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EARLY PROTECTION BY VACCINES IN BURNS

R. J. JONES

From the MRC Industrial Injuries and Burns Unit, Birmingham Accident Hospital, Bath Row, Birmingham B15 LNA

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SUMMARY.—Groups of mice, inoculated once a week for 3 weeks with vaccines from culture filtrates of *Pseudomonas aeruginosa* and *Proteus mirabilis* and slime from *Klebsiella aerogenes*, were protected against lethal infections with the homologous strains injected intraperitoneally after burning.

A single injection of pseudomonas and proteus vaccines also protected burned and unburned mice against lethal, homologous, i.p. infections as early as 24–48 hr after vaccination. The klebsiella vaccine did not induce early protection. A single injection of a trivalent vaccine, made by combining pseudomonas, proteus and klebsiella vaccines in equal proportions, protected burned mice against lethal *K. aerogenes*, *Ps. aeruginosa* and *Pr. mirabilis* infections.

Mice which were protected against homologous infections had no detectable agglutinins in their sera until three days after the protective response had been demonstrated.

MOST research on vaccines for the protection of patients with burns has been directed against *Ps. aeruginosa* (Feller, 1966; Jones, Jackson and Lowbury, 1966; Markley and Smallman, 1968; Jones, 1968; 1969*a*; and Alexander Fisher, MacMillan and Altemeier, 1969), an organism which has proved difficult to control by antibacterial chemotherapy (Lowbury, Kidson, Lilly, Ayliffe and Jones, 1969; Lowbury and Jackson, 1970). The application of new methods of topical chemoprophylaxis has caused a reduction in the incidence and severity of *Ps. aeruginosa* infections, but there has not been a comparable success in the control of infections by *K. aerogenes* and some other Gram-negative bacilli (Cason, Jackson, Lowbury and Ricketts, 1966). Experiments have shown that strains of *Pr. mirabilis* and *K. aerogenes*, isolated from patients with burns, can be almost as lethal for burned mice as some strains of *Ps. aeruginosa* (Jones, 1970). The persistent risk of *Ps. aeruginosa* infections in burned patients, especially to those with extensive burns, and the selection of potentially pathogenic strains of other species of Gram-negative bacilli by topical application of some chemotherapeutic agents has prompted this investigation into the possibility of using vaccines as an alternative or complementary method of controlling *Ps. aeruginosa*, *Pr. mirabilis* and *K. aerogenes* infections of burns.

In previous studies (Jones, 1968; 1969*a*, *b*) the preparation of vaccines from culture filtrates of *Ps. aeruginosa* was described: these vaccines protected mice against lethal infections by different serotypes of *Ps. aeruginosa*. In studies reported here attempts have been made to prepare similar vaccines from culture filtrates of *Pr. mirabilis* and *K. aerogenes*. In the earlier studies, the protection-inducing properties of the pseudomonas vaccines were assessed in mice which

had been vaccinated by 3 weekly injections before being given a lethal infective challenge. If burned patients are to benefit from vaccination against Gram-negative bacilli it is important that injection of the vaccine, immediately after burning, should have a protective effect during the first 3 or 4 weeks before grafting, when they are especially at risk from infection. In this study the possibility of obtaining early protection against *Ps. aeruginosa*, *Pr. mirabilis* and *K. aerogenes* infections of burned mice has been studied.

MATERIALS AND METHODS

Strains of bacteria.—The strain of *Ps. aeruginosa* (P14) was obtained from the National Collection of Type Cultures and was exceptionally virulent for burned mice (Jones *et al.*, 1966; Jones, 1970). The strain of *Pr. mirabilis* (2332) was isolated from a patient with burns. The strain of *K. aerogenes* (2628) was isolated from a blood culture taken at autopsy of a burned patient who died with a klebsiella septicaemia. Both strains were virulent for burned mice on i.p. injection (Jones, 1970).

Preparation of vaccines.—(1) Pseudomonas vaccine was extracted from a culture filtrate of *Ps. aeruginosa* (P14) by a method described by Carney and Jones (1968). The vaccine, previously referred to as Fl, had $\overline{MW} > 200{,}000$ and contained several different antigenic determinants (Jones, 1969*b*).

(2) A vaccine was prepared from a strain of *Pr. mirabilis* using the same cultural and fractionation methods as were used in preparing the pseudomonas vaccine.

(3) Several vaccines were prepared from *K. aerogenes*. One vaccine was extracted from a culture filtrate of *K. aerogenes* using the same methods as have been described for extracting vaccines from culture filtrates of *Ps. aeruginosa* and *Pr. mirabilis*. During the preparation of this vaccine, a viscous material was found in the continuous flow head of the centrifuge (MSE. HS-18), and it appeared to be loosely attached to the deposited bacterial cells. The viscous material was separated from the bacteria, dialysed for 24 hr against running tap water and freeze-dried (slime vaccine).

Vaccines were also prepared from whole cell suspensions of *K. aerogenes* by killing a standard suspension, containing $3{\cdot}0 \times 10^9$ organisms per ml., as estimated by Brown's Opacity Tube method, with heat, phenol, formaldehyde and alcohol. Heated vaccine: 5·0 ml. of suspension was placed for 3 hr in a water bath containing boiling water. Phenolized vaccine: 0·25 ml. of 40 per cent phenol solution was added to 5·0 ml. of suspension. Formolized vaccine: 0·01 ml. of 40 per cent formalin (neutral) was added to 4·0 ml. of suspension giving a final concentration of formaldehyde of 0·1 per cent. Alcoholized vaccine: 15 ml. of absolute alcohol was added to 5·0 ml. of suspension, the solution was centrifuged and the deposited bacteria were resuspended in 5·0 ml. saline. Vaccines were incubated at 37° for 24 hr, then inoculated into nutrient broth and on to nutrient agar containing horse blood, to test the sterility of the vaccines.

Vaccination of mice with culture filtrate vaccines.—Male, albino, inbred, Schofield mice were used throughout these experiments.

Frequency of dosage and route of inoculation.—Mice were inoculated with vaccines from culture filtrates of *Ps. aeruginosa*, *Pr. mirabilis* and *K. aerogenes* in 2 ways. In one series of experiments mice, weighing 10–12 g. at the beginning of the experiment, were vaccinated once a week for 3 consecutive weeks before challenge. In another series of experiments mice weighing 20 g. were injected once with a dose of vaccine equivalent to a single weekly injection dose as was used in the previous series of experiments. Mice were vaccinated i.p. in all experiments.

Dosage.—Lyophilized culture filtrate vaccine (0·5 mg.) was dissolved in 10 ml. of saline containing 0·5 per cent formaldehyde. The formolized vaccine solution was diluted 1/20 with saline and mice were inoculated with 1·0 ml. of the diluted solution. This dosage was equivalent to 2·5 μg./25 g. mouse or 0·1 mg./kg. mouse, and was the dosage shown by Jones (1968) to be most effective in protecting mice against homologous challenge.

Vaccination of mice with suspensions of bacteria.—Killed suspensions of *K. aerogenes* were inoculated once a week for 3 consecutive weeks into mice weighing 10–12 g. at the beginning of the experiment. Suspensions (0·1 ml.) of *K. aerogenes* (previously described) were inoculated once a week for the first 2 weeks; 0·2 ml. of suspensions was inoculated in the 3rd week.

Vaccination of burned mice.—Burned mice which received single inoculations of vaccines were injected immediately after burning while the mice were still anaesthetized. Dose, route of injection and frequency of dosage as stated above for unburned mice.

Vaccination of burned mice with a trivalent vaccine.—Culture filtrate vaccine (0·5 mg.) from *Ps. aeruginosa*, P14 and from *Pr. mirabilis* and slime from *K. aerogenes*, were each dissolved in a separate 10 ml. of saline containing 0·5 per cent formalin. To 2 ml. of each of the 3 solutions were added 34 ml. saline giving a final concentration of each vaccine of 2·5 μg./ml. A volume of 1·0 ml. of the final dilution (0·3 mg. of trivalent vaccine/kg. mouse) was inoculated immediately after burning.

Burning.—Dorsal surfaces of mice weighing 20 g. were depilated by plucking. After anaesthetizing the mice with ether, 2 brass blocks, which had been heated in boiling water, were applied for 10 sec. to the bare dorsal surface. The burn was whole-skin-thickness and covered approximately 5 per cent of the total body surface (Jones and Lawrence, 1964).

Infection.—Burned and unburned mice were infected i.p. with 1·0 ml. of saline suspensions of bacteria. The suspensions were made from cultures grown for 18 hr at 37° on nutrient agar containing horse blood. The number of bacteria in the suspensions was estimated by using Brown's Opacity Tubes (Wellcome).

The LD_{100} for burned and unburned mice of *Ps. aeruginosa*, *Pr. mirabilis* and *K. aerogenes* was determined in previous experiments (Jones, 1970), but in one series of experiments where groups of burned mice were challenged 1, 2, 3, 7, 10 and 14 days after vaccination, the LD_{100} for all 3 organisms had to be increased day by day; the infecting doses used in this experiment are shown in Table I.

TABLE I.—*LD_{100}* of Gram-negative Bacilli for Burned Mice*

Strain of bacteria	Number of bacteria inoculated i.p. into burned mice on the following days after burning					
	1	2	3	7	10	14
Ps. aeruginosa P14	$1{\cdot}4\times10^{7}$	$1{\cdot}4\times10^{7}$	$1{\cdot}4\times10^{7}$	$1{\cdot}4\times10^{8}$	$1{\cdot}4\times10^{8}$	$2{\cdot}1\times10^{8}$
Pr. mirabilis (2332)	$1{\cdot}4\times10^{8}$	$2{\cdot}1\times10^{8}$	$4{\cdot}2\times10^{8}$	$4{\cdot}2\times10^{8}$	$4{\cdot}2\times10^{8}$	$5{\cdot}6\times10^{8}$
K. aerogenes (2628)	6×10^{8}	$1{\cdot}2\times10^{9}$	$1{\cdot}2\times10^{9}$	$1{\cdot}2\times10^{9}$	$1{\cdot}2\times10^{9}$	$1{\cdot}2\times10^{9}$

* Estimated from Brown's Opacity Tubes.

Mice which were injected with a trivalent vaccine after burning were challenged i.p., 3 days after vaccination, with 1·0 ml. of saline suspension containing: $1{\cdot}4\times10^{7}$ orgs/ml. of *Ps. aeruginosa* P14; $1{\cdot}4\times10^{8}$ orgs/ml. of *Pr. mirabilis* and $6{\cdot}0\times10^{8}$ orgs/ml. of *K. aerogenes*, and also with a challenge in which all 3 strains were included. The combined challenge was made by preparing saline suspensions containing 3 times the number of organisms stated above for each species. Similar volumes of each suspension were mixed together and 1·0 ml. doses of the mixed suspension was used for challenge.

Titration of agglutinins.—Pairs of vaccinated mice were exsanguinated by cardiac puncture on days 1, 2, 3, 7, 10 and 14 after vaccination. Pooled samples of serum from the pairs of mice were stored at −21°, so that all titrations could be carried out on one occasion. All sera from vaccinated burned and unburned mice were first screened for homologous agglutinin content by the slide agglutination method, and sera which showed positive agglutination by the slide method were also titrated for agglutinins by a tube dilution method.

Slide agglutination.—A colony from an 18 hr/agar culture was emulsified in one drop of saline on a glass slide. One drop of homologous serum was added to the emulsified suspension. The serum and suspension were mixed by rocking the slide. Agglutination which occurred within 5–15 sec. of mixing was called 3+ and 2+ respectively. Agglutination which occurred between 15–30 sec. of mixing was +; no agglutination after 30 sec. of mixing was called −ve.

Tube agglutination.—Formolized suspensions of homologous strains, containing $3{\cdot}5\times10^{9}$ organisms/ml., were added in equal volumes to doubling dilutions of serum in $3\times\frac{1}{2}$ in. tubes, using methods of Jones and Lowbury (1963).

RESULTS

Protection against homologous infection in mice vaccinated weekly for 3 weeks before challenge.—Table II shows the value of vaccines, prepared in a similar way from culture filtrates of *Ps. aeruginosa*, *Pr. mirabilis* and *K. aerogenes*, in protecting burned mice against homologous lethal challenge. In this experiment mice were vaccinated once a week for 3 weeks before being burned and challenged with a lethal i.p. infection. None of the mice vaccinated against *Ps. aeruginosa*, P14, and *Pr. mirabilis* infections died after receiving a homologous infective challenge which killed all unvaccinated controls. The *K. aerogenes* culture filtrate vaccine gave mice no significant protection against lethal i.p. challenge; 7/10 of mice vaccinated with *K. aerogenes* vaccine died, compared with 9/10 of the unvaccinated controls.

TABLE II.—*Protection of Mice, Inoculated with Culture Filtrate Vaccines Before Burning, Against Homologous Infection*

	Challenge			
	Organism	Route	Dose	Mortality
Ps. aeruginosa (P14) Fl	*Ps. aeruginosa* P14	i.p.	7×10^6	0/10
Unvaccinated controls	*Ps. aeruginosa* P14	i.p.	7×10^6	10/10
Pr. mirabilis (2332) Fl	*Pr. mirabilis* (2332)	i.p.	7×10^7	0/10
Unvaccinated controls	*Pr. mirabilis* (2332)	i.p.	7×10^7	10/10
K. aerogenes (2628) Fl	*K. aerogenes* (2628)	i.p.	3×10^8	7/10
Unvaccinated controls	*K. aerogenes* (2628)	i.p.	3×10^8	9/10

TABLE III.—*Protective Efficacy of Klebsiella Vaccines Against Homologous* K. Aerogenes (2628) *Infection of Burned Mice*

	Challenge			
Vaccine	Route	Organisms	Dose	Mortality
Heated bacteria	i.p.	*K. aerogenes* (2628)	3×10^8	3/10
Formolized bacteria	,,	,,	,,	2/10
Phenolized bacteria	,,	,,	,,	2/10
Alcoholized bacteria	,,	,,	,,	0/10
Fl (culture filtrate fraction)	,,	,,	,,	7/10
Slime fraction	,,	,,	,,	0/10
Unvaccinated controls	,,	,,	,,	9/10

Following the failure of the klebsiella culture filtrate vaccine to protect mice against homologous challenge, other vaccines, prepared by killing standard suspensions of *K. aerogenes* by heat, formaldehyde, phenol and alcohol and a crude slime fraction, were inoculated into groups of mice once a week for 3 weeks. The protection-inducing properties of the vaccines were then assessed as before by i.p. challenge after burning. Nine out of 10 unvaccinated control mice injected i.p. after burning with *K. aerogenes*, died (Table III). Two groups showed that good protection had been induced; these had been vaccinated with an alcoholized suspension of *K. aerogenes* and with material from the slime fraction. Although few mice died in the groups of mice vaccinated with the heat-killed, phenolized and formolized suspensions of *K. aerogenes*, many of the mice which did not die, looked ill for several days after challenge, indicating that the vaccines were only weakly protective.

Since the slime fraction from *K. aerogenes* culture filtrate not only protected mice against infection but also was as soluble as the pseudomonas and proteus culture filtrate vaccines, it was used in the following experiments to investigate early protective respones of mice after single inoculations of vaccines.

Early protection of unburned mice by vaccines.—Groups of unburned mice were inoculated with single doses (2·5 μg./mouse) of vaccines from culture filtrates of *Ps. aeruginosa* and *Pr. mirabilis*, and from slime of *K. aerogenes*. On days 1, 2, 3, 7, 10 and 14 after vaccination, mice were injected i.p. with LD_{100} of homologous strains to see how soon after vaccination protective immunity developed. Table IV shows the LD_{100} of *Ps. aeruginosa*, *Pr. mirabilis* and *K. aerogenes* in unburned

TABLE IV.—*Mortality of Unburned Mice Infected I.p. With* Ps. aeruginosa (*P*14), Pr. mirabilis (2332) *and* K. aerogenes (2628)

Infecting organism	Deaths in groups of 5 mice Challenge dose 10^9	10^8	10^7	10^6	10^5	10^4
Ps. aeruginosa P14	5	5	5	3	1	0
Pr. mirabilis (2332)	5	5	2	0	0	0
K. aerogenes (2628)	5	5	3	2	0	0

TABLE V.—*Vaccination of Unburned Mice Against Homologous* Ps. aeruginosa (*P*14) Pr. mirabilis (2332) *and* K. aerogenes (2628) *Infections*

Vaccine: Organism from which vaccine was made	Type	Challenge: Organism	Dose	Route	Mortality: Deaths in groups of 5 mice challenged on the following days after vaccination 1	2	3	7	10	14
Ps. aeruginosa (P14)	Fl	*Ps. aeruginosa* (P14)	7×10^7	i.p.	5/5	0/5	0/5	0/5	0/5	0/5
Unvaccinated controls		*Ps. aeruginosa* (P 14)	7×10^7	i.p.	5/5	5/5	5/5	5/5	5/5	5/5
Pr. mirabilis (2332)	Fl	*Pr. mirabilis* (2332)	7×10^8	i.p.	5/5	1/5	0/5	1/5	4/5	5/5
Unvaccinated controls		*Pr. mirabilis* (2332)	7×10^8	i.p.	4/5	5/5	5/5	5/5	5/5	5/5
K. aerogenes (2628)	Slime fraction	*K. aerogenes* (2628)	12×10^8	i.p.	3/5	3/5	4/5	4/5	3/5	4/5
Unvaccinated controls		*K. aerogenes* (2628)	12×10^8	i.p.	4/5	5/5	5/5	5/5	4/5	4/5

mice, which was found by inoculation of groups of 5 mice i.p. with a range of 10-fold dilutions in saline of suspensions of the organisms. The LD_{100} selected for use in the following experiments were: 7×10^8 organisms/ml. for *Ps. aeruginosa*; 7×10^8 organisms/ml. for *Pr. mirabilis* and 12×10^8 organisms/ml. for *K. aerogenes* as estimated from Brown's Opacity Tubes.

The mice inoculated with pseudomonas and proteus vaccines showed evidence of early protective responses to the vaccines (Table V); the klebsiella slime fraction failed to induce an early protective response in the mice.

Mice which received a single inoculation of the pseudomonas vaccine survived a homologous challenge, administered 2 days after vaccination, which killed all

unvaccinated controls. Mice challenged 3, 7, 10 and 14 days after receiving a single injection of pseudomonas vaccine also survived lethal challenges which killed all unvaccinated controls.

Mice, inoculated with a single dose of proteus vaccine, were able to survive LD_{100} challenge, 3 days after vaccination (Table V). The protective response in the group of mice inoculated with the proteus vaccine was found to decline progressively, as shown by the results in mice given LD_{100} challenges 7, 10 and 14 days after vaccination: $\frac{1}{5}$ vaccinated mice, challenged 7 days after vaccination, died; $\frac{4}{5}$ mice died, when challenged 10 days after vaccination and all mice died when challenged 14 days after vaccination.

The protective response of unburned mice to a single inoculation of klebsiella vaccine was poor; although the group of mice challenged 2 days after vaccination seemed marginally protected ($\frac{2}{5}$ surviving a LD_{100} challenge), none of the other groups of mice which were challenged 3, 7, 10 or 14 days after vaccination showed evidence of the development of an effective protective response.

Early protection of burned mice by vaccines.—Groups of burned mice were inoculated with a single dose (2·5 μg./mouse) of vaccine from culture filtrates of *Ps. aeruginosa* and *Pr. mirabilis* and slime from *K. aerogenes*. On days 1, 2, 3, 7, 10 and 14 following burning and vaccination, mice were injected intraperitoneally with LD_{100} of homologous strain (Jones, 1970) to see how soon after vaccination protective immunity developed.

All mice, inoculated after burning with a single injection of pseudomonas vaccine, were protected against homologous LD_{100} challenges given on 1, 2, 3, 7, 10 and 14 days after vaccination (Table VI).

TABLE VI.—*Vaccination of Burned Mice Against Homologous*, Ps. aeruginosa (*P*14) Pr. mirabilis (2332) *and* K. aerogenes (2628) *infections*

Vaccine		Challenge			Mortality: Deaths in groups of 5 mice challenged on the following days after vaccination					
Prepared from	Type	Organism	Dose	Route	1	2	3	7	10	14
Ps. aeruginosa (P14)	Fl	*Ps. aeruginosa* (P14)	Variable†	i.p.	0/5	0/5	0/5	0/5	0/5	0/5
Unvaccinated controls		*Ps. aeruginosa* (P14)	,,	,,	5/5	5/5	5/5	5/5	5/5	5/5
Pr. mirabilis (2332)	Fl	*Pr. mirabilis* (2332)	,,	,,	1/5	0/5	0/5	0/5	0/5	0/5
Unvaccinated controls		*Pr. mirabilis* (2332)	,,	,,	4/5	3/5	5/5	5/5	2/5*	2/5*
K. aerogenes (2628)	Slime material	*K. aerogenes* (2628)	,,	,,	2/5	2/5	1/5	4/5	3/5	3/5
Unvaccinated controls		*K. aerogenes* (2628)	,,	,,	4/5	4/5	4/5	5/5	4/5	5/5

* The 3 mice which did not die were very ill.
† See Methods.

Following inoculation of the proteus vaccine, the burned mice developed early protection against homologous LD_{100} challenges (Table VI); on the 2nd, 3rd and 7th days after vaccinations burned mice resisted lethal challenges which killed all the unvaccinated controls. The infecting dose of *Pr. mirabilis*, inoculated into both vaccinated and unvaccinated mice 10 and 14 days after burning and vaccination, killed only $\frac{2}{5}$ of the unvaccinated controls. The 3 mice in the control groups

which survived the challenges on each occasion became ill and by the end of the experiment (14 days after vaccination) were emaciated. The mice in the vaccinated groups which received the same challenge showed no sign of illness on the 10th and 14th days after vaccination.

The number of deaths found in groups of burned mice challenged 1, 2, 3, 7, 10 and 14 days after vaccination with the homologous *K. aerogenes* strain (Table VI) suggest that the klebsiella vaccine induced a weak protective response in the burned mice. Fewer burned mice died in the groups challenged on the first 3 days after vaccination compared with the number of deaths in groups of mice challenged 7, 10 and 14 days after vaccination, showing that even though the protective response overall was weak, it was more effective directly after burning than 7, 10 and 14 days after burning and vaccination.

The protective response following a single inoculation of the klebsiella slime fraction contrasts with the good protection obtained following a course of immunization with the slime fraction (Table III).

Early protection of burned mice with a trivalent vaccine.—A group of mice which were injected with a trivalent vaccine (equal parts by weight of pseudomonas and proteus culture filtrate vaccine and slime from klebsiella) after burning were given lethal i.p. challenges 3 days after vaccination. The challenges consisted of lethal doses of individual strains of bacteria used in preparing the trivalent vaccine together with a combined challenge, which contained the sum of the organisms of each individual challenge combined into one challenge.

All mice vaccinated after burning with the trivalent vaccine were protected against doses of bacteria which killed most of the unvaccinated controls (Table VII).

TABLE VII.—*Vaccination of Burned Mice Against* Ps. aeruginosa (*P*14) Pr. mirabilis (2332) *and* K. aerogenes (2628) *With a Trivalent Vaccine*

Treatment of burned mice	Deaths in groups of 5 mice challenged 3 days after vaccination with			
	Ps. aeruginosa (P14)	*Pr. mirabilis* (2332)	*K. aerogenes* (2628)	Combined challenge
Vaccinated with Trivalent vaccine	0/5	0/5	0/5	0/5
Not vaccinated	4/5 (1·2)*	5/5 (1·8)	4/5 (1·5)	5/5 <(1·0)

* Mean survival time in days of mice which died.

The lethality of the challenge dose is shown by the short time in which the injected bacteria killed the unvaccinated mice; all mice which died, did so within 2 days of challenge; the combined challenge killed all unvaccinated controls in less than 24 hr.

The trivalent vaccine induced an early protective response in the mice against *K. aerogenes*, a response which the klebsiella vaccine on its own failed to accomplish (Table V).

Agglutinin titres in sera from vaccinated burned and unburned mice.—Pooled serum was obtained from groups of burned and unburned mice 1, 2, 3, 7, 10 and 14 days after inoculation with single doses of *Ps. aeruginosa* and *Pr. mirabilis* culture filtrate vaccines and *K. aerogenes* slime fraction.

Weak agglutination, as shown by the slide agglutination method, was found in

TABLE VIII.—*Homologous Agglutination in Serum from Vaccinated Burned and Unburned Mice, as Shown by Slide Agglutination Technique*

Vaccine	Condition of mice at time of vaccination	Day after vaccination when serum obtained					
		1	2	3	7	10	14
Ps. aeruginosa (P14) Fl	Burned	−	−	−	+++	++	++
	Unburned	−	−	−	+	−	−
Pr. mirabilis (2332) Fl	Burned	−	−	−	−	±	−
	Unburned	−	−	−	+	−	−
K. aerogenes (2628) slime	Burned	−	−	−	−	−	−
	Unburned	−	−	−	+	−	−

+++ agglutination appeared within 5 sec.
++ agglutination appeared within 15 sec.
+ agglutination appeared within 15–30 sec.
± agglutination appeared after 30 sec.
− no agglutination.

undiluted serum of groups of unburned mice vaccinated 7 days previously with *Ps. aeruginosa*, *Pr. mirabilis* and *K. aerogenes* vaccines (Table VIII). Doubling dilutions of these 3 sera showed titres which were all $<1/2$. No agglutinins were found in sera of unburned mice obtained 1, 2, 3, 10 and 14 days after inoculation with any of the 3 vaccines, or in the serum of burned mice on days 1, 2 and 3 in spite of the high degree of protection found in some of the mice at these times.

Sera from burned mice obtained 7, 10 and 14 days after injection of the pseudomonas vaccine rapidly agglutinated the homologous strain of *Ps. aeruginosa* on the slide. Doubling dilutions of these sera gave titres of 1/2, $<1/2$ and $<1/2$ respectively for sera obtained 7, 10 and 14 days after vaccination. Only one other sample of serum obtained from vaccinated burned mice showed a positive slide agglutination reaction. This sample was obtained from mice which had been inoculated 10 days previously with the proteus vaccine and gave a titre of 1/2 by the tube dilution test.

Serum from burned mice injected with the trivalent vaccine, showed no agglutinins to *Ps. aeruginosa*, *Pr. mirabilis* and *K. aerogenes*, 3 days after vaccination when protection against lethal challenge by these organisms was found.

DISCUSSION

The first part of the study showed that vaccines from culture filtrates of *Ps. aeruginosa* and *Pr. mirabilis* and slime from *K. aerogenes* protected burned mice against homologous LD_{100} challenge. In these experiments mice were given weekly injections of the vaccine for 3 weeks before burning and infection. The second part showed that single injections of pseudomonas and proteus vaccines also induced early protective responses in both burned and unburned mice; the klebsiella vaccine failed to induce an early protective response in either burned or unburned mice. A single injection of a trivalent vaccine, made by combining pseudomonas, proteus and klebsiella vaccines in equal proportions, induced early protection in burned mice against *K. aerogenes* as well as *Ps. aeruginosa* and *Pr. mirabilis* challenges. In these experiments the lethal challenges were inoculated 3 days after injection of the trivalent vaccine either as an individual challenge or as a combined *Ps. aeruginosa*, *Pr. mirabilis* and *K. aerogenes* challenge.

Early protection by pseudomonas, proteus and trivalent vaccines was initiated in mice by a single injection of vaccine and was highly effective even when the vaccine was administered after burning. With the pseudomonas and proteus vaccines all but one burned mouse survived homologous LD_{100} challenges administered i.p. 24 hr after vaccination. With the pseudomonas vaccine subsequent LD_{100} challenges of mice in burned and unburned groups given 3, 7, 10 and 14 days after vaccination showed that this high level of resistance to lethal injections was maintained by the vaccinated mice for the duration of the experiment (14 days). The proteus vaccine induced a similar high degree of early protection, maintained for 14 days, against homologous LD_{100} challenge in burned mice, but the same dose of vaccine in unburned mice gave complete protection to the mice for only 7 days after vaccination, and thereafter the protective response gradually disappeared from the vaccinated mice and was undetectable in mice given lethal challenges 14 days after vaccination.

Early protection, shown in the experiments, occurred in the absence of detectable, homologous, circulating agglutinins; agglutinins did not appear in the serum of vaccinated mice until 3–4 days after an effective protective response had been demonstrated. In unburned mice given single injections of pseudomonas and proteus vaccines, agglutinins appeared in low titre on one occasion only (7 days after vaccination) and were not found in the serum of mice 1, 2, 3, 10 and 14 days after vaccination. However mice injected with the pseudomonas vaccine were immune to lethal challenges 2, 3, 10 and 14 days after vaccination, occasions when no agglutinins were found in the serum. Burned mice injected with pseudomonas and proteus vaccines were also completely protected against lethal homologous infections at times when no agglutinins were detectable in the serum. The trivalent vaccine induced no agglutinins against any of the 3 species of bacteria, *Ps. aeruginosa*, *Pr. mirabilis* and *K. aerogenes*, from which it was made. These experiments show that agglutinin titres would not make a reliable index of early protection in vaccinated mice, and suggest that agglutinating antibodies play little part in early protective responses following vaccination.

It is perhaps surprising that burned mice, which are more susceptible than unburned mice, to bacterial infections (Rosenthal, Millican and Rust, 1957; Millican, Evans and Markley, 1966; Jones, 1970), were able to develop protective responses more quickly than unburned mice. Some light is thrown on this paradox by Millican *et al.* (1966) who showed that burning does not block certain immunological responses of mice to bacterial antigens. Their work, however, does not explain why the protective response was more rapid in burned than in unburned mice. The findings of these studies suggest that burning makes mice more responsive to bacterial vaccines and enables vaccinated mice to resist infections more successfully than unburned mice.

It was encouraging to find that the trivalent vaccine induced an early protective response in burned mice against *K. aerogenes*, since the klebsiella vaccine on its own failed to induce protection. From these experiments it was not clear whether the pseudomonas and/or proteus vaccines had a synergistic effect on the klebsiella vaccine or whether the pseudomonas or proteus vaccines induced protection against *K. aerogenes* infections: the specificity of early protective responses to vaccines will be examined in future experiments.

These experimental results provide no evidence of the mechanisms by which early protective responses operate. It is possible that the vaccines halt or reverse

the leucopenia that occurs in some severe infections after burning (Sevitt 1957). The vaccines may act on cellular mechanisms of immunity, enhancing the phagocytic competence of leucocytes, or possibly stimulating leucocytosis, they may induce non-specific factors of resistance (Weinstein, Waitz and Came, 1970), or alternatively they may stimulate production of non-agglutinating antibacterial antibodies. It is possible that the non-appearance of agglutinating antibodies in the serum of mice showing the early protective response holds the key to the understanding of this mechanism of resistance in mice. If agglutinating antibodies had appeared in the serum of vaccinated mice at the same time as the protective response appeared, the protection might have been attributed to an anamnestic or booster reaction. Current investigations of bactericidal, leucocytic and passive protective responses of vaccinated mice suggest that the early protective response can be explained by the presence of a protective factor in the serum of vaccinated mice. An examination of the specificity of the protective factor against a range of Gram-negative bacilli will also be made to show the practical usefulness of vaccination with a limited number of antigens in prophylaxis.

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