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Immunogenicity of a Chaotropically Extracted Protective Antigen(s) of *Pasteurella multocida* Type A (Bovine Origin) Against Experimental Pasteurellosis in Mice

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Immunization of mice with a potassium thiocyanate extract of *Pasteurella multocida* type A (bovine strain) imparted complete protection against an experimental challenge infection with $1{\cdot}6 \times 10^3$ colony-forming units of the homologous strain. Formalin-treated vaccine (one or two doses) was much less effective. Significantly higher bactericidal antibody titres were observed in the sera of mice immunized with the extract compared with those given the vaccine. The bactericidal activity was localized in the IgM and IgG fractions. The extract consisted of protein(s) and carbohydrate(s) and on immunoelectrophoresis displayed at least three antigenically distinct components.

INTRODUCTION

The importance of *Pasteurella multocida* in the aetiology of animal diseases such as haemorrhagic septicaemia of cattle, pneumonic pasteurellosis of cattle and fowl cholera in chickens and turkeys is well recognized (Bains, 1955; Carter, 1967). Pasteurellosis in cattle in North America occurs mainly in the pneumonic form, and *P. multocida* and *P. haemolytica* are the two species most frequently isolated from active cases of disease. While considerable data are now available on *P. haemolytica* infections and immunity in mice and cattle (Thomson & Gilka, 1974), information has only recently become available on *P. multocida* infection and immunity in mice (Baba, 1977; Collins, 1973). *Pasteurella multocida* involved in pneumonic pasteurellosis in cattle and fowl cholera is Carter's type A, in which capsular substance is composed of hyaluronic acid polymers (Carter & Rundell, 1975). The capsule of *P. multocida* type B is a glycoprotein (Bains, 1955), extractable with chaotropic ions such as potassium thiocyanate (KSCN). The latter has been found to protect against experimental pasteurellosis in mice (Bains, 1955; Mukkur & Nilakantan, 1972) and haemorrhagic septicaemia in cattle (Dhanda, 1958; Mukkur & Nilakantan, 1972). The purpose of the present investigation was to compare the protective properties in mice of a formalin-treated intact cell vaccine with those of KSCN-extracted soluble antigen(s) of *P. multocida* type A.

METHODS

Maintenance and serotyping of the organism. The strain of *P. multocida* used in this study was originally isolated from the lung of a cow suffering from acute bronchopneumonia; it has been identified as Carter's type A (Carter & Rundell, 1975) and Heddleston's serotype 3 (Heddleston *et al.*, 1972). The organism was

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maintained on 5% fresh human blood agar (FBA) at 4°C, subcultured once a week and passaged through mice every 4 weeks to maintain it in the fully encapsulated form.

Preparation of P. multocida vaccine. Pasteurella multocida was harvested from plates of FBA with 0·3% (v/v) formalin/saline solution. The resultant suspension was incubated at 37°C for 12 h, a procedure which killed the organisms; they were then washed at least three times with saline and finally resuspended in saline to give the desired bacterial count spectrophotometrically (Bausch & Lomb Spectronic 20). An A_{600} of 0·25 corresponded to $2{\cdot}30 \pm 0{\cdot}45 \times 10^8$ bacteria ml^{-1}.

Preparation of KSCN extract. The procedure was modified from that of Bains (1955) in that 'live bacteria' rather than acetone-dried cells were used. Briefly, *P. multocida* was grown on FBA for 48 h and harvested in 0·5 M-KSCN/0·08 M-NaCl (Fisher Scientific Co., Toronto, Canada). The suspension was incubated in a shaking water-bath at 37°C for 5 h and then sedimented by centrifugation at 17300 *g* for 30 min. The supernate was filtered through a 0·8 μm Millipore filter, dialysed exhaustively against 0·32 M-NaCl/0·01 M-Tris/HCl, evaporated under reduced pressure and stored frozen at −20°C until required.

Spectral analysis of the extract revealed absorption maxima at 226, 276 and 418 nm. A standard curve relating the A_{276} of the extract to the protein concentration, determined by the method of Lowry *et al.* (1951), was constructed and used to determine the protein concentration in the extract used in the various immunization experiments.

Toxicity of the KSCN extract. To determine the toxicity of the extract, 31·25, 62·5, 125 or 250 μg extract protein were injected intraperitoneally (i.p.) into groups of 10 mice. The mice were then kept under observation for 4 weeks and their mortality and morbidity were recorded daily.

Schedule of immunization. Groups of 15 mice were immunized according to the following schedules, the whole scheme being repeated four times: (i) one i.p. inoculation with formalin-treated *P. multocida* vaccine to give a dose of either 10^6, 10^7, 10^8, 4×10^9 or 10^{10} colony-forming units (c.f.u.); (ii) two i.p. inoculations with formalin-treated *P. multocida* vaccine administered 2 weeks apart, the first dose consisting of 10^6 c.f.u. and the second 10^7 c.f.u.; (iii) one i.p. inoculation with 1·0 ml KSCN extract containing either 31·25, 62·5 or 125 μg protein ml^{-1}; (iv) one i.p. inoculation of a mixture of 0·5 ml KSCN extract (125 μg protein ml^{-1}) and 0·5 ml formalin-treated *P. multocida* vaccine containing 2×10^8 c.f.u. ml^{-1}.

Determination of LD_{50}. The LD_{50} of *P. multocida* was determined at the start of the project for both the i.p. and intranasal (i.n.) routes. Bacterial suspensions were adjusted spectrophotometrically, appropriately diluted to contain 3 to 10^8 c.f.u. *P. multocida* ml^{-1} (as judged by direct plating) and inoculated by the appropriate route into groups of 10 mice; the deaths were recorded for up to 1 week post-challenge. Post-mortem examinations were performed on the dead animals, and *P. multocida* were isolated from the lungs and spleen and identified according to established criteria (Carter, 1967). For challenge by the i.p. route, 1·0 ml bacterial suspension was injected. For challenge by the i.n. route, mice were anaesthetized with ether and 0·01 ml bacterial suspension (10^3 to 10^8 c.f.u. ml^{-1}) was instilled into their nares using a tuberculin syringe fitted with a blunt, polished 27 gauge needle. Extreme care was taken to avoid injury to the nares and any mouse whose nasal mucus membrane was visibly injured was discarded. The number of c.f.u. in spectrophotometrically standardized suspensions and their dilutions were determined by plating on trypticase soy agar (TSA; Difco). The LD_{50} was calculated by the method of Reed & Muench (1938). The LD_{50} for both routes was determined three times.

Challenge experiments. Ten of the immunized mice in each group were challenged i.p. and/or i.n. with various numbers of c.f.u. homologous *P. multocida* at 1 or 4 weeks post-immunization. The challenge dose is expressed in c.f.u. because of the variation observed in the LD_{50} of *P. multocida*. Again, the numbers of c.f.u. in the spectrophotometrically adjusted bacterial suspensions used for challenge were determined by plating on TSA plates.

Determination of bactericidal titre. For the bactericidal assay, a reaction mixture consisting of 200 to 400 c.f.u. *P. multocida*, serial two- or tenfold dilutions of an antiserum, and guinea pig complement was incubated at 37°C for 30 min. (The quantity of each reagent in the reaction mixture was either 0·2 or 0·5 ml.) At the end of the incubation period, 0·1 ml samples of the reaction mixture were plated on TSA plates; these were incubated at 37°C and the surviving pasteurellae were counted after 24 h. A graph of the percentage of c.f.u. against the antiserum dilution was then plotted to determine the bactericidal titre. Generally, the graph was either horizontal or had a slightly negative slope up to a certain antiserum dilution (e.g. 1:20), and then the slope became positive for the succeeding dilutions (which in this case would be 1:40, 1:80, 1:160 etc.). Bactericidal titre was defined as the lowest dilution of antiserum at which the slope of the graph was positive. It is emphasized here that the determination of bactericidal titres by the method of Kenny & Herzberg (1968), in which the titre is defined as the dilution of antiserum that kills 50% of the bacteria in the original inoculum, could not be used because less than 50% kill was observed when the antiserum used was from the group immunized with formalin-treated vaccines. Sera from five mice within each immunization regimen were pooled for bactericidal tests. As each experiment was carried out in quadruplicate, the titres obtained were the average of four readings.

Immunoelectrophoresis. The KSCN extract was subjected to immunoelectrophoresis according to Scheidegger (1955). The antisera used were rabbit antiserum to formalin-treated *P. multocida* and rabbit antiserum to the KSCN extract of this organism.

Chemical analysis. The total protein content was determined by the method of Lowry *et al.* (1951) and total carbohydrates by the α-naphthol reaction (Dische, 1955).

Fractionation of mouse sera. To isolate the IgM and IgG fractions, $(NH_4)_2SO_4$-precipitated mouse sera were separated on columns of Bio-Gel P-300 equilibrated with 0·32 M-NaCl/0·01 M-Tris/HCl, pH 8·0.

Reduction and alkylation. Immunoglobulin fractions were treated with 2-mercaptoethanol and alkylated with sodium iodoacetate (Mukkur & Inman, 1971).

Statistical analysis. The multiple comparison test (Dunnett, 1964) was used to compare the immunizing efficiency of the formalin-treated *P. multocida* vaccine with that of the extract at a significance level of 5%.

RESULTS

LD_{50} of *P. multocida*

In three separate experiments, the LD_{50} of *P. multocida* was 5, 3 and 7 c.f.u. on challenge by the i.p. route, yielding an average LD_{50} of $5 \pm 2{\cdot}8$ c.f.u. When challenged by the i.n. route, the LD_{50} was $6{\cdot}2 \times 10^4$, $3{\cdot}8 \times 10^4$ and 5×10^4 c.f.u. in three individual experiments, yielding an average of $5{\cdot}0 \pm 2{\cdot}1 \times 10^4$ c.f.u.

Immunizing efficiency of one dose of formalin-treated P. multocida vaccine

Regardless of the number of formalin-treated c.f.u. used for immunization, all the immunized mice were protected on i.p. challenge infection with 15 c.f.u. homologous *P. multocida* given 4 weeks post-immunization (Table 1). However, the degree of protection decreased as the challenge dose was increased, for when the challenge infection was increased to $1{\cdot}5 \times 10^2$ and $1{\cdot}5 \times 10^3$ c.f.u., the survival rate was reduced to 53 to 63% and 10 to 20%, respectively. On i.n. challenge with $1{\cdot}5 \times 10^5$ c.f.u., the survival rate was the same as that recorded for an i.p. challenge with $1{\cdot}5 \times 10^3$ c.f.u. (Table 1).

Immunizing efficiency of two doses of formalin-treated P. multocida vaccine

The protection against both i.p. and i.n. challenge infection given 4 weeks post-immunization afforded by two doses of formalin-treated vaccine was not significantly greater than that provided by one dose, with the exception of the i.p. challenge dose of $1{\cdot}5 \times 10^3$ c.f.u. homologous *P. multocida.* Here the survival rate in the group of mice receiving two doses was $50 \pm 10\%$ in contrast to 10 to 20% for those receiving only one dose ($P < 0{\cdot}05$).

Toxicity and gross chemical composition of the KSCN extract

None of the mice injected with the extract, in amounts ranging from 31·25 to 250 μg protein, died as a direct consequence of its administration. While the amount of protein extracted from 4×10^9 live *P. multocida* was determined to be 125 μg, the total carbohydrate content was calculated to be 40 μg. However, the amount of lipopolysaccharide in the extract was not determined.

Immunizing efficiency of the KSCN extract

When mice immunized i.p. with 1·0 ml KSCN extract containing either 31·25, 62·5 or 125 μg protein ml^{-1} were challenged i.p. with 10, 10^2 and 10^3 c.f.u. and i.n. with 10^6 c.f.u. homologous *P. multocida* given 4 weeks post-immunization, all were protected. Therefore, for the subsequent studies, mice immunized with 62·5 μg extract protein were used.

While all the mice immunized with the extract resisted i.p. challenge infections with 16, $1{\cdot}6 \times 10^2$ and $1{\cdot}6 \times 10^3$ c.f.u. homologous *P. multocida* at 4 weeks post-immunization, the

Table 1. *Percentage survival of mice immunized intraperitoneally with one dose of formalin-treated P. multocida vaccine containing various numbers of colony-forming units on challenge infection with the homologous P. multocida at 4 weeks post-immunization*

All experiments were done in quadruplicate with a group of 10 mice for each experiment. None of the unimmunized control mice survived any of the above challenge infections.

Challenge dose* (c.f.u.)	Route of challenge	Percentage survival (±S.E.) No. of c.f.u. used for immunization				
		10^6	10^7	10^8	4×10^9	10^{10}
15	i.p.	100±0	100±0	100±0	100±0	100±0
$1{\cdot}5\times10^2$	i.p.	55±5	63±4	60±0	60±4	53±2
$1{\cdot}5\times10^3$	i.p.	10±0	20±4	20±0	20±10	10±0
$1{\cdot}5\times10^5$	i.n.	20±4	10±0	20±10	10±0	10±0

i.p., Intraperitoneal; i.n., intranasal.
* Based on the count of colony-forming units (c.f.u.).

Table 2. *Percentage survival of mice immunized intraperitoneally with 62·5 μg KSCN extract protein on challenge infection with the homologous P. multocida at 1 and 4 weeks post-immunization*

All experiments were done in quadruplicate with a group of 10 mice for each experiment. None of the unimmunized control mice survived any of the above challenge infections.

Challenge dose* (c.f.u.)	Route of challenge	Percentage survival (±S.E.) Interval post-immunization	
		1 week	4 weeks
16	i.p.	50±0	100±0
$1{\cdot}6\times10^2$	i.p.	53±2	100±0
$1{\cdot}6\times10^3$	i.p.	40±0	100±0
$1{\cdot}6\times10^4$	i.p.	ND	70±10
$1{\cdot}6\times10^6$	i.n.	ND	100±0
$1{\cdot}6\times10^7$	i.n.	ND	60±4

i.p., Intraperitoneal; i.n., intranasal; ND, not done.
* Based on the count of colony forming units (c.f.u.).

survival rate at 1 week ranged between 40 and 53% depending on the challenge dose. After i.n. challenge with $1{\cdot}6\times10^6$ c.f.u., none of the immunized mice died (Table 2).

Immunizing efficiency of a vaccine consisting of a mixture of KSCN extract and formalin-treated P. multocida

When mice immunized with a mixture of KSCN extract (62·5 μg protein) and 1×10^6 formalin-treated c.f.u. *P. multocida* were challenged i.p. 4 weeks post-immunization with $1{\cdot}3\times10^3$ c.f.u. homologous *P. multocida*, all survived. However, on challenge with $6{\cdot}5\times10^3$, $1{\cdot}3\times10^4$ or $1{\cdot}3\times10^5$ c.f.u. homologous *P. multocida*, the survival rates were 80, 60 and 40%, respectively. On i.n. challenge with $1{\cdot}3\times10^7$ c.f.u. homologous *P. multocida*, 50% of the immunized mice survived.

Bactericidal titres of mouse sera following immunization with formalin-treated P. multocida vaccines and KSCN extract

The sera obtained from mice 4 weeks after immunization with the different schedules and examined for their bactericidal titre against the homologous *P. multocida* showed that the KSCN extract alone was the most powerful immunogen and that one dose of formalin-

Table 3. *Bactericidal titres of mouse sera* 4 *weeks after immunization with formalin-treated P. multocida vaccines and/or KSCN extract of P. multocida*

Immunization schedule	Immunization dose (c.f.u. and/or μg protein)	Bactericidal titre ($\pm$S.E.)
Formalin-treated *P. multocida*	10^6	20 ± 2
	10^7	20 ± 2
	10^8	20 ± 2
	4×10^9	20 ± 3
	10^{10}	5 ± 0
	10^6 followed by 10^7 2 weeks later	50 ± 4
KSCN extract	62·5 μg	200 ± 35
Formalin-treated *P. multocida* plus KSCN extract	$10^6+62\cdot5$ μg	86 ± 7

Table 4. *Localization of bactericidal activity in mouse antisera to the KSCN extract of P. multocida collected* 6 *weeks post-immunization*

Immunoglobulin fraction	Concn (μM)	Percentage survival of *P. multocida* in the bactericidal test
Native IgM	1·94	40
Native IgG	12·8	42
2ME-treated IgM	10	90
2ME-treated IgG	12·8	40

2ME, 2-Mercaptoethanol.

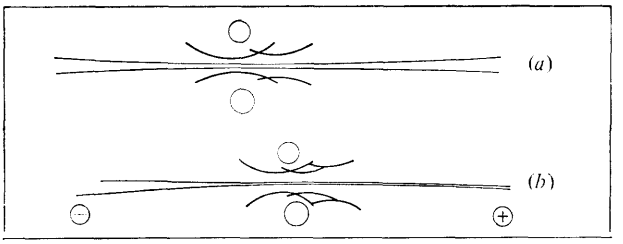

Fig. 1. Line drawing of the immunoelectrophoretic patterns of the KSCN extract of *P. multocida* developed with rabbit antiserum to (*a*) formalin-treated *P. multocida* and (*b*) KSCN extract. All the wells contained KSCN extract.

treated *P. multocida* vaccine was the least effective (Table 3). Two doses of formalin-treated *P. multocida* vaccine were little better than one. The addition of formalin-treated *P. multocida* vaccine reduced the efficacy of the extract alone, as judged by a reduction both in the survival rate following homologous *P. multocida* challenge (date not shown) and in the bactericidal titre (Table 3).

Localization of antibody activity and immunoelectrophoretic analysis

Both IgM and IgG isolated from mouse antiserum to the KSCN extract of *P. multocida* obtained 6 weeks post-immunization possessed bactericidal activity (Table 4). Treatment of IgM with 2-mercaptoethanol resulted in the expected reduction of bactericidal activity (Kenny & Herzberg, 1968). The KSCN extract subjected to immunoelectrophoresis showed the presence of three arcs on development with rabbit antiserum to the extract. However, only two arcs were discernible on development with rabbit antiserum to formalin-treated *P. multocida* (Fig. 1).

DISCUSSION

Practically no significant differences were observed between the immunizing efficiency of one and two doses of the formalin-treated *P. multocida* vaccine, as judged by the responses to challenge and the development of bactericidal antibody titres. These results support the earlier conclusions of Collins (1973), although the level of protection achieved in mice immunized with two doses of formalin-treated *P. multocida* vaccine was not the same. This could be attributed to differences in the origins of the strains used in the two investigations.

Mice immunized with the KSCN extract of *P. multocida* were significantly more resistant to i.p. and i.n. challenge infection with homologous *P. multocida* at 4 weeks post-immunization than those immunized with either one or two doses of formalin-treated *P. multocida* vaccine. However, no further improvement in the degree of protection against pasteurellosis was observed when the KSCN extract and *P. multocida* were used together as immunogens. In fact, the humoral antibody response of mice so immunized was characterized by significantly lower bactericidal titres than those observed in mice immunized with the extract alone (Table 3). One could argue that the lower bactericidal titres obtained with sera from mice immunized with less than 4×10^9 c.f.u. of formalin-treated *P. multocida* were due to the administration of KSCN-extractable protein in a quantity less than 62·5 μg. For example, 10^9 c.f.u. *P. multocida* would contain 31·25 μg KSCN-extractable protein. But the same argument is not applicable to those groups of mice immunized with formalin-treated *P. multocida* containing 4×10^9 or 10^{10} c.f.u. The observed immunosuppression might conceivably be due to antigenic competition (Schwab, 1975) or partial denaturation and/or modification of some vital protective antigen(s) as a result of formalin treatment.

The importance of antibodies in immunity to pasteurellosis in mice was recently suggested by Collins (1973), who reported a successful transfer of immunity against *P. multocida* infection in mice by passive transfer of serum antibodies. Although the contribution of respiratory immune mechanisms, such as the role of nasal secretory IgA and specific activated alveolar macrophages, in immunity to pasteurellosis in mice has not been investigated, enhancement of the rate of specific bacterial clearance in immunized mice has been reported (Thomson & Gilka, 1974).

Since the capsular substance of *P. multocida* type A has previously been reported to be composed of hyaluronic acid polymers and not glycoproteins as for type B strains (Bains, 1955; Dhanda, 1958; Prince & Smith, 1966), it will be interesting to establish the location of the protective antigens extracted with KSCN. The physico-chemical characterization of the extract, including the isolation of the immunogenic principle and its relationship to the free endotoxin isolated recently from an avian strain of *P. multocida* (Rebers & Heddleston, 1974), is under investigation at the present time.

Previous studies (Bains, 1955; Dhanda, 1958) suggested that immunity to pasteurella infections was capsular-type specific and more recent studies have suggested that it is somatic serotype specific (Heddleston & Rebers, 1972). It would, therefore, be ideal to obtain an antigen preparation that could be used as an immunogen to impart immunity not only against the homologous but also heterologous serotypes of *P. multocida*. Investigations in this laboratory have recently demonstrated cross-protection between *P. multocida* type A (bovine origin) and *P. haemolytica* serotype 1 (Mukkur, 1977). Cross-protection was also demonstrated between two avian serotypes of *P. multocida* type A in chickens (Gaunt *et al.*, 1978). As the KSCN-extracted protective antigen(s) from all the 15 avian serotypes of *P. multocida* type A showed, on gel diffusion analysis, at least two precipitin lines which were antigenically identical with each other, the extract might contain some antigens that are common to different serotypes of *P. multocida* type A and *P. haemolytica* serotype 1 (other strains of *P. haemolytica* have not yet been tested). This raises hope for the development of a cross-protecting vaccine for *P. multocida* type A infections.

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