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PASSIVE PROTECTION OF MICE AGAINST *PSEUDOMONAS AERUGINOSA* BY SERUM FROM RECENTLY VACCINATED MICE

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SUMMARY.—Serum of mice which had been inoculated 3 days previously with a vaccine (P14F1) prepared from a strain of *Pseudomonas aeruginosa* P14 gave passive protection to unvaccinated mice against a lethal i.p. challenge by *Ps. aeruginosa* P14. The serum which protected mice against *Ps. aeruginosa* P14 did not agglutinate suspensions of this organism. Its capacity to protect unvaccinated mice was removed by absorption with *Ps. aeruginosa* P14, but not by absorption with an unrelated strain of *Ps. aeruginosa*; passive protective capacity was removed also by absorption with a strain of *Klebsiella aerogenes* but not by absorption with a strain of *Proteus mirabilis*. Serum from recently vaccinated mice had no bactericidal activity against the immunizing strain; heparinized blood taken from such mice usually showed no bactericidal action, but there was consistently poorer growth of the pseudomonas in blood from recently vaccinated mice, or from mice passively protected by serum from the recently vaccinated, than in blood from unvaccinated mice.

ACTIVE immunization with vaccines has been reported to have protective value in burned patients against *Pseudomonas aeruginosa* (Feller, 1966; Alexander, Fisher, MacMillan and Altemeier, 1969) and in burned animals against *Ps. aeruginosa* and *Proteus mirabilis* (Markley and Smallman, 1968; Jones, 1968, 1971) and against *Klebsiella aerogenes* (Jones, 1971). Though active immunity might not be expected to develop quickly enough to protect a patient already burned at the time when immunization is started, some evidence of early prophylaxis by pseudomonas vaccines has already been reported (Alexander *et al.*, 1969). Markley and Smallman (1968) and Jones (1971) have shown that burned mice are protected against lethal challenge by a pseudomonas vaccine as early as 24 hr after the first injection of vaccine.

In this paper we report some experiments on the mechanism of early resistance of vaccinated mice. We have investigated the passive transfer of protection to unvaccinated mice by serum of mice which had recently received a single injection of pseudomonas vaccine, and studied the bactericidal action of blood and serum from mice actively and passively protected in these ways.

MATERIALS AND METHODS

Pseudomonas vaccine.—The vaccine was prepared from a culture filtrate of *Ps. aeruginosa* P14 by the methods of Carney and Jones (1968); the fraction used was called P14F1. Mice were injected i.p. with a single dose of P14F1 vaccine (0·1 mg./kg. mouse) in 1·0 ml. of saline.

Serum from vaccinated mice.—Groups of 50 mice (Schofield, male, approx. 20 g.), each injected 3 days previously with a single dose of pseudomonas vaccine (0·1 mg. P14F1/kg. mouse), were anaesthetized with Nembutal (1·0 ml. of a 1/20 solution in physiological saline

by i.p. injection) and exsanguinated by cardiac puncture. The pooled blood was kept at 4° for 30 min. Serum was removed from the clot after centrifugation at 3000 r.p.m. for 15 min., filtered through a G.S. Millipore membrane (pore size, 0·2 μm), and stored at −21° until used.

Tests of passive protection.—Tests were made for protection of unvaccinated mice in the following manner. Serum (0·5 ml.) from vaccinated mice was inoculated i.p. into each unvaccinated mouse; groups of 3–5 protected mice were challenged 4 hr after passive immunization with a lethal i.p. dose ($2{\cdot}1 \times 10^8$ *Ps. aeruginosa* P14 in 1·0 ml. of saline); groups of unprotected mice and of mice inoculated with serum from unvaccinated mice were challenged in the same way. The challenge dose had previously been shown to cause death in 100 per cent of unprotected mice (Jones, 1970). The serum from one group of vaccinated mice was heated at 56° for 30 min. to inactivate complement before tests for passive transfer of resistance.

Tests for bacterial absorption of serum taken from vaccinated mice.—*Ps. aeruginosa* P14 serotype 6c, *Ps. aeruginosa* B4 serotype 2/5, *Pr. mirabilis* 2332 and *K. aerogenes* (2628) were each grown at 37° for 18 hr on nutrient agar. 3 ml. saline suspensions of each organism were made containing $2{\cdot}1 \times 10^9$ bacteria/ml. of *Ps. aeruginosa* P14 and B4, $3{\cdot}0 \times 10^9$ bacteria/ml. of *K. aerogenes* 2628 and $3{\cdot}5 \times 10^9$ bacteria/ml. of *Pr. mirabilis* 2332, as estimated from Brown's Opacity Tubes. The suspensions were centrifuged at 10,000 r.p.m. for 15 min. at 4°, then resuspended in 1·5 ml. of saline. Each suspension was added to 1·5 ml. of serum from mice vaccinated 3 days previously. After mixing by shaking, the tubes were left for 1 hr at 20° and shaken intermittently. The serum suspensions were centrifuged and filtered as before.

To test whether the protective properties of the serum had been removed by bacterial absorption, groups of 3 mice were inoculated i.p. with 0·5 ml. amounts of serum absorbed separately with each strain of bacteria and control groups of 3 mice were inoculated with unabsorbed serum; all mice were challenged i.p., 4 hr after passive immunization, with a lethal dose ($2{\cdot}1 \times 10^8$ of *Ps. aeruginosa* P14 in 1·0 ml. of saline). Two identical experiments were made.

Tests for bactericidal action of serum.—Amounts (0·02 ml.) of a thrice washed 24 hr broth culture of *Ps. aeruginosa* P14, resuspended in Ringer's solution, were placed in sterile 3 in. × $\frac{1}{2}$ in. tubes containing 0·5 ml. of serum from mice vaccinated 3 days previously with a single injection of pseudomonas vaccine P14F1. A control group, containing the same suspension of *Ps. aeruginosa* P14 with 0·5 ml. of serum from unvaccinated mice, was also prepared. Tubes were sealed with cotton wool impregnated with paraffin wax, incubated at 37° in a water bath and shaken frequently. At 0, 2, 6 and 24 hr after incubation, surface viable counts were made of the number of bacteria present by the method of Miles, Misra and Irwin (1938) on blood agar containing 4 per cent agar.

Tests for bactericidal action or growth of bacteria in blood from unvaccinated mice and from mice vaccinated 3 days previously.—Blood was obtained from groups of 3, 5 or 12 anaesthetized animals by cardiac puncture. Mice were anaesthetized by i.p. administration of 1·0 ml. of a 1/20 Nembutal solution in saline, and blood was collected in a plastic tube coated on its inside with lithium heparin.

A saline suspension of bacteria adjusted to contain approximately 5×10^5 bacteria/ml by Brown's Opacity Tubes, was added in 0·1 ml. amounts to 1·5 ml. heparinized blood in a sterile 3 in. × $\frac{1}{2}$ in. tube. Experiments were made with *Ps. aeruginosa* P14 and with strains of *Pr. mirabilis*, *Pr. vulgaris*, *Pr. morganii* and *K. aerogenes*. A surface viable count was made on blood agar of 0·1 ml. of the suspension of bacteria in blood, immediately after adding the bacteria to the blood. The bacteria and blood mixture was then incubated at 37°, with intermittent shaking, and after varying periods of incubation, depending on the conditions of the experiments described, the numbers of bacteria in the blood were again counted, using a surface viable counting method (Miles *et al.*, 1938; Colebrook, Lowbury and Hurst, 1960). The incubation time in most experiments was 5 hr.

RESULTS

Passive protection of unvaccinated mice by serum from recently vaccinated mice

Table I summarizes the results of 2 experiments which show that unvaccinated mice passively immunized with serum of recently vaccinated mice were well protected against homologous infection. Heating the serum of vaccinated mice

TABLE I.—*Passive Protection of Unvaccinated Mice with Serum from Mice Vaccinated 3 Days Previously with Pseudomonas Vaccine P14F1*

Passive protection	Challenge	Mortality Expt. 1	Mortality Expt. 2
Serum from vaccinated mice	*Ps. aeruginosa* P14	0/5	0/5
Heated serum from vaccinated mice	,,	1/5	—
Serum from unvaccinated mice	,,	5/5	5/5
Unprotected controls	,,	5/5	5/5

at 56° for 30 min. before it was injected into unvaccinated mice had little or no effect on the protective properties of the serum. Serum from unvaccinated mice gave no passive protection.

Serum of vaccinated mice, which showed the passive protective effects described above, caused no agglutination of *Ps. aeruginosa* P14.

Effect of bacterial absorption of serum taken from recently vaccinated mice on its passive protective action

All mice in the control groups which were passively immunized with unabsorbed serum survived a challenge which killed all unprotected controls (Table II).

TABLE II.—*Passive Immunization of Mice with Serum of Vaccinated Mice Absorbed with Gram-negative Bacilli*

Bacteria used to absorb serum	Challenge organism (4 hr after passive immunization)	Mortality in groups of 3 mice Expt 1	Expt 2
Ps. aeruginosa P14 serotype 6c	*Ps. aeruginosa* P14	3	3
Ps. aeruginosa B4 serotype 2/5	,,	0	0
Pr. mirabilis 2332	,,	0	0
K. aerogenes 2628	,,	3	3
Unabsorbed serum	,,	0	0
Unprotected controls	,,	3	3

Serum absorbed with the homologous *Ps. aeruginosa* P14 and with *K. aerogenes* 2628 failed to protect mice against challenge by *Ps. aeruginosa* P14, showing that these bacteria had absorbed the protective factor from the serum of recently vaccinated mice. A strain of *Pr. mirabilis* 2332 and a serologically unrelated strain of *Ps. aeruginosa* did not remove the protective factor from the serum of vaccinated mice, as shown by the fact that serum absorbed with these bacteria protected groups of mice against lethal *Ps. aeruginosa* P14 challenge.

Tests for bactericidal activity of serum from recently vaccinated mice

The mechanism by which serum transfers passive protection to unvaccinated mice might be due to its activation of the defence mechanisms of the mice into

TABLE III.—*Tests for Bactericidal Activity of Serum from Vaccinated and Unvaccinated Mice Against* Ps. aeruginosa *P14*

Time after mixing serum with bacteria when sample taken (hr)	Counts of *Ps. aeruginosa* P14/ml. in serum from Unvaccinated mice	Vaccinated mice
0	$3{\cdot}9\times10^{4}$	$5{\cdot}1\times10^{4}$
2	$3{\cdot}66\times10^{4}$	$3{\cdot}9\times10^{4}$
6	$1{\cdot}101\times10^{6}$	$1{\cdot}041\times10^{6}$
24	$3{\cdot}6\times10^{9}$	$7{\cdot}2\times10^{8}$

which it is injected (*e.g.* by opsonization) or to its bactericidal activity. Table III shows that serum from mice which were fully protected against *Ps. aeruginosa* P14 3 days after receiving an injection of pseudomonas vaccine P14F1 did not show any appreciable bactericidal activity against the immunizing strain of *Ps. aeruginosa*, but allowed vigorous growth of the bacteria within the first 6 hr.

Tests for bactericidal activity of whole blood from recently vaccinated and from passively protected mice

Since passive protection with serum from recently vaccinated mice did not apparently depend on a serum bactericidin, tests for bactericidal action were made on heparinized whole blood.

TABLE IV.—*Tests for Bactericidal Activity of Blood of Mice Vaccinated with a Pseudomonas Vaccine (P14F1) Against 5 Strains of* Ps. aeruginosa

Challenge strains of bacteria		Counts of visible bacteria/ml after five hours incubation with blood from Unvaccinated mice		Vaccinated mice	
	Serotype	0 hr	5 hr	0 hr	5 hr
Ps. aeruginosa	P14	$1 \cdot 14 \times 10^6$	+ + +	$1 \cdot 14 \times 10^6$	$1 \cdot 47 \times 10^{2}$*
Ps. aeruginosa	P14	$4 \cdot 8 \times 10^5$	$4 \cdot 275 \times 10^7$	$4 \cdot 8 \times 10^5$	$9 \cdot 225 \times 10^5$
Ps. aeruginosa	P2AB	$5 \cdot 25 \times 10^5$	$2 \cdot 475 \times 10^7$	$5 \cdot 25 \times 10^5$	$1 \cdot 3575 \times 10^7$
Ps. aeruginosa	Type 11 R	$3 \cdot 4 \times 10^5$	$1 \cdot 275 \times 10^7$	$3 \cdot 4 \times 10^5$	$8 \cdot 55 \times 10^6$
Ps. aeruginosa	Type 11 S	$3 \cdot 15 \times 10^5$	$3 \cdot 3 \times 10^7$	$3 \cdot 15 \times 10^5$	$1 \cdot 1625 \times 10^7$

* Viable count at 6 hr.

Table IV shows the results of tests with *Ps. aeruginosa*. In one experiment blood of the vaccinated mouse had a strong bactericidal activity against the immunizing strain, while blood of the unvaccinated mice allowed apparently unimpeded growth. Other experiments showed no bactericidal activity of the blood from vaccinated mice either against the immunizing strain or against serologically unrelated strains, but bacterial growth was in each experiment somewhat greater in the blood of mice which had not been vaccinated. Similar small or marginal differences were shown in growth of other species of Gram-negative bacilli in blood from mice vaccinated with P14F1 and from unvaccinated mice (Table V). Blood from unvaccinated mice which were passively protected

TABLE V.—*Bactericidal Activity of Blood of Mice Vaccinated with a Pseudomonas Vaccine P14F1 Against 4 Different Strains of Proteus and a Single Strain of* K. aerogenes

Challenge strain of bacteria	Counts of viable bacteria/ml after 5 hr incubation with blood from Unvaccinated mice		Vaccinated mice	
	0 hr	5 hr	0 hr	5 hr
Pr. mirabilis 2332	$4 \cdot 8 \times 10^3$	$1 \cdot 7 \times 10^5$	$4 \cdot 8 \times 10^3$	$1 \cdot 02 \times 10^5$
Pr. mirabilis 450	$5 \cdot 85 \times 10^5$	$6 \cdot 9 \times 10^7$	$5 \cdot 85 \times 10^5$	$4 \cdot 725 \times 10^7$
Pr. morganii 496	$3 \cdot 25 \times 10^5$	$1 \cdot 575 \times 10^7$	$3 \cdot 25 \times 10^5$	$9 \cdot 075 \times 10^6$
Pr. vulgaris 645	$3 \cdot 25 \times 10^5$	$2 \cdot 4 \times 10^6$	$3 \cdot 25 \times 10^5$	$2 \cdot 175 \times 10^6$
K. aerogenes 2628	$2 \cdot 8 \times 10^3$	$6 \cdot 25 \times 10^3$	$2 \cdot 8 \times 10^3$	$1 \cdot 05 \times 10^4$

TABLE VI.—*Bactericidal Activity of Blood of Mice Passively Immunized with Serum from Vaccinated and Unvaccinated Mice*

Time after passive immunization when blood sample was taken	Counts of *Ps. aeruginosa* P14/ml. in blood from			
	Unvaccinated mice (controls)		Vaccinated mice	
	0 hr	5 hr	0 hr	5 hr
30 min.	$3{\cdot}55\times10^5$	$1{\cdot}875\times10^7$	$3{\cdot}55\times10^5$	$3{\cdot}975\times10^7$
4 hr	5×10^5	$1{\cdot}6\times10^6$	5×10^5	$1{\cdot}425\times10^5$
24 hr	$3{\cdot}4\times10^5$	$2{\cdot}175\times10^6$	$3{\cdot}4\times10^5$	$1{\cdot}65\times10^6$

with serum from recently vaccinated mice showed slight but transient bactericidal activity against the immunizing strain (Table VI).

In an attempt to find reasons for the variable results of different experiments shown in Table IV, we have tested the bactericidal activity of blood with different sizes of bacterial inoculum ($6{\cdot}4\times10^6$–$6{\cdot}4\times10^2$ bacteria per ml.), and found no evidence that inoculum size influences the results of such tests. Nor was any difference found in the bactericidal activity of blood from mice bled on the first, second, third and fourth day after vaccination.

These findings suggest that the factor causing early active protection does not depend on enhancement of bactericidal action by circulating leucocytes, though this may sometimes occur both in the vaccinated animal and, to a smaller extent, in animals passively protected by serum from animals during early active protection.

Leucocytes, erythrocytes and haemoglobin levels in blood of vaccinated mice after infection

A group of mice which had been vaccinated 3 days previously with a single injection of pseudomonas vaccine P14F1 and a group of unvaccinated mice were infected intraperitoneally with a lethal, homologous challenge (*Ps. aeruginosa* P14). During the 4 hr of the experiment the infected vaccinated mice remained alert and healthy; the fur of the infected unvaccinated mice became ruffled and the mice became progressively more lethargic and looked ill. Total leucocyte counts were carried out on samples of 3 mice from each group, before infection and again $\frac{1}{2}$, 1, 2 and 4 hr after infective challenge. Counts of leucocytes, erythrocytes and haemoglobin levels were estimated before and 4 hr after infective challenge.

The mean leucocyte and erythrocyte counts and haemoglobin levels of infected vaccinated and unvaccinated mice during the 4 hr period are shown in Table VII.

TABLE VII.—*Mean Total Leucocyte and Erythrocyte Counts per mm³ and Haemoglobin Levels in Vaccinated and Unvaccinated Mice Infected with 10 LD_{100} of* Ps. aeruginosa *P14*

Time after infection when sample taken (hr)	Unvaccinated infected mice			Vaccinated infected mice		
	WBC (10^3 per mm.³)	RBC (10^6 per mm.³)	Hb Haemoglobin (g. per cent)	WBC (10^3 per mm.³)	RBC (10^6 per mm.³)	Hb Haemoglobin (g. per cent)
0	9·4	7·8	13·3	1·3225	8·58	13·6
$\frac{1}{2}$	4·025	—	—	6·175	—	—
1	4·925	—	—	6·45	—	—
2	4·8	—	—	12·45	—	—
4	2·2	8·02	15·6	6·15	7·81	14·1

The mean leucocyte count of infected unvaccinated mice fell from 9400–2200/mm^3 —*i.e.* below the normal range (4000–12,000/mm.^{3}, mean 8000); the mean leucocyte counts of infected, vaccinated mice fluctuated within the normal range. No apparent change in erythrocyte counts or haemoglobin concentration of blood occurred in the same period of time.

DISCUSSION

In a previous paper (Jones, 1971) it was shown that a single injection of a pseudomonas vaccine (P14F1) induced protection of mice against i.p. challenge 3 days later with the homologous strain of *Ps. aeruginosa* P14. The experiments described here show that serum from mice which had been actively protected in this way conferred homologous protection upon unvaccinated mice into which the serum was injected. The factor in the serum which caused this passive protection was not destroyed by heating the serum at 56° for 30 min. to inactivate complement, but it was removed by absorption with the homologous strain of *Ps. aeruginosa* P14; absorption with an unrelated strain of *Ps. aeruginosa* and with a strain of *Proteus mirabilis* did not remove the factor conferring passive protection against *Ps. aeruginosa* strain P14, but a member of another species (*K. aerogenes*) was as effective as the homologous *Ps. aeruginosa* in removing this factor. This was consistent with the observation (to be reported later) that P14F1 vaccine did not give early protection against the whole range of serotypes of *Ps. aeruginosa*, but gave some protection against members of other species of bacteria; similar observations have been made by Fisher, Devlin and Gnabasik, 1969.

The mechanism by which vaccination induces early and passively transferable protection has not yet been clearly defined. Fox and Lowbury (1953) found no bactericidal activity in the agglutinating sera prepared by immunizing rabbits with strains of *Ps. aeruginosa*. The sera from recently vaccinated mice which conferred passive protection were also found to have no bactericidal activity, but they were without agglutinating properties for the immunizing strain; agglutinins did not appear until the 8th day after the injection of vaccine. As the serum was not bactericidal, its protective action must be attributed to an enhancement of the defence mechanism of the recipient animal, probably through an opsonic mechanism; specific opsonic action was shown by Fox and Lowbury (1953) in the serum of rabbits immunized and protected against *Ps. aeruginosa*.

A comparison of the growth of bacteria in whole blood from vaccinated and unvaccinated mice showed a consistently poorer growth of the homologous *Ps. aeruginosa* P14 and also of *K. aerogenes* in the former than in the latter. In one experiment the blood of vaccinated mice showed a strong bactericidal effect; evidence of a transient bactericidal effect was also found in the blood of passively protected mice. The irregularity of these findings has not been explained yet. It seems likely that the main cellular mechanism through which mice achieve early protection following vaccination is by opsonic effects on phagocytic cells in the tissues. There was some evidence that a leucopenia was induced following infection in the unvaccinated but not in the vaccinated mice.

Early responses to antigenic stimulus have been described by Bauer and Stantsky (1961) and by Uhr and Finkelstein (1963), and are shown to be associated with increases in IgM. The IgM molecule has been shown by Robbins, Kenny and Suter (1965) and by Rowley and Turner (1966) to have 300–500 times greater

opsonizing activity than a molecule of IgG, and Gupta and Reid (1961) have reported that IgM was 120 times as active as IgG in sensitizing bacteria to complement-dependent bactericidal action of phagocytes. Opsonic action has been found to be associated with protective action against bacterial challenge (Smith, Barnett, May and Sanford, 1967).

Studies in progress have shown that the protective factor in the serum of recently vaccinated mice was present only in fractions containing molecules similar in size to IgM. Our findings in the early active and passive protection of mice are consistent with a mechanism of this kind rather than with the early non-specific protection against Gram-negative bacilli which can be obtained by injection of endotoxin (Sultzer, 1968). No endotoxin has been found in the P14F1 vaccine (J. Cameron, personal communication). The absorption of the protective factor from the serum of vaccinated mice by the homologous strain of *Ps. aeruginosa* is additional evidence in support of the view that it is an antibody. Further studies on the mechanism by which the protection is brought about are in progress.

REFERENCES

ALEXANDER, J. W., FISHER, M. W., MACMILLAN, B. G. AND ALTEMEIER, W. A.—(1969) *Archs Surg.*, **99**, 249.

BAUER, D. C. AND STANTSKY, A. B.—(1961) *Proc. natn. Acad. Sci., U.S.A.*, **47**, 1667.

CARNEY, S. A. AND JONES, R. J.—(1968) *Br. J. exp. Path.*, **49**, 395.

COLEBROOK, L., LOWBURY, E. J. L. AND HURST, L.—(1960) *J. Hyg., Camb.*, **58**, 357.

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

FISHER, M. W., DEVLIN, H. B. AND GNABASIK, F. J.—(1969) *J. Bact.*, **98**, 835.

FOX, J. E. AND LOWBURY, E. J. L.—(1953) *J. Path. Bact.*, **65**, 533.

GUPTA, J. D. AND REID, C. E.—(1967) *J. Immun.*, **98**, 1093.

JONES, R. J.—(1968) *Br. J. exp. Path.*, **49**, 411.—(1970) *Br. J. exp. Path.*, **51**, 53.—(1971) *Br. J. exp. Path.*, **52**, 100.

MARKLEY, K. AND SMALLMAN, E.—(1968) *J. Bact.*, **96**, 867.

MILES, A. A., MISRA, S. S. AND IRWIN, J. O.—(1938) *J. Hyg. Camb.*, **38**, 732.

ROBBINS, J. B., KENNY, K. AND SUTER, E.—(1965) *J. exp. Med.*, **122**, 385.

ROWLEY, D. AND TURNER, K. J.—(1966) *Nature, Lond.*, **210**, 496.

SMITH, J. W., BARNETT, J. A., MAY, R. D. AND SANFORD, J. P.—(1967) *J. Immun.*, **98**, 336.

SULTZER, B.—(1968) *J. infect. Dis.*, **118**, 340.

UHR, M. AND FINKELSTEIN, M. S.—(1963) *J. exp. Med.*, **117**, 457.