Difference in Binding of Killed and Live *Streptococcus pneumoniae* Serotypes by C-Reactive Protein

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The binding and opsonic properties of C-reactive protein (CRP) for various species of bacteria were investigated. CRP bound more avidly to killed than to live *Streptococcus pneumoniae*, the binding varying among various serotypes; CRP hardly bound to a number of other bacterial species studied. CRP enhanced complement-dependent phagocytosis of live *S. pneumoniae* by granulocytes but did not enhance the phagocytosis of live *Staphylococcus aureus* or group B streptococci. We suppose that CRP may serve as an opsonin for killed bacteria and bacterial debris but is probably not an important opsonin for live bacteria other than *S. pneumoniae*.

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INTRODUCTION

C-reactive protein (CRP) is one of the major acute-phase proteins. A thousandfold increase in the serum level can be observed in patients with marked tissue damage due to severe bacterial infection, trauma, burns and neoplasms [1]. CRP is thought to play a role in host defence by opsonization, for example, *Candida albicans* and various types of *Streptococcus pneumoniae* [2–4]. CRP binds strongly to bacterial phosphocholine moieties in a Ca$^{2+}$-dependent manner [1, 2, 5] and activates the classical pathway of complement [6, 7]. In the presence of CRP, complement-dependent opsonization of *S. pneumoniae* type 27 and pneumococcal polysaccharide-coated erythrocytes has been reported [8, 9]. However, enhanced phagocytosis was only found for live *S. pneumoniae* type 27 with capsular bound CRP and C3 [10] and not for live *S. pneumoniae* type 4 which binds C3 in the cell wall in the presence of CRP [11].

In vivo, administration of CRP has been shown to increase survival of mice infected with various types of *S. pneumoniae* [12–14]. Moreover, an increased resistance to *S. pneumoniae* infections in CRP transgenic mice has been demonstrated recently [15]. However, it is not known whether this CRP-mediated protection also occurs for other pathogens.

The aim of the present study was to evaluate the binding of CRP to various strains of live and killed bacteria and to determine whether CRP can enhance the phagocytosis of bacteria by human granulocytes.

MATERIALS AND METHODS

*Micro-organisms.* *Staphylococcus aureus* (type 42D), *Staphylococcus epidermidis*, *Escherichia coli* (054), *S. pneumoniae* types 3, 14, 19, 23 and 27, *Haemophilus influenzae* type b, *Streptococcus pyogenes* and group B streptococci were used. *Streptococcus pneumoniae*, group B streptococci and *Streptococcus pyogenes* were cultured at 37°C overnight in brain heart infusion (BHI, Oxoid Ltd, Basingstoke, UK) medium; *S. aureus*, *S. epidermidis*, *H. influenzae* and *E. coli* were cultured at 37°C overnight in Nutrient Broth no. 2 (Oxoid Ltd). The bacteria were harvested by centrifugation at 1500 g for 10 min at room temperature, washed twice with PBS (pH 7.4) and resuspended at a concentration of approximately $1 \times 10^7$/ml bacteria in Hanks' balanced salt solution (HBSS; 1.2 mM Ca$^{2+}$) containing 0.1% (w/v) gelatin (gelatin–HBSS).

For the enzyme-linked immunosorbent assay (ELISA) the bacteria were heat-killed (1 h at 70°C), centrifuged at 1500 g for 10 min, washed three times with PBS and resuspended in PBS at a concentration of $1 \times 10^7$/ml.

*Granulocytes.* Granulocytes were isolated from the blood of healthy adult donors by dextran sedimentation of erythrocytes as described [16], and suspended in gelatin–HBSS (1.2 mM Ca$^{2+}$) at a final concentration of $1 \times 10^7$ cells/ml.
Serum. Human serum (HS; 2 mm Ca\(^{2+}\)) was obtained from a healthy donor with blood group AB and aliquots of serum were stored at −70°C. Antibacterial antibodies present in the serum were absorbed by incubating 100 μl human serum with 2 × 10^9 killed bacteria, used in this study, on ice for 30 min (HS\(_{ads}\)). HS\(_{ads}\) was inactivated by heating at 56°C for 30 min (HS\(_{inact}\)). Rabbit antiserum against CRP was raised in our laboratory. Anti-bacterial antibodies present in the rabbit preimmune serum and in the anti-CRP serum were adsorbed by incubating 300 μl 2% (v/v) serum with 1 × 10^9 of the bacteria under study at 37°C for 30 min under slow rotation (4 r.p.m.). Preimmune rabbit serum served as the control for non-specific binding of serum.

**Binding of CRP to bacteria.** Purified CRP, isolated as described [17], was kindly provided by Prof. John Volanakis (University of Alabama, Birmingham, AL, USA). Binding of CRP to live or killed bacteria in suspension was studied by incubating approximately 5 × 10^7 bacteria in 500 μl PBS with 500 μl CRP (1 mg/ml in PBS) on ice for 30 min (pH 7.4). After centrifugation at 1500 g at 4°C, the amount of residual CRP present in the supernatant was determined by ELISA (limit of detection 10 ng/ml).

**Quantification of residual CRP in the supernatant.** Microwells (Titertek, Flow Laboratories, Zwanenburg, the Netherlands) were coated with 100 μl of sheep anti-human CRP (Organon Technika Corporation, Durham, NC, USA; 1 μg/ml) in 0.1 M sodium carbonate buffer (pH 9.6) by overnight incubation at room temperature. After three washes with PBS containing 0.05% (v/v) Tween-20 (PBS/Tween) the wells were incubated with 100 μl PBS/Tween containing 0.1% inactivated (30 min at 56°C) newborn calf serum (NBCS) at 37°C for 30 min. After washing, the wells were incubated at 37°C for 1 h with 100 μl samples containing CRP in PBS/Tween and 1% heat-inactivated NBCS (PBS/Tween/NBCS). Next, the wells were washed again and incubated at 37°C for 1 h with 100 μl of rabbit anti-human CRP serum diluted in PBS/Tween/NBCS [1 : 500]. After washing, binding of CRP was assessed by subsequent incubation of the wells at 37°C for 1 h with swine anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Nordic Immunological Laboratories, Tilburg, the Netherlands) diluted [1 : 500] in PBS/Tween/NBCS. After washing with PBS/Tween, the wells were incubated with 100 μl 3,3',5,5'-tetramethylbenzidine (TMB, 100 mg/l, Sigma, St. Louis, MO, USA) as substrate for the HRP, and the reaction was stopped after 20 min. by adding 25 μl 4N H\(_2\)SO\(_4\) [18]. Optical densities (OD) were read within 30 min, at 450 nm using a Titertek Multiscan (Titertek, Flow Laboratories). In these experiments, serial dilutions of purified CRP were used to construct standard curves.

**Phagocytosis assay.** Phagocytosis of bacteria by human granulocytes was evaluated by measuring the decrease in the number of viable extracellular bacteria [16]. Granulocytes (5 × 10^6/ml) were incubated with 5 × 10^7 live bacteria/ml in a final volume of 450 μl in gelatin–HBSS containing 10% (v/v) HS\(_{ads}\) at 37°C under slow rotation (4 r.p.m.) and samples were taken at selected time-points. The number of viable extracellular bacteria was determined by plating serial dilutions of the supernatant obtained after differential centrifugation of granulocytes and bacteria.

**Statistical analysis.** Binding assay data were analysed using Mann–Whitney U-test. Analysis of variance (ANOVA) was used for the phagocytosis assay data. *P*-values < 0.05 were considered significant.

**RESULTS**

First the binding of CRP to killed bacteria was investigated. In preliminary experiments no differences in binding of CRP to heat-killed or formaldehyde-killed bacteria (data not shown) were found. All further experiments were performed with heat-killed bacteria. The results showed that the binding of CRP to killed *S. pneumoniae* types 14, 19, 23 and 27 varied between 47 and 79% (Table 1). However, low or no binding of CRP to killed *S. pneumoniae* type 3, *S. pyogenes*, *S. aureus*, *S. epidermidis*, *E. coli*, *H. influenzae* type b, and group B streptococci was found (Table 1). Binding of CRP to live *S. pneumoniae* was markedly lower than to killed bacteria (Table 1). In the presence of 10 mm EDTA (pH 7.4) no binding of CRP to killed *S. pneumoniae* types 14 and 27 was observed (data not shown), which indicates that binding of CRP to these bacteria is Ca\(^{2+}\)-dependent [19].

Next we studied whether CRP bound to live bacteria could enhance phagocytosis. The results showed that CRP promoted phagocytosis of *S. pneumoniae* type 27 in the presence of 10% HS\(_{ads}\) (Fig. 1). Bacterial aggregation was not observed (not shown). However, CRP did not enhance phagocytosis of these pneumococci in the presence of HS\(_{ads}\) (Fig. 1). In the absence of serum, CRP did not enhance phagocytosis of live *S. pneumoniae* type 27 by human granulocytes (data not shown). Phagocytosis of live *S. pneumoniae* type 14 by granulocytes in the presence of CRP and HS\(_{ads}\) was less than that of *S. pneumoniae* type 27 (Fig. 2). Phagocytosis of live *S. aureus* or group B streptococci was not enhanced by CRP in the presence of human serum (Fig. 2).

**DISCUSSION**

The results of this study showed that CRP binding varied markedly between various serotypes of killed *S. pneumoniae* and that live *S. pneumoniae* bind less CRP than killed *S. pneumoniae*. This difference in binding of killed bacteria is not dependent on the killing procedure since no difference in binding of CRP to heat-killed or formaldehyde-killed bacteria was

| Table 1. Binding of CRP (% bound) to various live and killed bacteria |
|--------------------------|-------------------------|------------------|-----------|
| **Bacteria**             | **Killed**              | **Live**         | **P value** |
| *S. pneumoniae* type 3   | 20 ± 14                 | 17 ± 13          | 0.25      |
| *S. pneumoniae* type 14  | 47 ± 9                  | 23 ± 22          | 0.16      |
| *S. pneumoniae* type 19  | 79 ± 9                  | 37 ± 12          | 0.001     |
| *S. pneumoniae* type 23  | 72 ± 13                 | 52 ± 10          | 0.01      |
| *S. pneumoniae* type 27  | 74 ± 12                 | 31 ± 7           | 0.003     |
| Group B Streptococci    | 14 ± 18                 | nd               |           |
| *S. pyogenes*            | 3 ± 4                   | nd               |           |
| *S. aureus*              | 6 ± 6                   | 0                |           |
| *S. pyogenes*            | 9 ± 11                  | nd               |           |
| *E. coli*                | 19 ± 10                 | nd               |           |
| *H. influenzae* type b   | 16 ± 9                  | nd               |           |

Binding of CRP to live or killed bacteria. Binding is determined by ELISA. Values are expressed as the mean ± SD of six experiments. nd = not determined.
observed. Furthermore, it had been reported already that the heat-killing procedure does not affect CRP-binding [2]. In dead bacteria, modification or disruption of the normal configuration of the outer structure may have occurred. Consequently, components that may serve as ligands for CRP are exposed by killed bacteria and not by live intact bacteria. The differences in CRP-binding capacity of killed \textit{S. pneumoniae} may be due to a variable content of membrane and/or capsular ligands for CRP, in particular phosphocholine (PC) [2]. PC is a membrane component of \textit{S. pneumoniae}. Only limited quantitative information on the PC content in bacteria is available [20, 21]. \textit{Haemophilus influenzae} contains 0.001–0.045 pg/CFU PC-containing antigens, \textit{Streptococcus} group G contains 1.8 pg/CFU, while no PC-containing antigen was found in \textit{S. pyogenes}, group B streptococcus, \textit{S. aureus} and \textit{E. coli} [22]. These data agree well with our results: no PC-containing antigens according to the literature and we found no CRP binding. The observation that binding of CRP to \textit{S. pneumoniae} type 3 is low relative to that with \textit{S. pneumoniae} type 27 is in agreement with an earlier study using radiolabelled CRP [23]. The finding that several Gram-positive micro-organisms other than \textit{S. pneumoniae} and Gram-negative micro-organisms, e.g. \textit{E. coli} and \textit{H. influenzae} type b, bound only a small amount or no CRP has also been reported by others [2, 24].

Phagocytosis of live \textit{S. pneumoniae} types 27 and 14 by granulocytes was enhanced in the presence of CRP and serum. Despite the fact that binding of CRP by live \textit{S. pneumoniae} types 14 and 27 was similar (23% versus 31%) a difference in phagocytosis of these bacteria was observed. This might be due to a difference in binding of C3-activation products [25]. The finding that phagocytosis of live \textit{S. pneumoniae} by CRP was only enhanced in the presence of fresh serum demonstrated that complement is required for the opsonic activity of CRP. Our finding that CRP did not enhance phagocytosis of \textit{S. aureus} and group B streptococci can be explained by the lack of binding of CRP to these bacteria.

Which role has CRP as opsonin \textit{in vivo}? Since CRP binds more avidly to killed than to live \textit{S. pneumoniae} we assume that CRP may play a role in disposing of killed bacteria, and of bacterial and cellular debris which is formed during various types of tissue injury [4, 26]. It seems unlikely that CRP serves as an important opsonin for bacteria other than \textit{S. pneumoniae} serotypes since the binding of CRP to other killed bacterial species studied is rather low.

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