Comparison of the role of tyrosine residues in human IgG and rabbit IgG in binding of complement subcomponent C1q

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Treatment of covalently cross-linked or heat-aggregated oligomers of human IgG with 4 mM-tetranitromethane abrogated their C1q-binding activity. In contrast, tetranitromethane modification of rabbit IgG oligomers, under identical conditions, had no effect upon their C1q-binding activity. The tetranitromethane treatment led to nitration of about ten tyrosine residues per IgG molecule in both species, and the modification was specific for tyrosine residues. Reduction of the nitrated protein with $\text{Na}_2\text{S}_2\text{O}_4$ did not lead to recovery of C1q-binding activity in human IgG oligomers or to loss of activity in rabbit IgG oligomers. Tryptic peptides from the nitrated proteins were isolated and a peptide containing nitrotyrosine-319 was recovered from human IgG, as well as peptides from both species corresponding to the region around nitrotyrosine-278. These data are consistent with the inactivation of C1q-binding activity in human IgG being the result of nitration of tyrosine-319; the rabbit IgG is unaffected by nitration because position 319 is phenylalanine. The evidence supports the C1q-receptor site proposed by Burton, Boyd, Brampton, Easterbrook-Smith, Emanuel, Novotny, Rademacher, van Schravendijk, Sternberg & Dwek [(1980) *Nature* (London) **288**, 338–344]: residues 316–338.

INTRODUCTION

The classical complement pathway is activated when its first component C1 interacts via its C1q subcomponent with the Fc regions of aggregated IgG. This interaction provides the link between the recognition of antigen by immunoglobulin and its destruction, mediated by the classical complement pathway (reviewed in [1]). The precise location of the C1q-binding site on IgG is not yet known, despite being the object of many investigations; these studies have used techniques such as enzymic digestion [2,3], amino acid sequence comparison, solvent exposure and charge characteristics of potential binding sites [4,5], properties of specific inhibitors of the interaction [4–11] and chemical modification of amino acid residues on IgG [4,6,7,12–17]. Consideration of the data arising from these studies has led to two regions of the $\text{C}_{\text{H}}2$ domain being proposed as possible C1q-binding sites: residues 275–295 (site A [8,18]) and residues 316–338 (site B [4,16]).

A comparison of the amino acid sequences for the two putative C1q-receptor sites on rabbit IgG with those on human IgG (Fig. 1) reveals an important difference: the tyrosine residue at position 319 (within site B) in human IgG is absent from the corresponding sequence of rabbit IgG, although both species possess Tyr-278 in site A. This difference was exploited, in an attempt to discriminate between the two candidate receptors, by examining the effect of treatment with the tyrosine-selective reagent tetranitromethane on the C1q-binding activity of both human IgG and rabbit IgG. Demonstration of a loss of C1q-binding activity in human IgG (but not rabbit IgG) following specific chemical modification of tyrosine residues under identical conditions would support a role for Tyr-319 (in human IgG) in the C1q-binding process, in agreement with site B.

In the present paper we report that nitration of about ten tyrosine residues per human IgG (but not rabbit IgG) molecule abrogates its C1q-binding activity. Inactivation was likely to be associated with nitration of Tyr-319 within site B. A preliminary account of this work was presented at the 12th Annual Lorne Conference on Protein Structure and Function, Lorne, Vic., Australia, on 11 February 1987.

MATERIALS AND METHODS

Materials

Tetranitromethane was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Dithiobis(succinimidylpropionate) and phenyl isothiocyanate were obtained from Pierce Chemical Co., Rockford, IL, U.S.A. Poly(ethylene glycol) 8000 and $\text{Na}_2\text{S}_2\text{O}_4$ were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A., and May and Baker, Dagenham, Essex, U.K., respectively. Porapak Q was purchased from Waters Chromatography Division, Melbourne, Vic., Australia. Enzymobeads (containing lactoperoxidase and glucose oxidase) were bought from Bio-Rad Laboratories, Richmond, CA, U.S.A., and Na^{125}I was supplied by Amersham International, Amersham, Bucks., U.K. All solutions were prepared with reverse-osmosis water (Millipore Corp., Milford, MA, U.S.A.).

Human C1q

C1q was purified from human serum (courtesy of the Red Cross Blood Bank, Sydney, N.S.W., Australia) and radiolabelled with ^{125}I with the use of lactoperoxidase/glucose; both procedures were performed according to the methods of Tenner *et al.* [19].

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Fig. 1. Candidate C1q-receptor sites on rabbit IgG and human IgG

The amino acid sequences (one-letter code) are given for the currently favoured C1q-binding sites on both rabbit IgG and human IgG.

IgG preparations

IgG was partially purified from serum by repeated precipitation with 18 % (w/v) Na_2SO_4 . This material was further purified by ion-exchange chromatography on a DEAE-Sephacel column (25 cm $\times$ 4 cm) equilibrated in 0.02 M-sodium phosphate buffer, pH 8.0 or 6.3, for human IgG or rabbit IgG respectively. Chemically cross-linked oligomers of IgG were made by the method of Wright *et al.* [20] with the use of dithiobis(succinimidylpropionate), and purified on a Sephacryl S-300 column (90 cm $\times$ 2 cm) equilibrated in phosphate-buffered saline (0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.2). Fractions containing oligomers (predominantly trimers and tetramers) were pooled for use in this study. Heat-induced aggregation of IgG was achieved by incubating IgG (20 mg/ml) at 63 °C for 20 min in phosphate-buffered saline. The supernatant obtained after centrifugation (9000 *g* for 10 min at 20 °C) was designated heat-aggregated IgG. Insoluble immune complexes were prepared from rabbit anti-ovalbumin IgG as described by Emanuel *et al.* [16].

Assays of C1q-binding activity

The assay described previously [21], based on differential protein precipitation with poly(ethylene glycol), was used to assess the C1q-binding activity of soluble IgG oligomers. The binding of C1q to insoluble immune complexes was measured in the same way, except for the omission of poly(ethylene glycol).

Tetranitromethane modification of IgG

A fresh stock solution of 10 mM-tetranitromethane in phosphate-buffered saline was prepared by shaking vigorously 12 μl of 8.37 M-tetranitromethane in 10 ml of phosphate-buffered saline. Human IgG and rabbit IgG aggregates (typically 7 μM) were incubated at 37 °C for 1 h in phosphate-buffered saline in the presence of tetranitromethane at concentrations up to 4 mM. Excess reagent was removed by extensive dialysis of the samples against phosphate-buffered saline.

Stoichiometry of tetranitromethane modification of IgG

The extent of nitration was determined spectrophotometrically. Nitrated samples were diluted in 0.1 M-borate buffer, pH 9.0, and their absorbance at 428 nm was monitored; concentrations of nitrotyrosine residues were determined assuming $\epsilon = 4100 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [22]. Protein concentrations were determined assuming $A_{280} = 1.4$ for a 1 mg/ml solution of IgG [23] or from radioactivity

measurements when trace amounts of ^{125}I -labelled IgG were added after nitration but before dialysis.

Dithionite treatment

Nitrated protein was incubated at room temperature for 5 min with 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$ in phosphate-buffered saline. The extent of reduction (from nitrotyrosine to aminotyrosine residues) was determined from the loss of absorbance at 428 nm and the concomitant gain at 288 nm assuming $\epsilon_{288} = 2200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [24]; samples were dialysed against phosphate-buffered saline before being assayed for C1q-binding activity.

SDS/PAGE

Electrophoresis was done by the method of Laemmli [25] and gels were silver-stained by the method of Wray *et al.* [26].

Amino acid analysis

This was performed with a Waters Pico-Tag analysis system, according to the manufacturer's instructions, on samples that had been hydrolysed in 6 M-HCl for 24 h at 110 °C *in vacuo*.

H.p.l.c.

H.p.l.c. was performed in an LKB 2150/1/2 system obtained from LKB, Bromma, Sweden. Plots and calculations were recorded with a LDC/Milton Roy CI-10 integrator and Sekonics thermal printer. Separations were achieved on an LKB Lichrosorb C_{18} (10 μm particle size) reverse-phase column (25 cm $\times$ 0.4 cm).

Tryptic digestion

Nitrated protein (10 mg/ml in phosphate-buffered saline) was incubated at 37 °C with 0.01 M-dithiothreitol for 1 h; the reaction mixture was then made 0.02 M with respect to iodoacetamide and incubated at room temperature in the dark for 30 min. The reduced and alkylated IgG was then separated from the reaction products by extensive dialysis against 0.1 M-ammonium bicarbonate buffer, pH 8.4. The nitrated protein (approx. 5 mg/ml in 0.1 M-ammonium bicarbonate buffer, pH 8.4) was incubated at 37 °C for 1 h with trypsin (1 % by wt. of protein) and then for a further 30 min with another 1 % of trypsin.

Porapak Q chron.atography of nitrotyrosine-containing peptides

Affinity purification of nitrotyrosine-containing peptides was carried out by using a protocol based on the

Fig. 2. Effect of tetranitromethane treatment on the C1q-binding activity of IgG oligomers

(a) Chemically cross-linked human IgG (□ and ■) or rabbit IgG (○ and ●) oligomers were incubated in the presence (■ and ●) or in the absence (□ and ○) of 4 mM-tetranitromethane, then dialysed against phosphate-buffered saline, and the C1q-binding activity was determined as described in the Materials and methods section. (b) Heat-aggregated IgG oligomers were treated with 4 mM-tetranitromethane, then dialysed against phosphate-buffered saline, and the C1q-binding activity was determined as described in the Materials and methods section. The key to symbols is the same as for (a).

work of Niederwieser [27]. Tryptic digests of nitrated IgG were adjusted to pH 2 with HCl and then applied to a 2 ml column of Porapak Q equilibrated with 0.1 M-HCl at room temperature. The column was washed with approx. 10 ml of 0.1 M-HCl to remove unbound peptides. Nitrotyrosine-containing peptides were then eluted with acetonitrile/0.1 M-sodium acetate, pH 5 (1:1, v/v), and subjected to h.p.l.c. analysis.

RESULTS

Effect of tetranitromethane treatment on the C1q-binding activity of IgG

Treatment of chemically cross-linked aggregates of human IgG with the tyrosine-selective reagent tetranitromethane decreased their C1q-binding activity; no such activity loss was exhibited by chemically cross-linked rabbit IgG oligomers that had been treated identically (Fig. 2a). A similar trend was observed when the C1q-binding activity of heat-aggregated IgG oligomers was examined after tetranitromethane treatment, with human IgG but not rabbit IgG oligomers showing diminished activity upon nitration (Fig. 2b). The C1q-binding activity of rabbit IgG in the form of insoluble immune complexes was also unaffected by tetranitromethane modification (Fig. 3). Together, these results suggest the presence of critical tyrosine residues at or near the C1q-binding site of human IgG (but not rabbit IgG).

Specificity of IgG modification

The spectral changes observed upon nitration of both human IgG and rabbit IgG aggregates were consistent with the modification of tyrosine residues; the nitrated protein in alkaline conditions exhibited a maximum at 410 nm, which is characteristic of nitrotyrosine residues. Moreover, when these samples were subjected to amino acid analysis no novel amino acid residues except nitrotyrosine were detected. It would thus appear that tetra-

nitromethane is specific for tyrosine residues in both human IgG and rabbit IgG.

Stoichiometry of IgG modification

Table 1 shows the correlation of the extent of nitration of IgG aggregates with their C1q-binding activities. Nitration of an average of 10.2 tyrosine residues/molecule in both chemically cross-linked and heat-aggregated human IgG led to loss of up to 82% of its C1q-binding activity. On the other hand, modification of similar numbers of tyrosine residues in rabbit IgG aggregates had little effect on its C1q-binding activity.

Fig. 3. Effect of tetranitromethane treatment on the C1q-binding activity of immune complexes

Immune complexes were incubated with (●) or without (○) 4 mM-tetranitromethane, then washed with phosphate-buffered saline, and the C1q-binding activity was determined as described in the Materials and methods section.

Table 1. Correlation between the extent of nitration and the C1q-binding activity of IgG

Rabbit IgG and human IgG oligomers, treated with 4 mM-tetranitromethane (TNM), were assayed for their C1q-binding activity, and the extent of nitration was determined spectrophotometrically (as described in the Materials and methods section). Values for the tyrosine residues nitrated are given as mol of nitrotyrosine residues/mol of IgG. Values for C1q-binding activity are expressed as means $\pm$ S.E.M. for duplicate determinations.

	C1q-binding activity (%)	Tyrosine residues nitrated (mol/mol)
Rabbit IgG oligomers	82 $\pm$ 2	—
Chemically cross-linked + TNM	85 $\pm$ 3	11.9
Heat-aggregated + TNM	83 $\pm$ 2	9.3
Human IgG oligomers	83 $\pm$ 2	—
Chemically cross-linked + TNM	15 $\pm$ 3	10.2
Heat-aggregated + TNM	11 $\pm$ 4	10.4

Mechanism of the inactivation

The mechanism by which nitration of tyrosine residues in human IgG inactivates its C1q-binding sites was studied by examining the effects of treating the modified protein with $\text{Na}_2\text{S}_2\text{O}_4$. This reagent is able to reduce nitrotyrosine residues to aminotyrosine residues; this has a limited effect on the size of the incorporated group but restores the pK of the phenolic hydroxy group from approx. 7 in a nitrotyrosine residue to approx. 10 in an aminotyrosine residue [24].

Reduction of nitrotyrosine residues to aminotyrosine residues did not restore C1q-binding activity in human IgG aggregates, nor did it lead to loss of C1q-binding activity in rabbit IgG aggregates (Table 2). Controls were performed showing that $\text{Na}_2\text{S}_2\text{O}_4$ treatment of human IgG and rabbit IgG aggregates did not abrogate their C1q-binding activities. These observations imply that nitration of tyrosine residues in human IgG aggregates abrogates C1q-binding activity through the introduction of a bulky nitro side group causing steric hindrance of the C1q-IgG interaction rather than through alteration of the pK of the phenolic hydroxy groups of the tyrosine residues. Monitoring of the loss of A_{428} (due to nitrotyrosine residues) and the increase in A_{288} (due to aminotyrosine residues) showed that the $\text{Na}_2\text{S}_2\text{O}_4$ reduction of nitrotyrosine residues was complete within 5 min at room temperature.

It has been reported that tetranitromethane can lead to cross-linking of proteins, including rabbit IgG [28]. These observations were confirmed when SDS/polyacrylamide-gel electrophoresis was performed under reducing conditions to determine the extent of cross-linking; both human IgG and rabbit IgG oligomers were covalently coupled by tetranitromethane. The degree of cross-linking increased with increasing concentrations of both modifier and protein and at low pH. At pH 7.2, the pH at which tetranitromethane modification studies were performed, the extent to which IgG oligomers were cross-linked was estimated to be approx. 10%.

Table 2. Effect of dithionite on the C1q-binding activity of nitrated IgG oligomers

Rabbit IgG and human IgG oligomers, nitrated with tetranitromethane (TNM) as indicated in Table 1, were treated with $\text{Na}_2\text{S}_2\text{O}_4$ as described in the Materials and methods section. The C1q-binding activity and the extent of nitration were then determined as detailed in the Materials and methods section. Values for the tyrosine residues nitrated are given as mol of nitrotyrosine residues/mol of IgG. Values for the C1q-binding activity are given as means $\pm$ S.E.M. for duplicate determinations.

	C1q-binding activity (%)	Tyrosine residues nitrated (mol/mol)
Rabbit IgG oligomers	83 $\pm$ 3	—
Heat-aggregated + TNM	83 $\pm$ 6	9.3
Heat-aggregated + TNM + $\text{Na}_2\text{S}_2\text{O}_4$	82 $\pm$ 4	—
Human IgG oligomers	83 $\pm$ 2	—
Heat-aggregated + TNM	11 $\pm$ 4	10.4
Heat-aggregated + TNM + $\text{Na}_2\text{S}_2\text{O}_4$	13 $\pm$ 6	—

Fig. 4. Separation of nitrated tryptic peptides by h.p.l.c.

The nitrated tryptic peptides [derived from human IgG (a) and rabbit IgG (b) with ten nitrotyrosine residues per molecule] were prepurified on Porapak Q before h.p.l.c. These peptides were then applied to a C_{18} reverse-phase column developed with solvent A (0.01 M-acetate buffer, pH 5.0) and solvent B (acetonitrile) (see inset tabular matter for gradient conditions). The eluate was monitored at 360 nm and fractions were collected manually.

Isolation and identification of nitrotyrosine-containing peptides

In order to establish whether the loss of C1q-binding activity in human IgG following its treatment with

Table 3. Amino acid compositions of tryptic nitrated peptides

The nitrated peptides, isolated by h.p.l.c. and annotated as in Fig. 4, were subjected to amino acid analysis. The compositions for those peptides purported to be adjacent to Tyr-278 and/or Tyr-319 are listed. Values are given as mol of residue/mol of peptide normalized to 1.0 nitrotyrosine residue/peptide. The compositions for the expected tryptic fragments containing Tyr-278 or Tyr-319, from both human IgG and rabbit IgG, are tabulated to allow comparison. – indicates less than 0.2.

Amino acid	Composition (mol of residue/mol of peptide)						
	Peptide H1	Peptide H3	Peptide R5	Human IgG 318–320	Human IgG 318–322	Human IgG 275–288	Rabbit IgG 256–285
Asx	–	2.6	4.6	–	–	3.0	5.0
Thr	–	–	2.7	–	–	–	3.0
Ser	–	–	1.3	–	–	–	1.0
Glx	0.8	0.8	5.3	1.0	1.0	1.0	6.0
Pro	–	–	2.4	–	–	–	2.0
Gly	–	0.9	–	–	–	1.0	–
Ala	–	1.2	–	–	–	1.0	–
CyS*	1.1	–	1.0	–	1.0	–	1.0
Val	–	2.7	6.6	–	–	3.0	7.0
Met	–	–	–	–	–	–	–
Ile	–	–	0.8	–	–	–	1.0
Leu	–	–	–	–	–	–	–
Tyr (NO ₂)†	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Phe	–	1.1	0.7	–	–	1.0	1.0
His	–	1.3	–	–	–	1.0	–
Lys	1.9	1.2	–	1.0	2.0	1.0	–
Arg	–	–	1.2	–	–	–	1.0
Trp	N.E.	N.E.	N.E.	–	–	1.0	1.0

Abbreviation: N.E., not estimated.

* S-Carboxymethylcysteine or cysteic acid.

† Tabulated as tyrosine residues for theoretical compositions.

tetranitromethane arose from modification of Tyr-319 (in site B) or nitration of Tyr-278 (in site A), it was necessary to isolate and characterize nitrotyrosine-containing peptides from both human IgG and rabbit IgG.

In preliminary experiments, samples of nitrated human IgG were reduced and alkylated, and then treated with trypsin as indicated in the Materials and methods section. However, although five 360 nm-absorbing peptides could be detected when these digests were subjected to h.p.l.c. analysis, it was not possible to purify them from co-eluted unlabelled peptides.

Because of this problem, a new strategy was developed for affinity purification of the nitrotyrosine-containing peptides from the unlabelled peptide milieu that results from trypsin digestion. This method makes use of a Porapak Q liquid-chromatography column to retain only nitrated peptides. These labelled peptides are then recovered (> 95% yield) by elution with organic solvent before h.p.l.c. When the nitrated tryptic peptides from human IgG and rabbit IgG were purified on Porapak Q before h.p.l.c., the h.p.l.c. profiles shown in Fig. 4 were obtained. The amino acid compositions of these peptides are presented in Table 3. The expected tryptic peptide containing Tyr-319 (E-Y_{NO₂}-K) from human IgG was not found; however, a peptide containing one cysteine, two lysine, one glutamate and one nitrotyrosine residues was isolated. This composition fits uniquely the sequence for residues 318–322 of human IgG: E-Y_{NO₂}-K-C-K. Table 3 also shows that a peptide with amino acid composition

fitting a tryptic fragment surrounding Tyr-278 was isolated from both rabbit IgG and human IgG. These data suggest that this residue is not important for the binding of C1q by either species of IgG.

DISCUSSION

The present study was designed to distinguish between two candidate C1q-receptor sites on IgG from the IgG species' susceptibility to inactivation by modification of tyrosine residues. Although both rabbit IgG and human IgG contain Tyr-278 in site A, only human IgG has a tyrosine residue (Tyr-319) within site B. Thus strong evidence for site B containing the C1q-receptor site would be obtained by demonstrating that human IgG is inactivated when Tyr-319 is modified, and that rabbit IgG is insensitive to modification of tyrosine residues. The chemical modifier used, tetranitromethane, is reasonably specific for tyrosine residues, although the reaction with thiol groups and histidine, methionine and tryptophan residues has been reported [29–31]; significant reaction with these residues occurs only at alkaline pH. Reaction with thiol groups can also be discounted because there are no free thiol groups in IgG. These assumptions and the experimental data obtained (including appropriate spectral changes and amino acid composition) provide strong support for the specificity of tetranitromethane for IgG tyrosine residues.

The large decrease in C1q-binding activity experienced by human IgG oligomers upon tetranitromethane treat-

ment was in marked contrast with the lack of effect in nitrated aggregates of rabbit IgG (Fig. 2). These observations suggest the presence of critical tyrosine residues at or near the C1q-binding site of human IgG (but not rabbit IgG); thus the data support site B with the unique Tyr-319. The possibility of nitration-induced conformational change in human IgG (other than at the C1q-binding site) being the cause of inactivation is remote because, as shown in Table 1, rabbit IgG aggregates, which were nitrated to a similar extent (approx. 25% of all tyrosine residues), remained active.

The modification with tetranitromethane of tyrosine residues in human IgG has been reported previously by Johnson & Thames [17]. In that paper it was noted that modification of an average of 7.3 tyrosine residues per human IgG molecule led to a 72% decrease in anti-complementary activity; our data are in agreement with these observations. This study by Johnson & Thames [17] differed from the present work in that it relied on adsorbing the modified IgG on latex beads before the haemolytic assay, did not measure directly C1q-binding activity, examined only human IgG and did not identify the residues modified.

The reaction of tetranitromethane with tyrosine produces 3-nitrotyrosine, nitroformate and two protons; the pK_a of the phenolic hydroxy group subsequently changes from approx. 10 to approx. 7 owing to the effect on the ring electrons caused by substitution of the nitro group for the hydrogen atom [24]. Thus the loss of C1q-binding activity in nitrated aggregates of human IgG may be the result of two factors. First, steric hindrance by the nitro group (introduced in place of a hydrogen atom) could disrupt the appropriate interactions required for C1q-IgG binding. Alternatively, or concomitantly, the change in pK_a of the phenolic group from 10 to 7 may lead to loss of C1q binding through generation of unfavourable charge interactions between C1q and human IgG aggregates. Dithionite reduction of the nitrated protein, which restores the pK of a modified residue to approx. 10, failed to restore lost C1q-binding activity to nitrated human IgG oligomers and did not affect the C1q-binding activity of nitrated rabbit IgG oligomers. This implies that steric hindrance by the bulky nitro group is the cause of human IgG inactivation, rather than alteration of the charges of modified tyrosine residues.

The small degree of tetranitromethane-mediated cross-linking of IgG oligomers that occurred under the conditions used was judged to be irrelevant to this study because the same (minor) extent of cross-linking occurred in both human IgG and rabbit IgG oligomers. Also, it would be expected that further aggregation of oligomers would lead, if anything, to a small increase rather than the observed large decrease in C1q-binding activity of nitrated human IgG oligomers.

Two criteria needed to be met for the isolation and identification of nitrotyrosine-containing peptides. First, under the acidic conditions of peptide separation by reverse-phase h.p.l.c., these peptides should be distinguishable from those without modified tyrosine residues. Secondly, the peptides should have non-ambiguous amino acid composition so that confident identification can be made from the amino acid analysis. The isolation of the nitrotyrosine-containing tryptic peptides from the digests of nitrated IgG was greatly facilitated by the use of Porapak Q chromatography. Removal of the unlabelled peptides (which had confused the initial amino acid

analysis) by this 'affinity' step meant that the results of further characterization (h.p.l.c. and amino acid analyses) were easier to interpret. The tryptic digest of nitrated human IgG yielded a peptide whose amino acid composition fitted uniquely the sequence for residues 318–322. The assignment of this peptide was made easier by the presence of the carboxymethylcysteine, since there are only 11 such residues per half IgG molecule. A possible explanation for the fact that trypsin did not cleave after Lys-320 is that this residue is flanked by bulky groups: the carboxymethylcysteine residue at position 321 and the nitrotyrosine residue at position 319. In addition, peptides corresponding to those expected for the tryptic fragments containing Tyr(NO₂)-278 were isolated from digests of both human IgG and rabbit IgG, implying that the region adjacent to this residue is not important for C1q binding. As identification of the other nitrotyrosine-containing peptides was not made, we cannot rule out the possibility that modification of residues outside of the C_H2 domain was responsible for the differential effects exerted by nitration of human IgG and rabbit IgG. However, we are not aware of any reports of modification of residues outside the C_H2 domain directly affecting the C1q-binding activity of IgG.

We have shown previously that modification of histidine residues (presumably His-324) on rabbit IgG abrogated its C1q-binding activity [21]. These findings reinforce the conclusion drawn from the current work: Tyr-319 is involved in the binding of C1q by human IgG and thus the C1q-receptor site on IgG is encompassed within the site proposed by Burton *et al.* [4], namely residues 316–338.

Since submission of this paper, Duncan & Winter [32] have reported similar conclusions about the location of the C1q-binding site, based on the effects of site-directed mutagenesis of human IgG₁.

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