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# Antibody-Independent Interaction of the First Component of Complement with Gram-Negative Bacteria

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The interaction of the first component of complement with two serum-sensitive strains of *Escherichia coli* and *Klebsiella pneumoniae* was studied. It could be demonstrated that highly purified $C\bar{1}$, free of immunoglobulin G and immunoglobulin M, binds to *E. coli* or *K. pneumoniae*. C1 binding was also found with specifically absorbed human serum, after incubation of bacteria with normal serum in the presence of ethylenediaminetetraacetate or agammaglobulinemic serum; the number of C1 molecules taken up by the bacteria was not influenced, indicating that C1 binding was independent of naturally occurring antibodies. C1 bound to bacteria was still able to cleave C4, the natural substrate of C1. From these observations, it is concluded that C1 in an enzymatically active state can be bound directly to bacteria independently of antibody.

Normal human serum exhibits bactericidal and bacteriolytic properties with respect to some strains of gram-negative rods; moreover, it opsonizes gram-positive cocci (8). These reactions are thought to be mediated by antibodies and complement (9). More recently, it has been shown that serum from C4-deficient guinea pigs is able to kill gram-negative rods, probably by activation of the alternative pathway of complement (1, 18). Furthermore, it was demonstrated that C1 via its subcomponent C1q directly interacts with bacterial lipopolysaccharides (LPS) and lipid A independently of antibody; the C1 esterase, $C1\bar{s}$, is not affected by these bacterial constituents (10).

Based on these observations, the interaction of the first complement component with two serum-sensitive strains of *Escherichia coli* and *Klebsiella pneumoniae* was studied. Evidence is provided that purified $C\bar{1}$ as well as serum C1 is bound to the bacteria in an esterolytically active form. The bound $C\bar{1}$ molecules, however, were only partially detectable by the C1 fixation and transfer test. Specifically absorbed serum, serum from an agammaglobulinemic patient, and highly purified $C\bar{1}$, free of immunoglobulin G (IgG) and IgM, were used as sources of C1 to demonstrate an antibody-independent interaction of C1 with the bacteria.

The nomenclature used in this paper follows that recommended by the World Health Organization Committee on Complement Nomenclature (19).

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## MATERIALS AND METHODS

**Sera.** Five-tenth-milliliter aliquots from a pool of normal human sera and from individual sera were kept at −60°C.

**Bacteria.** Two serum-sensitive strains of gram-negative rods were used: *E. coli* and *K. pneumoniae* H, which had been isolated from routine specimens. The bacteria were taken from an 18-h broth culture, washed with Veronal-buffered saline (VBS) with sucrose (VBS-S) buffer, counted in a Neubauer chamber, and diluted in VBS-S to $2 \times 10^8$/ml.

**Absorption of human serum with bacteria.** Bacteria prepared as described above were diluted to $5 \times 10^9$/ml. A 1-ml amount of the bacterial suspension was centrifuged, and the supernatant was discarded. The pellet was then resuspended in 1 ml of pooled human serum and incubated for 60 min at 0°C. The bacteria were centrifuged, and the supernatant serum was reabsorbed. Altogether the serum was absorbed three times.

**Complement reagents and assays.** Buffers (VBS with ethylenediaminetetraacetate [EDTA] or VBS-S), preparation of cell intermediates ([EA], EAC4, etc.), and molecular titration of C1 and C4 have been described by Rapp and Borsos (16). All complement titrations were done in the microliter system (17), and the results are expressed as $z$-values or effective molecules per milliliter.

$C\bar{1}$ was purified by double zonal ultracentrifugation according to Colten et al. (5). The particular $C\bar{1}$ preparation used in the experiment yielded $7.8 \times 10^{11}$ effective $C\bar{1}$ molecules per ml, and appropriate dilutions were made from this as described in the text.

Functionally pure C2gp and C4hu were purchased from Cordis Corp., Miami, Fla.

**Determination of IgG and IgM contents of the C1 preparation and of the agammaglobulinemic serum.** IgG contents were kindly determined by A. Morell, Institut für klinisch-experimentelle Tumorfor-

schung, Bern, Switzerland, using a double radioimmunoassay. The C$\bar{1}$ preparation was free of IgG (less than 1 μg/ml); the agammaglobulinemic serum yielded 0.40 mg of IgG1, 0.24 mg of IgG2, 0.007 mg of IgG3, and 0.01 mg of IgG4 per ml. Determination of IgM was done according to Prellwitz et al. (15). Neither in the C$\bar{1}$ preparation nor in the agammaglobulinemic serum could IgM be demonstrated (less than 2 μg/ml).

**C1 transfer test.** The C1 transfer test was done according to Borsos and Rapp (2), with the following minor modifications. In the first (fixation) stage, the C1 source (either as a whole-serum dilution or in purified form) was incubated for 30 min at 0°C with the respective bacteria (diluted to $2 \times 10^8$/ml in VBS-S [$\mu = 0.065$]), using siliconized glass tubes to prevent binding of C1 to the glass surface. The bacteria were then transferred to fresh sets of glass tubes, washed four times, and resuspended in the original volume.

For the second (transfer) stage, 0.1-ml samples of serial dilutions of the bacterial suspension in VBS ($\mu = 0.15$) were incubated with 0.1 ml of EAC4 in VBS for 15 min at 30°C. Subsequently, 0.1 ml of C2gp in sucrose buffer ($\mu = 0.04$) was added, and incubation was continued for 10 min at 30°C. Finally, 1.0 ml of C-EDTA (gp serum diluted 1:33 in VBS-EDTA) was added and incubated for another 60 min at 37°C, and the degree of hemolysis was determined spectrophotometrically at 412 nm. The number of effective C1 molecules was subsequently calculated. Controls included incubation of the respective C1 source in the absence of bacteria followed by the same test procedure.

**C4 inactivation.** A 5-ml amount of bacterial suspension ($2 \times 10^8$/ml) in VBS-S were preincubated for 30 min at 0°C with equal volumes of diluted serum or a dilution of purified C1. Treated bacteria were then washed, transferred to fresh tubes, and resuspended in 5 ml of VBS-S. Samples, 0.5 ml, of the bacterial suspension were incubated for 30 min at 30°C with 0.5 ml of an appropriate dilution of partially purified human C4, and the remaining C4 activity was subsequently determined.

Controls included: (i) buffer plus diluted C4hu; (ii) C1 source without bacteria (similar to the C1 transfer test) plus diluted C4hu; (iii) bacteria without C1 source to exclude inactivation of C4 by untreated bacteria (this control was done separately from the experiments proper).

## RESULTS

**Fixation of highly purified C$\bar{1}$ to bacteria.** To control the following experiments with serum C1, in a first set of experiments bacteria were interacted with highly purified C$\bar{1}$ (see Materials and Methods). A suspension of $2 \times 10^8$ *K. pneumoniae* per ml was incubated with equal volumes of a dilution of purified C$\bar{1}$ for 30 min at 0°C, followed by the C$\bar{1}$ transfer test. The same experiment was also carried out with *E. coli*. The results of this experiment (Table 1) show that C$\bar{1}$ is readily bound under these conditions. However, only 10% of the C$\bar{1}$ molecules exposed to *K. pneumoniae* and only 5% of C$\bar{1}$ exposed to *E. coli* were detected with the fixation and transfer test. This finding raises the question of whether all C$\bar{1}$ molecules bound to the bacteria are transferred under these conditions. Therefore, the number of C$\bar{1}$ molecules available to *E. coli*, the number of C$\bar{1}$ molecules transferred from *E. coli* to EAC4, and the number of C$\bar{1}$ molecules remaining in the supernatant after incubation with *E. coli* were determined and compared to sensitized erythrocytes (EA). The results summarized in Table 2 indicate that 690 C$\bar{1}$ molecules (47.7%) of the 1,447 available C$\bar{1}$ molecules (100%) were taken up by the bacteria, but that only 202 C$\bar{1}$ molecules (29.3%) of the bound C$\bar{1}$ were measured by the transfer test.

**Fixation of C1 to bacteria with serum as the C1 source. (i) Normal human serum.** A standardized population of gram-negative bacteria was exposed to normal human serum at 0°C. Subsequently, the number of fixed C1 molecules was determined. The serum was used in its native form as well as after having been absorbed thrice with the respective bacteria; this was done to remove antibodies which might be present in the serum.

Five-tenths milliliter of a suspension of $2 \times 10^8$ *E. coli* per ml was incubated for 30 min at 0°C with either a 1:1 or a 1:10 serum dilution.

TABLE 1. *Binding of highly purified human C$\bar{1}$ to K. pneumoniae and E. coli*

| Organism[a] | No. of C$\bar{1}$ molecules/bacterium[b] | No. of C$\bar{1}$ molecules bound/ml[c] |
|---|---|---|
| *K. pneumoniae* | 1,000 | $1.5 \times 10^{10}$ |
| | 200 | $2.4 \times 10^9$ |
| *E. coli* | 300 | $2.0 \times 10^9$ |
| | 60 | $4 \times 10^8$ |

[a] $2 \times 10^8$ bacteria per ml.
[b] Incubation for 30 min at 0°C.
[c] Determined by the C$\bar{1}$ transfer test.

TABLE 2. *Comparison of the binding of C$\bar{1}$ to E. coli or EA and detection of the bound C$\bar{1}$ with the C$\bar{1}$ transfer test*[a]

| Binding to: | Effective C$\bar{1}$ molecules | | Total | |
|---|---|---|---|---|
| | C1 transfer | Supernatant | No. | % |
| *E. coli* | 202 | 757 | 959 | 66.3 |
| EA | 644 | 853 | 1,497 | 103.5 |
| Buffer (control) | | 1,447 | 1,447 | 100 |

[a] A total of $2 \times 10^8$ bacteria or EA per ml were incubated with purified C$\bar{1}$ for 30 min at 0°C; afterwards the cells were centrifuged. C$\bar{1}$ bound to the cells and that left in the supernatant were determined and compared to buffer-treated controls.

Afterwards, the bacteria were washed and transferred to fresh tubes. Finally, the number of bound C1 molecules per milliliter of the bacterial suspension was determined by the C1 transfer test (Table 3). It can be seen from the data in Table 3 that the number of bound C1 molecules does not substantially differ in the experiments with untreated and absorbed serum.

If normal serum contains antibodies that mediate C1 fixation, it must be expected that pretreatment of the bacteria with normal serum in the presence of EDTA should increase the number of antibody-dependent C1 binding sites in the C1 binding experiment. To exclude the presence of sensitizing antibodies in normal serum, the following experiment was done. A suspension of *E. coli* ($2 \times 10^8$/ml) was preincubated with undiluted serum containing EDTA at a final concentration of 0.02 M or with buffer at 0°C for 30 min and washed twice. Afterwards, these bacteria were exposed to a 1:20 dilution of normal or absorbed serum as the C1 source. The reaction mixture was incubated for 30 min at 0°C. Finally, the amount of bound C1 was determined. The data in Table 3 indicate that the number of bound C1 molecules is not increased when bacteria are preincubated with normal or absorbed serum.

**(ii) Agammaglobulinemic serum.** In this series of experiments, the serum from a patient with agammaglobulinemia was taken as the C1 source, and the binding of C1 to *E. coli* was determined. No substantial decrease of the number of bound C1 molecules was found in this experiment in comparison with normal serum (Table 3).

**C4 consumption by $C\bar{1}$ bound to bacteria.** The question arises as to whether the C1 molecules as bound from native serum by the surface of bacteria are activated in analogy to the well-known C1 activation after the fixation of this component to immune aggregates (3). This problem was studied by interacting bacteria that previously had been exposed to serum C1 with a preparation of purified human C4. If these bacteria contained fixed C1 in its active form, a demonstrable C4 decrease should be expected in the fluid phase.

An appropriate dilution of partially purified human C4 was incubated for 30 min at 30°C with equal volumes of bacteria after previous exposure of the latter to diluted normal serum. As a control experiment, C4 was exposed also to bacteria that had been pretreated with purified, i.e., activated, $C\bar{1}$. After the incubation with C4, the bacteria were spun down, and the supernatant was tested for remaining C4 activity (Table 4). It can be seen from the data in Table 4 that C4 was inactivated only in those samples in which the bacteria had been previously interacted with serum or $C\bar{1}$ in its purified form.

Those bacteria that had been exposed to normal serum exhibited a C4 consumption entirely comparable to those that had been interacted before with activated $C\bar{1}$. This allows the conclusion that C1 from serum is already activated or became activated after its fixation to the bacterial cell wall.

To exclude the possibility that the observed consumption of C4 is due to some enzymes re-

TABLE 3. *Number of C1 molecules bound to the surface of E. coli after incubation with normal serum, absorbed serum, or serum from a patient with agammaglobulinemia*

| Pretreatment of bacteria[a] | No. of C1 molecules bound[b] |
|---|---|
| NS, 1:1 | $2.2 \times 10^9$ |
| NS, 1:10 | $1.5 \times 10^9$ |
| Absorbed serum, 1:1[c] | $1.6 \times 10^9$ |
| Abssorbed serum, 1:10[c] | $1.1 \times 10^9$ |
| NS, 1:20 | $5.1 \times 10^8$ |
| NS, 1:20-EDTA | $4.0 \times 10^8$ |
| Absorbed serum, 1:20,[c]-EDTA[d] | $3.8 \times 10^8$ |
| NS, 1:10 | $3.8 \times 10^9$ |
| Patient serum, 1:10 | $3.0 \times 10^9$ |

[a] 30 min at 0°C. NS, Normal serum.

[b] Determined per milliliter of the bacterial suspension ($2 \times 10^8$ bacteria per ml).

[c] Absorbed three times with packed *E. coli*; see text.

[d] Bacterial sensitization was carried out with undiluted serum containing EDTA at a final concentration of 0.02 M; after the bacteria were washed, they were exposed to a 1:20 dilution of normal or absorbed serum.

TABLE 4. *Consumption of partially purified human C4 by K. pneumoniae or E. coli preincubated with serum or highly purified* $C\bar{1}$

| Pretreatment[a] | Hemolytic C4 activity ($z$-value) of purified C4 after incubation[b] with: | |
|---|---|---|
| | Buffer | Pretreated bacteria |
| *K. pneumoniae* | | |
| NS, 1:18 | 0.9 | 0.07 |
| 300 $C\bar{1}$ molelcules/bacterium | 0.82 | 0.25 |
| *E. coli*[c] | | |
| NS, 1:54 | 0.522 | 0.094 |
| 150 $C\bar{1}$ molecules/bacterium | 0.506 | 0.149 |

[a] For 30 min at 0°C. NS, Normal serum.

[b] 30 min at 30°C.

[c] $2 \times 10^8$ bacteria per ml.

leased from the bacterial cells, untreated *E. coli* and *K. pneumoniae* ($2 \times 10^8$/ml in VBS-S) were incubated with diluted human C4 for the same time, followed by a test for C4; no consumption of C4 was obtained in these controls (*z*-values: buffer control, 0.427; *E. coli*, 0.477; *K. pneumoniae*, 0.460).

## DISCUSSION

Recently, we presented evidence that LPS of gram-negative bacteria as well as the lipid A portion of LPS interact directly with the first component of complement, $C\bar{1}$ (10). The ligand of $C\bar{1}$ to LPS or lipid A was shown to be the subcomponent C1q; the subcomponent $C1\bar{s}$, the C1 esterase, was not affected (10). This antibody-independent interaction of C1 or C1q with LPS or lipid A was, in the meantime, confirmed by other investigators (12).

The purpose of the present study was to test whether purified $C\bar{1}$ and C1 in serum are also bound to intact gram-negative bacteria independently of antibody. We could demonstrate that C1 from serum, as well as purified $C\bar{1}$, was taken up by serum-sensitive *E. coli* and *K. pneumoniae* (Tables 1 and 3). The C1 fixation and transfer test used for measuring bacteria-bound $C\bar{1}$ was found to be not as useful as for $C\bar{1}$ bound to antigen-antibody complexes (i.e., EA); this indicates that $C\bar{1}$ is bound to bacteria more strongly than to EA (Table 2). This interpretation has been confirmed in studies with LPS-coated erythrocytes; it was found that about 98% of the employed $C\bar{1}$ was bound to LPS-coated erythrocytes, but only 5% was detectable in the transfer test (R. Thesen and M. Loos, manuscript in preparation). The binding of $C\bar{1}$ or serum C1 was found to be antibody independent, indicating a direct binding of C1 to the bacteria tested. Binding of C1 was found regardless of whether normal human serum, human serum previously adsorbed with the respective bacterial strain, or agammaglobulinemic human serum was tested as the C1 source (Table 3).

Recent reports have shown that bacterial LPS as well as intact gram-negative bacteria are able to activate the complement system via the alternative pathway, leading to a consumption of the six terminal components (1, 7, 18).

Endotoxin-coated erythrocytes activate the classical sequence C1, C4, and C2 in the presence of gamma-2-globulin (13). $C\bar{1}$ can also directly be bound with its subcomponent C1q to LPS or lipid A without antibodies (10, 12). Furthermore, we reported that C1 is absorbed to *Mycoplasma pneumoniae* in the absence of antibodies; the direct interaction with C1 had even more biological consequences than the activation of the alternative pathway (4). In addition, a direct interaction with the first component of complement has been shown for RNA viruses (6), lymphocytes (6), and heart mitochondria (14). The experiments presented in this study once more support the finding that a direct interaction of C1 with biological substances is a common phenomenon.

Although we demonstrated that C1 is taken up by bacteria, no conclusion can be made as to which components of the membrane are involved in the binding of C1. The finding that $C\bar{1}$ bound to bacteria is still able to consume C4 supports the interpretation that C1 is bound via its subcomponent C1q, similarly to the interaction of LPS and C1q (10). Studies investigating whether LPS in the intact bacterial cell wall is the binding site for C1 are in progress.

The consumption of C4 by bacteria that previously had been exposed to serum C1 indicates that C1 molecules are bound in their active form. It cannot be decided whether C1 taken up by bacteria was already present in serum in its activated form, as described in an earlier study (11), or whether activation took place after binding to the bacteria. C4 consumption, however, was only found when the bacteria were preincubated with either purified $C\bar{1}$ or serum as a source of C1; buffer-treated bacteria had no effect on C4 (Table 4). It is known from the immunohemolytic system that the C1 subcomponent $C1\bar{s}$, the C1 esterase, cleaves C4 into C4b and C4a; the C4b fragment becomes directly bound to the cell membrane independently of antibody and $C\bar{1}$. The direct interaction of bacteria with C1 may be of biological importance for the early defense against microbial infections.

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