C3 binds covalently to the C γ 3 domain of IgG immune aggregates during complement activation by the alternative pathway

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Ovalbumin–antiovalbumin IgG immune aggregates were incubated with normal human serum in the presence of iodo[1- 14 C]acetamide, in conditions in which only the alternative pathway of complement was activated. The [14 C]C3b–IgG covalent complexes formed were digested with pepsin, and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Covalent complexes of [14 C]C3–Fd and [14 C]C3–pFc' were visualized, demonstrating that, during complement activation by the alternative pathway, C3 is covalently incorporated into the C γ 3 domain of IgG, as well as into the Fd region. The C γ 2 domain becomes protected from pepsin action by the bound C3b. All the covalent linkages between C3 and the IgG were sensitive to hydroxylamine. When [14 C]C3–pFc' covalent complexes were treated with 1 M-NH $_2$ OH and loaded onto a Bio-Gel P-4 column, a radioactive peak of 3 kDa was obtained. The material released from [14 C]C3–pFc' and [14 C]C3–F(ab') $_2$ complexes after treatment with 1 M-NH $_2$ OH was mixed and analysed in the Bio-Gel P-4 column. A similar radioactive peak of 3 kDa was obtained. When this peak, either from [14 C]C3–pFc' alone or from the mixture of [14 C]C3–F(ab') $_2$ and [14 C]C3–pFc', was fractionated by h.p.l.c., virtually the same radioactive peptide profile was obtained, indicating that very similar C3 peptides remained covalently bound to both regions (Fab and C γ 3) of the antibody molecule. It is suggested that C3 bound to the C γ 3 domain of IgG may interfere with the Fc–Fc interactions of immune aggregates and thus may be involved in several biological properties displayed by these complement-activating aggregates.

INTRODUCTION

It is well established that upon activation of the complement system by immune aggregates, the C1, C4 and C3 components of complement become tightly bound to the aggregates (Heidelberger, 1941; Müller-Eberhard & Biro, 1963; Müller-Eberhard & Lepow, 1965; Takahashi *et al.*, 1977; Goers & Porter, 1978; Campbell *et al.*, 1980; Ziccardi, 1984). C1 subcomponents C1r and C1s are linked via the bound C1q and are readily removed by treatment with buffers containing EDTA (Dodds *et al.*, 1978). Subcomponent C1q is more strongly bound, since buffers of extreme pH containing a high salt concentration are required to remove it from the aggregates (Arlaud *et al.*, 1979). A major breakthrough was the demonstration that activation of complement by IgG immune aggregates results in the covalent binding of C3 (and C4) to the aggregates (Campbell *et al.*, 1980; Gadd & Reid, 1981b). The formation of covalent bonds between IgG and C3 (and probably C4) is essential for the solubilization of preformed or deposited immune aggregates (Takata *et al.*, 1984) and the inhibition of immune precipitation (Schifferli *et al.*, 1980; Hong *et al.*, 1984). The precipitation reaction between antigen and antibody probably always takes place in the presence of complement *in vivo*. In these processes, both the antigen and the antibody are targets for the covalent binding of C3 (Anh-Tuan *et al.*, 1984; Takata *et al.*, 1984).

Fujita *et al.* (1977) reported that a C3-cleaving activity was formed on the surface of immune aggregates containing ovalbumin and rabbit F(ab') $_2$ after incubation with human serum. Gadd & Reid (1981a) have shown that immune aggregates containing IgG or F(ab') $_2$ antibody caused exactly the same extent of utilization of alternative pathway components, as judged by haemolytic assays, when incubated with human serum under conditions allowing only alternative pathway activation. These data suggested that there was no involvement of the Fc region of IgG in the activation of complement by the alternative pathway and that the site involved in C3b binding was probably located on the Fab region of the IgG molecule. Covalent binding of C3 to the Fd region of heavy and light chains was subsequently demonstrated by several groups (Gadd & Reid, 1981b; Hong *et al.*, 1984; Takata *et al.*, 1984; Antón *et al.*, 1986). Once bound to the antibody, C3b becomes protected from the regulatory action of factors H and I (Fries *et al.*, 1984), although CR1 present on the erythrocyte membrane can overcome this protection, facilitating the proteolytic action of factor I (Fries *et al.*, 1985). C3b covalently bound to F(ab') $_2$ immune aggregates is degraded in serum much faster than when it is bound to IgG complexes, suggesting that the Fc regions are involved in providing a protective environment for C3b bound to the immune aggregates (Antón *et al.*, 1986).

There are conflicting data concerning the nature of the

Abbreviations used: DID, double immunodiffusion; Gdn-HCl, guanidine-hydrochloride; IEP, immunoelectrophoresis; NHS, normal human serum; PBS, phosphate-buffered saline; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis.

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covalent link formed between C3b and IgG. The most convincing evidence supports the theory that C3b is linked to IgG through an ester bond which is sensitive to hydroxylamine (Takata *et al.*, 1984; Antón *et al.*, 1986). However it has also been reported that C3b can form amide bonds (Gadd & Reid, 1981*b*) and that the C3b-IgG covalent binding is destroyed by alkali treatment but not by hydroxylamine treatment (Hong *et al.*, 1984).

Here we show that upon activation of the alternative pathway of complement by IgG immune aggregates in serum, C3b becomes covalently bound to the C γ 3 domain of IgG, in addition to the Fab region, and that the C γ 2 domain is protected by the bound C3b from the proteolytic action of pepsin. All the covalent linkages between C3b and IgG, either on the Fab or on the Fc regions, are sensitive to hydroxylamine when the reaction takes place in the presence of denaturants (guanidine-HCl).

MATERIALS AND METHODS

Materials

Human plasma employed for the isolation of complement components C3 and C1q was obtained from the blood bank of the Fundación Jiménez Díaz (Madrid). Fresh human serum was obtained from healthy donors from this Department. Divalent ions were chelated by passing the serum through a Chelex-100 (Bio-Rad) column, and then Mg $^{2+}$ ions were added to a final concentration of 5 mM and immediately frozen at -80°C (NHS/Chelex). The effectiveness of this procedure was checked by measuring [in an atomic absorption spectrophotometer (Perkin Elmer Mod. 305B)] the concentration of Ca $^{2+}$ ions, which was reduced from 2.12 mM to 0.097 mM (97% depletion); and that of Mg $^{2+}$ ions, which was reduced from 0.798 mM to 0.01 mM (98.7% depletion), after the passage of serum through the Chelex-100 column. DEAE-Sephadex CL-6B, QAE-Sephadex-A50, SP-Sephadex, DEAE-Sephacel, Sephadex G-200 and G-50, CNBr-activated Sepharose 4B and a Mono Q HR 5/5 column were from Pharmacia. Ovalbumin (grade V), Coomassie Brilliant Blue R and di-isopropyl phosphofluoridate were from Sigma. Pepsin (E.C. 3.4.23.1.) was from Worthington. Cellex D, Bio-Gel P-4, Enzymobeads and all chemicals for SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) were from Bio-Rad. Iodo[1- ^{14}C]acetamide (53 mCi/mmol) and ^{125}I (16.6 mCi/ μg of iodine) were from Amersham.

Isolation of complement components

C3 was prepared as previously described (Antón *et al.*, 1986) following the method of Sim *et al.* (1981). It was stored at -80°C in 25 mM-potassium phosphate buffer, pH 7.0, containing 5 mM-EDTA and 250 mM-NaCl. Native C3 was repurified from C3 preparation by f.p.l.c. (Pharmacia) using a Mono Q column. This method separates C3 (H $_2$ O), degraded and aggregated C3 from native C3 (Sánchez-Corral & Vivanco, unpublished work). Purity of C3 was examined by SDS/PAGE, double immunodiffusion (DID) and immunoelectrophoresis (IEP). Haemolytic assays for C3 were carried out according to the method in common use in our laboratory (Sánchez-Corral & Vivanco, unpublished work). C3 was functionally, immunochemically and electrophoretically homogeneous. The protein concentration of C3 preparations was determined from their

absorbance at 280 nm considering an $A_{1\text{cm}}^{0.1\%}$ of 0.97 (Tack & Prahl, 1976). Radiolabelling of C3 with iodo[1- ^{14}C]acetamide was performed as described by Janatova *et al.* (1980). After labelling, excess of iodo[^{14}C]acetamide was removed by dialysis against 0.01 M-phosphate buffer/0.15 M-NaCl, pH 7.2 (PBS) containing 20 mM-nonradioactive iodoacetamide.

Human C1q was purified as previously described (Alcolea *et al.*, 1986). Radioiodination was performed by the peroxidase method (Tenner *et al.*, 1981). A specific activity of $4-6 \times 10^5$ c.p.m./ μg was usually obtained. Purity of C1q preparations was checked by DID and SDS/PAGE and the functional activity assessed by haemolytic assay (Tenner *et al.*, 1981) and by its ability to interact with immune aggregates (Emanuel *et al.*, 1982).

IgG antibodies and antigen-antibody aggregates

The production of rabbit antiovalbumin antiserum, the purification of specific IgG antiovalbumin by affinity chromatography and the preparation of the F(ab') $_2$ fragment have been described in detail (Albar *et al.*, 1981; Antón *et al.*, 1986). IgG was labelled with ^{125}I by the chloramine-T method (McConahey & Dixon, 1966), with a specific activity of 1×10^6 c.p.m./ μg .

Ovalbumin-antiovalbumin aggregates were formed by incubating rabbit IgG at equivalence with optimum amounts of antigen for 60 min at 37°C and 48 h at 4°C in PBS, pH 7.2. The precipitates were washed three times in the same buffer and finally resuspended in 0.15 M-NaCl, 0.01% NaN $_3$ and 1 mM-di-isopropyl phosphofluoridate.

SDS/polyacrylamide-gel electrophoresis

SDS/PAGE was performed as in Laemmli (1970). The running gels used were 10–15% gradients. Two-dimensional gel electrophoresis was carried out basically according to Law & Levine (1977). Standards of high and low M_r from Bio-Rad were used to estimate the apparent M_r of the bands.

Autoradiography of gels containing samples labelled with ^{125}I was carried out by exposing the dried gels to Kodak X-Omat-GRS films (Kodak) at -70°C for different periods of time. Fluorography of gels containing ^{14}C -labelled samples was performed according to Bonner & Laskey (1974), using Kodak X-Omat-S films.

Preparation of immune aggregates bearing covalently-bound C3

IgG immune aggregates containing 0.7 nmol of antibody (105 μg) were washed twice with a 50 mM-Tris/HCl buffer, pH 7.2, containing 0.15 M-NaCl and depleted of Ca $^{2+}$ and Mg $^{2+}$ with Chelex 100; and incubated for 15 min at 37°C with 790 μl of NHS/Chelex. The depletion of Ca $^{2+}$ and the addition of Mg $^{2+}$ ions to serum prevents activation of the classical pathway but allows the activation of the alternative pathway. After the incubation period the reaction was stopped with 1 ml of ice-cold Tris/HCl buffer and the precipitates were washed three times with the same buffer. Control experiments were performed in the presence of 20 mM-EDTA instead of Chelex-100 treatment to remove Ca $^{2+}$ and Mg $^{2+}$. When appropriate, the aggregates were resuspended in sample buffer for SDS/PAGE. In some experiments ^{125}I -C1q (5 μl ; 0.85 mg/ml, 23 500 c.p.m./ μg) was included in the NHS/Chelex or NHS/EDTA in

order to detect the possible incorporation of C1q in the immune aggregates. In a different set of experiments investigating C3b binding to immune aggregates, 6.25 μ Ci of iodo[1- 14 C]acetamide (54 mCi/mmol) were added to NHS/Chelex. After the incubation time (15 min at 37 °C) the precipitates were washed extensively with PBS containing 40 mM-cold iodoacetamide. The iodo[1- 14 C]acetamide is incorporated in the free —SH group of nascent C3b which is released from native C3 during the activation step. Binding of labelled C3b to immune aggregates can then be measured.

Pepsin digestions

Covalent complexes of [14 C]C3–IgG formed as described above were washed, resuspended in 0.1 M-sodium acetate buffer, pH 4.6, and digested with pepsin at an enzyme/substrate ratio of 1:50. The choice of the amount of pepsin and the length of digestion (24 h) was based on the results from preliminary experiments and on the well-known conditions of rabbit IgG digestion with pepsin (Nisonoff *et al.*, 1960). At the end of the digestion, samples were centrifuged 11 500 *g*, 5 min (microfuge Heraeus B) and both the precipitate, [14 C]C3–F(ab')₂, and the supernatant, [14 C]C3–pFc', were collected and resuspended in sample buffer for SDS/PAGE. In some cases the precipitate was resuspended in 200 μ l 0.1 M-NaHCO₃, pH 9.6, containing 2.5 M-guanidine/HCl and 1 M-NH₂OH. The supernatant from the same experiment was mixed with 50 μ l of 0.2 M-NaHCO₃ buffer, pH 9.6, containing 5 M-guanidine/HCl and 100 μ l of 2 M-NH₂OH. Both samples were incubated for 2 h at 37 °C. After this treatment, samples were dialysed against 100 mM-NH₄HCO₃ over MF-Millipore filters (Type VS, 0.025 μ m) in Petri dishes for 30 min at room temperature, lyophilized and resuspended in sample buffer for SDS/PAGE.

Purified C3 that had been treated with 1 M-hydroxylamine in the presence of iodo[1- 14 C]acetamide was dialysed against 0.1 M-acetate buffer, pH 4.6, and digested with pepsin in the same conditions employed with the immune aggregates or at a lower enzyme/substrate ratio. Digested samples were analysed by SDS/PAGE and gel filtration.

Gel filtration on Bio-Gel P-4

Samples of soluble C3 treated with NH₂OH and labelled with iodo[1- 14 C]acetamide were digested with pepsin at an enzyme/substrate ratio of 1:100 and loaded onto a gel filtration column (0.9 cm $\times$ 60 cm) of Bio-Gel P-4 equilibrated in 0.1 M-NH₄HCO₃ buffer. This column was used because preliminary experiments showed that a small C3 radioactive fragment was generated by pepsin digestion of C3 which was not detected by SDS/PAGE and fluorography. Supernatants from [14 C]C3–IgG aggregates digested with pepsin and treated with 1 M-NH₂OH were also fractionated in the Bio-Gel P-4 column. The column was calibrated with serum, glucagon and dinitrophenyl-Lys, and fractions of 0.5 ml were collected.

High performance liquid chromatography

Peaks obtained in the Bio-Gel P-4 column were pooled, lyophilized and analysed by reverse phase h.p.l.c. using a Waters instrument equipped with a μ BondaPak C18 column (0.39 cm $\times$ 30 cm), equilibrated in 10 mM-ammonium acetate buffer, pH 6.5. A linear gradient up

to 30 % acetonitrile was used for 55 min at a flow rate of 1.5 ml/min, and 0.6 ml fractions were collected. Immediately before and after the gradient, 20-fraction sets were collected at initial and final conditions, respectively. Finally, the column was washed with 100 % acetonitrile and the wash was collected for 20 additional fractions. Radioactive peptides were detected by counting aliquots of each fraction added to 4 ml of Aquasol (New England Nuclear) in a Beckman LS 2800 liquid scintillation counter.

RESULTS

Enzymic digestion of immune aggregates carrying covalently-bound C3

IgG immune aggregates carrying covalently-bound [14 C]C3 were digested with pepsin for 24 h at 37 °C. After this period, the mixture was centrifuged and the precipitate and the supernatant were resuspended in buffer for SDS/PAGE and electrophoresed on a 10–15 % gradient gel under reducing conditions (Fig. 1a). Lane 1 in this figure corresponds to IgG immune aggregates and lane 2 to aggregates incubated with NHS/Chelex, with no pepsin digestion (control). In lane 2 there are high-*M_r* bands formed by covalent linkage of the α -chain of C3 with the heavy and light chains of the antibody (Antón *et al.*, 1986). In addition, there are bands corresponding to the β chain of C3, heavy chain of IgG, ovalbumin, a 44 kDa fragment of the α chain of C3 (α -44) and a heterogeneous band of about 30 kDa, mainly corresponding to the light chain of the antibody molecule. This heterogeneous band is bordered by two intense edges (Fig. 1a) which correspond to C1q chains (see below). The precipitate and the supernatant of the digested complexes are shown in lanes 6 and 7 respectively. Lanes 4 and 5 show the precipitate and the supernatant of control immune aggregates incubated for 24 h under the same conditions, but without pepsin. The three main bands of lane 6 correspond to the heavy chain of IgG, ovalbumin and Fd + light chain. A faint band is also detected at the position of the β chain of C3. In the supernatant (lane 7, Fig. 1a) this band is more intensely stained. There is a minor band of heavy chain and a doublet at an *M_r* of about 33 000, which probably corresponds to a C3 fragment (see below). The most intense band of this lane corresponds to the pFc' fragment, appearing at an *M_r* of about 12 000. Similar experiments were performed incubating immune aggregates with NHS/EDTA instead of NHS/Chelex, and the corresponding precipitate and supernatant are shown in tracks 8 and 9. F(ab')₂ immune aggregates alone (lane 12) and incubated with NHS/Chelex (lane 10) are also included as references. It is also worth pointing out the difference in intensity of heavy chains bands (Fig. 1a) in control (lane 8) and in immune aggregates that have incorporated C3 (lane 6). In the latter, more intact residual heavy chain remains, suggesting that the covalently-bound C3 could be protecting the heavy chain from enzymic action of pepsin. Alternatively, the presence of C3 in the immune aggregates could be decreasing the actual enzyme/substrate ratio.

The fluorography of the gel from Fig. 1(a) is presented in Fig. 1(b), in which lanes are numbered to correlate with Fig. 1(a). Lanes 2 and 4 show that only high-*M_r* bands and residual α' chain of C3 (α -65) appear labelled,

Fig. 1. (a) Analysis of pepsin digested IgG immune aggregates bearing covalently bound C3 by SDS/PAGE (10–15% gradient gel) under reducing conditions; (b) fluorography of pepsin digested IgG immune aggregates bearing covalently bound C3

(a) Lane 1, control IgG immune aggregates; lane 2, aggregates incubated with NHS/Chelex; lane 3, markers of M_r ; lanes 4 and 5, precipitate and supernatant respectively of immune aggregates incubated with NHS/Chelex for 15 min at 37 °C and 24 h at 37 °C with sodium acetate buffer, but without pepsin (see text); lanes 6 and 7, precipitate and supernatant, respectively, obtained after the pepsin digestion of immune aggregates incubated with NHS/Chelex; lanes 8 and 9, precipitate and supernatant respectively obtained from immune aggregates incubated with NHS/EDTA and digested with pepsin, as a control; lane 10, F(ab')₂ immune aggregates incubated with NHS/Chelex; lane 11, markers of M_r ; lane 12, control F(ab')₂ immune aggregates. (b) Tracks are numbered as in (a). The displacement of pFc', Fd+light chain and heavy chain bands, in comparison with the corresponding stained bands (a), is indicated.

indicating that most of the radioactivity is incorporated into the α' chain of C3 forming high- M_r complexes with the antibody chains. In lane 6, the major labelled band corresponds to Fd+light chain, but it is shifted to an M_r about 3000 higher than in the stained gel (Fig. 1a, lane 6). The same shift is observed with the heavy chain. A similar situation is observed in lane 7, in which the pFc' band is slightly moved to an M_r of about 15000. This suggests that similar C3 fragments are bound to F(ab')₂ and pFc', increasing their M_r by 3000. By comparing the intensities of Fd+light chain (lane 6) and pFc' (lane 7) bands in Fig. 1(b), a higher incorporation of C3b to the Fab region can be deduced. However, it should be noted

Fig. 2. Analysis of C1q binding on the pepsin digestion of immune aggregates

Lane 1, stained C1q. The remaining bands correspond to an autoradiograph containing ¹²⁵I-C1q (lanes 2–5) or ¹²⁵I-IgG (lanes 6–10). Lane 2, ¹²⁵I-C1q; lane 3, buffer; lane 4, immune aggregates incubated with NHS/EDTA and ¹²⁵I-C1q; lane 5, immune aggregates incubated with NHS/Chelex and the same amount of ¹²⁵I-C1q as in lane 4; lane 6, ¹²⁵I-IgG immune aggregates; lanes 7 and 8, precipitate and supernatant respectively of the pepsin digestion of ¹²⁵I-IgG immune aggregates incubated with NHS/Chelex; lanes 9 and 10, precipitate and supernatant respectively of the pepsin digestion of the same amount of ¹²⁵I-IgG immune aggregates as in lane 7, incubated with NHS/EDTA.

that the distribution of radioactivity between the supernatant and the precipitate does not correspond with that observed in the fluorography (Fig. 1b). Nearly the same number of d.p.m. were detected in the supernatant and in the precipitate in five independent assays. This strongly suggests that C3 may also be bound to the C γ 2 domain which is digested by pepsin to small peptides that are not detected in the gel.

It is remarkable that all the high- M_r bands present in lane 2 (Fig. 1b) were totally digested by pepsin. There were no bands in lanes 6 and 7 (Fig. 1b) corresponding to [¹⁴C]C3-ovalbumin complexes (lane 2, Fig. 1a,b). Thus, this radioactive band should be contributing to the radioactivity detected in the supernatant.

Involvement of C1q in the immune aggregates

Another complement component that could be protecting the heavy chain of the antibody is C1q, which interacts with the C γ 2 domain, the most susceptible region of IgG to pepsin action. The two intense bands which border the Fd+light chain broad band in lane 2 of Fig. 1(a), closely correspond to the M_r of C1q chains, suggesting the presence of C1q in this band. A similar experiment to that described in Fig. 1(a,b) was performed including ¹²⁵I-C1q in the NHS/Chelex or ¹²⁵I-IgG in the immune aggregates, and the result is shown in the autoradiography of Fig. 2. Lane 1 shows a stained gel strip, corresponding to C1q. The same sample, but labelled with ¹²⁵I, is shown in lane 2. Immune aggregates incubated with ¹²⁵I-C1q in the presence of

Fig. 3. Effect of hydroxylamine treatment on the [^{14}C]C3-F(ab') $_2$ and [^{14}C]C3-pFc' covalent complexes

Fluorography of a 10–15% gradient gel. Lanes 1 and 7, [^{14}C]C3 control (C3 α chain); lane 2, [^{14}C]C3-F(ab') $_2$ (precipitate) previous to NH_2OH treatment; lane 3, [^{14}C]C3-pFc' (supernatant) previous to the NH_2OH treatment; lane 4, buffer; lane 5, [^{14}C]C3-pFc' after incubation with NH_2OH ; lane 6, [^{14}C]C3-F(ab') $_2$ after incubation with NH_2OH .

NHS/EDTA or NHS/Chelex appear in lanes 4 and 5, respectively, indicating that C1q is forming part of the aggregates in both cases. A higher incorporation of [^{125}I]-C1q in the presence of EDTA (lane 4) is evident. Lane 6 corresponds to immune aggregates containing [^{125}I]-IgG, as a control. [^{125}I]-immune aggregates were incubated with NHS/Chelex and digested with pepsin. The precipitate is shown in lane 7 and the supernatant in lane 8. When NHS/EDTA and [^{125}I]-IgG immune aggregates were used (lane 9, precipitate; lane 10, supernatant) a remarkable difference was observed with regard to the remaining intact heavy chain. These data suggest that heavy chains that have incorporated C3 in addition to C1q (lane 7), are partially protected from pepsin action, unlike those with only C1q (lane 9).

Effect of hydroxylamine treatment on the C3-IgG covalent complexes

The experiments shown in Fig. 1(a,b) suggested that a small C3 fragment was bound to the Fd and to the C γ 3 domain of IgG, causing both bands to appear at an M_r about 3000 higher than that corresponding to the fragments alone. The precipitate and the supernatant of IA incubated with NHS/Chelex in the presence of iodo[1- ^{14}C]acetamide and digested with pepsin, were then treated with 1 M-hydroxylamine in the presence of 2.5 M-Gdn/HCl. The addition of the denaturant was

essential for releasing the C3 fragment bound to F(ab') $_2$ and pFc' fragments. The fluorography of one such experiment is shown in Fig. 3. Lane 2 corresponds to the precipitate, [^{14}C]C3-F(ab') $_2$. The bulk of the radioactivity is incorporated in the Fd+light chain band. Residual heavy chain is also visualized. When this sample was treated with 1 M- NH_2OH and Gdn/HCl, the result shown in lane 6 was obtained. Most of the radioactivity was released by the NH_2OH . The labelled peptide carrying the [^{14}C]acetamide is small, and was removed while dialysing. A similar situation was found on analysis of the supernatant. The radioactivity incorporated in the pFc' band (lane 3), which should correspond to [^{14}C]C3-pFc' complexes, is released by NH_2OH treatment (lane 5). These results indicate that a small fragment of C3 of about 3 kDa, which includes the thioester region and hence carries the [^{14}C]acetamide, is generated by pepsin digestion of [^{14}C]C3-IgG complexes. The fragment is linked by a covalent bond which is sensitive to hydroxylamine, most probably an ester bond. Two strips of an SDS/PAGE gradient gel, one containing [^{14}C]C3-F(ab') $_2$ and the other containing [^{14}C]C3-pFc', were treated with hydroxylamine and subjected to a second-dimension gel, similar to the first. The radioactive bands observed in the first dimension could not be detected in the second, even when they were exposed for long periods of time (data not shown).

Nature of the C3 fragment covalently bound to the pFc' fragment

Native C3 which had been treated with hydroxylamine in the presence of iodo[1- ^{14}C]acetamide and extensively dialysed, was digested with pepsin at enzyme/substrate ratios of 1:100 or 1:50 for 24 h, and loaded into a Bio-Gel P-4 column. The radioactive profiles obtained are shown in Fig. 4. Peak I eluted in the excluded volume and peaks II and III eluted at an M_r of 3000 and 2000 respectively. On fluorography of an SDS/PAGE gel, peak I showed two bands with an M_r of about 33000 and 35000 (Fig. 4, inset), whereas peaks II and III were not detected in the gel.

When [^{14}C]C3-pFc' complexes were treated with 1 M-hydroxylamine and Gdn/HCl, and loaded into the P-4 column, the radioactive profile shown in Fig. 5 was obtained. The main peak elutes at the same position as peak II in Fig. 4. Complexes of [^{14}C]C3-F(ab') $_2$ and [^{14}C]C3-pFc', incubated with NH_2OH and Gdn/HCl as before, were dialysed and the diffusates were mixed and lyophilized. Afterwards, the mixture was loaded on the Bio-Gel P-4 column, and virtually the same profile was generated (Fig. 5). Fractions of peaks II and III in Fig. 4 were pooled, lyophilized and fractionated by h.p.l.c. on a μ Bondapak C18 column. The resulting peaks are shown in Fig. 6, in which a main radioactive peak elutes in fraction 66 of pool II, while pool III shows a major peak at fraction 20. Peaks from Fig. 5 were separated in pools I and II as indicated in the Figure and fractionated by h.p.l.c. The radioactive peptidic profiles of pools A-II and B-II are shown in Fig. 7. Pool A-II gives a radioactive profile with a main peak at fraction 49, whereas pool B-II produces a similar radioactive profile but with a major qualitative difference in the peak at fraction 29. Likewise, the radioactive peptidic profiles of pools A-I and B-I are shown in Fig. 8. Both pools A-I and B-I gave virtually the same radioactive profile, with minor quantitative differences (around fractions 84–87).

Fig. 4. Gel filtration on Bio-Gel P-4 of [^{14}C]C3 samples digested with pepsin

Digestions were performed at an enzyme/substrate ratio of 1:50 (—) or 1:100 (----). Inset: fluorography of a 10% gel. Lane 1, undigested [^{14}C]C3; lane 2, [^{14}C]C3 digested with pepsin at an enzyme/substrate ratio of 1:100; lane 3, peak I from [^{14}C]C3 digested at an enzyme/substrate ratio of 1:50; lane 4, peak II from the same sample as lane 3. Arrows indicate markers used for the calibration of the column: V_0 , excluded volume; Gluc, glucagon (3 kDa).

Fig. 5. Elution profile on Bio-Gel P-4 of complexes of [^{14}C]C3-pFc' alone or [^{14}C]C3-F(ab') $_2$ plus [^{14}C]C3-pFc' after NH_2OH treatment

A (—) corresponds to [^{14}C]C3-pFc' complexes and B (----) to [^{14}C]C3-F(ab') $_2$ plus [^{14}C]C3-pFc' complexes. Fractions of 0.5 ml were collected and aliquots were counted as described in the Materials and methods section. Fractions corresponding to A-I, A-II, B-I and B-II peaks were pooled, lyophilized and fractionated by h.p.l.c.

These data support the theory that the same C3 fragments are generated on the Fab and pFc' regions of IgG when C3-IgG immune aggregates are digested with pepsin. However, the pepsin digestion of C3 in solution produces fragments of comparable size but apparently different composition to those generated on the immune aggregates.

Fig. 6. Reverse phase h.p.l.c. analysis of pools II (—) and III (----) from Fig. 4

Fig. 7. Reverse phase h.p.l.c. of pools A-II (—) and B-II (----) from Fig. 5

Fig. 8. Reverse phase h.p.l.c. of pools A-I (—) and B-I (----) from Fig. 5

DISCUSSION

Interaction of complement with immune aggregates in serum induces tremendous alterations in the aggregates in terms of their molecular weight, composition and biological functions (Takahashi *et al.*, 1980). Although several complement components become bound to the complexes in this process, the covalent incorporation of C3b into the antigen-antibody lattice is the critical event responsible for two of the major complement functions: the solubilization and the inhibition of precipitation of immune aggregates. During the complement-immune aggregates interaction, C3 convertase produced by the alternative pathway is assembled on the immune precipitates (Fujita *et al.*, 1977; Takahashi *et al.*, 1977) and the subsequent reaction between this complex-bound C3 convertase and serum C3 leads to the deposition of a large number of activated C3 fragments on the precipitates (Takahashi *et al.*, 1978). The formation of the C3 convertase on the immune aggregates implies that the antibody molecule is an activator of complement in which C3b is protected from the enzymatic action of factors I and H. Considering that immune aggregates containing F(ab')₂ or whole IgG are able to consume complement to a comparable extent (Gadd & Reid, 1981a), it is accepted that the assembly of the C3 convertase takes place on the Fab region of the antibody. However, it seems likely that every domain of the antibody molecule is able to interact with C3b. In fact, here we have shown that C3 becomes covalently bound to the C γ 3 domain of IgG (Fig. 1b, lane 7) in addition to the Fd region (Fig. 1b, lane 6). From the intensities of Fd + light chain and pFc' bands (Fig. 1b) it seems that the majority of the bound C3 molecules are bound to the Fab region. However, the same number of d.p.m. were measured in the supernatant and in the precipitates. This strongly suggests that C3b is also bound to the C γ 2 domain. Considering that the number of Fab regions is twice that of the Fc regions, the C γ 2 domain appears to take up, proportionally, a significant number of C3b molecules. These C3b molecules would be responsible for the protection of heavy chains from pepsin action (Fig. 1a, lane 6; Fig. 2, lane 7). In addition, C3b bound to the C γ 2 domain may fulfill the reported regulatory action on C1 (Ziccardi, 1986), preventing further incorporation of C1q on the aggregates. Altogether, these data suggest several questions: (1) Are

all bound C3b molecules able to form the C3 convertase? (2) Is the C3b fixed to the Fab region the only one protected from the action of factors H and I? (3) Is the C3b bound to the Fab or to the Fc regions responsible for the solubilization and/or the inhibition of precipitation? It is well documented that the initial C3b deposition on the immune aggregates occurs very rapidly but that there is no solubilization until the number of C3b molecules deposited is very high (Takahashi *et al.*, 1977). It is conceivable that C3b bound to the C γ 3 and C γ 2 domains of IgG could disrupt the Fc-Fc interactions, which are known to play a major role in immune precipitation (Moller & Bak, 1984). Several other properties of the immune aggregates-bearing C3b, such as the lack of interaction with Fc receptors (Eden *et al.*, 1973) or the regulatory effect on C1 (Ziccardi, 1986), could also be explained by the presence of C3b in the Fc region of the antibody. The participation of the C γ 2 domain as an acceptor site for C3b was only indirectly assessed by pepsin digestion of C3-IgG complexes, because this domain is split into small peptides by the enzyme. Besides, it is apparent that C1q is always bound to immune aggregates in serum, if the antibody belongs to the adequate isotype. This means that some C γ 2 domains will be covered with C1q so that the binding of C3 to the C γ 3 domain will be favoured, although most probably there is a competition between C3 and C1q for the binding to the C γ 2 domain. In fact, when C1q was incubated with immune aggregates in the presence of NHS/EDTA (Fig. 2, lane 4), without C3 competition, a higher amount of C1q was incorporated in the aggregates, than when both components were present simultaneously (Fig. 2, lane 5). In addition, the incorporation of C1q into the immune aggregates does not protect the heavy chain of IgG from the proteolytic action of pepsin but the covalent binding of C3 does (Figs. 1a, 2). C3 bound to the C γ 3 domain could also be protecting the adjacent C γ 2 domain by steric hindrance or, more probably, C3 may interact covalently with the C γ 2. Hence IgG immune aggregates that activate complement by both pathways can be viewed as antigen-antibody lattices in which C1q is covering the C γ 2 domains of some antibody molecules and C3 (and probably C4) is covalently bound all over the antibody molecule and the antigen. The functional implications of the binding of C3b to different regions of the antibody need further investigation.

Pepsin digestion of C3 produces two major fragments containing the thioester region: one of about 33-35 kDa, easily detected by SDS/PAGE (Fig. 4, inset) and another of about 3 kDa only detectable by gel filtration (Fig. 4). The inability to detect small C3 fragments by SDS/PAGE is not a novel event (Harrison & Lachmann, 1980). This small fragment of 3 kDa or a related one was generated by pepsin digestion of C3-IgG immune aggregates. Virtually the same radioactive profile was obtained on the Bio-Gel P-4 and by h.p.l.c. analysis from samples of [¹⁴C]C3-pFc' alone treated with NH₂OH or of [¹⁴C]C3-F(ab')₂ plus [¹⁴C]C3-pFc' treated in the same way (Fig. 5). The inclusion of Gdn/HCl in the reaction mixture was essential for removing the radioactivity, so that the attachment point of the 3 kDa fragment was not accessible to NH₂OH in the absence of denaturants. These data strongly suggest that the same fragments of C3 are bound to the Fab and on the pFc' regions, and there does not seem to be any specific protection from the action of pepsin on C3 bound to either IgG region.

However, it is worth noting that pepsin is able to generate fragments of C3 lower than 3 kDa (pool III, Fig. 4), so that the C3 fragment detected on the immune aggregates is not a terminal fragment. The 3 kDa fragment generated by pepsin digestion of soluble C3 produces a symmetrical peak on gel filtration (Fig. 4) and a main peak on h.p.l.c. (Fig. 6). However, the C3 fragment released either from the F(ab')₂ or pFc' fragments gave rise to an heterogeneous peak on the same P-4 column (Fig. 5), indicating the presence of C3 fragments of different sizes on the digested C3-immune aggregates.

In summary, we have shown that C3 becomes covalently bound to multiple sites on rabbit IgG upon activation of complement by the alternative pathway. All the attached molecules could be removed by hydroxylamine, indicating that every C3 molecule was linked to the immunoglobulin by an ester bond. Of particular interest was the finding that some C3 molecules were bound to the Cγ3 domain of the antibody. The presence of C3 on the Fc areas of immune aggregates allows the understanding of several biological properties displayed by these aggregates which have activated the complement system.

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