Outer membrane proteins of bovine strains of *Pasteurella multocida* type A and their doubtful role as protective antigens

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Summary. Outer membranes were prepared by the Sarkosyl method from 30 strains of *Pasteurella multocida* and the closely related Taxon 13, which had been isolated from cattle. The patterns of the outer membrane proteins (OMPs) on SDS-PAGE were generally similar to one another, though the four major proteins (*a–d*) varied somewhat in molecular mass; these patterns allowed the strains to be arranged into 12 groups. Taxon 13 strains and typical *P. multocida* strains were indistinguishable, both types being found within the same group. Mice were vaccinated with heat-killed bacteria of three strains and challenged with 10 LD₅₀ of homologous and heterologous live bacteria, representing groups based on OMP patterns; the best protection was afforded by strain W674, which protected against nine of the 17 challenge strains; but there was no correlation between protection and PAGE pattern. Pre-vaccination and pre-challenge sera were used in immunoblotting to probe OMPs from protective and non-protective strains. All three vaccines produced antibody to proteins *a* and *d*; these proteins appeared to be common to all strains, varying in molecular mass but not in overall antigenic expression. The antibody response to the other two major OMPs appeared to be PAGE-group specific. There was no correlation between protection and the antigen pattern seen by immunoblotting.

Introduction

Pasteurella multocida is a small gram-negative, saccharolytic, indole-positive, facultatively anaerobic coccus. Carter's capsular serotype A is associated with several animal diseases of world-wide importance such as bovine and porcine pneumonia, fowl cholera and rabbit septicaemia.^{1–3} Type A can be distinguished from other types^{4,5} by indirect haemagglutination (IHA) and hyaluronidase decapsulation (HYD). Similar organisms⁶ that do not produce indole and are unable to ferment mannitol and sorbitol have been designated Taxon 13.

Many studies of the pathogenic mechanisms and immunogenicity of surface extracts of *P. multocida* serotypes have been made with the aim of developing vaccines.^{7–11} However, effective vaccines have been developed only for types B and E, the haemorrhagic septicaemia serotypes.¹² Lugtenburg *et al.*^{13,14} have shown a correlation between cell-

envelope-protein type and pathogenicity of strains, presumably type D,¹⁵ isolated from swine atrophic rhinitis. For type A strains, an experimental vaccine for rabbits has been reported recently. It is based on a potassium thiocyanate extract of whole bacteria¹⁶ and the protective immunogen has been identified as a 37.5-Kda outer membrane protein (OMP).¹⁷

The aim of this study was to analyse the OMP profiles of *P. multocida* type A and the related Taxon 13 strains isolated from bovine pneumonia, to determine the heterogeneity of this group, and to attempt to define a protective immunogen in a mouse septicaemia model, by investigation of the immunogenicity of the OMPs.

Materials and methods

Bacteria

With the exception of the reference strain NCTC 10322 which was obtained from the National Collection of Type Cultures, London NW9 5HT, all the strains were isolated at Veterinary Investigation Centres from the lungs of calves with pneumonia, and were identified

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provisionally as *P. multocida*. They were sent to the Moredun Research Institute (MRI) on blood-agar plates, and subcultured there once on plates of Dextrose Starch Agar (DSA; Gibco). After overnight incubation, pure cultures were suspended in skimmed milk and stored by freeze drying. Strains were typed by IHA and HYD,²⁰ and identified further by biochemical reactions in a combination of conventional tube tests and API 20E test strips. The IHA technique was similar to that of Carter,^{4, 18} except that glutaraldehyde-fixed sheep erythrocytes were used instead of human group O cells.¹⁹ Tests were performed in 50- μ l volumes in U-shaped wells of microtitration plates (Sterilin) with rabbit antiserum kindly supplied by Dr V. Norring (Statens Veterinære Serum Laboratorium, Denmark). From the 93 strains submitted, 30 were selected for this study: 24 of *P. multocida* type A, and six of Taxon 13. More details of the bacterial identification have been published elsewhere.²¹

Vaccine strains and preparation of vaccine

In a preliminary study,²² vaccine preparations from nine strains, containing capsule, lipopolysaccharide, formalin-killed bacteria or heat-killed bacteria, were tested for protective ability against homologous and heterologous challenge with 10 LD₅₀ of live bacteria. The heat-killed cell vaccines of *P. multocida* strains W674 and W829 afforded the best heterologous protection. These two strains, and a Taxon 13 strain (X120) which gave no protection, were selected for the present study.

Preparation of vaccine. Freeze-dried bacteria were plated on DSA and incubated overnight at 37°C in a moist sealed chamber to enhance hyaluronic acid production.²³ Several iridescent colonies, which represent the wild-type (virulent) phenotype, were inoculated into 30 ml of Brain Heart Infusion Broth (BHIB; Oxoid) and incubated overnight at 37°C in an orbital shaker. The culture was transferred to 1 L of BHIB in a 2-L conical flask and kept for 6 h at 37°C in an orbital incubator. The cells were harvested by centrifugation at 12 000 *g* for 20 min at 20°C, washed once in phosphate buffered saline pH 7.4 (PBS) and finally resuspended in 10 ml of PBS. They were killed in a water bath at 68°C for 90 min and, after sampling for sterility, they were freeze-dried. Vaccine consisted of dried bacteria (final concentration 5 mg/ml) in equal volumes of water and Bayol F (Esso) containing Arlacel (Sigma) 10%.

Immunisation and challenge of mice

Swiss white mice of both sexes, bred at MRI, were 4–6 weeks old at the beginning of an experiment. Groups of 10 mice were given two doses of 0.2 ml of vaccine subcutaneously, 14 days apart. Vaccinated mice and non-vaccinated controls were challenged intraperitoneally with 10 LD₅₀ of a 6-h broth culture of the organism, 14 days after the second vaccination; and deaths were recorded up to 48 h after challenge. The LD₅₀ was

determined previously by the method of Reed and Muench;²⁴ it ranged from 7.5×10^6 to 1.2×10^8 organisms.

A 50- μ l sample of blood was collected from the tail vein of each mouse pre-vaccination and pre-challenge. The blood from each group was pooled, and the serum was stored at –20°C.

Isolation of outer membrane (OM)

A modification of the method of Filip *et al.*,²⁵ for the preparation of OM from *E. coli*, was used. Confluent growth from each of five DSA plates was washed off with 5 ml of 0.01 M HEPES buffer, pH 7.4. The bacteria were deposited by centrifugation (20 000 *g*, 15 min), resuspended in 20 ml of buffer, and disrupted in a French pressure cell (Aminco, Silver Springs, MD, USA) at 6000 psi. After removal of unbroken cells by centrifugation (10 000 *g*, 15 min), c. 400 μ l of Sarkosyl (sodium N-lauroyl sarcosinate; Sigma; 30% w/v) was added to give a final concentration of 0.7%. The OM was deposited by centrifugation at 50 000 *g* for 1 h, washed in distilled water, resuspended in 1 ml of distilled water, and stored at –20°C.

SDS-PAGE and immunoblotting

OM proteins were analysed on slab gels 10% with Laemmli buffers,²⁶ and stained with Coomassie Blue.

The method of Hancock and Poxton²⁷ was used for immunoblotting. Separated proteins were transferred to nitrocellulose membranes (Sartorius, 0.2 μ m pore-size), allowing transfer to proceed for 16 h at 12 V. For immunodetection by the BioRad system, the mouse sera were diluted 1 in 100, and the rabbit anti-mouse IgG-horseradish peroxidase conjugate (ICN Immunochemicals) was diluted 1 in 500.

Results

SDS-PAGE of outer membrane protein (OMP)

Fig. 1 shows the Sarkosyl OM preparations from 30 strains of *P. multocida* type A and Taxon 13, analysed by SDS-PAGE, and stained with Coomassie Blue. Overall, the patterns are very similar, revealing two, three or four major proteins (labelled *a*, *b*, *c* and *d*) with molecular masses (*M_r*) in the range 36.5–19 Kda, though each protein varied in *M_r* over a limited range. By visual examination, the strains could be arranged in 12 groups based on the mobility of these major OMPs (table I).

Proteins *b* and *d* were the most strongly stained and were present in all strains. The *M_r* of protein *b* was in the range 34.5–29.5 Kda. Protein *c* showed the most constant *M_r*—26 Kda in the majority of strains. Protein *a* was absent or incompletely

Fig. 1. SDS-PAGE gels of Sarkosyl OM preparations from strains of *P. multocida* and Taxon 13, * stained with Coomassie Blue; a, b, c, d represent major OMPs. (a) Track 1, strain X200; 2, W829; 3, X1113; 4, W571; 5, X198; 6, X1016; 7, X195; 8, W599; 9, W674; 10, X1043; 11, NCTC 10322; 12, W819; 13, X969; 14, W590; 15, W833; 16, X109. (b) Track 16, strain X109; 17, X12; 18, X9; 19, W828; 20, X699; 21, X1122; 22, X918; 23, X1053; 24, X110; 25, X120; 26, W577; 27, X483; 28, W971; 29, W820; 30, X1049.

Table I. Grouping of 24 strains of *P. multocida* and six strains of Taxon 13 with patterns produced by major OMPs on SDS-PAGE as shown in fig. 1

Group	Tracks (fig. 1)	Strains of <i>P. multocida</i> or Taxon 13
1	1 2 3	X200 W829 X1113
2	4 6 7	W571 X1016 X195
3	8 9	W599 W674
4	10	X1043*
5	5 11	X198 NCTC10322
6	12 13	W819* X969
7	14	W590*
8	15 16 17 18 19	W833 X109 X12 X9 W828
9	20 21	W669 X1122
10	22 23 24 25	X918 X1053 X110 X120*
11	26	W577*
12	27 28 29 30	X483 X971 W820* W1049

* Six strains of Taxon 13.

resolved in strains X200, W829, X1113, W571, X198, X1016, X195 and NCTC 10322; where present, its M_r ranged from 36.5 to 33.5 Kda. The M_r of protein *d* ranged from 21 to 19 Kda.

There were no features in the OMP patterns which allowed differentiation between the typical *P. multocida* and the Taxon 13 strains.

Mouse protection

Groups of mice were vaccinated with heat-killed cells of strains W674 and W829 (*P. multocida*) and strain X120 (Taxon 13). Vaccinated mice and non-vaccinated controls were challenged with 10 LD₅₀ of the homologous strain and 16 heterologous strains representing most of the groups defined by SDS-PAGE of the OMPs (table II). Strain W674 appeared to afford the best protection—at least 50% protection against nine of the 17 challenge strains. Strain W829 gave this degree of protection against four strains, but the Taxon 13 strain X120 protected only against strain X9. Strain X120 was also the only strain that did not afford homologous protection at this level. There was no apparent correlation between the SDS-PAGE group pattern and protection.

Demonstration of antibody response to the major OMPs in vaccinated mice by immunoblotting

For each of the three groups of vaccinated mice, seven strains (against some of which vaccination was protective and some non-protective) were selected for investigation by immunoblotting with pre-vaccination and pre-challenge sera. With the pre-vaccination sera, immunoblots showed no

reaction. In the pre-challenge sera, however, the mice had responded to at least two of the major OMPs; but there was no correlation between immunoblot pattern and protection. Proteins *a* and *d* were the most immunogenic. Immunoblots with pre-challenge sera from mice vaccinated with strain W829 are shown in fig. 2b. There was a relatively uniform response to both *a* and *d* proteins; but this did not depend upon the M_r being the same as that of the vaccine strain. It appears that there are common epitopes on all the *a* proteins and similarly on all the *d* proteins. The response to the other two major proteins was much more variable and appeared to be PAGE-group specific, e.g., strains W829 and X1113 in tracks 1 and 3 of fig. 2 both belong to PAGE group 1 (table I). There was, however, no correlation between the patterns and protection: vaccine W829 protected against the strains in tracks 1, 2, 3 and 6, but not against the strains in tracks 4, 5 and 7, although the immunoblots in tracks 4 and 6 are indistinguishable.

Discussion

The patterns produced on SDS-PAGE by Sarkosyl extracts of *P. multocida* are similar to the patterns produced by the OMPs of other gram-negative bacteria, in which there are a small number of major proteins and several minor ones.²⁸ The four major proteins which we have labelled *a*–*d* have allowed us to arrange strains of *P. multocida* into groups (table I). It is apparent from this grouping that strains of Taxon 13 share profiles of major OMPs with strains of *P. multocida*, and we suggest that these taxa cannot be distinguished on this basis.

Table II. Percentage protection of mice in groups of 10 vaccinated with heat-killed organisms and challenged with strains of *P. multocida* or Taxon 13

Vaccine strain	Percentage protection* against challenge with																
	<i>P. multocida</i> strain										Taxon 13 strain						
	W674 (3)†	W829 (1)	W669 (9)	W571 (2)	X9 (8)	X12 (8)	X110 (10)	X198 (5)	X971 (12)	X1016 (2)	X1053 (10)	X1113 (1)	X1122 (9)	X120 (10)	W577 (11)	W590 (7)	W819 (6)
W674	70	50	50	0	20	60	60	20	20	0	50	60	80	70	0	20	20
W829	50	90	0	0	60	0	0	...	10	0	30	50	40	0	0	0	20
X120	40	30	0	0	60	20	20	...	10	0	20	0	40	20	0	20	20

* Percentage protection = $\frac{\text{number of vaccinated survivors} - \text{number of non-vaccinated survivors}}{10} \times 100$

† In parenthesis : SDS-PAGE group no.—see table I.

Fig. 2. (a) SDS-PAGE gels of Sarkosyl OM preparations from strains of *P. multocida* and Taxon 13* against which mice were or were not protected by vaccination with strain W829 (see table II). Track 1, strain W829 (protected); 2, W674 (protected); 3, X1113 (protected); 4, X12 (not protected); 5, W577* (not protected); 6, X9 (protected); 7, X120* (not protected). (b) Corresponding immunoblot probed with pre-challenge serum from mice vaccinated with heat-killed cells of strain W829. (a, b, c, d represent major OMPs).

When mice were vaccinated with heat-killed cells they produced an antibody response to two or more of the major OMPs (fig. 2b). This response was a combination of species-specific and PAGE-group-specific antibodies, but the latter did not correlate with protection. The proteins were antigenic in mice but not protective. Moreover, although proteins may differ from one another in M_r , they did not necessarily differ antigenically. This finding applies also to other groups of closely related species, e.g., some of the major OMPs of Enterobacteriaceae share epitopes but differ in M_r .²⁹

Our conclusion is that, in this mouse model of pasteurellosis, the major OMPs are not protective antigens, and therefore may be unlikely candidates for vaccines. However, this appears to contradict

the findings of Lu *et al.*¹⁷ who showed that the 37.5 Kda OMP (presumably our protein *a*) was protective against homologous challenge in a rabbit model; but in a mouse model, protection was afforded against heterologous challenge only if the challenge strain was shown, by probing with a monoclonal antibody, to express the 37.5-Kda antigen.³⁰ Protection was not provided against strains lacking this antigen. Thus, they showed that this OMP can be a protective immunogen in the strains that possess it; but they concede that other antigens may be more important as vaccine candidates, because only 24% of their strains of *P. multocida* expressed this antigen.

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