Antigenic analysis of iron-regulated proteins in *Pasteurella haemolytica* A and T biotypes by immunoblotting reveals biotype-specific epitopes

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The antigenic relationships of the iron-regulated proteins (IRPs) in *Pasteurella haemolytica* A and T biotype strains were examined by SDS-PAGE and immunoblotting. *P. haemolytica* cells of the A biotype, grown under conditions of iron-limitation, expressed two IRPs, of 35 and 70 kDa. All T biotype strains expressed IRPs with slightly different molecular masses of 37 and 78 kDa. Immunoblotting of all 16 *P. haemolytica* serotypes was carried out using a panel of polyclonal and monoclonal antibodies raised against serotype A2 antigens. Polyclonal antibodies revealed inter-serotype cross-reactivity towards the 35 and 70 kDa IRPs within the A biotype but no cross-reactivity against a T biotype protein in the 78 kDa region. Monoclonal antibody against the 35 kDa antigen reacted only with the A biotype 35 kDa IRP. Identical profiles were obtained for 10 field isolates of serotype A2, further emphasizing the antigen conservation within the A biotype. These findings reinforce the view that the A and T biotypes of *P. haemolytica* should be considered as separate species and suggest that IRPs from single A and T biotype strains incorporated into a vaccine might provide cross-protection against all *P. haemolytica* serotypable strains. Similar studies on the IRPs of 10 untypable strains revealed some of these to have different antigenic reactivities from those observed within the A and T biotypes.

Introduction

Pasteurellosis, caused by *Pasteurella haemolytica*, is a serious, often fatal respiratory disease in sheep, cattle and goats (Gilmour, 1980). Predisposing factors are implicated in the disease (Harris, 1974; Davies *et al.*, 1980), but the ultimate cause of death is considered to be pneumonia caused by *P. haemolytica*.

Isolates of *P. haemolytica* fall into two distinct biotypes (A and T) depending on their morphological and biochemical characteristics. Sixteen serotypes based upon capsular antigen diversity can be identified by an indirect haemagglutination test (IHA). Serotypes 3, 4, 10 and 15 belong to the T biotype, and 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14 and 16 are of the A biotype (Biberstein *et al.*, 1960; Fraser *et al.*, 1982; Fodor *et al.*, 1988). In addition, approximately 12% of all isolates are untypable in the IHA typing system.

Proper management and vaccination are the accepted means of control for the disease. Vaccines containing cell extracts of serotypes A1, A6 and A9 conferred a considerable degree of protection on specific-pathogen-free (SPF) lambs against homologous, but not heterologous challenge (Gilmour *et al.*, 1983). However, protection against the A2 serotype, which is the predominant ovine isolate from field cases, has proven more difficult to demonstrate with cell extract vaccines.

Solid immunity against the A2 serotype was demonstrated when SPF lambs recovered from experimental pasteurellosis caused by serotype A2 were subsequently challenged (Donachie *et al.*, 1986). Sera from these sheep contained antibodies directed against iron-regulated proteins (IRPs). These proteins were demonstrated in bacteria collected from host tissue and body fluids, and in bacteria grown *in vitro* under iron-depleted conditions (Donachie & Gilmour, 1988). Moreover, IRPs were absent or only weakly expressed in cells grown in iron-replete medium. Recently, antibodies to these antigens presented on 'in vitro' grown cells have been shown to significantly enhance protection against A2 challenge in the SPF lamb experimental system (Gilmour *et al.*, 1991). Predominant IRPs of *P. haemolytica* A2 at

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Abbreviations: dp, 2,2'-dipyridyl; IHA, indirect haemagglutination; IRP, iron-regulated protein; NB, nutrient broth; SPF, specific-pathogen-free.

apparent molecular masses of 100, 70 and 35 kDa have been identified using SDS-PAGE (Donachie & Gilmour, 1988; Lainson *et al.* 1991).

The presence of antibodies specific for IRPs in the serum of immune animals recovered from infection and enhancement of vaccine efficacy by these proteins implicates them as protective antigens. As *P. haemolytica* contains 16 serotypes, shared antigenic epitopes among IRPs could be significant in possible heterologous immunity to infection.

This paper reports the cross-reactivity of antibodies against two of these iron-regulated proteins and discusses the relevance of cross-protection in vaccine development.

Methods

Bacterial strains and growth conditions. All strains used for the purpose of this comparative study were field isolates obtained from cases of ovine and bovine pasteurellosis and maintained at Moredun Research Institute as reference strains stored at -70°C . The details are shown in Table 1. Bacteria were subcultured and identified by the IHA test (Biberstein *et al.*, 1960). When required, 10 ml seed cultures were initiated in nutrient broth (NB, Gibco No. 2) and 6 h later, inoculated into 1 l flasks of iron-depleted media obtained by the addition of 2,2'-dipyridyl (dp, 150 μM , Sigma). Cells were grown at 37°C for 18 h with shaking, then harvested by centrifugation and washed twice in PBS (0.01 M-sodium phosphate, 0.15 M-NaCl, pH 7.4). Bacterial suspensions were adjusted to give protein concentrations of 2 mg ml $^{-1}$ as determined by the Pierce BCA Protein Assay Reagent.

Preparation of bacterial envelopes. Bacterial envelopes were prepared as described by Donachie & Gilmour (1988). Briefly, washed cells were sonicated in an MSE sonicator (150 watt, MK2) for three 30 s pulses at maximum power with intermittent cooling. The sonicate was then centrifuged at 4000 g and the supernatant fluid centrifuged at 30000 g for 1 h to pellet envelopes. Envelopes were resuspended in 0.1 M Tris/HCl, pH 7.4.

SDS-PAGE and immunoblotting. *P. haemolytica* proteins were separated by SDS-PAGE, using a modification of the method described by Laemmli (1970). A discontinuous system comprising resolving and stacking gels of 10 and 4% (w/v) acrylamide, respectively, was used. Prior to electrophoresis, bacterial preparations were boiled for 5 min in a 50% (v/v) solution of a sample buffer containing 4% (w/v) SDS and 8% (v/v) mercaptoethanol. Separated proteins were stained with Coomassie blue R250 (Sigma).

Proteins were electrophoretically transferred to Immobilon PVDF Transfer Membrane (Millipore) by the method of Sharp & Herring (1983). The membrane was blocked with 50% (v/v) horse serum in Blot Wash Buffer (BWB) comprising 0.5 M-NaCl, 0.01 M-EDTA and 0.5% (v/v) Tween 20 in PBS, for 1 h at 37°C . After three 5 min washes in BWB, the membrane was incubated at 37°C for 1 h with either hybridoma supernatant containing monoclonal antibody or specific lamb antisera at a 1:50 dilution in BWB followed by three 5 min washes in BWB. Bound antibody was then detected by incubation with horseradish-peroxidase-conjugated anti-mouse or sheep IgG (SAPU, Law Hospital, Carlisle) for 1 h at 37°C . The membrane was washed as described above, rinsed with distilled water and conjugate detected by the precipitation of the oxidized diaminobenzidine, 1 mg ml $^{-1}$ in 0.1 M Tris/HCl, pH 7.4 (Sigma). The reaction was stopped by rinsing in tap water.

Table 1. *P. haemolytica* strains used for cross-reactivity studies

Strain	Serotype	Source
D0652	A1	Ovine
A713	A2	Ovine
A635	T3	Ovine
D0693	T4	Ovine
R341B	A5	Ovine
D0135	A6	Ovine
D0800	A7	Ovine
D0200	A8	Ovine
D0775	A9	Ovine
D0770	T10	Ovine
B561	A11	Ovine
D0132	A12	Ovine
D0804	A13	Ovine
N513	A14	Ovine
D0685	T15	Ovine
N236	A16	Ovine
A713	A2	Ovine
Y578	A2	Ovine
A609	A2	Ovine
E0200	A2	Ovine
E674	A2	Ovine
T884	A2	Ovine
E0284	A2	Ovine
Y510	A2	Ovine
D1036	A2	Ovine
A461	A2	Bovine
D0203	A2	Bovine
B0131	Untypable	Ovine
P704C	Untypable	Bovine
T1291B	Untypable	Ovine
T1255A	Untypable	Ovine
T1262A	Untypable	Ovine
S622G	Untypable	Ovine
S622K	Untypable	Ovine
T12910	Untypable	Ovine

Monoclonal antibody. A monoclonal antibody specific for a 35 kDa IRP in *P. haemolytica*, described by Lainson *et al.* (1991), was used in immunoblotting.

Antiserum. Antisera from SPF lambs recovered from an experimental A2 serotype infection (Donachie *et al.*, 1986) were pooled and used in immunoblotting to compare the cross-reactivity of *P. haemolytica* proteins.

Antibodies to the 70 kDa A2 serotype IRP were raised in SPF lambs by immunization with the 70 kDa band excised from polyacrylamide gels after SDS-PAGE separation of envelopes. The bands were macerated in 0.1 M Tris/HCl, pH 7.4, and Alhydrogel (Superfos) added to a final concentration of 0.3% (w/v). Each excised strip containing approximately 100 μg protein was homogenized in Alhydrogel. SPF lambs were vaccinated subcutaneously at weekly intervals with 100 μg protein.

Polyclonal antiserum to the 35 kDa A2 serotype IRP was raised in sheep following two subcutaneous injections of purified protein (100 μg per dose) obtained by affinity chromatography. Briefly, monoclonal anti-35 kDa antibody was coupled to a standard Sepharose 4B column (Pharmacia) and a sonicated lysate of iron-depleted *P. haemolytica* A2 cells in 0.5 M-NaCl was passed through the column. Bound antigen was eluted with 0.1 M-glycine/HCl, pH 2.3.

Results

Fig. 1 shows the SDS-PAGE-separated whole-cell lysates of *P. haemolytica* serotypes A2 and T3 grown in iron-depleted and iron-replete media. Two A2 serotype IRPs having molecular masses of 35 and 70 kDa are less prominent in cells grown in iron-replete medium but are prominent bands in cells grown in iron-depleted medium. Similarly, two T3 serotype IRPs of 37 and 78 kDa are preferentially expressed in cells grown under conditions of iron limitation. The profiles of A and T biotypes show distinct differences, which are most obvious in the 20–50 kDa region. The lower of the two T biotype IRPs is slightly displaced from the A biotype 35 kDa IRP and is seen at 37 kDa. Additionally, the higher molecular mass T biotype IRP is evident at 78 kDa compared to 70 kDa in the A biotype. Coomassie-blue-stained profiles of the A and T biotype envelopes indicate that the 70 and 78 kDa proteins are located in the membranes while the 35 kDa IRP is not (unpublished observations).

Immunoblot analysis was performed to determine whether antibodies raised in sheep against experimental *P. haemolytica* A2 infection exhibited cross-reactivity with other serotypes. Immunoblots of lysates from iron-depleted and iron-replete cells of serotypes 1–4 were reacted with an A2 convalescent serum pool (Fig. 2). Much cross-reactivity is exhibited and antibodies specific for the lower of the two IRPs (35 and 37 kDa) are observed in both biotypes. No cross-reactivity is evident against a 70 kDa protein in the T serotypes and there is no reaction with the 78 kDa IRP. Furthermore, polyclonal antiserum raised in lambs against the A2 70 kDa protein excised from polyacrylamide gels reacts with a complementary protein present in envelopes of the A biotype, but not the T biotype (Fig. 3). A band at 66 kDa, again only in the A biotype, is also recognized by this antiserum pool.

When immunoblots of iron-regulated A and T cell lysates were probed with polyclonal anti-35 kDa antiserum, reaction was observed with a single protein (Fig. 4a). Antibody reactivity towards the T biotype strains was with the 37 kDa antigen. Although antibody cross-reactivity with the T biotype protein was easily discernible, the intensity was reduced when compared to A biotype reactions.

A murine monoclonal antibody against the 35 kDa iron-regulated protein exhibited cross-reactivity with all the A biotype strains (Fig. 4b). No reaction at 35 kDa was observed with the T serotypes. However, some contained a weakly reacting band of <29 kDa (Fig. 4b, lane 4T).

The reactivity of specific antibody to 70 and 35 kDa antigens with ten A2 field isolates revealed identical

Fig. 1. SDS-PAGE of *P. haemolytica* A2 and T3 whole-cell proteins stained with Coomassie blue, showing the response to iron limitation. Arrowheads indicate A2 serotype 35 and 70 kDa IRPs, and T3 serotype 37 and 78 kDa IRPs.

Table 2. Reactivities of eight untypable isolates of *P. haemolytica* with polyclonal and monoclonal antibodies

A positive reaction with a monoclonal (mAb) or polyclonal antibody (pAb) is indicated by +.

Strain no.	Antibody specificity			Proposed biotype
	Anti-35 kDa mAb	Anti-35 kDa pAb	Anti-70 kDa pAb	
1	—	+	—	T
2	+	+	—	?
3	—	+	+	?
4	+	+	+	A
5	—	+	—	T
6	—	+	+	?
7	+	+	+	A
8	—	+	—	T

Fig. 2. Immunoblot of *P. haemolytica* whole cells, serotypes 1-4, grown in iron-replete and iron-depleted conditions (dp) reacted with pooled sera taken from lambs recovered from *P. haemolytica* A2 infection. Arrowheads indicate A1 serotype 35 and 70 kDa IRPs, and T3 serotype 37 and 78 kDa IRPs.

Fig. 3. Immunoblot of envelope preparations of *P. haemolytica* serotypes 1-16 grown under conditions of iron-limitation, reacted with sheep polyclonal antiserum raised against the A2 70 kDa IRP excised from polyacrylamide gels. The arrowhead indicates the 70 kDa protein.

reactions with the two proteins in all eleven strains. Reactions were comparable with the standard A2 strain (Figs 5a and b).

Whole-cell proteins from eight untypable *P. haemolytica* strains were separated by PAGE and examined in immunoblotting using the monoclonal antibodies and polyclonal antisera to the 35 kDa IRP and also with polyclonal anti-70 kDa antiserum to identify cross-reacting proteins (Table 2).

Five of the eight untypable strains examined gave reactivities identical to either the A or the T biotype. The remaining three strains could not be placed in either biotype on the basis of these reactivities. Strain 2, for

example, had a protein that reacted with the 35 kDa monoclonal antibody but lacked a protein which reacted with the anti-70 kDa polyclonal antiserum.

Discussion

To assess the degree of conservation of antigenic epitopes in and between the serotypes of *P. haemolytica*, immunoblotting with specific antibody was performed. The observations demonstrate that inter-serotype cross-reactivity exists against both the 35 and 70 kDa IRPs within the A biotype. Although the polyclonal anti-35 kDa (A2) specific antiserum reacts with a T biotype

Fig. 4. (a) Immunoblot of *P. haemolytica* whole cells, serotypes 1–16, grown under conditions of iron-limitation reacted with polyclonal anti-35 kDa (A2) antiserum. For comparison, arrowheads indicate A8 serotype 35 kDa protein and T10 serotype 37 kDa protein. (b) Similar immunoblot reacted with culture supernatant from anti-35 kDa (A2) monoclonal cell line (12/16-6-11). The arrowhead indicates the 35 kDa protein.

Fig. 5. (a) Immunoblot of A2 field isolate envelope proteins reacted with sheep polyclonal antiserum raised against the A2 (A713) 70 kDa IRP excised from polyacrylamide gels. Lanes: 1, standard A2 strain; 2–11, strains Y578–D0203 as shown in Table 1. The arrowhead indicates the 70 kDa protein. (b) Immunoblot of A2 field isolate whole-cell proteins reacted with culture supernatant from anti-35 kDa (A2, A713) monoclonal cell line (12/16-6-11). Lanes are as in (a). The arrowhead indicates the 35 kDa protein.

protein, the lack of reactivity of the specific anti-35-kDa (A2) monoclonal antibody indicates that the T biotype protein, although sharing common epitopes, is not identical. Mosier *et al.* (1989), have correlated the presence of serum antibodies to a 35 kDa antigen with resistance to experimental A1 challenge. It is not known whether this antigen is the same as the 35 kDa antigen described here. The weak reactivity of serotype A11

35 kDa antigen compared to other A serotypes with the monoclonal antibody indicates that A11 differs from other A serotypes. Strains of this serotype have previously been shown to be different from other A serotypes in such characteristics as production of neuraminidase and sialoglycoprotease (Frank & Tabatabai, 1981; Otulakowski *et al.*, 1983).

The reactivity of the anti-70 kDa (A2) polyclonal

antiserum with the 70 kDa A biotype protein, but not the 78 kDa T biotype protein, indicates that these two proteins are antigenically distinct. In addition, a second non-iron-regulated antigen is recognized in A biotypes. Presumably, this was excised from the gels along with the 70 kDa protein. Why it should also be biotype-specific is intriguing. It is interesting that both anti-T10 and anti-A2 convalescent sera react with the T biotype 78 kDa protein (unpublished observations). This could be explained by the presentation of native antigen *in vivo* whereas the anti-70 kDa polyclonal serum was raised by immunization with denatured 70 kDa material. The shared epitopes may be labile in the solubilizing buffer.

It is clear from the reactions of A2-specific polyclonal and monoclonal antibodies with A and T whole-cell immunoblots, that conservation of antigenic proteins is maintained throughout the A biotype. Further evidence of antigenic conservation is obtained from antibody reactivity with a selection of ten A2 field isolates in which no variation was observed.

Deneer & Potter (1989) showed antigenic cross-reactivity between the IRPs of serotypes 1–12 using A1 convalescent calf serum. Although unable to show the IRPs as being biotype-dependent, they did speculate on the antigenic divergence of serotype 10.

Little is known of the pathogenicity and immunogenicity of untypable *P. haemolytica* strains. Since they make up a sizeable percentage of field isolates it seems prudent to determine the cross-reactivity of the major antigens for the purposes of possible vaccine inclusion. Specific antibody reactivity towards the panel of untypable strains indicates that several strains show characteristics of either one or both biotypes. These results indicate that there is antigenic diversity within the untypable strains and reinforce the findings of Donachie *et al.* (1984) that distinct serogroups exist among the untypable strains and that these are not merely serogroups deficient in capsular antigen. The diversity of antigenic epitopes observed in untypable strains makes it unlikely that a vaccine containing the antigens of only one serogroup would immunize against a significant proportion of untypable serogroups.

Our results suggest that two IRPs with apparent molecular masses of 35 and 70 kDa are conserved within the A biotype of *P. haemolytica*. The observed cross-reactivity of polyclonal and monoclonal antibodies to the IRPs by immunoblotting suggests firstly that cross-protective immunity could occur within the A biotype and, therefore, for vaccine purposes the antigens expressed by a single A biotype strain may be sufficient to confer protection against A biotype infection. Secondly, these findings give further evidence that the A and T biotypes of *P. haemolytica* may represent different species.

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References

- BIBERSTEIN, E. L., GILLS, M. & KNIGHT, H. (1960). Serological types of *Pasteurella haemolytica*. *Cornell Veterinarian* **50**, 283–300.
- DAVIES, D. H., DAVIS, G. B. & PRICE, M. L. (1980). A longitudinal serological survey of respiratory virus infections in lambs. *New Zealand Veterinary Journal* **28**, 125–127.
- DENEER, H. G. & POTTER, A. A. (1989). Iron-repressible outer-membrane proteins of *Pasteurella haemolytica*. *Journal of General Microbiology* **135**, 435–443.
- DONACHIE, W. & GILMOUR, N. J. L. (1988). Sheep antibody responses to cell wall antigens expressed *in vivo* by *Pasteurella haemolytica* serotype A2. *FEMS Microbiology Letters* **56**, 271–276.
- DONACHIE, W., FRASER, J., QUIRIE, M. & GILMOUR, N. J. L. (1984). Studies on strains of *Pasteurella haemolytica* not typable by the indirect haemagglutination test. *Research in Veterinary Science* **37**, 188–193.
- DONACHIE, W., BURRELLS, C., SUTHERLAND, A. D., GILMOUR, J. S. & GILMOUR, N. J. L. (1986). Immunity of specific pathogen-free lambs to challenge with an aerosol of *Pasteurella haemolytica* biotype A serotype 2. Pulmonary antibody and cell responses to primary and secondary infections. *Veterinary Immunology and Immunopathology* **11**, 265–279.
- FODOR, L., VARGA, J., HAJTOS, J., DONACHIE, W. & GILMOUR, N. J. L. (1988). Characterization of a new serotype of *Pasteurella haemolytica* isolated in Hungary. *Research in Veterinary Science* **44**, 399.
- FRANK, G. H. & TABATABAI, L. B. (1981). Neuraminidase activity of *Pasteurella haemolytica* isolates. *Infection and Immunity* **42**, 1119–1122.
- FRASER, J., LAIRD, S. & GILMOUR, N. J. L. (1982). A new serotype (biotype T) of *Pasteurella haemolytica*. *Research in Veterinary Science* **32**, 127–128.
- GILMOUR, N. J. L. (1980). *Pasteurella haemolytica* infections in sheep. *Veterinary Quarterly* **2**, 191–197.
- GILMOUR, N. J. L., MARTIN, W. B., SHARP, J. M., THOMPSON, D. A., WELLS, P. W. & DONACHIE, W. (1983). Experimental immunization of lambs against pneumonic pasteurellosis. *Research in Veterinary Science* **35**, 80–86.
- GILMOUR, N. J. L., DONACHIE, W., SUTHERLAND, A. D., GILMOUR, J. S., JONES, G. E. & QUIRIE, M. (1991). Vaccine containing iron-regulated proteins of *Pasteurella haemolytica* A2 enhances protection against experimental pasteurellosis in lambs. *Vaccine* **9**, 137–140.
- HARRIS, G. (1974). An outbreak of pasteurellosis in lambs. *Veterinary Record* **2**, 84–85.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature, London* **227**, 680–685.
- LAINSON, F. A., HARKINS, D. C., WILSON, C. F., SUTHERLAND, A. D., MURRAY, J. E., DONACHIE, W. & BAIRD, G. D. (1991). Identification and localization of an iron-regulated 35 kDa protein of *Pasteurella haemolytica* serotype A2. *Journal of General Microbiology* **137**, 219–226.
- MOSIER, D. A., SIMONS, K. R., CONFER, A. W., PANCIERA, R. J. & CLINKENBEARD, K. D. (1989). *Pasteurella haemolytica* antigens associated with resistance to pneumonic pasteurellosis. *Infection and Immunity* **57**, 711–716.
- OTULAKOWSKI, G. L., SHEWEN, P. E., UDOH, A. E., MELLORS, A. & WILKIE, B. N. (1983). Proteolysis of sialoglycoprotein by *Pasteurella haemolytica* cytotoxic culture supernatants. *Infection and Immunity* **42**, 64–70.
- SHARP, J. M. & HERRING, A. J. (1983). Sheep pulmonary adenomatosis demonstration of a protein which cross-reacts with the major core proteins of Mason-Pfizer monkey virus and mouse mammary tumour virus. *Journal of General Virology* **64**, 2323–2327.