride were repeated from d to g. Time intervals: c to d, 3 min; d to e and e to f, 4 min; f to g, 10 min.

Fig. 3 demonstrates the similarity of the time course of the inhibition for all stimulants tested. In Table 1 the relative inhibitory effect of β-haemolysin against the 5 stimulants has been calculated from experiments in which the same concentration of toxin was used. β-Haemolysin appears to antagonize 5-hydroxytryptamine and nicotine more effectively than acetylcholine.

TABLE 1

RELATIVE INHIBITORY ACTIVITY OF β-HAEMOLYSIN AGAINST VARIOUS SMOOTH MUSCLE STIMULANTS

All experiments were carried out with 0.1 i.u./ml. of β-toxin, on guinea-pig ileum, stored in the refrigerator for 24 hr. The stimulants were applied in equiactive doses

Stimulant	Relative inhibitory activity of β-haemolysin
Acetylcholine	1
Histamine	0·85
5-Hydroxytryptamine	1·35
Nicotine	1·4
Barium chloride	1·15

As with Colisan, application of a stimulant in amounts 10 to 20 times in excess of the standard dose provoked a contraction even at the height of the inhibition, thus demonstrating its competitive character.

For an understanding of the fact that full inhibition by β-haemolysin appears only after the first washing, it should be recalled that an immediate stimulant effect is observed when peptone alone is added to the organ bath, and that prompt recovery ensues when the bath fluid is replaced by fresh Locke solution. In contrast, α-haemolysin adheres to the intestine and can be removed only slowly (see Discussion).

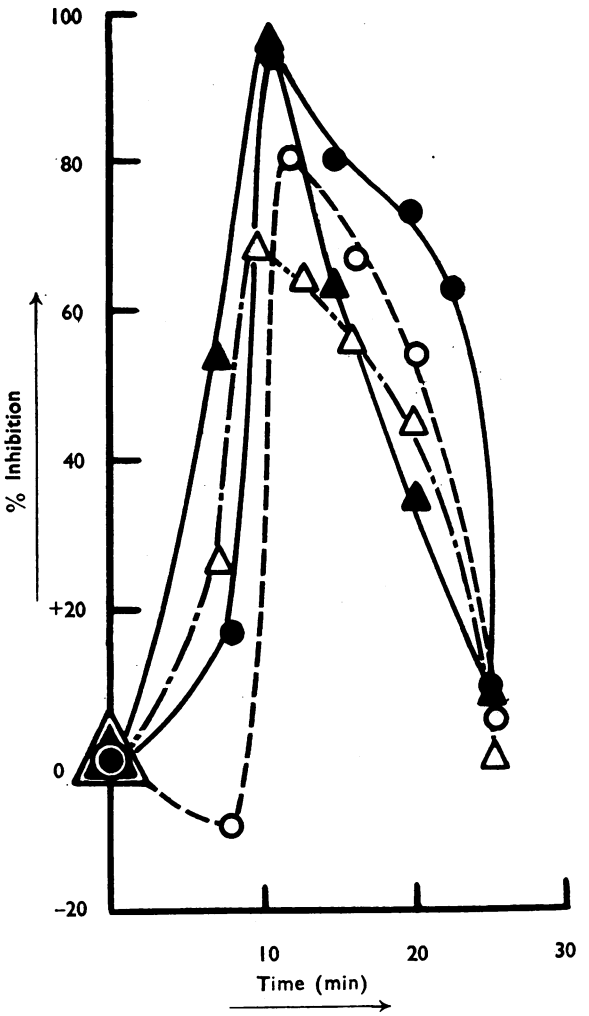

Fig. 3. Time course of inhibition of smooth muscle stimulants by β-haemolysin. In these experiments, 0.1 i.u./ml. of β-haemolysin and equiactive concentrations of the stimulants were used. The intestine was stored for 24 hr in the refrigerator before use. Ordinate: % inhibition. Abscissa: time in min. ●——●, 5-hydroxytryptamine. ▲——▲, nicotine. △——△, histamine. ○——○, barium chloride.

Comparison of the haemolytic activity of Colisan and staphylococcus haemolysins. The similarity of action of β-haemolysin and Colisan on smooth muscle suggested that these two bacterial principles may resemble each other also in other biological actions. β-Haemolysin is characterized by its inertness against rabbit erythrocytes, but it is highly active against sheep red blood corpuscles if interaction at 37° C is followed by cold incubation. However, Colisan revealed a different pattern of activity: it haemolyses sheep erythrocytes with about one quarter its efficiency

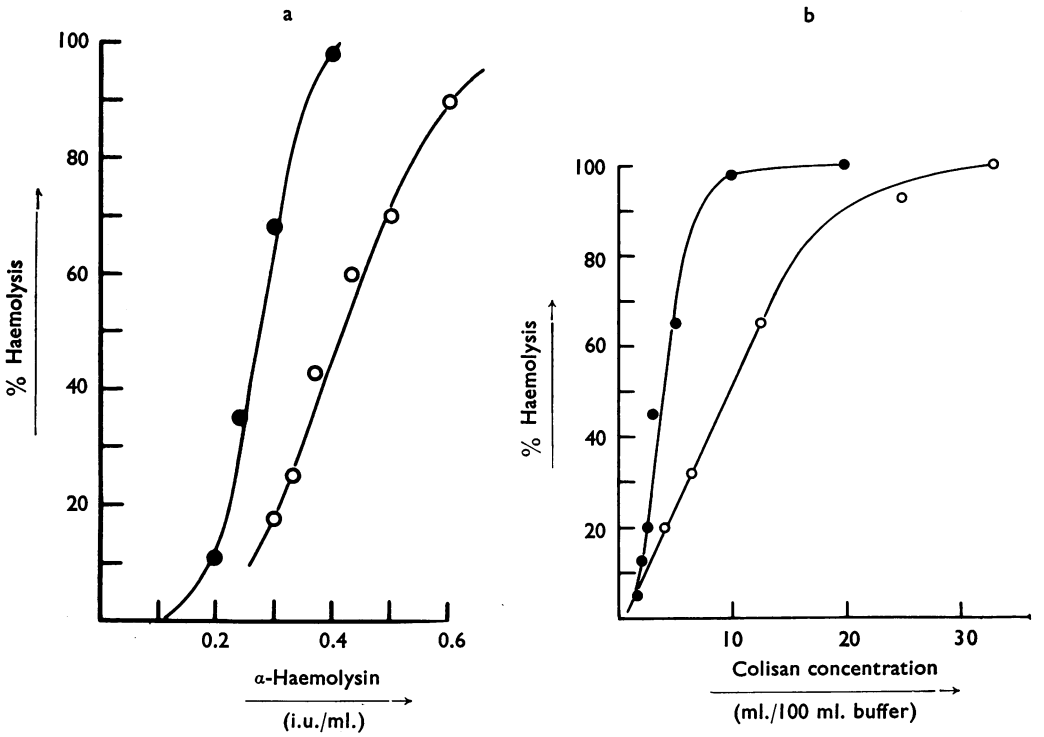

Fig. 4. Haemolytic activity against rabbit (● —— ●) and sheep (○ —— ○) erythrocytes. Incubation for 15 min at 37°. (a) Ordinate: % haemolysis; abscissa: α-haemolysin (i.u./ml.). (b) Ordinate: % haemolysis; abscissa: Colisan. From the central linear portions of these curves, the H_{50} values of Table 2 were derived.

against rabbit's cells, but the haemolytic action is not enhanced at all by subsequent cold treatment. In this respect, Colisan resembles staphylococcus α-toxin. Comparison of Colisan and α-toxin (Fig. 4) reveals indeed that in both cases the

TABLE 2

RELATIVE ACTIVITIES OF STAPHYLOCOCCUS α- AND β-HAEMOLYSIN AND OF COLISAN IN VARIOUS BIOLOGICAL TESTS

Because the bacterial extracts used do not represent pure substances, all results were expressed as % of the paramecicidal activity of the same material

Activity measured	Colisan	β-Haemolysin	α-Haemolysin
1. Lysis of *Paramecium caudatum*	100	100	100
2. Amoebicidal action against *Entamoeba histolytica*	5–10	4	25
3. H_{50}, rabbit erythrocytes	2–4	0	5,500
H_{50}, sheep erythrocytes	0·5–1·0	800	3,750
4. Antagonistic action against acetylcholine-induced contractions of guinea-pig ileum	50	50	10,000

dose–activity curve for haemolysis of rabbit cells exhibits a steeper slope than the corresponding plot for sheep erythrocytes. Likewise, the ratio of H_{50} for the two haemolytic effects is of the same magnitude (see Table 2).

Activity of haemolysins against protozoa. The above finding induced us to search for antiprotozoal activity among the staphylococcus toxins. β-Haemolysin kills both paramecium and entamoeba, the ratio of P:A titre being similar to that of Colisan. α-Toxin exhibits only weak antiprotozoal properties, but the ratio P:A (=4) is significantly lower than for β-toxin or Colisan (see Table 2). It is therefore improbable that the slight antiprotozoal activity of α-toxin is due to contamination with small amounts of β-haemolysin.

DISCUSSION

The experiments reported demonstrate the close similarity of action of Colisan and β-haemolysin on smooth muscle cells. The broad antagonistic spectrum of these two bacterial principles against smooth muscle stimulants supports the view that both act directly on the contractile cell. Both combine slowly with their substrate and can be removed only by prolonged washing. These features clearly distinguish β-haemolysin and Colisan from α-toxin. The latter attaches itself very quickly to smooth muscle, as contact for 30 to 60 sec is sufficient to produce the characteristic spastic contraction which passes 30 to 60 min later into a state of atony (Thal & Egner, 1961). The attachment of α-haemolysin is irreversible (Brown, Prichard & Quilliam, 1959).

On the other hand, the haemolytic properties of Colisan resemble those of α-toxin and differ from those of β-haemolysin. These differences may indicate that each bacterial extract contains a mixture of active substances which are responsible for the different biological effects. But it appears also possible that only a single active principle is present. In this case one may assume that structural differences of the active materials determine their specific mode of combination with various receptors. Only fractionation experiments will supply information on this point.

The cytocidal effect of both haemolysins and of Colisan against paramecium is much stronger than against entamoeba. This difference may—at least in part—be due to the fact that the culture medium for paramecium is essentially free from polymeric substances which could adsorb the active principles. Indeed, we have observed that the P:A value can be raised by using semisolid media for growing entamoeba instead of the liquid substrate described.

The combination of lytic effects on single cells with actions on smooth muscle organ poses the question whether a single mechanism may be responsible for these seemingly unrelated biological actions. The spasmolytic effect of Colisan and β-haemolysin develops slowly. About the same incubation period is required to immobilize paramecium by extracts with a high P-titre. It thus appears possible that in both actions a slow change of membrane permeability may take place. It should be recalled that Thal & Egner (1961) also concluded from their observations on α-toxin that it probably attacks the cell membrane directly. The present finding that protozoal cells undergo lysis, when treated with staphylococcal haemolysins,

opens a new experimental approach to the problem of the mechanism of action involved.

This work is taken in part from a Ph.D. thesis of S. L., submitted to the Faculty of Science, The Hebrew University, Jerusalem, Israel. This research was supported by the B. de Rothschild Foundation for the Advancement of Science in Israel.

REFERENCES

ANDERSON, K., JAMES, D. M. & MARKS, J. (1954). The action of staphylococcal toxins on isolated rabbit intestine. *J. Hyg. (Lond.)*, **52**, 492–501.

BERGMANN, F., REITLER, R., CHAIMOVITZ, M. & BERGMANN, D. (1960). Pharmacological study of a new antibiotic of bacillary origin. *Brit. J. Pharmacol.*, **15**, 313–318.

BROWN, D. A., PRICHARD, B. N. C. & QUILLIAM, J. P. (1959). Some pharmacological properties of the α-toxin of *Staphylococcus pyogenes*. *Brit. J. Pharmacol.*, **14**, 59–67.

JACKSON, A. W. & LITTLE, R. M. (1957). Staphylococcal toxins. I, Factors affecting the hemolytic activity of alpha toxin. *Canad. J. Microbiol.*, 3, 47–54.

JACKSON, A. W. & MAYMAN, D. (1958). Staphylococcal toxins. IV, Factors affecting hemolysis by β-lysin. *Canad. J. Microbiol.*, **4**, 477–486.

KELSEY, J. C. & HOBBS, B. C. (1954). Studies on the effect of staphylococcal culture filtrates on isolated rabbit gut. *J. Hyg. (Lond.)*, **52**, 502–509.

THAL, A. P. & EGNER, W. (1961). The site of action of the staphylococcus α-toxin. *J. exp. Med.*, **113**, 67–81.