THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*

IX. Its Aggressins and their Mode of Action

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THE earlier literature (see Smith and Keppie, 1955) indicated that at least two factors were involved in the aggressin action of *Bacillus anthracis* ; one was extracellular and related to the immunizing activity of certain strains and the other appeared to be connected with the capsule. Work described in the present series of papers (reviewed Smith, 1958) has shown this broadly to be true and has established the nature of the compounds involved. The extra-cellular, oedema-forming, lethal toxin of *B. anthracis* (Smith, Keppie and Stanley, 1955) which is the basis of immunity to anthrax (Stanley and Smith, 1963) has an aggressin action in the early stages of infection (Smith, Zwartouw and Harris-Smith, 1956). This toxin consisted of three components—factors I, II and III—which were purified and chemically investigated ; they were neither lethal nor oedema-producing when injected singly but fully toxic when injected together (Smith, Tempest, Stanley, Harris-Smith and Gallop, 1956 ; Thorne, Molnar and Strange, 1960 ; Stanley, Sargeant and Smith, 1960 ; Sargeant, Stanley and Smith, 1960 ; Stanley and Smith, 1961 ; Smith and Stanley, 1962 ; Stanley and Smith, 1963).

The capsule of *B. anthracis* contains polyglutamic acid (Tomscik, 1956). Samples of this material showing no evidence of heterogeneity were isolated from *B. anthracis* grown *in vivo*, and from the body-fluids of infected guinea-pigs (Zwartouw and Smith, 1956 ; Smith, Zwartouw and Harris-Smith, 1956) ; it was neither toxic nor immunogenic (Smith and Gallop, 1956). This paper describes the aggressin activity of a toxic mixture of the three recently purified factors of the toxin and of sodium polyglutamate, *i.e.* their ability to enhance the virulence *in vivo* of an otherwise ineffective dose of *B. anthracis*, to interfere with the phagocytosis of phagocytosis-sensitive *B. anthracis*, and to interfere with the bactericidal action of horse serum and extracts of guinea-pig leucocytes on *B. anthracis*. Furthermore evidence is presented which indicates that when the toxin and polyglutamic acid interfere with phagocytosis of *B. anthracis*—which is perhaps the main defence against this organism—they do so by differing mechanisms.

In addition to the toxin and capsular polyglutamic acid, Smith, Zwartouw and Harris-Smith (1956) showed that a third aggressin might contribute to the overall activity. An impure lipoprotein fraction (Protein *A*) obtained from *B. anthracis* grown *in vivo*, (a) rendered *B. anthracis* more resistant to phagocytosis, (b) was absent from a non-capsulated avirulent strain of *B. anthracis* and (c) was non-immunogenic. This material was obtained from extracts of whole capsulated organisms. It was not therefore possible to assess its importance in the overall aggressin activity, because it was not known whether the active material was

hidden in the soma, or present in the capsule or on the surface, where it could act effectively. This paper, summarizes unsuccessful attempts to demonstrate Protein *A* in the capsule and hence leaves the question of its importance as an aggressin undecided.

MATERIALS AND METHODS

Sodium polyglutamate from B. anthracis *grown* in vivo.—The two samples described by Zwartouw and Smith (1956) were used; they were obtained from the cellular substance and from the body-fluids of infected guinea-pigs, but only one set of results is recorded below, since they behaved identically.

The mixture of purified factors I, II and III of the anthrax toxin.—The final preparations of factors I, II and III were described by Stanley and Smith (1961), Smith and Stanley (1962) and Stanley and Smith (1963). Concentrated solutions of the factors were mixed in the proportions factor I 4 μg., factor II 40 μg. and factor III 8 μg. (these are the amounts of material which form a mixture fully lethal for mice; Stanley and Smith, 1961; Smith and Stanley, 1962) and dilutions of this mixture were examined immediately in the tests for aggressins.

Protein A from B. anthracis *grown* in vivo.—This was described by Smith, Zwartouw and Harris-Smith (1956).

Tests for aggressins

Ability to enhance the virulence of B. anthracis (*Pasteur II*) *in guinea-pigs.*—This test was described by Smith and Gallop (1956). The concentration of material in the highest dilution (0·2 ml.) showing activity has been quoted.

Antiphagocytic activity

Two tests were used in this work, utilising the polymorphs of normal guinea-pig blood (PMN), and phagocytosis—sensitive *B. anthracis* (spores of strain NP incubated in tryptic meat broth for 20 min. at 37°). These tests were based on:

Viable counts of the numbers of bacteria surviving phagocytosis by treated and untreated phagocytes.—This was the standard assay for antiphagocytic activity described by Smith and Gallop (1956), which had been used in preference to the following test for the routine examination of fractionation samples (Smith and Gallop, 1956; Smith, Zwartouw and Harris-Smith, 1956).

The phagocytic-index of treated and untreated phagocytes.—This test was described by Keppie, Smith and Harris-Smith (1953). In control samples the guinea-pig phagocytes ingested the organisms and the mean phagocytic-index was usually 8–10. Addition of aggressin decreased the phagocytic-index and the statistical treatment (by our colleague Mr. S. Peto) of results (100 phagocytes counted for each determination of mean phagocytic-index) from more than 50 experiments showed that a difference of 1·5 or more in the mean phagocytic-index was significant.

Dilutions of the materials were examined in these tests and in Table I the lowest concentration of materials (in the total test-mixture of 1 ml.) showing significant activity, has been quoted.

Anti-bactericidal activity

Two tests have been used utilising the bactericidal properties of normal horse serum and of extracts of guinea-pig white blood cells.

Serum.—Freeze-dried horse serum was reconstituted and diluted so that 90 per cent of the test inoculum was killed in the control tubes under the following conditions.

Mixtures of the diluted serum (0·4 ml.) and of the dilutions of the test substance (0·4 ml. in Locke) were made in screwcap bottles (5 ml.). After standing 30 min. at room temperature a suspension (0·2 ml.) of newly germinated anthrax spores (strain NP, incubated in tryptic meat broth for 20 min. at 37° and subsequently diluted to 3×10^5 per ml. in gelatin-Locke) was added. Viable counts of the control samples and test mixtures were made immediately, and after rotation for 3 hr. at 37°. Active materials completely interfered with the bactericidal activity and the lowest concentration of material (in the total test-mixture,

1 ml.) showing significant activity (*i.e.* double the percentages of surviving organisms relative to the control samples) has been quoted in Table I.

White blood-cell extract.—Large guinea-pigs (800 g.) received intraperitoneally 2 per cent w/v wheat starch (15 ml.). After 18 hr., the animals were killed and their peritoneal cavities were washed out with 0·5 per cent gelatin-saline (50 ml. per animal) containing heparin (3 I.U. per ml.). The WBC were collected by centrifugation, washed three times with gelatin) saline, suspended in water ($2·5 \times 10^8$ per ml.) and frozen ($-70°$) and thawed (room temperature) three times. This suspension of disintegrated white blood cells was used in the same way as described for horse serum, and the results have been quoted similarly.

RESULTS

The aggressin activity of the mixture of factors I, II and III of the anthrax toxin and of sodium polyglutamate

The results in Table I show that both the toxin-mixture and the sodium polyglutamate have the following biological activities. They enhance the virulence of *B. anthracis in vivo*, they are antiphagocytic, and they are anti-bactericidal towards both horse serum and extracts of guinea-pig white blood cells. Similar results were obtained in all the antiphagocytic and anti-bactericidal tests, if the virulent N.P. strain of *B. anthracis* was replaced by the avirulent Sterne strain.

TABLE I.—*Aggressin Activity of Mixtures of the Three Factors of the Anthrax Toxin and of Sodium Polyglutamate*

	Lowest active concentration (per cent w/v)			
	Toxin-mixture			
Test for aggressin*	factor I	factor II	factor III	Sodium polyglutamate
Virulence enhancing activity *in vivo*	0·025	0·25	0·05	2·0
Anti-phagocytic activity in guinea-pig blood				
1. Viable count of unphagocytosed organisms	0·003	0·03	0·006	0·8
2. Phagocytic-index	0·003	0·03	0·006	0·8
Antibactericidal activity				
1. Horse serum	0·001	0·01	0·002	0·03
2. Extract of guinea-pig white blood-cells	0·001	0·01	0·002	0·03

* For details of the tests—see Methods.

On a weight basis, the toxin mixture was the more active, but *in vivo* the polyglutamate may play as large a role in invasion, as the toxin, since it is concentrated around the organism as a capsule and not—as is the toxin—dispersed in the surrounding environment.

The mode of action of the toxin mixture and of sodium polyglutamate in preventing phagocytosis

The toxin mixture and sodium polyglutamate inhibited phagocytosis by different mechanisms.

The disabling of phagocytes by the toxin mixture.—The mixture of the three factors of the anthrax toxin, but not sodium polyglutamate, exerted a direct

harmful effect on the phagocytes which persisted after removal of the toxin mixture. The following experiments were based on the tests for antiphagocytic activity.

Gelatin-Locke (0·4 ml.) containing the toxic mixture or sodium polyglutamate was added to fresh defibrinated guinea-pig blood (0·4 ml.) and the mixture was rotated at 37° for 1 hr. The mixture was cooled to 0–2° and the blood cells removed by centrifugation (2000 r.p.m., 17 cm. radius, 15 min.) and washed twice at 0–2° with gelatin-Locke (2 × 5 ml.). The cell deposits (*ca.* 0·2 ml.) were made up to 0·6 ml. with gelatin-Locke (0·4 ml.)—in Table II it will be seen that one control tube contained the aggressin throughout the subsequent phagocytosis; it was added at this stage—and fresh serum (0·2 ml.) from defibrinated blood was added. After rotating at 37° for ½ hr. the suspension of cells (0·8 ml.) was used as the mixture of blood and experimental sample in both tests for antiphagocytic activity (see Methods). The aggressins were tested at concentrations equal to, or slightly above the minimal active concentrations indicated in Table I. The results shown in Table II clearly indicate the harmful effect of

TABLE II.—*The Disabling of Guinea-pig Phagocytes by a Mixture of the Three Factors of the Anthrax Toxin but not by Sodium Polyglutamate*

Aggressin (w/v per cent in 0·4 ml.)	Treatment* of phagocytes (vol: 0·8 ml. in pretreatment, 1·0 ml. in final phagocytosis)	Phagocytosis tests†	
		Mean phagocytic-index	Average per cent of organisms surviving phagocytosis
Toxin mixture			
Factor I (0·008)	Control	10·7	39
Factor II (0·08)	Pretreatment with aggressin	8·4	58
Factor III (0·016)	Aggressin present during phagocytosis	5·8	63
Sodium polyglutamate (2·0)			
	Control	10·8	39
	Pretreatment with aggressin	10·4	39
	Aggressin present during phagocytosis	9·3	74

These results are the average of 2–4 similar experiments.

* For details of pretreatment see methods.

† For details of these two separate tests see methods. A difference of 1·5 or more in mean phagocytic index was significant; and also a difference 10–15 in average percentage of the organisms surviving phagocytosis (Smith and Gallop, 1956).

the toxin-mixture on the phagocytes and the innocuous nature of sodium polyglutamate. Similar results were obtained if the virulent NP strain of *B. anthracis* in these tests was replaced by the avirulent Sterne strain.

The increased resistance to phagocytosis of B. anthracis *(strain NP) after treatment with sodium polyglutamate*

B. anthracis (strain NP) was treated with sodium polyglutamate or with the toxin mixture at 0–2°; after removal of the aggressin the organisms were tested

TABLE III.—*Increase in Phagocytosis-resistance of* B. anthracis *(strain NP) when treated with Sodium Polyglutamate but not the Toxin Mixture*

Pretreatment of *B. anthracis*		Average (per cent) of organisms						
		Washed organisms			Unwashed organisms			
Sample	Conc. (per cent) in contact with organisms at 0–2°	(a) control	(b) expt	(b–a)	control	(a) corrected* control	(b) expt	(b–a)
Sodium polyglutamate	1·0	27	46	19	32	43	54	11
Toxin mixture								
Factor I .	0·008	49	50	1	49	51	43	−8
Factor II .	0·08							
Factor III .	0·016							

The results are the average of 3–5 similar experiments.

The details of the test involving the determination of phagocytosis-resistance of *B. anthracis* after pretreatment with the aggressin and its subsequent removal, were described by Smith, Zwartouw and Harris-Smith (1956).

* For details of this correction see Smith, Zwartouw and Harris-Smith (1956).

for resistance to phagocytosis. The details of the test were the same as those described by Smith, Zwartouw and Harris-Smith (1956) and the results are given in Table III. Sodium polyglutamate, but not the toxin mixture, appeared to be adsorbed on the surface of organisms of strain NP rendering them more resistant to phagocytosis. In these tests with strain NP, the preliminary growth ($\frac{3}{4}$ hr.) of the organism in serum (see Smith, Zwartouw and Harris-Smith, 1956) was important. Organisms grown in tryptic meat broth show no significant ability to add on the protective coat of sodium polyglutamate. A precursor to the capsule might be necessary for adsorption of the polyglutamic acid. The absence of this precursor might explain the fact that the non-capsulated avirulent Sterne strain of *B. anthracis* could not be pretreated with sodium polyglutamate as described above.

The anti-opsonic effect of sodium polyglutamate.—The aggressin effect of the polyglutamate appeared to be the prevention of opsonization by normal serum.

This conclusion was reached from tests in which the materials were used to interfere with the preliminary opsonization of *B. anthracis* (strain NP) by normal guinea-pig serum prior to phagocytosis of the washed organisms by serum-free WBC. Interference with opsonization resulted in a reduced phagocytic index. The details of the test were as follows :—Spores of the " NP " strain were incubated at 37° in tryptic meat broth for 20 min. and then cooled to 0–2° : the mixture which was kept at 0–2° for 1 hr. for opsonization, consisted of a suspension (0·3 ml.) of the organisms (number = $\times 30$ the number of PMN to be added later), a solution (0·3 ml.) of the material in gelatin-Locke, and a batch of guinea-pig serum (0·6 ml.) which had been found to be suitable for opsonization : the organisms were washed twice in gelatin-Locke (4 ml.) and resuspended in (0·45 ml.). The mixture for the phagocytosis-stage was the suspension (0·3 ml.) of treated organisms, a suspension (0·3 ml.) of guinea-pig WBC which has been washed free from serum with gelatin-Locke (0·15 ml.) : it was rotated in waxed tubes for 45 min. after which time stained films were prepared from the buffy layer.

In two experiments with two different batches of sodium polyglutamate, organisms which had been opsonized in the presence of this material (1 per cent

w/v—in the total volume—1·2 ml.—used for opsonization) produced—on subsequent phagocytosis—mean phagocytic indices of 9·3 and 5·0 compared with corresponding indices in control samples of 11·9 and 10·8 respectively.

Unsuccessful attempts to demonstrate the presence of Protein A *in the Capsule of* B. anthracis *grown* in vivo

A rabbit received intravenously, successive doses of protein *A* over approx. 2 months until samples of its serum formed a specific precipitate with a solution of protein *A*. This antiserum to protein *A* was used in two series of experiments.

A suspension of *B. anthracis* grown *in vivo* was not agglutinated by this rabbit anti-serum to protein *A* but it was by a rabbit serum containing antibody to polyglutamic acid. Furthermore, the antiserum to protein *A* would not produce in the capsule of *B. anthracis* any precipitinogen patterns observable with the phase contrast microscope and similar to those formed by a serum containing antibody to polyglutamic acid (Tomscik, 1956 ; confirmed by us).

Hence, there is still no evidence that protein *A* is a surface or capsular product of *B. anthracis* and therefore an effective aggressin of this pathogen.

DISCUSSION

The main host defence mechanisms against anthrax appear to be phagocytosis (Adami, 1909), and extracellular lysis by an anthracidal substance present in the serum of some species and found in certain leucocyte extracts (Cromartie, Bloom and Watson, 1947 ; Bloom, Watson, Cromartie and Freed, 1947). Their relative importance no doubt varies from species to species of host. The two main aggressins produced by *B. anthracis*—the extracellular toxin and capsular polyglutamic acid—have been shown to cause interference with both these defence mechanisms and also to enhance the virulence of *B. anthracis in vivo*. It was gratifying that a mixture of the three purified factors of the anthrax toxin prepared *in vitro*, had all the facets of the aggressin activity of the impure toxin and of its components produced *in vivo* (Smith, Keppie and Stanley, 1955 ; Smith and Gallop, 1956 ; Smith, Zwartouw and Harris-Smith, 1956). The immunizing activity of the components of the toxin (Stanley and Smith, 1963) emphasizes their aggressin activity since the protection of animals against anthrax appears to rely on the ability of the antibodies to abort the infection at the very early, *i.e.* the invasive stage of the disease. (Cromartie, Bloom and Watson, 1947 ; Cromartie, Watson, Bloom and Heckly, 1947.)

The contrasting modes of action of the toxin and sodium polyglutamate in inhibiting phagocytosis fit in with their location in the diseased host. The toxin is excreted by the bacteria and dispersed widely in the body-fluids of the host ; it is active as an aggressin at low concentration and has been shown to have a directly harmful effect on the phagocytes. On the other hand, sodium polyglutamate is concentrated around the organisms as a capsule. This will compensate for the fact that it is not as active—on a weight basis—as are the mixed components of the toxin, and accords well with its probable role in preventing phagocytosis by inhibiting opsonization.

There is circumstantial evidence that polyglutamic acid is not the only component of the anthrax capsule. First, Tomscik (1956) pointed out the immuno-

logical difference between capsulated *B. anthracis* containing polyglutamic acid and cultures of uncapsulated *B. subtilis* containing a slime of polyglutamic acid. The former will produce polypeptide antibody in a rabbit, the latter will not, although the polypeptide from *B. subtilis* is precipitable by the antiserum prepared from *B. anthracis*. Tomscik (1956) suggested that in *B. anthracis* the capsular polypeptide may form a protein complex. Second, Kent, Record and Wallis (1957) showed that ordinary diffusive forces would preclude the retention in the capsule of massive amounts of polyglutamic acid as isolated. To stay in the capsule, either the natural polyglutamic acid must be of much larger molecular size or it must be constrained in some way perhaps by electrostatic forces and hydrogen bonding between it and the cell surface and/or by some protein framework. Third, Smith and Gallop (unpublished observations) have been unable to remove the capsule of *B. anthracis* by an enzyme preparation known to hydrolyse the sodium polyglutamate isolated from *B. anthracis* grown *in vivo*.

It would have been gratifying if we could have obtained some evidence that protein *A* (Smith, Zwartouw and Harris-Smith, 1956) was also in the capsule of *B. anthracis*, but we have been unable to do so. Hence, the relevance of the activity of protein *A* in aggressin-assays to the overall aggressin activity of the intact organism *in vivo* is still unknown.

SUMMARY

Both the mixture of factors I, II and III of the anthrax toxin and capsular sodium polyglutamate enhanced the virulence of *B. anthracis in vivo*, interfered with the phagocytosis of *B. anthracis* by guinea-pig phagocytes, and inhibited the bactericidal activity of horse serum and extracts of guinea pig white blood-cells.

On a weight basis, the toxin mixture was more active in these tests than capsular sodium polyglutamate and this accords with the respective location of the two aggressins in *B. anthracis* infection.

The inhibition of phagocytosis produced by the toxin mixture results from its direct harmful effect on the phagocytes whereas the polyglutamate acts by inhibiting the opsonization of organisms by normal serum.

The relevance of the aggressin activity of protein *A* (Smith, Zwartouw and Harris-Smith, 1956) to the overall aggressin activity of *B. anthracis in vivo* is unknown, since the protein could not be proved to be on the surface or in the capsule.

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