

Papers

# Detection of C-polysaccharide in serum of patients with *Streptococcus pneumoniae* bacteraemia

S H Gillespie, M D Smith, A Dickens, J G Raynes, K P W J McAdam

**Abstract**

**Aim**—To investigate the fate of *Streptococcus pneumoniae* C-polysaccharide antigen in serum in patients with *S pneumoniae* bacteraemia.

**Method**—In vitro dissociation experiments were performed to demonstrate that C-polysaccharide was masked by ligands in normal and acute phase serum. Serum samples from 22 patients with *S pneumoniae* bacteraemia were treated to dissociate immune complexes and then tested for C-polysaccharide by enzyme linked immunosorbent assay (ELISA).

**Results**—C-polysaccharide antigen was masked in normal and acute phase serum but could be released by EDTA treatment and detected by ELISA. Antigen was found in six patients ranging in concentration from 2.5 to 200 ng/ml. Patients with detectable antigen were more likely to die than those in whom antigen was not detected.

**Conclusion**—This study demonstrates that C-polysaccharide antigen commonly circulates in patients with *S pneumoniae* bacteraemia but its presence is masked by ligands present in serum.

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*Streptococcus pneumoniae* is the commonest bacterial cause of acute community acquired pneumonia.<sup>1</sup> Bacteraemia is an important life-threatening complication of *S pneumoniae* induced pneumonia which still has approximately 30% mortality despite the availability of antibiotics to which this organism is usually sensitive.<sup>2</sup>

Although the bacterial capsule is an essential determinant of pathogenicity,<sup>2</sup> it is not toxic to the host. Its main activity is to inhibit phagocytosis by preventing contact between the phagocyte surface and complement components deposited on the bacterial cell wall.<sup>3</sup>

C-polysaccharide is the major cell wall polysaccharide antigen of *S pneumoniae*. Native C-polysaccharide is a ribitol teichoic acid composed of a repeating oligosaccharide subunit made up of  $\beta$ -D-glu p-1-3- $\alpha$ -acetoamido-tri-

deoxygalactosamine p-1-4- $\alpha$ -D-GalNAc p-1-3- $\beta$ -D-GalNH<sub>2</sub> p-1-1'-ribitol-5-phosphate.<sup>4</sup> The immunodominant epitope is made up of the two phosphorylcholine residues attached to the oligosaccharide subunit.<sup>4</sup> The *S pneumoniae* lipoteichoic acid, known as the F antigen, has a similar polysaccharide structure and also contains phosphorylcholine.<sup>4</sup>

The importance of C-polysaccharide in the pathogenesis of inflammation in *S pneumoniae* infection is being increasingly recognised. C-polysaccharide and F antigen activate the alternative complement pathway<sup>5,6</sup> via the phosphorylcholine component. C-polysaccharide is the active cell wall component, as removal of teichoic acid from peptidoglycan diminishes complement activation by cell wall components. The plasma membrane is also a weak activator of the alternative pathway, and this activity resides in the lipoteichoic acid F antigen.<sup>6</sup> The components of the complement cascade generated by interaction with C-polysaccharide are thought to be crucial in the generation of an inflammatory reaction in the alveoli<sup>7</sup> and the meninges.<sup>8</sup> Studies of inflammation in the rabbit model indicate that those fractions of killed *S pneumoniae* which contain teichoic acid polymers induce inflammation when injected into the cisterna magna.<sup>9</sup> Organisms with high spontaneous loss of phosphorylcholine containing components induce a greater inflammatory response in the rabbit meninges.<sup>9</sup>

The fate of C-polysaccharide in the body is unknown. There is only a single report of serum C-polysaccharide antigen in a splenectomised patient with fulminant bacteraemia.<sup>10</sup> There is also a single report of C-polysaccharide antigen being found in urine.<sup>11</sup> We postulated that C-polysaccharide antigen was present in serum but masked by ligands such as anti-phosphorylcholine antibody and C-reactive protein (CRP). We have now shown that this is the case experimentally and in a series of patients with *S pneumoniae* bacteraemia.

**Methods**

**IMMUNOASSAYS**

Microtitration plates (M29A Dynatech, UK) were coated with a 1 in 2000 dilution of a

Department of Medical Microbiology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF  
S H Gillespie  
A Dickens

Department of Clinical Microbiology, University College Hospital, Gower Street, London WC1E 6AU  
M D Smith

Department of Clinical Sciences, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT  
J G Raynes  
K P W J McAdam

Correspondence to: Dr S H Gillespie.

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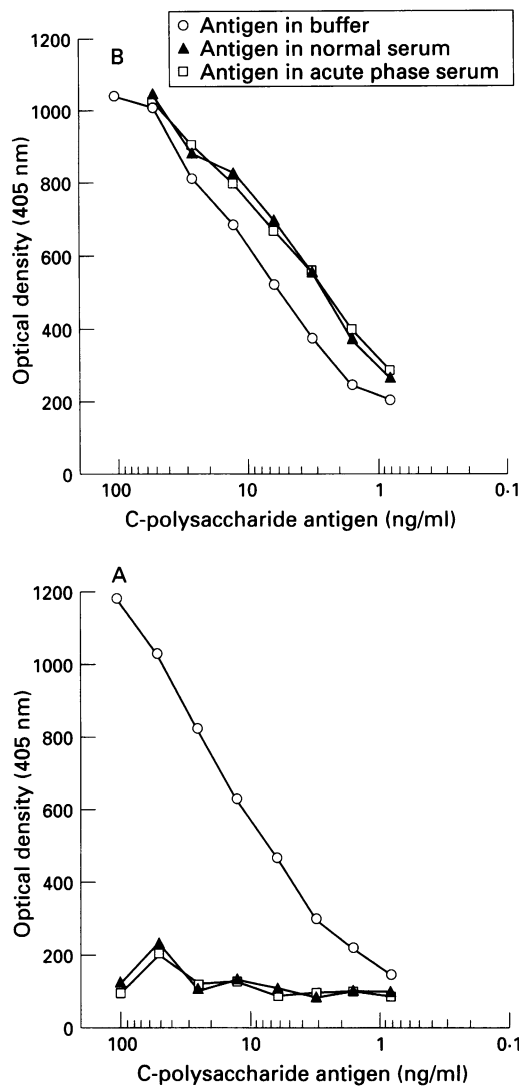


Figure 1 Detection of dilutions of C-polysaccharide antigen in buffer, normal serum and acute phase plasma (A) and in buffer, normal serum and acute phase plasma after treatment with EDTA (B).

mouse IgM monoclonal antibody directed against phosphorylcholine (5/88 Universal Biologicals, UK) in 0.06 M bicarbonate buffer (pH 9.6) by overnight incubation at 4°C. After washing with 10 mM Tris containing 0.15 M NaCl and Tween-20 (0.05%) and 1 mM CaCl<sub>2</sub> (pH 7.6) (TBSTC) samples were added and incubated for one hour at room temperature. CRP was purified and conjugated with horseradish peroxidase by the periodate method.<sup>12</sup> After further washes, 100 µl CRP conjugate (1.7 µg/ml), diluted in TBSTC, was added and the plates incubated for three hours at room temperature. Then 2,2'-azino-di-3-ethyl-benzthiazoline sulphonate (ABTS) peroxidase substrate (Kirkegaard-Perry, Gaithersburg, Maryland, USA) was added after the plates were washed four times and the optical density read at 405 nm using an automated enzyme linked immunosorbent assay (ELISA) reader (Titertek Multiscan MC, Flow Laboratories, UK). Antigen concentration was quantified by comparison with an antigen standard curve diluted in TBSTC using known quantities of purified C-polysaccharide antigen (Dr J Lui, Rockefeller University).<sup>13</sup>

#### DISSOCIATION OF IMMUNE COMPLEXES

Known quantities of purified C-polysaccharide were added to TBSTC, normal human serum and acute phase plasma, and were incubated at 37°C for one hour. Doubling dilutions were made in TBSTC and aliquots of 100 µl were added in triplicate to wells coated with the 5/88 monoclonal antibody as described previously.<sup>13</sup> Each dilution was tested before and after immune complex dissociation using the C-polysaccharide antigen capture ELISA described earlier.

Antigen-antibody complexes were dissociated by EDTA treatment as described previously.<sup>14</sup> Samples were mixed with an equal volume of 0.2 M EDTA, pH 8.0, boiled for five minutes and centrifuged at 18 000 × g for five minutes.

Serum samples from patients admitted to the Royal Free Hospital, Whittington and University College Hospitals, London, with culture positive bacteraemic *S pneumoniae* infection were collected and processed as described earlier. The details of the patients' presentation, underlying condition, therapy, and outcome were recorded using a standard protocol from the clinical record. Serum samples submitted for serology of parasitic infection from 36 patients without evidence of respiratory disease served as controls.

#### Results

Detection of dilutions of C-polysaccharide antigen in TBSTC buffer, normal serum and acute phase plasma are illustrated in fig 1A. It shows that C-polysaccharide antigen cannot be detected in acute phase when it is added to normal serum. The results of assays following immune complex dissociation are illustrated in fig 1B. These data suggest that the standard antigen dilution curve can be reconstituted by treatment of acute phase and normal serum with EDTA.

Serum samples from 22 patients (13 males and nine females; mean age 63 years, median 69 years, range seven months to 92 years) with acute *S pneumoniae* bacteraemia were examined. Pneumonia was the main condition in 17 cases and conformed to a lobar pattern in eight. In four patients bacteraemia was a complication of meningitis and in one bacteraemia was present without evidence of localised infection. A predisposition to infection was found in six patients with chronic obstructive airways disease, in three following acute myocardial infarction and congestive cardiac failure, and in one with immunosuppression due to azothioprim and steroid therapy.

There was a fatal outcome in five patients, directly related to *S pneumoniae* infection in three. A single fatal case was associated with acute pancreatitis and *Proteus* sp. bacteraemia, and in another death occurred suddenly 12 days after acute myocardial infarction.

C-polysaccharide antigen was found in serum from six of the 22 patients with *S pneumoniae* bacteraemia in concentrations ranging from 2.5 to >200 ng/ml. Of these six patients,

Outcome of *S pneumoniae* bacteraemia and antigen in serum

Outcome	Antigen positive	Antigen negative
Died	3	2*
Survived	3	14

\* One patient died suddenly 12 days after an acute myocardial infarction during acute infection. Another had severe pancreatitis and coincident *Proteus* septicaemia.

three died and three recovered. Of the two fatal cases in whom no antigen was detected, a coincident serious life-threatening complication was present as described earlier. These data are summarised in the table.

Data were analysed using the EPI info statistical package, (Public Domain Software, Centers for Disease Control, Atlanta, Georgia, USA). The presence of detectable antigen was associated with a 3.4-fold increased risk of death (95% confidence interval 0.97–11.97;  $p=0.1$  tailed Fisher exact test).

None of the samples from the 36 control patients had a positive result in the C-polysaccharide antigen capture assay. This suggests that C-polysaccharide antigen detection in serum has a sensitivity of 27% and a specificity of 100%.

### Discussion

The fate of *S pneumoniae* capsular antigens has been studied but the mechanism by which the body breaks down microbial polysaccharide antigens is obscure. During in vitro and in vivo growth, *S pneumoniae* soluble and insoluble type specific capsular polysaccharide antigens are produced. Once solubilised, capsular antigens readily cross serous membranes and are excreted in the urine.<sup>15</sup> Capsular polysaccharide found in urine has a lower molecular weight than antigen found in the circulation. The electrophoretic mobility differs and double immunodiffusion shows only lines of partial identity with vaccine antigen.<sup>16</sup> It is suggested that in vivo degradation occurs with the effect that antigens of progressively lower molecular weights are excreted in the urine. It is presumed that the mass of capsular antigen generated during infection is slowly degraded and antigenuria may persist for several weeks. The fate of other polysaccharide antigens including C-polysaccharide is not known. The results reported in fig 1 suggest that both normal human serum and acute phase plasma have sufficient binding capacity to inhibit the immunological detection of C-polysaccharide. The detection of C-polysaccharide is prevented by phosphorylcholine binding ligands and when serum is treated with EDTA the antigen is released and can be detected. This study confirms this also happens in vivo as C-polysaccharide antigen can be found in the serum of patients with *S pneumoniae* bacteraemia. The presence of C-polysaccharide in serum has only previously been reported in a premortem specimen of a splenectomised patient with fulminant bacteraemia<sup>10</sup> where the quantity of antigen may have overcome the circulating ligand binding capacity. C-polysaccharide antigen has also

been reported in urine samples from patients with bacteraemia but only when concentrated 50-fold.<sup>11</sup> The results reported here together with the report of C-polysaccharide in urine suggest that C-polysaccharide antigenaemia is a common event in severe *S pneumoniae* bacteraemia.<sup>10 11</sup>

In their seminal paper, Austrian and Gold<sup>17</sup> demonstrated the continuing high mortality associated with *S pneumoniae* bacteraemia stating "Some persons are at higher risk of death from pneumococcal infection which was not influenced by antimicrobial therapy in the first 48 hours following hospital admission". Patients with fulminant disease show profound hypocomplementaemia with consumption of the components of the alternative complement pathway. It has also been shown that in patients with complicated *S pneumoniae* infection alternative complement pathway factors were significantly depleted when compared with patients with uncomplicated infection.<sup>18</sup> In view of the importance of teichoic acid components in generating inflammation by activation of the alternative complement pathway the presence of C-polysaccharide antigenaemia demonstrated in our bacteraemic patients supports the idea that this antigen has an important role in the pathogenesis of this disease.

Demonstration of circulating C-polysaccharide may also explain the contradictory results of some protection studies using phosphorylcholine binding ligands. Rabbit polyclonal and mouse monoclonal antibodies directed against phosphorylcholine have been shown to protect mice from fatal challenge<sup>19</sup> and this has also been shown for CRP.<sup>20</sup> One explanation for this protection was thought to be by enhancement of opsonisation.<sup>18</sup> C-polysaccharide is situated deep within the capsule and is not available for opsonisation and therefore opsonisation experiments have indicated that antibodies directed against phosphorylcholine, in contrast to those directed against C-polysaccharide, do not promote phagocytosis for most *S pneumoniae* serotypes<sup>21</sup> and that the opsonising efficacy of serum can be correlated with levels of anti-capsular but not anti-phosphorylcholine antibody.<sup>21</sup> It is possible that the mechanism of protection associated with phosphorylcholine binding ligands is due to its ability to bind free C-polysaccharide and prevent overwhelming activation of the complement cascade.

In this study C-polysaccharide was detected in the serum of five of 20 patients with bacteraemic *S pneumoniae* infection. This is similar to the proportion of patients with *S pneumoniae* bacteraemia in whom capsular polysaccharide can be detected.<sup>22 23</sup> C-polysaccharide antigen was found in only six of 22 patients with *S pneumoniae* bacteraemia although all of the control serum samples were negative for this antigen. As a diagnostic test, this is equivalent to a sensitivity of 27% and a specificity of 100%. Although this is the first report of the use of C-polysaccharide antigen detection in serum by lysis of immune complexes it is clear that this method is specific but does not provide any further advantages to capsular antigen de-

tection methods already reported.<sup>22 23</sup> Further studies are underway to determine the importance of C-polysaccharide antigen concentration with respect to outcome.

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