The Three Factors of Anthrax Toxin: their Immunogenicity and Lack of Demonstrable Enzymic Activity

BY J. L. STANLEY AND H. SMITH

Microbiological Research Establishment, Porton, Salisbury, Wiltshire

(Received 27 September 1962)

SUMMARY

A lethal mixture of the three factors (I, II, III) of anthrax toxin had none of the following enzyme activities: adenosine triphosphatase, alkaline or acid phosphatase, catalase, collagenase, ribonuclease, desoxyribonuclease, gelatinase, hyaluronidase, lecithinase, lipase, proteinase. The mode of action of anthrax toxin is unknown. Although factor II is the only factor which is immunogenic when injected alone, the addition of factor I results in an increased immunizing activity; however, the activity of this mixture can be decreased by adding factor III. The possible effect of these results on preparations used to immunize against anthrax is discussed.

INTRODUCTION

The specific, lethal and oedema-forming toxin of *Bacillus anthracis* (Smith, Keppie & Stanley, 1955; Harris-Smith, Smith & Keppie, 1958; Thorne, Molnar & Strange, 1960) consists of three components (Smith *et al.* 1956; Thorne *et al.* 1960; Stanley, Sargeant & Smith, 1960; Sargeant, Stanley & Smith, 1960; Stanley & Smith, 1961; Smith & Stanley, 1962). The three factors (I, II, III) have been purified. The final preparation of factor I appeared to be a single serological component but factors II and III were serologically heterogeneous. The three factors were neither lethal nor oedema-producing when injected alone. When mixed together, all three acted synergistically in lethality tests in mice, but factor III inhibited to some extent the otherwise large oedema-producing activity of mixtures of factors I and II; a summary of the relationship between these factors in toxicity tests was given in Table 8 of Stanley & Smith (1961). The present paper describes the failure to show in mixtures of the three purified factors any significant enzymic activity which might be the basis for their toxicity. It also describes the ability of these factors and mixtures of them to immunize guinea pigs against infection with *B. anthracis*, and summarizes attempts to separate the major serological component of purified factor II (the main immunogen).

METHODS

Analytical methods (including ultracentrifugation and paper electrophoresis). These were described by Stanley & Smith (1961).

Purified factors I and III of the anthrax toxin. These were prepared as described by Stanley & Smith (1961) and Smith & Stanley (1962).

Purified factor II of the anthrax toxin. This was prepared from filtrates of cultures

containing the toxin of *Bacillus anthracis* (Thorne *et al.* 1960) by two methods. (a) By the method of Strange & Thorne (1958) which involves ammonium sulphate precipitation, precipitation at pH 4.2 and chromatography on an alumina C_γ-Celite column. The properties of this immunogenic material were described by Strange & Thorne (1958) and Sargeant *et al.* (1960); it appeared to contain two serological components B and C. (b) By fractionation on diethylaminoethyl (DEAE) cellulose. Culture filtrate (4 l.) was dialysed (18 hr.) against 0.01 *I* phosphate buffer (pH 7.4) and applied to a column (4.5 cm. diam.; 4 cm. long) of DEAE cellulose (5 g.) which had been equilibrated with 0.1 *I* phosphate buffer (pH 7.4). No pressure was applied and the flow rate was about 30 ml./min. The column was eluted with: (1) 0.1 *I* phosphate buffer (60 ml.; pH 7.4); (2) 0.15 *I* phosphate buffer (90 ml.; pH 7.4) and (3) 0.2 *I* phosphate buffer (60 ml.; pH 7.4). Factor II (60 ml., 0.04 % protein) was eluted by buffer (2). The final preparation was concentrated by dialysis against Carbowax (20 M) and showed no evidence for heterogeneity in the ultracentrifuge (0.5 % (w/v) dialysed against 0.05 *I* phosphate buffer (pH 7.4)) and on paper electrophoresis (1 mg. examined in 0.2 *I* barbitone buffer, pH 8.6)). After exhaustive dialysis against water the freeze-dried product had N 14.2 % (cf. Strange & Thorne, 1958). This preparation of factor II was indistinguishable from the preparation described above in: (1) the assay for factor II activity (Stanley *et al.* 1960), (2) gel-diffusion against 'spore' and 'antigen' antisera which indicated the presence of two serological components (Sargeant *et al.* 1960) and (3) tests for immunogenicity (Smith & Gallop, 1956).

Serological precipitation in gels. The methods and antisera ('spore' H533 and 'antigen' H25) used were described by Sargeant *et al.* (1960).

Test for immunogenicity. Guinea pigs were immunized as described by Smith & Gallop (1956); the total amount of preparation given in the three equal doses of the immunization course has been quoted in the tables below together with the number of animals surviving subsequent challenge with 1000 lethal doses (LD) of *Bacillus anthracis*, strain N.P., spores. In many experiments, no unvaccinated control animals survived this challenge.

Tests for enzymic activity

A mixture of purified factor I (4 μ g.), purified factor II (40 μ g.) and purified factor III (9 μ g.) was examined in the following tests for enzyme activity, in each of which a known active enzyme preparation was included for comparison.

Adenosine triphosphatase. By the method of Kielley & Meyerhof (1950) with adenosine triphosphate (Boehringer & Soehne, Mannheim) as substrate and the enzyme of red cells for comparison.

Acid and alkaline phosphatase. By the method of King (1951) with disodium phenylphosphate (British Drug Houses Ltd.) as substrate and the phosphatases of red cells and serum for comparison.

Catalase. This was detected by its ability to decompose hydrogen peroxide; crystalline catalase (Boehringer & Soehne, Mannheim) was used for comparison.

Collagenase. By the method of Oakley *et al.* (1946) with hide powder coupled with azocoll as substrate and the filtrate of a 72 hr. culture of *Bacillus cereus* for comparison.

Desoxyribonuclease. By the method of Kunitz (1950) with desoxyribonucleic acid

(prepared from calf thymus by our colleague S. Lovett) as substrate and a sample of desoxyribonuclease (Light and Co., Ltd.) for comparison.

Ribonuclease. By the method of Kunitz (1946) with a sample of ribonucleic acid (Boehringer & Soehne, Mannheim) as substrate and a preparation of ribonuclease (Light and Co., Ltd.) for comparison.

Gelatinase. By the method of Strange & Thorne (1958) on X-ray film, with a filtrate of a 72-hr. culture of *Bacillus cereus* for comparison.

Hyaluronidase. By the method of Alburn & Whitley (1951) with potassium hyaluronate (Allen & Hanbury, Ltd.) as substrate and hyaluronidase (Evans Medical, Ltd.) for comparison.

Lecithinase. By the method of Macfarlane & Knight (1941) with egg yolk media as the substrate and filtrate from a 72-hr. culture of *Bacillus cereus* for comparison.

Lipase. By the method of Bier (1955) with Tween 20 as the substrate and a sample of lipase (Light and Co., Ltd.) for comparison.

Proteinase. By the method of Kunitz (1947) with Bacto isoelectric casein (Difco Laboratories) as substrate and crystalline trypsin (Armour Laboratories) for comparison.

RESULTS

Lack of demonstrable enzymic activity in mixtures of factors I, II and III of the anthrax toxin

In tests, in which known enzyme preparations were active, a mixture of factors I, II and III of anthrax toxin had no adenosine triphosphatase, phosphatase (acid or alkaline), catalase, collagenase, desoxyribonuclease, gelatinase, hyaluronidase, lecithinase, lipase, proteinase or ribonuclease activity. Hence we have not identified any enzymic basis for the toxic activity of these factors. A mixture of factors I and II obviously increased capillary permeability since it produced oedema when injected intradermally. Addition of factor III decreased the local oedema production by a mixture of factors I and II (Stanley & Smith, 1961). It seemed possible that factor III was a spreading factor leading to a quick dispersal of factors I and II, hence to a diminution in their local effect. However, no evidence for the presence of a spreading factor (by the method of Hechter, 1947) or of a permeability increasing substance (by the method of Miles & Wilhelm, 1955) was obtained when factor III was examined in rabbits.

Immunogenicity for guinea pigs of mixtures of factors I, II and III of anthrax toxin

When the three factors were injected singly, only factor II protected guinea pigs against subsequent infection with *Bacillus anthracis* (Tables 1, 2). However, the addition of factors I and III to factor II produced some interesting results. These mixtures were not given in sufficient strength to kill any guinea pigs and since all vaccinations were done subcutaneously there were no overt signs of oedema production. The results in Tables 1 and 2, which were analysed by our colleague Mr S. Peto, show that addition of factor I to factor II increased the immunizing power of the latter, but that addition of factor III to a mixture of factor I and II decreased their combined activity. On the other hand, addition of factor III to factor II did not seem to affect the immunizing activity of the latter (Table 2).

It was also possible that a mixture of factors I and III had some immunizing activity (Table 2).

Table 1. *Effect of factor I of anthrax toxin on the immunogenicity of factor II*

This table includes the results of five experiments; sections *A*, *B* and *C* include the results of two similar experiments and sections *D* and *E* the results of five similar experiments.

Antigens injected* (μ g.)		Immunizing activity in guinea pigs.	
Factor I	Factor II	No. of survivors No. injected	= %
<i>A</i> 120	—	0 15	Nil
	30	0 25	Nil
	10	0 25	Nil
	3	0 25	Nil
<i>B</i> —	600	14 25	56
	30	16 25	64
<i>C</i> —	200	10 25	40
	10	17 25	68
<i>D</i> —	60	22 60	37
	3	27 40	67
<i>E</i> —	6	18 60	30
	3	24 40	60

* For details of tests of immunizing activity in guinea pigs see Methods; the total dose of antigen (given subcutaneously in 3 equal doses) is quoted; animals were challenged with 1000 lethal doses of virulent *Bacillus anthracis* strain N.P. No control animals survived.

Attempts to separate the major serological component in factor II of anthrax toxin

The final preparation of factor II was a mixture of two serological components, B and C (Sargeant *et al.* 1960); B appeared to be associated with factor II activity but the relative proportions of components B and C in the final preparation was unknown (see Sargeant *et al.* 1960). Since this preparation of factor II was the only factor of the anthrax toxin which was immunogenic when injected alone, attempts were made to separate the two components so that each could be examined for factor II activity and immunogenicity. These attempts, described below, were only partially successful. Component C was obtained free from component B by the purification of fractions discarded during the fractionation of factor I (Stanley & Smith, 1961); it proved to have no factor II or immunogenic activity at the con

centrations examined. This supported the view of Sargeant *et al.* (1960), that component B was responsible for the factor II activity and indicated that component B was also the immunogen.

The preparation of component C was sufficiently pure to be used for estimations of the relative proportions of components B and C in factor II by comparative titrations on serological diffusion plates. Component B proved to be the major

Table 2. *Effect of factor III of anthrax toxin on the immunogenicity of factors I and II and their mixture*

This table includes the results of four similar experiments for each comparison of factors I and II (or their mixture) with and without the addition of factor III. For details of tests of immunizing activity in guinea pigs see Methods.

Antigens injected* (μ g.)			Immunizing activity in guinea pigs.*	
I	II	III	No of survivors No. injected	= %
—	—	300	0 10	Nil
—	—	100	0 10	Nil
—	—	30	0 10	Nil
—	—	3	0 29	Nil
3	—	—	0 35	Nil
3	—	3	7 50	14
—	60	—	15 40	38
—	60	3	10 29	34
—	60	30	7 20	35
—	6	—	19 78	24
—	6	3	21 79	27
—	6	9	5 20	25
3	60	—	28 40	70
3	60	3	23 40	58
3	60	30	2 20	10
3	6	—	35 60	58
3	6	3	20 49	41
3	6	9	4 30	13

* See Table 1.

component in the preparation of factor II but all subsequent attempts to decrease significantly its contamination with component C failed.

Preparation of component C. The serological detection and characterization of component C has been described elsewhere (Sargeant *et al.* 1960); in gel-diffusion plates it precipitated with 'antigen' (H25) antiserum but not with 'spore' (H 533) antiserum. Although, during the original filtration of toxic cultures, component C passed the filters to be present in the final preparation of factor II, some component C was retained on the filters to be eluted with factor I. It was from fractions discarded during the fractionation of the latter (see Stanley & Smith, 1961; the 0.1 I and 0.12 I phosphate buffer eluates from the DEAE-cellulose column), from two batches of filter eluate (2×200 ml. $\equiv 2 \times 50$ l. original culture) that component C was obtained. The fractions, containing component C and some component B and factor I, were bulked and left at 0° for 16 days. A precipitate formed and after removal of this, the soluble material contained component C but had a much decreased content of component B. The solution was diluted with water (3 vol) and applied to a column (2 cm. diam., 1 cm. length) of DEAE-cellulose (0.2 g.) which had been equilibrated with 0.1 I phosphate buffer (pH 7.4). Component C was eluted with 0.2 I phosphate buffer (pH 7.2) containing 0.2 M-NaCl (4 ml.). A sample of this material was dialysed against water and freeze-dried to obtain the content of non-dialysable matter.

For paper electrophoresis (pH 8.6, 0.2 I barbitone buffer) the final solution was concentrated by dialysis against carbowax; 1 mg. of material showed a single band migrating to the anode. In serological diffusion plates, the final preparation produced the C line against 'antigen' antiserum (see below) but 4 μ g. produced no B line and this indicated (by comparative titration with factor II which contained serological component B) that contamination with component B was less than 5%. The preparation of component C contained 104% protein (calculated with reference to an ovalbumin standard) and on serological diffusion plates 0.24 μ g. was the minimum quantity that formed the C line against 'antigen' (H25) antiserum. It (40 μ g.) had no activity in the assay for factor II, and in the test for immunogenicity total doses of 60 and 6 μ g. did not protect guinea pigs (cf. factor II in Table 1).

Estimation of content of component C in the final preparation of factor II. The difficulty was that the B and C lines formed by factor II with 'antigen' (H25) antiserum overlaid one another so that individual titrations could not be observed directly (Sargeant *et al.* 1960). Estimations were done by two methods using antigen (H 25) antisera: (1) wells containing serial descending dilutions of factor II were alternated with those containing the preparation of component C (1 μ g.) which formed a line which ran into the line formed by the higher concentrations of factor II. The amount of factor II in the first dilution which showed the absence of component C (i.e. the C line from adjacent wells crossed the B line of the factor II preparation and continued to the factor II wells) was noted and compared with the amount of component C preparation (0.12 μ g.) which just did not form a C line. (2) An antiserum (R-1; Sargeant *et al.* 1960) which inhibited the formation of the B line by preparations of factor II (see Sargeant *et al.* 1960) but had no effect on the formation of the C line (by comparative titrations with the preparation of component C), was added (equal volumes) to serial dilutions of the preparation of factor II. The minimal amounts of factor II which formed a C line under these conditions

were compared with the corresponding amounts of the preparation of component C examined under the same conditions. Both methods of estimation agreed in indicating that the amount of component C in several batches of the final preparation of factor II was not more than 15–30 %. The second method of estimation was used in subsequent work.

Attempts to decrease the amount of component C in preparations of factor II. Exhaustive attempts to reduce significantly the content of component C in the final preparation of factor II by fractionating the latter on diethylaminoethyl DEAE-cellulose, hydroxyapatite or on alumina Cγ celite columns failed. Since components B and C were so alike in chemical behaviour attempts were made to avoid the necessity of separating them by obtaining initial culture filtrates with a lower relative content of component C. Filtrates of cultures, taken after different times of incubation were examined in the hope that components B and C might be formed at significantly different rates or at different stages in growth. Several flasks of medium (250 ml.; Thorne *et al.* 1960) were inoculated with *Bacillus anthracis*, Sterne strain, 2.5×10^7 spores, and incubated at 37°. After 8, 12, 18, 24 and 36 hr., samples of the culture were filtered. Immediately each sample (at 8 hr., 1 l.; at 12 hr., 500 ml.; at 18, 24 and 36 hr., 250 ml.) was dialysed for 4 hr. against phosphate buffer (pH 7.4, 0–2°) and fractionated for factor II on DEAE-cellulose (2 g.) as described in Methods. Subsequent serological analysis indicated that the factor II preparations obtained from the 8, 12, 18, 24 and 36 hr. filtrates contained not more than 30, 30, 12, 17 and 17 %, respectively, of component C. Although the content of component C could be varied by varying the time of incubation, it was not significantly decreased from the normal figure (15–30 %) for various preparations of factor II obtained from 24 hr. filtrates.

DISCUSSION

Although some knowledge is now available about the chemistry of the components of the anthrax toxin, their mode of action is unknown. We have been unable to identify any enzymic activity in a mixture of the three factors of anthrax toxin which might explain its lethal effect. There is no explanation for the synergic activity of the three factors in lethality tests (see Stanley & Smith, 1961; table 8), for the dramatic effect of mixtures of factors I and II on the capillaries *in vivo* which results in the production of so much local oedema, or for the effect of factor III on the mixture of I + II in decreasing its local oedema production and increasing its lethality. It would be satisfying to think that the complexing action of factor I on metals had some connexion with its role in the toxic mixture but its non-replacement by EDTA (Stanley & Smith, 1961) was discouraging in this respect. The three factors of the toxin do not appear to be complexed in any way, at least when they appear extracellularly. Thus, the individual factors could be detected (in gel diffusion plates against 'spore' antiserum) in the plasma of guinea pigs dying of anthrax and in cultures *in vitro* which had not been filtered (which separates the factors) but only centrifuged to remove most bacteria and concentrated for examination by dialysis against Carbowax.

The immunizing activities of the various factors of the anthrax toxin are important in relation to practical vaccination against anthrax. Although the factor II preparation was the only factor which was immunogenic when injected

alone, it was clear that the addition of the other factors influenced the overall immunogenicity, e.g. addition of factor I increased the immunizing activity of factor II but the combined activity of I+II was decreased by adding factor III. However, the addition of factor III alone to factor II seemed to have no significant effect on the immunizing activity at the concentrations examined. It is almost impossible to say what is the optimum mixture of the three factors which will produce the maximum immunogenicity, for the following reasons. First, the large amount of material and the number of animals which would be needed for comprehensive titrations of various mixtures of the three factors (cf. toxicity tests, Stanley & Smith, 1961); the unforeseen results which might emerge from such titrations are indicated by the appearance of some immunizing activity in mixtures of factors I and III (see Table 2). Secondly, the low slope of the dose-response curve in these tests for immunogenicity (cf. a similar response with products from *Pasteurella pestis*, Keppie *et al.* (1960) and from *Brucella abortus*, Smith *et al.* (1962)). The preparation used at present for vaccinating man against anthrax is prepared from a culture filtrate (Belton & Strange, 1954) which contains factors II and III. It would appear that addition of factor I (which is removed during the filtration) and removal of factor III would improve the vaccine, if the resulting mixture could be detoxified. The latter should not present much difficulty in view of the extreme lability of the toxic manifestations of factors I and II (Stanley *et al.* 1960; Stanley & Smith, 1961). In this respect, it is interesting that an impure preparation containing factor I of anthrax toxin produced *in vivo* (Stanley *et al.* 1960) was a good immunogen (60 µg. protected over 50 % of the guinea pigs in the standard test for immunogenicity), and later Sargeant *et al.* (1960) showed it to contain, in addition to factor I, component B (i.e. the immunogen in the factor II preparation) in a detoxified but antigenic form.

We are indebted to Dr J. Keppie and Mr K. Witt for help with the tests for immunogenicity and to Mr R. Blake for excellent technical assistance.

REFERENCES

- ALBURN, H. E. & WHITLEY, R. W. (1951). Factors affecting the assay of hyaluronidase. *J. biol. Chem.* **192**, 379.
- BELTON, F. C. & STRANGE, R. E. (1954). Studies on a protective antigen produced *in vitro* from *Bacillus anthracis*; medium and methods of production. *Brit. J. exp. Path.* **35**, 144.
- BIER, M. (1955). Lipases. *Meth. Enzymol.* **1**, 627.
- HARRIS-SMITH, P. W., SMITH, H. & KEPPIE, J. (1958). Production *in vitro* of the toxin of *Bacillus anthracis* previously recognized *in vivo*. *J. gen. Microbiol.* **19**, 91.
- HECHTER, O. (1947). Studies on spreading factors. I. The importance of mechanical factors in hyaluronidase action in skin. *J. exp. Med.* **85**, 77.
- KEPPIE, J., COCKING, E. C., WITT, K. & SMITH, H. (1960). The chemical basis of the virulence of *Pasteurella pestis*. III. An immunogenic product obtained from *Past. pestis* which protects both guinea pigs and mice. *Brit. J. exp. Path.* **41**, 577.
- KIELLEY, W. W. & MEYERHOF, O. (1950). Studies on adenosinetriphosphatase of muscle. III. The lipoprotein nature of the magnesium-activated adenosinetriphosphatase. *J. biol. Chem.* **183**, 391.
- KING, E. J. (1951). *Microanalysis in Medical Biochemistry*, p. 69. London: J. and A. Churchill Ltd.

- KUNITZ, M. (1946). A spectrophotometric method for the measurement of ribonuclease activity. *J. biol. Chem.* **164**, 563.
- KUNITZ, M. (1947). Crystalline soya-bean trypsin inhibitor. II. General properties. *J. gen. Physiol.* **30**, 291.
- KUNITZ, M. (1950). Crystalline desoxyribonuclease. I. Isolation and general properties. Spectrophotometric method for the measurement of desoxyribonuclease activity. *J. gen. Physiol.* **33**, 349.
- MACFARLANE, M. G. & KNIGHT, B. C. J. G. (1941). The biochemistry of bacterial toxins. I. The lecithinase activity of *Clostridium welchii* toxins. *Biochem. J.* **35**, 884.
- MILES, A. A. & WILHELM, D. L. (1955). Enzyme-like globulins from serum reproducing the vascular phenomena of inflammation. I. An activable permeability factor and its inhibitor in guinea-pig serum. *Brit. J. exp. Path.* **36**, 71.
- OAKLEY, C. L., WARRACK, G. H. & VAN HEYNINGEN, W. E. (1946). The collagenase (κ toxin) of *Clostridium welchii* type A. *J. Path. Bact.* **58**, 229.
- SARGEANT, K., STANLEY, J. L. & SMITH, H. (1960). The serological relationship between purified preparations of factors I and II of the anthrax toxin produced *in vivo* and *in vitro*. *J. gen. Microbiol.* **22**, 219.
- SMITH, H. & GALLOP, R. C. (1956). The chemical basis of the virulence of *Bacillus anthracis*. VI. An extracellular immunising aggressin isolated from exudates of infected guinea pigs. *Brit. J. exp. Path.* **37**, 144.
- SMITH, H., KEPPIE, J., PEARCE, J. H. & WITT, K. (1962). The chemical basis of the virulence of *Brucella abortus*. IV. Immunogenic products from *Brucella abortus* grown *in vivo* and *in vitro*. *Brit. J. exp. Path.* **43**, 538.
- SMITH, H., KEPPIE, J. & STANLEY, J. L. (1955). The chemical basis of the virulence of *Bacillus anthracis*. V. The specific toxin produced by *B. anthracis* *in vivo*. *Brit. J. exp. Path.* **36**, 460.
- SMITH, H. & STANLEY, J. L. (1962). Purification of the third factor of the anthrax toxin. *J. gen. Microbiol.* **29**, 517.
- SMITH, H., TEMPEST, D. W., STANLEY, J. L., HARRIS-SMITH, P. W. & GALLOP, R. C. (1956). The chemical basis of the virulence of *Bacillus anthracis*. VII. Two components of the anthrax toxin; their relationship to known immunising aggressins. *Brit. J. exp. Path.* **37**, 263.
- STANLEY, J. L., SARGEANT, K. & SMITH, H. (1960). Purification of factors I and II of the anthrax toxin produced *in vivo*. *J. gen. Microbiol.* **22**, 206.
- STANLEY, J. L. & SMITH, H. (1961). Purification of factor I and recognition of a third factor of the anthrax toxin. *J. gen. Microbiol.* **26**, 49.
- STRANGE, R. E. & THORNE, C. B. (1958). Further purification studies on the protective antigen of *Bacillus anthracis* produced *in vitro*. *J. Bact.* **76**, 192.
- THORNE, C. B., MOLNAR, D. M. & STRANGE, R. E. (1960). Production of toxin *in vitro* by *Bacillus anthracis* and its separation into two components. *J. Bact.* **79**, 250.