Biochem. J. (1989) 257, 865–873 (Printed in Great Britain)

Interaction between complement subcomponent C1q and bacterial lipopolysaccharides

Abdellah ZOHAIR, Serge CHESNE,* Richard H. WADE and Maurice G. COLOMB*
Laboratoire d'Immunochimie, INSERM Unité 238, alliée au CNRS, Université J. Fourier et DRF-Grenoble, et Laboratoire de Biologie Structurale, DRF-Grenoble, Grenoble, France

The heptose-less mutant of *Escherichia coli*, D31m4, bound complement subcomponent C1q and its collagen-like fragments (C1qCLF) with K_a values of 1.4×10^8 and 2.0×10^8 M^{-1} respectively. This binding was suppressed by chemical modification of C1q and C1qCLF using diethyl pyrocarbonate (DEPC). To investigate the role of lipopolysaccharides (LPS) in this binding, biosynthetically labelled [^{14}C]LPS were purified from *E. coli* D31m4 and incorporated into liposomes prepared from phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [PC/PE/LPS, 2:2:1, by wt.]. Binding of C1q or its collagen-like fragments to the liposomes was estimated via a flotation test. These liposomes bound C1q and C1qCLF with K_a values of 8.0×10^7 and 2.0×10^7 M^{-1}; this binding was totally inhibited after chemical modification of C1q and C1qCLF by DEPC. Liposomes containing LPS purified from the wild-strain *E. coli* K-12 S also bound C1q and C1qCLF, whereas direct binding of C1q or C1qCLF to the bacteria was negligible. Diamines at concentrations which dissociate C1 into C1q and $(C1r, C1s)_2$, strongly inhibited the interaction of C1q or C1qCLF with LPS. Removal of 3-deoxy-D-manno-octulosonic acid (2-keto-3-deoxyoctonic acid; KDO) from *E. coli* D31m4 LPS decreases the binding of C1qCLF to the bacteria by 65%. When this purified and modified LPS was incorporated into liposomes, the C1qCLF binding was completely abolished. These results show: (i) the essential role of the collagen-like moiety and probably its histidine residues in the interaction between C1q and the mutant D31m4; (ii) the contribution of LPS, particularly the anionic charges of KDO, to this interaction.

INTRODUCTION

The first step in the activation of the classical complement pathway is the binding of the C1 component via C1q to immune complexes (IC) (Porter & Reid, 1979). This activation leads to the generation of several biological activities that initiate inflammation and facilitate the interaction with various effector cells. In the absence of specific antibodies, C1q can bind to many macromolecules (Cooper, 1985), such as DNA, RNA tumour virus, heparin, polyanions, lipopolysaccharides (LPS) of Gram-negative bacteria and various biological surfaces.

In a direct antibody-independent activation of the classical complement pathway by Gram-negative bacteria, several authors agree on the role of lipid A in the binding and activation of C1 (Loos *et al.*, 1974; Morrison & Kline, 1977; Cooper & Morrison, 1978), involving an interaction with the C1q subcomponent of C1.

In the case of the antibody-independent activation of C1 by the Gram-negative D31m4 heptose-less mutant of *Escherichia coli* K-12, this interaction involves C1q and more specifically its collagen-like region (Aubert *et al.*, 1985).

The present paper analyses, at the molecular level, the interaction between C1q and *E. coli* D31m4. We have determined the region of the C1q molecule involved and the potential amino acid residues concerned with this interaction. Anionic charges of LPS are also shown to be important in this interaction.

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 by Arlaud *et al.* (1979). Collagenase, pepsin, DNAase and RNAase were obtained from Boehringer. Iodogen was from Pierce. Sepharose 4B, Sephadex G-50, Sephacryl 300 were from Pharmacia, CM 52 cellulose was from Whatman. Yeast extract and Bio-polytone were purchased from Bio-Merieux, Craponne, France. Phenylglyoxal (PG), diethyl pyrocarbonate (DEPC), bovine serum albumin (BSA), KDO, phosphatidylcholine (PC) from egg and phosphatidylethanolamine (PE) from *E. coli* were obtained from Sigma. Polyamines were from Merck. ^{14}C-labelled glucose and ^{3}H-labelled DEPC were from Centre d'Energie Atomique, Gif-sur-Yvette, France, $Na^{125}I$ (100 mCi/ml), L-3-phosphatidyl[*N-methyl*-^{3}H]choline, 1,2-dipalmitoyl (1 mCi/ml) were from The Radiochemical Centre, Amersham, Bucks.,

Abbreviations used: BSA, bovine serum albumin; C1qCLF: collagen-like fragments of complement subcomponent C1q; C1qGF, globular fragments of C1q; DEPC, diethyl pyrocarbonate; IC, immune complexes; KDO, 3-deoxy-D-manno-octulosonic acid; LPS, lipopolysaccharides; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phenylglyoxal; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; SUV, small unilamellar vesicles.

* To whom correspondence and reprint requests should be sent, at the following address: DRF/LBIO/ICH, CENG 85X, 38041 Grenoble Cédex, France.

U.K. Yeast alcohol dehydrogenase, horse spleen apoferritin and ox liver catalase were obtained from Calbiochem.

Methods

Bacteria and cultivation. *E. coli* K-12 S was obtained from Institut Pasteur, Paris, France. The deep-rough heptose-less strain *E. coli* D31m4 (Boman & Monner, 1975) was generously given by Dr. H. G. Boman, Department of Microbiology, University of Stockholm, Stockholm, Sweden. The structural characteristics of lipopolysaccharides of both strains are represented in Fig. 1. The strains were cultured in a rich medium containing glucose (Prehm *et al.*, 1975).

The stability of the mutant strain was tested from its requirement for histidine, proline and tryptophan, its ampicillin-resistance (Boman *et al.*, 1971) and its non-viability in the presence of 0.1% sodium deoxycholate (Rosner *et al.*, 1979). Exponential-phase cultures were prepared by inoculating 1 ml of fresh overnight culture into 500 ml of rich medium, and incubating for 3 h in an agitating water bath at 37 °C.

The bacterial concentration was determined from A_{600} (an absorbance of 1.0 corresponded to densities of 1.0×10^9 cells/ml for *E. coli* K-12 S and of 2.0×10^8 cells/ml for *E. coli* D31m4). Cells were harvested by centrifugation at 2100 *g* for 15 min, then washed twice with 5 mM-sodium veronal buffer, pH 7.4, 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 ($DGVB^{++}$), and pellets were stored at −80 °C. Before use, pellets were thawed in the presence of $DGVB^{++}$, washed twice, and resuspended in the same buffer at the required density.

Biosynthetic ^{14}C radiolabelling and purification of ^{14}C-labelled LPS from bacteria. Bacteria were harvested by centrifugation from 50 ml of exponential-phase culture in the presence of [U-^{14}C]glucose (540 μCi; final specific radioactivity 1.96 μCi/μmol), as described above, washed twice with distilled water and freeze-dried. LPS (or [^{14}C]LPS) were purified as described by Galanos *et al.* (1969).

Obtention of the lipid A moiety by removal of KDO from purified ^{14}C-labelled LPS of *E. coli* D31m4. A portion (50 mg) of ^{14}C-labelled LPS in acetic acid 1% (v/v) was incubated for 3 h at 100 °C, neutralized with NH_4OH, then centrifuged at 200000 *g* for 90 min. The pellet was washed three times with distilled water and freeze-dried. The resulting lipid A moiety was resuspended in distilled water for the titration of KDO (Karkhanis *et al.*, 1978) and phosphorus (Itaya & Michio,

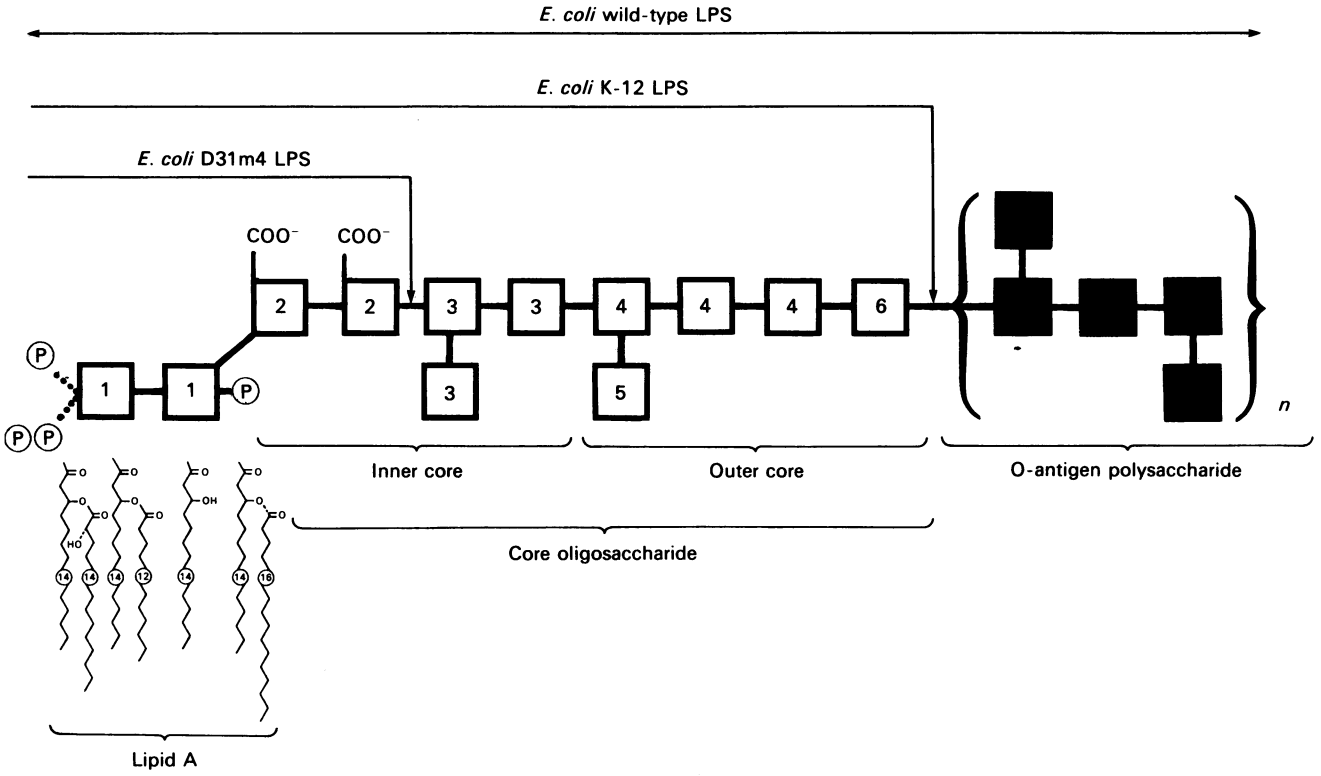

Fig. 1. Schematic representation of LPS from *E. coli*

In the *E. coli* wild-type, the hydrophobic lipid A region is connected to the hydrophilic O-antigen polysaccharide by the core oligosaccharide. The K-12 strain of *E. coli* completely lacks the O-antigen moiety, and loss of the major part of the core conducts to the deep rough heptose-less mutant as D31m4. Numbered squares: 1, D-glucosamine; 2, 3-deoxy-D-manno-octulosonic acid (KDO); 3, L-glycero-D-mannoheptose; 4, D-glucose; 5, D-galactose; 6, *N*-acetyl-D-glucosamine. Black squares indicate O-antigen oligosaccharide units; further abbreviations: P, phosphate group; P-P, pyrophosphate group; COO^-, carboxylate group of KDO. The lipid A backbone structure, glucosaminyl-$\beta(1 \rightarrow 6)$glucosamine, is substituted to different extents by saturated fatty acids, including lauric (dodecanoic) acid, myristic acid and β-hydroxymyristic acid (Rosner *et al.*, 1979). For the KDO content of *E. coli* D31m4, see Strain *et al.* (1983); for the outer-core portion of *E. coli* K-12, see Jansson *et al.* (1981).

1966) or in chloroform for the preparation of liposomes containing lipid A. The supernatant from the centrifugation was also used for the titration of KDO and phosphorus.

Removal of KDO from *E. coli* D31m4. A pellet of *E. coli* D31m4 corresponding to a density of 4.0×10^8 cells/ml was resuspended in acetic acid 1% (v/v). Portions of this suspension were incubated at 100 °C for a period of 1, 2 or 3 h. At the indicated time, samples were removed and bacteria were centrifuged at 2100 *g* from 15 min at 15 °C. Pellets were resuspended and washed three times in $DGVB^{++}$ for protein-binding assays or in distilled water for the titration of KDO. Acetic acid supernatants were also tested for KDO.

Complement components. C1q and proenzymes C1r and C1s were purified as described by Arlaud *et al.* (1979, 1980*b*), C1q collagen-like fragments (C1qCLF) and C1q globular head fragments (C1qGF) according to Sasaki & Yonemasu (1983). Purified C1q, C1qCLF, C1r and C1s were determined from their A_{280} by using $A^{1\%}_{1cm}$ values of 6.8 (Reid *et al.*, 1972), 2.1 (Siegel & Schumaker, 1983), 11.5 and 9.5 (Sim *et al.*, 1977) respectively. M_r values were taken to be 410000 for C1q, 190000 for C1qCLF, 85000 for C1r, 85000 for C1s and 48000 for C1q globular-head fragments. The C1 complex was reconstituted from $(C1r, C1s)_2$ and ^{125}I-labelled C1q (or ^{125}I-labelled C1q modified by DEPC) in a molar ratio 2:1 (w/w), incubated for 30 min at 0 °C in 5 mM-triethanolamine/145 mM-NaCl/5 mM-$CaCl_2$, pH 7.4. C1q or C1qCLF were labelled with ^{125}I by the Iodogen method described by Fraker & Speck (1978), and labelled proteins were kept at 4 °C.

Preparation of insoluble ovalbumin–anti-ovalbumin immune complexes (IC). ICs were prepared as described by Arlaud *et al.* (1979), and were determined from their A_{280} ($A^{1\%}_{1cm}$ 13.0).

Phospholipid vesicles. Small unilamellar vesicles (SUV) were prepared from PC and PE [PC/PE 1:1 (w/w)] as described by Huang & Kennel (1979). Addition of LPS or [^{14}C]LPS to PC/PE [LPS/PC/PE 1:2:2 (by wt.)] gave similar vesicles, as seen by elution profile on a Sepharose 4B-CL column and cryoelectron microscopy using the frozen-hydrated specimen-preparation technique (Dubochet *et al.*, 1985).

Discontinuous sucrose gradient ultracentrifugation. Samples containing lipid vesicles and proteins (see under 'Binding assay' below) were floated through 35% (w/v) sucrose as described (Tschopp *et al.*, 1982). A 0.5 ml portion of the sample, adjusted to 40% (w/v) sucrose, was placed on top of a 0.6 ml cushion of 65% (w/v) sucrose, overlaid with 2.2 ml of 35% (w/v) sucrose and 0.6 ml of buffer [5 mM-veronal/70 mM-NaCl/0.5% (w/v) BSA, pH 7.2], then ultracentrifuged at 110000 *g* for 15 h at 4 °C (Beckman SW 60 rotor).

SDS/PAGE. Gels containing 10 or 12.5% acrylamide were prepared as described by Fairbanks *et al.* (1971). Samples were prepared as described previously (Arlaud *et al.*, 1980*a*). Proteins were stained with Coomassie Blue R 250.

Sucrose-density-gradient ultracentrifugation. Samples were sedimented at 4 °C for 15 h (110000 *g*) as described by Martin & Ames (1961). After centrifugation, fractions (120 μl) were collected from the top of the tubes and the protein was estimated from radioactivity and Coomassie Brilliant Blue G 250 staining as described by Bradford (1976). Yeast alcohol dehydrogenase (7.6 S), ox liver catalase (11.4 S) and horse spleen apoferritin (17.6 S) were used as standards for the estimate of $s_{20,w}$.

Binding assays. Bacteria/^{125}I-labelled proteins (C1q or C1qCLF). Unless otherwise specified, binding was initiated by the incubation of 10^7 cells with ^{125}I-labelled protein (C1q or C1qCLF) in 200 μl of $DGVB^{++}$ for 45 min at 0 °C. Control tubes without bacteria were handled in parallel. The suspension was transferred to 0.75 ml of 5% (w/v) sucrose in 5 mM-triethanolamine/HCl buffer/70 mM-NaCl, pH 7.4, and centrifuged at 9800 *g* for 15 min at 4 °C. Radioactivities of the pellet (containing cell-bound ^{125}I-labelled protein) and of the supernatant (soluble ^{125}I-labelled protein) were measured.

^{125}I-labelled protein incorporation in lipid vesicles. The required amount of vesicles and ^{125}I-labelled protein (C1q or C1qCLF) were incubated in 200 μl of 5 mM-Veronal buffer/70 mM-NaCl/0.5% (w/v) BSA, pH 7.2, for 30 min at 25 °C. Control tubes, with ^{125}I-labelled protein or vesicles were handled in parallel. Then the samples were transferred to a discontinuous sucrose gradient as described previously.

Chemical modification of C1q or C1qCLF. Chemical modifications were made in presence of BSA in order to avoid non-specific binding of C1q (or C1qCLF) to reaction vessels during the modification procedure and assay. SDS/PAGE and sucrose-gradient-centrifugation controls showed that cross-linking of C1q (C1qCLF) to BSA or internal cross-linking of C1q (C1qCLF) did not occur. (*a*) DEPC modification of C1q or C1qCLF. A 20 μg portion of ^{125}I-labelled C1q (or C1qCLF) [2×10^4 c.p.m./μg] was incubated with the desired amount of DEPC at 37 °C for 10 min in 200 μl of 10 mM-phosphate/150 mM-NaCl/0.5% (w/v) BSA, pH 7.2. At this stage, L-histidine was added to the solution (twice the molarity of the DEPC), and the protein was isolated as described by Penefsky (1977), by gel filtration–centrifugation on Sephadex G-50 equilibrated with 10 mM-phosphate/150 mM-NaCl/0.5% (w/v) BSA, pH 7.2. Regeneration of histidine was performed by incubation of the modified protein before the gel-filtration–centrifugation step with 750 mM-hydroxylamine for 30 min at 25 °C in the same buffer. (*b*) Phenylglyoxal modification of arginine residues of C1q. A 20 μg portion of ^{125}I-labelled C1q (2×10^4 c.p.m./μg) was incubated with 2 mM-PG for 20 min at 37 °C in 200 μl of 20 mM-phosphate/150 mM-NaCl/0.5% (w/v) BSA, pH 8.4. Arginine was added to the solution to 16 mM, and ^{125}I-labelled C1q was isolated by gel filtration–centrifugation on Sephadex G-50 in the same buffer.

DNAase or RNAase digestion of the outer membrane surface of bacteria. Aliquots of *E. coli* D31m4 suspension ($A_{600} = 1.0$) were incubated with the appropriate enzyme (0–100 μg of DNAase or 0–500 μg of RNAase) in 1 ml of $DGVB^{++}$ containing 150 mM-NaCl (and 5 mM-$MgCl_2$ in the case of DNAase), for 30 min at 37 °C. Then the bacteria were washed three times with $DGVB^{++}$; binding assays were as described above.

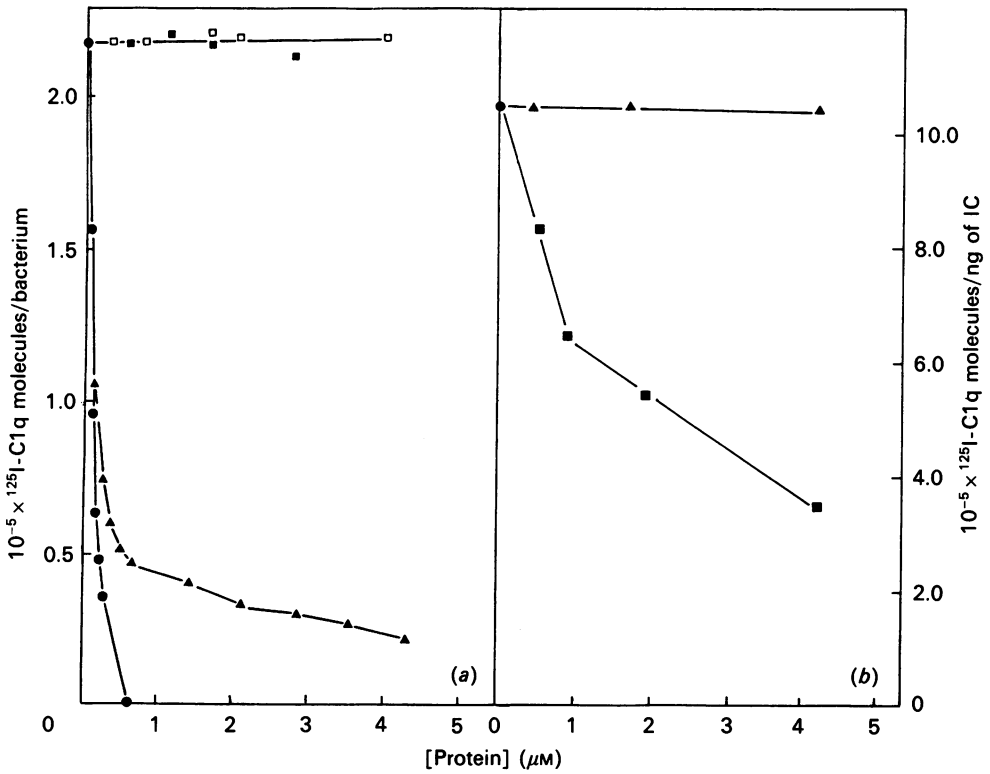

Fig. 2. Competition exerted by (*a*) C1q, C1qCLF, C1qGF and BSA on the binding of ^{125}I-labelled C1q to *E. coli* D31m4, and (*b*) C1qCLF (▲) or C1qGF (■) on the binding of ^{125}I-labelled C1q to IC

(*a*) Bacteria (10^7 cells) were incubated with ^{125}I-C1q (2 nM) in the presence of increasing quantities of unlabelled C1q (●), C1qCLF (▲), C1qGF (■) or BSA (□). Binding was assayed as described in the Materials and methods section. (*b*) IC (12.5 μg) were incubated with ^{125}I-labelled C1q (24 nM) in the presence of increasing amounts of unlabelled C1qCLF (▲) or C1qGF (■) for 30 min at 37 °C. Binding was assayed as described in the Materials and methods section.

RESULTS

Binding of ^{125}I-labelled C1q to *E. coli* D31m4

In a previous detailed study we showed that ^{125}I-labelled C1q binds to the heptose-less mutant *E. coli* D31m4 (150000–300000 molecules per bacterium) at 70 mM-NaCl equivalent ionic strength and at pH 7.4 with K_a of 1.4×10^8 M^{-1} (Aubert *et al.*, 1985). This binding was reversed by unlabelled C1q or the collagen-like fragments (C1qCLF) prepared from C1q (Fig. 2*a*). Neither purified C1q globular-head fragments (C1qGF) nor bovine serum albumin at a concentration of 4 μM were able to displace bound ^{125}I-labelled C1q from the bacteria. The same concentration of C1q globular-head fragments inhibited the binding of C1q to ovalbumin–anti-ovalbumin IC by 70% (Fig. 2*b*). Binding of ^{125}I-labelled C1qCLF to *E. coli* D31m4 was a concentration-dependent and saturable process, with affinity parameters comparable with those estimated for the whole C1q molecule (Aubert *et al.*, 1985), with an average of 150000 molecules per bacterium and a K_a of 2.0×10^8 M^{-1}.

Chemical modification of selective residues

The chemical modification of ^{125}I-labelled C1q by an arginine-selective reagent, PG, did not alter the binding of C1q to *E. coli* D31m4, whereas it led to a 40% decrease of the binding to ovalbumin–anti-ovalbumin IC.

The chemical modification of ^{125}I-labelled C1q by

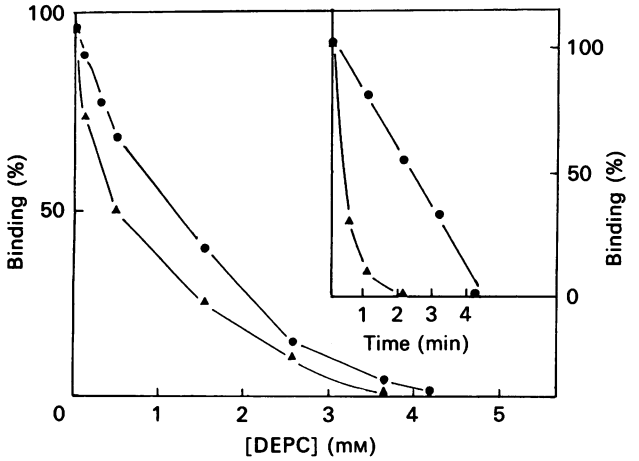

Fig. 3. Modification of C1q or C1qCLF by DEPC: effect on binding to *E. coli* D31m4

^{125}I-labelled C1q (122 nM) or ^{125}I-labelled C1qCLF (263 nM) was treated with increasing amounts of DEPC (0–4 mM) for 10 min as described in the Materials and methods section. Binding of 2 nM-C1q (●) or 4.2 nM-C1qCLF (▲) to 10^7 cells of *E. coli* D31m4 was assayed. Inset: the same concentrations of ^{125}I-labelled C1q or ^{125}I-labelled C1qCLF (see above) was treated with DEPC (2.4 mM) as a function of time. Binding of 2 nM-C1q (●) or 4.2 nM-C1qCLF (▲) to 10^7 cells of *E. coli* D31m4 was assayed.

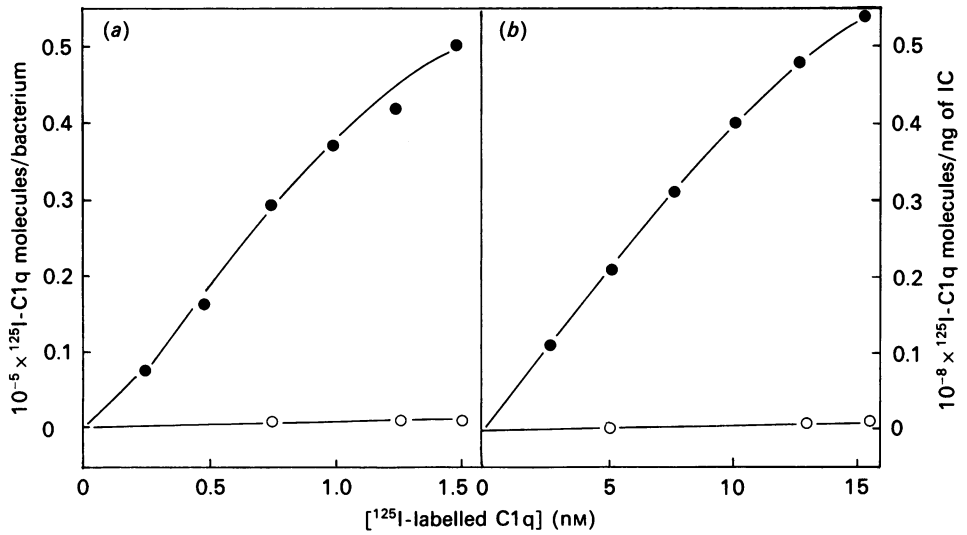

Fig. 4. Modification of C1q by DEPC: effect on the binding to *E. coli* D31m4 (*a*) or ovalbumin–anti-ovalbumin IC (*b*)

Increasing amounts of ^{125}I-labelled C1q, previously modified with 5 mM-DEPC (○) or not modified (●), were incubated with (*a*) *E. coli* D31m4 (10^7 cells) or with (*b*) ovalbumin–anti-ovalbumin IC (25 μg), then the binding was measured.

5 mM-DEPC strongly suppressed the binding of ^{125}I-labelled C1q to *E. coli* D31m4 (Figs. 3 and 4*a*), as well as to ovalbumin–anti-ovalbumin IC (Fig. 4*b*). After incubation of the modified protein in the presence of 750 mM-hydroxylamine [known to regenerate histidine from its DEPC adduct (Miles, 1977)], this inhibition was partially reversed (30%) only in the case of IC. The DEPC modification of C1qCLF completely inhibited the binding of these fragments to *E. coli* D31m4 (Fig. 5). When purified C1q ($s_{20,w}$ 10.5) was incubated with $(C1r, C1s)_2$ ($s_{20,w}$ 8.8) and submitted to centrifugation in a sucrose gradient, a single sedimentation coefficient $s_{20,w}$ of 16.0 was observed, as expected for the resulting C1 complex. By contrast, when DEPC-modified C1q was incubated with $(C1r, C1s)_2$ under the same conditions, two distinct sedimentation coefficients of 10.5 S for DEPC-treated C1q and 8.8 for $(C1r, C1s)_2$ were observed.

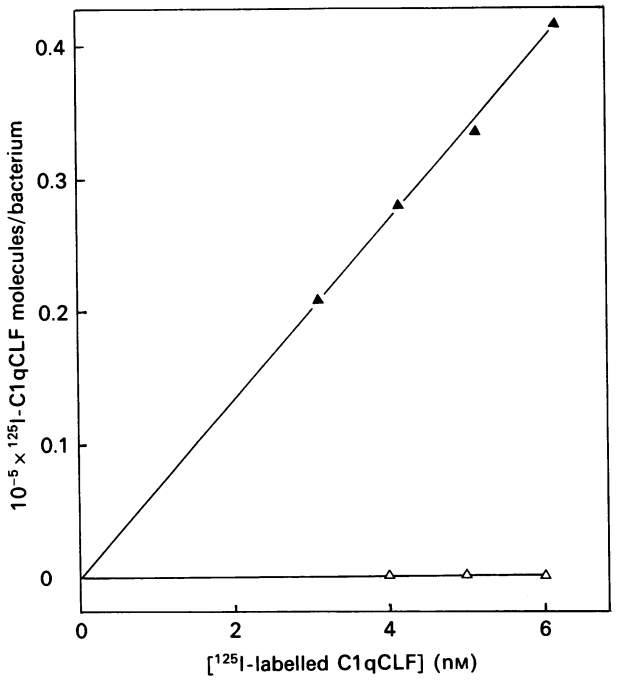

Fig. 5. Modification of C1qCLF by DEPC: effect on the binding of *E. coli* D31m4

Increasing amounts of ^{125}I-labelled C1qCLF were incubated with 10^7 cells of *E. coli* D31m4, then the binding was measured as described in the Materials and methods section. △, DEPC-modified C1qCLF (5 mM-DEPC); ▲, untreated C1qCLF.

Binding of ^{125}I-labelled C1q (or C1qCLF) to liposomes

C1q or C1qCLF did not bind to liposomes without LPS (Fig. 6), whereas the binding of C1q (Figs. 7*a* and

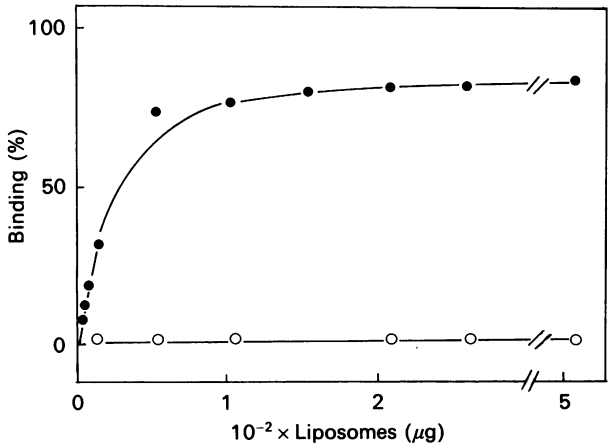

Fig. 6. Binding of ^{125}I-labelled C1q to liposomes

^{125}I-labelled C1q (24 nM) was incubated with increasing amounts of (●) liposomes containing LPS from *E. coli* D31m4 or (○) liposomes without LPS. Binding was measured as described in the Materials and methods section.

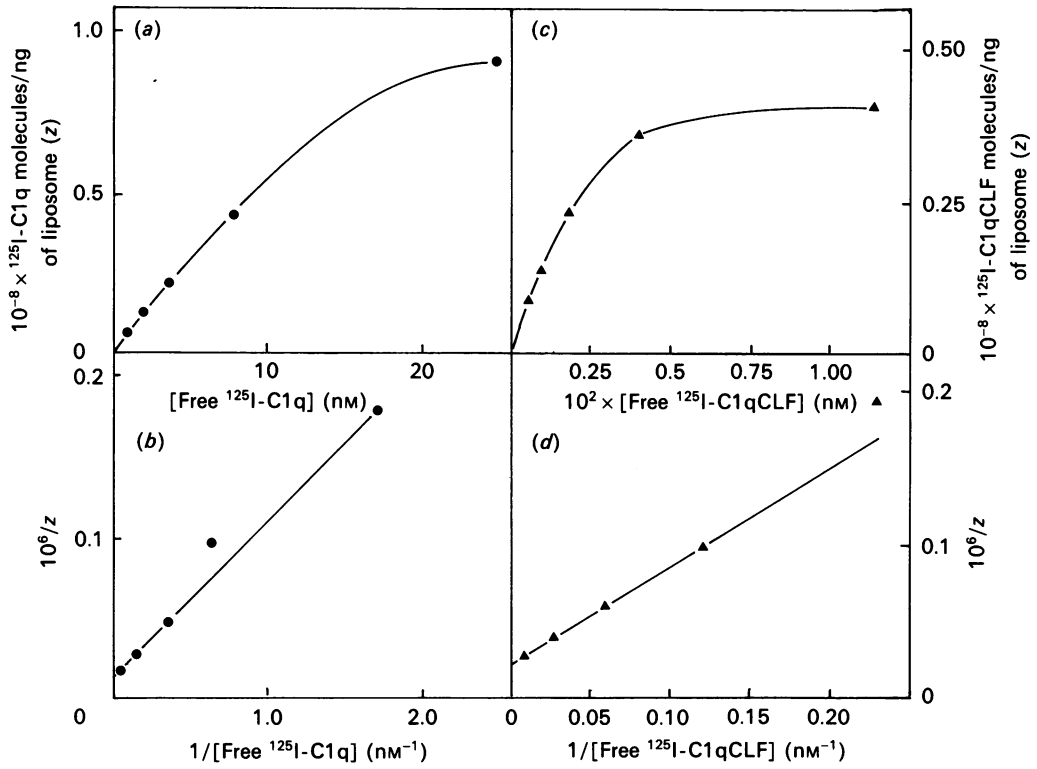

Fig. 7. Binding of ^{125}I-labelled C1q (●) or ^{125}I-labelled C1qCLF (▲) to liposomes containing LPS from *E. coli* D31m4

Liposomes (50 μg, containing LPS from *E. coli* D31m4) were incubated with increasing amounts of ^{125}I-labelled C1q or ^{125}I-labelled C1qCLF (0–5 μg). Binding was measured as described in the Materials and methods section. (*a*) and (*c*) Direct plots of the binding of C1q and C1qCLF; (*b*) and (*d*) corresponding Lineweaver–Burk plots.

7*b*) or C1qCLF (Figs. 7*c* and 7*d*) to liposomes containing LPS from *E. coli* D31m4 was a concentration-dependent and saturable process. Determination of the binding parameters gave an average of 1.25×10^8 and 0.8×10^8 molecules per ng of liposomes–LPS respectively, and with K_a values of 8.0×10^7 and 2.0×10^7 M^{-1} respectively for C1q and C1qCLF. When liposomes containing LPS from *E. coli* K-12 S were used, C1q or C1qCLF bound with an average of 6.25×10^8 and 5.00×10^8 molecules per ng of liposomes–LPS respectively, and with K_a values of 2.4×10^8 and 1.0×10^7 M^{-1} respectively.

After treating the LPS with 1% (v/v) acetic acid at 100 °C for 3 h the titration of KDO in the pellet and supernatant showed that there was no detectable KDO in the lipid A moiety, and all the KDO detected in the same amount of untreated LPS was found in the supernatant. However, this treatment could also affect the phosphate groups of the lipid A moiety. Therefore, a phosphate titration of the supernatant and pellet was performed and showed that the phosphate groups were not affected by this treatment. Similarly, using the same test (KDO titration), it could be shown that when D31m4 bacteria were treated with 1% (v/v) acetic acid at 100 °C for increasing times, the KDO decreased in the pellet and increased in the supernatant (Fig. 8). After 3 h treatment there was 8% residual KDO in the pellet.

The binding of ^{125}I-C1q to liposomes containing the lipid A moiety was 90 times less than that to LPS-liposomes. The ^{125}I-C1qCLF no longer bound to the liposomes containing the lipid A moiety. Tests of binding of C1qCLF to bacteria treated with 1% (v/v) acetic acid at 100 °C showed that there was a strong decrease in binding (65%) after 3 h treatment (Fig. 8).

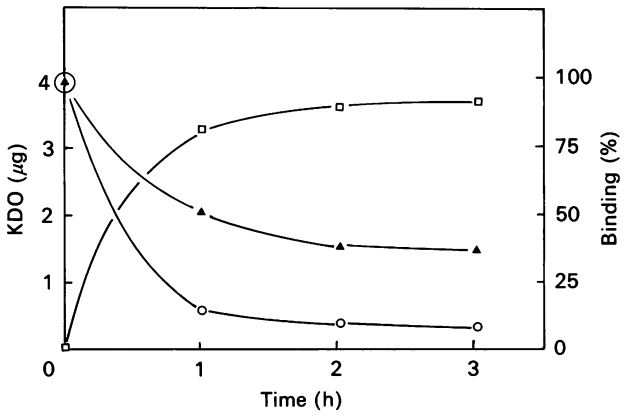

Fig. 8. Acid treatment of *E. coli* D31m4 at 100 °C: effect on the binding of C1qCLF

E. coli D31m4 in 1% (v/v) acetic acid (A_{600} 2.0) were incubated at 100 °C for 1, 2 or 3 h. Portions were centrifuged at 2100 *g* for 15 min at 15 °C and KDO was measured in the pellets (○) and in the supernatants (□). Binding of C1qCLF to acid-treated bacteria was measured as described in the Materials and methods section (▲).

Table 1. Effect of diamines on the binding of ^{125}I-labelled C1qCLF to *E. coli* D31m4 or to liposomes containing LPS from *E. coli* D31m4

Bacteria [*E. coli* D31m4 (10^7 cells)] or liposomes containing LPS from D31m4 (50 μg) were incubated with 52 nM-^{125}I-labelled C1qCLF and 20 mM-diamines. Binding was measured as described in the Materials and methods section.

	Binding	
Diamine	Molecules of ^{125}I-C1qCLF per bacterium	Molecules of ^{125}I-C1qCLF per ng of liposomes–LPS
–	8.3×10^4	8×10^7
Diaminopropane	1.75×10^4	0
Diaminobutane	1.3×10^4	0
Spermidine	0	0

Effect of DEPC on the binding of C1q or C1qCLF to liposomes–LPS

After DEPC modification of histidine residues (Miles, 1977), the binding of ^{125}I-labelled C1q (or C1qCLF) to liposomes containing LPS from *E. coli* D31m4 or K-12 S was completely abolished, as was the binding of modified C1q to *E. coli* D31m4 (Fig. 4*a* and Fig. 5).

Effect of diamines on the binding of C1qCLF to *E. coli* D31m4 or liposomes containing LPS from this mutant

Incubation of ^{125}I-labelled C1qCLF with *E. coli* D31m4 or with liposomes–LPS (Table 1) in the presence of 20 mM-diamines, particularly spermidine, strongly abolished the binding of the protein. It must be emphasized that this concentration is far below the concentration (100 mM) of diamines required to block the binding of C1q to immunoglobulin aggregates (Lin & Fletcher, 1978) and to ovalbumin–anti-ovalbumin IC (Emmanuel *et al.*, 1982). At this concentration (20 mM), C1 was shown to be dissociated into its own moieties, C1q and $(C1r, C1s)_2$ (Villiers *et al.*, 1984).

DISCUSSION

The C1q subcomponent of C1 binds to the heptoseless mutant D31m4 of *E. coli* at pH 7.4 and at physiological ionic strength with an affinity constant (K_a of 1.4×10^8 M^{-1}) (Aubert *et al.*, 1985). The following experimental results argue in favour of specific C1q-binding sites on the external leaflet of the outer membrane of *E. coli* D31m4: (i) the binding is concentration-dependent and saturable (Aubert *et al.*, 1985); (ii) the presence of BSA (5 μM) does not modify the binding; (iii) nuclease treatment of the bacteria does not modify the interaction either [this test excludes the possible interaction of C1q with contaminating nucleic acid (Comis & Easterbrook-Smith, 1985*a*)].

C1q appears to fix to *E. coli* D31m4 by its collagen-like region as: (i) C1q collagen-like fragments decrease the binding of C1q to the bacteria by 80%; (ii) globular-head fragments (5 μM) have no effect on the C1q binding to the mutant D31m4 (the same concentration of globular-head frgaments lowers C1q binding to ovalbumin–anti-ovalbumin IC by 70%); (iii) C1qCLF can bind directly to *E. coli* D31m4 (150000 molecules per bacterium) and with an affinity constant essentially the same as for the whole C1q molecule (Aubert *et al.*, 1985). Treating C1q with PG (2.0 mM), an arginine-residue-specific reagent, has no effect on the binding to *E. coli* D31m4, whereas the binding to ovalbumin–anti-ovalbumin IC decreases by 40%. Thus C1q arginine residues do not appear involved in the interaction with *E. coli* D31m4.

As illustrated in Figs. 3 and 4, the treatment of C1q with DEPC totally abolishes the binding of C1q both to *E. coli* D31m4 and IC. Previous data (Lin & Fletcher, 1978; Easterbrook-Smith, 1983; Comis & Easterbrook-Smith, 1985*b*) have shown that the C1q histidine residues are involved in the binding to IC. C1qCLF treated with DEPC no longer bind to *E. coli* D31m4 (Fig. 5). In addition, this chemical modification of C1q prevents the reconstruction of C1 from its purified subcomponents C1q and $(C1r, C1s)_2$.

These observations point to a predominant role of histidine residues in the collagen part of C1q, both in the binding of C1q to D31m4 outer membrane and to the $(C1r, C1s)_2$ moiety of C1. The following elements appear to support this assessment: (i) the specificity of DEPC to form carbethoxyl groups with nitrogen side chains of histidine residues has been reported (Miles, 1977). Increase of the absorbance at 242 nm was indeed observed upon addition of DEPC to C1q, reflecting the specific carbethoxylation of histidine residues; (ii) other reactivities of DEPC have been described with tyrosine, tryptophan and cysteine which can be ruled out in the present case; a decrease in A_{278} was not observed upon addition of DEPC to C1q, which argues against a reactivity of tyrosine in this case; it is known, from sequence studies, that tryptophan residues are absent in the collagen part of C1q; the only cysteine residue present in each A, B and C chain of the collagen fragment is involved in disulphide bonding. However, another secondary reactivity of DEPC with lysine residues has been described that cannot be ruled out in the present experiments in view of the high amount of hydroxylysine and lysine in the collagen part of C1q.

It remains that the two likely candidates for the binding of DEPC are His-44 in the B chain and His-58 in the C chain, in the 'kink' region of C1q (Reid, 1979), the integrity of which is essential for C1q to interact with $(C1r, C1s)_2$ in C1 (Colomb *et al.*, 1984; Schumaker *et al.*, 1986). The absence of reversibility by hydroxylamine, under the conditions described in the Materials and methods section, could be explained either in terms of bivalent reactivity of the two histidine residues with large excess of DEPC (Miles, 1977) or in terms of irreversible cross-linking of the two histidine residues when they are in the close vicinity of each other.

In the case of Gram-negative bacteria such as *E. coli* it has been suggested that C1q binds to the LPS component of the outer membrane and in particular to lipid A (Cooper, 1985).

The incorporation of *E. coli* D31m4 or K-12 S LPS into artificial liposomes has enabled us to analyse in detail the interaction between C1q and *E. coli* D31m4. From the results given in Figs. 6, 7(*a*) and 7(*c*), it appears quite clear that it is the *E. coli* D31m4 LPS which bind C1q and its collagen-like fragments. Since only a small

amount of C1q is bound to the wild-strain *E. coli* K-12 S (8% compared with D31m4 in our experiments), we tested whether this binding difference also exists between liposomes containing LPS extracted from *E. coli* K-12 S and liposomes containing LPS from *E. coli* D31m4. The results show that the liposomes containing LPS from *E. coli* K-12 S strongly bind C1q and C1qCLF. This difference in binding between the bacteria and the corresponding liposomes can be explained by the accessibility of the lipid A–KDO moiety to C1q or C1qCLF: (i) *E. coli* K-12 S LPS consists of lipid A and a polysaccharide core (Jansson *et al.*, 1981), whereas the LPS of the heptose-less mutant consists of lipid A and only two KDO units (Strain *et al.*, 1983); (ii) the LPS density on the outer membrane of *E. coli* is 10^5 LPS molecules/μm^2 (Kulpa & Leive, 1978), whereas on the surface of a liposome [ϕ (average diameter) = 40 nm] the LPS density is 10^3–10^4 LPS molecules/μm^2 as estimated from the incorporation of *E. coli* D31m4 ^{14}C[LPS]. It is thus probable that the lipid A–KDO region is masked on the membrane surface of *E. coli* K-12 S, but that it is exposed on the liposomes. As C1q and C1qCLF do not bind to LPS incorporated into liposomes after DEPC treatment, it appears that DEPC-sensitive residues of the collagen-like region of C1q are involved in the binding, as observed with bacteria. However, in this last case the involvement of other bacterial membrane components cannot be excluded. The nature of the binding of C1q or C1qCLF to LPS could involve the positive histidine residues on C1q or C1qCLF on one hand and the negative carboxy KDO or phosphate groups of LPS on the other hand. We have used two approaches to test this hypothesis.

(i) Diamines can be used to neutralize the negative charges (KDO and phosphate). Diamines at a concentration of 20 mM, in particular spermidine, totally inhibit the binding of C1q and C1qCLF to *E. coli* D31m4 and to liposomes containing LPS from this mutant (Table 1). The same results were obtained with *E. coli* K-12 S. These results are entirely consistent with those concerning the stability of C1, as these diamines are known to dissociate C1 into C1q and $(C1r, C1s)_2$ (Villiers *et al.*, 1984); they confirm that, in both studies, the collagen-like moiety of C1q is involved. They represent a different reactivity of C1q which has been shown to bind to immunuglobulin aggregates through its globular heads (Cooper & Morrison, 1978; Bragado *et al.*, 1982; Emmanuel *et al.*, 1982; Van Schravendijk & Dwek, 1982) and to IC. In this last case, ionic interaction has also been demonstrated, but here high concentrations (100 mM) of diamines are required to block the binding (Lin & Fletcher, 1978; Allan *et al.*, 1979; Emmanuel *et al.*, 1982).

(ii) The KDO groups can be removed from LPS by hydrolysis with 1% acetic acid at 100 °C. Our results show clearly that the binding of C1q collagen-like fragments to *E. coli* D31m4 is directly related to the presence of KDO in the outer membrane (Fig. 8). In the case of liposomes containing lipid A there is no binding whatever of C1q collagen-like fragments, and it is clear that both KDO groups of the LPS from D31m4 mutant are required to bind C1q or C1q collagen-like fragments.

In conclusion, C1qCLF binding to *E. coli* outer membrane appears to be due mainly to the interaction of cationic residues (probably histidine residues) of the protein and the polyanionic KDO moiety of the lipopolysaccharide. Further studies based on proteolytic peptide purification and sequencing should give precise evidence for the amino acid residues involved in the binding.

We thank Miss Christiane Cazaux and Miss Anne-Marie Cottaz for typing the manuscript.

REFERENCES

Allan, R., Rodrick, M., Knobel, H. R. & Isliker, H. (1979) Int. Arch. Allergy Appl. Immunol. **58**, 140–148
Arlaud, G. J., Sim, R. B., Duplaa, A. M. & Colomb, M. G. (1979) Mol. Immunol. **16**, 445–450
Arlaud, G. J., Chesne, S., Villiers, C. L. & Colomb, M. G. (1980*a*) Biochim. Biophys. Acta **616**, 105–115
Arlaud, G. J., Villiers, C. L., Chesne, S. & Colomb, M. G. (1980*b*) Biochim. Biophys. Acta **616**, 116–129
Aubert, B., Chesne, S., Arlaud, G. & Colomb, M. G. (1985) Biochem. J. **232**, 513–519
Boman, H. G. & Monner, D. A. (1975) J. Bacteriol. **121**, 455–464
Boman, H. G., Jonsson, S., Monner, D., Normark, S. & Bloom, G. D. (1971) Ann. N.Y. Acad. Sci. **182**, 342–357
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Bragado, R., Lopez de Castro, J. A., Juarez, C., Albar, J. B., Garcia, P. A., Ortiz, F. & Vivanco-Martinez, F. (1982) Mol. Immunol. **19**, 579–588
Colomb, M. G., Arlaud, G. J. & Villiers, C. L. (1984) Complement **1**, 69–80
Comis, A. & Easterbrook-Smith, S. B. (1985*a*) FEBS Lett. **185**, 105–108
Comis, A. & Easterbrook-Smith, S. B. (1985*b*) Biochim. Biophys. Acta **842**, 45–51
Cooper, N. R. (1985) Adv. Immunol. **37**, 151–207
Cooper, N. R. & Morrison, D. C. (1978) J. Immunol. **120**, 1862–1868
Dubochet, J., Adrian, M., Lepault, J. & McDowall, A. W. (1985) Trends Biochem. Sci. **10**, 143–146
Easterbrook-Smith, S. B. (1983) Biosci. Rep. **3**, 135–140
Emmanuel, E. J., Brampton, A. D., Burton, D. R. & Dwek, R. A. (1982) Biochem. J. **205**, 361–372
Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) Biochemistry **10**, 2606–2617
Fraker, P. J. & Speck, J. C., Jr. (1978) Biochem. Biophys. Res. Commun. **80**, 849–857
Galanos, C., Lüeritz, O. & Westphal, O. (1969) Eur. J. Biochem. **9**, 245–249
Huang, C. & Kennel, S. J. (1979) Biochemistry **18**, 1702–1707
Itaya, K. & Michio, U. (1966) Clin. Chim. Acta **14**, 361–366
Jansson, P., Lindberg, A. A., Lindberg, B. & Wollin, R. (1981) Eur. J. Biochem. **115**, 571–577
Karkhanis, D., Zeltner, J. Y., Jackson, J. J. & Carlo, D. J. (1978) Anal. Biochem. **85**, 595–601
Kulpa, C. F. & Leive, L. (1978) J. Bacteriol. **126**, 467–477
Lin, T. Y. & Fletcher, D. S. (1978) Immunochemistry **15**, 107–117
Loos, M., Bitter-Suermann, D. & Dierich, M. (1974) J. Immunol. **112**, 935–940
Miles, E. W. (1977) Methods Enzymol. **47**, 431–442
Martin, R. G. & Ames, B. N. (1961) J. Biol. Chem. **236**, 1372–1379
Morrison, D. C. & Kline, L. F. (1977) J. Immunol. **118**, 362–368
Penefsky, H. S. (1977) J. Biol. Chem. **252**, 2891–2899
Porter, R. R. & Reid, K. B. M. (1979) Adv. Protein. Chem. **33**, 1–71

Prehm, P., Stirm, S., Jann, B. & Jann, K. (1975) Eur. J. Biochem. **5**, 41–55
Reid, K. B. M. (1979) Biochem. J. **179**, 367–371
Reid, K. B. M., Lowe, D. M. & Porter, R. R. (1972) Biochem. J. **130**, 749–763
Rosner, M. R., Tang, J. Y., Barzilay, I. & Khorana, H. G. (1979) J. Biol. Chem. **254**, 5906–5917
Sasaki, T. & Yonemasu, K. (1983) Biochim. Biophys. Acta **742**, 122–128
Schumaker, V. N., Hanson, D. C., Kilchherr, E., Phillips, M. L. & Poon, P. H. (1986) Mol. Immunol. **23**, 557–565
Siegel, R. C. & Schumaker, V. N. (1983) Mol. Immunol. **20**, 53–66
Sim, R. B., Porter, R. R., Reid, K. B. M. & Gigli, I. (1977) Biochem. J. **163**, 219–227
Strain, S. M., Fesik, S. W. & Armitage, I. M. (1983) J. Biol. Chem. **258**, 13466–13477
Tschopp, J., Müller-Eberhard, H. J. & Podack, E. R. (1982) Nature (London) **298**, 538
Van Schravendijk, M. R. & Dwek, R. A. (1982) Mol. Immunol. **19**, 1179–1185
Villiers, C. L., Arlaud, G. J. & Colomb, M. G. (1984) Eur. J. Biochem. **140**, 421–426

Received 21 March 1988/11 July 1988; accepted 6 October 1988