Immunoblotting and culture positive endocarditis

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Abstract

Serum samples from patients with endocarditis due to *Streptococcus mutans*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Streptococcus lactis* and a "nutritionally" variant streptococcus were immunoblotted against antigenic extracts from all five species. In *S mutans* endocarditis there was an endocarditis specific pattern of IgM against bands of 220, 200, and 190 kilodaltons. In *S pneumoniae* IgM against antibody of a molecular weight greater than 150 kilodaltons was specific to endocarditis. In *S agalactiae* IgM against bands at 82, 71, and 66-67 kilodaltons was endocarditis specific. In *S lactis* endocarditis specific IgM was present against antigenic bands at 105, 66, 61 and 58 kilodaltons. With the "nutritionally" variant streptococcus it was impossible to distinguish between cases of endocarditis and controls.

Immunoblotting has been used to diagnose both culture negative and enterococcal endocarditis.^{1,2} In *Enterococcus faecalis* endocarditis there was a strong IgM response to *E faecalis* antigenic bands of 112, 88-90, and 45-47 kilodaltons and a strong IgG response to 88-90, and 45-47 kilodaltons bands. Recovery from *E faecalis* endocarditis correlated with a decline in the serum IgM against these bands. Similar patterns were determined for *Enterococcus faecium*, *Streptococcus bovis*, and *Streptococcus sanguis*. These patterns were successfully applied to cases of culture negative endocarditis.

Infective endocarditis is a clinical diagnosis which can be supported by the demonstration of vegetation on echocardiograms. Confirmation is made by blood cultures but these may be negative in up to 10% of cases.³ This is thought to be either due to previous antibiotic treatment or to the presence of a fastidious causative organism. It is therefore important to assess whether measuring serum antibody by immunoblotting can be used to diagnose these culture negative cases. To do this it is necessary to establish background data from culture positive cases. This is especially important in the case of nutritionally variant streptococci (NVS) where previous work using enzyme linked immunosorbent assays failed to detect antibodies in 26% of the cases of endocarditis and produced a false positive result in 7% of patients infected with a different micro-organism.⁴

Methods

PREPARATION OF ANTIGEN

Streptococcus mutans (No 263, Culture Collection, Manchester) *Streptococcus agalactiae* (No 204, Culture Collection, Manchester), *Streptococcus lactis* (isolate from a patient with endocarditis), and *Streptococcus pneumoniae* (patient isolate) were cultured in Brain Heart Infusion broth for 48 hours at 37° C in a rotary shaker. The NVS (patient isolate) required the addition of cysteine and pyridoxine.

Cultures were pelleted by centrifugation at 2500 × *g* for 15 minutes, resuspended in 10 mM TRIS-HCl (pH 7.5) and repelleted. The organisms were fragmented in an Xpress at -20° C. The disintegrated cells were centrifuged at 10000 × *g* for 10 minutes. The supernatant was used in further experiments and stored at -20° C. The protein concentration was measured by the Lowry method.⁵

SODIUM DODECYL POLYACRYLAMIDE GEL ELECTROPHORESIS

The supernatants were solubilised in 2.6% sodium dodecyl sulphate and 1.2-mercaptoethanol at 100° C for five minutes. Electrophoresis of these preparations was carried out on a 10% polyacrylamide gel in a discontinuous buffer system.⁶ Protein (10 µg) was applied to each well. Molecular weight standards were the Amersham Rainbow markers. These were myosin 200 000; phosphorylase B 92 500; bovine serum albumin 69 000; ovalbumin 46 000; carbonic anhydrase 30 000; trypsin inhibitor 21 500; lysozyme 14 300.

IMMUNOBLOTS

Proteins were transferred on to nitrocellulose paper in a buffer containing 25 mM TRIS, 192 mM glycine, and 20% methanol (pH 8.3). Transfer was performed at 25° C with a current of 500 milliamperes for 30 minutes in a transphor cell (LKB Laboratories). Free protein sites were saturated by incubation in 3% bovine serum albumin (Sigma) in buffered saline (0.9% NaCl and 10 mM TRIS, pH 7.4) at 4° C overnight. The nitrocellulose membrane was then incubated at 25° C for two hours with the patient's sera diluted 1 in 10 in 3% bovine serum albumin and 0.05% Tween 20 in buffered saline. After washing five times for 30 minutes in 0.9% saline and 0.05% Tween 20 the nitrocellulose was incubated for one hour at 25° C with alkaline phosphatase conjugated anti-human IgM or IgG (Sigma). The conjugate was diluted 1 in 1000 in 3% bovine serum albumin in buffered saline. After washing as described above, the

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Table 1 Details of antibody responses against *S mutans*

Band apparent molecular weight	<i>S mutans</i> endocarditis (n = 2)		Endocarditis controls (n = 10)		Septicaemia controls (n = 10)	
	IgM	IgG	IgM	IgG	IgM	IgG
220	2	2		1		1
200	2	2				
190	2	2		1		1
140	1					
125		2	2	1	4	4
115	2	2	2	2	1	
105	2	2		1		4
86	2	1				
78	1	1	2	2		1
72-74	2	2	1	2	2	3
69	2	2		1	3	1
64	2	2	1	1		
58-59	1	2	4	2		
47	1	2	1	3		1
45		2	1	1	1	

nitrocellulose was incubated for 15 minutes at 25° C with buffer (100 mM TRIS-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl) containing a mixture of 66 µl/10 ml of nitroblue tetrazolium (NBT 50 mg/ml in N, N-dimethylformamide 70%) and 33 µl/10 ml of 5-bromo-4-chloro-3 indolyl phosphate (BCIP 50 mg/ml in N, N-dimethylformamide 70%). The reaction was stopped by washing in water. Immunoblots were examined and those where the antibody response by reflectance densitometry (Chromoscan 3; Joyce Loeb) was greater than 40 mm were counted as positive.

TEST SERA

Controls

Each case of streptococcal endocarditis was examined against the control sera which came from patients with septicemias due to *Pseudomonas aeruginosa* (n = 3), *Candida albicans* (n = 3), *Klebsiella aerogenes* (n = 1), *Staphylococcus aureus* (n = 1) and *Staphylococcus epidermidis* (n = 2). In the case of *S pneumoniae* sera were also examined from five cases of invasive aspergillosis.

Controls with endocarditis

Each new patient with endocarditis was

examined against a pool of 10 patients with streptococcal endocarditis. This comprised *Enterococcus faecalis* (n = 3), *Streptococcus mitis* (n = 1), *Streptococcus sanguis* (n = 4), *Streptococcus bovis* (n = 1), *Streptococcus mitior* (n = 1).

Patients with endocarditis

All patients had clinical, echocardiographic, and cultural evidence of endocarditis, with at least two positive sets of blood cultures. These were 10 cases in all, comprising *S mutans* (n = 2), *S pneumoniae* (n = 2), *S agalactiae* (n = 2), *S lactis* (n = 1) and NVS (n = 3). All cases of endocarditis were confirmed both by clinical culture and positive blood cultures.

Results

Streptococcus mutans

Immunoblotting identified 15 antigenic bands which varied in molecular weight from 45 to 220 kilodaltons (table 1). IgM against a cluster of three antigenic bands at 220, 200, and 190 kilodaltons was specific to active endocarditis. (fig 1). The overall titre of antibody was highest in the patients with *S mutans* endocarditis.

Streptococcus pneumoniae

Immunoblotting showed 22 separate antigenic bands with molecular weights varying between 47.5 and 190 kilodaltons. There was considerable IgG in patients with endocarditis, controls with streptococcal endocarditis, and septicaemic controls. The amount of IgM was highest in the patients with *S pneumoniae* endocarditis. IgM against bands with a molecular weight of between 150 and 190 kilodaltons was specific to endocarditis. The pattern differed between the two cases with the exception of the band at 190 kilodaltons which was common to both cases (table 2). IgM against a band at 123 kilodaltons was also specific to pneumococcal endocarditis. Concentrations of IgM against the band at 100 kilodaltons was high in both cases of endocarditis (fig 2, tract 1). This was also present at a lower titre in both control groups. A second serum sample taken after the patient had been successfully treated by antibiotics was available in one of the cases of pneumococcal endocarditis. This showed a relatively constant titre of IgG (fig 2, tracts 2 and 4) while the IgM against bands at 190, 100, and 90 kilodaltons fell profoundly (fig 2, tract 3). Thus, although the presence of IgM against the band at 100 kilodaltons was not diagnostic of active pneumococcal endocarditis, it did drop with successful treatment.

Streptococcus agalactiae

Immunoblotting detected 20 antigenic bands with a molecular weight varying from 30 to 185 kilodaltons. Both the total titres of IgM and IgG were higher in the two cases of endocarditis. IgM against bands at 82, 71 and 66-67 kilodaltons and IgG against bands at 34-36, 31.5-33, and 30 kilodaltons were specific to endocarditis (table 3) (fig 3).

Figure 1 Tracts 1 and 2, IgM responses in the two cases of *S mutans* endocarditis against *S mutans*. Tracts 3 and 4, corresponding IgG responses.

Table 2 Details of antibody responses against *S pneumoniae*

Band apparent molecular weight	<i>S pneumoniae</i> endocarditis (n = 2)		Endocarditis controls (n = 10)		Septicaemia controls (n = 10)	
	IgM	IgG	IgM	IgG	IgM	IgG
190	2	1				
181	1					
177	1	2		4		1
169	1					
160	1	1				
150	1	1		4	1	6
130	1	1		7	1	2
123	2	1				
120	1	1	2	7	3	5
110	1	2	4	5	7	6
100	2	2	1	10		11
90	2	2		9		10
85	1	2		3		1
82		2	1	2		5
80		1		1		2
68	1	1		1		3
66		1		1		
65		1		1		
58	1	1		1		4
56						
53-54	1	2		1		
47-5		1		1		
40		2				

Streptococcus lactis

Immunoblotting showed six antigenic bands with molecular weights varying between 47–105 kilodaltons. Antibody against this streptococcus is rare among the control population and the IgM concentration of the test case was specific (table 4) (fig 4).

Nutritionally variant streptococcus

Immunoblotting showed 10 antigenic bands with molecular weights varying between 36·5

Figure 2 Tract 1, IgM before, tract 2, IgG before, tract 3, IgM after and tract 4, IgG after successful antibiotic treatment of pneumococcal endocarditis. The post treatment IgG titres against *E faecium* and *E faecalis* are shown in lanes 5 and 6.

Figure 3 Tract 1 (IgM) and tract 2 (IgG) of a case of *S agalactiae* endocarditis against *S agalactiae* antigen.

and 155 kilodaltons. The titre of IgM compared with IgG was higher in both control populations and the three cases of endocarditis. It was impossible to distinguish two of the cases of NVS endocarditis from the control population (Table 5) (fig 5). Figure 5, tract 1, shows where the IgM titre in a case of endocarditis due to *E faecalis* was higher than that in the case of NVS endocarditis (tract 3). The only exception to this was when the nutritionally variant isolate was examined against the serum sample from the corresponding patient. This showed a much higher titre of IgM with bands at 155, 140, 105, 92, 89, 88, 70, 54, 41·5, and 36·5 kilodaltons (Table 5) (Fig 5, tract 5 IgM, tract 6 IgG). This pattern was seen in neither of the other two cases of endocarditis nor in any of the control sera.

Discussion

The application of immunoblotting to a further five species of streptococcus has permitted the delineation of endocarditis specific patterns.

Table 3 Details of antibody responses against *S agalactiae*

Band apparent molecular weight	<i>S agalactiae</i> endocarditis (n = 2)		Endocarditis controls (n = 10)		Septicaemia controls (n = 10)	
	IgM	IgG	IgM	IgG	IgM	IgG
185	1	2		1		
172	1	2	2	3		
147	2	2	3	5		4
122		2				
92	1	1	1	3		
86	2	2	3	5		
84		2	1	2		
82	2	2				
77	2	2		5	3	1
74	1	2		1	1	1
71	2	2				
66-67	2	2				
64	1	2	1			
57	1	1		1		
51.5	1	1				
39-41	1		1			
38		1	1	1		
34-36	1	2				
31.5-33		2				
30	1	2				

Table 4 Details of antibody response of patients against *S lactis*

Band apparent molecular weight	<i>S lactis</i> endocarditis (n = 1)		Endocarditis controls (n = 10)		Septicaemia controls (n = 10)	
	IgM	IgG	IgM	IgG	IgM	IgG
105	1		1			
90	1	1	2			1
66	1					
61	1		2		2	1
58	1					
47	1	1			1	

The overall titre of antibody in the corresponding case of endocarditis is usually higher than both the endocarditis and the septicaemic controls. This is especially true for IgM and agrees with earlier findings.⁷ Homologous fluorescent streptococcus antibody titres equal to or greater than 400 were found in 14 out of 15 patients with endocarditis caused by viridans streptococci when the patient's own blood culture isolate was the antigen. In the case of pneumococcal endocarditis where there were sequential sera the titre of IgM fell with successful treatment. There is therefore a correlation between successful antibiotic treatment and an immunological marker (falling serum IgM) which matches the disappearance of the micro-organism. Current markers either measure the sensitivity of the bacteria to antibiotics, for example, minimum inhibitory concentration, minimum bacteriocidal concentration—the presence of the antibiotic in the patient (such as back-titration)—or are non-specific (such as falling erythrocyte sedimentation rate). The former also requires the original isolate, which is not available in cases of culture negative endocarditis.

In the case of *S mutans* a triplet of high molecular weight antigenic bands at 220, 200, and 190 kilodaltons was specific to endocarditis. An antigen, designated Antigen B, which is a major glycoprotein present on the cell surface of *Streptococcus mutans*, has been described.⁸ It has been implicated in the bacterial adherence to dental surfaces. Furthermore,

monkeys immunised with the protein were protected against dental caries.⁹ It has been shown to have a cross-reaction with human heart¹⁰ and to share epitopes with human IgG heavy chains.¹¹ It produced two antigenic bands on immunoblotting with apparent molecular weights of 195 and 74 kilodaltons. The band at 195 kilodaltons is likely to correspond with one of those to which antibody specific to *S mutans* endocarditis has been produced. This suggests that this antibody would cross-react with human IgG, and in part would explain the autoimmune manifestations of *S mutans* endocarditis.¹¹

Antibody against *S pneumoniae* is much commoner in the sera of all the patients examined than would be expected. This presumably reflects the high incidence of pneumococcal infection. As with *S mutans* IgM, against the high molecular weight bands, in this case IgM between 150 and 190 kilodaltons was specific to endocarditis. The band at 100 kilodaltons was especially interesting as although antibody against this was found in control sera, this was of a low titre. The resolution of pneumococcal endocarditis saw a drop in the titre of IgM against bands at 190, 100, and 90 kilodaltons.

In the case of the *S agalactiae* the antibody titre in the cases of endocarditis was far higher than in the control sera. It was thus possible to differentiate endocarditis specific bands at 82, 71, and 66-67 kilodaltons. In the single case of *S lactis* endocarditis immunoblotting showed a far higher titre of IgM in the patient than in the controls. This is because *S lactis* infection is rare so that the background level of antibody is low.

The picture with NVS endocarditis was more complicated. Where the isolate and the serum came from the same case of endocarditis there was a high titre of IgM. In the other two patients the titre of IgM was lower, and, indeed, as seen in fig 5, a case of *E faecalis* endocarditis produced a higher titre. This is disappointing as it means that the diagnosis of culture negative endocarditis due to NVS cannot be made by immunoblotting against a single antigenic extract. This confirms the results of earlier work where enzyme linked immunosorbent assays were used to detect antibody in patients with endocarditis.⁴ These results can in part be explained by the description of three separate serotypes of NVS.¹² There may be strain or serotype specific antigens which would explain the multiple antigenic bands detected in the IgM when the patient's own serum was matched to the test strain. A conserved band at 91.7 kilodaltons has been described¹³ which may be related to the antigenic bands at 88 and 89 kilodaltons. Antibody against this occurred in all three patients with NVS endocarditis. Unfortunately, it was also common in the control sera.

In summary, this paper demonstrates that there are endocarditis specific antibody patterns for *S mutans*, *S agalactiae*, *S lactis* and *S pneumoniae*. The background level of antibody in control sera is highest in patients when tested against the *S pneumoniae* antigenic extract. The

Figure 4 Tract 1 (IgM) and tract 2 (IgG) of a case of *S lactis* endocarditis against *S lactis* antigen.

Table 5 Details of antibody response of against “nutritionally variant” *Streptococcus*

Band apparent molecular weight	NVS endocarditis (patients own strain)		NVS endocarditis (n = 2)		Endocarditis controls (n = 10)		Septicaemia controls (n = 10)	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
155	1						3	1
140	1						3	
105	1							
92	1	1			1		3	
89	1	1	2	2	1	2	7	1
88	1		2	1	3	2	5	
70	1					1	3	
54	1				1		1	
41.5	1				1			
36.5	1

Figure 5 Tracts 1 and 2, IgM and IgG of *E faecalis* endocarditis against NVS antigen. Tracts 3 (IgM) and 4 (IgG) of NVS (different patient) against NVS antigen. Tracts 5 (IgM) and 6 (IgG) of NVS (corresponding patient) against NVS antigen.

test has problems with NVS and this is probably due to the serological heterogeneity among isolates. This might be overcome by immunoblotting the sera against isolates representing serotypes 1, 2, and 3. These data

will now be applied to culture negative cases of endocarditis.

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