Comparison of Cell Surface Antigen Extracts From Two Serotypes of Pasteurella haemolytica

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Cells of Pasteurella haemolytica serotypes A1 and A6 were extracted with sodium salicylate and the chemical and antigenic composition of both extracts determined. The extracts were concentrated by ultrafiltration and the serotype antigen, measured by the indirect haemagglutination test, was estimated to have a molecular weight between 100000 and 300000. The chemical composition of sodium salicylate extracts (SSEs) from both serotypes was similar, having protein, carbohydrate, fatty acid and phosphorus present in the ratio 10:1:0.5:0.1. SDS-PAGE of both SSEs gave similar profiles with at least 48 bands present. These results suggest that sodium salicylate removes the outer membrane of P. haemolytica. Crossed immunoelectrophoresis indicated that a major serotype-specific antigen was present in SSEs of both strains. This antigen was extracted from the SSE with hot phenol/water and analysed by gas chromatography. The sugar composition of A1 and A6 phenol/water extract (PWE) was qualitatively identical although some differences in proportions were observed. A1 and A6 PWE antigens protected mice against homologous serotype challenge and A6 PWE protected against heterologous (A1) challenge.

INTRODUCTION

Pasteurella haemolytica is the causal organism of enzootic pneumonia and septicaemia in sheep. There are at least 15 serotypes of P. haemolytica (Fraser et al., 1982) and these fall into the two biotypes described by Smith (1961).

Antigens from a number of A biotype strains have been obtained by extraction of cells with sodium salicylate (Gilmour et al., 1979) and vaccines containing these antigens have been shown to confer protection against homologous but not heterologous serotype challenge in sheep (Gilmour et al., 1979, 1983) and in mice (Evans & Wells, 1979).

Burrells et al. (1983) reported considerable cross-reactivity in ELISA between sodium salicylate-extracted antigens from different serotypes and Donachie et al. (1983), using the same method, demonstrated that serotype specificity resided in the polysaccharide antigens. Apart from these reports very little is known about the chemical, physical and antigenic composition of sodium salicylate extracts.

We describe here the results of chemical and in vitro immunological analyses of sodium salicylate extracts from P. haemolytica serotypes A1 and A6.

METHODS

Bacteria. Pasteurella haemolytica biotype A, serotypes 1 and 6 (A1 and A6), strains FA1 and FA6, were isolates from sheep with pneumonic pasteurellosis. They were stored lyophilized or in nutrient broth at −70 °C.

Abbreviations: IHA, indirect haemagglutination; KDO, ketodeoxyoctonic acid; PWE, phenol/water extract; SSE, sodium salicylate extract.

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Preparation of sodium salicylate extracts (SSEs). Strains of *P. haemolytica* A1 and A6 were removed from storage, checked for purity and serotype identity and inoculated into 50 ml Oxoid no. 2 broth. After incubation at 37 °C for 18 h with agitation, 15 ml of the resultant culture were inoculated into 1:51 of the same medium and further incubated at 37 °C for 6 h with agitation. Optical density measurements were made with a nephelometer, previously correlated with viable counts, to check that growth had reached approximately 10^6 live bacteria ml⁻¹. The bacteria were sedimented by centrifugation at 4200 g for 40 min at 4 °C and resuspended in one tenth of the broth culture volume in 1 m-sodium salicylate. This suspension was shaken at 37 °C for 3 h and the bacteria were removed by centrifugation at 28000 g for 40 min at 4 °C. The clear supernate was dialysed against a dilute phosphate/saline buffer (PBS: 0·02 m-sodium phosphate; 0·03 m-sodium chloride; pH 7·6) for 48 h. It was then concentrated by ultrafiltration through Diaflo (Amicon, Lexington, Mass., USA) XM300 and XM100A membranes and any slight sediment removed by centrifugation. From a 31 culture approximately 30 ml of an opalescent and slightly viscous concentrate was obtained. All concentration procedures were carried out at 4 °C and the final concentrated extract stored at −20 °C or lyophilized. In later preparations the SSE was concentrated before dialysis without any apparent effect on the final product.

Preparation of phenol/water extracts (PWEs). SSEs were further extracted with phenol by a method similar to that described by Westphal et al. (1952). SSE (20 mg) was suspended in 4 ml distilled water and added to an equal volume of 90% (v/v) phenol. The mixture was heated at 68 °C for 10 min with shaking, cooled in an ice bath and then centrifuged at 28000 g for 30 min. The aqueous layer was removed, dialysed against running tap water for 24 h and lyophilized.

Antisera. Antisera were raised against washed whole cells of *P. haemolytica* A1 and A6 in New Zealand White rabbits using the procedure described by Fraser et al. (1982).

Indirect haemagglutination (IHA) test. A sample of the concentrated SSE or PWE was diluted in PBS in 1:0 ml volumes. Ox red blood cells, fixed in 1% glutaraldehyde in PBS (v/v) (Shirai et al., 1975), were added to the sample dilutions to give a final concentration of 0·5% (v/v) and the suspensions incubated at 37 °C for 30 min. The cells were washed three times in PBS and resuspended to their original 2·0 ml volumes. A homologous rabbit antisem at a previously determined optimal dilution was dispersed in 25 µl volumes into the wells of a U-bottomed microtitre plate (Cookes Engineering Co., Alexandria, Va., USA). After addition of 25 µl sensitized cell suspension per well the plate was allowed to stand at room temperature for 2 h and examined for haemagglutination. Positive and negative controls were included. IHA titres were expressed as reciprocals of the highest dilution of SSE or PWE which produced haemagglutination.

Crossed immunoelectrophoresis (CIE). The procedure was essentially that described by Weeke (1973a). The SSE and PWE antigens were examined by CIE on 5 x 5 cm squares of Gelbond film (FMC Corporation, Marine Colloids Division, Bio Products, Rockland, Me., USA) using barbital/glycine/Tris buffer pH 8·8 (Weeke, 1973b). Buffer was used undiluted (ionic strength 0.08) in the electrode reservoirs, but in gels and in sample buffer it was diluted 1 in 4 with distilled water. Electrophoresis was at 20 V cm⁻¹ for 45 min in the first dimension and 4 V cm⁻¹ for 18 h in the second. Gels were pressed, washed and stained with Coomassie blue as described by Weeke (1973b).

Chemical assays. Total dry weight of the SSE and PWE was determined by freeze drying after complete dialysis against water. Total protein was estimated by the Lowry method with bovine serum albumin as standard. Total carbohydrate was determined by the phenol/sulphuric acid method described by Dubois et al. (1956) with glucose as standard. Total phosphorus was determined by the method of Chen et al. (1956). For the estimation of fatty acid content freeze-dried samples of SSE (10 mg) were hydrolysed with 6 M-HCl (2 ml) at 105 °C for 4 h. The hydrolysates were then repeatedly extracted with diethyl ether (Clarke et al., 1967), the residue dissolved in chloroform, and the fatty acid content determined by the copper colorimetric method of Duncombe (1963) with palmitic acid as standard.

Ketodeoxyxonic acid (KDO) and heptose were measured by the method of Osborn (1963).

Vaccines. Aqueous suspensions of SSE (1·18 mg ml⁻¹) and PWE (0·44 mg ml⁻¹) of A1 and A6 were emulsified with equal volumes of Freund's complete adjuvant (Difco).

Vaccination and challenge. Six-week-old C57 black mice randomly allocated to groups of ten, were inoculated subcutaneously with 0·1 ml vaccine at day 0 and 14. For each serotype, vaccinated and unvaccinated groups of mice were challenged at 28 d after the initial vaccination according to the method of Evans & Wells (1979), except that no mice were killed immediately after challenge. Briefly, mice were challenged intraperitoneally with 0·5 ml of a mixture of bacterial cell suspension in mucin (1:4, v/v). The challenge doses for A1 and A6 were 1·3 × 10⁶ colony forming units (c.f.u.) ml⁻¹ and 2·9 × 10⁵ c.f.u. ml⁻¹ respectively. The mice were killed 6 h after challenge, their livers removed and the number of bacteria in each liver assessed by viable counting (Miles et al., 1938). Counts from the livers of vaccinated and control mice were compared by the Mann–Whitney ranking test (Snedecor & Cochran, 1967).

Gas chromatography (GC). PWEs were hydrolysed with 2 m-hydrochloric acid at 100 °C for 3 h. The monosaccharides were analysed as alditol acetates prepared by the method of Poxton & Cartmill (1982) on a Pye Unicam 104 chromatograph using a column containing 3% (w/w) OV-225.
RESULTS

SSE was prepared from *P. haemolytica* serotype A1 (strain FA1) and serotype A6 (strain FA6) by the ultrafiltration method. Antigenic activity for A1, estimated as the IHA titre against homologous rabbit antisera, was determined at each step in the concentration procedure. Samples were titrated in serial doubling dilutions from 1 in 5 to 1 in 640 in 1 ml volumes of PBS. Loss of antigenic activity in sediments during clarification by centrifugation was less than 1% of the total activity. Table 1 shows the specific IHA titres of the dialysed SSE before ultrafiltration, the ultrafiltrate, and the concentrated preparation of two different A1 SSE preparations passed through Diaflo XM100A or XM300 membranes. Antigenic activity was almost completely retained by the XM100A filter but appreciable amounts of antigen passed through the XM300 filter.

Both A1 and A6 SSE had a high degree of antigenicity when titrated against their respective homologous antisera in the IHA test. At a concentration of 2.5 mg ml⁻¹ the SSEs of A1 and A6 had titres of 1 in 1000 and 1 in 5000 respectively.

The chemical analyses for A1 and A6 SSEs (Table 2) indicate a strong similarity in chemical composition with both preparations containing protein, carbohydrate, fatty acid and phosphorus in roughly the same ratio (10:1:0.5:0.1).

The antigens of *P. haemolytica* A1 and A6 SSE were visualized by CIE. The homologous antibody/antigen reactions for A1 and A6 SSE were similar in that one major antigen was present in each of the SSEs (Fig. 1a, b). When the loading of the A6 SSE was increased to that of the A1 SSE (50 μg) three other precipitin lines were observed in the homologous antisera gel. However, this resulted in a very diffuse and distorted precipitin line for the major antigen (result not shown). SSE antigens were also run in CIE with an intermediate gel containing heterologous antisera between the antigen and the homologous antisera-loaded gel. The major antigen for both SSEs passed through the intermediate gel and precipitated only in the homologous antisera gel (Fig. 1c, d). The major antigens would appear to be serotype-specific. When A6

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**Table 1. IHA titres of two different SSE preparations from *P. haemolytica* A1 before and after ultrafiltration through XM300 and XM100A membranes**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific titre of fraction (titre/sample volume)</th>
<th>XM300 membrane</th>
<th>XM100A membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysed SSE before ultrafiltration</td>
<td>0.39</td>
<td>0.39</td>
<td>2.6</td>
</tr>
<tr>
<td>Concentrate</td>
<td>100</td>
<td>&gt;490</td>
<td></td>
</tr>
<tr>
<td>Ultrafiltrate</td>
<td>0.245</td>
<td>&lt;0.021</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Composition of sodium salicylate and phenol/water extract prepared from *P. haemolytica* serotypes A1 and A6**

<table>
<thead>
<tr>
<th>Component</th>
<th>Serotype A1</th>
<th>Serotype A6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SSE</td>
<td>PWE</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>Protein</td>
<td>57.9</td>
<td>6.3</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>6.0</td>
<td>14.8</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>2.8</td>
<td>10.8</td>
</tr>
<tr>
<td>Heptose</td>
<td>ND</td>
<td>6.6</td>
</tr>
<tr>
<td>KDO</td>
<td>ND</td>
<td>0.6</td>
</tr>
</tbody>
</table>
SSE at an equivalent loading to A1 SSE was run with an intermediate gel the three minor antigens were held back by the intermediate gel indicating that these antigens are common to A1 and A6 (result not shown).

The PWEs of both A1 and A6 had comparable levels of antigenicity when measured by IHA (1 in 1000 and 1 in 2000 respectively at concentrations of 1 mg ml\(^{-1}\)).

Both A1 and A6 PWEs were rich in polysaccharide and had little protein compared to their respective SSEs (Table 2). Both contained heptose and small amounts of KDO. When the PWEs of A1 and A6 were examined by GC the sugar compositions were similar, with glucose, galactose, mannose, glucosamine, galactosamine and two presumptive heptoses present in the molar ratios 1.8:1.5:0.15:1.0:1.5:1.5:2.4 for A1 PWE and 3.3:1.3:2.1:1.0:0.4:0.4:0.35 for A6 PWE. The presumptive heptoses were later confirmed by mass spectrometry.

PWEs of SSE were also run on CIE. The antigenic profiles obtained when these preparations were run through a blank intermediate gel into a homologous antiserum gel were similar to those found for the SSE preparations. One major peak was visible for each serotype (Fig. 2a, b). In addition two minor peaks were present in the intermediate gel of the A1 PWE run and one minor peak was visible at the interface of the two gels in the A6 PWE run. When the intermediate gel contained heterologous antiserum the major antigens of both serotypes were unaffected and precipitated in the homologous antiserum gel (Fig. 2c, d). One of the two minor
Surface antigens of Pasteurella haemolytica

Fig. 2. CIE of (a) A1 PWE (10 µg) run against 100 µl A1 antiserum, with blank intermediate gel (IG); (b) A6 PWE (10 µg) run against 100 µl A6 antiserum with blank IG; (c) A1 PWE (10 µg) run against 100 µl A1 antiserum with IG containing 50 µl A6 antiserum and (d) A6 PWE (10 µg) run against 100 µl A6 antiserum with IG containing 50 µl A1 antiserum.

Fig. 3. SDS-PAGE of sodium salicylate extracts (300 µg) of P. haemolytica serotypes A1 and A6 on 10% acrylamide gels stained with Coomassie blue.
antigens of A1 PWE was apparently unaffected and the other antigen failed to precipitate (Fig. 2c). The minor antigen present in A6 PWE was precipitated within the intermediate gel indicating some reaction with the heterologous antiserum (Fig. 2a).

Analysis of the proteins present in A1 and A6 SSE by SDS-PAGE (Fig. 3) showed that the two serotypes contained a similar pattern of polypeptides. At least 48 bands were present with one major band at around 51000.

Table 3 shows that PWE from both serotypes conferred significant protection on mice challenged with homologous serotype culture. When challenged with the heterologous serotype, A6 PWE protected against A1 (P = 0.05) but A1 PWE did not protect against A6. The degree of protection against homologous serotype challenge afforded by SSE (significant at 0.001 level) in each case was greater than that of the PWE (both significant at 0.01 level).

**DISCUSSION**

Sodium salicylate may remove a whole complex of lipopolysaccharide (LPS) and protein from the outer surface of *P. haemolytica* cells. As the antigenic activity determined by IHA titres is high in the supernate after extraction and this activity is retained after ultrafiltration through an XM100A membrane but not an XM300 membrane (Table 1) it is possible to suggest an apparent molecular size of between 100000 and 300000 for the antigenic complex in the A1 and A6 serotypes. Chemical analyses of both SSEs showed them to be similar.

SDS-PAGE analysis of the SSEs further illustrates the close similarity of the two serotypes in structure as there are no major differences between them in the number or migration of polypeptide bands present. This contrasts with a previous study by Thompson & Mould (1975) where proteins extracted with phenol/acetic acid and water from the two serotypes produced different profiles on electrophoresis in polyacrylamide gels.

The profiles obtained by SDS-PAGE indicate that the SSE of both serotypes contains a large number of proteins. The exact origin of these proteins is unknown but it is likely that outer membrane proteins are present.

CIE of the SSEs from A1 and A6 detected a number of antigens. In each case a dominant serotype-specific antigen was present and in A6 SSE three minor antigens which were also common to A1 were sometimes observed by precipitation with heterologous antisera in an intermediate gel. The serotype-specific antigen, as detected by CIE and IHA, of each serotype was extractable with hot phenol/water and shown to be largely polysaccharide. The presence of heptose and KDO, which are generally regarded as markers for LPS, make it likely that the LPSs of *P. haemolytica* A1 and A6 are present in the PWE preparations and visualized in CIE.

The identical monosaccharide composition of A1 and A6 PWEs revealed by GC suggests that the antigenic differences seen in CIE and IHA are a result of either the linkages between sugars or the proportion of sugars in the polysaccharides, and are not due to completely different components.
However, CIE indicated that the PWEs were not pure preparations of the serotype antigens as, for each serotype, minor antigenic peaks were held back in intermediate gels containing heterologous antiserum. These cross-reacting antigens may be present in the small amount of contaminating protein or may be distinct polysaccharide components. Further purification of the serotype antigen with immunosorbent techniques has so far been unsuccessful.

The finding that the PWEs of both A1 and A6 were protective in mice against homologous challenge and, in the case of A6 PWE, against heterologous challenge indicates that the serotype-specific protection reported for SSE vaccines by Evans & Wells (1979) probably resides in the serotype antigen extracted by the phenol/water. The one-way cross-protection seen with A6 PWE against A1 challenge could be explained by the presence of antigenic determinants within A6 PWE which are shared with A1 PWE and important in protection against A1. Other determinants which are not shared may be important in protection against A6.

The degree of protection afforded by PWE was less than that provided by SSE which may indicate that the immunogenicity of the serotype antigen is enhanced by close association with other outer membrane components such as proteins. Karch & Nixdorff (1981) have shown that a combination of outer membrane protein and LPS from Proteus mirabilis gave rise to increased immune responses to both components compared to those obtained when they were injected individually into mice.

The possibility that antigens other than the serotype antigens are important in protection is supported by the work of Biberstein & Thompson (1965) and Knight et al. (1969) who showed that cross-protection unrelated to the serotype antigen does occur between Pasteurella haemolytica serotypes. Non-specific protection against Salmonella typhimurium by rough LPS preparations of P. haemolytica A7 has been reported by Rimsay et al. (1981). On the other hand, available evidence suggests that, in sheep, protection is serotype-specific (Gilmour et al., 1979).

If the components involved in the immune response to P. haemolytica can be identified and characterized it may be possible to select strains which produce a more effective antigenic complex for inclusion in pasteurella vaccines.

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