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Virulence factors of *Francisella tularensis*

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SUMMARY

The mechanism causing viable *Francisella tularensis* to lose virulence in aerosols has been investigated. Fully virulent organisms were found to be encapsulated and avirulent organisms from aged aerosols, decapsulated. Capsules were also removed by suspension of *F. tularensis* in hypertonic sodium chloride. The resulting naked, but viable, organisms were predominantly avirulent for guinea-pigs challenged intraperitoneally. Capsular material and cell walls were found to contain large amounts of lipid, about 50 and 70% (w/w) respectively, and to differ in lipid and sugar composition. Isolated capsular material was not found to contain a lethal toxin for mice or guinea-pigs, or to induce an immunological response in these animals to fully virulent *F. tularensis*.

INTRODUCTION

As aerosols of the highly virulent Schu S4 strain of *Francisella tularensis* age, the viable fraction of the population loses virulence by the respiratory route for mice (Schlamm, 1960), guinea-pigs (Hood, 1961), monkeys (*Macaca mulatta*) and man (Sawyer *et al.* 1966). Investigation of components in culture media and spray fluids indicated that the chloride ion in particular was involved in the mechanism responsible for this phenomenon (Hood, 1961).

The factors responsible for the virulence of *Francisella tularensis* have eluded identification despite previous investigation by many workers (Larson, Bell & Owen, 1954; Ormsbee, Bell & Larson, 1955; Ormsbee & Larson, 1955; Shepard, Ribi & Larson, 1954; Stefanye, 1961; Guss, 1970; Nutter, 1971; Hambleton, Evans, Hood & Strange, 1974). In the present study the action of chloride ions has been more clearly defined and utilized to produce avirulent organisms and isolated material for detailed examination in further attempts to identify the factors responsible for the virulence of *F. tularensis*.

MATERIALS AND METHODS

Micro-organism

Francisella tularensis strain Schu S4 (Eigelsbach, Braum & Herring, 1951) was used throughout the study. It was grown as previously described (Hambleton *et al.* 1974) in the liquid medium, modified casein hydrolysate, 'MCPH' (Mills, Berthelsen, Donaldson & Wilhelm, 1949).

Aerosols

Aerosols were produced by a Collison atomizer (May, 1973) from undiluted cultures of *F. tularensis*, approximate concentration 2×10^{10}/ml, and consisted predominantly of particles 1–2 μm in diameter. The methods used for the ageing, collection and viable assay of aerosols and for animal (guinea-pig) exposure were similar to those previously described (Hood, 1961). In some experiments water-washed spores of *Bacillus globigii* var *niger* were added to the spray fluid to establish the proportion of viable *F. tularensis* in aerosols by the method described by Harper, Hood & Morton (1958). Aerosols were held at temperatures within the range 10–15 °C and at 85–90 % relative humidity.

Animals

Porton mice (25 g) or 350–400 g guinea-pigs (D/Hartley) were used for tests of virulence, toxicity and immunogenicity.

Virulent and avirulent F. tularensis

Virulent organisms (LD 50 value 1, guinea-pig, respiratory) were obtained from aerosols aged about 1 s or from fresh cultures. Avirulent organisms (LD 50 values > 100) were obtained from 20 h aged aerosols and were also produced by suspension in hypertonic sodium chloride.

Morphology

F. tularensis was examined in an electron microscope (Philips 300). The organisms obtained from aerosol samples collected in cysteine broth (Hood, 1961) were killed by the addition of formaldehyde (1 %, w/v) to the sample, washed with water by centrifugation, resuspended to a suitable concentration and negatively stained with 1 % (w/v) phosphotungstic acid at pH 7·2. Organisms separated from culture fluid were similarly treated to serve as controls.

Preparation and analysis of bacterial material

F. tularensis separated from culture medium by centrifugation was resuspended ($1–3 \times 10^{10}$ organisms/ml) in 10 % (w/v) sodium chloride solution at pH 6·4–6·8 and stored at 4 °C for 3–7 days. Two materials were obtained, one was revealed in the supernatant fluid after centrifugation (2000 ***g***, 30 min) as an opalescent material and was separated from the sodium chloride by dialysis against water. The second was a wall preparation from the deposited bacteria above using a method similar to that described by Shepard *et al.* (1954). The two materials were analysed for fatty acid, sugar and amino acid content.

Fatty acids. Dried (*in vacuo*) dialysed weighed samples of the materials were methanolysed in 2 ml of anhydrous methanolic HCl (2 M) for 7 h at 70 °C and the methyl esters of the fatty acid components were extracted with petroleum ether (40–60° fraction). The extract was concentrated to 0·4 ml and samples analysed by gas liquid chromatography (g.l.c.). Components were separated on columns of 10 % polyethylene glycol adipate on Chromosorb W at a temperature of 175 °C and 10 %

Apiezon L at 195 °C. The eluted components were detected with a dual flame ionization detector and identified by comparison with the elution patterns of authentic standards.

Sugars. These were converted to alditol acetates and the products analysed by g.l.c. Dialysed material was hydrolysed for 16 h at 100 °C in 0·5 M-H_2SO_4, neutralized with barium hydroxide and the supernatant fluid, separated by centrifugation, was dried *in vacuo*. The sugars were reduced to alditols with sodium borohydride solutions as described by Abdel-Akher, Hamilton & Smith (1951) for 6 h at room temperature. The reaction was stopped by adding acetic acid (to pH 5–6) and the boron removed, as methyl borate, by evaporation from methanol (containing approximately 1 % (w/v) HCl). The residue was acetylated (Sawardeker, Sloneker & Jeanes, 1965) with 0·4 ml of a mixture of pyridine and acetic acid anhydride (1:1 v/v) at 120 °C for 4 h. Finally the pyridine and acetic anhydride were removed by evaporation *in vacuo* and the residue was dissolved in chloroform. Samples of the solution were analysed by g.l.c. on a 1·542 m column of 3 % OV 225 on Chromosorb W HP at 205 °C. Sugars were identified by comparison with authentic standards run under identical conditions.

Amino acids and amino sugars. Dialysed material was hydrolysed with 6 M-HCl for 24 h at 110 °C for analysis of amino acids and with 4 M-HCl for 6 h at 110 °C for analysis of amino sugars. Samples of the hydrolysates were analysed with an amino acid autoanalyser (Technicon TSM1).

RESULTS

Morphology

Fully virulent *F. tularensis* taken directly from the culture medium or from aerosols after 1 s ageing were morphologically similar. Each organism was completely surrounded by a well defined, relatively thick (0·02–0·04 μm) electron transparent outer structure. Typical examples of groups and single organisms (at higher magnification) are shown (Plates 1 and 2) from samples obtained from aerosols. In contrast, predominantly avirulent *F. tularensis* collected from aerosols aged for 20 h were found to have lost most or all of this outer structure (Plates 3 and 4). An occasional organism could be found in these samples of organisms which still retained small portions of outer structure (Plate 5). In the enlarged electron micrographs (Plates 3 and 4) two wall membranes can be observed but with the degree of resolution obtained it does not appear that ruptures were present in the wall membranes of the organism. In fact the similarity in shape of virulent and avirulent organisms suggested that the residual structure maintained the integrity of the organism.

Bacteria in very old cultures (6 months' storage at 4° C) containing a high proportion of avirulent bacteria (Hood, 1961) were similar in appearance to the avirulent bacteria in 20 h aerosols.

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Table 1. *Viability of* F. tularensis *before and after treatment with* 10 % (*w/v*) *sodium chloride solution*

Batch	Viable bacteria/ml ($\times 10^{10}$) Before treatment	After treatment (3–5 days)
1	1·14	1·19
2	1·65	1·57
3	0·96	1·16
4	1·05	0·93

Physical effect of sodium chloride

The concentration of sodium chloride in the aerosol particles disseminated from spray fluids containing about 0·5 % salt and equilibrated at 85 % relative humidity is about 10 % (w/v) (Hood, 1961). Such a hypertonic solution could be responsible for the physical removal of the outer structure from virulent *F. tularensis*. This hypothesis was tested by observing the effect of suspending the organisms in 10 % (w/v) sodium chloride solution. The suspensions were stored at 0–4 °C and, after centrifugation (2000 ***g***, 30 min), the supernatant fluids were examined daily for opalescence. Electron microscopic examination of the deposited organisms showed that most of their outer structures were removed after exposure to the sodium chloride solution for 3–7 days; stripping was indicated by an increase in the opalescence of the supernatant fluid. Assay of the bacteria before and after exposure, however, showed that no significant change in viability had occurred (Table 1). The dry weight (after dialysis against water) of the material removed was approximately 10 % of the equivalent dry weight of the whole bacteria – about 100 μg was obtained from 10^{10} bacteria. A 1 % (w/v) sodium chloride solution was found to be about 25 % as efficient as a 10 % solution in removing the material from the organisms.

The morphological appearance of the outer structure of the virulent *F. tularensis* and its removal without apparent impairment of the organisms' viability is a characteristic of classical bacterial capsules (Duguid, 1951; Tomcsik, 1956; Wilkinson, 1958). In view, however, of the conflicting reports concerning encapsulation of this organism (Ota, 1936; O'Hara, 1940; Hesselbrock & Foshay, 1945; Eigelsbach, Chambers & Coriell, 1946) the nature of the apparent capsular material was investigated. An important point to be established was whether the released material was similar in chemical composition to that of the bacterial wall or to that of capsules from other bacterial species.

Analysis of outer structure and wall

Outer structures of virulent *F. tularensis* were removed in 10 % (w/v) sodium chloride as above and dialysed against several changes of water before being analysed. The resulting naked organisms were used to obtain walls for analysis.

Fatty acids, reducing sugars and amino acids accounted for over 90 % (dry weight) of the composition of the wall and the capsule (Tables 2 and 3). The

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Table 2. *Analysis of wall and capsule material*

	Wall		Capsule	
Sugar	$\mu g/100\ \mu g$	Total sugar (%)	$\mu g/100\ \mu g$	Total sugar (%)
Glucose	6·12	43·7	tr	—
Galactose	0·51	3·64	tr	—
Heptose	3·38	24·1	Nil	—
Mannose	0·54	3·85	4·45	16·6
Rhamnose	1·26	9·0	7·35	36
? dideoxy	0·93	6·6	4·2	20
? dideoxy	1·26	9·0	5·4	26·5
Total	14·0	—	Total 21·4	—
Fatty acid		Total fatty acid (%)		Total fatty acid (%)
β-OH 10:0	22·4	30·4	tr	—
α-OH 12:0	7·0	9·5	tr	—
12:0 (ibr)	8·4	11·4	tr	—
14:0	5·6	7·6	tr	—
β-OH 14:0	7·7	10·5	tr	—
α-OH 14:0	tr	—	20·1	31·3
16:0	9·8	13·3	30·6	48
18:0	7·7	10·5	Nil	—
Total	68·8	—	Total 50·7	—
Glycerol	9·18	—	Glycerol 2·7	—

concentrations of total lipids were high in each material (69 and 51 % (w/w) respectively). Total sugars (21 %, w/w) and amino acids (35 %, w/w) in the capsule exceeded those found in the wall (14 and 7·6 % respectively).

The similarity of the amino acid spectra and the proportions of individual amino acids in both wall and capsule suggest a similar structure in their 'protein' moiety. Detailed analysis of the sugar and fatty acid composition revealed considerable qualitative and quantitative differences between the two materials: of the total wall sugar over 40 % was glucose and 24 % heptose whereas neither of these sugars were found in appreciable amounts in the capsule. Mannose (16 %), rhamnose (36 %) and two unidentified – possibly dideoxy – sugars (45 %) accounted for most (97 %) of the total sugar in the capsule.

The capsular lipid consisted almost entirely of two fatty acids – saturated straight chain 16:0 (48 %) and α-OH 14:0 (31 %). Only a trace of α-OH 14:0 was present in the wall which contained β–OH 10:0 (30 %) as its principal fatty acid together with six other fatty acids (totalling about 60 %) in similar proportions.

In spite of the likely degree of error in determining total weights of components derived from 200–300 μg quantities of material it can be concluded from the results that the chemical composition of the capsule is significantly different from that of the wall.

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Table 3. *Analysis of wall and capsule material (amino acids)*

Amino acid	Wall		Capsule	
	μg/100 μg	Total amino acids (%)	μg/100 μg	Total amino acids (%)
Aspartic	1·26	16·5	5·64	16·5
Threonine	0·404	5·3	2·12	6
Serine	0·52	6·8	2·78	8
Glutamic	0·774	10	4·64	13·5
Proline	0·194	2·5	1·07	3
Glycine	0·342	4·4	1·65	5
Alanine	0·497	6·5	2·41	7
Cystine	Nil	.	Nil	.
Valine	0·099	1·3	2·08	6
DPA/Methionine	1·18	15·5	nil	.
Isoleucine	0·226	3	2·15	6
Leucine	0·452	6	3·05	9
Tyrosine	0·163	2	1·3	4
Phenyl alanine	0·44	6	1·62	5
Glucosamine	+	.	.	.
Histidine	0·411	5	0·96	3
Ornithine	Nil	.	Nil	.
Lysine	0·512	6·5	2·43	7
Arginine	0·156	2	1·25	4
Galactosamine	+	.	+	.
Total	7·63	Total	35·15	

Virulence of F. tularensis *decapsulated in sodium chloride solution*

The virulence of *F. tularensis* decapsulated by storage in sodium chloride was investigated.

Naked organisms isolated from preparations of capsular material in 10% (w/v) sodium chloride solution were resuspended in 1% (w/v) sodium chloride and LD 50 values via the peritoneal route in guinea-pigs and mice were determined.

The virulence of the organisms in each of three such suspensions tested was depressed for the guinea-pig: LD 50 values (95% confidence limits in parentheses) of 15 (8–49), 29 (9–223) and 315 (limits not obtainable) were obtained compared with about one organism in the original cultures. No loss of virulence for mice was found; LD 50 values of about one organism were obtained for each suspension.

These results are similar to the findings with naked bacteria obtained from aged aerosols – loss of virulence for the guinea-pig (i.p.) but no loss for mice (i.p.) (Hood, 1961).

Toxicity and immunogenicity of capsular material

The toxicity of viable *F. tularensis* has been demonstrated (Moody & Downs, 1955; Nutter & Myrvik, 1966). Injections of about 10^9–10^{10} organisms kill mice, guinea-pigs and rabbits in < 24 h. Toxicity is destroyed by treatment of the organism with heat, phenol or formaldehyde (Gordon, Donaldson & Wright, 1964; Nutter & Myrvik, 1966) which could explain why the active toxic factors have not been isolated by conventional extraction methods. Previous attempts to isolate

Table 4. *Response of mice to challenge (i.p.) with decapsulated* F. tularensis

(Accumulative deaths/total challenged.)

	Days after challenge			
Dose ($\times 10^4$)	2	3	4	5
1400	1/8	8/8	—	—
140	0/8	4/8	8/8	—
14	0/8	6/8	8/8	—
1·4	0/8	6/8	6/8	8/8

Table 5. *Response of guinea-pigs to challenge (i.p.) with decaspulated* F. tularensis

(Accumulative deaths/total challenged)

	Days after challenge								
Dose ($\times 10^4$)	2	3	4	5	6	7	8	9	10
1000	1/10	6/10	10/10	—	—	—	—	—	—
100	1/10	4/10	9/10	10/10	—	—	—	—	—
10	0/10	5/10	8/10	9/10	10/10	—	—	—	—
1	0/10	1/10	5/10	7/10	8/10	10/10	—	—	—
0·1	0/10	0/10	7/10	7/10	7/10	7/10	8/10	9/10	9/10

immunogens from this organism have also been unsuccessful (Ormsbee *et al.* 1955; Stefanye, 1961; Guss, 1970; Nutter, 1971; Hambleton *et al.* 1974). Since loss of the capsule and decrease in virulence appear to be related, toxic activity may reside in the capsule. An unconventional method of isolation of capsular material after removal from bacteria by exposure to hypertonic sodium chloride, might be less destructive, and permit isolation of an undegraded native endotoxin or immunogen.

Toxicity

Capsular material suspended in hypertonic sodium chloride which had been centrifuged at 2000 ***g*** for 30 min contained about 10^5 viable bacteria/ml, fully virulent for mice (see above).

Attempts to sterilize the material by filtration through a membrane filter (Millipore GSWP, 0·22 μm pore size) or by centrifugation at 17000 ***g*** failed because both capsular material and organisms were retained by the membrane and also sedimented at this ***g***. However, because of the strong possibility of destruction of toxicity by conventional methods of sterilization contaminated material was included in the tests. Animals injected with organisms (decapsulated) only, suspended in 1% sodium chloride, served as controls for these tests.

Apart from tests in two rabbits (see below), all tests were made in mice and guinea-pigs by intraperitoneal injection of the total material suspended in 1% sodium chloride. Preliminary tests also included animals injected with capsular material sterilized by heat (60 °C) or formaldehyde (1%) treatment. Some of these animals were used subsequently for immunogen detection.

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Table 6. *Response of guinea-pigs and mice to challenge (i.p.) with* F. tularensis *capsular material* and effect of actinomycin D (mice)*

(Accumulative deaths/total challenged)

	Days after challenge									
	2	3	4	5	21	2	3	4	5	21
	Mice					Guinea-pigs				
(*a*)	0/10	6/10	10/10	—	—	0/8	3/8	6/8	8/8	—
(*b*)	0/10	0/10	0/10	0/10	0/10	0/8	0/8	0/8	0/8	0/8
(*c*)	0/10	0/10	1/10	2/10	2/10	—	—	—	—	—
(*d*)	0/10	0/10	1/10	3/10	3/10	—	—	—	—	—

(*a*) Unsterilized capsular material giving 5×10^3 viable *F. tularensis* to each animal.
(*b*) Capsular material sterilized by filtration.
(*c*) Capsular material sterilized by filtration; mice pre-injected with 12·5 μg of actinomycin D.
(*d*) Mice receiving 12·5 μg actinomycin D only.

* 800 μg of capsular material/animal.

Control animals (Tables 4 and 5) challenged with 10^6 organisms were not apparently distressed for at least 2 days; all mice susequently developed a fatal tularaemia. In guinea-pigs the number of survivors during the 3-week period after challenge reflected the increased LD 50 value of decapsulated organisms in this animal.

The response of those animals challenged with approximately 100 μg of capsular material (derived from about 10^{10} organisms) containing 10^4–10^6 viable organisms was not apparently different from that of control animals receiving similar doses of organisms only.

None of the animals injected with sterilized capsular material were apparently affected for at least three weeks. One preparation of material containing no viable bacteria because it had been stored for 3 months at 4 °C, failed to elicit any apparent response in two 3 kg rabbits (New Zealand) when given doses of 500 μg intravenously.

The results indicated that the capsular material was non-toxic. However, since the material used for these preliminary tests had been stored for at least 2 weeks it was possible that lack of toxicity was due to degradation of a labile toxin.

Fresh material was prepared to investigate this possibility. It was isolated from freshly grown bacteria, resuspended to a concentration of $1\text{–}3 \times 10^{11}$/ml, in 10 % (w/v) sodium chloride solution, and stored at 4 °C for 3 days. Centrifugation of the suspension (2500 ***g***, 45 min) decreased the viable bacterial concentration in the supernatant fluid containing capsular material to about 10^4/ml. Two preparations were made from this: (*a*) a concentrated suspension of capsules (using negative pressure dialysis) containing the equivalent of 1600 μg dry wt capsular material/ml in 1 % (w/v) sodium chloride solution; (*b*) to a portion of (*a*) an equal volume of 4 % (w/v) sodium deoxycholate was added to dissolve the capsular material and thus enable it to pass through a bacterial filter (Millipore membrane, 0·2 μm average pore size). The filtrate was immediately dialysed against water to remove the

Table 7. *Response of mice and guinea-pigs to challenge (i.p.) with virulent* F. tularensis* *after prior† treatment with capsular material*

(Accumulative deaths/total challenged.)

Material injected	Days after challenge					
	2	3	4	5	6	7
			Mice			
100 μg capsular material	0/10	0/10	1/10	10/10	—	—
800 μg capsular material	0/9	0/9	2/9	9/9	—	—
800 μg capsular material and 12·5 μg actinomycin D	0/8	0/8	0/8	8/8	—	—
None (controls)	0/10	6/10	10/10	—	—	—
			Guinea pigs			
100 μg capsular material	0/10	0/10	1/10	4/10	8/10	10/10
None (controls)	0/8	0/8	6/8	8/8	—	—

* Approximately 1000 viable cells in 0·1 ml 1 % sodium chloride solution.
† Capsular material injected 3 weeks before challenge.

deoxycholate. During this process reappearance of opalescence was observed indicating a reaggregation of the capsule material molecules. The dialysis residue (concentrated by negative pressure) was resuspended in 1 % (w/v) sodium chloride solution to contain about 1600 μg/ml (dry weight) of material.

These preparations, (*a*) and (*b*), were injected (i.p.) into mice and guinea-pigs of both sexes in doses of approximately 800 μg of material. The results (Table 6) indicated that neither preparation was toxic. The animals injected with the material containing some viable bacteria survived apparently in good health for 2–3 days after challenge before they showed the symptoms of a tularaemia infection followed by death in a similar period to that found in (control) animals receiving a similar dose of bacteria only. Animals receiving the sterilized preparation exhibited no signs of distress for at least 3 weeks after challenge.

Potentiation of toxicity

Pieroni, Broderick, Bundeally & Levine (1970) reported that actinomycin D administered to mice immediately after injection of a typical endotoxin increased their sensitivity to the endotoxin 100000-fold. A sterile preparation of capsular material (filtered after solution in sodium deoxycholate and then dialysed) prepared as described above was injected into mice immediately after they were injected with 12·5 μg of actinomycin D (contained in 0·1 ml of 1 % (w/v) sodium chloride solution). The dose of capsular material received by each animal was equivalent to about 800 μg dry weight. Control mice received actinomycin D plus saline only. No deaths occurred in test or control animals during the first 3 days post-challenge and a similar number of animals died in each group during the following 4 days – presumably due to the toxicity of the actinomycin D (Table 6).

Immunogenicity of capsular material

Animals (mice and guinea-pigs) surviving for 3 weeks after injection of capsular material sterilized by heat (60 °C; 30 min) or by filtration (after solution in sodium deoxycholate solution) were used to test for immunogenicity of the capsular material.

The animals were challenged (i.p.) with approximately 1000 lethal doses of fully virulent (Schu 4) *F. tularensis*. Their response was compared with that of untreated (control) animals of similar weight to the test animals. The results (Table 7) indicated that the capsular material was not significantly protective against the challenge dose. Mice previously treated with either 100 or 800 μg and guinea-pigs with 100 μg of material survived only a day or so longer than the control animals.

DISCUSSION

Capsules and sodium chloride

The relative ease with which the capsules are removed from aged *F. tularensis* in fluids containing sodium chloride has probably contributed to the failure of other workers (Hesselbrock & Foshay, 1945; Eigelsbach *et al.* 1946; Shepard *et al.* 1954) to observe that this strain of *F. tularensis* at least is normally encapsulated. Their suspensions of the organism were prepared for examination in fluids containing sodium chloride. In a more recent examination (Pavlova, Meshcheryakova & Emelyanova, 1967) of several strains of *F. tularensis* including the present one, capsules were observed in a proportion of the bacteria in each of the preparations examined. These authors prepared the bacteria by washing in buffer and distilled water. The mechanism involved in capsule removal by sodium chloride is not fully understood but experiments (Zimmerman, 1969) in which *Serratia marcescens* was suspended in salts of various strengths (including sodium chloride at 10 %) suggest the following: at high sodium chloride concentration differential shrinkage of the membranes occurs due to a variation in salt permeability to produce spaces between the various structures external to the cytoplasmic membrane. It seems likely that a similar mechanism will operate when *F. tularensis* is exposed to high concentrations of sodium chloride giving a different osmotic gradient between wall and capsule. This would induce a lack of cohesion and result in capsule detachment upon rehydration. Organisms in aerosol particles produced from a fluid containing about 0·5 % (w/v) sodium chloride will, after equilibration to 85 % relative humidity, be exposed to about 10 % (w/v) (Hood, 1961) causing a similar effect to that occurring in organisms suspended *in vitro* in strong solutions of this salt.

F. tularensis *wall*

Chromatographic analyses of material from the walls of *F. tularensis* have revealed striking differences between its composition and that from the walls of other gram-negative bacteria. In contrast to the usual 10–20 % proportion of lipid found in these organisms (Salton, 1964) the *F. tularensis* wall consists of about 70 % lipid and the principal fatty acid is β-OH 10:0 (30 %) and not the straight chain

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16:0 which is the principal fatty acid in most gram-negative organisms (Cho & Salton, 1966). The remaining fatty acids consist almost entirely of relatively similar proportions of α-OH 12:0, ibr 12:0, β-OH 14:0, 16:0 and 18:0. The presence of α-hydroxy fatty acids is unusual in gram-negative bacteria, but they have been reported in *Pseudomonas aeruginosa* (Hancock, Humphreys & Meadow, 1970), *Azotobacter agilis* (Kaneshiro & Marr, 1963) and *Rhodomicrobium vanniellii* (Park & Berger, 1967) organisms taxonomically unrelated to *F. tularensis*. The walls of the two species of organisms to which *F. tularensis* has previously been assumed to relate – *Yersinia pestis* and *Brucella* species – have been analysed for their fatty acid composition (Tornabene, 1973; Bobo & Eagen, 1968). *Yersinia pestis* is typical of gram-negative organisms in containing the straight chain 16:0 as the principal fatty acid. *Brucella abortus* is similar to *F. tularensis* in that this fatty acid is not the principal one and both organisms contain similar proportions of 18:0 fatty acid. There are, however, highly significant differences, qualitative and quantitative, between lipid composition of the walls of *F. tularensis* and *Brucella abortus* which add support to the decision of taxonomists to place *F. tularensis* in a separate genus of uncertain affiliation.

Toxicity

In previous attempts to isolate antigens from *F. tularensis* the methods used were those commonly employed for the preparation of endotoxins from *Enterobacteriaceae* (Ormsbee *et al.* 1955; Stefanye, 1961; Guss, 1970; Nutter, 1971; Hambleton *et al.* 1974), e.g. extraction with phenol, acetone, water saturated with ether or trichloroacetic acid. Without exception, the products obtained were poor immunogens and non-toxic or at least lacked the typical property of classical endotoxins – lethality for rabbits (Stefanye, 1961; Nutter, 1971). Most of the extract procedures were preceded by or included washing of the bacteria in sodium chloride solution. Since the amount of capsular material removed (and discarded) would be influenced by the culture media and age of the bacterial suspension the amount of capsular material in the final products used for their tests is unknown. In the present investigation neither toxic nor immunogenic activity was found in capsular material isolated by the relatively mild treatment with sodium chloride solution. This suggests that the main and perhaps sole function of the capsule in the pathogenesis of tularaemia is to protect the organism against cellular host defence mechanisms.

Virulence and encapsulation

It has been clearly demonstrated that the cellular factors in the host are the prime defence mechanisms of resistance to *F. tularensis* in the initial period of infection in normal animals and the relative virulence of strains of this organism is paralleled by their ability to proliferate intracellularly in mononuclear phagocytes (Buddingh & Womack, 1941; Shepard, 1959; Stefanye, Tresselt & Spero, 1961; Thorpe & Marcus, 1964*a*, *b*, 1965; Nutter & Myrvik, 1966). The identification of a capsule surrounding the present strain of *F. tularensis* and the apparent correlation found between decapsulation and virulence, at least for the guinea-pig, is strong

evidence that the capsule plays a vital role in initiating infection by this organism. Its presence may account, for example, for the recently reported (Proctor, White, Ayala & Canonico, 1975) organisms' complete resistance to phagocytosis by neutrophiles.

A direct correlation between LD 50 value of predominantly avirulent populations and their proportion of fully capsulated organisms was not obtained. All of these organisms were apparently completely or partially decapsulated suggesting that all of them were of lowered virulence and the increase in LD 50 value could be simply a reflexion of a variation in the bactericidal activity of the hosts' phagocytes. The possibility of partial loss of capsule from already weakened structures during the final preparation required for electron microscopic observation could not be ruled out because direct observation of capsules in the light microscope is precluded by their small size.

The lipid component is similarly high in both capsule and wall in this organism, 51 and 68% (w/w) respectively, but decapsulated organisms are predominantly avirulent. This suggests that one or both of the two fatty acids, of which the capsular lipid is largely composed, 16:0 and α-OH 14:0, could be responsible for the organisms' defence against the hosts' bactericidal mechanisms. The exceptionally high, possibly unique, concentration of an α-OH fatty acid found in the capsule of the present strain of *F. tularensis* suggests that this fatty acid in particular may be associated with the extreme resistance of fully virulent (encapsulated) organisms to the intracellular (mononuclear cells) bactericidal factors in normal, non-immune, hosts.

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EXPLANATION OF PLATES

Plate 1

Fully virulent *Francisella tularensis* showing well defined capsules; collected from aerosol about 1 s old. × 45000.

Plate 2

Francisella tularensis from aerosol aged about 1 s. × 105750.

Plate 3

Francisella tularensis from predominantly avirulent 20 h aged aerosol; decapsulated. × 39000.

Plate 4

Decapsulated *Francisella tularensis* from 20 h aged aerosol. × 74000.

Plate 5

Partially decapsulated *Francisella tularensis* from predominant avirulent 20 h aged aerosol. × 81000.

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(*Facing p.* 60)

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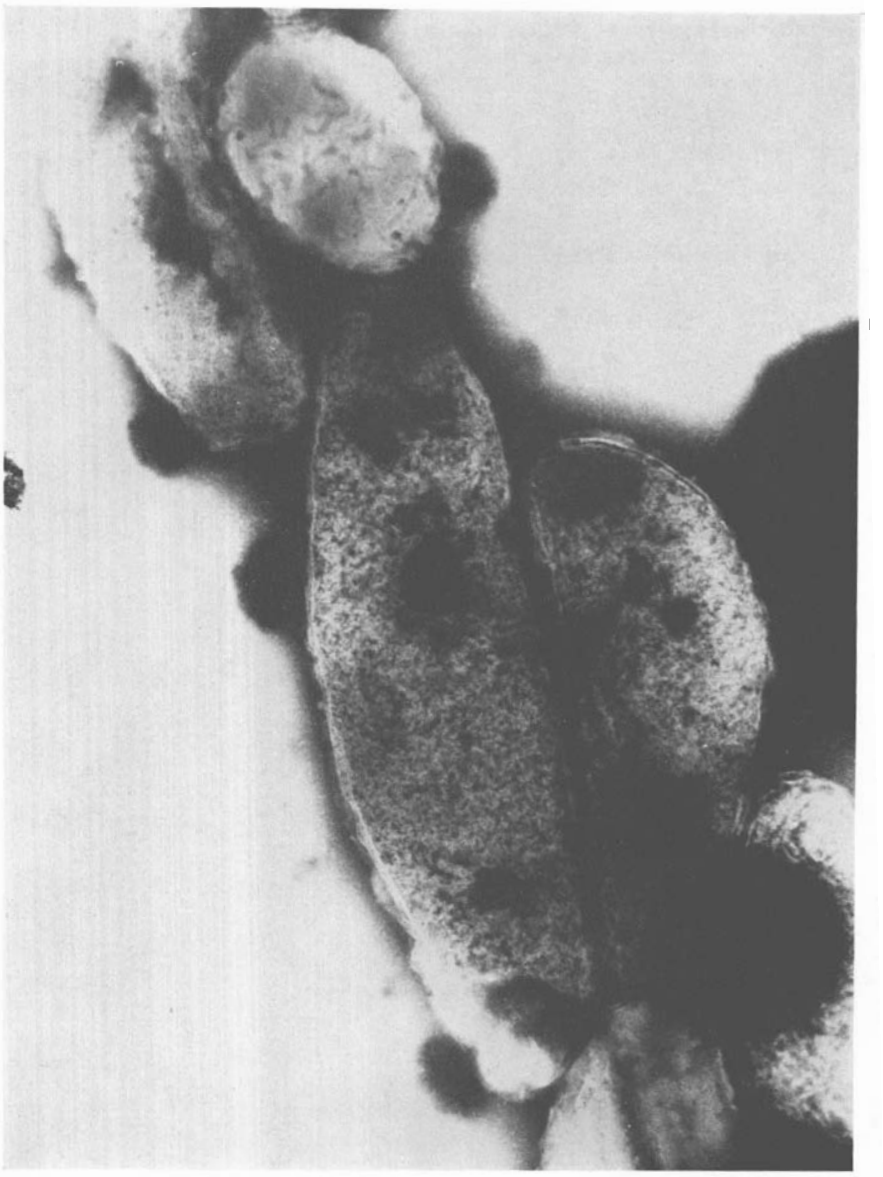

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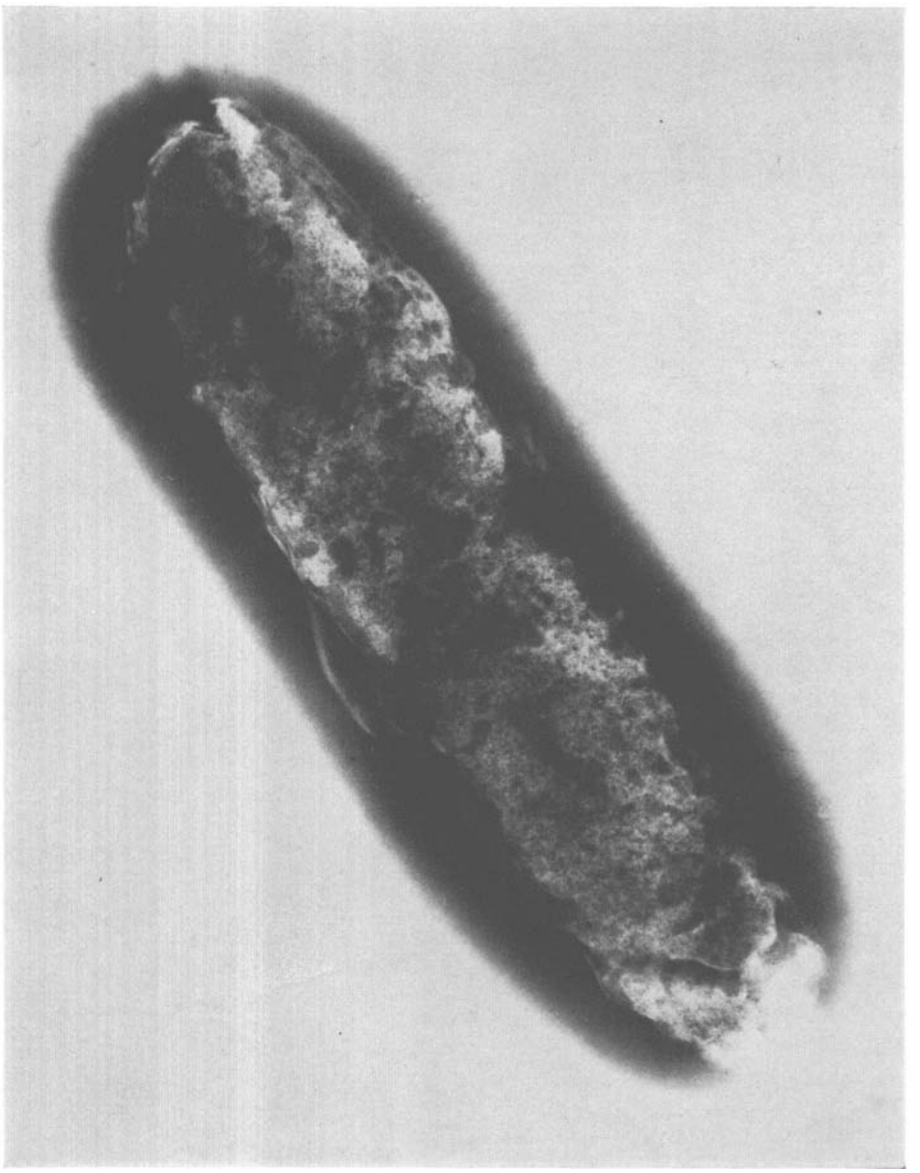

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