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Antibody-independent interaction between the first component of human complement, C1, and the outer membrane of *Escherichia coli* D31 m4

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The heptoseless mutant of *Escherichia coli*, *E. coli* D31 m4, binds C1q and C1 at 0 °C and at low ionic strength (I 0.07). Under these conditions, the maximum C1q binding averages 3.0×10^5 molecules per bacterium, with a K_a of 1.4×10^8 M^{-1}. Binding involves the collagen-like region of C1q, as shown by the capacity of C1q pepsin-digest fragments to bind to *E. coli* D31 m4, and to compete with native C1q. Proenzyme and activated forms of C1 subcomponents C1r and C1s and their Ca^{2+}-dependent association $(C1r–C1s)_2$ do not bind to *E. coli* D31 m4. In contrast, the C1 complex binds very effectively, with an average fixation of 3.5×10^5 molecules per bacterium, and a K_a of 0.25×10^8 M^{-1}, both comparable with the values obtained for C1q binding. C1 bound to *E. coli* D31 m4 undergoes rapid activation at 0 °C. The activation process is not affected by $C\bar{1}$-inhibitor, and only slightly inhibited by *p*-nitrophenyl *p'*-guanidinobenzoate. No turnover of the $(C1r–C1s)_2$ subunit is observed. Once activated, C1 is only partially dissociated by $C\bar{1}$-inhibitor. Our observations are in favour of a strong association between C1 and the outer membrane of *E. coli* D31 m4, involving mainly the collagen-like moiety of C1.

INTRODUCTION

Activation of the classical complement pathway involves, as a primary event, activation of C1, a Ca^{2+}-dependent complex of subcomponents C1q, C1r and C1s (Reid & Porter, 1981). C1 can be activated by immune complexes and aggregated immunoglobulins, and by a variety of non-immunoglobulin-containing compounds and biological surfaces (Porter, 1980; Esser, 1982, Loos, 1982).

One example of these activators is the lipopolysaccharide of Gram-negative bacteria: the lipid A backbone binds and activates C1, independently of antibody (Loos *et al.*, 1974; Morrison & Kline, 1977; Cooper & Morrison, 1978). Loos *et al.* (1978) showed that C1 bound by certain serum-sensitive strains of Gram-negative bacteria is enzymically active and able to cleave C4, the natural substrate of C1. Betz & Isliker (1981) have also demonstrated that a semi-rough mutant of *Escherichia coli*, the galactose epimerase-deficient strain *E. coli* J5, binds and activates C1, and that this step initiates a bactericidal reaction in the presence of complement components C2–C9. Studies by Tenner *et al.* (1984) have defined the characteristics of C1 binding and activation by *E. coli* J5, and the nature of the control exerted by $C\bar{1}$-inhibitor on C1 activation and $C\bar{1}$ activity.

The studies reported in the present paper were initiated to characterize the antibody-independent binding and activation of C1 by the deep-rough mutant of *E. coli*, the heptoseless strain *E. coli* D31 m4, from which the lipopolysaccharide structure is current being studied (Strain *et al.*, 1983). In contrast with the results obtained by Clas & Loos (1981) with *Salmonella minnesota* R forms, and by Tenner *et al.* (1984) with *E. coli* J5, our results indicate that C1 interaction with *E. coli* D31 m4 only involves C1q, and is mediated by the collagen-like region of this protein.

MATERIALS AND METHODS

Materials

Outdated human citrated plasma was obtained from the Centre de Transfusion Sanguine, Grenoble, France. Serum was prepared from plasma and stored as described previously (Arlaud *et al.*, 1979*a*). Di-isopropyl phosphorofluoridate and type VI soluble collagen were obtained from Sigma Chemical Co. *p*-Nitrophenyl *p'*-guanidinobenzoate hydrochloride was from Merck. Antibiotic 3, yeast extract and Bio-polytone were purchased from BioMerieux, Craponne, France. $Na^{125}I$ (1.55 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Lactoperoxidase (purified grade) was obtained from Calbiochem.

Bacteria and cultivation

E. coli K-12S was obtained from Dr. R. Devoret, Gif-sur-Yvette, France. The deep-rough heptoseless strain *E. coli* D31 m4 and its parental strains D31, D3 and D2 (Boman & Monner, 1975) were generously given by Dr. H. G. Boman, Department of Microbiology, University of Stockholm, Stockholm, Sweden. The strains were cultured in a rich medium (Prehm *et al.*, 1975). Mutant strains were periodically tested for histidine, proline and tryptophan requirements and for ampicillin-resistance (Boman *et al.*, 1971). *E. coli* D31 m4 was also tested for non-viability in the presence of 0.1% (w/v) sodium deoxycholate (Rosner *et al.*, 1979). Exponential-

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phase cultures were prepared by inoculating 1 ml of fresh overnight culture into 500 ml of rich medium, and incubating for 3 h in a shaking water bath at 37 °C. Bacterial concentration was determined from absorbance at 600 mn (A_{600} = 1.0 corresponded to densities of 1×10^9 cells/ml for *E. coli* K-12S and of 2×10^8 cells/ml for *E. coli* D31 m4). Cells were harvested by centrifugation at 2100 *g* for 15 min, then washed twice with 5 mM-sodium Veronal buffer, pH 7.5, containing 70 mM-NaCl, 0.15 mM-$CaCl_2$, 0.5 mM-$MgCl_2$, 2.5% (w/v) glucose and 0.5% (w/v) gelatin ($DGVB^{++}$ buffer), and pellets were stored at −20 °C. Before use, pellets were thawed in the presence of $DGVB^{++}$ buffer, washed twice and resuspended in the same buffer at a density of 10^8 cells/ml.

Complement components

C1q, activated $C\overline{1r}$ and $C\overline{1s}$ were purified as described by Arlaud *et al.* (1979*b*). Proenzyme C1r and C1s were prepared by a modified technique (Arlaud *et al.*, 1980*b*). $C\overline{1}$-inhibitor was purified as described by Reboul *et al.* (1977). Purified C1r ($C\overline{1r}$), C1s ($C\overline{1s}$) and C1q were determined from their absorbance at 280 nm, by using respectively $A^{1\%}_{1cm}$ = 11.5, 9.5 (Sim *et al.*, 1977) and 6.8 (Reid *et al.*, 1972). M_r values were taken to be 85000 for C1r ($C\overline{1r}$), 85000 for C1s ($C\overline{1s}$) and 410000 for C1q. The tetrameric $(C1r\text{–}C1s)_2$ and $(C\overline{1r}\text{–}C\overline{1s})_2$ complexes were reconstructed by mixing C1r ($C\overline{1r}$) and C1s ($C\overline{1s}$) in a 1:1 molar ratio in the presence of 1 mM-$CaCl_2$. Pepsin-digest fragments of C1q were obtained as described by Reid (1976).

C1 subcomponents were labelled with ^{125}I by the method originally described by Heusser *et al.* (1973), as modified by Arlaud *et al.* (1980*a*). C1 was reconstituted by incubating C1q with either C1r and ^{125}I-labelled C1s, or ^{125}I-labelled $(C1r\text{–}C1s)_2$, in the $(C1r\text{–}C1s)_2$/C1q ratio 1.16:1 (w/w), for 30 min at 0 °C in $DGVB^{++}$ buffer.

Binding assay

Unless otherwise specified, binding reaction was initiated by incubation of 1.2×10^6–1.05×10^8 cells with approx. 5×10^{11} effective ^{125}I-labelled C1q molecules in 200 μl of $DGVB^{++}$ buffer for 45 min at 0 °C. Control tubes without bacteria were handled in parallel. The suspension was layered on to 0.75 ml of 5% (w/v) sucrose in 70 mM-NaCl/5 mM-triethanolamine/HCl buffer, pH 7.4, and centrifuged at 9800 *g* for 10 min at 4 °C. Radioactivities of the pellet (containing cell-bound ^{125}I-labelled C1q) and of the supernatant (containing unbound ^{125}I-labelled C1q) were measured.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Reduced samples were prepared by incubation in 4 M-urea/1% (w/v) sodium dodecyl sulphate/50 mM-dithiothreitol/100 mM-Tris/HCl buffer, pH 8.0, for 1 h at 37 °C, then alkylated with 140 mM-iodoacetamide for 20 min at 37 °C. Gels containing 5% (w/v) acrylamide were prepared as described by Fairbanks *et al.* (1971) and run at 5 mA per gel. Protein staining with Coomassie Blue and destaining was according to the method of Weber & Osborn (1969). Gels loaded with ^{125}I-iodinated proteins were cut into 1 mm slices for direct counting of radioactivity in an MR 480 Kontron counter. Activation of C1 was estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of reduced samples, with measurement of the conversion of the single-chain proenzyme proteins into the characteristic A and B chains of their activated forms, of respective apparent M_r 57000 and 35000 ($C\overline{1r}$) or 57000 and 28000 ($C\overline{1s}$). When C1 was reconstructed from ^{125}I-labelled C1s, the A and B chains of $C\overline{1s}$ exhibited 75% and 25% of the radioactivity incorporated in the proenzyme protein; when C1 was reconstructed from ^{125}I-labelled $(C1r\text{–}C1s)_2$, the B chains

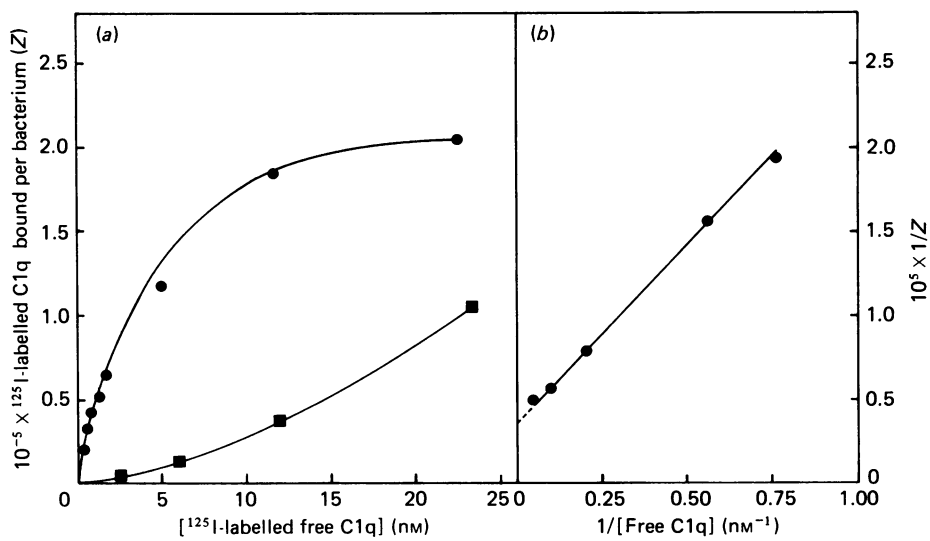

Fig. 1. Binding of ^{125}I-labelled C1q to *E. coli* D31 m4 as a function of free C1q concentration

(*a*) Increasing concentrations of ^{125}I-labelled C1q were incubated with *E. coli* D31 m4 at densities of 6.0×10^6 (●) or 1.05×10^8 (■) cells/ml. Binding was measured as described in the Materials and methods section. (*b*) Lineweaver–Burk plot of the binding measured at the density of 6.0×10^6 bacteria/ml.

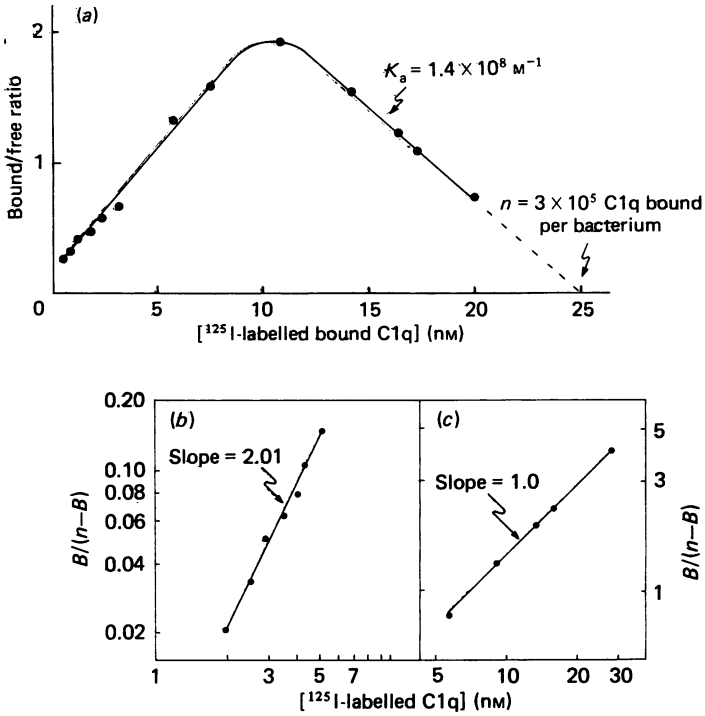

Fig. 2. (*a*) Scatchard plot of the binding of ^{125}I-labelled C1q to *E. coli* D31 m4, and (*b*) and (*c*) Hill plots of the binding data presented in (*a*)

Bacteria (5×10^7 cells/ml) were incubated with various concentrations of ^{125}I-labelled C1q (0.68–19.6 μg/ml). Binding was measured as described in the Materials and methods section. The right-hand side of the curve was used to determine the association constant, K_a, and the number of binding sites per cell, *n*.

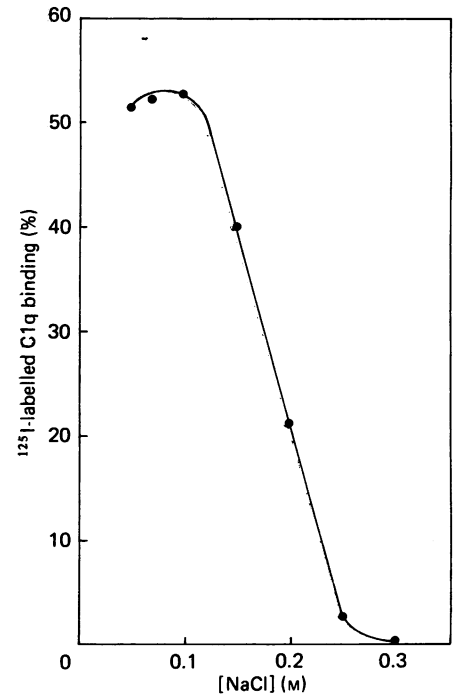

Fig. 3. Effect of ionic strength on the binding of ^{125}I-labelled C1q to *E. coli* D31 m4

Bacteria (5×10^7 cells/ml) were incubated with ^{125}I-labelled C1q (0.8 μg) in the presence of increasing concentrations of NaCl. Binding was measured as described in the Materials and methods section.

of $C\overline{1r}$ and $C\overline{1s}$ represented respectively 57% and 4%, and the A chains 13% and 26%, of the total bound label (Villiers *et al.*, 1982).

RESULTS

Binding of C1q to *E. coli* D31 m4

As illustrated in Fig. 1(*a*), binding of ^{125}I-labelled C1q to *E. coli* D31 m4 was a concentration-dependent saturable process, suggesting the presence of a discrete number of C1q-binding sites on the outer bacterial membrane. Increasing the bacterial density from 6.0×10^6 to 1.05×10^8 cells/ml led to binding curves of sigmoïdal shape. Representation as a Lineweaver–Burk plot (Fig. 1*b*) allowed an estimate of the maximum number of C1q-binding sites at 2.8×10^5 per cell. The affinity of C1q for the membrane binding sites was estimated by using a Scatchard analysis of the binding data. As shown in the typical Scatchard plot presented in Fig. 2(*a*), a $\wedge$-shaped curve was obtained, suggesting a positive binding-site co-operativity at low C1q fixation (corresponding to the left-hand side of the curve). The effect was confirmed by the Hill plot derived from the binding data, which showed two slopes, of values 2.0 (positive co-operative binding; Fig. 2*b*) and 1.0 (non-co-operative binding; Fig. 2*c*). The Scatchard analysis (Fig. 2*a*) indicated the presence of 3.0×10^5 binding sites per cell, corresponding to the value estimated from Fig. 1(*b*), with an association constant (K_a) of 1.4×10^8 M^{-1}. Decreasing the bacterial density was found to decrease the observed binding-site co-operativity, which was abolished for density values below 10^6 bacteria/ml.

Identical binding results were obtained either for fresh cultures of *E. coli* D31 m4 or frozen–thawed bacteria. Parallel experiments performed with the wild-type strain *E. coli* K-12 S or with the semi-rough mutant strains D31, D3 and D2 showed a nearly complete lack of binding of ^{125}I-labelled C1q (0.3–2.0% of the binding obtained with *E. coli* D31 m4).

Kinetic studies of the binding of ^{125}I-labelled C1q to *E. coli* D31 m4 indicated that more than 50% of the binding was achieved within 5 min, whereas maximum binding required 45–60 min. Comparable kinetics were observed when binding was performed either at 0 °C or at ambient temperature, and the amount of membrane-bound C1q remained stable for several hours in these conditions.

The effect of ionic strength on the binding of ^{125}I-labelled C1q to *E. coli* D31 m4 is illustrated in Fig. 3. Optimal binding was obtained at NaCl concentrations 70–100 mM, whereas increasing the NaCl concentration above 100 mM readily decreased C1q binding, which was abolished at concentrations above 250 mM.

The specificity of *E. coli* D31 m4 membrane binding sites for C1q was studied by measuring the ability of unlabelled C1q, the collagenous fragments obtained by pepsin digestion of C1q, and soluble collagen from human placenta, to compete with ^{125}I-labelled C1q for binding sites on the bacterial membrane. As shown in Fig. 4, unlabelled C1q efficiently competed with ^{125}I-labelled

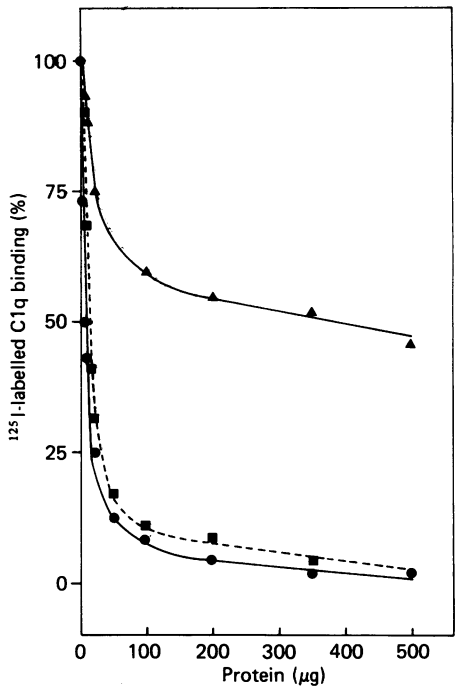

Fig. 4. Competition of ^{125}I-labelled C1q binding to *E. coli* D31 m4 by unlabelled C1q, the collagen-like fragments of C1q, and soluble collagen

Bacteria (5×10^7 cells/ml) were incubated with ^{125}I-labelled C1q (0.95 μg) in the presence of increasing amounts of unlabelled C1q (●), or the collagen-like fragments of C1q (■), or soluble collagen (▲). Binding was assayed as described in the Materials and methods section.

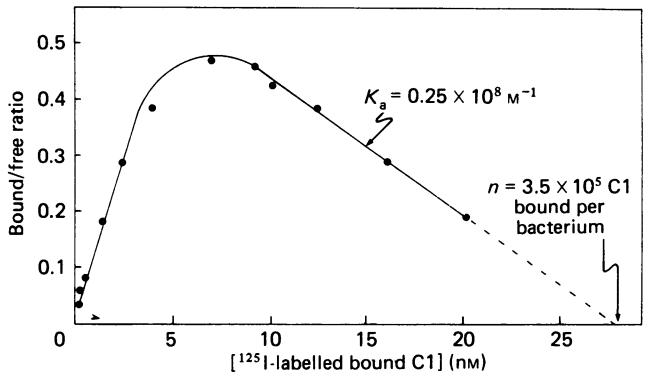

Fig. 5. Scatchard plot of the binding of ^{125}I-labelled C1 to *E. coli* D31 m4

Bacteria (5×10^7 cells/ml) were incubated with various concentrations of ^{125}I-labelled C1 (0.22–8.30 μg/ml) reconstituted from C1q and ^{125}I-labelled $(C\bar{1}r\text{–}C\bar{1}s)_2$ (see the Materials and methods section). Binding was measured as described in the Materials and methods section. The right-hand side of the curve was used to determine the association constant, K_a, and the number of binding sites per cell, n.

C1q, and nearly completely prevented its binding at high concentration. Soluble collagen also competed with ^{125}I-labelled C1q, although 50% of the binding still occurred in the presence of 5 mg of this protein/ml. In the same conditions, the collagenous fragments of C1q were nearly as efficient competitors as the native molecule, thus clearly indicating that the binding was mediated by the collagen-like region of C1q. ^{125}I-labelled collagenous fragments of C1q were shown to bind to *E. coli* D31 m4: Scatchard analysis of the binding gave a ∧-shaped curve comparable with that obtained for native C1q (see Fig. 2*a*), and indicated the presence of 1.2×10^6 binding sites/cell, with an association constant (K_a) of 4×10^7 M^{-1}.

Binding of C1 to *E. coli* D31 m4

Under the same conditions as used for C1q binding (see the Materials and methods section), isolated ^{125}I-labelled C1 subcomponents C1r and C1s, whether in their proenzyme or activated forms, did not significantly bind to *E. coli* D31 m4, and prior binding of C1q to the bacteria did not modify this result. In the same way, both proenzyme and activated forms of the ^{125}I-labelled Ca^{2+}-dependent complex $(C\bar{1}r\text{–}C\bar{1}s)_2$ proved to be unable to bind directly to *E. coli* D31 m4. Prior binding of C1q to the bacteria did not allow subsequent binding of the activated $(\overline{C1r}\text{–}\overline{C1s})_2$ complex, whereas the proenzyme species $(C\bar{1}r\text{–}C\bar{1}s)_2$ was able to bind to C1q-coated cells.

This result was confirmed and made more precise by the use of proenzyme, ^{125}I-labelled C1, reconstituted in the fluid phase either from C1q and ^{125}I-labelled $(C\bar{1}r\text{–}C\bar{1}s)_2$, or from C1q, C1r and ^{125}I-labelled C1s (see the Materials and methods section). In both cases, reconstituted C1 was able to bind to *E. coli* D31 m4; the Scatchard analysis of the binding (Fig. 5), comparable with that obtained for C1q alone (see Fig. 2*a*), indicated 3.5×10^5 binding sites/cell, with an association constant (K_a) of 0.25×10^8 M^{-1}. Binding of ^{125}I-labelled C1 to *E. coli* D3 m4 showed an ionic-strength-dependence comparable with that observed for C1q (see Fig. 3), with a sharper maximum at NaCl concentration 80 mM, and complete inhibition of the binding at concentrations above 250 mM.

Activation of C1 by *E. coli* D31 m4

After incubation for 60 min at 0 °C, analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of C1 bound to *E. coli* D31 m4 indicated that 60% of the molecules were activated, as judged from the activation state of either C1s (Fig. 6*a*) or C1r (results not shown). Parallel analysis performed on supernatants showed that, after the same incubation period, unbound C1 molecules were still in their proenzyme state (Fig. 6*b*). Kinetic experiments indicated that activation proceeded very rapidly, 60% of the C1 molecules being activated after 5 min at 0 °C. At this temperature, the activation level reached a plateau (70% activation after 90 min), whereas 100% activation was obtained after 30 min at 37 °C.

Pretreatment of bacteria with serine-proteinase inhibitors (di-isopropyl phosphorofluoridate, *p*-nitrophenyl *p'*-guanidinobenzoate) had no effect on C1 binding or activation. Addition of inhibitors during incubation showed that di-isopropyl phosphorofluoridate and $C\bar{1}$-inhibitor had no effect on C1 activation, whether incubation was performed at 0 °C or at 37 °C. However, at both temperatures, 1 mM-*p*-nitrophenyl *p'*-guanidinobenzoate induced slight inhibition (20–30%) of C1 activation.

In order to study the control of C1 activity by

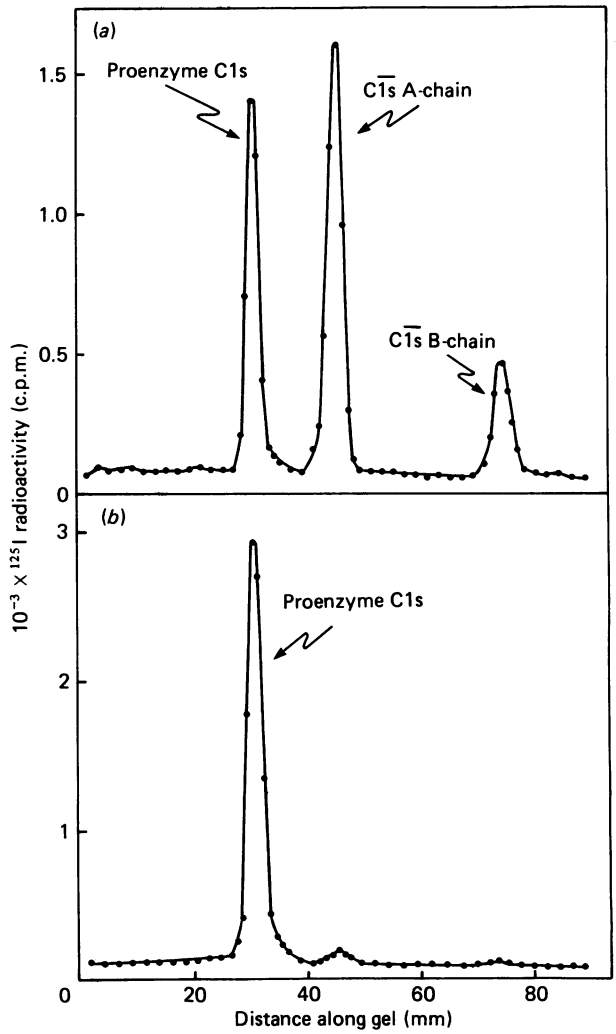

Fig. 6. Activation of ^{125}I-labelled C1 by *E. coli* D31 m4

Bacteria (7.5×10^7 cells/ml) were incubated for 60 min at 0 °C with ^{125}I-labelled C1 (17.5 μg/ml) reconstituted from C1q, C1r and ^{125}I-labelled C1s (see the Materials and methods section). After centrifugation, unbound C1 contained in the supernatant was reduced and alkylated, and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (*b*). Cell-bound C1 was released by 50 μl of DGVB^{++} containing 10 mM-EDTA and 200 mM-NaCl, and analysed as described for unbound C1 (*a*).

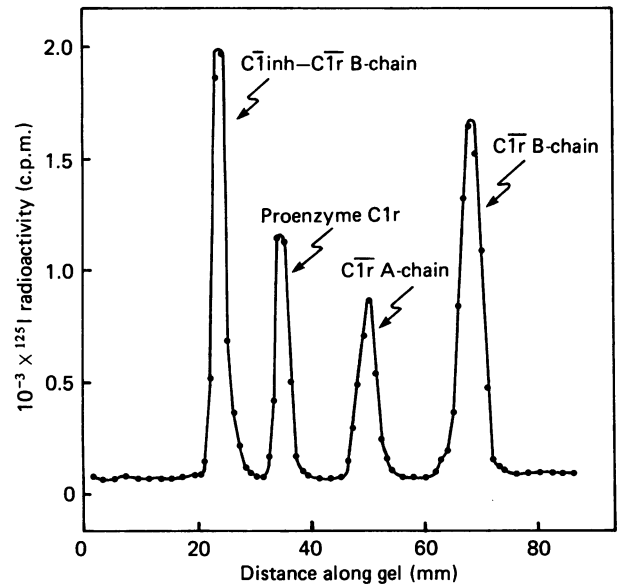

Fig. 7. Control of C1̄ activity by C1̄-inhibitor

Bacteria (7.5×10^5 cells/ml) were incubated for 60 min at 0 °C with ^{125}I-labelled C1 (18.8 μg/ml) reconstituted from C1q and ^{125}I-labelled $(C1r–C1s)_2$ (see the Materials and methods section). Excess C1̄-inhibitor (100 μg/ml) was added and incubation carried out for 30 min at 37 °C. After centrifugation, cell-bound ^{125}I-labelled C1 contained in the pellet was released by 50 μl of DGVB^{++} containing 10 mM-EDTA and 200 mM-NaCl, reduced and alkylated, and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

C1̄-inhibitor, C1 reconstituted from C1q and ^{125}I-labelled $(C1r–C1s)_2$ was used. C1 obtained by this method is heavily labelled on the B-chain moiety of C1̄r, whereas the corresponding region of C1s is poorly labelled (see the Materials and methods section). After incubation of ^{125}I-labelled C1 with *E. coli* D31 m4 for 1 h at 0 °C, cell-bound C1 was incubated with excess C1̄-inhibitor either at 0 °C or at 37 °C. After 1 h at 0 °C, no release of radioactivity could be measured in the fluid phase. In contrast, incubation of cell-bound C1 with C1̄-inhibitor for 30 min at 37 °C induced the release of 30% of the cell-bound radioactivity. Analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the material released in the fluid phase indicated that radioactivity was contained in C1̄ inhibitor–C1̄r B-chain complexes, generated from reduction and alkylation of C1̄-inhibitor–C1̄r complexes (results not shown). The same analysis performed after reduction and alkylation of the material still bound to the bacteria (Fig. 7) showed that 40% of C1r molecules had reacted with C1̄-inhibitor, as evidenced from their occurrence as C1̄ inhibitor–C1̄r B-chain complexes, whereas 60% of C1r (recovered as C1̄r B chain) had not reacted with C1̄-inhibitor.

DISCUSSION

These studies show that proenzyme C1, the first component of human complement, and its subcomponent C1q both interact with the heptoseless mutant *E. coli* D31 m4 in the absence of antibody. C1q binds to *E. coli* D31 m4 with a high affinity (K_a 1.4×10^8 M^{-1}) comparable with the values obtained for C1q binding to other cellular or subcellular membranes (Storrs *et al.*, 1981, 1983).

Optimal C1q binding occurs at ionic strength below physiological value (I 0.07–0.10), and binding is readily inhibited at high ionic strength (I 0.3), thus showing a similar ionic-strength-dependence as observed for C1q binding to heart mitochondrial membranes (Storrs *et al.*, 1981). C1q binding to *E. coli* D31 m4 appears to be mediated via the collagenous portion of the molecule, as judged from: (i) the competition exerted by soluble collagen and collagen-like fragments of C1q on the binding of ^{125}I-labelled C1q to *E. coli* D31 m4, (ii) the capacity of ^{125}I-labelled collagen-like fragments of C1q to bind to *E. coli* D31 m4, with affinity parameters (K_a, number of sites) comparable with those estimated for the whole C1q molecule. Such a binding thus appears

different from that observed by Storrs *et al.* (1983) on heart mitochondrial membranes, as those authors reported that the presence of high concentrations of soluble collagen resulted in only a slight decrease in the membrane binding of ^{125}I-labelled C1q. In the same way, it has been shown that C1q binding to IgG complexes involves the globular heads of the protein (Hughes-Jones & Gardner, 1979). However, C1q binding via its collagen-like region to different cell types, such as human platelets (Wautier *et al.*, 1977), human peripheral blood mononuclear cells (Tenner & Cooper, 1980) or human macrophages U 937 (Arvieux *et al.*, 1984), has been reported.

C1q binding only occurs for the deep-rough heptoseless mutant *E. coli* D31 m4, and no significant binding is observed for parental strains D2, D3 and D31, or the wild-type strain K-12 S. These strains, unlike *E. coli* D31 m4, contain part of the polysaccharide structure of the membrane lipopolysaccharide. Binding of C1q to *E. coli* D31 m4 could thus arise from increased accessibility of the lipid A region of the mutant, in agreement with previous studies (Loos *et al.*, 1974; Morrison & Kline, 1977; Cooper & Morrison, 1978) showing that binding of C1 by isolated bacterial lipopolysaccharide involves the lipid A region of these compounds and the C1q subcomponent of C1. However, involvement of another membrane component in C1q binding cannot be excluded.

It is noteworthy that Scatchard analyses of the binding of C1q or collagen-like fragments of C1q to *E. coli* D31 m4 indicated positive binding-site co-operativity. It is not known whether this effect, which was decreased or abolished either at high C1q concentration or at low bacterial density, reflects a real co-operativity or simply arises from the cellular nature of one of the ligands.

As individual subcomponents C1r and C1s and the Ca^{2+}-dependent subunit $(C1r–C1s)_2$ were not able to bind to *E. coli* D31 m4, it is likely that binding of the C1 complex to the bacterial membrane is also mediated via the C1q subunit, probably through the collagen-like region of this subunit. This is in agreement with the finding that both C1q binding and C1 binding exhibit comparable association constants (1.4×10^8 M^{-1} and 0.25×10^8 M^{-1} respectively) and binding-sites number (3.0×10^5 and 3.5×10^5 molecules/cell respectively). In the same way, both processes are characterized by comparable ionic-strength-dependence. Moreover, it is noteworthy that integrity of the C1 complex was a strict prerequisite for binding of the $(C1r–C1s)_2$ subunit to *E. coli* D31 m4. Thus binding of proenzymic C1 [C1q–$(C1r–C1s)_2$] and hybrid C1 [C1q–$(C1r–C\overline{1}s)_2$], both reconstructed in the fluid phase (Villiers *et al.*, 1982), were obtained, whereas no binding of the activated $(C\overline{1}r–C\overline{1}s)_2$ subunit occurred in the presence of C1q, owing to the lack of reconstruction of fully activated C1 in the fluid phase (Villiers *et al.*, 1982). Therefore binding of C1 to the membrane of *E. coli* D31 m4 appears different from that described in the case of *Salmonella minnesota* (Clas & Loos, 1981), where additional binding to a C1 subcomponent besides C1q was postulated, or in both cases of *E. coli* J5 (Tenner *et al.*, 1984) and murine leukaemia virus (Bartholomew & Esser, 1980), where additional binding of C1s was demonstrated.

C1 activation by *E. coli* D31 m4 was assessed from the use of ^{125}I-labelled C1 reconstituted from either C1q, C1r and ^{125}I-labelled C1s, or C1q and ^{125}I-labelled $(C1r–C1s)_2$, these two systems allowing a study of the conversion of ^{125}I-labelled proenzyme C1s or C1r respectively into their active forms. Most of the activation studies were conducted at 0 °C, conditions where no spontaneous activation of C1 was detectable. At this temperature, C1 activation by *E. coli* D31 m4 proceeded rapidly, and reached 60% after 5 min, a maximum activation of 70% being obtained on prolonged incubation. No release or exchange of the activated $(C\overline{1}r–C\overline{1}s)_2$ subunit or isolated $C\overline{1}r$ and $C\overline{1}s$ subcomponents occurred upon activation, as assessed from the analysis of incubation supernatants, which showed that unbound C1 was entirely proenzyme. As observed in the case of *E. coli* J5 (Tenner *et al.*, 1984), C1 activation by *E. coli* D31 m4 was not prevented by the regulatory protein $C\overline{1}$-inhibitor. It is noteworthy that *p*-nitrophenyl *p'*-guanidinobenzoate slightly inhibited C1 activation, although the inhibition (20–30%) was low compared with that exerted by this inhibitor on spontaneous activation of C1 (Ziccardi, 1982) or C1r (Arlaud *et al.*, 1980*b*), and on C1 activation by antibody–antigen aggregates (Dodds *et al.*, 1978). From both kinetic and inhibition characteristics, *E. coli* D31 m4 thus appears to be a very efficient activator, comparable with *E. coli* J5 (Tenner *et al.*, 1984), although no turnover of the $(C1r–C1s)_2$ subunit of C1 was observed, in contrast with the results obtained by Kilchherr *et al.* (1982) in the case of IgG-mediated C1 activation.

Although $C\overline{1}$ inhibitor did not prevent C1 activation by *E. coli* D31 m4, it was able to react at 37 °C with the activated complex bound to the bacterial membrane. This was assessed fom the formation of ^{125}I-labelled $C\overline{1}$-inhibitor–$C\overline{1}r$ complexes and $C\overline{1}$-inhibitor–$C\overline{1}s$ complexes, identified by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. It is thus likely that reaction of $C\overline{1}$ inhibitor with activated C1 bound to *E. coli* D31 m4 led to the formation of $C\overline{1}$-inhibitor–$C\overline{1}r$–$C\overline{1}s$–$C\overline{1}$-inhibitor complexes, as previously found for C1 bound to other activating systems (Arlaud *et al.*, 1979*a*; Sim *et al.*, 1979; Ziccardi & Cooper, 1979). However, the effect of $C\overline{1}$-inhibitor was partial, as only 60% of activated C1 reacted with the inhibitor, and only half of these reactive molecules were disrupted upon reaction, the rest remaining bound to the bacterial membrane. This result may reflect some heterogeneity of the C1 population bound to the bacterial membrane, which could arise from different degrees in the interaction between the C1 molecules and the cell membrane, and/or differences in the accessibility of these molecules to $C\overline{1}$-inhibitor.

Our results raise the question of the possibility of different alternatives in the binding and/or activation mechanisms of C1 by bacteria in the absence of antibody.

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