Immunology, 1972, **23**, 889.

Passive Protective Properties of Serum Fractions from Mice Inoculated with an Anti-Pseudomonas Vaccine

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(*Received 12th February* 1972)

Summary. Serum from mice injected intraperitoneally with a single dose of pseudomonas vaccine passively protected other mice against a lethal intraperitoneal *Pseudomonas aeruginosa* challenge. Early protection was given mainly by the IgM fraction of serum of vaccinated mice; 6–14 days after vaccination protection was given mainly by the IgG fraction.

INTRODUCTION

Passive immunization as a means of controlling *Pseudomonas aeruginosa* infections of burns has been widely reported (Millican, Rust and Rosenthal, 1957; Jones, Jackson and Lowbury, 1965; Feller, 1966; Markley and Smallman, 1968; Jones, Lilly and Lowbury, 1971), and it is generally believed that gamma globulin (IgG) is the class of antibody responsible for the protective capacity of antipseudomonas serum (Millican *et al.*, 1957; Bjoronson and Michael, 1970).

Recent experiments have shown that it was possible to protect mice against lethal *Ps. aeruginosa* infection with serum obtained from mice only 3 days after a single injection of vaccine; a time when increased levels of specific antipseudomonas IgG were not expected (Jones *et al.*, 1971).

Studies on responses of animals to a primary antigenic stimulus by Robbins, Kenny and Suter (1965), Pike, Schultze and Chandler (1966) and Smith, Barnett, May and Sandford (1967), show that IgM appears in the serum of vaccinated animals before IgG.

In this study, we have investigated changes which occur in the passive protective properties of IgM and IgG fractions from mice which have received a single injection of a pseudomonas vaccine.

MATERIALS AND METHODS

Vaccine

A high molecular weight substance (P14F1 vaccine) was extracted from a culture filtrate of *Ps. aeruginosa*, P14 by gel filtration, using methods described by Carney and Jones (1968).

Ps. aeruginosa (P14) was grown for 5 days at 37° in 20 l of a synthetic dialysable medium. The bacteria were removed from the culture by centrifugation and positive pressure filtration; the volume reduced to 200 ml on a cyclone evaporator and unused medium was removed by ultrafiltration through cellophane. The remaining non-dialysable bacterial

products were fractionated by gel filtration through Sephadex G-200; the vaccine (P14F1) was the first fraction excluded from the Sephadex. It had a mol. wt of 6×10^6.

Serum from vaccinated mice

Schofield, male, albino mice were inoculated intraperitoneally with a single injection of 1·0 ml of a saline solution of 0·1 mg/Kg mouse weight of P14F1 vaccine. At various times after vaccination ten or twenty mice were anaesthetized by i.p. injection of 1·0 ml of 1/20 Nembutal in saline solution, and exsanguinated by cardiac puncture. The pooled blood sample was kept at 4° for ½ hour, serum was removed after centrifugation at 3000 rev/min for 5 minutes, filtered through a GS Millipore membrane (pore size 0·2 μm) and stored at −21°.

Gel filtration of serum from vaccinated mice

In pilot studies serum was obtained from mice 3 days after a single injection of P14F1 vaccine. 1·0 ml of serum was fractionated on a column of Sephadex G-200, 2·5× 42 cm, flow rate 10 ml/hour using phosphate-saline (1 part isotonic NaH_2PO_4—Na_2H_4OP buffer, pH 7·4 to nine parts isotonic saline solution) as the eluant containing 0·05 per cent of sodium azide as a preservative. The effluent from the column was monitored automatically by UV absorption (LKB Uvicord 4701A) at 254 nm and by refractometry (Waters Model R-4). The 5·0 ml fractions which were collected were regrouped into three fractions (called fractions A, B and C) to include all fractions in each of the main absorption peaks A, B and C (see Fig. 1). Each of the combined fractions was concentrated to 3·0 ml by ultrafiltration through cellophane and dialysed against sterile saline to remove sodium azide.

In the main experiments, to avoid formation of precipitates in the eluted fractions, ultrafiltration and dialysis stages were omitted for fractions found in peak A. In these experiments 1·0 or 3·0 ml of serum from vaccinated mice was fractionated on a freshly packed column of Sephadex G-200 of similar dimensions to the one described above. The same buffer without preservative was used as eluant. The effluent was monitored as before. Each 5·0 ml fraction in peak A (Fig. 1) was examined in a Pye Unicam SP500 S.2 spectrophotometer. The fraction which showed the highest UV absorption at 280 nm was used in the passive protection tests.

Passive protection experiments

(1) In pilot studies groups of three mice were inoculated intraperitoneally, 1·0 ml/mouse, which combined fractions A, B and C respectively. Two to 3 hours later mice were each challenged intraperitoneally with $1LD_{100}$ of *Ps. aeruginosa*, P14. The lethal *Ps. aeruginosa*, P14 challenge was a saline suspension (1·0 ml) containing $2{\cdot}1 \times 10^6$ bacteria (Jones, 1970, 1972). The immunizing properties of four batches of pooled serum were tested.

(2) Pooled serum was collected from groups of ten mice 1, 2, 3, 4, 5, 7, 10 and 14 days after vaccination. A single peak A fraction (containing IgM at ⅛ of its concentration in the original serum) and combined peak 13 fractions (containing IgG at ½ of its concentration in the original serum) isolated from each serum were sterilized by positive pressure filtration. The whole serum and IgM and IgG fractions of each serum were serially diluted by doubling dilutions from ½ to $\frac{1}{1536}$ in sterile Hanks's balanced salt solution. Groups of six or nine mice, depending on availability of fractions, were inoculated 1·0 ml

per mouse with the dilutions of serum: 2–3 hours later the mice, together with unimmunized controls, were challenged intraperitoneally with $1LD_{100}$ *Ps. aeruginisa*, P14.

(3) 6·0 ml pooled serum, obtained from mice 4 days after vaccination, was fractionated on Sephadex G-200. Forty-five mice were passively immunized (1·0 ml/mouse) with a 1/2 dilution in Hanks's BSS of the peak IgM fraction. Two to 3 hours later groups of three immunized mice and groups of three unimmunized control mice were respectively challenged intraperitoneally with $1LD_{100}$ of fifteen serologically different strains of *Ps. aeruginosa*. The serotypes used for challenge were: 1, 2A, 2A2B5C, 3, 5C, 5D, 6A, 6B, 6C, 8, 9, 10, 11, 14 and NT (non-typable); these strains were isolated from burns and other hospital sources; the lethal dose of each serotype was determined previously (Jones, 1972).

Titration of agglutinins

Sera were first screened for homologous agglutinins by a slide agglutination method (Jones 1971) and sera which showed positive agglutination by the slide method were titrated for agglutinins by a tube dilution method in which formolized suspensions of the homologous *Ps. aeruginosa* was used (Fox and Lowbury, 1953).

Titration of haemagglutinins

Sera were heated at 56° for 30 minutes to inactivate complement; 0·5 ml of these sera was absorbed for 30 minutes at room temperature with 0·04 ml packed, thrice washed, sheep erythrocytes to remove antibodies to sheep erythrocytes. To 0·2 ml volumes of a series of doubling dilutions of the absorbed serum (from undiluted to 1 in 128) was added 0·2 ml of a 0·2 per cent suspension of sheep cells sensitized with the pseudomonas P14F1 vaccine. Sensitized cells were prepared by methods described by Jones and Lowbury (1963) in which 0·1 ml of packed sheep erythrocytes was sensitized with 1·0 ml of vaccine solution (1 mg/ml) at 37° for 2 hours. The sensitized erythrocytes were washed three times in saline and resuspended in saline to give a 0·2 per cent suspension. Haemagglutination was read after 18 hours at room temperature.

RESULTS

GEL FILTRATION OF SERUM FROM VACCINATED MICE

Fig. 1 shows an elution curve resulting from the fractionation of 1·0 ml of serum from a vaccinated mouse on a column of Sephadex G-200. Effluent from the column was monitored by refractive index; the areas beneath the peaks are proportional to the amount of protein present. The proteins in the serum were separated out into four peaks A, B, C and D. Macroglobulins, including IgM, were found in peak A, IgG molecules in peak B, molecules with molecular weights similar to albumin in peak C and residual substances of low molecular weight in peak D (Andrews, 1965).

All sera obtained from unvaccinated and vaccinated mice, except serum obtained 3 days after vaccination, produced an elution curve similar to Fig. 1. The elution curve of serum taken 3 days after vaccination had the same distribution of peaks A, B, C and D as Fig. 1, but peak A appeared as a shoulder on the leading edge of Peak B.

PASSIVE PROTECTION AGAINST *Ps. aeruginosa* INFECTION WITH SERUM AND SERUM FRACTIONS FROM VACCINATED MICE

Four separate pilot experiments were carried out with serum obtained 3 days after

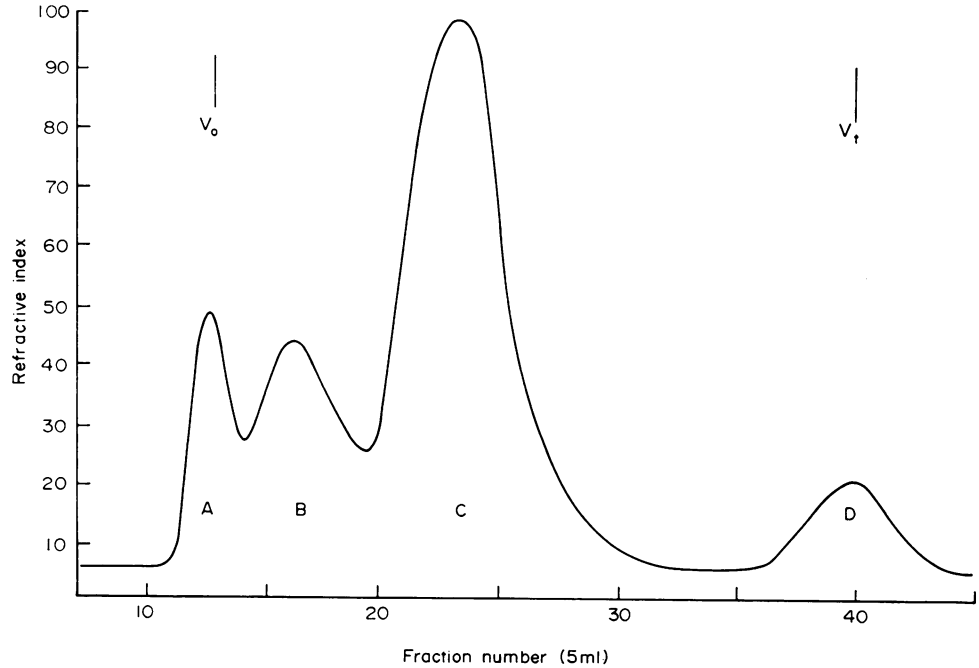

FIG. 1. Elution curve resulting from fractionation of 1·0 ml of serum from vaccinated mice on Sephadex G-200. Selected fractions from peaks A, B and C were used to immunize mice passively against a lethal *Ps. aeruginosa* infection.

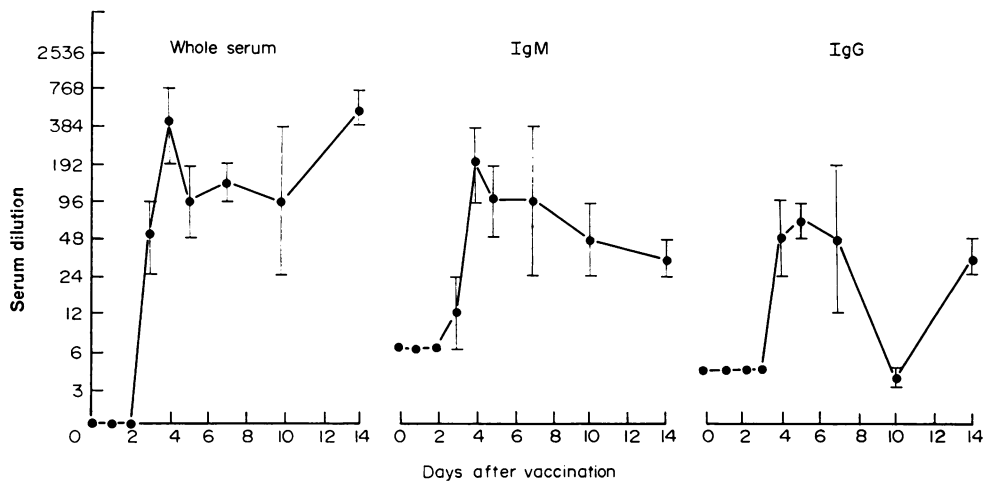

FIG. 2. Passive protective properties of dilutions of serum, IgM and IgG fractions of serum against a $1LD_{100}$ *Ps. aeruginosa* infection. Serum was obtained from mice on different days after vaccination. The upper limit shown for each sample is the dilution of serum (or serum fraction) which failed to protect mice against the challenge; the lower limit is the dilution which protected a group of nine mice against the same challenge; the continuous line is drawn mid-way between the two limits, to illustrate changes in protection with time after vaccination. The initial dilutions of IgM and IgG fractions were $\frac{1}{6}$ and $\frac{1}{8}$ with respect to whole serum.

vaccination; this serum was previously shown to protect mice against *Ps. aeruginosa*, P14 (Jones *et al.*, 1971). After gel filtration, the fractions beneath peaks A, B and C were combined, dialysed and reduced in volume. None of the groups of mice passively immunized with any of the combined fractions A, B or C, were consistently protected against a $1LD_{100}$ *Ps. aeruginosa*, P14 challenge.

It seemed possible that the protective capacity of the serum fractions had been altered by the concentration procedures. In the remaining experiments mice were passively immunized with fractions taken directly from the column.

Fig. 2 shows the passive protective properties of serum and serum fractions obtained from mice at different periods of time after vaccination.

No protection was found in serum or serum fractions (IgM and IgG) from unvaccinated mice (Day 0 in Fig. 2) or from serum taken 1 or 2 days after vaccination.

TABLE 1

TITRES OF HAEMAGGLUTINS AND AGGLUTININS IN THE SERUM OF VACCINATED MICE

Day after vaccination when serum obtained	Titre of antibody*	
	Haemagglutinins	Agglutinins
0	<4	<2
1	<4	<2
2	<4	<2
3	8	<2
4	128	<2
5	64	<2
7	8	2
10	4	2
14	4	2

* Reciprocals of serum dilutions.

Three days after vaccination protection was found both in the whole serum and IgM fraction, but not in the IgG fraction. Protection in the IgG fraction was first detected 4 days after vaccination; the same day when maximum levels of protection were found in the whole serum and IgM fraction. After the 4th day of vaccination, a gradual decrease in the protective capacity of subsequent IgM fractions was found.

Protection in the IgG fractions reached its maximum level 5 days after vaccination, remained at this level for nearly 3 days and then fell to a low level 10 days after vaccination, but rose again on the 14th day of vaccination.

While protection in IgM or IgG fractions was showing a tendency to decrease between 5–10 days after vaccination, the amount of protection in whole serum showed little change. Fourteen days after vaccination a new overall high level of protection was found in the whole serum: a corresponding but smaller increase in the protective capacity of the IgG fraction was also found.

Table 1 shows that the titre of antibodies to vaccine-coated sheep RBC in the whole serum of vaccinated mice correlated well with the early passive protective response. The highest titre of haemagglutinin occurred in serum 4 days after vaccination—the time when the maximum passive protective response in the IgM was found (Fig. 2). Low titres of agglutinins specific for the strain of pseudomonas used for making the vaccine

were first detected in serum taken 7 days after vaccination. Agglutinins appeared 4 days after protection was found in the IgG fraction showing that agglutinins were poor indicators of protection after vaccination.

SPECIFICITY OF PROTECTIVE RESPONSE OF IgM FRACTION

To test the specificity of the protection induced by passively immunizing mice with IgM fraction, groups of mice immunized with peak IgM fraction obtained from serum 4 days after vaccination were challenged with a range of different serotypes of *Ps. aeruginosa*. Table 2 shows that passive protection was homologous and monospecific.

TABLE 2

PASSIVE PROTECTION OF MICE AGAINST DIFFERENT SEROTYPES OF *Ps. aeruginosa* BY AN IgM FRACTION FROM MICE RECENTLY VACCINATED WITH SEROTYPE 14

Treatment of mice	Serotypes of *Ps. aeruginosa* used for challenge													
	1	2A	2A, 2B, 5C	3	5C	5D	6A	6B	6C	8, 9	10	11	14	NT
Immunized	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Unimmunized	−	−	−	−	−	−	−	−	−	−	−	−	−	−

\+ All mice survived challenge. − No mice survived challenge.

DISCUSSION

A single injection of pseudomonas vaccine produced changes in the serum proteins of mice which in turn improved the passive protective potential of the serum. Protection was first detected in the serum 3 days after vaccination and was still present 11 days later. Passive protection tests with IgM and IgG fractions obtained from pooled sera at different times after vaccination, showed early passive protective properties due predominantly to IgM fractions and later protection due to IgG fractions. The sequential production of IgM then IgG found in these experiments suggests that an orthodox primary immunological response follows vaccination.

In experiments (Jones, 1972) in which active protection was studied it was found that 4 days after injection of pseudomonas vaccine (P14F1) actively protected mice could resist $1LD_{100}$ i.p. challenges from nine different serological types of *Ps. aeruginosa*. In our present study where we tried to passively transfer this widespread protection by passively immunizing mice with the IgM fraction taken from serum of mice 4 days after vaccination, we found that we could only protect mice against the homologous strain. This suggests that the peak IgM fraction contained only part of the full protective response and implies that the non-specific part of the protective response, found in actively protected mice, was probably cell-bound.

For burned patients who need early protection against *Ps. aeruginosa* infections, some consideration might be given to ways of broadening the range of protection given by IgM. In our burns unit there are usually never more than four different serotypes of *Ps. aeruginosa* present at any one time. A polyvalent vaccine, which incorporated vaccines active against the current pseudomonads would ensure that actively immunized patients would produce early IgM with a useful protective function. To monitor this early protection in the serum of vaccinated mice we found passive haemagglutination tests of more value than agglu-

tination tests. Haemagglutinins were first detected when passive protection tests first showed protection developing in the IgM fraction whereas agglutinins were not found until 3 days later.

Knowledge of the protective potential of different classes of immunoglobulin is necessary if active and passive immunization against *Ps. aeruginosa* are to be attempted in patients with burns. Even though our experiments showed that quite large amounts of IgM with specific protective properties were produced early after vaccination it is doubtful whether IgM would have practical value in passive immunization against *Ps. aeruginosa*. Alexander, Fisher and MacMillan (1971) showed that pseudomonas sepsis could develop in the presence of IgM in burned patients vaccinated with a pseudomonas vaccine: in the presence of IgG, specific for the infecting immunotype of *Ps. aeruginosa*, pseudomonas sepsis was minimized. IgM is physically less stable than IgG. IgG has a longer half-life in humans than IgM. Thus for preparing a serum for passive immunization against *Ps. aeruginosa* vaccinating procedures which favour the production of IgG antibodies will be needed. Work is in progress to find a vaccination method which will produce IgG with widespread protective activity against strains of *Ps. aeruginosa*.

REFERENCES

ALEXANDER, J. W., FISHER, M. W. and MACMILLAN, B. G. (1971). 'Immunological control of Pseudomonas infection in burned patients: a clinical evaluation.' *Arch. Surg.*, **102**, 31.

ANDREWS, P. (1965). 'The gel filtration behaviour of proteins related to their Molecular weights over a wide range.' *Biochem. J.*, **96**, 595.

BJORONSON, A. B. and MICHAEL, J. G. (1970). 'Biological activities of rabbit IgM and IgG antibodies to *Pseudomonas aeruginosa*.' *Infect. Immun.*, **2**, No. 4. 453.

CARNEY, S. A. and JONES, R. J. (1968). 'Biochemical and immunochemical properties of culture filtrates of virulent and avirulent strains of *Pseudomonas aeruginosa*.' *Brit. J. exp. Path.*, **49**, 395.

FELLER, I. (1966). *Research in Burns* (Ed. A. B. Wallace and A. W. Wilkinson) Edinburgh (Livingstone), p. 470.

FOX, J. E. and LOWBURY, E. J. L. (1953). 'Immunity to *Pseudomanas pyocyanea* in man.' *J. Path. Bact.*, **65**, 519.

JONES, R. J. (1970). 'Passive immunisation against Gram E-negative bacilli in burns.' *Brit. J. exp. Path.*, **51**, 53.

JONES, R. J. (1971). 'Early protection by vaccine in burns.' *Brit. J. exp. Path.*, **52**, 100.

JONES, R. J. (1972). 'Specificity of early protective responses induced by pseudomonas vaccines.' *J. Hyg. (Camb)*. **70**, 343.

JONES, R. J., JACKSON, D. M. and LOWBURY, E. J. L. (1965). 'Antiserum and antibiotic in the prophylaxis of burns against *Pseudomonas aeruginosa*.' *Brit. J. plast. Surg.* **19**, 43.

JONES, R. J., LILLY, H. A. and LOWBURY, E. J. L. (1971). 'Passive protection of mice against *Pseudomonas aeruginosa* by serum from recently vaccinated mice.' *Brit. J. exp. Path.*, **52**, 264.

JONES, R. J. and LOWBURY, E. J. L. (1963). 'Staphylococcal antibodies in burned patients.' *Brit. J. exp. Path.*, **44**, 576.

MARKLEY, K. and SMALLMAN, E. (1968). 'Protection by vaccination against *Pseudomonas* infection after thermal injury.' *J. Bact.*, **96**, 867.

MILLICAN, R. C., RUST, J. and ROSENTHAL, S. M. (1957). 'Gammaglobulin factors protective against infections from *Pseudomonas* and other organisms.' *Science*, **126**, 509.

PIKE, R. M., SCHULTZE, M. L. and CHANDLER, C. H. (1966). 'Agglutinating and precipitating capacity of rabbit anti-*Salmonella typhosa* IgG and IgM antibodies during prolonged immunisation.' *J. Bact.*, **92**, 880.

ROBBINS, J. B., KENNY, K. and SUTER, E. (1965). 'Isolation and biological activities of rabbit IgM and IgG anti-*Salmonella typhimurium* antibodies.' *J. exp. Med.*, **122**, 385.

SMITH, J. W., BARNETT, J. A., MAY, R. D. and SANDFORD, J. P. (1967). 'Comparison of the opsonic activity of IgG and IgM anti-Proteus globulins.' *J. Immunol.*, **98**, 336.