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FACTORS AFFECTING THE IMMUNOGENICITY OF *PASTEURELLA HAEMOLYTICA* IN MICE

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ABSTRACT

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An appreciable level of immunity from intraperitoneal infection with *Pasteurella haemolytica* was established in mice by using a vaccine prepared in a conventional bacteriological culture medium, with aluminium hydroxide gel as adjuvant. The level of immunity could not be elevated by using bacteria grown in tissue culture media, enriched brain heart infusion broth, the addition of serum to the media or by using bacteria that had been harvested in the logarithmic growth phase.

Although various extracts of the bacteria elicited a distinct immunity, the immunogenicity of vaccines containing bacteria could not be enhanced by augmentation with those products.

The potential application of the vaccine in cattle and sheep is discussed.

INTRODUCTION

Smith (1959) was the first to report on the successful immunization of mice against intraperitoneal infection with *Pasteurella haemolytica* with a vaccine prepared from bacteria grown on sheep blood agar sedimented with potassium aluminium sulphate and incorporated into an oil adjuvant. Success has since also been reported by Mayr, Wizigmann, Schels & Plank (1969) and Goedemans (1970), who also used bacteria grown on blood agar plates. Such vaccines, however, have not readily been universally applied.

P. haemolytica possesses a number of characteristics that should be taken into account when the production and formulation of a vaccine are contemplated. The capsular material may be an important immunogen and promising results have been recorded with the use of chemical extracts of *P. haemolytica* as immunizing antigens. One of the most successful has been the use of sodium salicylate extracts (Gilmour, Martin, Sharp, Thompson & Wells, 1979; Wells, Gilmour, Burrell & Thompson, 1979; Gilmour, Angus, Donachie & Frazer, 1982). Potassium thiocyanate (Mukkar, 1977; Tadayon & Lauerman, 1981) and saline (Gentry, Corstvet & Panciera, 1982) have likewise given good results.

Recently it was shown that *P. haemolytica* produces a cytotoxin (Benson, Thomson & Valli, 1978) which impairs phagocytosis (Markham & Wilkie, 1980), is specific for bovine leucocytes (Shewen & Wilkie, 1982) and depresses the liberation of chemotactic factors (Markham, Ramnaraine & Muscoplat, 1982). This toxin is a highly immunogenic protein and, since it has the characteristics of a virulence factor, it may be an effective agent for immunization against *P. haemolytica* (Himmel, Yates, Lauerman & Squire, 1982).

Brain heart infusion broth (BHI) as such (Wilkie & Norris, 1976; Markham *et al.*, 1982) or BHI, augmented with other nutrients (Berggren, Baluyut, Simonson, Bemrick & Maheswaran, 1981), is commonly used to grow cells for toxin production. RPMI 1640 culture medium (Baluyut, Simonson, Bemrick & Maheswaran, 1981; Shewen & Wilkie, 1982) has also been used with success. It is evident, however, that the toxin is primarily produced during the logarithmic growth phase (Berggren *et al.*, 1981; Baluyut *et al.*, 1981; Shewen & Wilkie, 1982; Markham *et al.*, 1982).

With the abovementioned considerations in mind, our objective was to investigate the optimum cultural conditions for the production of an effective vaccine against *P. haemolytica*.

MATERIALS AND METHODS

Bacterial strains

P. haemolytica Strain I29 Type A1 was obtained from Dr E. L. Biberstein[1], while Strains 9479 (Type 1) and 9637 (Type 1) were isolated from cases of fatal bovine pneumonia.

Culture media

The following culture media were used in this study: Blood tryptose agar (BTA) containing 10 % bovine blood; Eagle's, McCoy's and RPMI 1640 tissue culture media; brain heart infusion broth (BHI)[2]; BHI broth plus 10 % sheep serum; BHI plus yeast extract and N–Z amine (Berggren *et al.*, 1981); B & J medium (Bain & Jones, 1958) and B & J medium plus 10 % sheep serum. The media were made up according to the instructions of the manufacturers or publication, and distributed in 500 mℓ volumes in 4 ℓ Roux flasks. The media were sterilized either by filtration or autoclaving for 20 min at 120 °C.

Preparation of experimental vaccines

The Roux flasks were inoculated with bacteria harvested from a 24-hour-old blood tryptose agar plate culture. The flasks were then shaken for 18 h in a horizontal position at 37 °C. The packed cell volume was determined by centrifugation of 5,0 mℓ of culture in a Hopkin's tube for 30 min at 3000 g and the culture, after being inactivated by the addition of 0,3 % formalin, was allowed to stand at room temperature for 24 h. When logarithmic phase growth was required, the flasks were inoculated with 50 mℓ of an 18 h culture/500 mℓ and shaken for 6 h. Depending on the yield and the requirements of the experiment, the cultures were either concentrated by centrifugation or diluted with the corresponding culture medium to give a density of 0,25 % or 1,0 % packed cells. In most instances, 10 mℓ of an 11 % potassium alum solution was added as adjuvant to 100 mℓ of vaccine. In one experiment $A1(OH)_3$ gel[3] at the rate of 12,5 mℓ per 100 mℓ was added and the material stirred at room temperature for 60 min.

When dry bacteria or extracts were used, the required quantity of material was dissolved in B & J medium and the solution treated as outlined above.

Preparation of dry bacteria and extracts

Dry bacteria were prepared from cultures grown on filtered Eagle's medium. The cells were deposited by centrifugation, washed once with saline, resuspended in a small quantity of distilled water and lyophilized.

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[3] Rehsorptar, Armour Pharmaceutical Company, Kaukanee, Illinois, USA

Phenol and Veronal extracts were prepared as described previously (Cameron, Pienaar & Vermeulen, 1980). Sodium salicylate extracts were prepared by the method of Wells *et al.* (1979). The final extract, however, was not concentrated by ultrafiltration but dialyzed against distilled water for 48 h at 5 °C, concentrated by perevaporation and lyophilized. Potassium thiocyanate extracts were prepared as described by Mukkur (1977) and likewise concentrated and dried.

Water extractable antigens were prepared on the basis of the findings of Gentry *et al.* (1982). Thirty-five grams of packed cells (harvested from 7 Roux flasks) were suspended in 30 mℓ of distilled water and stirred for 60 min at 37 °C. The cells were then removed by centrifugation at 10 400 *g* for 30 min and he supernatant fluid freeze-dried.

For the preparation of vaccines containing cells plus water extract, the following procedure was followed: One litre of culture was heated for 60 min at 60 °C and subsequently centrifuged to sediment the cells. The supernatant fluid was dialyzed against distilled water and concentrated to 100 mℓ by perevaporation. The cells were resuspended in saline containing 0,5 % formalin to a density of 10 % packed cells and kept at room temperature for 24 h. Two-and-a-half mℓ of this suspension was added to the concentrated supernatant fluid and the mixture adsorbed to $Al(OH)_3$ as outlined above.

Immunization and challenge of mice

Six-week-old conventional male albino mice were used in all the experiments. Forty mice were used to assay every vaccine. They were each given 2 subcutaneous injections of 0,2 mℓ at an interval of 2 weeks and challenged 14 days after the 2nd injection. When concentrated vaccine was used, 0,02 mℓ was injected into the foot pad.

Groups of experimental mice in each experiment as well as 40 nonimmunized control mice were challenged essentially as described by Evans & Wells (1979). Bacteria for challenge purposes were grown on BTA plates, washed off with saline and the density adjusted nephelometrically to approximately 5×10^8 organisms/mℓ. Fivefold dilutions, ranging from 1:5 to 1:125 of this suspension, were made in tryptone water, and 1 part of each of the dilutions as well as the undiluted suspension was mixed with 4 parts of freshly prepared gastric mucin. The 40 mice in each experimental group were divided into 4 groups of 10 and challenged with the respective dilutions by intraperitoneal injection of 0,5 mℓ of the material. Deaths were recorded after 72 h. The death patterns in the control groups were not always identical and, consequently, deaths were only tabulated for the dilutions at which the deaths in the control group did not represent excessive challenge.

RESULTS

Immunogenicity of strains

The results of an experiment in which the immunogenicity of 3 strains, of *P. haemolytica* Type 1, which were grown in B & J broth and precipitated with alum, was compared are given in Table 1. All 3 strains gave a good immunity to one another. Strain 9479, however, provided slightly better immunity than the other 2 and was therefore selected for further experiments.

TABLE 1 Comparison of the immunogenicity of 3 strains of *P. haemolytica* Type 1

Vaccine strain	Challenge strains	Surviving mice/6 challenged with 1:5 dilution of standardized suspension	Total
I29	I29 9479 9637	4 4 4	12
9479	I29 9479 9637	5 5 5	15
9637	I29 9479 9637	3 5 5	13
Non-immunized controls	I29 9479 9637	1 3 0	4

Effect of cultural conditions on immunogenicity

Culture medium. The results of an experiment in which the immunogenicity of vaccines that were prepared from bacteria grown on different culture media are given in Table 2. In this experiment Eagle's medium without sheep serum and Bain & Jones's medium with 10 % sheep serum gave the best results. These media were examined further in subsequent experiments.

TABLE 2 The influence of different media on the immunogenicity of *P. haemolytica*

Medium used for vaccine production	Yield of packed cells %	Surviving mice/10			Total/ 30
		Dilution of challenge material			
		Undiluted	1:5	1:25	
Eagle's[1]	0,9	1	6	9	16
Eagle's + 10% SS[1]	1,5	1	2	7	10
McCoy's + 10% SS[1]	0,1	1	1	5	7
RPMI 1640[1]	0,5	1	7	7	15
Bain & Jone's[2]	0,8	2	3	6	11
Bain & Jones's + 10% SS	0,9	2	5	8	15
Blood tryptose agar	—	1	3	7	11
BHI + 10% SS[2]	0,8	1	1	7	9
BHI + YE & NZ Amine[2]	0,5	3	5	7	15
Non-immunized controls	—	1	1	5	7

SS = Sheep serum BHI = Brain heart infusion broth (Difco).
[1] = The medium was sterilized by filtration
[2] = The basic medium was sterilized by autoclaving

TABLE 3 Effect of serum added to Eagle's and Bain & Jones's medium on the immunogenicity of *P. haemolytica*

Medium variations	Member of surviving mice/10				Total/ 40
	Dilution of challenge material				
	Undil	1:5	1:25	1:125	
Eagle's with 10% SS	1	2	8	9	20
Bain & Jones's with 10% SS	2	7	9	9	27
Mean for media with serum					23,5
Eagle's without serum	3	7	9	10	29
Bain & Jones's without serum	2	6	7	10	25
Mean for media without serum					27
Non-immunized controls	1	4	2	4	11

SS = Sheep serum

TABLE 4 Comparison of steam sterilized and filtered Eagle's and Bain & Jones's medium

Medium variations			Number of surviving mice/10				
			Dilution of challenge material				Total/40
			Undil	1:5	1:25	1:125	
Eagle's medium	Filtered	pH 7,0	4	7	7	10	28
		pH 8,0	1	7	9	9	26
	Autoclaved	pH 7,0	1	2	7	9	19
		pH 8,0	0	2	8	7	17
Mean for Eagle's medium							22,5
Bain & Jones's medium	Filtered	pH 7,0	1	3	10	10	24
		pH 8,0	2	2	7	8	19
	Autoclaved	pH 7,0	1	1	2	9	13
		pH 8,0	3	1	6	6	16
Mean for Bain & Jones's medium							18
Non-immunized controls			1	0	2	1	4

TABLE 5 Immunogenicity of *P. haemolytica* for mice in a combined vaccine

Cultural conditions for *P. haemolytica* component	Nature of vaccine	Packed cell volume of *P. haemolytica* in final vaccine %	Surviving mice/10 after challenge				Total survivors/40
			Dilution of challenge material				
			Undil	1:5	1:25	1:125	
Eagle's autoclaved	Polyvalent	0,25	0	3	8	10	21
Eagle's filtered	Polyvalent	0,25	1	4	8	10	23
Eagle's filtered	Monovalent	0,25	2	0	5	8	15
Eagle's filtered	Monovalent	1,0	0	4	5	9	18
Non-immunized controls	—	—	0	1	0	4	5

TABLE 6 Comparison of various experimental *P. haemolytica* vaccines with 2 commercial products

Nature of vaccine				Surviving mice/10			Total survivors/30
Culture medium	Incubation time	Adjuvant	Route	Dilution of challenge material			
				1:5	1:25	1:125	
Bain & Jones's	24 h	$Al(OH)_3$[1]	s.c.	2	8	10	20
Eagle's	24 h	$Al(OH)_3$[1]	s.c.	0	7	10	17
Bain & Jones's	24 h	Alum	s.c.	2	5	10	17
Eagle's	24 h	Alum	s.c.	0	5	8	13
Eagle's	6 h	Alum	s.c.	2	6	9	17
Eagle's	24 h	None	f.p.	2	6	10	18
Commercial[2]	?	?	s.c.	1	2	7	10
Commercial[3]	?	?	s.c.	4	7	9	20
Non-immunized controls				1	1	3	5

[1] Rehsorptar- — Armour Pharmaceutical Co.
[2] Rhivax 3 + P, — Pitman Moore Inc., Washington Crossing, N.J., USA
[3] OVIVAC - P — Hoechst (UK) Ltd, Walton Manor, Bucks, England
s.c. = subcutaneous
f.p. = foot pad 0,02 mℓ

Addition of serum. The effect of serum added to either Eagle's or Bain & Jones's medium was re-examined, the results of which are given in Table 3. As in the previous experiment, Eagle's medium without serum gave better results than Eagle's medium with serum, whereas the reverse was found with Bain & Jones's medium. Overall, the addition of serum to the culture medium did not improve the potency of the vaccine prepared.

Effect of sterilization. The results of experiments in which the influence of the method of sterilization on Eagle's and Bain & Jones's media at different pH values were detemined, are given in Table 4.

Filtered Eagle's medium gave the best results irrespective of the initial pH. The difference between filtered and autoclaved medium was also evident in the case of Bain & Jones's medium. Overall, Eagle's medium gave somewhat better results than Bain & Jones's medium, but the difference was small when both media were steam sterilized.

Immunogenicity of P. haemolytica *in a combined vaccine*

According to the results shown in Table 5, the immunogenicity of *P. haemolytica* Type 1 is not adversely affected when it is incorporated into a vaccine containing also 2 strains of *P. multocida* and a strain of *P. haemolytica* Type 2. In fact, it would appear that its immunity is slightly enhanced by the presence of these organisms.

Effect of adjuvant, growth time and route of immunization

The results of an experiment in which the immunogenicity of various vaccine formulations were compared are given in Table 6.

Neither short-term cultivation nor intradermal (foot pad) application had any beneficial effect. As in the previous experiments, Bain & Jones's and Eagle's medium gave comparable results. In this instance, however, the former was slightly better. $Al(OH)_3$ gel likewise was very slightly better as an adjuvant than potassium alum, and the results obtained with it were identical with the degree of immunity afforded by one of the commercial products. Subsequent experiments using other serotypes of *P. haemolytica* showed that a locally prepared $Al(OH)_3$ gel was as effective as the commercial product (Cameron & Bester, 1983, unpublished results).

Immunogenicity of chemical extracts

To compare quantitatively the immunogenicity of chemical extracts it was necessary first to determine the immunogenicity of dry cells. The results of such an experiment are given in Table 7.

TABLE 7 Quantitative estimation of the immunogenicity of dry *P. haemolytica* cells

Vaccine concentration mg/mℓ	Surviving mice/10		Total survivors/20
	Dilution of challenge material		
	1:5	1:25	
125	3	5	8
25	3	9	12
5	5	9	14
1	3	8	11
0,2	1	6	7
0,04	0	6	6
Non-immunized controls	2	1	3

The best immunity was obtained at a concentration of 5 mg/mℓ. However, for practical reasons and to best demonstrate the possible advantage of extracts, a concentration of 1 mg/mℓ was selected for comparative purposes in the next experiment.

Table 8 shows the results of an experiment in which the immunogenicity of extracts was compared with that of dry and wet whole cells. It is evident that, apart from the phenol and water extracts, none of the preparations were particularly more potent than the whole cells.

Although the water extract itself gave a good immunity, it did not enhance the immunity afforded by cells alone. This is evident from the results of a comparative experiment shown in Table 9.

TABLE 8 The immunogenicity of chemical extracts of *P. haemolytica* cultured in Eagle's medium

Extract	Concentration mg/mℓ	Surviving mice/10			Total survivors/30
		Dilution of challenge material			
		1:5	1:25	1:125	
Phenol	1,0	7	9	10	26
Veronal	1,0	3	4	8	15
Sodium salicylate	1,0	2	6	8	16
KSCN	1,0	4	7	10	21
Water	1,0	5	10	10	25
Dry cells	1,0	2	7	9	18
Wet cells	10[1]	2	4	9	15
Controls	—	0	3	2	5

[1] Equavalent to 1% packed cells

In all instances, vaccines prepared from cells alone afforded better protection than vaccines composed of cells and augmented with concentrated extracts.

DISCUSSION

The results presented in this study show that mice can be successfully immunized against intraperitoneal infection with a conventionally produced vaccine to which potassium alum is added as an adjuvant. Similar results were obtained with a commercial aluminium hydroxide gel, and subsequent studies, using other *P. haemolytica* serotypes, showed that a locally prepared product was as good as the commercial product and, in mice, the use of an oil adjuvant vaccine did not have any advantage over $Al(OH)_3$ absorbed vaccines (Cameron & Bester, 1983, unpublished results). Wells *et al.* (1979), however, found oil adjuvant vaccines to be superior to $Al(OH)_3$ adsorbed products in sheep in terms of antibody response. This aspect should be further investigated with our preparations, particularly because species differences are known to occur (Tadayon & Lauerman, 1981).

Intraperitoneal infection in mice is useful for initial studies on the immunogenicity of *P. haemolytica*, but success with this model does not necessarily indicate that the product will be equally effective in the target species. Nevertheless, appreciable success has been recorded with respect to the protection against pneumonic infection in cattle (Wilkie & Markham, 1979; Newman, Corstvet & Panciera, 1982) and sheep (Gilmour *et al.*, 1979; Gilmour, Sharp, Donachie, Burrells & Frazer, 1980), but these results need to be confirmed with our products.

Apart from septicaemia and pneumonia, which are common syndromes in sheep (Gilmour, 1978; Gilmour, 1980) mastitis (blue udder) in South Africa is a serious problem (Van Tonder, 1983, personal communication).

TABLE 9 Comparison of vaccines containing cells only with vaccines containing cells augmented with heat extracted antigen adsorbed to aluminium hydroxide gel

Nature of vaccine	Culture medium	Surviving mice/10			Total survivors/30
		Dilution of challenge material			
		1:5	1:25	1:125	
Cells only	Eagle's	0	7	10	17
	Bain & Jones's	2	8	10	20
	BHI	1	6	8	15
Total surviving mice/90					52
Cells plus heat extracted antigen	Eagle's	1	3	8	12
	Bain & Jones's	2	1	4	7
	BHI	1	1	6	8
Total surviving mice/90					27
Non-immunized controls		1	1	3	5

Effective immunization against this syndrome would require the transfer of antibodies into the udder cavity. To what extent this can be achieved is not quite clear. Wells, Evans, Burrells, Sharp, Gilmour, Thompson & Rushton (1979) showed that antibodies alone are not sufficient to impart a passive immunity to lambs. However, the same group (Gilmour *et al.*, 1980) have since shown that passive immunity is effective, a finding that was subsequently supported by the studies of Cowan & McBeath (1983). It therefore seems likely that effective immunity could be induced to udder infections, but this has yet to be proved experimentally.

The multiplicity of serotypes of *P. haemolytica* involved in ovine pasteurellosis (Thompson, Frazer & Gilmour, 1977; Gilmour, 1978; Gilmour, 1980) poses a serious problem for the formulation of effective vaccines. There is evidence of a degree of cross-protection among serotypes (Knight, Biberstein & Allison, 1969) and the neutralization of the cytotoxin is not absolutely serotype specific (Shewen & Wilkie, 1983). The evidence that immunity is type specific is, however, far more convincing (Biberstein & Thompson, 1965; Gilmour *et al.*, 1979). The necessity of including all the serotypes that prevail in a particular country or area is therefore quite evident (Rodger, 1982; Gilmour, 1982). It has been shown, however, that in the case of *P. multocida* the inclusion of too many strains in a vaccine can adversely affect the immune response to all of them (Cameron & Bester, 1983). Preliminary results reported in the present study suggest that this is not necessarily the case with *P. haemolytica*, but this observation must also be established in sheep.

Finally, note should be taken of the use of live vaccines injected intradermally (Anon., 1980). This may be applicable in cattle, but it would be impracticable in sheep. However, we were unable to demonstrate any advantage of injecting inactivated bacteria into the foot pads of mice. Wilkie & Norris (1976) also showed that antibody response was independent of the route of immunization. The recent report on the effective immunization of calves, using a chemically altered strain of *P. haemolytica* Type 1 (Kucera, Wong & Feldner, 1983), is also a significant advance.

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