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Requirement for an Additional Serum Factor Essential for the Antibody-Independent Activation of the Classical Complement Sequence by Gram-Negative Bacteria

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Killing of *Salmonella minnesota* and *Salmonella typhimurium* S and R strains in serum of nonimmune humans and guinea pigs was drastically reduced in the selective absence of C1q, C1r, Ca^{2+}, C4, or C2, the components of the classical complement pathway. Binding of $C\bar{1}$ and C1q to the S form and six different core-deficient R mutant strains became stronger the shorter the lipopolysaccharide molecule. $C\bar{1}$ and C1q had, under physiological conditions, no affinity to the serum-resistant S forms, whereas these components were bound by the serum-sensitive R forms with high affinity. However, a mixture of the individual complement components C1-C9, which rapidly lysed sensitized erythrocytes, did not kill the serum-sensitive bacteria. Isolated C1 bound to these bacteria cleaved fluid-phase C4 but did not convert C2. C2 turnover could be detected only when serum was used as a source of C1 or C4, indicating that an additional serum component is necessary for the antibody-independent bactericidal effect. Functional tests indicated that this factor is a euglobulin which mediates binding of C4 to the bacteria even in the absence of C1 or after treatment with EDTA. Binding of C4 followed by the generation of C4b sites as acceptors for C2 was a prerequisite for the killing of the bacteria. The factor could not be replaced by immunoglobulin G or immunoglobulin M, nor was it blocked by preincubation with anti-immunoglobulin G or anti-immunoglobulin M.

The strong bactericidal effect of normal human serum and normal guinea pig serum (GPS) against smooth (S) and rough (R) forms of *Salmonella* strains was reduced in the selective absence of C1q, C1r, Ca^{2+}, C4, and C2 (3). These observations indicate that the bactericidal activity is mediated by C1 and the components of the classical complement (C) pathway (C1, C4, C2) and is independent of antibodies. Some gram-negative bacteria interact directly with the first component of complement, C1, and its subcomponent C1q (2, 4, 6). Comparative studies of *Salmonella minnesota* and *Salmonella typhimurium* S and R forms showed a high binding affinity of C1 to the serum-sensitive rough mutants, whereas the serum-resistant S forms had no affinity under physiological conditions (4). The high affinity of C1 to the R forms seemed to be due to additional binding structures on the outer membrane besides lipopolysaccharide (LPS), i. e., proteins, which are more susceptible on R forms than on S forms (5, 12). The additional binding structures involve a different binding mechanism of C1 to the bacteria, as shown by Bartholomew and Esser in the direct interaction of C1 with capsid proteins of retroviruses (1).

MATERIALS AND METHODS

S. minnesota wild type (S form S-1114) and the Re mutant (R-595, SF-1167) were kindly provided by G. Schmidt, Max Planck Institut, Freiburg, Federal Republic of Germany. The bacteria were taken from an 18-h broth culture, washed, counted in a Neubauer chamber, and adjusted to 2×10^8 microbes per ml.

The LPS preparation was obtained from C. Galanos, Max Planck Institut. Sheep erythrocytes were prepared and coated with LPS by the method of Schlecht and Westphal (10).

Pooled normal GPS was stored at −25°C. A 1-ml amount of serum was incubated with 2×10^9 bacteria for 1 h at 37°C; the bacteria were removed by centrifugation, and the remaining C components were tested in the supernatants and compared with unabsorbed controls. The preparation of complement components and the interaction of complement with bacteria have been previously described (4). The methods of preparing sheep erythrocytes (E) and antibodies (A), the cellular intermediates (EA and EAC4), and Veronal-buffered saline (VBS) with 4% sucrose (VBS-S) or with EDTA (VBS-EDTA) and the procedure of the hemolytic assays have been described by Rapp and Borsos (9).

The antisera versus bacterial surface structures (O antigen: anti-O21; H antigens: anti-Hb and anti-Henx) and human immunoglobulin G (IgG) and IgM standards were purchased from Behring Institut, Marburg, Federal Republic of Germany.

TABLE 1. Consumption of complement components in GPS by the S and Re forms of *S. minnesota* at different ionic strengths

Complement component	Complement consumption (effective molecules per 2×10^8 bacteria)[a]			
	S form		Re form	
	$\mu = 0.065$	$\mu = 0.15$	$\mu = 0.065$	$\mu = 0.15$
C1	351	28	926	1,187
C4	315	206	1,373	1,341
C2	30	5	162	134
C3	65	153	64	150
C5	477	633	274	479
C6	262	ND[b]	159	ND
C7	323	ND	32	ND
C8	352	ND	199	ND
C9	254	ND	130	ND

[a] Bacteria were incubated for 60 min at 37°C.
[b] ND, Not determined.

C2-deficient human serum was kindly provided by N. Day, Memorial Sloan-Kettering Cancer Center, New York. We obtained the C4-deficient guinea pigs from M. Frank, NIAID, Washington, D. C.

For detection of C2 turnover, 2×10^8 bacteria were incubated with ca. 1,000 effective $C\bar{1}$ molecules per cell (9) for 10 min at 30°C. The cells were washed twice, the same number of C4 molecules was added, and the solution was incubated for another 10 min at 30°C. Such pretreated bacteria were used to test the consumption of C2 in the presence of the test sample.

For isolation of the serum components, the euglobulin fraction was separated from normal GPS by dialysis against distilled water. The precipitate was suspended in VBS buffer (pH 7.4; $\mu = 0.15$) containing 0.15 M NaCl and 0.02 M EDTA, followed by filtration of the protein solution on a column of Sephadex G-200. The fractions were tested for their functional activity in C2 turnover.

Protein fractions were analyzed by polyacrylamide gel electrophoresis in slab gels by the method of Lugtenberg et al. without sodium dodecyl sulfate and mercaptoethanol (7). Electrophoresis was carried out in a Bio-Rad apparatus model 220 under a constant voltage of 100 V. Gels were stained with Coomassie blue R250 (Serva, Heidelberg, Federal Republic of Germany) by the method of Weber and Osborn (13) and destained for 4 h in a solution of 7.5% acetic acid–5% methanol by diffusion.

RESULTS

Absorption of pooled normal GPS for 60 min at 37°C with S and Re forms of *S. minnesota* resulted in the reduction of the complement activity. This suggested the dependency of the serum-mediated killing on complement components. Therefore, the number of all components after absorption of 1:10 diluted (0.065 μ) and concentrated (0.15 μ) GPS with 2×10^8 bacteria (60 min; 37°C) was determined (Table 1). C3 to C9, the components that can be activated via the alternate as well as the classical pathway, were consumed by both bacterial strains in similar amounts. The classical components C1, C4, and C2 had a higher turnover rate after absorption of serum with the serum-sensitive Re form than with the serum-resistant S form, confirming the dependency of the serum bactericidal effect on the classical complement components (3). At 0.065 and 0.15 μ, the Re form bacteria bound ca.

TABLE 2. Antibody-independent lysis of LPS-coated erythrocytes by native and purified complement components[a]

Source of complement components[b]				% Lysis of LPS-coated erythrocytes
$C1/C\bar{1}$	C4	C2	C3 to C9	
C4-deficient GPS (w)	p (w)	p (w)	C-EDTA	71
C2-deficient HS	C2-deficient HS (w)	p (w)	C-EDTA	96
C2-deficient HS	C2-deficient HS		C2-deficient HS	1
p (w)	p (w)	p (w)	C-EDTA	0

[a] LPS-coated erythrocytes (1.3×10^8 per ml), prepared by the method of Schlecht and Westphal (10), were incubated with serum C1 or with purified $C\bar{1}$ for 10 min at 30°C. The cells were then incubated with C4, C2, and C-EDTA (GPS diluted 1:50 in VBS-EDTA).
[b] p, Purified; HS, human serum; w, cells were washed in VBS-S. Number of effective molecules per cell: $C1/C\bar{1}$, 1,000; C4, 500; C2, 500.

TABLE 3. C2 consumption after incubation of bacteria with purified C1 and purified or native C4[a]

Components	C2 titer (effective molecules per cell)
Buffer	907
Re form[b]	937
Re $C\bar{1}$,[c] C4[d]	922
Re $C\bar{1}$, C4[e]	36

[a] C2 was added after washing and incubated for 10 min at 30°C.
[b] 2×10^8 cells per ml.
[c] ca. 1,000 effective $C\bar{1}$ molecules per cell.
[d] ca. 500 effective C4 molecules per cell.
[e] Diluted GPS (1:5 in VBS-EDTA) was used as source for C4 (ca. 500 effective molecules per cell).

1,000 $C\bar{1}$ molecules per cell with high affinity. In contrast, the S form absorbed only 28 molecules under physiological conditions. The 351 effective $C\bar{1}$ molecules which were consumed by the S form at 0.065 μ were fixed in a reversible way: $C\bar{1}$ can be split off and transferred to EAC4 at μ = 0.15. C4 was consumed more efficiently the more $C\bar{1}$ was bound to the bacteria. The incubation of the Re bacteria with serum resulted in the consumption of C2, whereas only minimal C2 turnover induced by $C\bar{1}$, which was activated by the S form, could be detected.

Since the C1 binding was found to be important for serum-mediated killing (4), the activation of the whole classical pathway by the bacteria and the interaction with the purified components of the classical sequence were further investigated. In preceding experiments, erythrocytes coated with purified LPS of the Re form of *S. minnesota* were shown to bind and activate C1, which was able to cleave C4; however, no C2 consumption was detectable. This observation disagreed with the results obtained in serum after absorption with the R form bacteria (Table 1). Therefore, LPS-coated erythrocytes were incubated with purified or native C1 ($C\bar{1}$), C4, and C2. The native components were absorbed from sera diluted 1:10 in VBS (0.15 μ) with a selective complete defect in one component; thus, the complement cascade was interrupted (Table 2). The purified components were supplied in concentrations comparable to those in serum: about 1,000 $C\bar{1}$, 500 C4, and 500 C2 effective molecules per bacterium.

Seventy-one percent of the target cells (LPS-coated erythrocytes) were lysed when C1 was absorbed from C4-deficient GPS and the cascade was completed with purified components. A higher degree of lysis (96%) was obtained when a C2-deficient patient serum was used as a source of C1 and C4. In contrast, no lysis of LPS-coated erythrocytes was found when purified C1 ($C\bar{1}$), C4, and C2 were used, although the hemolytic potency of these components was proven with sensitized erythrocytes (EA). Therefore, these experiments indicate that an additional serum component was necessary for cell lysis, as the complement sequence could not be built up with purified complement components.

Similar results were obtained with S and Re forms of *S. minnesota* (Table 3). Consumption of purified C2 was not detectable when untreated bacteria or bacteria pretreated with purified $C\bar{1}$ (ca. 1,000 effective molecules per cell) and C4 (ca. 500 effective molecules per cell) were used. In other reaction mixtures, the bacteria were pretreated with purified $C\bar{1}$ and incubated in C-EDTA (0.2 ml of GPS and 0.8 ml of VBS-EDTA; ca. 500 effective C4 molecules per cell) for 15 min at 30°C to absorb C4, comparable to the preparation of EAC4 (9). The cells were then washed twice in VBS-EDTA and twice in VBS-S. Purified $C\bar{1}$ which had been removed by EDTA-treatment was restored. After washing, C2 was added, and the solution was incubated for 10 min at 30°C; C2 consumption was tested in the supernatants. In these experiments, C2 was only converted when EDTA-chelated serum was used as a source of C4. The control with untreated bacteria showed that C4 could be absorbed by the microbes from EDTA-treated serum independent of the presence of $C\bar{1}$.

For further studies, normal GPS was separated on Sephadex G-200. By this procedure, we obtained the four characteristic serum peaks. Each peak was tested for mediating activity in

TABLE 4. Test of serum fractions of normal GPS (Sephadex G-200) for a factor which mediates the C2 turnover

Components	No. of C2 molecules remaining after incubation[a] of the reaction mixtures with:				
	Peak 1	Peak 2	Peak 3	Peak 4	Buffer
S C1, C4[b] + C2	0.2	456	95	94	97
Re C1, C4 + C2	0.0	300	79	61	97
C2 titer	0	194	0	0	0

[a] 10 min at 30°C.
[b] About 1,000 effective $C\bar{1}$ and C4 molecules per cell, purified from GPS, were incubated with 2×10^8 bacteria for 10 min at 30°C.

TABLE 5. C2 activity after incubation with bacteria, purified C1, C4, C2, and several other serum fractions or components

Incubation mixture addition[a]	% Remaining C2 activity
Buffer	100
α_2-Lipoprotein	96
Pseudoglobulin fraction	142
Euglobulin fraction	3
Peak 1 + buffer (Sephadex G-200)	1
Anti-O21[b]	105
Anti-Hb[c]	82
Anti-Henx[c]	93
IgG	89
IgM	94

[a] The basic incubation mixture contained bacteria, C1, C4, and C2.

[b] Antiserum versus the somatic antigens of the bacteria (group O 21).

[c] Antisera versus flagella antigens.

C2 consumption after incubation of the Re mutant of *S. minnesota* with purified $\overline{C1}$ and C4 (Table 4). Complete C2 turnover could be detected in the presence of peak 1, the void volume fraction of the column. This fraction also contained IgM, α_2-macroglobulin, α_2-lipoprotein, hemoglobulin-haptoglobulin complexes, C1, and C3. Therefore, IgM and α_2-lipoprotein were also tested for C2-converting activity and were found to be functionally ineffective (Table 5).

To rule out the participation of (natural) antibodies against *S. minnesota*, defined antibodies against the O and H antigens of *S. minnesota* (O antigen: anti-O21; H antigens: anti-Hb and anti-Henx) as well as pools of human IgG or human IgM were tested for activity in mediating C2 turnover. The participation of these antibodies in the consumption of C2, which might be caused by a component mediating the fixation of C4 to the bacterial cell, could be excluded. These interpretations are supported by the experiments of Borsos et al. (IX International Complement Workshop, November 1981, Key Biscayne, Fla.). These authors provided evidence for the lack of C4 binding by human IgM during activation of the classical pathway.

C2-converting activity was detected in the first peak after separation of normal GPS on a Sephadex G-200 column. The pool which mediated C2 consumption (peak 1) was further analyzed by polyacrylamide gel electrophoresis without sodium dodecyl sulfate and mercaptoethanol (Fig. 1). The component mediating the activation of the whole classical complement cascade by bacteria was detected in a major protein band after slicing and elution of the gel. Since, under these experimental conditions, the macromolecular C1 complex does not enter into the gel, it is not possible that $\overline{C1}$ alone is able to initiate C2 consumption. This is also in agreement with the data presented in Table 2.

DISCUSSION

Incubation of the S and R forms of *S. minnesota* with serum caused the killing of the serum-sensitive strains on one hand and a large reduction of the serum components of the classical complement pathway on the other hand. The strong bactericidal effect of normal GPS against these bacteria was decreased in the selective absence of C1, C4, or C2. These observations indicate that the serum sensitivity of bacterial strains is caused by the effective activation of classical complement components.

The low C1-binding affinity of the serum-resistant S form and the high binding affinity of the serum-sensitive R forms support the findings of an antibody-independent C1-mediated serum bactericidal killing (3, 4). C1 was directly bound and activated by the R form bacteria; C4 was cleaved by the active C1 esterase, but no C2 turnover was measurable. The latter observation was in contrast with the C1, C4, and C2 dependency of the bactericidal activity of serum. Therefore, an additional serum component, different from antibodies, must be required. This confirms the earlier observations of Skarnes, who investigated the antibacterial potential of serum from young rabbits and guinea pigs (8, 11). The killing of the rough strains in the absence of "natural" antibodies required complement and at least one additional nonspecific component. However, in this complement-mediated bactericidal system, the early components of human complement (C1, C4, C2) plus certain serum euglobulins were able to kill pathogenic strains of *Shigella sonnei* (11), and no late-acting components were necessary in contrast to the

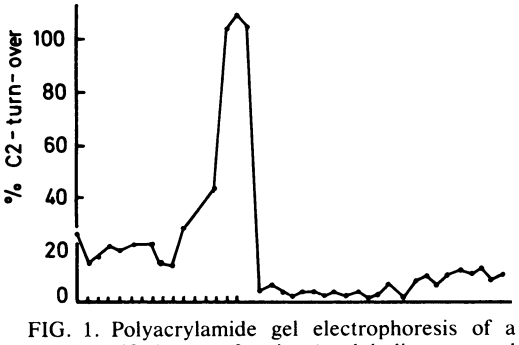

FIG. 1. Polyacrylamide gel electrophoresis of a partially purified serum fraction (euglobulin separated on Sephadex G-200) essential for the sequence of the classical complement cascade. 100% ≅ 180 effective C2 molecules.

results of our experiments, in which all complement components were essential (Table 2).

The additional serum component was partially purified from GPS. Functional tests provided evidence that the factor is a protein which mediates the binding of C4 to the bacterial membrane in the absence of antibodies. In antibody-mediated classical pathway activation, receptors on guinea pig erythrocytes specific for the fourth component of human complement were demonstrated by Wilson et al. (14). The additional component was detectable in C4-deficient GPS and in C2-deficient human serum via its activity in mediating C4 fixation. C4 could be absorbed by bacteria from serum without previous C1 binding in a tight manner that endured washing. The binding of C4 must be presupposed for its function as a C2 acceptor and consequently for the cleavage of C2 by C1 esterase that is already cell-bound and activated. Therefore, C1 absorption with high affinity by the R forms seems to be decisive for a serum-mediated bactericidal effect toward serum-sensitive forms of gram-negative bacteria.

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