

# Obtention and Assay of Rabbit Anti-*Pseudomonas* Serum

HORACIO F. LABORDE AND CARMEN LEGNANI DE FAJARDO

Laboratorio de Microbiología, Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay

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Inoculation of rabbits with a nonliving anti-*Pseudomonas* vaccine induced appreciable levels of agglutinating antibodies against strains of *P. aeruginosa* included or not included in the vaccine. Serum obtained from vaccinated rabbits was able to confer temporary protection to mice against challenge with homologous or heterologous strains of *Pseudomonas*. When two or three doses of serum were used, all mice survived the challenge dose for more than 48 hr, but some of the animals died 10 days after challenge. When five doses of serum were used, all mice survived this 10-day period, and even 4 months later they did not show any sign of infection. Serum treatment temporarily inhibited *Pseudomonas* activity and allowed for the activation of the immunogenic mechanisms of the animals. This was corroborated by the fact that mice treated with three doses of serum and surviving the challenge dose for more than 20 days were immune against a second challenge. Anti-*Pseudomonas* gamma globulin conferred a lower degree of protection against homologous or heterologous strains of *Pseudomonas*.

*Pseudomonas aeruginosa* is a virulent bacterium often found in burn, surgical, lactant infant, premature infant, and gynecological wards. In burn wards, for example, *Pseudomonas* septicemia can produce a mortality rate of 32% in infants (6) and 36% in adults (9). Any strain is potentially pathogenic and, from a therapeutic view point, its most distinctive characteristic is its resistance to the action of antibiotics or sulfa drugs. In previous work (2, 7), we studied the effectiveness of different kinds of nonliving anti-*Pseudomonas* vaccines in inducing immunity in mice against challenge with one lethal dose of virulent strains of *Pseudomonas*. We also found (*unpublished data*) that the inoculation of mice with serum obtained from rats immunized with these vaccines was capable of conferring immunity against *Pseudomonas*.

The present paper reports experiments in which mice treated with immune anti-*Pseudomonas* serum or gamma globulin survived the inoculation of a lethal dose of virulent strains of *P. aeruginosa*. Rabbits injected with nonliving anti-*Pseudomonas* polyvalent vaccines were used as the source of immune serum.

## MATERIALS AND METHODS

**Bacterial strains.** All strains of *P. aeruginosa* were isolated from human patients and were of the smooth, green-pigmented, cytochrome oxidase-positive type. The strains employed for vaccine preparation or for the challenge dose were grown on nutrient agar

(Difco) at 37 C for 24 hr and were passaged through mice prior to use in the experiments. *Pseudomonas* strains 49 IH, 9465, 6018, 7887, 4037 and 480 were obtained from the Instituto de Higiene, Montevideo, Uruguay. *Pseudomonas* strains 59, 154, and 157 were from our own collection. *Pseudomonas* strains 72F1 (Instituto de Higiene) and L10 (Hospital de Niños, La Plata, Argentina) were used for heterologous challenge.

The heterogeneity of the strains was ascertained by serological and immunological tests.

**Monovalent vaccine preparation.** Strain 59 was used for vaccine preparation, as described in a previous note (7).

**Polyvalent vaccine preparation.** Cultures of strains 59, 157, 154, 49 IH, 9465, 6018, 7887, 4037, and 480 were suspended together (15 mg of wet cells of each strain) in 5 ml of 0.5% phenol saline. After standing for 24 hr at 37 C, the suspension was adjusted with 0.5% phenol saline to a concentration of 10 mg/ml. The resulting vaccine was checked for sterility by culturing suspensions on nutrient agar, fluid thioglycolate, and Sabouraud dextrose agar.

**Mice vaccination tests for mono- or polyvalent vaccines.** Vaccination schedules and immunity tests have been described (2, 7).

**Rabbit immunization procedures.** The antiserum against *Pseudomonas* was obtained from rabbits which were injected with 1 ml of the polyvalent vaccine three times weekly for 3 weeks and daily during the 4th week. Injections were made subcutaneously in the upper flanks, and no pyrogenicity or other pathological symptoms appeared after inoculation. Exsanguination was carried out, under pentobarbital

anesthesia, by cardiac puncture 8 days after the last injection. The blood was allowed to clot at 37 C for 30 min and then was left at 4 C for 24 hr. After centrifugation, the serum was removed and immediately used in protection tests, serological tests, or for electrophoretic fractionation.

**Electrophoretic fractionation of immune serum.** Purified immune gamma globulin for protection tests was obtained by a preparative method developed in this laboratory.

Electrophoresis of 11-ml samples was performed with an apparatus from Buchler Instruments, Inc., Fort Lee, N.J. Difco agar (bacteriological grade, purified in this laboratory), 2% in Veronal buffer (pH 8.6 and 0.5 ionic strength), was used in trays (19 by 20 cm) as a layer 1.5 cm thick. The trough for the sample (17 cm long, 0.5 cm wide, 1.3 cm deep) was made at equal distances from both electrodes. For electrophoresis, a constant voltage of 110 v, giving a current of 110 ma, was maintained for 16 hr at room temperature. The gamma globulin fraction was removed from the gel with a handpress. It was dialyzed for 72 hr against tap water and was used for inoculation. The concentration of gamma globulin was approximately one-third of that found in serum.

**Serological tests.** Agglutination titers in rabbits and in serum-treated mice were determined by a method similar to that of Christie (1). Titers were measured against *P. aeruginosa* strains 157, 49 IH, 72F1, and L10. The antigens were prepared by suspending a 24-hr culture of *Pseudomonas* in physiological saline at an opacity equivalent to McFarland tube no. 10. One drop of this suspension was mixed with one drop of the diluted serum on a glass slide. After 10 min at room temperature, the mixture was examined for clumping. Titers for the agglutination tests were expressed as the reciprocal of the final serum dilution in which agglutination was obtained.

**Challenge.** The challenge dose consisted of 0.2 ml of a *Pseudomonas* suspension equivalent in opacity to McFarland tube no. 2 or no. 3, depending on the strain; when administered intraperitoneally, it was fatal to 100% of the control mice in less than 48 hr.

**Mice protection tests.** Mice of both sexes, weighing 15 to 20 g, were used in all tests. The following experiments were done. (i) For treatment with two doses of serum, mice were inoculated intraperitoneally with 0.2 ml of antiserum; 16 hr later they were reinoculated intraperitoneally with 0.2 ml of antiserum, and after 2 hr they were challenged with 1 LD<sub>100</sub> of a *Pseudomonas* strain homologous (strain 157) or heterologous (strains 72F1 and L10) to the polyvalent vaccine. (ii) For treatment with three doses of serum, the same inoculation plan as that used in (i) was followed, and a third dose of 0.2 ml of serum was administered subcutaneously 4 hr after the inoculation of the challenge dose. (iii) For treatment with five doses of serum, the same schedule as that used in (ii) was employed, and complementary 0.2-ml doses of serum were inoculated 5 and 10 days after challenge.

As a complementary test, mice treated with three doses of serum and which survived the inoculation of the lethal dose for more than 20 days were reinocu-

lated with 1 LD<sub>100</sub> of a strain used, or one not used, for antiserum preparation.

The determination of the protective activity of purified immune gamma globulin was carried out following the same inoculation schedule as that used in (ii).

The capacity to protect mice against 1 LD<sub>100</sub> of a virulent *Pseudomonas* strain was used as an estimate of the immunity conferred by serum or gamma globulin, or induced by vaccines.

## RESULTS

**Serological tests.** Serum from rabbits inoculated with polyvalent vaccine agglutinated all *Pseudomonas* strains tested, including those not employed for vaccine preparation. Titers of 320 or 640 were routinely obtained.

Mice treated with three doses of rabbit anti-*Pseudomonas* serum showed low agglutination titers against strains used in the vaccine and also against the strains not included in the vaccine. Antibody levels were followed for 22 days from the beginning of treatment. Figure 1 shows the response of several experimental groups of animals, and each point of the curve represents the titer of sera pooled from six mice. It is important to note that there were no significant differences in the agglutination titers against homologous or heterologous strains. The values of the agglutination titers rose to a peak at 16 to 48 hr after the beginning of the treatment. However, all titers were considerably diminished after the 8th day and had disappeared after the 22nd day. When five doses of serum were used, similar titer peaks

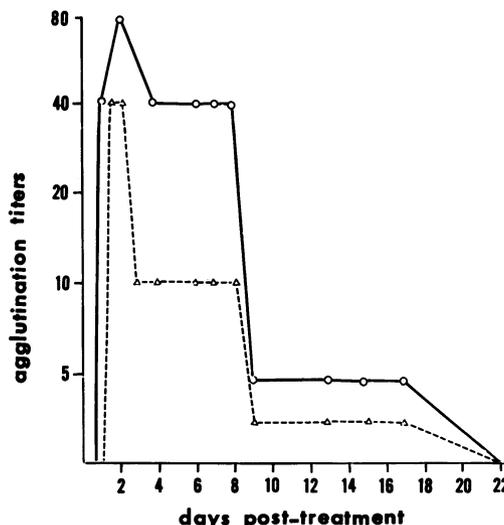


FIG. 1. Serum agglutination levels against strains of *Pseudomonas*, in mice treated with three doses of rabbit anti-*Pseudomonas* serum. Symbols: ○, homologous strains; △, heterologous strains.

of 80 were obtained at days 6 and 11 from the beginning of the treatment. By this method, protective levels were maintained for approximately 20 days (instead of 10 when two or three doses were used), and detectable levels lasted a month.

**Immunological tests.** In tests in which two doses of rabbit anti-*Pseudomonas* serum were used before challenge with homologous or heterologous strains (157 or 72F1, respectively), 100% survival was obtained during the first 10 days after challenge. Similar results were obtained when mice treated with three doses of serum were challenged with homologous (*Pseudomonas* 157) or heterologous (*Pseudomonas* 72F1 and L10) strains (Table 1). Therefore, the agglutinating activity against heterologous strains, demonstrated in serological reactions, was paralleled by the protective immunity observed when a heterologous strain of *Pseudomonas* was used for the challenge dose.

The gamma globulin obtained from immune rabbit serum conferred low protection to mice challenged with homologous or heterologous strains of *Pseudomonas* (Table 1).

The resistance to a challenge dose, of mice treated with two or three doses of serum, persisted for at least 10 days, but after this period some of the mice died. Therefore, the five-dose serum treatment was attempted to determine whether it would result in a prolongation of the survival period. This treatment restored the waning circulating antibodies to an effective level and insured 100% survival of the challenged mice (Table 2). Four months after challenge, no ill effects were apparent. The anatomopathological examination showed neither organic anomalies nor lesions at the sites of the inoculation.

Mice which had been treated with three doses of serum and had survived the challenge dose for more than 20 days became immune against *Pseu-*

*domonas*. When another LD<sub>100</sub> of a homologous or heterologous strain was inoculated 1 month after the first challenge, 90 to 100% survival was obtained (Table 3).

Table 4 shows the results obtained when mice vaccinated with mono- or polyvalent vaccines, or treated with immune serum, were challenged with a strain homologous or heterologous to those used for vaccine or serum preparation. When a heterologous challenge was used, the immunity afforded by vaccines was small for the polyvalent type and nonexistent for the monovalent type. On the other hand, passive immunity conferred by serum insured 100% survival of the animals.

TABLE 2. *Survival of mice treated with two, three, or five doses of immune serum and challenged with homologous strain 157*

No. of doses	No. of deaths/no. challenged	
	5 days	20 days
2	0/20	2/20
3	0/36	3/36
5	0/20	0/20

TABLE 3. *Survival of mice treated with serum, challenged with strain 157, and reinoculated with another challenge dose of the same strain*

Mice	First challenge	Second challenge <sup>a</sup>
	(no. of deaths/ no. challenged)	(no. of deaths/ no. challenged)
Treated (three doses).....	3/36	2/32
Controls.....	10/10	10/10

<sup>a</sup> One month after first challenge.

TABLE 1. *Protective activity of two or three doses of rabbit anti-*Pseudomonas* serum or gamma globulin*

Inoculum	Challenge tests (no. of deaths/no. challenged)		
	strain 157 <sup>a</sup>	strain 72F1 <sup>b</sup>	strain L10 <sup>b</sup>
Serum			
Two doses.....	0/20	0/25	
Three doses.....	0/56	0/30	0/29
Gamma globulin			
Three doses.....	9/20		6/20
Controls.....	15/15	15/15	15/15

<sup>a</sup> Strain included in the vaccine.

<sup>b</sup> Strain not included in the vaccine.

TABLE 4. *Comparative results obtained with mice vaccinated, or treated with three doses of serum, when challenged with strains homologous or heterologous to the vaccine*

Inoculum	No. of deaths/no. challenged		
	Strain 157 <sup>a</sup>	Strain 72F1 <sup>b</sup>	Strain L10 <sup>b</sup>
Monovalent vaccine <sup>c</sup> .....	10/10		
Polyvalent vaccine....	3/28	8/10	
Serum.....	0/30	0/30	0/30
Controls.....	10/10	10/10	10/10

<sup>a</sup> Strain homologous to the polyvalent vaccine.

<sup>b</sup> Strain heterologous to the polyvalent vaccine.

<sup>c</sup> Prepared with strain 59.

## DISCUSSION

It has been demonstrated that *P. aeruginosa* infection in animals or man provokes an antigenic response that can be detected by immunization or agglutination tests (4). Graber et al. (5) showed that sera from burned patients confer a small degree of protection against *Pseudomonas* infection in mice. Fox and Lowbury (3) found, in burned patients, that agglutination titers against *Pseudomonas* increase during the acute period of the infection.

The present studies indicated that polyvalent anti-*Pseudomonas* vaccines given to rabbits induced agglutinating antibodies against homologous and heterologous strains of *Pseudomonas*. The use of nonliving vaccines to obtain the immune serum seems to be a better method than the inoculation of living *Pseudomonas*, which usually kills a high percentage of the rabbits (8).

Mice treated with rabbit anti-*Pseudomonas* serum showed low and transient agglutination titers against homologous and heterologous strains of *Pseudomonas*. Consequently, it was of interest to determine whether this agglutinating activity would be accompanied by an effective passive immunity. It was found that protection afforded by two or three doses of immune serum was 100% effective during the first 10 days after challenge (with strains employed or not employed for vaccine preparation), but that after this period some of the mice died. This fact can be correlated with the observed diminution of serum agglutination titers, that decreased from 40 to 4 by the 9th day after the inoculation of serum.

When the number of doses was increased to five, all mice survived the 10- to 20-day period; moreover, the animals were healthy even 4 months after challenge.

It was also demonstrated that treated mice surviving for more than 20 days after challenge became immunized against a new challenge dose of *Pseudomonas* strains homologous or heterologous to the vaccine. Thus, it can be assumed that the first challenge dose acted, as a result of the extended protection afforded by serum, as a live vaccine and not as an LD<sub>100</sub>. This is corroborated by the previously demonstrated fact (7) that non-vaccinated mice surviving the inoculation of live *Pseudomonas* became, 15 to 20 days after inoculation, immunized against challenge with 1 LD<sub>100</sub> of a strain homologous or heterologous to the one inoculated.

Millican and Rust (8) demonstrated that poliomyelitis immune gamma globulin (human) has a low protective activity against *Pseudomonas* infection. Therefore, we considered it worthwhile to investigate the protective properties of purified anti-*Pseudomonas* gamma globulin. The poor results obtained may have been due to the dilution suffered by this globulin during the electrophoretic purification. Considering the advantages of purified gamma globulin treatment, we are trying to obtain a more concentrated solution.

A point of clinical interest which we would like to make concerns the possible use of anti-*Pseudomonas* serum in the treatment of *Pseudomonas* infection. As demonstrated, anti-*Pseudomonas* serum confers protection against homologous or heterologous strains, whereas monovalent vaccines do not. Moreover, antiserum protection is not dependent on the immunogenic mechanisms, which, in some patients, may be impaired.

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