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Specificity of the protective response induced by the slime layer of *Pseudomonas aeruginosa*

By A. MATES* and P. ZAND
Department of Life Science, Bar-Ilan University, Ramat-Gan, Israel

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SUMMARY

Active protection against *Pseudomonas aeruginosa* could be induced in mice by immunization with either the phenol killed cells or the alcohol precipitated fraction of the slime layer, or the ribosomal vaccine preparation. Passive protection could also be induced by injecting into mice antisera prepared in rabbits against these bacteria. This protection was due to the production of antibodies in reaction to the slime layer; the absorption of these antibodies by the slime caused the loss of protection. The fact that mice were also protected by vaccination with strains other than those used for challenging was attributed to the presence of an antigenically similar slime. Passive protection towards a heterologous strain, even one with an antigenically similar slime layer, was dependent on the dose of the challenging injection.

INTRODUCTION

In recent years, much attention has been given to the development of immunotherapeutic measures in order to supplement or replace the chemotherapeutic treatment of individuals who have been or could be infected with *Pseudomonas aeruginosa*. The need for such an approach arose because of the increased number of infections caused by this organism (Finland, 1970), especially following burns, and its resistance to the inhibitory action of antibacterial drugs. Recently progress has been made in preventing colonization of burns by the use of topical chemoprophylaxis. In this way the chance of invasive septicaemia has been reduced, but occasionally fatal infections have occurred even following this treatment (Lindberg *et al.* 1965).

Previous studies have shown that infection due to *Pseudomonas aeruginosa* could be prevented by immunization with the whole cells in man (Feller, 1967) and animals (Fisher, Devlin & Gnabasik, 1969). Protection could also be induced in animals by an ethanol-precipitated fraction of the slime layer (Alms & Bass, 1965), and various culture filtrates (Jones, 1968; Johnston & Syeklocha, 1972). However, as Markley (1967) and most other investigators have pointed out, in order that immunotherapy may effectively protect against infection with pseudomonas, a vaccine should prevent infection by many strains of the bacteria. Fisher *et al.* (1969) have shown that a vaccine obtained from one strain of *P. aeruginosa*

* Present address: Department of Microbiology, Queen's University, Kingston, Ontario, Canada.

could protect mice against up to 21 % of other strains tested. On this basis he developed the immunotype scheme based on seven strains which protected mice against more than 90 % of the strains isolated from a wide variety of sources. In a much more closed environment, Pierson & Feller (1970) have shown that 76 % of 555 clinically isolated strains reacted with a monovalent serum.

Although numerous investigators have dealt with the antigen which could induce protection most of them have done so using one strain. It is the purpose of this study to identify the antigen which could induce protection, and to analyse its distribution among various strains. The data obtained from the *in vitro* studies were applied *in vivo* by testing the active protection due to immunization with this antigen, and passive protection due to vaccination with the antibody to this antigen.

MATERIALS AND METHODS

Bacterial strains

P. aeruginosa strains from human infections were obtained from Prof. D. Sompolinski, Assaf Harofe, Israel Government Hospital, Zriffin. The strains were maintained on tryptic soy agar (DIFCO) slants and were transferred once a week.

Preparation of vaccine

Phenol-killed vaccine (Laborde & de Fajarodo, 1965). The *P. aeruginosa* strain used for vaccine preparation was grown overnight on nutrient agar (DIFCO) at 37° C. The bacteria were harvested with 0·5 % phenolized saline and incubated in this solution overnight at 37° C. The suspension was tested for sterility and adjusted to 200 Klett units at 660 nm. in a Klett Summerson spectrophotometer. This is equivalent to 2×10^9 organisms/ml.

Alcohol precipitated fraction from slime (*APF*) (Alms & Bass, 1967). *P. aeruginosa* was grown on slants of brain heart infusion (BHI) agar (DIFCO) overnight at 37° C. The bacteria were harvested from the slant and inoculated into BHI broth. After incubation at 37° C. for 5 hr., 5 ml. of this culture were transferred to BHI agar in Roux culture bottles and incubated overnight at 37° C. The bacteria were harvested by gentle agitation with 5 ml. of 0·15 M-NaCl solution per bottle, and were then removed by centrifugation at 7300 ***g*** for 1 hr. The supernatant was again centrifuged at 27,000 ***g*** for 1 hr. to remove any debris of cells. Sodium acetate (10 % w/v) and glacial acetic acid (1 % w/v) were then added to this supernatant which contained the crude slime. The slime was precipitated by adding an equal volume of alcohol by drops at 4° C. to this solution. The alcohol-precipitated fraction of the slime was collected by centrifugation at 27,000 ***g*** for 1 hr. The precipitate was washed once with distilled water and then dissolved in 0·15 M-NaCl and left for 72 hr. at 4° C. This solution was afterwards dialysed for 48 hr. against distilled water and then lyophilized and stored at 4° C.

Ribosomal vaccine (Youmans & Youmans, 1966). *P. aeruginosa* was grown in a shaker water bath at 37° C. for 16 hr. The bacteria were washed twice with saline and were then suspended in 0·55 M sucrose phosphate buffer (0·01 M pH 7·0) con-

taining 0·03 M-$MgCl_2$; they were then ruptured in a cold French press. The broken cells were centrifuged at 26,300 ***g*** for 15 min.; four-fifths of the supernatant was transferred and spun in Spinco Model L at 46,900 ***g*** for 10 min. Four-fifths of the supernatant from this centrifugation was again centrifuged at 144,700 ***g*** for 3 hr. This supernatant was then resuspended in phosphate buffer pH 7·0, 0·01 M at a concentration of 50 mg. wet weight/ml. To a volume of ribosomal fraction an equal volume 0·5 % sodium dodecylsulphate (SDS) was added; the mixture was then left for 1 hr. at room temperature and afterwards was incubated overnight at 4° C. The SDS precipitate was removed by centrifugation at 36,000 ***g*** for 20 min. and four-fifths of the supernatant was again centrifuged at 144,700 ***g*** for 3 hr. in order to precipitate the immunizing fraction.

Mice

Female white mice of an outbreed stock were supplied by the Hebrew University colony, Jerusalem. The mice weighed between 18 and 20 *g* at the beginning of each experiment. The animals were allowed food and water freely.

Immunization procedure

Mice were injected once intraperitoneally with 0·2 ml. of either the organism suspended in phenol saline or with slime, or with ribosomal preparations. The animals were observed daily and were challenged one week later.

Preparation of challenge inoculum

Pseudomonas aeruginosa was grown on nutrient agar (DIFCO) for 24 hr. at 37° C. The bacteria were harvested from the plates, washed twice and then resuspended in saline to a concentration of 200 Klett units at 660 nm. in a Klett Summerson spectrophotometer which is equivalent to 2×10^9 organisms/ml. as determined by plate counts. Except when otherwise stated, 0·2 ml. of this suspension (10 LD50) were injected into unimmunized animals (which served as controls) and immunized animals of the same age. The unimmunized animals died within 24 hr.

Passive protection test

Sera were prepared in rabbits by immunizing them with a series of four intramuscular injections of the antigen in complete Freund adjuvant. A week after the last inoculation the animals were bled, and the sera were separated and stored at −20° C. In order to test the protective ability of the serum, the animals were injected i.p. with 0·2 ml. of serum undiluted or diluted 4 hr. before a challenge.

Antibody determination

Immunodiffusion technique. Slides 7 × 7 cm. were covered with 4 ml. of 1 % Noble agar (DIFCO). After drying at 37° C., 10 ml. of 1 % Noble agar in saline containing 0·05 % merthiolate were added. Wells were made at equal distances of 6 mm. from the central well and 0·1 ml. of antigen or antibody were added to each

well. The slides were incubated for 2 days after which they were washed for 3–4 days with saline. The precipitin line was stained with azocarmine.

Passive haemagglutination. The alcohol precipitated fraction of the slime was bound to sheep red blood cells by glutaraldehyde using a modification of the method described by Avrameas, Taudou & Chuilon (1969). Ten ml. of 2% glutaraldehyde in 0·1 M pH 7·2 phosphate saline buffer was added to 2 ml. of 50% washed sheep red blood cells in the same buffer, and the mixture was left at room temperature for 1 min. To this mixture we added 20 ml. of 0·1 M-NaH_2PO_4 which contained 0·15 M-NaCl, and this mixture was centrifuged at 5000 ***g*** for 5 min. The packed cells were then washed with the second solution and resuspended to a volume of 2 ml. (50% packed cells). Two ml. of slime (6 mg. slime/ml.) in 0·1 M-Na_2HPO_4 (pH 8·5) which contained 0·15 M-NaCl were added to these cells and this was left at room temperature for 2 hr. The sheep red blood cells which were thus coated with the slime were washed twice with phosphate buffered saline pH 7·2 and their concentration was adjusted to 2·5%.

In order to carry out haemagglutination titrations, 0·05 ml. of 2·5% sensitized cells were added to 0·5 ml. of twofold dilutions of the serum in 1% normal rabbit serum. The tubes were incubated in a water bath at 37° C. for 1 hr. and overnight in a cold room. The antibody titre was determined as the highest dilution which showed agglutination.

RESULTS

The effect of the dose and type of vaccine on the protective response of the mouse after a single intraperitoneal injection

Groups of ten mice were injected intraperitoneally with the various vaccine preparations obtained from *P. aeruginosa* strain 647. One week after vaccination the mice were challenged with 10 LD50 of the same strain and the surviving mice were counted 24 hr. afterwards. Table 1 shows that all three preparations of the antigen could protect mice against the homologous strain of *P. aeruginosa*. The various types of vaccine differed primarily in the amounts which were needed in order to produce immunization. To obtain full protection against challenge with 10 LD50 of the bacteria it was necessary to immunize a mouse with at least either 2×10^8 organisms or 100 μg. slime or 40 μg. ribosomal preparation.

Passive protection of unvaccinated mice by serum obtained from immunized rabbits

Sera obtained from rabbits immunized with the various vaccine preparations previously described, were administered intraperitoneally to the mice. Four hours after receiving the various serum dilutions the mice were challenged with 10 LD50, and the surviving mice were counted after 24 hr. Table 2 shows that the serum obtained from rabbits immunized with either the phenol-killed vaccine or the slime could afford complete protection at a 1/10 dilution, whereas the serum obtained from the rabbit immunized with the ribosomal fraction was less potent. When these various sera were examined for the presence of precipitating antibody, produced in response to the slime layer, it could be seen that this presence was

Table 1. *Protection of mice against* Pseudomonas aeruginosa *by different vaccine preparations*

Immunizing agent	Vaccine dose/ mouse	Death/ total	Survivors %
Phenol-killed bacteria	4×10^7 bacteria	7/10	30
	1×10^8 bacteria	3/10	70
	2×10^8 bacteria	0/10	100
	4×10^8 bacteria	0/10	100
Alcohol-precipitated fraction of the slime	10 μg.	8/10	20
	50 μg.	7/10	30
	100 μg.	0/10	100
	200 μg.	0/10	100
Ribosomal fraction of the bacteria	10 μg.	10/10	0
	25 μg.	7/10	30
	40 μg.	0/10	100
	75 μg.	0/10	100
Control	—	10/10	0

Table 2. *Protection conferred on mice by their passive immunization with rabbit antisera obtained against various preparations of* Pseudomonas aeruginosa *antigens*

Immunizing agent	Antiserum dilution	Presence of precipitating antibody to the slime layer	Passive haem-agglutination titre to slime	Death/ total	Survivors %
Phenol-killed bacteria	1/10	+	1/1280	0/10	100
	1/100	—	1/80	5/10	50
	1/1000	—	1/10	9/10	10
Alcohol-precipitated fraction of the slime	1/10	+	1/1280	0/10	100
	1/100	—	1/80	4/10	60
	1/1000	—	1/10	9/10	10
Ribosomal fraction of the bacteria	1/1	—	1/160	2/10	80
	1/10	—	1/10	9/10	10
Unimmunized	1/1	—	—	9/10	10

associated with complete protection. Using the much more sensitive method of passive haemagglutination, we were able to measure quantitatively the amount of antibody even in the absence of precipitating antibody. The data presented in this experiment show a relationship between the titre of antibody as measured by the latter method, and the degree of protection of the mouse against challenge with 10 LD50 of bacteria.

The effect of the alcohol-precipitated fraction of the slime on the virulence of the bacteria

Schwartzmann & Boring (1971) showed that addition of small amounts of slime to the bacteria inhibited phagocytosis as measured by phagocytic killing of the organism. When the slime was added to the bacteria and they were then

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Table 3. *Effect of alcohol-precipitated fraction of the slime on the virulence of* Pseudomonas aeruginosa *to mice*

No. of organisms	LD 50	mg. slime	Death/ total	Survivors %
—	—	0·1	0/10	100
—	—	0·2	0/10	100
—	—	1·0	0/10	100
1×10^8	1	—	6/10	40
6×10^6	1/16	—	2/10	80
6×10^6	1/16	1·0	8/10	20
6×10^6	1/16	0·2	6/10	40

Table 4. *Effect of absorption by the alcohol-precipitated fraction of the slime on the protection of the mice by the immune antisera*

Antisera prepared to	Slime absorption	pHA* titre	Death/ total	Survivors %
Phenol-killed bacteria	—	1/1024	0/10	100
	+	1/10	10/10	0
Alcohol-precipitated fraction of the slime	—	1/1024	0/10	100
	+	1/10	10/10	0

* Passive haemagglutination of the slime.

injected intraperitoneally into the mice the virulence of the bacteria was increased. Table 3 shows that a small amount of slime (0·2 mg.), which by itself is not toxic and is even able to immunize the mice against challenge with the bacteria, significantly increased the virulence of the inoculum of the bacteria. An increase in the amount of slime added to the bacteria caused a further increase in virulence.

The effect of absorption of antisera by the slime fraction on their protective power

The data presented above indicated that the slime fraction when injected together with the bacteria increased the latter's virulence. The results from Table 2 would indicate that there is a relation between anti slime antibody and the degree of protection. We therefore tested the effect of absorption of the antibody by the slime layer. For this purpose 6 mg. of slime were added to 1 ml. of the serum and incubated at 37° C. for 30 min. After incubation the mixture was centrifuged at 27,000 ***g*** for 1 hr. to remove antigen-antibody complex. After centrifugation the sera were tested for remaining antibody activity by the sensitive method of passive haemagglutination. Table 4 shows that complete removal of the antibody by the slime fraction corresponded to the loss of protection afforded by the sera against challenge with 10 LD50 of the bacteria.

Specificity of the protective response of mice vaccinated with Pseudomonas aeruginosa *strain* 647

Table 5 summarizes results of experiments in which mice vaccinated with phenol-killed *P. aeruginosa* 647 were challenged after one week with 10 LD50 of

Table 5. *Specificity of the protection in mice after a single intraperitoneal injection of* Pseudomonas aeruginosa 647 *phenolized vaccine*

Strain	Precipitation with anti-slime 647	Death/ total	Survivors %
647	+	0/10	100
C-18	+	0/10	100
1179	+	0/10	100
31	+	3/10	70
352	+	4/10	60
1214	+	3/10	70
R-1634	+	4/10	60
214	+	2/10	80
M-995	−	10/10	0
F-7	+	0/10	100
1416	+	5/10	50
M-876	−	10/10	0
3444	+	3/10	70
C-11	+	0/10	100
109	+	0/10	100
119x	+	0/10	100
629	−	10/10	0
68	+	2/10	80
62–213	+	2/10	80
2185	+	0/10	100
9060	+	1/10	90
22613	+	0/10	100
643	−	10/10	0
1316	+	0/10	100

the various strains. Of the 24 strains tested complete protection was obtained against 10 strains, partial protection against 10 and no protection at all against four strains. The crude slimes obtained from these 24 strains were tested by gel diffusion against antiserum obtained by inoculation with the alcohol precipitated fraction of the slime of strain 647. We observed that only the slimes of those strains which did not show a precipitin reaction with the slime of the immunizing strain (strain 647) were able to kill all the mice which were immunized with this organism. However, complete protection was not observed against all these strains whose slime reacted with the anti-slime of the immunizing vaccine; this is probably due to the heterogeneity of the slime layer. From the results presented in Plate 1 we can see that even though there is a cross reaction between the slimes against the homologous strain we obtained two lines of precipitation. As we observed in the large group of strains tested, the probability is that there are two components of the slime, one which cross-reacts with other strains and one which is strain specific. When a larger group of strains obtained from the same hospital was tested, we found antigenic similarities to strain 647 in 121 of 130 strains tested. Further trials to identify the remaining nine strains by preparing anti-slime against one of them were unsuccessful.

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Table 6. *Passive protection of unvaccinated mice with serum from rabbits vaccinated with the alcohol-precipitated fraction of slime from* Pseudomonas aeruginosa 647

			Challenging dose					
			10 LD50			1LD50		
Strain	Active protection to 10 LD50	Anti-body titre by pHA/ mouse*	serum treated animals	control	protec-tion	serum treated	control	protec-tion
647	100	1/800	0/10	10/10	100	0/10	3/10	100
629	0	1/800	10/10	10/10	0	4/10	5/10	20
1316	100	1/800	10/10	10/10	0	0/10	4/10	100
109	100	1/800	7/10	10/10	30	0/10	4/10	100
C-18	100	1/800	8/10	10/10	20	0/10	4/10	100
119x	100	1/800	10/10	10/10	0	0/10	5/10	100

*Passive haemagglutination of slime.

Passive cross-protection among Pseudomonas aeruginosa *strains with similar slime layer.*

The data presented above suggested that some degree of protection could be achieved by active immunization of mice with slime which would cross react with slime of bacteria used for challenge. When antibody prepared against the slime of *P. aeruginosa* 647 was transferred to mice (Table 6) and they were then challenged with 10 LD50 complete protection was observed only against the homologous strain. Towards other strains little or no protection at all was observed against this challenging dose, even though protection was obtained by active immunization. However, when the challenging dose was lowered protection was achieved against these heterologous strains. Even at this lower dose almost no protection was observed against a strain whose slime did not react with the antibody.

DISCUSSION

The serological heterogeneity of *P. aeruginosa* has long been recognized and several serological schemes have been proposed (Habs, 1957; Verder & Evans, 1961). However, attempts to correlate protection against challenge with the bacteria, to the various serotype schemes present were unsuccessful. Most of these investigators tried to measure agglutinating antibody (Jones, 1968) or antibody to the lipopolysaccharide fraction of the bacteria (Gaines & Landy, 1955). Recently Alms & Bass (1965, 1967*a*, *b*) have shown that protection of mice could be induced by a fraction of the slime layer. Using their results, based on the original observation of Liu, Abe & Bates (1961), we were able to show that passive protection afforded by an antiserum against challenge with *P. aeruginosa* correlated with the presence of antibody to the alcohol precipitated fraction of the slime. These antibodies were detected by the sensitive method of passive haemagglutination as developed in our laboratory. Furthermore, removal of these antibodies from the serum by absorption by the slime layer fraction caused this antiserum

to lose its protective properties for the mouse from challenge with the bacteria, even though the antiserum was prepared to the whole bacteria and not only to the slime.

Based on these observations, that antibodies to the slime layer were probably the most important factor in immunity to *P. aeruginosa* infection in mice, we developed a system based on antigenic compatibility among the various strains. The data presented in Table 5 were similar to those observed by other investigators (Fisher *et al.* 1969; Bass & McCoy, 1971; Jones, 1972) who found that active immunization with one strain might protect mice from challenge with a wide variety of serologically unrelated strains. In our system this kind of protection was related to antigenic similarity of the slime layers. Complete protection was obtained in 40% of the strains tested, which is relatively high compared to the results obtained by Fisher *et al.* (1969), who, however, got their strains from a wide variety of sources. The partial protective power of the immunizing strain against some of the strains with which their slime cross-react may be dependent on the challenging dose. When the dose was decreased a complete protection was observed also against those strains with which only partial protection had been previously obtained. However, against those strains whose slimes did not cross-react no protection at all was observed even at the level of one LD50.

The conflicting results observed in those studies which attempt to evaluate the use of gamma globulin from convalescent patients (Feingold & Oski, 1965; Stone, Graber, Martin & Kolb, 1965; Feller & Pierson, 1968) to protect against infection with *P. aeruginosa* must be assessed on the basis of two points. First, one must know if the gamma globulin used for protection has antibody against the slime layer of the bacteria which infected the patient. Secondly, the degree of infection at the time when the antiserum was administered must not be above the degree which this serum is able to handle.

Thus the presence of an immunotype scheme based on the antigenicity of the slime layers can be used for preparing a vaccine and an antiserum. The amount of antibodies to this antigen, as measured by passive haemagglutination, could be the index of the protective power of a serum against infection with *P. aeruginosa* with an antigenically similar slime layer.

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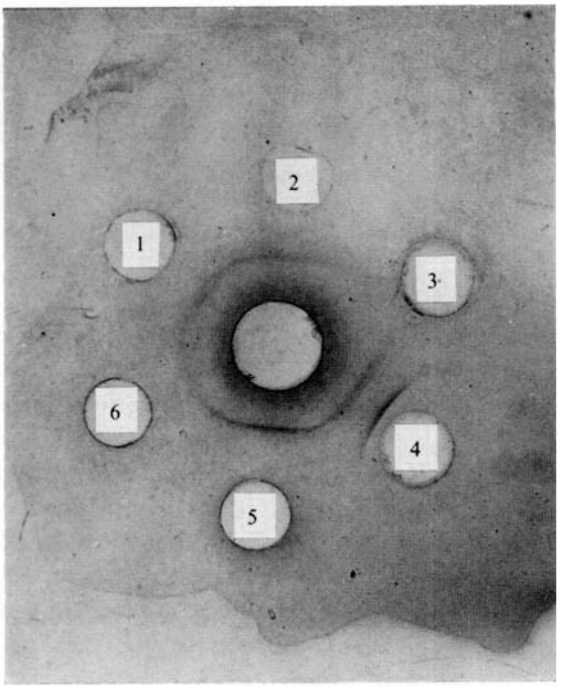
EXPLANATION OF PLATE

Immunodiffusion of rabbit anti *Pseudomonas aeruginosa* slime. In the centre, anti-*P. aeruginosa* 647 slime. In the surrounding wells slime from *P. aeruginosa* strains: 1-352; 2-214; 3-629; 4-647; 5-68; 6-109.



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