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# FORMULATION OF AN EFFECTIVE *PASTEURELLA MULTOCIDA* VACCINE FOR SHEEP

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ABSTRACT

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An effective vaccine for the immunization of sheep against *Pasteurella multocida* infection was prepared from *P. multocida* Strain D4 (Type D) and a selected strain of *P. multocida* Type A. Provided an adequate concentration of bacteria was used, the vaccine thus formulated induced antibodies in sheep that protected mice not only against the vaccine strains but also against infection by a number of heterologous Type A and Type D strains as well as untypable strains.

A locally prepared $A1(OH)_3$ gel was found to be an effective adjuvant.

## INTRODUCTION

It has been shown that there is not consistent correlation between the immunological relationship of *Pasteurella multocida* Type A strains and their serological identity. Conversely, it has been found that a degree of cross-immunity may exist between serologically unrelated strains, and it was postulated that *P. multocida* Types A & D strains may possess a common immunizing antigen which is not revealed by conventional serological tests. (Cameron, Engelbrecht & Vermeulen, 1978; Cameron, Pienaar & Vermeulen, 1980). The practical problems posed by these findings with respect to the formulation of an effective vaccine could not be solved by the use of polyvalent vaccines containing numerous strains, highly concentrated vaccines or chemical extracts (Cameron & Bester, 1983). However, in the course of these studies, a particular strain, namely, *P. multocida* Strain D4 (Type D), was identified, which afforded immunity against a number of heterologous strains.

The object of this study was to further investigate the use of this strain as a potential universal immunizing antigen and to follow the immune response in sheep of various vaccine formulations.

## MATERIALS AND METHODS

### *Bacterial strains*

All the reference strains used in this study have already been employed and described in earlier publications (Cameron *et al.*, 1978; Cameron *et al.*, 1980; Cameron & Bester, 1983). The additional strains used were isolated from cases of fatal pneumonia.

### *Experimental animals*

*Mice:* Conventional male albino mice (4–6 weeks old) were used for the passive protection tests on the sheep sera.

*Sheep:* Young adult Dorper rams (6–12 months of age) were used to prepare the hyperimmune antisera against the various strains. Young Merino wethers were used in the experiments that were designed to determine the optimal concentration of Strain D4 antigen and in the experiment in which the value of different adjuvants was investigated.

### *Hyperimmune antiserum*

Hyperimmune antisera to the *P. multocida* Strains D4, 8473 and 4009 were raised in sheep as previously described (Cameron & Bester, 1983).

### *Preparation of vaccines and immunization of sheep*

For the experiment in which the optimal concentration of *P. multocida* Strain D4 antigen was determined, the organism was grown in 500 mℓ volumes of Bain & Jones's (B & J) medium (Cameron *et al.*, 1978) in Roux shake flasks for 18 h at 37 °C. The cultures were inactivated by the addition of 0,5 % formalin and the packed cell volume determined by centrifuging an aliquot in Hopkins tubes for 30 min at 3 000 *g*. The cultures were then centrifuged and, depending on the final packed cell volume required, the necessary volume of supernatant fluid was removed. The suspensions were subsequently precipitated by the addition of 100 mℓ of an 11 % potassium alum solution per ℓ to give a final concentration of 1,0 %.

For the preparation of the oil adjuvant vaccine the cell density was adjusted to 1,0% and then emulsified by means of a syringe with an equal volume of Freund's incomplete adjuvant (Difco)*.

The sheep were divided into 4 groups of 6 each and pre-bled for serum. Each animal, at a 4-week interval, was given 2 subcutaneous injections of 2,0 mℓ each of the appropriate vaccine and bled 14 days after the 2nd injection.

To compare the efficacy of various adjuvants and cultural conditions on the immunogenicity of polyvalent vaccines, 4 different products were composed. *P. multocida* Strains D4 and 8473 (Type A), *P. haemolytica* Strains J28 (Type 2) and 01193 (Type 6) were cultured and inactivated as outlined above. To avoid toxicity of the final product, the cells were collected by centrifugation and resuspended in 0,15 M phosphate buffered saline pH 7,2, containing 0,02 % formalin and 1:10 000 merthiolate, to give a packed cell volume of 2,0 %. Equal volumes of each strain were mixed, divided into 3 aliquots (A, B & C) and treated further.

To aliquot A was added 3 mℓ of a 10 % solution of potassium alum and 1,5 mℓ of a 7,4 % KOH solution per 100 mℓ of bacterial suspension. This solution was stirred for 30 min at room temperature and stored at 4 °C.

Aluminium hydroxide gel† (12,5 mℓ/mℓ) was added to aliquot B. The same volume of an $A1(OH)_3$ gel prepared at the Institute (50 % gel) was added to aliquot C. The solution was stirred for 30 min and stored at 4 °C.

Vaccine D was prepared exactly like vaccine A, except that the bacteria were grown in Eagle's tissue culture medium instead of B & J medium.

Seven sheep were used per group for each vaccine. They were pre-bled and given 2 subcutaneous injections of 2,0 mℓ each at an interval of 4 weeks. They were bled 4 weeks, 3 months and 6 months after the 2nd injection. At this time a 3rd booster injection was given, and the sheep were again bled 14 days later.

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*Passive immunity tests in mice*

The sera of each bleeding from each experimental group of sheep were pooled and tested in mice as described previously (Cameron *et al.*, 1978). In the 2nd experiment it was found that the sera collected before immunization were toxic for the mice to the extent that mice which received such sera were more susceptible to infection than mice which had received no serum at all. This effect, however, could largely be overcome by injecting 0,5 mℓ volumes of serum intraperitoneally instead of 0,2 mℓ intravenously.

## RESULTS

*Spectrum of protection afforded by hyperimmune antiserum*

The ability of hyperimmune sheep antiserum to *P. multocida* Strain D4 (Type D) to protect mice from infection by a range of heterologous *P. multocida* isolates is shown in Table 1.

From the results it is evident that very good protection was established against the 6 Type D strains that were tested. Although to a lesser degree, the serum also protected mice from infection by 4 out of the 7 Type A strains that were tested. No protection, however, was demonstrable to Strains 4009 and 8473. Effective protection was also esablished for 3 non-typable strains and, as was anticipated, the serum did not protect against *P. multocida* Types B and E.

*Effect of concentration of antigen on the immune response of sheep to* P. multocida *Strain D4*

The results of an experiment in which vaccines containing different concentrations of antigen are given in Table 2.

Whereas hyperimmune antiserum gave a wide spectrum of cross-protection, serum from sheep that had been immunized with a vaccine that contained 0,25 % packed cells of Strain D4 gave only a degree of homologous immunity but afforded no protection of any consequence to the heterologous strains. However, sera from sheep that had been immunized with vaccines containing either 0,5 % or 1,0 % packed cells of Strain D4 afforded appreciable protection to heterologous strains. It is thus evident that, to exploit the properties of Strain D4, a high level of homologous immunity is required before a wide spectrum of heterologous immunity can be demonstrated. Antigen prepared in Freund's incomplete adjuvant was ineffective.

*Protection afforded by* P. multocida *Strains 8473 (Type A)*

According to the results in Table 1, Strain D4 hyperimmune sheep antiserum, amongst others, did not protect mice against Strains 4009 and 8473. Hyperimmune antisera were prepared against them and their homologous and heterologous immunity is shown in Table 3. Strain 4009 antiserum did not give a good homologous immunity and was not investigated further. Strain 8473 antiserum, however, gave good homologous protection and also protected against Strain 4009 and a bovine isolate (Strain 9974). On the other hand, Strain 8473 antiserum did not protect against Strain SI(A) and only poorly protected against Strain 15121(A). The latter strains, however, were well protected against by Strain D4 antiserum (Table 1).

*Effect of adjuvants on the immune response*

According to the results given in Table 4 the best response was obtained with vaccines A and C. These vaccines were produced on B & J medium and respectively contained potassium alum with KOH or $A1(OH)_3$, prepared at the Institute.

After the 2 initial injections the titres were still comparatively high 3 months later, but virtually no protective effect of the sera could be demonstrated 6 months post-immunization. A booster injection given at this stage resulted in a substantial stimulation of mouse protective antibodies in the sheeps' sera.

This response was particularly marked with respect to the vaccine strains, but the most gratifying finding was that these sera also protected mice against 2 unrelated Type A strains as well as 2 non-typable, yet pathogenic,

TABLE 1 Cross protection of hyperimmune *P. multocida* D4 sheep antiserum against heterologous strains

| Protection afforded by D4 hyperimmune antiserum | | | | | | | |
|---|---|---|---|---|---|---|---|
| Type D strains | | Type A strains | | Untypable strains | | Other strains | |
| Strain No. | Logs protection | Strain No. | Logs protection | Strain No. | Logs protection | Strain No. | Logs protection |
| D4 | 4,0 | SI | 1,2 | 1676 | 2,5 | 921 (type B) | 0,8 |
| D2 | 5,1 | 7675 | 1,1 | 74 | 2,4 | 7630 (type E) | 0,0 |
| D II | 3,2 | 4009 | 0,0 | 17785 | 2,2 | | |
| D6 | 2,9 | 135 | 3,1 | | | | |
| 33191 | 4,2 | 8467 | 0,6 | | | | |
| NM | 5,0 | 8473 | 0,0 | | | | |
| | | 15121 | 1,2 | | | | |

TABLE 2 Protection afforded by various Strain D4 vaccines against heterologous strains

| Challenge strains | Nature of vaccine | | | | |
|---|---|---|---|---|---|
| | Hyperimmune antiserum | 0,25 % pcv alum precipitated | 0,5 % pcv alum precipitated | 1,0 % pcv alum precipitated | 0,5 % incomplete Freund's adjuvant |
| | Logs protection | | | | |
| D4 (Type D) | 4,0 | 1,1 | 1,4 | 1,9 | 0,0 |
| SI (Type A) | 1,2 | 0,3 | 0,6 | 0,4 | nt |
| 7675 (Type A) | 1,1 | 0,0 | 2,2 | 2,4 | nt |
| 135 (Type A) | 3,1 | 0,0 | 1,1 | 1,7 | nt |
| 8467 (Type A) | 0,6 | 0,0 | 1,2 | 0,8 | nt |
| 15121 (Type A) | 1,2 | 0,2 | 0,0 | 0,4 | nt |
| 1676 (Non-typable) | 2,5 | 0,0 | 1,7 | 1,8 | nt |
| 74 (Non-typable) | 2,4 | 0,0 | 4,7 | 4,4 | nt |
| 17785 (Non-typable) | 2,2 | 0,0 | 2,3 | 2,9 | nt |

TABLE 3 Protection afforded by Strain 8473 Type A and Strain 4009 Type A hyperimmune sheep antiserum against homologous and heterologous strains

| Hyperimmune antiserum | Logs protection | | | | | |
|---|---|---|---|---|---|---|
| | Challenge strains | | | | | |
| Strain No. | 8473 (A) | 4009 (A) | SI (A) | 15121 (A) | 9539 (A) bovine | 9974 NT bovine |
| 8473 | 2,0 | 2,1 | 0,0 | 0,8 | 0,3 | 1,2 |
| 4009 | nt | 0,5 | nt | nt | nt | nt |

nt = not tested

TABLE 4 Immune response of sheep to polyvalent vaccines containing different adjuvants

| Vaccine | Composition of vaccine | | Logs protection | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 4 weeks after 2nd injection | | 3 months after 2nd injection | | 6 months after 2nd injection | | 2 weeks after 3rd injection | | | | | |
| | Medium | Adjuvant | Strain No. | | Strain No. | | Strain No. | | Strain No. | | | | | |
| | | | D4 (D) | 8473 (A) | D4 (D) | 8473 (A) | D4 (D) | 8473 (A) | D4 (D) | 8473 (A) | 7675 (A) | 8467 (A) | 74 (NT) | 1676 (NT) |
| A | B & J | Alum & KOH | 2,6 | 3,3 | 1,6 | 0,9 | 0,1 | 0,0 | 2,3 | 3,2 | 1,4 | 0,6 | 2,0 | 1,8 |
| B | B & J | $Al(OH)_3$ (Rehsoptar) | 0,8 | 1,3 | 0,8 | 0,5 | nt | nt | nt | nt | nt | nt | nt | nt |
| C | B & J | $Al(OH)_3$ (OP) | 2,8 | 2,1 | 1,5 | 0,9 | 0,8 | 0,0 | 2,5 | 3,2 | 2,0 | 0,0 | 2,0 | 2,0 |
| D | Eagles | Alum + KOH | 1,1 | 1,5 | 1,0 | 0,0 | nt | nt | nt | nt | nt | nt | nt | nt |

NT = not typable

nt = not tested

strains of *P. multocida*. There are nevertheless still certain Type A strains (e.g. 8467) to which the composite vaccines did not afford protection.

## DISCUSSION

The usefulness of *P. multocida* Strain D4 (Type D), anticipated by Cameron & Bester (1983) as a universal immunogen, was confirmed by the results reported in this study. Not only did antiserum prepared against it in sheep protect mice against various other Type D strains, but it also afforded protection against both a variety of non-typable *P. multocida* isolates and a number of Type A strains.

Support for this contention is also found in the studies of Kucera, Wong & Eis (1981), who showed that a vaccine prepared from a mutant strain of *P. multocida* afforded immunity to various heterologous serotypes. The identity of the immunogenic antigen(s) of *P. multocida*, referred to previously (Cameron *et al.*, 1980), is not clear. Recent studies by Syuto & Matsumoto (1982) indicate that the protective antigen is a protein/carbohydrate complex. The cross-protecting factor, however, may be a different antigen. Using lysates of turkey-grown *P. multocida* in an attempt to identify this antigen, Brogden & Rimler (1983) found that various preparations afforded cross-protection but that the amount of protein they contained did not relate to the degree of protection.

Strain D4, however, did not afford protection against all Type A strains, for example, Strain 8473. On the other hand, antiserum and vaccine containing the latter strain did protect against certain Type A strains (e.g. 4009) against which D4 antiserum did not protect. It seems, therefore, that there are at least 3 categories of Type A strains: (i) those that are protected against by Strain D4 antiserum, (ii) those that show cross-protection with Strain 8473 but not D4 and (iii) those (e.g. Strain 8467) that have given inconsistent results.

Two injections of an aluminium hydroxide-adsorbed vaccine containing 0,5 % packed cells of *P. multocida* Strains D4 (Type D) and 8473 (Type A) and 2 serotypes of *P. haemolytica*, elicited a good antibody response in sheep. The immunity waned gradually, but a booster injection 6 months after initial immunization produced a marked anamnestic response. Assays done on the sera at this stage showed that the sheep contained antibodies that protected mice against a variety of, but not all, heterologous serotypes. It can therefore be deduced that the vaccine will be of marked value in protecting sheep against infections caused by a wide range of *P. multocida* strains.

The immune response to the *P. haemolytica* strains that were included in the polyvalent vaccine was not followed accurately. This organism is, however, a very important pathogen of sheep (Gilmour, 1978; Gilmour, 1980) and will be the subject of a further study.

## REFERENCES


BORGDEN, K. A. & RIMLER, R. B., 1983. Lysates of turkey-grown *Pasteurella multocida:* Effects of solubilizing agents on the immunologic properties of membrane vesicles. *American Journal of Veterinary Research*, 44, 428–432.

CAMERON, C. M., ENGELBRECHT, MARIA M. & VERMEULEN, ALETTA S. M., 1978. The serological and immunological relationship of Type Strains A and D of *Pasteurella multocida* to field isolates from sheep. *Onderstepoort Journal of Veterinary Research*, 45, 215–220.

CAMERON, C. M., PIENAAR, LORRAINE & VERMEULEN, ALETTA S. M., 1980. Lack of cross immunity among *Pasteurella multocida* Type A strains. *Onderstepoort Journal of Veterinary Research*, 47, 213–219.

CAMERON, C. M. & BESTER, FAITH J., 1983. The inefficacy of polivalent *Pasteurella multocida* vaccines for sheep. *Onderstepoort Journal of Veterinary Research*, 50, 101–104.

GILMOUR, N. J. L., 1978. Pasteurellosis in sheep. *Veterinary Record*, 102, 100–102.

GILMOUR, N. J. L., 1980. *Pasteurella haemolytica* infections in sheep. *Veterinary Quarterly*, 2, 191–198.

KUCERA, C. J., WONG, M. S. & EIS, M. S., 1981. Development of a chemically altered *Pasteurella multocida* vaccinal strain. *American Journal of Veterinary Research*, 42,1389–13984.

SYUTO, B. & MATSUMOTO, M., 1982. Purification of a protective antigen from a saline extract of *Pasteurella multocide*. *Infection and Immunity*, 37, 1218–1226.




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