Localization of the Site on the Complement Component C1q Required for the Stimulation of Neutrophil Superoxide Production*

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C1q, the recognition subunit of the classical complement pathway, interacts with specific cell surface molecules via its collagen-like region (C1q-CLR). This binding of C1q to neutrophils triggers the generation of toxic oxygen species. To identify the site on C1q that interacts with the neutrophil C1q receptor, C1q was isolated, digested with pepsin to produce C1q-CLR, and further cleaved with either trypsin or endoproteinase Lys-C. The resulting fragments were separated by gel filtration chromatography and analyzed functionally (activation of the respiratory burst in neutrophils) and structurally. Cleavage of C1q-CLR with endoproteinase Lys-C did not alter its ability to trigger neutrophil superoxide production. However, when C1q-CLR was incubated with trypsin under conditions permitting optimal cleavage, the ability of C1q-CLR to stimulate superoxide production in neutrophils was completely abrogated. Fractionation of the digests obtained with the two enzymes and identification by amino acid sequencing permitted localization of the receptor interaction site to a specific region of the C1q-CLR. Circular dichroism analyses demonstrated that cleavage by trypsin does not denature the remaining uncleaved collagen-like structure, suggesting that after trypsin treatment, the loss of activity was not due to a loss of secondary structure of the molecule. However, irreversible heat denaturation of C1q-CLR also abrogated all activity. Thus, a specific conformation conferred by the collagen triple helix constitutes the functional receptor interaction site. These data should direct the design of future specific therapeutic reagents to selectively modulate this response.

C1q shares an unusual macromolecular structure with certain other molecules also known to enhance uptake of specific pathogenic material (9–13). Like C1q, both pulmonary surfactant protein A (SP-A) and mannose-binding protein (MBP) have collagen-like sequences contiguous with noncollagen-like sequences, short NH₂-terminal domains containing interchain disulfide bonds, and a single disruption in the Gly-X-Y repeat pattern within the collagen-like sequence (13–15) causing a characteristic “kink” or bend in the tertiary structure. Like C1q, SP-A (9) and MBP (16) can enhance Fc receptor- and complement receptor-mediated phagocytosis. However, neither SP-A nor MBP by themselves stimulate superoxide (O₂⁻) production by polymorphonuclear leukocytes (17). In contrast to the readily detectable stimulation by C1q (6, 17), this suggests that the ligand interaction sites of the C1q receptor that triggers superoxide production (C1qRP₂) may differ from those sites of the receptor that enhance phagocytic capacity. Also consistent with the hypothesis that these receptors differ in some way is the observation that two monoclonal antibodies have been shown to inhibit the enhancement of phagocytosis mediated by C1q but do not block the production of superoxide by neutrophils (18, 19).

Very little is currently known about the ligand requirements for functional interaction with the C1q receptor on any cell types. It is known that the 176,000-Da collagen-like fragment of C1q mediates the functional interaction with most cells (4, 20), that C1r₂C1s₂ blocks that interaction (6), and that heat-aggregated C1q loses the ability to trigger B cell immunoglobulin secretion (21). In addition, it appears that a multivalent interaction is required for C1q to trigger a response upon binding to the cells (6, 21). It is not known whether this is due to a requirement for receptor clustering or a surface- or aggregate-induced conformational alteration. This requirement is similar to that seen with CR1, CR2, FcRII, and many other receptors (22, 23). If the receptor for C1q that triggers superoxide production differs from the one that enhances phagocytosis, it is probable that the interaction sites on the ligand, C1q, that mediate these responses are also distinct. Therefore, identification of the receptor interaction sites should permit the selective manipulation of the responses mediated by the different receptors; that is, to enhance phagocytosis without generating extracellular superoxide or to inhibit the production of superoxide without affecting monocyte phagocytic capacity. The present investigation characterizes a specific region on...
the collagen-like domain of C1q (C1q-CLR) that interacts with the neutrophil C1q receptor (C1qR_{O2}) to induce superoxide production. Enzymatic digestion, followed by functional analysis of the purified fragments and amino acid sequencing to unambiguously identify the cleavage sites generated, allowed the localization of a specific region of the C1q-CLR that is critical for the C1q-mediated neutrophil response. Furthermore, heat denaturation, as assessed by circular dichroism analyses, demonstrated that secondary structure of the collagen helix is required for the C1q-induced neutrophil superoxide response, indicating that a single linear amino acid sequence will not suffice for this interaction. Nevertheless, the data suggest that it may be possible to design synthetic antagonists to modulate this interaction and thus limit the production of toxic oxygen radicals.

RESULTS

Irreversible Denaturation of C1q-CLR Abrogates C1q-induced Superoxide Production by Human Neutrophils—It has been previously well established that the collagen-like portion of the C1q molecule binds to neutrophils and triggers superoxide generation (6, 17). Therefore, in all the present studies, C1q was treated with pepsin, and the pepsin-resistant C1q-CLR isolated by gel filtration was used as the functional ligand. To investigate parameters that could determine structural requirements for the functional interaction of C1q with its receptor, conditions that would lead to the loss of secondary structure as assessed by CD were explored. C1q-CLR in either 10 mM acetic acid, TBS, or TBS with 5 mM Ca^{2+}, pH 7.2, was heated to various temperatures, and the CD spectra were recorded. Fig. 1A shows that when heated in the acidic buffer used traditionally for collagen-like molecules, secondary structure, as assessed by the magnitude of the positive band at 224 nm characteristic of collagen triple helix, was rapidly lost in the 52°C range, similar to that previously reported by Brodsky-Doyele et al. (35). In contrast, when C1q-CLR was heated at pH 7.2 (in either the presence or the absence of Ca^{2+}), the loss of structure of the C1q-CLR was more gradual between 46 and 70°C.

To determine to what extent, if any, this denaturation was reversible and if this denaturation altered the ability of C1q-CLR to trigger neutrophil superoxide production, C1q-CLR was incubated at pH 7.2 for 30 min at 56, 79, and 100°C, temperatures known to induce increasing greatly loss of specific secondary structure, and then cooled to ambient temperature. The CD of the cooled material was then compared with that of an unheated control sample to determine if renaturation had occurred. In contrast to that seen with the acidic conditions used by Brodsky-Doyele and colleagues (35) (and repeated by us), the CD spectra of C1q-CLR heated to 56°C at pH 7.2 and subsequently recooled, was essentially identical to that of the untreated control, with the exception of some variability in the negative peak at 197 nm (Fig. 1B). This renaturation after heating was complete after an incubation of 45–60 min on ice. Heating the protein to 79°C, however, resulted in a greater irreversible loss of C1q-CLR secondary structure. Samples heated at 79°C and above did not renature even after days at 4°C (data not shown).

The effect of loss of secondary structure on the ability of C1q-CLR to trigger the generation of superoxide by neutrophils was then examined. Whereas irreversible loss of secondary structure...
structure by heating to 79 or 100 °C resulted in complete loss of the ability of C1q-CLR to trigger neutrophil O$_2^-$ production, C1q-CLR heated to 56 °C, followed by the recovery of secondary structure upon cooling, retained total functional activity as compared with the untreated C1q-CLR in its capacity to trigger O$_2^-$ (Fig. 2). In this experiment and all superoxide assays performed, neutrophils in uncoated control wells produced no superoxide, with a recording essentially identical to the 79°C/100°C samples (data not shown).

Trypsin Digestion of Reversibly Denatured C1q-CLR Abolishes the Neutrophil C1q Response—To investigate which region of the C1q-CLR molecule was required for C1q receptor-mediated triggering of the superoxide response in neutrophils, C1q-CLR was subjected to enzymatic cleavage. The fact that the C1q-CLR molecule could be reversibly denatured by heating at 50 °C was used to promote enzymatic cleavage by trypsin, which normally is not an efficient protease for collagen-like structures. C1q-CLR was heated to 50 °C, trypsin was added, and the incubation was continued at 50°C for 30 min. SDS-PAGE suggested that all chains of C1q-CLR were cleaved under these conditions (Fig. 3, lanes 3 and 7) compared with no enzyme controls (Fig. 3, lanes 2 and 6). To separate and characterize the resultant fragments, the digestion mixture was applied to a Superose 12 column, and the elution of the peak fractions was recorded (Fig. 4). When compared with the elution of undigested C1q-CLR sample (Fig. 4A, arrow; elution volume, 8.6 ml), the main fragment generated by trypsir cleavage was distinctly retained and thus significantly smaller in size (Fig. 4B, arrow; elution volume, 11.6 ml). This elution behavior is consistent with the evidence of cleavage seen by SDS-PAGE analysis but indicates that some of the multichain structure of C1q is maintained (albumin elutes at 12.6 ml). The major peak of both the undigested C1q-CLR and the trypsin-generated NH$_2$-terminal fragment of C1q-CLR (noted with the arrows in Fig. 4) were then assayed for the ability to trigger polymorphonuclear leukocyte superoxide production. The fragment resulting from trypsin digestion had very little activity as compared with the untreated or mock digested control (Fig. 5). This loss of activity did not reflect an inability of the trypsin-generated fragment to bind to the microtiter well surface as nearly equal molar amounts of the trypsin-digested, and untreated C1q-CLR were detected both by protein assay and by a more sensitive enzyme-linked immunosorbent assay using an anti-C1q monoclonal antibody. The data were consistent with loss of a critical sequence(s) required for the interac-

**Fig. 1.** Effect of heat treatment on the circular dichroism spectra of C1q-CLR. A, mean residue ellipticity at 224 nm of C1q-CLR in 10 mM acetic acid ( ■ ), TBS ( ○ ), or TBS plus 5 mM Ca$^{2+}$ ( ● ) was recorded as a function of temperature. B, complete CD spectra of C1q-CLR heated at the noted temperatures for 30 min followed by cooling for greater than 1 h to allow refolding. MRE is the mean residue ellipticity in degrees (deg)·cm$^2$·dmol$^{-1}$.

**Fig. 2.** Effect of loss of native secondary structure due to heat treatment on the ability of C1q-CLR to functionally interact with the neutrophil C1qRO$_2^-$. C1q-CLR was either left untreated (solid line, 4°C) or heated to 56°C (dashed line), 79°C (dotted line), or 100°C (dashed and dotted line) and allowed to cool for at least 45 min. The protein concentration was then adjusted to 30 μg/ml and tested for the ability to trigger superoxide production by neutrophils as described under “Materials and Methods.”

**Fig. 3.** Trypsin cleaves all three chains of C1q-CLR. SDS-PAGE analysis under nonreducing conditions of C1q-CLR (lanes 1 and 5), mock digested C1q-CLR (lanes 2 and 6), and after trypsin digestion (lanes 3 and 7) before (lanes 1–3) and after (lanes 5–7) purification via fast performance liquid chromatography gel filtration. Samples in lanes 6 and 7 are from the peak fractions designated by the arrows in Fig. 4 (A and B, respectively). Relative molecular mass markers are in lane 4.
Amino acid sequencing demonstrated that the earliest eluting peak from the trypsin-treated sample (Fig. 4B, pool a) contained the intact NH₂ termini of the A and C chains. The lack of the B chain NH₂ terminus is consistent with the previous identification of pyroglutamate at the NH₂ terminus of this chain (36). Treatment with pyroglutamate-aminopeptidase prior to NH₂-terminal sequence analysis allowed the quantitative identification of the predicted B chain residues Leu-Ser-(Cys)-Thr and thus verified that the B chain NH₂ terminus was present but blocked by this modified glutamine. The loss of activity was not due to digestion of the amino terminus of any of the three chains of the C₁q molecule. Furthermore, no evidence of any cleavage products associated with the main (NH₂-terminal) fragment after purification was detected, nor were trypsin autodigestion fragments contaminating this main protein peak. No cleavages by trypsin occurred in the first 31 amino acids of the A or C chain as sequencing through the first NH₂-terminal 31 residues on the A and C chains demonstrated a quantitative yield of each amino acid following the arginine and lysine residues (Fig. 6). Amino acid sequencing of the other fractions separated by the Superose column containing peptides of smaller size (Fig. 4B, pools b and c) allowed identification of multiple trypsin cleavage sites in the C₁q-CLR and C₁qRO₂. 

Fig. 4. Gel filtration demonstrates that C₁q-CLR is digested with trypsin. Typical Superose 12 column profile of C₁q-CLR (1 mg) incubated at 50 °C for 30 min without enzyme (A) or with trypsin (B) at E:S of 1:10. C is the profile of trypsin alone at the same concentration used in the digestion. The main fragment (designated by the open arrows) was used in the structural and functional assays. The areas labeled a, b, and c in B indicate the fractions pooled for amino acid sequencing and referred to in Table I. (The second peak in A is a nonprotein contaminant variably detected in fast protein liquid chromatography profiles.) The flow rate of the column was 0.5 ml/min.

Fig. 5. Trypsin digestion abrogates the stimulation of O₂ generation by C₁q-CLR. Neutrophils were added to microtiter wells coated with 100 µg/ml of the protein in the main peak from the Superose purification of C₁q-CLR that was untreated (solid line), mock digested (dotted line), or digested with trypsin (E:S, 1/10) (dotted line). Superoxide production by neutrophils was assayed by measuring reduction of cytochrome c. The data presented are from one experiment representative of three.

According to Reid and Thompson (36), all hydroxylysines are glycosylated (glucose-galactose disaccharide) except at positions B₅₀ and B₆₅. The NH₂ terminus of the B chain of C₁q-CLR was derived after treatment with pyroglutamate-aminopeptidase.
Cleavage site identification and sequences were based on enzyme site specificity and quantitative sequence data from analyses of both total digests (not shown) and pooled fractions derived from chromatography. All amino acid derivatives observed in each cycle were accounted for by the deduced sequences, no predicted amino acids derivatives were missing, and no other cleavages could be accounted for by the observed amino acid derivatives. The ratios of the amino acid derivatives observed were always those expected from the deduced sequences.

Table 1

| FPLC Pool | Sequence | Position | Cleavage site | Yield | Comment |
|-----------|----------|----------|---------------|-------|---------|
| Endo Lys-C | EDLCRAPD | A 1-8 | 160 NH₂ terminus | | |
| | NTGcGYGP | C 1-8 | 154 NH₂ terminus | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 66 Only Tyr in position 3 after Lys without Asp in position 4 | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 144 Only Met in position 3 after Arg/Lys | | |
| | NQPGFPS | C 59-66 | Lys⁵⁹ | 114 Only Asn in position 1 and Met in position 4 after Lys | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 86 Only Tyr in position 3 after Lys without Asp in position 4 | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 100 Only Phe in position 7 after Arg | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 70 Only Phe in position 7 after Arg | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 86 Only Phe in position 7 after Lys | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 62 Only Phe in position 7 after Arg | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 160 Only Phe in position 7 after Arg | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 100 Only Phe in position 7 after Lys | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 114 Only Phe in position 7 after Lys | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 58 Only Phe in position 7 after Arg | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 100 Only Phe in position 7 after Lys | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 48 Only Phe in position 7 after Arg | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 86 Only Phe in position 7 after Lys | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 38 Only Phe in position 7 after Arg | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 91 Only Phe in position 7 after Lys | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 25 Only Phe in position 7 after Arg | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 101 Only Phe in position 7 after Arg | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 96 Only Phe in position 7 after Arg | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 108 Only Phe in position 7 after Arg | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 86 Only Phe in position 7 after Arg | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 112 Only Phe in position 7 after Arg | | |
| | VGYGFPSS | A 60-67 | Lys⁵⁹ | 70 Only Phe in position 7 after Arg | | |
| | GPMKGpKG | B 66-73 | Lys⁵⁹ | 86 Only Phe in position 7 after Arg | | |

The lowercase p and k indicate hydroxylated proline and lysine, respectively, and the lowercase c indicates the known cysteine, which is not detected in the sequencing analysis.

Yields presented are from one set of data. Similar ratios were obtained in a second independent set of sequences for each enzyme.

Endoproteinase Lys-C Cleaves All Three C1q Chains but Has No Detrimental Effect on the Ability of the Molecule to Induce Superoxide.

Endo Lys-C cleaves all three chains of C1q-CLR, digestion with endo Lys-C (lanes 2 and 6) and C1q-CLR after digestion with endo Lys-C (lanes 4, 5, and 6) and after (lanes 1 and 2) purification via Superose gel filtration. Samples in lanes 1 and 2 are from the peak fractions designated by the arrows in Fig. 8 (B and A, respectively). The sample in lane 4 was preheated at 50°C for 30 min prior to the standard incubation for 2 h at 37°C. For comparison, trypsin-digested C1q-CLR is shown in lane 7. Molecular mass markers are in lane 3. All samples were electrophoresed under nonreducing conditions.

FIG. 7  Endo Lys-C cleaves all three chains of C1q-CLR. SDS-PAGE analysis of mock digested C1q-CLR (lanes 2 and 6) and C1q-CLR after digestion with endo Lys-C (lanes 1, 4, and 5) and before (lanes 4, 5, and 6) and after (lanes 1 and 2) purification via Superose gel filtration. Samples in lanes 1 and 2 are from the peak fractions designated by the arrows in Fig. 8 (B and A, respectively). The sample in lane 4 was preheated at 50°C for 30 min prior to the standard incubation for 2 h at 37°C. For comparison, trypsin-digested C1q-CLR is shown in lane 7. Molecular mass markers are in lane 3. All samples were electrophoresed under nonreducing conditions.

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provided evidence that the most NH₂-terminal cleavage occurred on the A chain after Arg⁴¹, on the B chain after Lys²⁹, and on the C chain after Arg¹ (Table 1 and Fig. 6). When tested for the ability to trigger superoxide production using the standard assay conditions, none of the fractions containing the smaller peptide fragments demonstrated any superoxide generating activity (data not shown).

Endoprotease Lys-C Cleaves All Three C1q Chains but Has No Detrimental Effect on the Ability of the Molecule to Induce Superoxide Production—Digestion of C1q-CLR with lower concentrations of trypsin (E:S, 1:100) resulted in a less degraded fragment as demonstrated by both Superose chromatography and SDS-PAGE (data not shown) and variable but significant activity in the neutrophil superoxide assay. Comparison of the sequence data of the fragments generated in each case revealed an apparent inverse relationship between the amount of superoxide generating activity and the percent of C1q-CLR that was cleaved at the most internal arginine residues (Arg⁴¹ in the A chain and Arg¹ in the C chain). Therefore, digestion with endo Lys-C, which, like trypsin, cleaves at lysine residues but, unlike trypsin, does not cleave at arginine, was assessed to determine if a population of homogeneously cleaved molecules could be generated and subsequently assessed for the ability to trigger superoxide products.

Analysis by SDS-PAGE demonstrated that specific peptide bonds of the C1q-CLR molecules were quantitatively and homogeneously cleaved by endo Lys-C at an E:S of 1:200 (Fig. 7). No additional cleavage occurred when higher amounts (E:S, 1:20) of endo Lys-C were added (data not shown) or when C1q-CLR was preheated to 50°C prior to digestion (Fig. 7, lane 4). The major proteolytic components were then separated and purified by Superose size exclusion chromatography. NH₂-terminal sequencing of the first eluting peak (Fig. 8B, pool a, corresponding to Fig. 7, lane 1) demonstrated that as in the trypsin digest, this peak contained an unaltered NH₂ terminus with no cleavage fragments remaining associated with the major C1q-CLR fragment generated. Sequencing of the later eluting peak areas (Fig. 8B, pools b and c) as well as the total
interacts with the collagen helix of C1q in the area of generation by C1q-CLR. Amino acid sequencing of the fragments generated by Lys-C digestion of C1q-CLR but is lost upon digestion with trypsin. Endo Lys-C digestion has no inhibitory effect on the stimulation of O2 generation by C1q-CLR. Primary structure of the fragment obtained from C1q-CLR digested with endo Lys-C (E:S, 1:200) retains full activity relative to the untreated C1q-CLR (solid line) or mock digested C1q-CLR (dashed and dotted line). Protein concentration used to coat the wells was 30 μg/ml.

Enzymatic Digestion of C1q-CLR Does Not Lead to a Loss of Secondary Structure of the Collagen Fibril—The loss of ability of C1q-CLR to trigger neutrophil superoxide production after trypsin treatment could be related to the loss of specific amino acid residues (positions A 38–59 and/or C 41–58). Alternatively, because the loss of collagen helical structure by heat denaturation abrogated all superoxide generation by C1q-CLR (Fig. 2), it is possible that digestion by trypsin also caused the denaturation or unwinding of the collagen helix leading to a loss of activity. Therefore, the CD spectra of the inactive fragment obtained from C1q-CLR by digestion with trypsin were recorded and compared with mock digested C1q-CLR. In addition, C1q-CLR digested with endo Lys-C was also analyzed by circular dichroism for comparison. No differences in the positive peak at 223 nm were seen after enzymatic digestion with either trypsin (Fig. 10A) or endo Lys-C (Fig. 10B) relative to the undigested material. Although small differences are detected in the CD in the 197–200 nm region, such differences are also detected between untreated samples. Therefore, the data suggest that the fragments remaining after enzymatic activity retained collagen helical secondary structure.

Each of these enzymes localized the most NH2-terminal cleavage sites for endo Lys-C to the COOH terminus of Lys59 of the A chain, Lys61 of the B chain, and Lys58 of the C chain, whereas trypsin cleaved on the COOH-terminal of Arg38 of the A chain, Lys61 of the B chain, and Arg41 of the C chain. CD analyses of the fragments after protease digestion demonstrated that peptide cleavage under these conditions, even close to the kink region, does not alter, to a major extent, the remainder of the collagen-like secondary structure. Thus, it appears that the C1qR prevents interaction of the collagen helix of C1q in the area of amino acid residues 38–60 of either the A or C chain. Alternatively, either two or all three correctly folded chains in this region may be required for effective receptor interaction.

Initial studies of heat-denatured C1q-CLR demonstrated that the ability of C1q to interact with the neutrophil C1q receptor and trigger superoxide production required some de-
The model of C1q (model B in from Kilchherr et al. (39)) was used to place the C1q A, B, and C chains in register. The C1q A, B, and C chain residues were superimposed on coordinates of the three-dimensional structure of a collagen triple helix peptide consisting of proline-hydroxyproline-glycine repeats (38) using the Biopolymer module in the Biosym Insight molecular modeling package. The amino acid side chains were rotated to match the well established favored rotamers for side chains.

There is no evidence for a significant population of single chain molecules, as there is no discernible shift in the position of the peaks detected by CD. These results suggest that the C1qRα2 interaction site on C1q requires some specific secondary structure rather than merely a linear sequence of amino acids for activity. A model for the spatial structure of this region of C1q based on the recently determined crystal structure of a synthetic collagen peptide (38) and the alignment of C1q chains proposed by Kilchherr et al. (39) is presented in Fig. 11. One feature of the model is that the three hydroxylysines that are known to be glycosylated (31) appear to be situated on one side of the helix nearly in the same plane. Although there is no direct evidence that these residues are important in the C1q receptor interaction, data of others demonstrate that carbohydrates often are of critical relevance to pattern recognition and thus host defense (40–42).

The region of the C1q molecule shown in this report to be of functional importance for triggering superoxide production in the neutrophil is different from that proposed by Malhotra et al. (43) to be the site of interaction of C1q with the calreticulin-like 56,000 M, C1q-binding protein, designated as cC1qR (C1q receptor interacting with the collagen-like region of C1q). These investigators deduced that the region on the NH2-terminal side of the kink in the C1q-CLR was the interaction site for cC1qR based on the comparisons of amino acid sequences and charge distributions among members of the family of molecules referred to as “defense collagens” (44) or “collectins” (45). This family of molecules, including MBP, SP-A, calprotectin, and C1q, have collagen-like sequence domains and usually display lectin-like activity (46). However, MBP and SP-A have not yet been shown to independently trigger O2 production as does C1q, and there has been no evidence reported that cC1qR is the receptor through which immobilized C1q triggers superoxide production. Therefore, it is likely that C1qRα2 is distinct from the calreticulin-like cC1qR and that the functionally relevant interaction site for triggering superoxide production is COOH-terminal to the kink region of the C1q molecule.

MBP and SP-A, like C1q, have been shown to stimulate the ingestion of pathogens (47–49) and enhance the phagocytic capacity of monocytes and macrophages (4, 9). Monoclonal antibodies to a 126,000 M, cell surface molecule (distinct from the calreticulin-like cC1qR) have been isolated that inhibit the MBP- (16), SPA-2, and C1q-mediated enhancement of phagocytosis (18, 19). These antibodies do not inhibit the production of superoxide by neutrophils (19), providing further evidence that the C1q receptor that modulates phagocytic activity on myeloid cells and C1qRα2 are distinct receptor complexes on these cells. Although the C1q binding component of C1qRα2 has not yet been identified, a recent study has used immunological and biochemical analysis to rule out several candidate surface molecules and additionally has obtained evidence implicating a multicomponent receptor complex, including CR3, in the transmembrane signaling involved in this response (50).

In 1983, Schumaker and co-workers (51) postulated a model of C1 macromolecular structure in which the tetramer C1r2C1s2 associates with the central portion of the C1q molecule within the cone defined by the C1q arms with the ends of C1r2C1s2 wrapping around the arms of the C1q hexamer at the region just above the kink region. This model was based on electron microscopy, molecular volume calculations, and symmetry considerations. The data presented here that localizes the neutrophil receptor interaction site above the kink support this model of Schumaker and co-workers (51) because our previous data demonstrated that the addition of C1r and C1s to C1q to form the complete C1 complex blocks the ability of C1q to stimulate superoxide production (6). These data then are consistent with the close association of C1rC1s with the region between residues 40–60 rather than with the region closer to the globular heads.

Finally, NH2-terminal amino acid sequencing of the intact C1q molecule and fragments produced by enzymatic digestion (Table I and Fig. 6) allowed the confirmation of several previously reported sequences throughout the collagen-like region of C1q (31). The sites of pepsin cleavage on each chain were clearly defined as in these preparations the COOH-terminal sequences were obtained in high yield without evidence of partial cleavages. In addition, we established the presence of a hydroxylated proline at position 75 in the A chain. Proline had been predicted by the cDNA sequence (30) but differed from the reported residue determined by amino acid sequencing (31). In addition, the lysines at amino acid 10 of chain A and at residue 19 of chain C reported by Reid (31) were obtained in a lower yield than expected and thus can be assumed to be partially hydroxylated. Interestingly, the peptide bond after the hydroxylsine at position B 65 was only partially cleaved by trypsin but was quantitatively cleaved by endo Lys-C. Although this was the only hydroxylsine that was cleaved by these proteases, its susceptibility was not due to heterogeneity

2. S. Ruiz and A. J. Tenner, unpublished data.

3. In the course of our analyses we noticed that the post-translational hydroxylation at residue 39 of the A chain precursor protein (corresponding to amino acid 17 in the mature protein) and residues 71 and 72 in the C chain precursor protein sequence (corresponding to residues 43 and 44 in the mature protein) were incorrectly entered in the Protein Identification Resource data base (accession number, PO 2747); our data clearly show that proline at position A 17 is hydroxylated but at position C 43 it is not hydroxylated, whereas the lysine at position C 44 must be hydroxylated, all in agreement with the protein sequence published by Reid and colleagues (30).
of the post-translational hydroxylation, because there was no evidence of a lysine detected upon amino acid sequencing. Whereas, unlike most other hydroxylsines in C1q-CLR, this residue is unglycosylated (31), carbohydrate-dependent steric hindrance cannot be the only factor determining cleavage, because the hydroxylsine at position B 50 is also not glycosylated but was never seen to be cleaved by either trypsin or endo Lys-C.

In summary, C1q functions both as the recognition molecule of C1, the initiator of the classical complement pathway, and as an element in the first line of defense (52, 53) by triggering the activation of the NADPH oxidase in neutrophils (17), resulting in the production of microbicidal oxygen radicals, and by enhancing the efficiency of phagocytic cells to clear pathogenic material. Because, as discussed above, the C1q receptor that mediates the enhacement of phagocytosis is known to be a major contributor to tissue destruction in many situations including myocardial infarction, pulmonary infection, and joint inflammation. If the C1q interaction with neutrophils contributes to the detrimental production of this toxic oxygen radical in inflammation, it would be clinically advantageous to inhibit this response. Once the interaction site involved in the C1q-mediated enhancement of phagocytosis is identified, the data presented here can be used to initiate the development of strategies for inhibiting the generation of the toxic O2 without compromising the ability of C1q to enhance the phagocytic capacity.

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REFERENCES

1. Cooper, N. R. (1985) Adv. Immunol. 37, 151–216
2. Ziccardi, R. J., and Cooper, N. R. (1979) J. Immunol. 123, 788–792
3. Bobak, D. A., Frank, M. M., and Tenner, A. J. (1988) Eur. J. Immunol. 18, 2001–2007
4. Bobak, D. A., Gaither, T. A., Frank, M. M., and Tenner, A. J. (1987) J. Immunol. 138, 1150–1156
5. Sorvillo, J. M., Gaglioti, I., and Pearlstein, E. (1986) J. Immunol. 136, 1023–1026
6. Tenner, A. J., and Cooper, N. R. (1982) J. Immunol. 128, 2547–2552
7. Hamada, A., and Greene, B. M. (1987) J. Immunol. 138, 1240–1245
8. Shingu, M., Yoshioka, K., Nobunaga, M., and Motomatu, T. (1989) Inflammation 13, 561–569
9. Tenner, A. J., Robinson, S. L., Borchelt, J., and Wright, J. R. (1989) J. Biol. Chem. 264, 13923–13928
10. Drickamer, K., Dordal, M. S., and Reynolds, L. (1986) J. Biol. Chem. 261, 6878–6887
11. Ezekowitz, R. A. B., Day, L. E., and Herman, G. A. (1988) J. Exp. Med. 167, 1034–1046
12. Voss, T., Elson, H., and Schafer, K. P. (1988) J. Mol. Biol. 203, 219–227
13. White, R. T., Damnn, D., Miller, J., Spratt, K., Schilling, J., Hawgood, S., Benson, B., and Cordell, B. (1985) Nature 317, 361–363
14. Bhandary, S. N., and Lynn, W. S. (1980) Biochim. Biophys. Acta 625, 343–355
15. Reed, K. B. M. (1983) Biochem. Soc. Trans. 11, 1–12
16. Taylor, A. J., Robinson, S. L., and Ezekowitz, R. A. B. (1999) Immunology 3, 485–494
17. Goodman, E. B., and Tenner, A. J. (1992) J. Immunol. 148, 3920–3928
18. Guan, E., Burgess, W. H., Robinson, S. L., Goodman, E. B., McTigue, K. J., and Tenner, A. J. (1991) J. Biol. Chem. 266, 20340–20345
19. Guan, E., Robinson, S. L., Goodman, E. B., and Tenner, A. J. (1994) J. Immunol. 152, 4005–4016
20. Tenner, A. J., and Cooper, N. R. (1980) J. Immunol. 125, 1658–1664
21. Daha, M. R., Klar, N., Hoekzema, R., and van Es, L. A. (1990) J. Immunol. 144, 1227–1232
22. Wüthrich, M., Uwatoko, S., and Mannix, M. (1989) Arthritis Rheum. 32, 544–551
23. Brunswik, M., J. June, C. H., Finkelman, F. D., and Mond, J. J. (1989) J. Immunol. 143, 1414–1421
24. Tenner, A. J., Lesavre, P. H., and Cooper, N. R. (1981) J. Immunol. 127, 648–653
25. Young, K. R., Ambrus, J. L., Jr., Malbran, A., Fauci, A. S., and Tenner, A. J. (1993) J. Immunol. 146, 3356–3364
26. Reed, K. B. M. (1976) Biochem. J. 155, 5–17
27. Siegel, R. C., and Schumaker, V. N. (1983) Mol. Immunol. 20, 53–66
28. Reed, K. B. M., Lowe, D. M., and Porter, R. R. (1972) Biochem. J. 130, 749–763
29. Schläger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
30. Sellar, G. C., Blake, D. J., and Reid, K. B. M. (1991) Biochem. J. 274, 481–490
31. Reid, K. B. M. (1979) Biochem. J. 179, 367–371
32. Lottenbergh, F., and Herschede, A. (1978) Hoppe-Seyler’s Z. Physiol. Chem. 359, 1611–1616
33. Boyum, A. (1976) Scand. J. Immunol. 5, Suppl. 9, 9–15
34. Long, C. G., Braswell, E., Zhu, D., Apigo, J., Baum, J., and Brodsky, B. (1993) Biochemistry 32, 11688–11695
35. Belia, J., Eaton, M., Brodsky, B., and Berman, H. M. (1994) Science 266, 75–81
36. Kirschner, E., Hofmann, H., Steigemann, W., and Engel, J. (1985) J. Mol. Biol. 186, 403–416
37. Konopski, Z., Smeets, B., Seljidi, E., and Eskeledt, T. (1994) Biochim. Biophysics Acta 1221, 61–65
38. Reid, K. B. M., and Thompson, E. O. P. (1978) Biochem. J. 173, 863–868
39. Klinger, M., Acton, S., Ashkenas, J., Pearson, A., Penman, M., and Resnick, D. (1993) J. Biol. Chem. 268, 4569–4572
40. Holmes, U., Malhotra, R., Sim, R. B., and ensenius, J. C. (1994) Immunol. Today 15, 67–74
41. Malhotra, R., Laursen, S. B., Willis, A. C., and Sim, R. B. (1993) Biochem. J. 283, 15–19
42. Krüger, M., Acton, S., Ashkenas, J., Pearson, A., Penman, M., and Resnick, D. (1993) J. Biol. Chem. 268, 5031–5041
43. Malhotra, R., Aharoum, J., Thié, S., and Sim, R. (1992) Eur. J. Immunol. 22, 1437–1445
44. Friis-Christiansen, P., Thié, S., Svehag, S., Dessau, R., Svendsen, P., Andersen, O., Laursen, S. B., and ensenius, J. C. (1990) Scand. J. Immunol. 31, 453–460
45. Iwaarden, J. F., Strijp, A. G., Elskamp, M. J. M., Welmers, A. C., Verhoef, J., and Greide, L. M. G. (1991) Am. J. Physiol. 261, L204–L209
46. Bobak, D. A., Washburn, R. G., and Frank, M. M. (1988) J. Immunol. 141, 592–597
47. Goodman, E. B., Anderson, D. C., and Tenner, A. J. (1995) J. Leuk. Biol. 58, 168–176
48. Poon, P. H., Schumaker, V. N., Phillips, M. L., and Strang, C. J. (1983) Mol. Biol. 168, 563–577
49. Ezekowitz, R. A. B. (1991) Curr. Opin. Immunol. 5, 59–65
