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PASSIVE IMMUNISATION AGAINST GRAM-NEGATIVE BACILLI IN BURNS

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SUMMARY.—When *Proteus mirabilis*, *Klebsiella aerogenes*, *Escherichia coli*, a virulent (P14) and an avirulent (P2AB) strain of *Pseudomonas aeruginosa* were inoculated on to the burned surfaces of mice, only *P. mirabilis* and *Ps. aeruginosa* (P14) invaded the burns and caused septicaemia and death.

When burned mice were inoculated i.p. with the same Gram-negative bacilli, *K. aerogenes* was found to be as pathogenic as *Pr. mirabilis; Ps. aeruginosa* (P14) was the most lethal organism and *Esch. coli* and *Ps. aeruginosa* (P2AB) only caused death when 7×10^9 organisms were inoculated.

Antisera, against the infecting strains, given to burned mice before infection prevented septicaemia and death in mice infected i.p. with *Ps. aeruginosa* (P14) and *Pr. mirabilis* but was less effective in protecting burned mice infected i.p. with *Esch. coli* and *K. aerogenes*.

In burned patients, clinical trials have shown that topical application of silver compounds reduces the incidence and severity of *Pseudomonas aeruginosa* infections (Moyer, Brentano, Gravens, Margraf and Monafo, 1965; Monafo and Moyer, 1965; Cason, Jackson, Lowbury and Ricketts, 1966), but has only a marginal prophylactic effect against other Gram-negative bacilli (Cason and Lowbury, 1968; Butcher, Margraf and Gravens, 1969; Stanford, Rapple and Fox, 1969).

Apart from *Ps. aeruginosa* the commonest Gram-negative bacilli isolated from burns treated with silver compounds are species of *Proteus*, *Klebsiella*, *Enterobacter* and *Escherichia* (Butcher *et al.*, 1969) and the clinical importance of these bacteria is emphasised by the fact that patients infected with these organisms sometimes die from septicaemia (Cason and Lowbury, 1968).

The limitations of silver compounds as therapeutic agents prompted a renewed interest in alternative methods of controlling infections in burns. Experiments in animals and man have shown that one very effective method of controlling *Ps. aeruginosa* invasion in burns is by specific antipseudomonas antibodies (Feller, 1966; Jones, Jackson and Lowbury, 1966; Markley and Smallman, 1968; Jones, 1968, 1969).

We report here a study on the value of specific antibodies prepared against *Proteus mirabilis*, *Klebsiella aerogenes* and *Escherichia coli* in protecting burned mice infected with these organisms at the time of burning. In a previous study (Jones *et al.*, 1966) it was found that the mortality of burned mice infected with strains of *Esch. coli* and *K. aerogenes* (7×10^8 organisms) was not significantly greater than that of the uninfected controls. We therefore studied effects of larger doses of bacteria and of i.p. infection of burned mice to see if *Esch. coli* and *K. aerogenes* were pathogenic by these methods of infection.

MATERIALS AND METHODS

Strains of bacteria.—The strains of *Pr. mirabilis* and *Esch. coli* were isolated from patients with burns. The strain of *K. aerogenes* was isolated from a blood culture at autopsy of a patient who died with a klebsiella septicaemia. The strains of *Ps. aeruginosa*—P14 and P2AB have been shown by previous experiments, to be virulent (P14) and avirulent for burned mice (Carney and Jones, 1968).

Suspensions used for infecting burned mice were prepared from overnight subcultures on nutrient agar. Loopfuls of organisms in 20 ml. saline were shaken for 5–10 sec. at full power on a shaker. The numbers of organisms in the suspensions were estimated using Brown's Opacity Tubes.

Infection of burned mice.—Mice were burned by applying brass blocks, heated in boiling water, to depilated dorsal surfaces of anaesthetised mice, using methods described by Jones and Lawrence (1964). The area burned was about 5 per cent of the body surface of a 25 g. mouse. The burns of mice were infected by spreading 0·1 ml. of a suspension of organisms in physiological saline over the surface of the burn 3 hr after burning (Jones *et al.*, 1966). In some experiments burned mice were infected i.p., by inoculating 1·0 ml. of a saline suspension of organisms into the peritoneal cavity 3–4 hr after burning.

Preparation of specific antisera.—Antiserum against *Pr. mirabilis*, *Esch. coli* and *K. aerogenes* was prepared by immunising rabbits by increasing i.v. dosage, using formolised suspensions and the immunisation schedule described by Jones and Lowbury (1963). Antiserum against *Ps. aeruginosa* (P14) was prepared against a culture filtrate fraction from the organism (Jones, 1969).

The rabbits in which the antisera were prepared were exsanguinated when the homologous agglutinin titres of the sera were between 1/2000–1/4000. The serum was filtered through a GS Millipore membrane and stored deep frozen. Before inoculation into mice the serum was again filtered through a sterile GS Millipore membrane.

RESULTS

Virulence of bacteria.—The mortality of mice in which burns received inocula ranging from 7×10^4 to 7×10^9 cells of *Pr. mirabilis*, *K. aerogenes* and *Esch. coli* is shown in Table I. These results are compared, in the same table, with the mortality of mice in which burns were infected with an invasive (P14) and a non-invasive (P2AB) strain of *Ps. aeruginosa*.

TABLE I.—*Mortality of Burned Mice Infected by Inoculation of Burns With Different Species of Gram-negative Bacilli*

Infecting organism	Numbers of deaths in groups of 5 mice occurring within 7 days of challenge dose in range						Mortality (per cent) for all challenge doses
	10^{9}*	10^{8}	10^{7}	10^{6}	10^{5}	10^{4}	
Ps. aeruginosa (P14)	5 (2·5)†	5 (3·4)	4 (4·2)	1 (2·0)	2 (3·5)	1 (4·0)	60·0
Pr. mirabilis	2 (1·5)	3 (5·0)	1 (2·0)	2 (2·5)	1 (4·0)	0	33·3
K. aerogenes	0	0	1 (2·0)	0	0	0	3·3
Esch. coli	1 (2·0)	0	1 (5·0)	0	0	0	6·6
Ps. aeruginosa (P2AB)	0	0	0	0	0	0	0

* For the estimated numbers of bacteria, these figures should be multiplied by 6 for *K. aerogenes*, 7 for *Ps. aeruginosa* and *Pr. mirabilis* and 7·5 for *Esch. coli*.

† Mean survival time of mice which died.

Only the strain of *Pr. mirabilis* and the invasive strain of *Ps. aeruginosa* (P14) were lethal for burned mice, and only when more than 10^7 organisms were spread over the surface of the burns. The strains of *K. aerogenes*, *Esch. coli* and the non-

invasive *Ps. aeruginosa* (P2AB) caused very few deaths, even when the burns were infected with inocula containing 10^9 organisms.

In contrast with these results, the mortality was 100 per cent in burned mice infected i.p. with 10^9 cells of *Pr. mirabilis*, *Esch. coli*, *K. aerogenes* and *Ps. aeruginosa* strains P14 and P2AB (Table II). When all mice in a single group died, the deaths usually occurred during the 24 hr following challenge, except in the group of mice infected with *Esch. coli*, which had a mean survival time of 1·8 days.

TABLE II.—*Mortality of Burned Mice Infected i.p. With Different Species of Gram-negative Bacilli*

Infecting organism	Number of deaths in groups of 5 mice occurring within 7 days of challenge dose								Mortality (per cent) for all challenge doses
	10^{9}*	10^{8}	10^{7}	10^{6}	10^{5}	10^{4}	10^{3}	10^{2}	
Ps. aeruginosa (P14)	5 (1·0)†	5 (1·0)	4 (2·0)	4 (1·5)	3 (2·0)	3 (2·6)	3 (2·6)	4 (3·0)	77·5
Pr. mirabilis	5 (1·0)	5 (1·0)	5 (1·2)	2 (2·0)	2 (2·0)	1 (3·0)	1 (3·0)	0	52·5
K. aerogenes	5 (1·4)	5 (1·0)	3 (2·0)	2 (2·0)	0	1 (2·0)	3 (3·6)	0	47·5
Esch. coli	5 (1·8)	3 (2·0)	0	0	0	0	1 (1·0)	0	22·5
Ps. aeruginosa (P2AB)	5 (1·0)	3 (2·0)	0	0	0	0	0	0	20·0

* For estimated numbers of bacteria, these figures should be multiplied by 6 for *K. aerogenes*, 7 for *Ps. aeruginosa* and *Pr. mirabilis* and 7·5 for *Esch. coli*.

† Mean survival time of mice which died.

The mean survival time of mice infected on the surface of the burn with 7×10^8 or 7×10^9 cells of *Ps. aeruginosa* (strain P14) was 3 times as long as that of burned mice challenged i.p. with the same numbers of organisms.

Protection of burned mice by antisera.—In the next experiment the protective value of antiserum was assessed in burned mice infected by local application of bacteria to the burns and by i.p. injection; the relative value of antiserum against infection by each of these routes was compared. In Table III the mice represented in the upper half of the table were infected i.p. after burning, while the mice in the lower half of the Table received surface infection. Both groups of mice were given

TABLE III.—*Protective Efficacy of Anti-pseudomonas Serum in Groups of Burned Mice Infected i.p. or on the Burns With Lethal Challenges of* Ps. aeruginosa *(P14)*

Antiserum		Infection			Mortality No. of mice which	
Volume given (ml.)	Type	Challenge organism	Route of challenge	No. of organisms	Died	Survived
0·5	Anti-pseudomonas	P14	i.p.	7×10^6	1	4
0·05	Anti-pseudomonas	P14	i.p.	7×10^6	0	5
0·005	Anti-pseudomonas	P14	i.p.	7×10^6	0	5
0·5	Unimmunised rabbit	P14	i.p.	7×10^6	5	0
Unprotected	Controls	P14	i.p.	7×10^6	5	0
0·5	Anti-pseudomonas	P14	Surface	7×10^8	0	5
0·05	Anti-pseudomonas	P14	Surface	7×10^8	0	5
0·005	Anti-pseudomonas	P14	Surface	7×10^8	0	5
0·5	Unimmunised rabbit	P14	Surface	7×10^8	3	2
Unprotected	Controls	P14	Surface	7×10^8	4	1

the same volume of serum at the time of burning, and infected with the same strain of bacteria, the highly virulent *Ps. aeruginosa* (strain P14).

Compared with unimmunised mice and mice inoculated with serum from an unimmunised rabbit, the mice passively immunised with the anti-pseudomonas serum were protected against septicaemia and death whether they were infected on the surface of the burn or by the i.p. route.

Since the route of infection apparently made little difference to the assessment of protection against *Ps. aeruginosa* by antiserum, it was considered that protection against the other species of Gram-negative bacilli could be satisfactory assessed by experiments on mice challenged i.p.

TABLE IV.—*Passive Protection With Homologous Antiserum of Burned Mice Challenged i.p. With* P. mirabilis, K. aerogenes *and* Esch. coli

Antiserum		Infection			Mortality / No. of mice which
Volume given (ml.)	Type	Challenge organisms	Route of challenge	No. of organisms	Died / Survived
0·5	Anti-proteus	*Pr. mirabilis*	i.p.	7×10^7	0 / 5
0·05	Anti-proteus	*Pr. mirabilis*	i.p.	7×10^7	0 / 5
0·005	Anti-proteus	*Pr. mirabilis*	i.p.	7×10^7	0 / 5
0·5	Unimmunised rabbit	*Pr. mirabilis*	i.p.	7×10^7	1 / 4
Unprotected	Controls	*Pr. mirabilis*	i.p.	7×10^7	4 / 1
0·05	Anti-klebsiella	*K. aerogenes*	i.p.	7×10^8	2 / 3
0·05	Anti-klebsiella	*K. aerogenes*	i.p.	7×10^8	3 / 2
0·005	Anti-klebsiella	*K. aerogenes*	i.p.	7×10^8	4 / 1
0·5	Unimmunised rabbit	*K. aerogenes*	i.p.	7×10^8	4 / 1
Unprotected	Controls	*K. aerogenes*	i.p.	7×10^8	5 / 0
0·5	Anti-escherichia	*Esch. coli*	i.p.	7×10^8	2 / 3
0·05	Anti-escherichia	*Esch. coli*	i.p.	7×10^8	3 / 2
0·005	Anti-escherichia	*Esch. coli*	i.p.	7×10^8	5 / 0
0·5	Unimmunised rabbit	*Esch. coli*	i.p.	7×10^8	3 / 2
Unprotected	Controls	*Esch. coli*	i.p.	7×10^8	5 / 0

In Table IV, one injection of antiserum at 3 different volumes (0·5, 0·05 and 0·005 ml.) was given to groups of 5 mice at the time of burning; 4–5 hr later the burned mice were infected i.p. with Gram-negative bacilli, previously found to be lethal (Table II). In each series, the antiserum used was one that had been prepared against the infecting strain. A group of unimmunised burned mice and a group given serum (0·5 ml.) from an unimmunised rabbit were included as controls for each of the infecting strains.

At all 3 immunising dosages, the antiserum against *Pr. mirabilis* was effective in preventing death after i.p. infection. 0·5 ml. of the anti-klebsiella serum reduced the mortality from *K. aerogenes* to about 40 per cent, but the smallest volume of antiserum (0·005 ml.) had no protective effect. In the groups of mice infected with *Esch. coli*, the largest volume (0·5 ml.) of homologous antiserum reduced the mortality to half of the unimmunised controls, but the smallest dose (0·005 ml.) of antiserum appeared to give less protection than the serum from an unimmunised rabbit.

DISCUSSION

The inoculation of suspensions of *Pr. mirabilis*, *K. aerogenes*, *Esch. coli* and *Ps. aeruginosa* (strains P14 and P2AB) on to the burned surfaces of mice confirmed the results of earlier studies (Jones *et al.*, 1966) in which only *Pr. mirabilis* and certain

strains of *Ps. aeruginosa* caused invasive infection of burns, with septicaemia and death. *K. aerogenes*, *Esch. coli* and an avirulent strain of *Ps. aeruginosa* (P2AB) did not invade or cause death even when very large numbers of bacteria were applied to the burn. Some strains of Gram-negative bacilli (*K. aerogenes*) which did not cause invasive infection from the burns of mice cause septicaemia and death in burned patients (Cason and Lowbury, 1968). The strains of *K. aerogenes* causing these infections may have been exceptionally virulent, or the patient may have been exceptionally deficient in cellular or humoral resistance, or possibly infection occurred by routes other than through the intact burn. In the experiments described above the strain of *K. aerogenes* was isolated from the blood of a burned patient at autopsy and therefore can be assumed to be of a virulent type. The animals were infected immediately after burning, when they are most susceptible to invasion (Millican, Rust and Rosenthal, 1957; Millican, Rosenthal, Rust and Jansky, 1963) and in a state of low resistance (Verder and Rosenthal, 1961; Jones and Lowbury, 1965). In these circumstances the organism was given optimum conditions to invade the burned mouse, but it did not; only when it was introduced i.p. into burned mice did the strain of *K. aerogenes* cause septicaemia and death. For the present it is only possible to speculate how klebsiella septicaemias originate in human burned subjects; perhaps the organism is able to multiply and invade, having gained access after operation or through intermediate colonisation of another organ.

In protection experiments in mice challenged by the i.p. route, it was found that passive immunisation with antisera against the infecting strains was more effective against the strains of bacteria (*Ps. aeruginosa* P14 and *Pr. mirabilis*) capable of invading burns than against *Esch. coli*, *K. aerogenes* and *Ps. aeruginosa* (P2AB). In mice inoculated i.p. it was necessary to inoculate larger numbers of the less virulent bacteria, *Esch. coli*, *K. aerogenes* and *Ps. aeruginosa* (P2AB) to kill the same numbers of mice as were killed by the smaller numbers of invasive bacteria, *Ps. aeruginosa* (P14) and *Pr. mirabilis*. Since the agglutinin titres of the immunising antisera against the infecting strains were similar, and since the volumes of antisera given to each group of mice were the same, it is hardly surprising that antisera gave less effective protection against *Esch. coli*, *K. aerogenes* and *Ps. aeruginosa* (P2AB) than against *Ps. aeruginosa* (P14) and *P. mirabilis*, because they were directed against larger numbers of organisms. In this conclusion it is assumed that agglutinin titre and protective antibody content of a serum are the same. The validity of this assumption was borne out by experiments of Jones and Lowbury (1965) who used a similar experimental procedure with *Ps. aeruginosa* as the infecting organism.

These preliminary experiments showed the advantages to be gained by giving antibacterial antibodies to infected burned mice. Against *Ps. aeruginosa* (P14) and *Pr. mirabilis* a single dose of antibacterial serum at the time of burning even at the smallest dosage (0·005 ml.) was enough to prevent death from septicaemia. Against *K. aerogenes* and *Esch. coli* the smallest volumes of antisera were not so effective in preventing septicaemic death, but the larger volumes (0·5 ml.) of antisera reduced the mortality by over one half. It is possible that larger or more frequent injections of antiserum may be required to achieve adequate protection against *K. aerogenes* and *Esch. coli*.

Passive immunisation by antiserum causes the risk of inducing allergic reactions and, occasionally anaphylactic shock; the benefits of antibacterial resistance might

be seriously offset by this hazard. Improvements in protection of burns against contamination, which have led to a large reduction in early infections, have made it seem less urgent to protect the patient by specific antisera, at this stage. But the time between burning and the delayed onset of infection, which is common today, might be used to immunise patients actively against the pathogenic bacteria they are likely to encounter in a burns ward. Studies are in progress to determine the most effective vaccines or immunogens for this purpose.

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