Immunoblot analysis of serological response to *Pseudomonas aeruginosa* septicaemia in man

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SUMMARY The occurrence of an outbreak of *Pseudomonas aeruginosa* septicaemias on an oncology ward permitted an analysis of antibody responses in patients who were all orally exposed to the same source of infection. Seven patients became septicaemic. Serial serum samples were immunoblotted against the homologous strain. Responses were compared with those of 16 other patients with septicaemias caused by other strains and 10 healthy controls. All 18 survivors produced increasing IgG or IgA antibody, or both, against a 35 000 dalton band, whereas these antibodies were usually absent or fell in titre in the five fatal cases. These antibodies were also lacking in sera taken just before a patient became septicaemic. This band had the electrophoretic characteristics of the outer membrane porin protein F.

During the past three decades *Pseudomonas aeruginosa* has emerged from relative obscurity to become a major opportunistic pathogen.¹ Other Gram negative bacilli have also increased in prevalence, but the number of fatalities due to *Paeruginosa* is usually the highest.²⁻³ Some variation in mortality rates probably reflects the geographical distribution of different strains as there is considerable diversity in their degree of virulence.⁴ In some centres mortality is as high as 50% in leukaemic patients.⁵

Host defence depends on specific antibody and polymorphonuclear phagocytes.⁶ This had led to great interest in the potential of immunotherapy and prophylaxis.^{4,5,7,8} Most vaccine preparations have been based on the lipopolysaccharide (LPS) component of *Paeruginosa*. Protection is serotype specific.⁹ These have had some success in burns units,^{10,11} but clinical trials among patients with cancer^{5,12-14} and those with cystic fibrosis¹³ gave equivocal results. The high incidence of side effects due to the endotoxic properties of the LPS molecule militates against the use of such vaccines in debilitated patients.^{5,12-14}

Other components of this micro-organism might form safer more effective vaccines in cancer patients. Examination of patients' serological responses to *Pseudomonas* could help to identify potential vaccine candidates. Many factors, however, determine the

outcome of an infection. Apart from host factors, such as the degree of debilitation of the patient, the virulence of *Paeruginosa* also depends on the strain and the route of challenge.^{4,15}

The occurrence of an outbreak of *Paeruginosa* septicaemias on a male oncology ward, caused by contaminated thymol mouthwash,^{16,17} provided an opportunity to compare serological responses occurring in a population who had all been exposed to the same strain via the same route. The antibody responses to many different components of this micro-organism were examined in a single assay system by combining immunoblotting with reflectance densitometry. Previously it has been shown that immunoblotting can be used as a sensitive quantitative solid phase immunoassay, reflectance densitometry being used to determine the intensity of the stained blots.¹⁸

Serial sera from other septicaemic patients infected with strains of *Paeruginosa* not part of the outbreak were also immunoblotted against the outbreak strain to determine whether they produced antibody to the same antigens as patients affected in the outbreak.

Patients and methods

One hundred serum samples were examined from 23 patients with *Paeruginosa* septicaemia, multiple serum samples (two to seven) being collected from each patient. All patients had clinical features of septicaemia corresponding to positive blood cultures.

Table 1 Details of patients affected by outbreak of *Pseudomonas aeruginosa* septicaemias in male oncology ward

Case number	Age (years)	Underlying disorders	Duration of neutropenia (days)		<i>Pseudomonas</i> infection
			<0.5	0.6-2.0 × 10 ⁹ /l	
1	17	Non-Hodgkin's lymphoma	12	3	Septicaemic
2	49	Non-Hodgkin's lymphoma	11	6	
3	47	Acute myeloblastic leukaemia	10	10	
4	21	Acute myeloblastic leukaemia	10	3	
5*	37	Non-Hodgkin's lymphoma	10	2	
6	48	Refractory anaemia with excess blasts	5	1	
7	44	Acute myeloblastic leukaemia	2	9	
8	37	Acute myeloblastic leukaemia	8	0	Infected
9	53	Non-Hodgkin's lymphoma	0	0	
10	43	Hodgkin's disease	0	0	
11	67	Acute myeloblastic leukaemia	2	6	Uninfected
12	48	Acute myeloblastic leukaemia	0	10	
13	54	Chronic lymphocytic leukaemia	0	0	

*Patient died.

Seven of the patients were part of an outbreak on an oncology ward at St Bartholomew's Hospital due to a strain of serotype 01 and phage type 7, 21, 44, 68, F8, 109 119X, 352, 1214, M4.^{16 17} Thymol mouthwash, which had been made up and distributed in communal jugs, was the likely source of this outbreak. Of the 13 patients on the ward, seven developed septicaemia (table 1). Their blood culture isolates were indistinguishable by conventional typing methods from the strain isolated from the thymol mouthwash. The same strain was cultured from the mouth or rectum, or both, of the septicaemic patients and also three patients on the ward who were colonised but not septicaemic. Sera from the infected patients and also from the three uninfected patients on the ward were examined. All the patients were adult males (average age 38 years, range 17 to 49 years) receiving cytotoxic chemotherapy for haematological malignancies. All were exposed to the same strain of *P aeruginosa* by the same route (oral). Septicaemic patients were treated with tobramycin and ticarcillin and clavulanate (timentin). One of them died from the infection.

The results from these patients were compared with those of the other 16 septicaemic adults with positive blood cultures who were not part of the outbreak and who were infected with different strains of *P aeruginosa*. Four of these patients died. Eight had haematological malignancies. Sera from 10 healthy blood donors were also examined.

PREPARATION OF IMMUNOBLOTS

The outbreak strain of *P aeruginosa* was grown aerobically at 37°C overnight on blood agar plates (Oxoid). It was harvested, washed in sterile distilled water, and ruptured in a Mickle cell disintegrator using glass balls for 10 minutes. The extract was spun at 12000 g for 10 minutes and the supernatant then

used, applying 50 µg to each well of a 10% acrylamide gel. A single batch of this preparation was used throughout these experiments. It was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting was performed as previously described.¹⁹ Briefly, transfer to nitrocellulose membranes was achieved in a trans-blotting chamber (BioRad Laboratories, Richmond, California), at 90 V and 350 mA for one and a half hours, using 25 mM Tris, 192 mM glycine in 20% methanol (pH 8.3). Nitrocellulose strips were then incubated overnight at 4°C in 3% bovine serum albumin (BSA) in buffered saline.

Each strip was then incubated for two hours at room temperature with the patient's serum, diluted 1/6 with 3% BSA, 0.05% Tween 20 (BioRad), in buffered saline. After washing in 0.9% saline and 0.05% Tween 20 strips were incubated for one hour at room temperature with alkaline-phosphatase conjugated goat antihuman IgM, IgG, or IgA (Sigma Chemical, St Louis, Missouri). The conjugate was diluted 1/1000 immediately before use in 3% BSA in buffered saline. After washing strips were incubated in a freshly prepared and filtered mixture of equal volumes of naphthol AS-MX phosphate (Sigma; 0.4 mg/ml in distilled water) and fast red TR salt (Sigma; 6 mg/ml in 0.2 M Tris, pH 8.2). Antigen-antibody binding was identified by deposition of red stain. The intensity of each band, measured by reflectance densitometry (Chromoscan 3; Joyce Loebl), was proportional to the amount of antibody present in the serum, as discussed by Towbin and Gordon.¹⁸

Sera from six patients with high titres of antibody to a 35 k band were also immunoblotted using the conditions recommended for protein F,^{20 21} in the SDS-PAGE system described by Lugtenberg *et al.*²²

Table 2 Antibody responses of septicaemic patients against seven pseudomonal antigens

Molecular weight (k)	Antibody titres	Immunoglobulin					
		IgM		IgG		IgA	
		Survived	Died	Survived	Died	Survived	Died
89	Increase	2		3	1	1	
	Constant	1		3	1	2	
	Decrease						1
	Undetectable	15	5	12	3	15	4
42	Increase			4	1	5	
	Constant	3	2	3	2	2	1
	Decrease						1
	Undetectable	15	3	11	2	11	3
35	Increase	7		13	1	14	
	Constant	3		5		2	
	Decrease		1		2		4
	Undetectable	8	4		2	2	1
34	Increase	4		4		7	
	Constant	2	1	4		2	
	Decrease				2		1
	Undetectable	12	4	10	3	9	4
32	Increase	3		4	1	8	
	Constant	2	1	4		1	
	Decrease				1		1
	Undetectable	13	4	10	3	9	4
31	Increase	2		5		8	
	Constant	2	1	2		1	
	Decrease				1		1
	Undetectable	14	4	11	4	9	4
28	Increase	2		5		8	
	Constant	1		2			
	Decrease						1
	Undetectable	15	5	11	5	10	4

Increase and decrease refer to a change in densitometry of > 30% and constant to changes of < 20%.

Using this system the extract was solubilised in 2% SDS-5% 2-mercaptoethanol at 100°C for five minutes and run on a 14% acrylamide gel.

Results

Twenty six antigenic bands (23 to 141 kilodaltons) were distinguishable in this preparation of *Paeruginosa* using the immunoblot technique. The antibody

responses to 19 bands remained constant in at least 22 of the 23 patients during the course of the septicaemias. Only three of these bands (45, 40, and 37 k) were recognised by more than five patients. The densitometry of antibodies binding to the remaining seven bands (89, 42, 35, 34, 32, 31 and 28 k) changed by > 30% during the course of the septicaemias (table 2). All 18 surviving patients produced increasing IgG or IgA antibodies, or both against the 35 k band, and at

Table 3 Densitometry of the antibody response in septicaemic patient in outbreak who survived

Molecular weight (k)	Serum (i)			Serum (ii)			Serum (iii)		
	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
42	96	78	27	95	86	99	93	166	157
35		41	12	32	51	76	50	126	108
34		20		19	29	35	25	96	71
32		10		10	12	26	12	55	39
31		17		2	19	15	17	51	28
28		2			2		6	25	12

Height of densitometry traces in mm for IgM, IgG, and IgA. Sera collected (i) one week before hospital admission; (ii) when the patient became clinically septicaemic; and (iii) during the recovery phase.

Fig 1 IgG and IgA response to *P aeruginosa* in septicaemic patient in outbreak who survived. Sera shown were taken (i) one week before hospital admission, (ii) when the patient became clinically septicaemic, and (iii) during recovery phase.

least eight of the surviving patients produced rising titres to bands of 34, 32, 31 and 28 k. Falling titres to these five bands were not observed during the septicaemic phase of any of the patients who recovered. Rising antibody titres to the 89 and 42 k bands were relatively uncommon (less than five patients). Table 3 and fig 1 show a representative example of the serological response to the homologous strain. This was a septicaemic patient from the outbreak who recovered. Initially there was relatively little (<50 mm) IgM, IgG, and IgA to the 35, 34, 32, 31 and 28 k bands, but titres rose over three-fold after the patient became septicaemic and during recovery. Similar serological responses to these bands occurred in surviving patients who were not part of the outbreak, whose sera were immunoblotted against the outbreak strain of *P aeruginosa*. The patient shown in

fig 1 also had increases in the antibodies to antigens of 36–42 k, but the rise in IgM to these bands was much less pronounced (table 3). In four of the five fatal cases IgG and IgA to bands 35, 34, 32, 31 and 28 k were either undetectable or faded terminally (table 2). The fifth fatal case showed a small rise in IgG to the 35 and 32 k bands with a concomitant disappearance of IgA to these antigens. Fig 2 shows representative immunoblots from the one patient in the outbreak who died. The IgG and IgA titres to bands 35, 34, 32 and 31 k rose little, if at all, when the patient became clinically septicaemic, and these fell over the next four days so that IgG and IgA were greatly reduced (<20 mm) in terminal sera (table 4). In contrast, the IgM titres to the 35, 34, 32 and 31 k bands remained high (>100 mm). This patient also had a high titre of pre-existing IgG

Table 4 Densitometry of antibody response in septicaemic patient in outbreak who died

Molecular weight (k)	Serum (i)			Serum (ii)			Serum (iii)		
	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
42	62	52	15	64	55	2	43	49	1
35	137	18	50	141	24	26	119	17	18
34	149	24	62	152	32	28	131	12	18
32	144	30	53	148	38	31	128	9	17
31	102	23	26	105	28	21	103	14	16
28	34			42			58		

Height of densitometry traces in mm for IgM, IgG, and IgA. Sera collected (i) six months before hospital admission; (ii) when the patient became clinically septicaemic; and (iii) four days later, a day before he died.

Fig 2 IgG and IgA response to *P aeruginosa* in septicaemic patient in outbreak who died. Sera shown were taken (i) six months before hospital admission, (ii) when patient became clinically septicaemic, and (iii) four days later, one day before he died.

Molecular weights (k) are given beside figs 1 and 2.

(148 mm) to the 71 k band but this remained constant at 150 (SD 15) mm

Early sera, taken when positive blood cultures were collected, were available from 14 of the septicaemic patients and provided information on the titres of antibodies at the onset of septicaemia. IgA to the 35 k band was invariably undetectable at this stage and also usually lacking to bands 34, 32, 31 and 28 k (table 5). IgG to the 35 k antigen was detectable in only two patients at this time and in each case was < 50 mm. In contrast, IgG and IgA to the 89 and 42 k bands were often present in these early sera.

Sera were collected from six patients in the outbreak who did not become septicaemic, despite being

exposed to the infected thymol mouthwash. The outbreak strain of *P aeruginosa* was found in the gastrointestinal tracts of three patients, and all three had a notable lack of IgA to bands 35, 34, 32, 31 and 28 k (table 5). Two of the infected patients had IgG to all five of these bands. The three other patients resisted gastrointestinal colonisation and they had IgA, as well as IgG, to the 35 k band and some of the 34, 32, 31 and 28 k bands.

Examination of sera from 10 healthy blood donors showed that IgM to pseudomonal antigens is not uncommon. Four of 10 subjects had IgM to the 35 k band but only one had either IgG (21 mm) or IgA (33 mm) to this band (table 5).

Table 5 Antibody present in early sera from 14 septicaemic patients, non-septicaemic patients in the outbreak and healthy controls

Molecular weight (k)	Septicaemic (early sera)			Non-septicaemic								
				Infected with outbreak strain 3			Not infected with outbreak strain 3			Healthy controls 10		
	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
89	2	8	3		1						5	
42	6	9	9	3	1	2	2			4		5
35	1	2			2		1	3	3	4	1	1
34	1	2	2		2			3	3	3		
32	2	3			2			3	2			
31	1	1			2			2	2		2	
28					2			3	1	1		

When SDS-PAGE conditions were adjusted to those recommended for outer membrane proteins, the 35 k band migrated to the 39 k position, characteristic of protein F.^{20 21} The bands of molecular weights 34, 32, 31 and 28 k also changed their relative mobilities when the conditions of electrophoresis and solubilisation changed. Their apparent molecular weights were comparable with those of the minor outer membrane proteins described by Hancock *et al.*^{20 21}

Discussion

The occurrence of an outbreak of *Paeruginosa* septicaemias on an oncology ward provided the opportunity to compare antibody responses in seven septicaemic patients, three asymptotically infected patients, and three uninfected patients, all of whom were exposed to a common oral source. Hancock *et al* used the immunoblotting technique qualitatively to assess the serological response of patients with cystic fibrosis to *Paeruginosa*.²¹ It could not be used to compare patients' responses quantitatively because of variations in the amount of some proteins present in different strains.²³ Because of the outbreak, it was possible to compare the responses to the homologous strain of 13 patients, multiple sera being assayed in parallel on a single gel. Sera from septicaemic patients infected with strains not associated with the outbreak were also assayed against the outbreak strain to determine whether antibody responses were type specific.

A relatively small number of bands were consistently associated with pronounced changes in antibody titres during the course of the septicaemias. These bands, of 35, 34, 32, 31 and 28 k, had the unusual electrophoretic characteristics of outer membrane proteins.^{20 21} There was surprisingly little evidence of a strong type specific response to LPS, although this could have been partly masked by the fact that after electrophoresis LPS appears as multiple bands over a wide range of molecular weights and not as a discrete component.²⁴ Antibodies to LPS seem to be protective in infections in experimental animals²⁵⁻²⁷; Pollack and Young correlated the development of these antibodies with survival in man.²⁸ In a later paper, however, Pollack *et al* found a significant increase in haemagglutinating titres to type specific LPS in only three of 17 infected patients.²⁹

The 35 k antigen seemed to be the major outer membrane protein, protein F, as its apparent molecular weight changed from 35 k on a 10% acrylamide gel to 39 k on a 14% gel using the conditions recommended for the characterisation of this protein.^{20 21} The 28-34 k bands were probably minor outer membrane proteins. The immunogenicity of outer membrane proteins from *Paeruginosa* has been shown in

mice³⁰ and man^{21 31}; they have the advantage over LPS of being highly conserved between different serotypes.²³ A preparation of protein F has been used as a protective vaccine in mice,³² and monoclonal antibodies to protein F passively immunised mice against *Paeruginosa*.³³

In this analysis all patients surviving the septicaemia produced IgG or IgA responses, or both, to protein F, and in four of the five fatal cases IgG and IgA were undetectable or faded terminally. This was not merely a reflection of the general state of the immune system as the antibody titres to many of the other bands remained constant during the course of the septicaemias. Moreover, in some fatal cases IgG and IgA to protein F fell, whereas IgM remained high and constant (table 4).

Antibodies developing during septicaemia may be a passive or correlative marker of survival rather than a prime determinant of ability to contain spread of infection. Some evidence to suggest that the antibody to protein F is protective is given by the analysis of antibodies present in patients who did not become septicaemic. Patients who became neither infected nor septicaemic had both IgA and IgG to the 35 k band. The three asymptotically infected patients invariably lacked IgA to this band; one of these patients was severely neutropenic (white cell count $<0.5 \times 10^9/l$) but had IgG to protein F, which may have inhibited systemic spread. Early sera from septicaemic patients in the outbreak invariably lacked IgA and usually IgG to protein F. Gastrointestinal infection is an important preliminary step to septicaemia in patients with cancer,³⁴ and it would be valuable to establish whether IgA to protein F can help prevent this.

Only some of the healthy controls had antibodies to the 35 k band, and usually this was of the IgM class, the presence of which was not associated with survival in septicaemic patients. This shows the contribution of other factors in preventing pseudomonas septicaemia, in particular an intact gastrointestinal mucosa and neutrophils.⁶ Table 1 illustrates that survival did not solely depend on the rate of recovery from neutropenia, four of the survivors being no less neutropenic than the patient who died in the outbreak. One of the infected patients who remained aseptically was also severely neutropenic for eight days.

Previously protein F antibodies have been shown by immunoblotting techniques in the serum of patients with cystic fibrosis and chronic pulmonary infection with *Paeruginosa*,²¹ and, using cross-immunoelectrophoresis, in one of four septicaemic patients with acute leukaemia.³¹ It has been postulated that the presence of these antibodies contributes to the rarity of bacteraemia in cystic fibrosis.²¹ This paper reports for the first time an association between

the production of antibody to protein F and survival from *Paeruginosa* septicemia in patients on an oncology ward. Pre-existing IgA and IgG to protein F was associated with absence of infection and septicemia, respectively, in similar neutropenic patients exposed to the same source of infection. This suggests that an alternative to LPS vaccines in such patients might be the outer membrane proteins of *Paeruginosa*, an approach which is already being pursued for *Haemophilus influenzae*³⁵ and *Neisseria gonorrhoeae*.³⁶

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