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RELATIONSHIP OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* PROTOPLASMIC TOXINS TO THE EXOTOXIN

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ABSTRACT

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Corynebacterium pseudotuberculosis (Buchanan, 1911) protoplasm was separated into two toxic fractions by means of gel chromatography. Toxin neutralization tests indicated that one of the fractions is probably identical to the exotoxin.

INTRODUCTION

Corynebacterium pseudotuberculosis (Buchanan, 1911) produces a number of biologically active substances which have been studied in some detail by numerous authors.

Petrie & McClean (1934) found that the exotoxin produced by *C. pseudotuberculosis* is lethal to guinea pigs in high doses and when smaller doses are injected intradermally it gives rise to erythema, oedema and necrosis in both guinea pigs and rabbits. Carne (1940) undertook a thorough study of the exotoxin and found that all 200 strains he examined produced the toxin. Guinea pigs were particularly susceptible to the lethal action of the toxin. Rabbits, on the other hand, reacted more severely to skin inoculations. Mice and rats again, were more resistant. Sheep also succumbed to subcutaneous injection of the toxin but large doses were required to produce lesions similar to those in guinea pigs. In the same study Carne showed that the exotoxins of all his strains were serologically identical. Doty, Dunne, Hokanson & Reid (1964) also found the exotoxins from different isolates to be identical.

Lovell & Zaki (1966a) have demonstrated that *C. pseudotuberculosis* exotoxin is lethal to white mice, and Jolly (1965) proved that the toxicity is due to increased local vascular permeability. He considers this activity to be an important facet in the pathogenesis of *C. pseudotuberculosis* infection.

C. pseudotuberculosis also produces a haemolysin which is capable of giving rise to haemoglobinuria or icterus when injected into sheep, particularly by the intravenous route (Carne, 1939). Similar findings have been reported by Robinson (1928). According to Carne (1939) the haemolysin is unrelated to the exotoxin and is not antigenic. It is thermolabile, oxygen sensitive and intimately associated with the bacterial cells. Zaki (1965) has, however, demonstrated haemolytic activity in culture supernatants free of bacteria and also found that the haemolytic activity can be neutralized by antiserum. He suggests that the exotoxin and haemolysin are identical.

In addition *C. pseudotuberculosis* produces substances which will inhibit staphylococcal beta haemolysin (Hartwigk, 1963) and potentiate the activity of staphylococcal delta haemolysin (Fraser, 1962). According to data presented by Lovell & Zaki (1966b) the haemolysin and these two substances appear to be identical.

The bacterial cells themselves also contain toxic products of which the surface lipid is probably the most well-known. Bull & Dickenson (1935) showed that removal of the lipid by successive extraction with acetone, ether and alcohol did not reduce the pyogenic activity of the organisms. Carne, Wickham & Kater (1956) also found that removal of the lipid did not affect the viability of the bacteria. The lipid is a waxlike substance and is toxic for guinea pigs. It contains corynomycolic acid (Diara & Pudles, 1959) which is similar to wax D of mycobacteria. Jolly (1966) demonstrated that the presence of lipid is associated both with virulence and the ability of *C. pseudotuberculosis* to grow as a pellicle on liquid media, and that the lipid probably also plays an important role in the pathogenesis of infection.

Cameron (1964) established that bacterial cells from which lipid has been removed and which have been killed with formalin retain the ability to produce sterile abscesses in guinea pigs when injected in sufficient quantities. Protoplasm derived from such cells is not toxic. Conversely, cells that are killed with phenol lose their ability to cause subcutaneous abscesses whereas their protoplasm remains toxic. From these results it was deduced that, apart from the toxic lipid, *C. pseudotuberculosis* cells contain a pyogenic factor as well as protoplasmic toxin(s).

It has also been shown (Cameron, 1964) that only bacteria which are grown under favourable cultural conditions possess the pyogenic factor and protoplasmic toxin and that the presence of these substances is correlated with their ability to induce a protective immunity. The presence of toxic substance(s) in the protoplasm and the immunogenic properties of the cells nevertheless seem to be unrelated because later experiments (Cameron & Buchan, 1966) revealed that the toxin(s) could be separated from the immunizing antigen by precipitation with 50 per cent saturated ammonium sulphate.

Further studies on the nature of the protoplasmic toxin(s) and its relationship to the exotoxin were conducted in this investigation.

MATERIALS AND METHODS

Preparation of protoplasm

C. pseudotuberculosis strain 137B was used in all the experiments. Large numbers of cells were produced and mechanically disrupted as described previously (Cameron & Swart, 1965; Cameron, Minnaar & Purdom, 1969). Unbroken cells and cell walls were separated from the protoplasm by centrifugation at 20,000 × *g* at 4°C for 20 min. Twenty millilitres of protoplasm was concentrated to 10 ml by perevaporation and 1 g streptomycin sulphate (dissolved in 5 ml sterile distilled water) added in order to precipitate the nucleoproteins (Baich & Vogel, 1962). The tenacious precipitate was then removed by centrifugation at 20,000 × *g* at 4°C for 20 min.

The supernatant fluid was either stored at —20°C or dialyzed against distilled water and lyophilized.

Preparation of exotoxin

Exotoxin was prepared as described by Lovell & Zaki (1966a). The culture supernatants containing exotoxin were concentrated twofold by perevaporating and

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stored at —20°C or dialyzed against distilled water and lyophilized.

Preparation of antisera

Unconcentrated protoplasm and twofold concentrated exotoxin were used to prepare protoplasm antiserum (PAS) and exotoxin antiserum (EAS). The solutions were treated with 0.5 per cent formalin until they no longer produced an inflammatory reaction when injected intradermally into rabbits. When toxoiding was complete they were emulsified with an equal volume of Freund's complete adjuvant.* Four rabbits were used for each preparation. Each rabbit received an initial dose of 10 ml antigen subcutaneously given at five different sites. A series of three 1 ml doses of antigen without adjuvant was administered intravenously 21, 22 and 23 days after the primary injection. Ten days later a second series of injections of 2 ml each was given and the rabbits bled ten days after the last injection. Concentrated sera used for immunodiffusion tests were prepared by dialysis against polyethylene glycol (40,000 M.W.).

Ammonium sulphate and alcohol fractionation of protoplasm

After treatment with streptomycin, 40 ml volumes of protoplasm were consecutively dialysed overnight at 4°C against solutions of ammonium sulphate (pH 7.0) to give final concentrations of 30 per cent, 50 per cent and 80 per cent saturation. The precipitates were collected after each step by centrifugation at 20,000 × *g* at 4°C, for 20 min, washed with the appropriate concentration of ammonium sulphate and dissolved in 10 ml 0.02 M phosphate buffer pH 7.2. The fractions were marked P30, P50 and P80 respectively and freed of residual ammonium sulphate by dialysis against 0.02 M phosphate buffer pH 7.2.

Subsequent alcohol fractionation of the P50 fraction was done by gradually adding 30 ml cold absolute ethanol to 10 ml P50 at 4°C. After standing at 4°C for 20 min the precipitate was removed by centrifugation at 20,000 × *g* for 20 min at 4°C, washed with 75 per cent cold ethanol and dissolved in 5 ml 0.02 M Tris-HCl buffer pH 8.2. It was designated fraction 3V. A further 30 ml of ethanol containing 0.2 per cent potassium acetate was added to the supernatant fluid. The precipitate obtained was collected as above and designated fraction 6V.

Exclusion chromatography of protoplasm and exotoxin

In the first experiments the gel was prepared in 0.02 M phosphate buffer pH 7.2 and the protoplasm was also dialyzed against the same buffer. Under these circumstances a considerable precipitate was formed. It was found that this could be avoided by using a 0.05 M phosphate buffer pH 8.0 instead.

Sephadex G200** was swollen in 0.05 M phosphate buffer pH 8.0 at 4°C and packed in 2.5 × 45 cm columns according to the manufacturer's recommendations. Ten millilitres twofold concentrated protoplasm or exotoxin were applied to the column and the flow rate adjusted to 30 ml per hour. Fractions (10 ml) were collected, the absorbancy at a wavelength of 280 mμ determined and the toxicity of each fraction assayed (*vide infra*). The toxic fractions were pooled, concentrated by perevaporation, dialyzed against 0.05 M phosphate buffer pH 8.0, recycled through Sephadex G200 and again concentrated and dialyzed against 0.05 M phosphate buffer pH 8.0.

Gel diffusion and immuno-electrophoresis of protoplasm and exotoxin

Ten millilitres of a 1 per cent Ionagar No. 2† solution in 0.85 per cent NaCl pH 7.0, containing 0.1 per cent sodium azide, were poured into plastic Petri dishes (8.5 cm diameter). After the agar had solidified, wells (8.0 mm diameter) were punched in the desired design. The wells were filled with the antigens and antisera (concentrated twofold) to be examined, and the plates kept in a moist chamber at 4°C until the precipitin lines were fully developed.

Preer diffusion tests were done as described by Cruickshank (1965).

Immuno-electrophoresis was done on microscope slides using Shandon immuno-electrophoresis equipment.†† One per cent agar in 0.05 M veronal buffer pH 8.2 was placed on the microscope slides and the same buffer was also used in the electrolyte chambers. Electrophoresis was carried out at 7 volts per cm (constant voltage) for 60 min. After addition of the antiserum (concentrated twofold) to the slot, the plates were placed in a moist chamber at 4°C until the precipitin lines were fully developed. Contact prints of the preparations were made after they had been dried and stained with amido black.

Estimation of toxicity and toxin neutralization tests

For estimation of toxicity the material was used either undiluted or diluted $^1/_5$ and $^1/_{25}$ in 0.85 per cent NaCl and 0.1 ml injected intradermally into rabbits. All tests were done in duplicate and the diameter of the inflammatory reaction was measured 18 h after injection.

In order to test the toxin neutralizing activity of antisera, serial dilutions of the antiserum were made in 0.85 per cent NaCl and an equal volume of toxin was added to each tube. When crude toxin was tested, a solution containing 2 mg per ml was used and when partially purified toxin was tested a solution was prepared which gave a reaction of approximately 2 cm diameter.

After standing at room temperature for 30 min, 0.1 ml of the toxin-antiserum mixtures were injected intradermally into rabbits and the diameter of the skin reactions recorded after 18 h.

Tests for leucocidal activity of exotoxin and protoplasmic toxins

The viability of polymorphonuclear cells was determined on the basis of their ability to exclude trypan blue.

Trypan blue solution was prepared by dissolving 0.1 g of powder in 10 ml Hanks' solution. Crystals which formed were removed by filtering the solution through Seitz EK asbestos pads.

The toxicity of the toxins was first tested in rabbits and then either concentrated or diluted so that 0.1 ml injected intradermally gave a skin reaction of approximately 2 cm diameter.

Rabbit peritoneal polymorphonuclear cells were collected as described previously (Cameron, 1969) and after washing, the concentration was adjusted to approximately 7.0×10^6 cells per ml in Hanks' solution.

Serial fivefold dilutions of the toxins were made in saline and 0.5 ml of the leucocyte suspension was added to each tube. The tubes were incubated at 37°C for 60 min, then centrifuged at 500 × *g* for 5 min to deposit the leucocytes which were resuspended in 0.5 ml Hanks' solution.

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**Pharmacia Fine Chemicals, Inc., Uppsala, Sweden
†Oxoid Ltd., London
††Shandon Scientific Co., Ltd., London

Two drops of cell suspension were mixed with two drops of trypan blue solution and the number of dead and live leucocytes counted microscopically. Twenty fields representing approximately 150 cells were counted for each test.

To determine the anti-leucocidal effect of serum, 0.5 ml of undiluted $^1/_5$ and $^1/_{25}$ dilutions of the sera were mixed with 0.5 ml toxin and allowed to stand at room temperature for 30 min. Hereafter 0.5 ml of the leucocyte suspension was added to each tube, the mixtures incubated at 37°C for 60 min, centrifuged at 500 × *g* for 5 min and the number of live and dead leucocytes counted as described above.

Results

Evaluation of antisera against crude exotoxin and protoplasm

In order to assess the purity of the different fractions by immunological means it was necessary to prepare an antiserum which would produce as many precipitin lines as possible. The four antisera prepared against both crude exotoxin and protoplasm were examined by gel diffusion and immuno-electrophoresis. The pattern of precipitin lines obtained with two of the best antisera is shown in Fig. 1. As shown in Fig. 2, the multitude of lines which developed between protoplasm P50 and PAS could be more clearly demonstrated by immuno-electrophoresis.

From these results it is apparent that protoplasm P50 contains at least ten antigens. A notable observation was that two strong precipitin lines were formed between exotoxin and PAS indicating that these antigens have a cellular origin and are released during lysis of the bacteria. The EAS, however, did not contain antibodies to these antigens but did form an additional third line with the exotoxin.

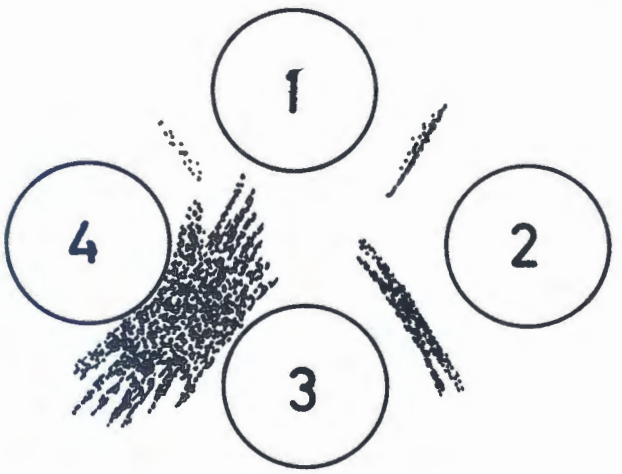

Fig. 1 Gel diffusion patterns obtained with protoplasm fraction P50, crude exotoxin and their corresponding antisera.
Well 1 = EAS Well 2 = Exotoxin
Well 3 = PAS Well 4 = P50

Toxicity of ammonium sulphate and alcohol fractions of protoplasm

The results of the toxicity estimations of the different fractions obtained by chemical methods are shown in Table 1. Fraction P50 was the most toxic but the other

Table 1 *Toxicity of protoplasm fractions prepared by ammonium sulphate followed by alcohol precipitation*

Toxin fraction	Diameter of skin reaction in cm		
	Dilutions		
	Undiluted	1/5	1/25
P30	1.4 1.5	1.3 1.2	1.0 1.0
P50	2.0 2.1	1.7 1.6	1.2 1.5
P80	0.9 1.0	0.8 0.9	0.5 0.6
3V	1.4 2.0	0.8 1.0	0.4 0.5
6V	0.8 1.0	0.5 0.8	0.4 0.2

Table 2 *Toxin neutralization tests with P50 fractions of exotoxin and protoplasmic toxins*

Toxin	Serum	Skin reactions in cm		
		Serum dilutions		
		Undiluted	1/5	1/25
Exotoxin P50. .	EAS 1	0.0	0.0	0.0
,, ,,	EAS 2	0.0	0.0	0.0
,, ,,	PAS 1	1.5	1.8	1.8
,, ,,	PAS 2	1.8	1.5	1.8
,, ,,	NRS	1.6	1.7	1.9
,, ,,	None	1.5	1.5	1.4
Protoplasm P50	EAS 1	0.0	0.0	0.0
,, ,,	EAS 2	0.0	0.0	0.2
,, ,,	PAS 1	1.3	1.3	1.4
,, ,,	PAS 2	1.4	1.3	1.4
,, ,,	NRS	1.3	1.2	1.2
,, ,,	None	1.4	1.2	1.2

EAS = Exotoxin antiserum
PAS = Protoplasm antiserum
NRS = Normal rabbit serum

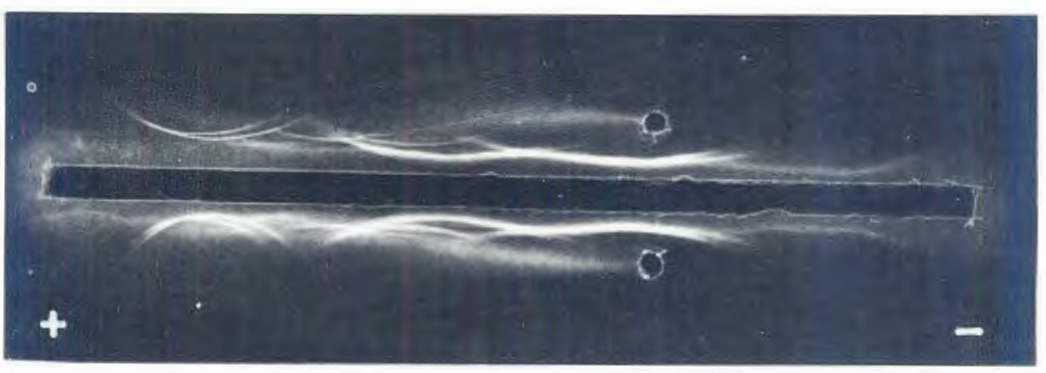

Fig. 2 Immunoelectrophoresis of protoplasm fraction P50 (5 times concentrated) against PAS antiserum (2 times concentrated)

ammonium sulphate fractions were also toxic and examination of these fractions by immuno-electrophoresis showed that they all contained most of the antigens present in the original protoplasm. This was particularly true for fraction P50. Further alcohol fractionation of P50 also yielded disappointing results and, as shown in Fig. 3, still contained a number of different antigens. Many of the fractions were poorly soluble and most of the toxic material was lost during the purification process. Consequently the small amount finally obtained did not warrant further examination.

Since protoplasmic toxin could not be purified by precipitation procedures, no further information regarding its relationship to the exotoxin could be obtained by immunodiffusion techniques.

The results of toxin neutralization tests aimed at elucidating the relationship between the exotoxin and protoplasmic fraction P50 are given in Table 2.

EAS completely neutralized both exotoxin and protoplasmic fraction P50 but PAS neutralized neither of the toxins despite the fact that, as already mentioned, it contained antibodies to numerous antigens.

Exclusion chromatography of exotoxin and protoplasm

In a further attempt to purify both products, and in order to avoid the excessive loss of material sustained during chemical fractionation, crude exotoxin and protoplasm were applied directly to Sephadex columns.

A typical elution curve for protoplasm is shown in Fig. 4. Toxicity assays on the fractions revealed that the protoplasm contains two toxins neither of which corresponds to any of the major protein peaks. Tubes 7 to 10 and 15 to 19 were pooled and designated Toxins A and B respectively. Toxic fractions obtained from three experiments were pooled, concentrated and recycled through Sephadex G 200.

It was observed that if Toxin A was dialyzed against 0.02 M phosphate buffer pH 7.2, a copious precipitate formed. This did not happen with Toxin B.

Crude exotoxin was similarly run through Sephadex G200. A typical elution curve is shown in Fig. 5.

The toxin was present in tubes 14 to 20 and this corresponds very closely to the elution position of protoplasmic Toxin B.

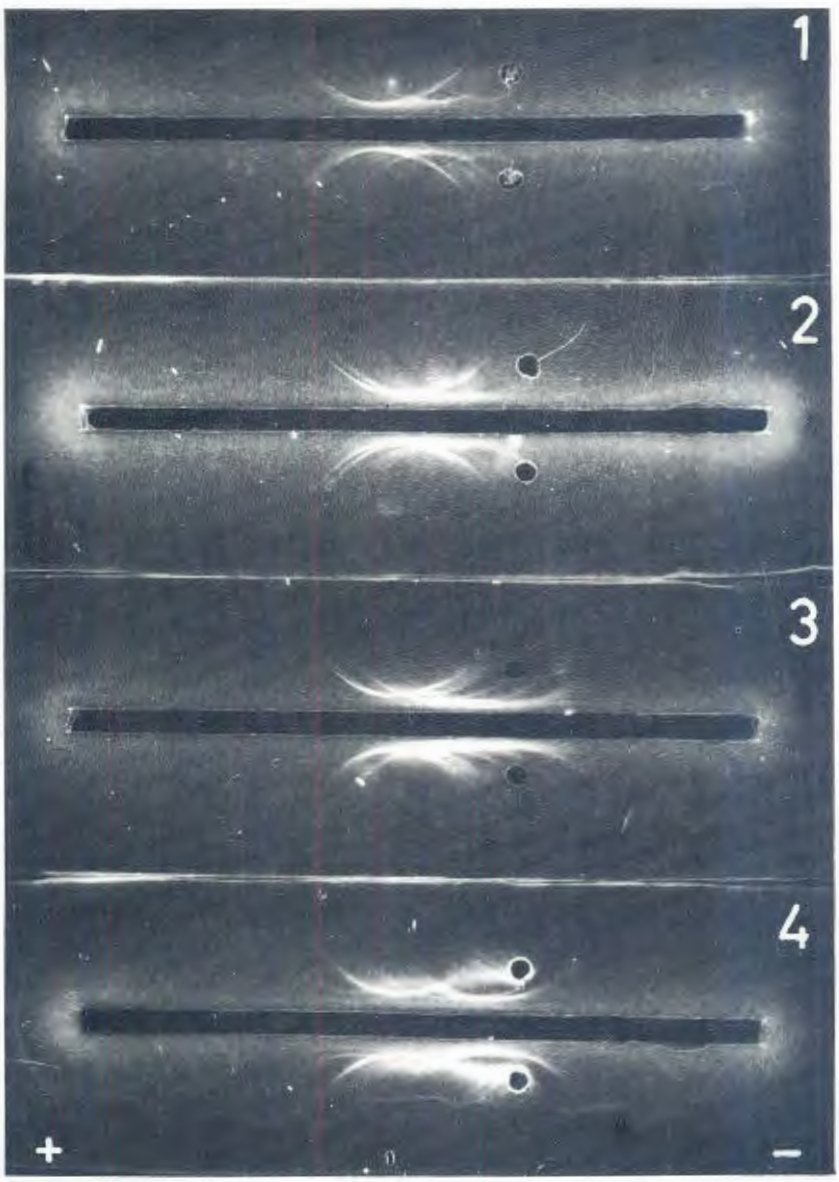

FIG. 3 Immunoelectrophoretic examination of ammonium sulphate and alcohol fractions of protoplasm. Slide 1 - Alcohol fraction 6V. Slide 2 - Alcohol fraction 3V. Slide 3 - Ammonium sulphate fraction P50. Slide 4 - Ammonium sulphate fraction P30

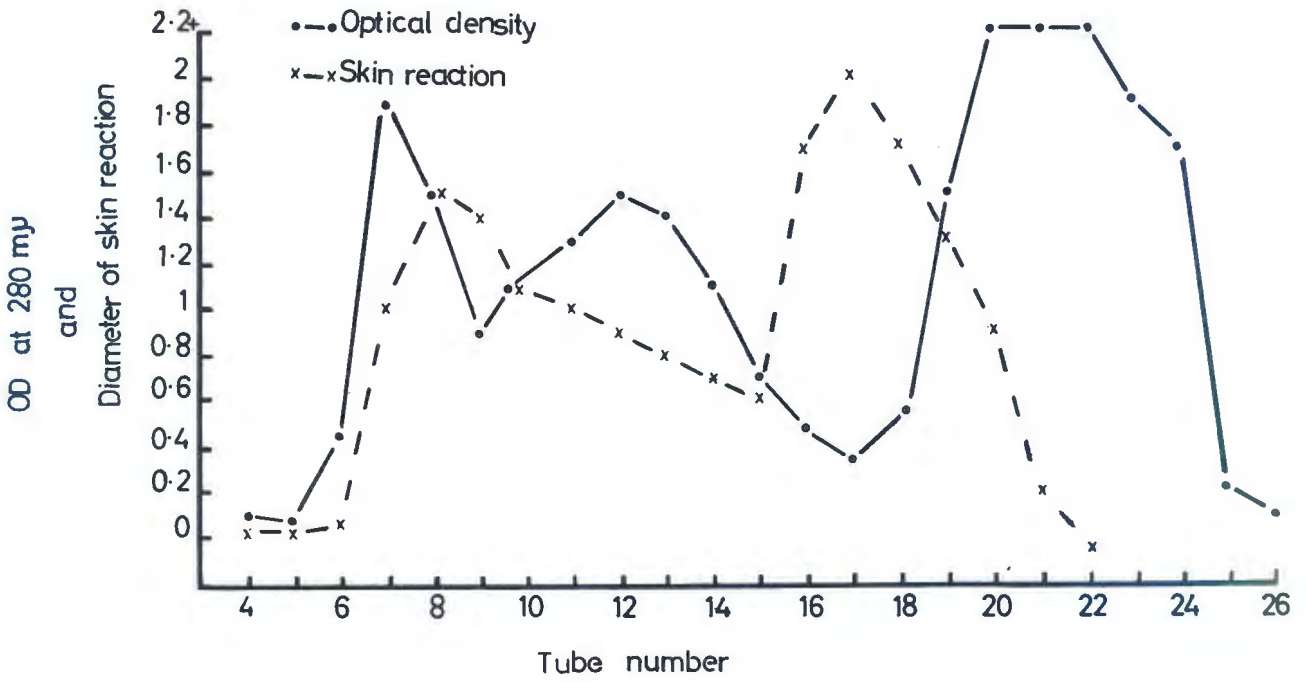

FIG. 4 Chromatography of protoplasm on Sephadex G200. Note discrepancies between protein peaks and fractions showing greatest toxicity. The same scale is used on the ordinate to indicate both optical density and diameter of skin reaction in cm. Toxin A = Tubes 7–10. Toxin B = Tubes 15–19

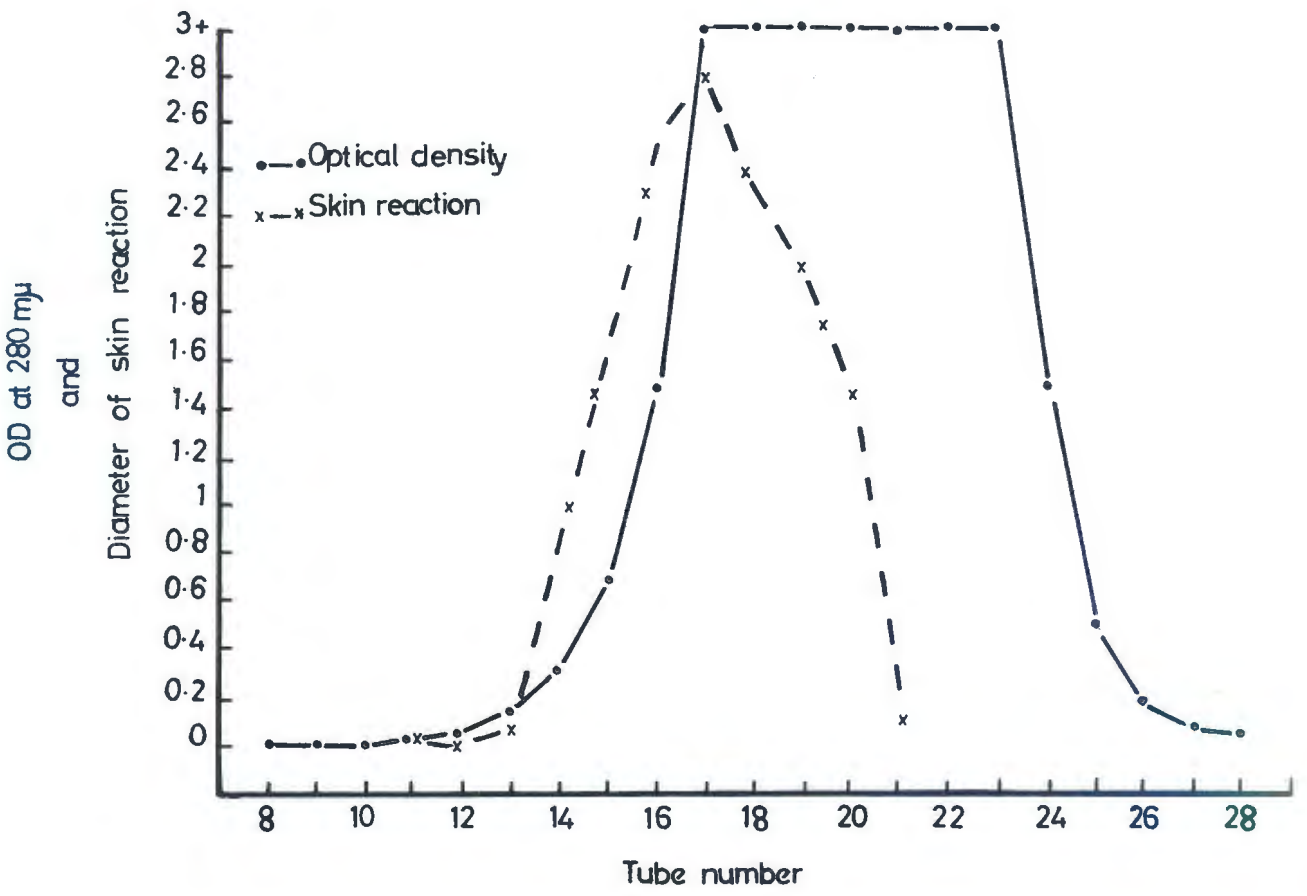

FIG. 5 Chromatography of exotoxin on Sephadex G200. Exotoxin is eluted before most of the protein

Toxin neutralization tests

The results of toxin neutralization tests with the three toxins are given in Table 3.

EAS neutralized both exotoxin and protoplasmic Toxin B, but not protoplasmic Toxin A. PAS on the other hand, had no demonstrable antitoxic effect on any of the toxins.

TABLE 3 *Toxin neutralization tests with Sephadex fractions of exotoxin and protoplasmic toxin*

Toxin	Anti-serum	Skin reactions in cm		
		Antiserum dilutions		
		Undiluted	1/5	1/25
Exotoxin	EAS	0.3	0.5	1.0
„	PAS	1.1	1.4	1.9
„	NRS	1.4	1.4	1.4
„	None	1.8	1.9	1.7
Protoplasmic Toxin A	EAS	1.9	1.5	1.4
„ „	PAS	1.5	1.8	1.8
„ „	NRS	1.2	1.4	1.8
„ „	None	1.7	2.0	2.0
Protoplasmic Toxin B	EAS	0.3	0.5	0.5
„ „	PAS	1.4	1.4	1.4
„ „	NRS	1.5	1.4	1.4
„ „	None	1.3	1.2	1.5
None	EAS	0.0	0.0	0.0
„	PAS	0.0	0.0	0.0
„	NRS	0.0	0.0	0.0
„	None	—	—	—

EAS = Exotoxin antiserum
PAS = Protoplasm antiserum
NRS = Normal rabbit serum

These findings indicate that the exotoxin and protoplasmic Toxin B are identical. As shown in Fig. 6, neither of the toxic fractions precipitated with EAS and consequently their identity could not be conclusively proved by gel diffusion. They did, however, produce precipitin lines with PAS but because this antiserum had no antitoxic activity, none of these precipitin lines can be equated with a toxin-antitoxin reaction.

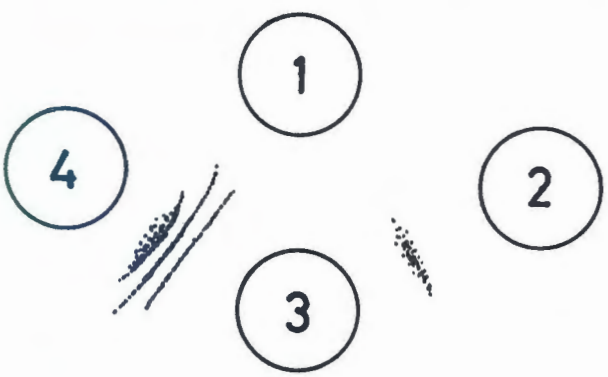

FIG. 6 Gel diffusion patterns of partially purified exotoxin and protoplasmic Toxin B against EAS and PAS.
Well 1 = EAS Well 2 = Exotoxin
Well 3 = PAS Well 4 = Protoplasmic Toxin B

Leucocidal activity of Exotoxin, Toxin A and Toxin B

A further parameter for examining the toxins was sought. Considering the necrotizing nature of the toxins, their leucocidal activity was tested. From Table 4 it can be seen that all the toxins are leucocidal. Toxin A was the most active. Exotoxin and Toxin B were less so, but approximately equally toxic.

TABLE 4 *Leucocidal activity of exotoxin and protoplasmic Toxins A and B*

Toxin	Percentage dead leucocytes			
	Dilutions			Average
	Undiluted	1/5	1/25	
Exotoxin	29.7	33.7	39.7	34.4
Protoplasmic Toxin A	99.0	65.9	61.1	75.7
Protoplasmic Toxin B	35.7	54.3	41.6	44.5
0.85% NaCl . . .	10.7			10.3
	10.7			
	9.4			

The antileucocidal activity of EAS and PAS on the exotoxin and Toxin B was used to examine the relationship between the two toxins. The results shown in Table 5 are somewhat difficult to interpret because the antisera used had some detrimental effect on the leucocytes. In general, however, they support those which were obtained by toxin neutralization tests.

TABLE 5 *Neutralization of leucocidal activity of exotoxin and protoplasmic Toxin B*

Toxin	Antiserum	Percentage dead leucocytes
Exotoxin	EAS	29.6
„	PAS	58.7
„	None	66.6
Protoplasmic Toxin B	EAS	17.6
„ „	PAS	34.3
„ „	None	68.5
None	EAS	47.0
„	PAS	50.9

EAS = Exotoxin antiserum
PAS = Protoplasm antiserum

DISCUSSION

In a previous paper (Cameron & Minnaar, 1969) it was reported that immunity against *C. pseudotuberculosis* could be obtained by immunizing guinea pigs and mice with whole dead bacteria. These, however, contain toxic substance(s) in the protoplasm which cause the development of sterile abscesses (Cameron, 1964). It was therefore of interest to examine the nature of the protoplasmic toxin(s), particularly concerning their relationship to the exotoxin.

Gel chromatography of protoplasm revealed that it contains two toxic substances. Toxin A is eluted from Sephadex G200 in the void volume and precipitates readily in 0.02 M phosphate buffer pH 7.2. It is not neutralized by either EAS or PAS. Toxin B is a lower molecular weight substance and its toxic activity is neutralized by EAS but not by PAS.

It therefore appears that protoplasmic Toxin B is identical to the exotoxin because both are eluted from Saphadex G200 in the same position and both are neutralized by the same antiserum. This antiserum, however, gave no precipitin lines with either of the toxins even when the more sensitive Preer tube technique was used.

Antiserum to protoplasm on the contrary had no toxin neutralizing activity but did produce precipitin lines in agar gel. This indicated that none of the lines observed can be equated with a toxin-antitoxin reaction.

The precipitin tests also revealed that the crude exotoxin and even the partially purified exotoxin produce precipitin lines with protoplasmic antiserum. This proves that the culture fluid contains antigens which are derived from the bacterial cells and provides further

evidence that the exotoxin found in the culture fluid is also a product of bacterial lysis. Similarly, it can be expected that crude exotoxin preparations could also contain other antigens, some of which may be responsible for inducing immunity. Therefore, unless an absolutely pure preparation of exotoxin can be produced, its immunizing activity can never be accurately assessed.

It has been possible to induce a solid immunity by using cell walls only (Cameron, Minnaar & Purdom, 1969). Hence, despite the fact that exotoxin plays a prominent role in the pathogenesis of *C. pseudotuberculosis* infection (Jolly, 1965), antitoxic immunity is probably of little consequence in establishing resistance to chronic infections, unless the exotoxin specifically originates from the cell wall.

Summary

Gel chromatography of *C. pseudotuberculosis* protoplasm revealed that it contains two toxic fractions designated A and B. Toxin B was shown to be identical to the exotoxin by means of toxin neutralization tests. Exotoxin preparations were also shown to contain antigens derived from the bacterial cells. These findings strongly support the contention of Petrie & McClean (1934) that the exotoxin is produced as a result of bacterial lysis.

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