Immunization of Mice and Chinchillas Against Pseudomonas aeruginosa

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SUMMARY

The possibility of production of an effective vaccine against Pseudomonas aeruginosa infections in fur-bearing animals was investigated. Twenty-three strains of Pseudomonas aeruginosa isolated from diseased chinchillas and mink were tested in mice for their immunogenic properties. Nineteen of these strains produced good immunity against homologous strains, and three of these produced also good immunity against heterologous strains. Of the remaining four strains two produced moderate immunity and two no immunity.

It was found that 0.05% or 0.5% formalin added to suspensions of Pseudomonas aeruginosa or ultrasonification of the suspension produced better results than 0.5% phenol, 0.3% alcohol or heat at 100°C for half an hour.

Chinchillas vaccinated with two doses of formolized Pseudomonas aeruginosa bacterins were immune for 36 weeks after the second dose, while all controls died within 48 hours after being challenged.

It was found that the protection afforded by the polyvalent bacterin extended beyond the strains included in the vaccine.

A field survey on 34 ranches which included over 7,700 chinchillas showed very promising and encouraging results.

RÉSUMÉ

Les auteurs ont étudié la possibilité de produire un vaccin efficace contre les infections à Pseudomonas aeruginosa chez les animaux à fourrures. On isola donc 23 souches de P. aeruginosa chez des chinchillas et des visons malades et on évalua leurs propriétés immunogéniques chez des souris. Dix-neuf de ces souches permirent l'obtention d'une immunité valable contre des souches homologues et trois d'entre elles contre des souches hétérologues également. Des quatre autres souches, deux ne donnèrent qu'une immunité moyenne, et les deux autres ne conférèrent aucune immunité.

On observa également que le fait d'ajouter de la formaline à 0.05% ou à 0.5% à la suspension de P. aeruginosa, ou encore en exposant cette suspension à des ultra-sons, donnèrent de meilleurs résultats que l'addition de phénol à 0.5%, d'alcool à 0.3% ou l'exposition à une température de 100°C pendant une demi-heure.

Les chinchillas vaccinés par deux doses de bactérine de P. aeruginosa formolée demeuraient immunisées pendant 36 semaines, après la seconde dose. Au contraire, tous les animaux-contrôles moururent dans les 48 heures qui suivirent une contamination. On remarqua que l'immunité obtenue à l'aide du vaccin polyvalent protégeait contre d'autres souches que celles comprises dans le vaccin. Une expérience sur le terrain, effectuée dans 34 ranches qui comprenaient plus de 7,700 chinchillas donna des résultats prometteurs et encourageants.

INTRODUCTION

Many attempts have been made to isolate and characterize the protective antigens of *Pseudomonas aeruginosa*. An important pathogenic factor of *Pseudomonas aeruginosa* appears to be in the slime layer (13), which acts as a capsule in preventing the phagocytosis of the organism. Alms and Bass (3) isolated a highly-protective antigen from the slime layer which produced good protection against challenge with the homologous, and partial protection against challenge with heterologous, strains of *Pseudomonas aeruginosa*. Alexander and his co-workers (1) tried several methods

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of purifying protective antigens from this organism. They found that a high molecular weight polysaccharide of very low toxicity immunized rats against lethal doses of homologous strains and, to a lesser degree, against challenge with heterologous strains. Filtrates of cultures or supernatant fluids of cultures were used successfully to immunize animals against *Pseudomonas aeruginosa* by several workers (1, 10, 11, 14).

Formolized bacterins have been used successfully in the prevention of *Pseudomonas aeruginosa* infections in goats (17), cattle (18), mink (5, 7, 9)) and chinchillas (6, 9). Although some successful attempts have been made to immunize fur-bearing animals against *Pseudomonas aeruginosa,* there have been no controlled laboratory studies on immunization.

MATERIALS AND METHODS

Strains of *Pseudomonas aeruginosa*

Twenty-three strains of *Pseudomonas aeruginosa,* which had been isolated from pathological materials submitted to the Fur-bearing Animal Diseases Laboratory at the Ontario Veteriary College, were used in these studies.

All strains were slime and pigment producers.

Preparation of Bacterins

Monovalent and polyvalent *Pseudomonas aeruginosa* bacterins were prepared from 23 strains isolated from mink and chinchillas. One colony of each strain of *Pseudomonas aeruginosa* from a blood agar culture was suspended in 5 ml of trypticase soy broth[1] and Roux flasks containing 125-150 ml of sterile trypticase soy agar[2] were inoculated by pouring the culture suspension over the surface of the agar. Sterile glass beads were added to the flasks, and the flasks were rocked horizontally to spread the culture suspension over the surface of the agar. The flasks were incubated in the inverted position for 48 hours at 37°C.

The growth was harvested by adding 20 ml of sterile normal saline solution to the flasks, and the flasks were rocked horizontally to remove the growth from the agar. The culture suspensions thus obtained were then inactivated by various chemical and physical agents, as outlined below.

All treated culture suspensions were tested for sterility by inoculating one blood agar plate with 1.0 ml of the suspension, and incubating the plates, agar down, at 37°C for 48 hours.

All bacterins were standardized to tube 3 of a Burroughs Wellcome nephelometer, to contain approximately 2.1 x 10^9 organisms/ml. Saline suspensions of living *Pseudomonas aeruginosa* organisms were inactivated by formalin, phenol, alcohol, heat, and ultrasonic vibrations.

Formolized bacterins — Formalin was added to the culture suspension to give final concentration of 0.5% or 0.05%. For field use, phenol was added to 0.5% formolized bacterins as a preservative, to give a final concentration of 0.5%.

Phenolized bacterins — Phenol alone was added to give a final concentration of 0.5%.

Alcohol-inactivated bacterins — Isopropyl alcohol was added to give a final concentration of 3.0%.

Heat-inactivated bacterins — Suspensions of organisms were heated at 100°C for one-half hour.

Ultrasonically-inactivated bacterins — Suspensions of organisms were inactivated by sonification at 10 kilocycles for one hour with a Fisher's ultrasonic probe.[3]

Experimental Animals

Mice — White Swiss mice weighing between 25 and 30 grams were obtained from the breeding colony at the Ontario Veterinary College, or from the Connaught Medical Research Laboratories in Toronto.

Chinchillas (Chinchilla laniger) — Chinchillas were obtained from several chinchilla ranches in Ontario which had no history of vaccination or recent losses from psuedomonas infections. The chinchillas were of both sexes, and over six months of age.

Immunization of Animals

Mice — Each mouse in these experiments

[1]Difco Laboratories, Detroit, Michigan.

[2]Difco Laboratories, Detroit, Michigan.

[3]Fisher Scientific Co. Ltd., Toronto, Canada.

TABLE I. Immunogenicity of Formolized Pseudomonas aeruginosa Bacterins

	Strains Used for Vaccination and Challenge					
Animals	316	470	19	32	403	B
Vaccinated mice	10/10[a]	7/10	7/10	10/10	0/10	2/10
Control mice	0/10	0/10	0/10	0/10	0/10	0/10

[a]Proportion of survivors to infected animals one week after challenge.

TABLE II. The Immunogenic Specificity of Pseudomonas aeruginosa Strains

Number of Strains Used for Bacterins	Challenge Strains	Number of Strains Producing		
		Good Immunity (75-100% Survivors)	Moderate Immunity (25-74% Survivors)	Poor or No Immunity (0-24% Survivors)
23	Homologous	19	2	2
	Heterologous	3	14	6

was vaccinated with two subcutaneous doses, each of 0.5 ml formolized bacterins, administered at a three-week interval.

Chinchillas — Chinchillas were vaccinated subcutaneously as described for each experiment.

Challenge of Animals

For each experiment challenge doses from mouse and chinchillas were calculated as follows:

One colony of *Pseudomonas aeruginosa* was suspended in each of several test tubes containing 5 ml of trypticase soy broth. The suspended cultures were mixed, and incubated at 37°C for varying periods. Dilutions of these cultures were made with sterile normal saline, and small groups of unvaccinated animals were challenged intraperitoneally with several dilutions to determine the infecting (Challenge) dose. The dilution selected for challenge contained approximately 10 minimal lethal doses (MLD) for mice, and approximately 1-3 MLD for chinchillas.

EXPERIMENTAL STUDIES AND RESULTS

Immunogenic Properties of *Pseudomonas aeruginosa* Strains

The purpose of these experiments was to study the immunogenicity (ability to stimulate increased resistance to infection) and immunogenic specificity of various strains of *Pseudomonas aeruginosa* in mice, by challenge of vaccinated mice with homologous and heterologous strains of *Pseudomonas aeruginosa.*

In the first experiment, six bacterins were tested for their ability to stimulate strain-specific homologous immunity. Ten mice were vaccinated with each monovalent *Pseudomonas aeruginosa* bacterin and each mouse was challenged three weeks after the last vaccination with approximately 10 MLD of the homologous strain.

The results of this experiment are presented in Table I.

Two of the six groups of vaccinated mice (316 and 32) were completely protected, whereas in two groups vaccinated with strains 470 and 19 only seven mice out of ten were protected. There was no evidence of immunity in the remaining two groups of vaccinated mice (403 and B).

This experiment demonstrated that not all strains of *Pseudomonas aeruginosa* stimulate good homologous immunity, and that some strains stimulate little or no immunity to challenge with the homologous strain of *Pseudomonas aeruginosa.* These findings are in accordance with those of Walker *et al* (19) and Fisher *et al* (8).

Immunogenic specificity of Pseudomonas aeruginosa strains — After it had been demonstrated that formolized *Pseudomonas aeruginosa* bacterins are, in most cases, capable of stimulating strain-specific immunity, the immunogenic specificity of bacterins prepared from 23 strains of *Pseudomonas aeruginosa* was studied.

Groups of mice were vaccinated with

each of 23 formolized pseudomonas bacterins. Each group of vaccinated mice was subdivided into four or more sub-groups, and each sub-group was challenged with approximately 10 MLD of homologous or heterologous strains of *Pseudomonas aeruginosa*.

Of the 23 strains of *Pseudomonas aeruginosa* studied, 19 (82%) showed good immunogenicity to challenge with the homologous strain (75-100% survival of vaccinated mice), two (9%) were moderately immunogenic (25-74% survival), and two were non-immunogenic (Table II).

Three of these 23 strains produced good immunity to heterologous challenge (85-100% survival), 14 (61%) were moderately immunogenic (25-74% survival), and six (26%) were poorly or non-immunogenic (0-24% survival) (Table II).

As was to be expected, better protection was afforded by homologous vaccines than by heterologous vaccines. These findings indicate that although some strains of *Pseudomonas aeruginosa* were capable of stimulating cross-protective immunity, homologous immunity among strains of *Pseudomonas aeruginosa* was greater than heterologous immunity. The question of immunogenic specificity is of great importance in vaccination studies, since it determines whether stock bacterins (monovalent or polyvalent) may be effective in the prevention of pseudomonas infections, or whether an autogenous bacterin must be prepared for each outbreak of pseudomoniasis.

The Effect of Physical and Chemical Inactivating Agents on the Immunogenicity of *Pseudomonas aeruginosa*

Several methods of inactivating *Pseudomonas aeruginosa* cultures were tested for their effects on the immunogenicity of this organism. After preliminary studies in mice, chinchillas were used for the evaluation of various bacterins prepared from strain 316, which had shown good immunogenic properties against challenge with homologous and heterologous strains of *Pseudomonas aeruginosa* in mice.

Cultures of *Pseudomonas aeruginosa* were exposed to various inactivating agents (formalin, phenol, isopropyl alcohol, heat, and ultrasonic vibrations), as outlined in "methods", and four chinchillas were vaccinated with two 0.5 ml doses of each bacterin administered at a three-week interval. Three weeks after the last vaccination, vaccinated chinchillas were challenged intraperitoneally with approximately 3 MLD of the homologous strain of *Pseudomonas aeruginosa*.

The results of this experiment are shown in Table III. All chinchillas used in these experiments were observed for a period of four weeks after challenge, but there were no further deaths or evidence of illness after 72 hours after challenge. The best results were obtained with formalin and ultrasonification as inactivating agents. Both 0.5% and 0.05% formalin produced the same results.

The Duration of Immunity in Chinchillas Vaccinated with Formalized *Pseudomonas aeruginosa* Bacterins

These experiments were done in an attempt to determine the duration of protective immunity in chinchillas vaccinated with formolized pseudomonas bacterins.

Eight chinchillas were vaccinated with two 0.5 ml doses of a standard formolized bacterin prepared from strain 316 at a three-week interval. They were divided into

TABLE III. Immunogenicity of Pseudomonas aeruginosa Cultures Inactivated by Various Chemical and Physical Agents

Type of Inactivating Agent Used for the Production of Bacterins	Time After Challenge in Hours		
	24	48	72
0.5% Formalin	4/4[a]	4/4	4/4
0.05% Formalin	4/4	4/4	4/4
0.5% Phenol	4/4	2/4	2/4
3.0% Alcohol	4/4	2/4	2/4
Heat (100° for 30 minutes)	2/4	1/4	1/4
Ultrasonification (10 KC for 60 minutes)	4/4	3/4	3/4
Controls	0/4	—	—

[a]Proportion of survivors to infected chinchillas.

TABLE IV. Duration of Immunity in Chinchillas Vaccinated With Formolized Pseudomonas aeruginosa Bacterins

Interval Between Last Vaccination and Challenge	Proportion of Survivors to Infected Chinchillas	
	Vaccinated	Controls
16 weeks	2/2	0/2
20 weeks	2/2	0/2
36 weeks	3/4	0/4

three groups, and one group was challenged 16 weeks, another 20 weeks, and the third group 36 weeks after the last vaccination, with approximately 1-3 MLD of the homologous strain of *Pseudomonas aeruginosa.*

The results of this experiment are summarized in Table IV.

From these findings, one may conclude that protective immunity in vaccinated chinchillas lasted for 36 weeks or longer. This is considerably longer than the three month period stated by Devos *et al* (6).

The Effect of Polyvalent Bacterins on Immunity in Chinchillas

Since the studies in mice had shown that some pseudomonas bacterins stimulate immunity not only against challenge with homologous, but also with heterologous strains, the use of polyvalent bacterins in chinchillas was studied. Two polyvalent formalized bacterins were tested in chinchillas against a virulent strain included in each bacterin in one group of animals, and against a virulent strain not included in the vaccine in another group of animals.

Polyvalent bacterin 'A' was prepared by mixing equal amounts of the 23 standard formolized bacterins which had been used in earlier experiments for immunogenicity studies in mice, and polyvalent bacterin 'B' was prepared from the three strains which had produced good immunity against both homologous and heterologous challenge in mice.

Chinchillas were vaccinated with two 0.5 ml doses of bacterin 'A' or bacterin 'B' at a three week interval, and were challenged with approximately 3 MLD of one strain which was included in the vaccine or one strain not included in the vaccine, 12 weeks after the last vaccination.

The results of this experiment are presented in Table V.

Most of the vaccinated chinchillas survived challenge, while unvaccinated controls all died.

TABLE V. The Immunogenicity of Polyvalent Formolized Bacterins in Chinchillas

Type of Vaccine	Proportion of Survivors to Infected Chinchillas after	
	Homologous Challenge	Heterologous Challenge
Vaccine A	9/12	8/12
Vaccine B	3/4	3/4
Controls	0/4	0/4

This experiment suggests that formolized polyvalent pseudomonas bacterins protect animals against strains which are not included in the preparation of the bacterins.

Evaluation of Protection Produced by *Pseudomonas aeruginosa* Bacterin in Chinchillas Exposed to Natural Infection

Formolized polyvalent and autogenous bacterins were distributed to chinchilla ranches with a questionnaire to evaluate their efficacy.

The recommended dose for vaccination was 0.5 ml for the first injection and 1 ml for the second injection, to be administered subcutaneously in the neck or inside thigh region at a ten to 14 day interval.

Vaccination of chinchillas younger than six to eight weeks of age was not recommended, unless there had been losses in this age group, in which case, the recommended doses were half the adult doses administered at the same time interval.

The owners were requested to evaluate the effects of vaccination at periods ranging from two months to one and a half years after the last vaccination. Over 7,700 chinchillas were vaccinated on 34 ranches from which replies had been obtained to the questionnaires.

Approximately one-half of the ranchers replied that some chinchillas had shown some listlessness or inappetence after vaccination, while the rest reported no apparent effects of vaccination on the behaviour of their chinchillas.

Although most ranchers reported beneficial results after vaccination, on some ranches, losses from pseudomonas infections continued, though to a lesser degree, after vaccination either with polyvalent and/or autogenous bacterin.

In one ranch, losses continued for several months after vaccination with an autogenous bacterin, though at a greatly reduced rate. Chinchillas autopsied showed very advanced lesions of pseudomoniasis, but few pseudomonas bacteria could be isolated in most cases, suggesting a chronic infectious process. This rancher later admitted to using a quaternary ammonium disinfectant (to which *Pseudomonas aeruginosa* is resistant) continuously in the drinking water for his chinchillas. Cultures from this drinking water had shown that it was heavily contaminated with *Pseudomonas aeruginosa,* and this contaminated water may,

therefore, have served as a continuous challenge to his chinchillas. Losses on this ranch stopped soon after the removal of this disinfectant from the drinking water, and supplying the fresh drinking water every day. Vaccination with a polyvalent bacterin, followed by an autogenous bacterin was applied.

In another case, sporadic losses continued from pseudomonas infections in spite of vaccination with autogenous and heterologenous vaccines. This rancher, however, had been periodically treating his chinchillas with chloramphenicol in the drinking water, and the resistance of *Pseudomonas aeruginosa* to this antibiotic may have accounted for the persistance of pseudomonas bacteria in his herd as a result of possible colonization with pseudomonas after oral antibiotic therapy.

The results of surveys such as these are often difficult to interpret, because of the extreme subjectivity of the replies. Many factors, such as management, sanitation, movement of animals from ranch to ranch and to shows, and the introduction of new animals on a ranch would have to be investigated thoroughly, preferably by a single investigator, before any definite conclusions could be drawn on the effects of vaccination on the incidence of pseudomonas infections in any herd of chinchillas.

The ideal approach to field evaluation studies on *Pseudomonas aeruginosa* bacterins would be to divide each herd into vaccinated and unvaccinated (control) groups, and to compare these groups for differences in morbidity and mortality. This method could, however, result in substantial losses to the rancher.

DISCUSSION

Microorganisms contain many different antigenic components of which only one or two may be particularly concerned with the immunogenicity of the organism. It is therefore important to try to identify, and certainly to preserve, these antigens in vaccine preparation, and in selecting strains for the production of vaccine, it is essential to select strains which contain immunogenic antigens. Nineteen of the 23 strains of *Pseudomonas aeruginosa* which were tested stimulated good immunity to homologous challenge. There may be different antigenic types within a species; consequently, immunization with one type does not necessarily protect against infection with other types. Only three of 23 strains of *Pseudomonas aeruginosa* tested stimulated good immunity against other strains. Laborde and de Fajardo (11) were unable to demonstrate cross-immunogenicity among strains of *Pseudomonas aeruginosa*. On the other hand, Liu *et al* (12) found that the slime-layer antigens and lethaltoxin antigens confer strain-specific immunity, while other extra-cellular-toxin antigens (protenase and lecithinase) stimulate species-specific immunity.

Since some strains produced protection not only against the same strains, but also against other strains of *Pseudomonas aeruginosa*, bacterins consisting of several strains were tested in chinchillas. These polyvalent bacterins protected chinchillas not only against strains included in the vaccine, but also against strains not included in the vaccine. The use of polyvalent bacterins for the treatment or prevention of pseudomonas infections has been recommended by several workers (2, 3, 13, 14, 16).

It has been found in preparing vaccines that certain protective antigens are labile to some chemical and physical agents which are used for killing suspensions of bacteria. Formolized bacterins appear to be more immunogenic than are bacterins treated by heat, alcohol or phenol. Similar results were reported by Carr *et al* (4) who found that formolized *Pseudomonas aeruginosa* bacterins were more immunogenic than heat or alcohol-treated bacterins. On the other hand, Millican *et al* (15) and Markley and Smallman (14) successfully immunized mice with pseudomonas bacterins which had been heated at 100°C for three hours, which suggests that carbohydrate antigens of *Pseudomonas aeruginosa* are immunogenic, a finding similar to that of Alexander *et al* (1) and Alms and Bass (3).

The duration of immunity is important in the determination of a vaccination schedule for field use on mink and chinchilla ranches. The only reference to the duration of protective immunity in chinchillas vaccinated against pseudomonas infections was made by Devos and co-workers (6), who stated that immunity lasts only for three months. On the other hand our

experiments showed that protective immunity in vaccinated chinchillas lasted 36 weeks.

Evaluation of formolized bacterins by means of field trials suggest that it was possible, by vaccination, to produce protection against pseudomonas infections, although other factors may also play an important role in the prevention of these infections. The most convincing evidence would be obtained if some groups of animals on fur ranches could remain unvaccinated, but this might have resulted in substantial losses of unvaccinated animals. For similar reasons, it would have been impractical to compare the immunogenicity of various pseudomonas bacterins under field conditions. However, laboratory studies and results obtained in the field provide considerable evidence of the efficacy of formolized pseudomonas bacterins in chinchillas. Although Haagsma and Pereboom (7), Collins and Donnelly (5), Devos *et al* (6) and Halen *et al* (9) have all successfully controlled pseudomonas infections in furbearing animals with autogenous formolized bacterins, we were also able to protect chinchillas by using vaccines prepared from heterologous strains of *Pseudomonas aeruginosa*.

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