

IDENTIFICATION OF THE PROTECTIVE AND TOXIC ANTIGENS OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS*

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INTRODUCTION

Cameron (1964) showed that the immunizing property of *Corynebacterium pseudotuberculosis* cells was closely associated with the toxicity of cell endoplasm and the ability of formalin-killed cells to produce sterile abscesses. He also found that the presence of toxic factors in the cells depended on the culture medium to a large extent. Furthermore Cameron & Swart (1965) developed a fluid medium and method of production of large quantities of highly toxic cells.

The purpose of this study was to determine whether the toxic factors in the cells are identical to the protective antigen(s) and to identify the protective antigen.

MATERIALS AND METHODS

Preparation of cell fractions

C. pseudotuberculosis strain 137 was grown for 48 hours, collected by centrifugation, washed twice with distilled water and freeze-dried (Cameron & Swart, 1965).

Endoplasm was obtained by ultrasonic disruption. A suspension of 10 gm cells per 100 ml 0.01 M sodium phosphate buffer pH 7.4, was mixed for 15 minutes by means of a magnetic stirrer and allowed to stand at 4° C for a further 15 minutes. The suspension was then cooled to -4° C and subjected to ultrasonic vibration for 2½ hours in a Branson model 575 sonifier with step horn. The output was set at 75 watts giving a flow of 4 amps. The temperature of the cooling fluid was never allowed to rise above 5° C. This method yielded maximal soluble protein.

Unbroken cell walls and other insoluble cell debris were removed by centrifugation at 20,000 g for 30 minutes. The supernatant fluid was drawn off and the sediment resuspended in half the original volume. This was again centrifuged and the supernatant fluid added to that obtained previously. The final pH of the solution was 7.0. This antigen solution was designated C 48.

Two ammonium sulphate precipitates were prepared as follows: To every 100 ml of the extract was added 16.3 gm crystalline ammonium sulphate and after thorough stirring its pH was adjusted to 7.0 with N NaOH. After standing for 2 hours at 4° C it was centrifuged at 20,000 g for 20 minutes and the supernatant fluid, designated S23, drawn off. The sediment, P23, was washed twice with an ammonium sulphate solution of the concentration as above and dissolved in 0.01M sodium phosphate buffer pH 7.4. This P23 fraction formed a colloidal solution and tended to precipitate out easily.

At this stage a further 18.86 gm ammonium sulphate per 100 ml extract was added to the S23 portion to give a final concentration of 35.16 gm/100 ml and the pH adjusted to 7.0. The solution was allowed to stand at 4° C for 15 minutes and the precipitate removed by centrifugation at 20,000 g for 20 minutes. The supernatant fluid was designated S50. After washing with an ammonium sulphate solution of the same concentration as above, the sediment was dissolved in 0.01M sodium phosphate buffer pH 7.4 and designated P50. The P50 precipitate dissolved readily to give a clear yellow solution.

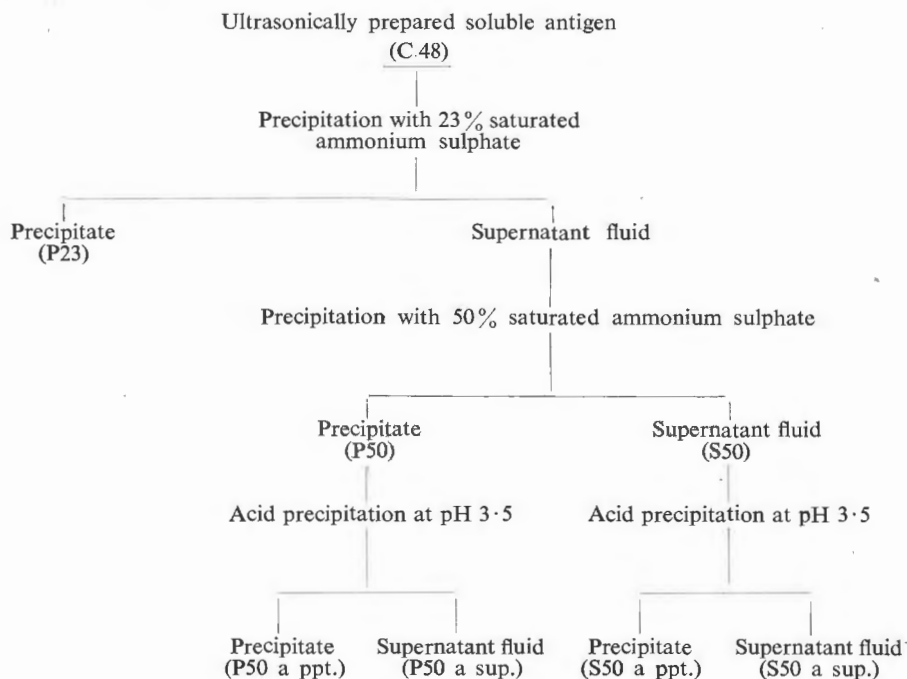
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All the fractions were dialyzed overnight against 15 litres 0·01M sodium phosphate buffer at 4° C. This was repeated twice to remove all residual ammonium sulphate.

The S50 and P50 portions were subjected to further acid fractionation by adjusting their pH to 3·5 with N HCl. In both a milky precipitate formed which was removed by centrifugation at 20,000 g for 30 minutes. After washing and redissolving of the precipitate, the pH of all four fractions was adjusted to 7·0 and marked S50 a. sup., S50 a ppt., P50 a sup. and P50 a ppt.

The procedure followed is shown schematically in Figure 1.

FIGURE 1.—Diagrammatic scheme for preparation of cell fractions



In order to maintain the original quantitative ratio of the different antigen fractions, the ammonium phosphate and acid precipitates were resuspended in volume of buffer equal to the volume from which they were prepared. When concentrated antigens were required, the fractions were freeze-dried and the dry material resuspended in the desired volume of buffer. All the fractions were stored at -20° C.

Protein determinations were done by the method of Lowry *et al.* (1951). For comparison a standard curve was set up with dilutions of albumin (N.B.C.) in veronal buffer pH 7·3 covering a range from 0 to 180 γ /ml. This was prepared from a stock solution containing 400 γ /ml.

Attempts to extract either toxic factors or protective antigens chemically from the cells were done by the following methods: acid (Dubos & Geiger, 1946), alkali (v. Heyningen & Gladstone, 1953), phenol water (Mergenhausen *et al.*, 1961), chloroform (Boivin & Mesrobeaunu, 1937), sodium desoxycholate (Barber & Soare, 1962), veronal buffer (Barber, 1961), T.U.F. (Maitland & Guerault, 1958), ether (Ribi *et al.*, 1961).

The toxicity of the fractions was determined by intradermal injection in rabbits (Cameron, 1964).

Preparation of antisera

Whole cell antigen was prepared by suspending 0.15 gm cells in 100 ml 0.5 per cent formol-saline and incubating at 37° C for 48 hours. Antigens for producing antisera to the different fractions were prepared from the original fractions. Formalin (0.5 per cent) was added to each fraction to kill all possible contaminants and inactivate any toxic factors. The P25, P50 and S30 fractions contained 0.6 mg protein per ml.

In preliminary experiments formolized antigen gave better results than phenol, acetone or alcohol treated antigen. Since there was an appreciable difference in antibody response in different rabbits, three rabbits were used for every antigen, and the serum giving the clearest precipitin lines used for further experiments.

Each animal was given 5 ml antigen subcutaneously and after three weeks the intravenous injections were started. These were given on the first three days of every alternate week, viz 1.0 ml the first week, 2.0 ml the third week and 3.0 ml during the fifth week. The rabbits were bled ten days after the last injection and the sera stored at -20° C.

Immunity experiments

Antigens used in the immunity experiments were prepared in the same way as those used for preparing antisera.

Groups of 25 guinea pigs were used to test the immunizing power of each antigen. Each guinea pig was given two injections of 1.0 ml each with an interval of three weeks. Half of each dose was given subcutaneously and the rest intramuscularly. They were challenged ten days after the last dose of antigen by injecting 0.1 ml of a 10⁻³ dilution of a cell suspension containing 0.1 ml packed cells per 100 ml. The material was administered subcutaneously on the abdomen. The guinea pigs were slaughtered six days after challenge and examined for the presence of abscesses. Figures of 4+, 3+, 1+ and 0 were allocated to each animal according to the extent and severity of the lesions. Unimmunized controls were similarly assessed and the difference in positive units between the control and immunized groups was expressed as the percentage protection.

Precipitin tests

Micro-precipitin tests were done on microscope slides covered with 5.0 ml 1.0 per cent Ion-agar (Difco) in distilled water. Sodium azide (0.5 per cent) was incorporated in the agar to control air contamination. Wells, 2.0 mm diameter and 4.0 mm apart, were made in the agar by means of a small cork bore and perspex template. One drop of reagent was placed in each well, the slides placed in petri dishes with a disc of moist blotting paper, and left in an incubator at 37° C overnight. The slides were examined in oblique light against a dark background for the presence of precipitin lines. This method gave excellent results.

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Immuno-electrophoresis

These tests were done in agar on microscope slides using a series of different buffers according to the method of Ward-Cox (1965).

EXPERIMENTAL RESULTS

The results of a typical experiment conducted to determine the immunizing and toxic properties of the main fractions are shown in Table 1.

TABLE 1.—*Comparison of immunizing properties and toxicity of cell fractions*

Vaccine or fraction	Toxicity for rabbits		Immunity in guinea pigs	
	Concentration mg/ml	Increase in skin thickness mm Dose 0.5 ml i.d. of a 1:5 dilution	Antigen concentration mg/ml	Overall % protection
Whole cells.....	—	n.t.	1.5	17.7
Complete soluble antigen (C48)	4.09	13.0	0.6	6.0
Fraction P23.....	1.62	6.0	0.6	7.0
Fraction P50.....	1.54	9.0	0.6	0.8
Fraction S50.....	0.92	1.0	0.6	12.6

i.d. = intradermal

n.t. = not tested

It is clear that fraction P23 and especially fraction P50 are responsible for the toxic effects of the complete soluble antigen, Fraction P23 has some protective value but P50 has virtually none. Fraction S50 is non-toxic and it gave far better protection than P23 and P50.

Initial experiments in which different techniques were used for demonstrating precipitin lines, showed that different patterns were obtained depending on the distance between the wells containing the antigen and the antiserum. Certain lines appeared when the antigen was undiluted, while they disappeared and others appeared with diluted antigen. For reproducible results, all tests have to be done on a standard-sized precipitin slide, only one selected antiserum used and the optimal concentration of each antigen accurately determined.

By testing dilutions of a preparation of C48 antigen against its homologous antiserum it was found to consist of three main precipitinogens. As can be seen in Plate 1, the X, A and B lines are visible at a 1:2 dilution of the antigen. At dilutions of 1:4 and 1:8 the X and A lines disappear and the B line separates clearly into B₁ and B₂ lines.

The lines were arbitrarily designated X, A and B to facilitate identification and reference.

The other fractions were concentrated by drying, diluted and similarly tested against the same serum and the protein content of the dilutions which gave the clearest lines determined.

The following values were obtained:

Fraction	Concentration of protein giving clearest precipitin lines
C48.....	5·675 mg/ml and 2·754 mg/ml
P50.....	0·60 mg/ml
P50 a sup.....	0·04 mg/ml
P50 a ppt.....	7·128 mg/ml
S50.....	0·66 mg/ml or higher
S50 a ppt.....	3·089 mg/ml

As previously stated, fraction P23 was very insoluble and no precipitin lines could be obtained with it, even at very high concentrations. The S50 a sup. fraction also contained no detectable precipitating antigen.

By cross precipitin tests shown in Plate 2 it was possible to demonstrate that fraction P50 contained antigens representing precipitin lines B₁ and B₂ and that fraction S50 contained antigens producing lines corresponding to A and B₂. The B₂ lines produced by fractions P50 and S50 did not join completely. This may be explained by the results of the following test in which it was found that the B₂ line is not single but composed of at least two components.

Plate 3 shows the lines obtained with P50 and its acid fractions. The precipitate contained only the antigen producing line B₂. Although not very clear on the photograph, this line consists of two components. This clearer differentiation probably resulted due to the absence of other precipitin lines. Fraction P50 a ppt. represented about 75 per cent of the protein content of the original P50 fraction and only produced a precipitin line when the protein content was about 7·0 mg/ml. It was shown to be non-toxic. The supernatant liquid retained both antigens, giving lines B₁ and B₂ and it produced distinct lines at a relatively low protein concentration, viz. 0·04 mg per ml and was toxic.

Acid precipitation of the S50 fraction did not result in any further separation of antigens. Both antigens giving the A and B₂ lines were present in the precipitate and, although the supernatant fluid retained a little protein, this fraction did not give rise to any precipitin lines.

The antigen corresponding to the X line could not be demonstrated in any of the fractions. It is suspected that it is present in the P23 fraction and that failure to produce a corresponding precipitin line was due to the poor solubility of this fraction.

Attempts to extract protective, toxic, or precipitating antigens by various chemical methods resulted in failure, but other techniques still have to be investigated (Tauber & Russel, 1961; Crowle, 1963; Fukushi, *et al.*, 1964).

Preliminary immuno-electrophoretic studies revealed the same antigens as were demonstrated by means of precipitin tests. The lines could be identified by means of parallel tests using whole C48 antigen and P50 and S50 fractions on opposite sides of a central channel containing antiserum. All the fractions migrated to the anode in the order X, B₁, A and B₂. The last two were often close together depending on the buffer used. No additional lines were observed and the two components of the B₂ fraction could not be distinguished.

DISCUSSION

The fact that formalin-killed *C. pseudotuberculosis* cells are able to produce sterile abscesses when injected into experimental animals, poses problems as far as vaccine production is concerned.

The results presented here, however, show that the toxic factors of the cells are distinct from the main endoplasmic protective antigen. Studies using column chromatography, such as have been done with *Brucella* (Glenchur *et al.*, 1963; Wolf & Live, 1964), would enable the preparation of more refined fractions. Chemical analysis of purified endotoxin and protective antigen may reveal that either one or the other could be extracted from the cells by chemical methods. This would facilitate the preparation of a vaccine devoid of toxic factors.

In certain bacterial species the protective antigen has been shown to be closely associated with the cell wall (Oprescu, 1961; Munoz, 1963; Sutherland 1963; Fregman & Smith, 1963; Griffiths & Mason, 1964; Omland, 1964). Therefore the protective properties of the cell wall and other antigen fractions of *C. pseudotuberculosis* must be thoroughly investigated before it can be stated without any doubt that the S50 fraction is the sole protective antigen.

Biological determination of immunity to *C. pseudotuberculosis* is tedious and, as it is relatively inaccurate, large numbers of experimental animals are required to obtain significant results. With the use of purified protective antigen it should be possible to detect protective antibody in the sera of immunized animals by means of precipitin or other serological tests *in vitro*.

SUMMARY

It has been shown that the toxic factors and the immunizing antigens in the cells of *C. pseudotuberculosis* are distinct. These different antigens can be partially separated by ammonium sulphate and acid precipitation and correspond to certain precipitinogens. The antigens were also successfully separated by electrophoresis.

The dominating protective antigen is present in the S50 fraction and is represented by the A line.

The toxic factor is present in the P50 and P50 a sup. fractions and corresponds to the B₁ line. The P23 (X line) fraction is somewhat toxic and is slightly immunogenic.

The antigen present in the P30 a ppt. fraction gives rise to the B₂ line and is neither protective nor toxic.

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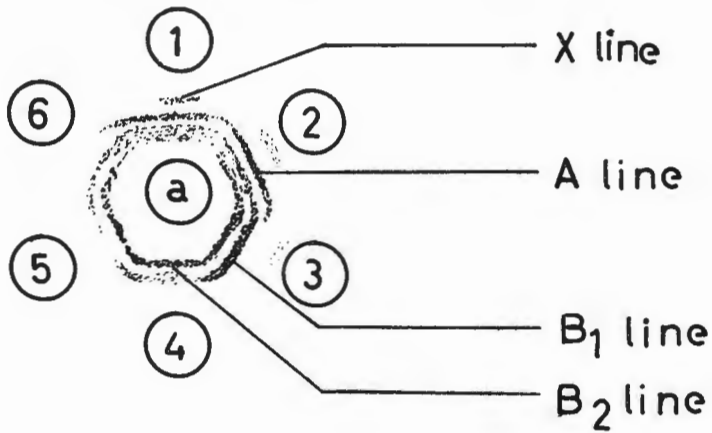
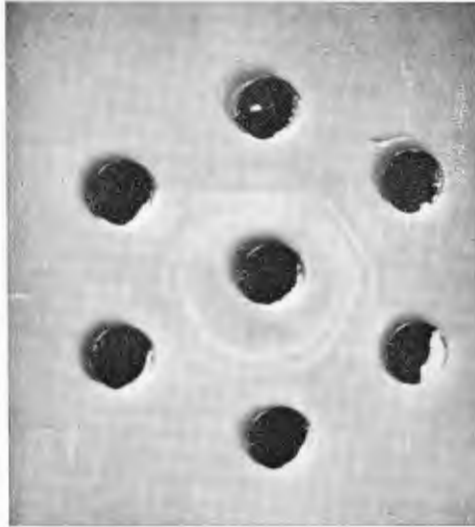


PLATE 1.—*Demonstration of various precipitinogens in C48 antigen.*

Well a—rabbit antiserum to C48 antigen.

Well 1—C48 antigen undiluted.

Well 2—C48 antigen 1:2 dilution (5·675 mg/ml).

Well 3—C48 antigen 1:4 dilution (2·754 mg/ml).

Well 4—C48 antigen 1:8 dilution.

Well 5—C48 antigen 1:16 dilution.

Well 6—C48 antigen 1:32 dilution.

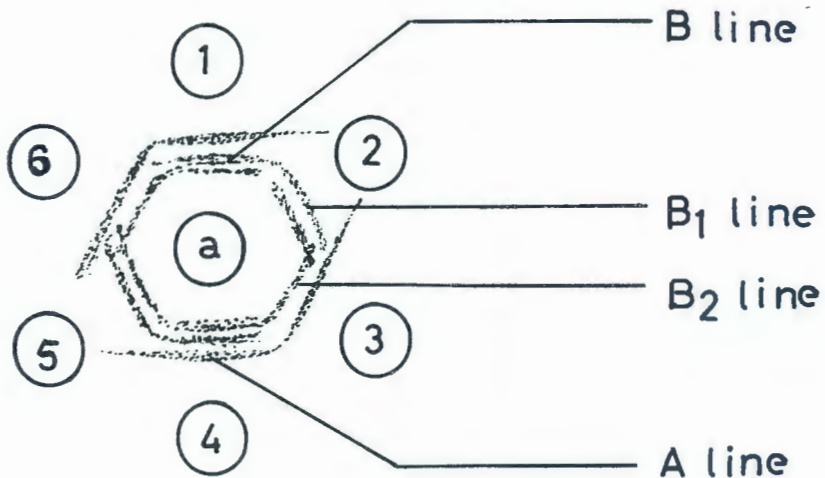


PLATE 2.—*Identification of precipitinogens present in fractions P50 and S50.*

Well a—rabbit antiserum to C48 antigen.

Wells 1 and 4—C48 antigen (5·675 mg/ml).

Wells 2 and 5—P50 fraction (0·6 mg/ml).

Wells 3 and 6—S50 fraction (0·66 mg/ml).

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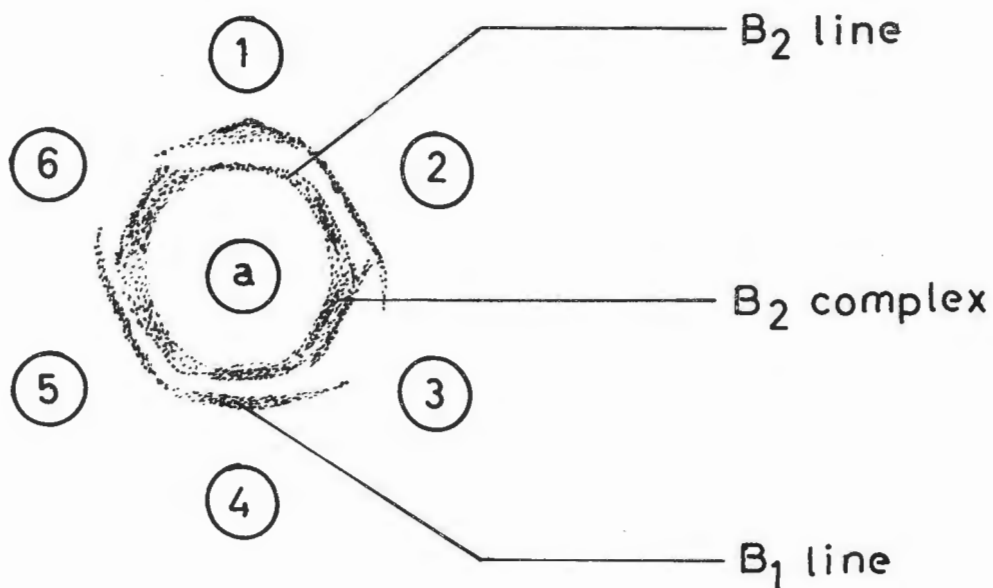
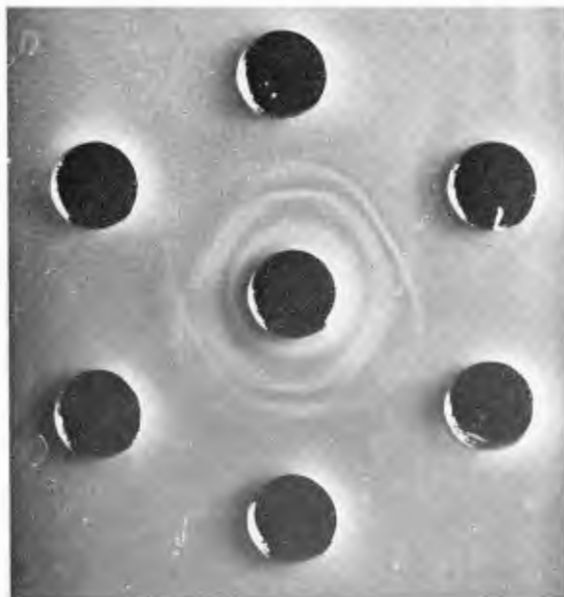


PLATE 3.—Precipitinogens present in fractions P50, P50 a sup., and P50 a ppt.

Well a—rabbit antiserum to C48 antigen.
 Wells 1 and 4—P50 fraction (0·6 mg/ml).
 Wells 2 and 5—P50 a sup. (0·04 mg/ml).
 Wells 3 and 6—P50 a ppt. (7·128 mg/ml).