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Detoxification of an immunogenic fraction from a culture filtrate of *Pseudomonas aeruginosa*

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INTRODUCTION

In a recent communication (Jones, 1968) it was shown that burned mice infected with virulent strains of *Ps. aeruginosa* were protected from septicaemia and death by active immunization with fractions separated from culture filtrates of *Ps. aeruginosa*. The mice were immunized during a 3-week period before burning and infection. The protective fractions contained molecules of high molecular weight and were separated from 5-day aerated batch cultures of *Ps. aeruginosa* by gel filtration through Sephadex G-200 (Carney & Jones, 1968).

Immunization with the Sephadex G-200 fraction protected mice against the strain from which the immunizing fraction had been made and also against strains of different serotype. It was found that a fraction from an avirulent serotype of *Ps. aeruginosa* protected burned mice against virulent strains of *Ps. aeruginosa* of different serotype (Jones, 1968).

The Sephadex-separated fractions killed mice at dosages 400 times those of the protective immunizing dosages (Carney & Jones, 1968). Their usefulness as potential human immunogens would be greatly enhanced if their lethal and toxic effects could be reduced without loss of protective properties. Further refinement of the Sephadex G-200 fractions by gel filtration through Sepharose-4B was shown by Jones (1968) to produce immunizing fractions that were less lethal.

In this study an attempt was made to detoxify an immunogenic fraction from a culture filtrate of a virulent strain of *Ps. aeruginosa* (strain B4), by treating the fraction with alcohol or formalin solutions.

A Sepharose-4B fraction was further refined by gel filtration through Sepharose-2B; this produced a fraction (*b*) that gave a single precipitation band on immunodiffusion (Carney & Jones, 1968). The lethal and toxic effects of treated and untreated Sepharose-2B fractions were determined by intraperitoneal inoculation of mice, and the protective properties of the fractions were assessed in burned mice using techniques described by Jones (1968).

MATERIALS AND METHODS

Isolation of the immunogenic fraction

The strain B4 from which the immunogen was separated was isolated from a patient with burns and had a phage-typing pattern of 21/68/Col.21/+ and a serotype of 2/5 (B.T. Thom, personal communication).

Ps. aeruginosa (B4) was grown for 5 days in an aerated batch culture containing 20 l. of a synthetic dialysable medium (Carney & Jones, 1968). After growth the bacteria were removed by centrifugation and positive pressure filtration (GS Millipore membrane); the volume of the culture was reduced to 1 l. by removing water at 20° C. in a circulatory cyclone evaporator; the unused medium constituents were removed by dialysis; the volume was further reduced to 100 ml. by ultrafiltration through cellophane and the contents of the sac were freeze-dried. The freeze-dried material was separated into fractions of different molecular size by gel filtration through G-200 Sephadex, Sepharose-4B and Sepharose-2B (Pharmacia Fine Chemicals, Uppsala). A fraction that was partly retained by Sepharose-2B, with a Kav value between 0·28 and 0·79, was used in this study.

Detoxification of pseudomonas immunogens

Alcohol precipitation. Thirty ml. of absolute alcohol containing a few drops of a saturated solution of sodium acetate were added to a solution of 10 mg of the Sepharose-2B fraction in 10 ml. of distilled water. After shaking the solution was left at 4° C. for 18 hr. and the precipitate was deposited by centrifugation. The precipitate was washed twice in absolute alcohol, dried at room temperature and redissolved in saline.

Formolization. Two methods were used.

(*a*) Ten mg. of the Sepharose-2B fraction from strain B4 was dissolved in 10 ml. of isotonic saline containing 0·1 % formalin solution and stored for 7 days at 4° C. before use.

(*b*) Ten mg. of the Sepharose-2B fraction from strain B4 was dissolved in 10 ml. of isotonic saline containing 0·5 % formalin solution. After 7 days storage at 4° C. the formolized fraction was dialysed overnight against saline.

Toxicity tests

The methods described by Carney & Jones (1968) were used.

Protection tests

Groups of mice, weighing 10–12 g. at the beginning of the experiment, were inoculated intraperitoneally with 0·1 mg./kg. of either formolized, alcoholized or untreated B4 Sepharose fraction, once a week for 3 consecutive weeks. Ten days after the last inoculation, mice were depilated and burned in a standard way (Jones & Lawrence, 1964), then challenged with 0·1 ml. of a saline suspension of *Ps. aeruginosa* containing 700×10^6 organisms, spread over the surface of the burn as described by Jones, Jackson & Lowbury (1966). Half of each group of immunized mice was challenged with the same strain of *Ps. aeruginosa* from which the fraction had been prepared (B4), the other half with a different virulent strain of *Ps. aeruginosa* (P14) of serotype 6*c* and phage typing pattern 16/13/24/F8/119X/352/1214/4 obtained from Dr M. T. Parker.

RESULTS

Toxicity of alcoholized, formolized and untreated Sepharose fractions from Pseudomonas aeruginosa *strain B4*

The number of mice which died after receiving a single intraperitoneal inoculation of various concentrations (80, 40, 20 10, 5 and 1·0 mg./kg.) of alcoholized, formolized (0·1 % and 0·5 %) and untreated Sepharose-2B fraction from strain B4, is shown in Table 1, together with LD50 of the four preparations, which were calculated from dose–response curves constructed from the percentage mortalities and dosages shown in Table 1.

Table 1. *Toxicity for mice of pseudomonas immunogens treated with alcohol and formalin solutions*

Treatment of immunogen	Dose of immunogen (mg./kg.)*						LD 50 of immunogen
	80	40	20	10	5	1	
Alcohol	3/3†	3/4	3/5	0/5	0/5	0/5	18·0
Formalin 0·1%	3/3	0/5	1/5	0/5	0/5	0/5	59·0
Formalin 0·5%	1/3	0/5	0/5	0/5	0/5	0/5	> 80·0
Untreated	3/3	2/4	1/5	0/5	0/5	0/5	40·0

* Given as a single intraperitoneal inoculation in 1·0 ml of saline.
† Number of mice dying/number inoculated.

Treatment of the Sepharose-2B fraction with 0·1 % and 0·5 % formalin solution was found to reduce its lethal effect for mice; the LD50 for the 0·1 % formolized fraction was 59·0 mg/kg, the LD50 for the 0·5 % formolized fraction was > 80 mg./kg. compared with an LD50 of 40 mg./kg. of the untreated fraction. Treatment of the Sepharose-2B fraction with alcohol lowered the LD50 of the fraction from 40 mg./kg. to 18 mg./kg., thereby increasing the lethal effect of the fraction more than twofold.

Even though few mice died in the groups of mice challenged with the 0·1 % formolized fraction, these mice, together with those challenged with the alcoholized and untreated fraction, showed external signs of illness—immobility, refusal of food and water, ruffled appearance and exudate in eyes (Plate 1)—at dosages of 10 mg./kg. and above. The external symptoms became worse during the 24 hr. period after challenge, reaching a maximum between 24 and 30 hr. after challenge. The mice which survived for 2 days returned to a normal rate of growth, reflected by a steady increase in body weight (Fig. 1), by the 3rd–5th day after a 40 mg./kg. challenge and by the 2nd day after a 1 mg./kg. challenge.

The groups of mice challenged with 40 mg./kg. of 0·5 % formolized fraction showed only slight external symptoms of illness 24 hr. after challenge and no signs of illness 48 hr. after challenge. Mice challenged with 1·0 mg./kg. of 0·5 % formolized fraction showed no external signs of illness. However, the body weights of mice challenged with both 40 mg./kg. and 1·0 mg./kg. of 0·5 % formolized fraction fluctuated in a similar way to the weights of mice receiving comparable dosages of

other preparations of Sepharose-2B fractions (Fig. 1), showing that even though the external symptoms resulting from the challenge had been reduced by treatment with 0·5 % formalin, the fraction still affected the mice systemically.

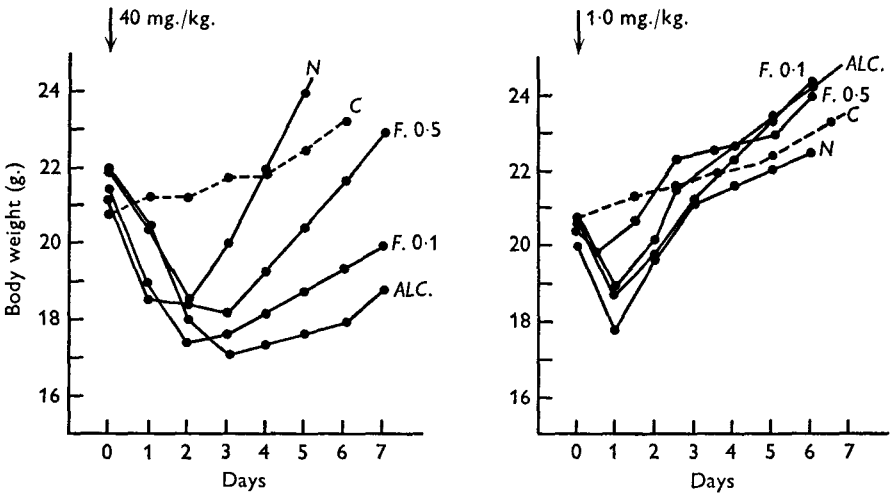

Fig. 1. Comparison of mean weights of groups of mice after intraperitoneal inoculation with fractions from a pseudomonas culture filtrate that have been treated with alcohol or formaldehyde. *C*, Uninoculated mice; *N*, mice inoculated with untreated fraction; *F* 0·5, mice inoculated with fraction treated with 0·5 % formalin solution; *F* 0·1, mice inoculated with fraction treated with 0·1 % formalin solution; *ALC*, mice inoculated with fraction precipitated with alcohol.

Protection against Pseudomonas aeruginosa *infection with pseudomonas immunogens*

Table 2 shows how effective four immunizing fractions, prepared from the Sepharose-2B fraction from strain B4, were in protecting burned mice from pseudomonas septicamia. Different groups of mice were immunized weekly for 3 weeks with formalin-treated (0·1 % or 0·5 %), alcohol-treated or untreated Sepharose-2B fraction. After immunization the mice were given whole-skin-thickness burns of approximately 5 % of body surface and challenged by infecting the surface of the burn with virulent strains of *Ps. aeruginosa*, B4 or P14. All mice given untreated or formalin-treated (0·1 % or 0·5 %) immunogen survived. The same challenge was found to kill 80 % of unimmunized burned control mice (Table 2). In the same experiment it was found that the alcohol-precipitated fraction was a less effective immunogen than the untreated or formolized fractions, as 20 % of the mice immunized with the alcohol-precipitated fraction then burned and challenged with strains P14 and B4 died. The mean survival times (Table 2) of the mice which were immunized with the alcohol-precipitated fraction indicates that the mice which died received some protection against pseudomonas invasion, since they survived 2–3 days longer than unimmunized mice which were burned and infected in a similar way.

Table 3 summarizes an experiment in which an attempt was made to find out

Table 2. *Protective efficacy of pseudomonas immunogens treated with alcohol and formalin*

Treatment of immunogen	Immunizing dose* (mg./kg.)	Infecting strain	Mouse mortality	Mean survival time days	No. of mice showing *Pseudomonas* in heart blood
Alcohol	0·1	B 4	2/9†	5·0	2
		P 14	2/10	6·0	2
Formalin 0·1 %	0·1	B 4	0/10	—	—
		P 14	0/10	—	—
Formalin 0·5 %	0·1	B 4	0/9	—	—
		P 14	0/10	—	—
Untreated fraction	0·1	B 4	0/9	—	—
		P 14	0/10	—	—
Unimmunized controls	None	B 4	8/10	3·7	8
		P 14	8/10	2·7	8

* Given once a week for three consecutive weeks before burning and infection.
† Number of mice dying/number treated.

Table 3. *Determination of the minimum protective dose of pseudomonas immunogen treated with* 0·5 % *formalin solution*

(The mice were all infected, after burning, with *Pseudomonas aeruginosa*, strain B 4)

Treatment of immunogen	Immunizing dose (mg./kg.)	Mouse mortality	Mean survival time days	No. of mice showing *Pseudomonas* in heart blood
None	0·1	1/10*	(5·0)	1
	0·01	1/10	(6·0)	1
	0·001	4/9	4·3	4
Formalin 0·5 %	0·1	0/10	—	—
	0·01	3/10	7·0	3
	0·001	1/10	(9·0)	1
Control	None	7/10	4·2	7

* Number of mice dying/number treated.

whether treatment of the protective Sepharose-2B fraction with 0·5 % formalin had altered its immunizing properties. Groups of mice were immunized with different dilutions (0·1, 0·01 and 0·001 mg./kg.) of 0·5 % formolized fraction and the protective properties of these fractions were compared with the protective properties of similar dilutions of untreated fractions, by challenging the immunized mice, after burning, with a suspension of *Ps. aeruginosa* (B 4) that killed 70 % of the unimmunized burned control mice.

The dose of untreated fraction that effectively protected 50 % (EPD 50) of the mice against pseudomonas septicaemia was approximately 0·001 mg./kg. mouse; the EPD 50 of the 0·5 % formolized fraction was less than 0·001 mg./kg. mouse.

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DISCUSSION

These experiments have shown that treatment with 0·5% formalin of a protective fraction from a culture filtrate of *Ps. aeruginosa* reduced both its lethal and systemic toxic effects and seemed to improve its protective properties for burned mice infected with virulent strains of *Ps. aeruginosa*. Other treatments—0·1% formalin solution and alcohol precipitation—were less effective than 0·5% formalin solution in reducing the lethal effects in mice.

Alcohol precipitation increased the lethal effects of the fraction for mice (LD 50 18 mg./kg.) and treatment with 0·1% formalin solution had only a small detoxifying effect on the fraction, raising the LD 50 from 40 mg./kg. to 59 mg./kg. After treating the fraction with 0·5% formalin solution only one mouse out of three was killed by the largest dose (80 mg./kg.) used in these experiments, and even though only small numbers of mice were used, the toxicity tests show that treatment with 0·5% formalin solution reduces the lethality by at least 50% compared with the untreated fraction.

Apart from reducing the lethal effects of the fraction, treatment with 0·5% formalin solution also reduced the duration of some of the systemic toxic side-effects of the fraction (exudates in eyes, ruffled appearances and lethargy).

In mouse protection experiments the lethal and toxic effects of the fractions were of little practical importance as the smallest protective doses used were well below (over 80,000 times) the lethal or systemic toxic doses. Mice immunized with 0·1 mg./kg. of untreated 0·1% and 0·5% formolized fractions given weekly for 3 weeks, received better protection against death from pseudomonas septicaemia than mice immunized with a similar dosage of alcoholized fraction. At lower immunizing doses (0·001 mg./kg.) the fraction treated with 0·5% formalin solution gave better protection against the strain from which it was prepared than the untreated fraction, and protected 9/10 mice from pseudomonas septicaemia compared with 5/9 mice that were immunized with the untreated fraction.

These preliminary experiments are encouraging since they suggest that simple chemical procedures may reduce the toxicity hazard of immunogens from culture filtrates of *Ps. aeruginosa* without impairing their immunizing properties, and this should considerably improve their usefulness as potential human immunogens.

SUMMARY

Mice immunized with a fraction from a culture filtrate of *Ps. aeruginosa*, then burned and infected with virulent serotypes of *Ps. aeroginosa*, were protected from septicaemia and death.

The immunogenic fraction killed mice at dosages 400 times those of the minimum protective dosages.

Treatment of the immunogenic fraction with 0·5% formalin reduced its lethal effect for mice and slightly improved its immunizing potency so that the difference between the smallest immunizing dose used and the smallest amount that killed mice was more than 80,000 doses.

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EXPLANATION OF PLATE

Appearance of mouse 2 days after intraperitoneal inoculation of 40 mg./kg. of a fraction separated by Sepharose-2B from a culture filtrate of *Pseudomonas aeruginosa* B4.