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THE SIGNIFICANCE OF THE ENDOTOXIN AND PYOGENIC FACTOR OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* IN IMMUNITY

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INTRODUCTION

Studies on *C. pseudotuberculosis* (the Preisz-Nocard bacillus) have revealed that it produces an exotoxin and contains a number of somatic antigens (Petrie & McClean, 1934; Bull & Dickenson, 1935; and Carne, 1940).

The exotoxin stimulates the production of antibodies protecting experimental animals against lethal doses of exotoxin. But it is not capable of stimulating immunity against infection with living organisms (Carne, 1956). Petrie & McClean (1934) postulated that the exotoxin has an intracellular origin and therefore antitoxin could possibly mitigate the effect of acute lethal infections as described by Robinson (1928, 1929) and Carne (1939) in sheep. They were able to protect horses against acute infection by repeatedly administering large doses of toxoid, toxin and killed and living organisms. These results, however, do not necessarily prove the identity of the exotoxin and any particular cellular component.

The pyogenic action of the organism has been studied by Bull & Dickenson (1935). They found that injection of formalin or heat killed cells, produced sterile abscesses, but could no longer cause acute death in guinea-pigs. Cells killed by toluol or chloroform, however, retained their lethal properties.

Carne (1956) described a toxic surface lipid. Removal of this lipid by ether extraction did not affect the viability of the organisms. It was not shown to be immunogenic.

The purpose of the present investigation was to examine the possibility of immunizing sheep against caseous lymphadenitis and epididymitis. As the exotoxin and surface lipid are of no consequence, the somatic antigens of *C. pseudotuberculosis* were investigated.

The factor responsible for abscessation by formalin killed cells was studied to determine what role it plays in immunity and how it could be effectively inactivated without destroying possible antigenic properties.

In order to obtain a high yield of cells on fluid medium, the organisms must be induced to grow as a pellicle (Carne, 1940). Experiments were, therefore, conducted to develop a medium on which a pellicle would form spontaneously.

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MATERIALS AND METHODS

(1) *Strains*

Strain 137B used in these studies, was obtained from Prof. H. R. Carne of the University of Sydney. It was originally isolated from an Australian sheep in 1950, passaged through a guinea-pig and subsequently stored freeze-dried in skimmed milk. Strains 5113C and 12624 were isolated from two cases of caseous lymphadenitis in goats in South Africa.

(2) *Media and Cultivation*

(*a*) *Solid medium:* Unless otherwise indicated, the organisms were grown on blood tryptose agar (bovine, Difco) in Mason tubes for 48 hours at 37°C. The cells were collected by means of a curved glass rod, washed three times in sterile physiological saline, freeze-dried and stored at 4°C until required.

(*b*) *Fluid cultures:* Nutrient beef broth used as the basic medium was prepared as follows: Lean beef and tap water in the proportion of 200 gm to 1 litre were boiled for 60 min, the broth siphoned off, filtered through butter muslin and cottonwool, and the volume restored. Peptone (Oxoid) 1 gm per 100 ml; NaCl 0·3 gm per 100 ml; $Na_2HPO_4.12H_2O$ 0·2 gm per 100 ml were then added and the broth simmered for 15 min. The pH was adjusted to 7·8 and the mixture sterilized by autoclaving at 15 lb/in^2 for 60 min in 20 litre quantities.

Various additional peptones, vitamin supplements and fermentable carbohydrates were added in different combinations and concentrations to the broth and tested for their ability to improve the yield.

The medium was prepared by adding unsterilized nutrients to presterilized broth, dissolved by heating and distributed in 500 ml amounts in large Roux flasks. The media were then sterilized by autoclaving at 15 lb pressure per in^2 for 30 min. With the flasks in a horizontal position, a ratio for surface area (cm^2) to volume (ml) of 0·833 was obtained, approximating Carne's (1940) recommendation.

A semi-synthetic medium suitable for use where facilities for preparing broth are not available, was also developed.

Its composition was as follows:—

Lab. Lemco (Oxoid)	3·00 gm
Proteose peptone (Difco)	10·00 gm
Yeast extract (Difco)	5·00 gm
" Tryptone " (Oxoid)	10·00 gm
NaCl	3·00 gm
$Na_2HPO_4.12H_2O$	5·71 gm
KH_2PO_4.anhydr	0·50 gm
Distilled water to	1,000 ml

The pH was adjusted to 7·8 and subsequently the medium was sterilized in 500 ml quantities by autoclaving at 15 lb pressure per in^2 for 30 min.

The growth from the fluid cultures was collected by centrifugation, washed, freeze-dried and weighed to determine the yield.

(3) *Preparation of endotoxin and cell wall suspensions*

The cells were disrupted by grinding with " Alundum " (90 mesh) combining the methods of McIlwain (1948), Schachman, Pardee & Stonier (1952) and Yoneda & Matsuda (1961).

(*a*) Dry cells were suspended in 0·5 per cent phenol-saline (0·5 gm cells to 2 ml fluid) in a porcelain mortar. To this suspension 3 gm of " Alundum 90 " was added and the mixture ground by hand with a pestle in an ice bath at 4°C for 30 min. Thirty ml cold phenol-saline was then added and the endoplasma, cell walls, intact cells and debris separated by differential centrifugation according to the method of Glenchur, Seal, Zinneman & Wendell (1963), and Takeya & Hisatsune (1963).

The ground cells in suspension were centrifuged at 4,000 g for 60 min, the supernatant collected and centrifuged at 10,000 g for 60 min to remove all cell walls. The endotoxin thus obtained was stored at −20°C in sealed vials.

(*b*) Cell wall suspensions were prepared by suspending the first sediment obtained in 30 ml phenol-saline and centrifuging at 3,000 g for 30 min. The supernatant containing cell walls, was collected by means of a syringe. The sediment was resuspended in 30 ml phenol-saline, again centrifuged at 3,000 g for 30 min and the supernatant collected. By this method a reasonable yield of cell walls containing no unbroken cells, was obtained. The cell walls were washed three times with saline by centrifuging at 10,000 g for 60 min, treated with Trypsin (Difco) by the method of Takeya & Hisutsane (1963), resuspended in the original volume and stored in sealed vials at −20°C.

(4) *Animal inoculation to determine the presence of endotoxin and pyogenic factors*

(*a*) Whole cell suspensions were prepared by suspending 0·15 gm of dry cells in 100 ml sterile saline.

These suspensions were treated as indicated in Table 5 and groups of four guinea-pigs injected subcutaneously with 0·5 ml and 1·0 ml respectively to determine pyogenicity. They were slaughtered six days after injection and examined for abscess formation. Cultures on blood tryptose agar were made from all abscesses.

(*b*) The toxicity of the endoplasm and cell walls was determined by injecting 0·5 ml of undiluted, 1/5 and 1/10 saline dilutions of the endotoxin intradermally into rabbits. The reactions were read after 48 hours by measuring the increase in skin thickness by means of calipers used for tuberculin tests. An increase of less than 0·5 mm without evidence of erythema and oedema, was regarded as negative.

Lethal activity was tested by injecting 1·0 and 2·0 ml of the products to be tested subcutaneously into separate guinea-pigs. Four guinea-pigs were used per dose and observed for six days before the reaction was assessed.

(*c*) The presence of exotoxin in culture filtrates was tested for in rabbits and guinea-pigs by the methods of Carne (1940).

(*d*) The pyogenic effect of cell wall suspensions was determined by subcutaneous injection of 1·0 ml and 0·5 ml into four guinea-pigs each. They were examined for the presence of abscesses at autopsy six days after injection.

(5) *Immunity tests*

(*a*) *Preparation of bacterins*

Dried cells of strain 137B were suspended in saline in the proportion of 1·5 gm cells to one litre of saline. Portions of this suspension were treated as follows:—

0·4% Formalin 48 hours	37°C
0·5% Phenol 48 hours	37°C
0·1% Beta-propiolactone	37°C
Autoclaving	120°C 30 min.

(*b*) *Immunization of guinea-pigs*

Eight groups of 30 guinea-pigs each were injected with the following bacterins:—

Formalin killed,
Phenol killed,
Beta-propiolactone killed,
Heat killed.

Each guinea-pig was given three injections of 0·5 ml subcutaneously at intervals of two weeks.

(*c*) *Challenge of immunized guinea-pigs*

Preliminary experiments to determine the I.D. 50 showed that when 0·1 ml of a cell suspension of 0·1 per cent packed cell volume density was diluted to 10^{-4} in saline and injected subcutaneously, an infection rate of approximately one I.D. 50 was obtained. The results obtained in these experiments are tabulated in Table 1.

TABLE 1.—*Determination of I.D.* 50

Dilution	1st Titration		2nd Titration		3rd Titration	
	Guinea-pigs infected	Guinea-pigs not infected	Guinea-pigs infected	Guinea-pigs not infected	Guinea-pigs infected	Guinea-pigs not infected
10^{-1}	6	0	6	0	6	0
10^{-2}	4	2	6	0	5	1
10^{-3}	3	3	4	2	5	1
10^{-4}	4	2	3	3	4	2
10^{-5}	2	4	3	3	2	4
10^{-6}	1	5	2	4	2	4

The immunized guinea-pigs and controls were challenged three weeks after the last injection. One I.D. 50 was used as the challenge dose throughout.

Twenty-five guinea-pigs of each group selected at random were sacrificed one week after challenge and examined for abscess formation.

The extent of abscessation was assessed as follows:—

Size of abscess—Diameter in mm	*Interpretation*
1– 4	1+
5– 8	2+
9–12	3+
13–16	4+

By this method it was also possible to take into account the *degree* of infection.

The percentage protection was taken as the difference between the tota number of positive units in the control and immunized groups.

Experimental Results

(1) *Factors influencing growth in fluid medium*

(i) *Growth factors*

None of the enrichments tested improved the growth to any appreciable extent when used alone. However, a combination of 2·0 per cent Tryptone (Oxoid) and 0·5 per cent Yeast extract (Difco) added to the basic broth medium, markedly improved the yield to an average density of 1·3747 gm dry cells per litre.

The semi-synthetic medium yielded an average of 0·8566 gm of dry cells per litre.

The required pellicle (Carne, 1940) develops spontaneously on both these media when they are correctly inoculated.

(ii) *Method of inoculation*

When the inoculum was removed from the surface of the blood tryptose agar with a curved glass rod and transferred to the production flasks in a dry condition, a pellicle formed and a satisfactory yield resulted. But when the inoculum was washed from the surface of the medium and introduced into the production flasks as a suspension, no pellicle resulted and the growth was poor. The same disappointing results followed when the medium used for the production of inoculum was at all moist.

(iii) *Sterilization*

Two methods of sterilization were tested. The enrichment ingredients may be added directly to the freshly prepared broth base and the complete medium sterilized or alternatively the broth base may first be sterilized, the enrichments added, and the complete medium re-sterilized. When the first procedure was followed the growth obtained was tenacious and the yield poor. The medium prepared according to the second method produced a granular pellicle and a high yield of cells was obtained.

A series of tests was carried out to determine whether tindalization of the medium instead of autoclaving would improve the yield (see Table 2). This, however, showed no advantages. On the contrary, autoclaved media gave a slightly higher yield and the cells also contained slightly more endotoxin. The same was also found with the semi-synthetic broth.

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TABLE 2.—*Influence of method of sterilization on yield after 72 hours incubation*

Medium	Method of Sterilization	Yield in gm/l (Average values)
Broth.....	Autoclaved. 15 lb 30'.....	1·3747
Broth.....	Tindalized 100° 60' for three consecutive days.....	1·2910
Semi-synthetic.....	Autoclaved. 15 lb 30'.....	0·8566
Semi-synthetic.....	Tindalized 100° 60' for three consecutive days.....	0·6910

(iv) *Incubation temperature and time*

The results of two experiments conducted to determine the optimal incubation period, are presented in Table 3.

TABLE 3.—*Influence of incubation time on yield*

Incubation Time (Hours)	Yield (gm/l)	
	1st Experiment	2nd Experiment
48.....	1·0063	1·3394
72.....	1·1095	1·9012
96.....	1·2004	1·5041
120.....	1·2061	1·309
144.....	0·9028	1·306

The highest density was obtained after 72 hours incubation. Thereafter the yield decreased. Best results were obtained when incubation was carried out at 37°C.

(v) *Other factors*

As the pellicle was readily broken by agitation, all the methods of aeration gave poor results. Incubation in an atmosphere of 20 per cent CO_2 did not improve the yield. Adjusting the pH in a series from 7·0 to 8·0 by 0·2 steps similarly had no effect.

All three strains, viz. 137B, 5113C, and 12624 used in this study, gave similar results.

(2) *Influence of conditions of cultivation and period of growth on the presence of endotoxin and the pyogenic factor*

Cells grown on blood-tryptose-agar and fluid medium for different periods were harvested as described, freeze-dried and resuspended in saline as explained above. Formalin was added to a concentration of 0·4 per cent and the flasks kept at 37°C for 48 hours when guinea-pigs were injected subcutaneously as indicated. Cells similarly cultivated were collected by centrifugation, washed, freeze-dried and endotoxin extracted as outlined under " Materials and Methods ". The results are given in Table 4.

TABLE 4.—*Endotoxin content and pyogenicity of cells variously cultivated*

Medium	Cultivation time (hours)	Dermal reaction in rabbits by untreated endotoxin			Abscess formation in guinea-pigs by formalized cells			
		Dilutions			Inoculum (density) 0·15 gm dry cells/100 ml			
		1/1	1/5	1/10	Dose (ml)			
		0·5	0·5	0·5	1·0	1·0	0·5	0·5
Bl.tr.ag.....	24	8	6	1	+	+	+	+
,, ...	48	9	6	1	+	+	+	−
,, ...	72	5	2	0	+	+	−	−
Fluid*	48	3	2	0	+	−	−	−
,, ...	72	4	1	0	±	−	−	−
,, ...	96	1	0	0	−	−	−	−
,, ...	120	0	0	0	−	−	−	−

* = Broth + 2% Tryptone and 0·5% Yeast extract.
\+ = Abscesses present.
− = No abscesses formed.
Figures denote increase in skin thickness in mm, 48 hours after injection.
Bl.tr.ag. = Blood tryptose agar.

Endotoxin could only be demonstrated in cells capable of producing abscesses when formalized. These two characteristics are clearly associated with each other and are present to a greater extent in cells derived from blood-tryptose-agar and in very young fluid cultures than in old cultures.

(3) *Effect of various treatments of cells on their pyogenicity and the persistence of endotoxin*

In this series of experiments, cells grown on blood-tryptose-agar for 48 hours at 37°C, were used. As above, dried cells were suspended in saline and subjected to the action of various chemicals and heat. Endotoxin and pyogenicity were tested for as indicated above. Table 5 shows the results obtained.

TABLE 5.—*Effect of various treatments on persistence of endotoxin and on pyogenicity*

Treatment of cells	Time (hours)	Temperature in °C	Dermal reaction in rabbits			Pyogenic action in guinea-pigs			
			Dilutions			Dose (ml)			
			1/1	1/5	1/10				
			Volume (ml)						
			0·5	0·5	0·5	1·0	1·0	0·5	0·5
0·4% Formalin	48	37	0	0	0	+	+	+	+
0·1% beta-propiolactone	48	37	0	0	0	±	−	−	−
0·5% Phenol	48	37	5	2	1	−	−	−	−
*Heat	1	60	5	1	1	+	+	+	+
Heat	½	121	1	0	0	−	−	−	−
Ether extraction and 0·4% Formalin	48	±25 (room)	0	0	0	+	+	+	+
	48	37							
Ether extraction and 0·5% Phenol	48	±25 (room)	7	2·5	1·5	−	−	−	−
	48	37							

* Organisms remained viable after treatment and produced active abscesses.
+ = Development of abscesses.
− = No abscesses observed.
Figures denote increase in skin thickness in mm, 48 hours after injection.

From these results it can be seen that the pyogenic factor is heat labile and destroyed by 0·1 per cent beta-propiolactone and 0·5 per cent phenol, but it is not affected by formalin. The endotoxin on the other hand, while also heat labile, is destroyed by formalin and beta-propiolactone, but not by phenol in the concentrations used. They are, therefore, clearly dissimilar in their resistance to chemical agents.

Cells from which the toxic surface lipid was removed by extraction with di-ethyl ether were still pyogenic after treatment with formalin and also produced endotoxin after phenol treatment. The pyogenic property of killed cells and the erythrogenic effect of the endotoxin are, therefore, not due to the surface lipid.

Trypsin treated cell walls were similarly tested. They gave neither a dermal reaction nor were they capable of producing abscesses.

The endotoxin obtained from cells suspended in 0·5 per cent phenol, was tested for lethal action by injecting 2 ml and 1 ml amounts subcutaneously into guinea-pigs. None of the experimental animals showed any adverse reaction.

(4) *Susceptiblity of endotoxin to various physical and chemical treatments*

For these experiments endotoxin was prepared from cells obtained from a 48 hour culture on blood-tryptose-agar. The usual procedure for extraction was followed and aliquots of the endotoxin treated as indicated in Table 6.

TABLE 6.—*Effect of various treatments on the activity of endotoxin*

Treatment	Dermal reaction in rabbits in mm		
	Dilutions		
	1/1	1/5	1/10
	Dose (ml)		
	0·5	0·5	0·5
Untreated	5	3·5	2
0·4% Formalin. 48 hours. 37°C	2	1	0
0·1% beta-propiolactone. 48 hours. 37°C	1	0	0
0·5% Phenol. 48 hours. 37°C	3	3	2
Heated. 60°C for 1 hour	1	0	0
Zeitz filtered. E.K.S.I.	1	0	0

The results obtained by subjecting endotoxin to various chemical treatments correspond to those obtained by treating whole cells with the same chemicals under similar conditions and thereafter extracting the endotoxin. The deleterious effect of beta-propiolactone, formalin and heat on the activity of the endotoxin is demonstrated.

Endotoxin is absorbed to a marked degree by filtration through Zeitz E.K.S.I. pads.

The effect of chemicals on the various cell fractions is summarized in Table 7.

TABLE 7.—*Characteristics of cell fractions*

Factor	Locality	Susceptibility to 121°C 30 min	Susceptibility to chemicals	Pathogenicity
Exotoxin	Extra-cellular	Destroyed	Toxoided by formalin	Erythrogenic and lethal
Endotoxin*	Endoplasm	Destroyed	Inactivated by formalin and beta-propiolactone, but not affected by phenol	Erythrogenic
Pyogenic factor*	Whole cell	Destroyed	Destroyed by phenol and beta-propiolactone, but not by formalin	Pyogenic
Surface wax	Cell surface	Unknown	Unknown	Toxic

* Present simultaneously, but only at certain stages of growth

(5) *Immunity tests in guinea-pigs*

The results of these experiments with cells variously cultivated and treated are shown in Table 8. The relationship between the endotoxin and pyogenic factor and immunizing properties are summarized in Table 9.

TABLE 8.—*Immunity tests in guinea-pigs*

Guinea-pig Number	Vaccines								
	Control	A_1	A_2	A_3	A_4	B_1	B_2	B_3	B_4
	Cultivation method and medium								
	—	Bl.tr. agar 48 h	Bl.tr. agar 48 h	Bl.tr. agar 48 h	Bl.tr. agar 48 h	Broth 72 h	Broth 72 h	Broth 72 h	Broth 72 h
	Method of inactivation								
	—	0·4% Formalin	0·5% Phenol	0·1% B.P.L.	Heat 15 lb 30 min	0·4% Formalin	0·5% Phenol	0·1% B.P.L.	Heat 15 lb 30 min
	Reactions of guinea-pigs after challenge								
1	1+	1+	—	—	—	3+	—	—	2+
2	—	—	—	1+	3+	3+	1+	—	—
3	—	—	—	1+	—	—	2+	—	2+
4	3+	—	—	—	1+	—	—	2+	1+
5	3+	1+	—	—	—	—	—	—	—
6	—	—	—	1+	—	2+	—	3+	—
7	—	—	—	—	—	—	—	—	—
8	—	—	—	1+	—	1+	—	—	—
9	2+	—	—	1+	—	—	—	2+	—
10	4+	1+	—	1+	1+	1+	—	—	—
11	—	—	1+	1+	—	—	3+	2+	—
12	—	—	—	—	1+	1+	—	—	2+
13	2+	—	1+	2+	—	2+	—	2+	—
14	4+	—	—	—	—	—	—	—	—
15	—	—	—	2+	—	—	2+	—	4+
16	3+	—	—	—	2+	—	1+	—	1+
17	—	—	—	—	2+	—	—	—	1+
18	2+	—	—	—	—	1+	—	2+	—
19	2+	—	—	—	—	3+	—	2+	2+
20	3+	—	1+	—	—	2+	1+	1+	2+
21	—	—	—	—	—	—	—	—	—
22	—	—	—	2+	2+	—	—	—	—
23	2+	—	—	1+	—	—	2+	—	—
24	2+	—	—	—	1+	—	3+	—	—
25	—	—	—	—	—	1+	—	1+	1+
Percentage protection compared with controls (see text).....	0	31	31	20	21	15	19	17	16

h = hours
\+ = abscesses developed
— = no observable reaction
Bl.tr.agar. = blood tryptose agar—Mason tubes
Broth = enriched broth in Roux flasks.

TABLE 9.—*Immunizing power of cells variously cultivated and treated in relation to endotoxin and pyogenic factors*

Medium and Method of Cultivation	Treatment of Cells	Endotoxin		Pyogenic factor		Immunizing power
		Before treatment	After treatment	Before treatment	After treatment	
Blood-tryptose-agar 48 hours 37°C	0·4% Formalin..	Present	Inactive	Present	Active	31%
	0·5% Phenol....	,,	Active	,,	Inactive	31%
	0·1% beta-propiolactone	,,	Inactive	,,	,,	20%
,,	Autoclaving 15 lb 30 min	,,	,,	,,	,,	21%
Broth 72 hours 37°C	0·4% Formalin..	Absent	Absent	Absent	Absent	15%
,,	0·5% Phenol....	,,	,,	,,	,,	19%
,,	0·1% beta-propiolactone	,,	,,	,,	,,	17%
,,	Autoclaving 15 lb 30 min	,,	,,	,,	,,	16%

DISCUSSION

The fluid media thus far employed for producing *C. pseudotuberculosis* exotoxin require laborious preparation and are unsuitable for producing a high yield of cells (Petrie & McClean, 1934; Bull & Dickenson, 1935; and Carne, 1940). A suitable fluid medium was, therefore, developed prior to the commencement of these studies.

Earlier authors (Petrie & McClean, 1934; Bull & Dickenson, 1935) surmised that *C. pseudotuberculosis* possessed a heat-labile endotoxin and a heat-stable pyogenic factor, both lethal, It was suggested that the endo- and exotoxins are identical (Petrie & McClean, 1934).

The results of the present investigations are summarized in Tables 5 and 6 and show that both the endotoxin and pyogenic factor are heat-labile. The former is destroyed at 60°C for one hour and the latter by autoclaving. They differ from one another in their susceptibility to chemical agents. The endotoxin is affected by formalin, but not by phenol, while the reverse is true for the pyogenic factor.

It was discovered that the two factors occur simultaneously but only in cells from young blood agar cultures and to a lesser degree in cells at an early stage of growth in fluid cultures. This phenomenon has not been recorded previously.

The observation that cells devoid of the toxic surface lipid still harbour endotoxin and the pyogenic factor, proves that both are distinct from this lipid.

The presence of endotoxin in extracts free of cell walls, has been shown. Neither endoplasm nor cell walls alone would produce abscessation, consequently the locality of the pyogenic factor is still unknown.

Petrie & McClean (1934) suggested that the exotoxin may have an intracellular origin. Experimental injection of large doses of endotoxin into guinea-pigs, however, failed to show any lethal activity. It is, therefore, unlikely that it is identical to the lethal exotoxin (Carne, 1940). This is, however, not conclusive proof and further serological investigations are indicated.

The possibility that either the endotoxin or the pyogenic factor is identical to the group antigen present in cells of *C. diphtheriae* and which gives cross-agglutination reactions with *C. pseudotuberculosis* (Cummings, 1954) requires investigation.

The results obtained with the immunity tests showed that all the bacterins, irrespective of method of cultivation and inactivation, have a basic immunizing power of 15 to 21 per cent in this specific trial. When they are treated by either formalin or phenol, cells which have been grown on blood-tryptose-agar and, therefore, possess both pyogenic factor and endotoxin, have an immunizing power of 31 per cent. One of the two toxic factors remains active by these procedures. When both factors in these cells are inactivated (heat and BPL) their immunizing power is no greater than that of cells devoid of these toxic factors.

Conclusions

The pyogenic factor retained in formalized cells is associated with the presence of phenol resistant endotoxin. These factors are only present to any appreciable extent in young cells cultured on blood-tryptose-agar. Cells from fluid cultures only occasionally contained endotoxin and their pyogenicity was very variable.

The results indicate that the pyogenic factor in cells is destroyed by 0·5 per cent phenol, 0·1 per cent beta-propiolactone and by autoclaving. Formalin in a concentration of 0·4 per cent kills the organisms under the circumstances described, but does not destroy the property of the dead cells to produce sterile abscesses. The endotoxin on the other hand is also heat labile but is affected by 0·4 per cent formalin, 0·1 per cent beta-propiolactone, but not by 0·5 per cent phenol.

As cells grown on blood-tryptose-agar have an appreciably greater immunizing power than those grown on fluid medium without blood, it is clear that both the pyogenic factor and the endotoxin are closely associated with immunity.

Summary

Two fluid media and methods for the cultivation of *C. pseudotuberculosis* are described. An average yield of 1·3747 and 0·8566 gm of dry cells per litre was obtained in the respective media.

An erythrogenic endotoxin and a pyogenic factor, occurring only in cells grown on blood tryptose agar, have been demonstrated. The endotoxin is inactivated by formalin, but not by phenol, while the reverse is true for the pyogenic factor.

Immunity tests in guinea-pigs have shown that cells containing these antigens have appreciably greater immunizing power than those without. This immunogenicity is reduced to a marked degree when the cells are killed by beta-propiolactone, but not by formalin or phenol.

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