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Re-examination of the Anomeric Configuration of the Galactose-(1-3)-Galactose Linkages in the Asparagine-linked Sugar Chains of Subcomponent C1q of Bovine Complement and Calf Thymocyte Plasma Membrane Glycoproteins

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The anomeric configuration of the terminal Gal1-3Gal linkages found in the asparagine-linked sugar chains of subcomponent C1q of bovine complement and of calf thymocyte plasma membrane glycoproteins were re-examined, and confirmed to be alpha by coffee bean α-galactosidase digestion. This contradictory result compared to the previous assignment could be explained by the finding that even very trace amounts (0.06 mU) of α-galactosidase contaminating the preparation of jack bean β-galactosidase were able to cleave the Galα1-3Gal linkage.

The asparagine-linked sugar chains with the Galβ1-3Galβ1-4GlcNAc sequence in their outer chain moieties were originally found in calf thymocyte plasma membrane glycoproteins [1-3], and then in rat erythrocyte membrane glycoproteins [4, 5] and C1q subcomponents of bovine [6], guinea pig and mouse complements [7]. However, recent attempts to characterize the enzyme, *N*-acetyllactosaminide β-galactosyltransferase, responsible for the formation of the Galβ1-3Galβ1-4GlcNAc sequence in calf thymus, were unsuccessful [8, 9]. Instead of the expected β-galactosyltransferase, *N*-acetyllactosaminide α(1-3)-galactosyltransferase activity was discovered, suggesting that the trisaccharide outer chain of the asparagine-linked sugar chains of calf thymocyte plasma membrane glycoproteins may not be Galβ1-3Galβ1-4GlcNAc but instead Galα1-3Galβ1-4GlcNAc [9, 10]. Recently, it has been also reported that the Galα1-3Galβ1-4GlcNAc sequence occurs in calf thyroglobulin [11], glycoprotein of

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Friend murine leukaemia virus [12], mouse Thy-1 glycoprotein [13], and glycopeptides from Ehrlich ascites tumor cells [14]. Therefore, we have re-examined the anomeric configuration of the terminal Gal1-3Gal linkage of the asparagine-linked sugar chains of bovine C1q and calf thymocyte plasma membrane glycoproteins.

Materials and Methods

Materials

Galβ1-4GlcNAcβ1-2Manα1-6[Galβ1-4GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4[Fucα1-6]-GlcNAc$_{OT}$ and GlcNAcβ1-2Manα1-6[GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4[Fucα1-6]-GlcNAc$_{OT}$ were prepared from human C1q as described previously [15]. Galβ1-4(2,5-anhydro-mannitol) was prepared from human asialotransferrin by hydrazinolysis and nitrous acid deamination [16] followed by NaB^3H_4 reduction. 2,5-Anhydro-mannitol was also prepared by nitrous acid deamination of glucosamine hydrochloride followed by NaB^3H_4 reduction.

β-Galactosidase and β-*N*-acetylhexosaminidase were purified from jack bean meal by the method of Li and Li [17]. Diplococcal β-galactosidase and β-*N*-acetylhexosaminidase were purified as described previously [17]. α-Galactosidase from coffee beans was purchased from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Galactose oxidase from *Dactylium dendroides* was purchased from Sigma Chemical Co., St. Louis, MO, USA. NaB^3H_4 (360 mCi/mmol) was from New England Nuclear, Boston, MA, USA.

Glycosidase Digestion

Samples were incubated with one of the following glycosidases: (1) jack bean β-gaiactosidase (2 units or 0.5 units) in 75 μl of 0.1 M sodium citrate-phosphate buffer, pH 3.5, at 37°C for 23 h or 42 h in either the presence or absence of 20 mg/ml of γ-D-galactonolactone; (2) coffee bean α-galactosidase (0.25 units) in 50 μl of 0.15 M sodium citrate-phosphate buffer, pH 6.5, containing 1 mg/ml of γ-D-galactonolactone at 25°C for 19 h. Other glycosidase digestions were performed as described previously [6].

Analytical Methods

Paper chromatography was carried out using the following solvents: I, ethyl acetate/pyridine/acetic acid/water, 5/5/1/3 by vol; II, ethyl acetate/pyridine/water, 12/5/4 by vol; III, 1-butanol/ethanol/water, 4/1/1 by vol. Bio-Gel P-4 (Bio-Rad, Richmond, CA, USA) column chromatography was performed by using a column (2 × 100 cm) as described previously [19].

Conversion of Asparagine-linked Sugar Chains of Bovine C1q and Calf Thymocyte Plasma Membrane Glycoproteins to Oligosaccharides

A plasma membrane fraction was prepared from calf thymocytes as described previously [20]. The dried membrane fraction containing 1.9 mg of protein, as determined by the method of Lowry *et al.* [21], was subjected to hydrazinolysis followed by *N*-acetylation

and $NaBH_4$ reduction according to the method previously described [22]. The oligosaccharides thus released were separated from amino acid derivatives and mucin-type oligosaccharides, if any, by paper chromatography using solvent I for 24 h. The asparagine-linked sugar chains were recovered from the area 0-5 cm from the origin by elution with water. The sugar chains from bovine C1q were prepared as described previously [6].

Tritium Labeling of Non-reducing Terminal Galactose Residues of Oligosaccharides

The oligosaccharides from calf thymocyte plasma membranes were heated in 0.5 ml of 0.01 N HCl at 100°C for 15 min to remove sialic acids. The hydrolysate was repeatedly evaporated to dryness after addition of water and then kept in a desiccator *in vacuo* over NaOH. The residue was then incubated with 27 units of galactose oxidase in 70 μl of 0.05 M sodium phosphate buffer, pH 7.0, at 37°C for 72 h, and passed through a column containing 0.5 ml of AG 50W-X12 (H^+) in the upper layer and 0.5 ml of AG 3 (OH^-) in the lower layer. After evaporation of the eluate to dryness, the residue was labeled with 0.17 mCi of NaB^3H_4 according to our previously reported method [23]. The product thus obtained contained 7.0×10^5 cpm of the radioactivity.

Release of Outer Chain Moieties from the Labeled Oligosaccharides

The hydrazinolysis-nitrous acid deamination method [16] with slight modifications was applied to release the outer chain moieties of oligosaccharides obtained from calf thymocyte plasma membrane glycoproteins. The tritium-labeled oligosaccharides were subjected again to hydrazinolysis at 105°C for 40 h for de-*N*-acetylation and evaporated to dryness *in vacuo* over concentrated H_2SO_4. The residue was deaminated in 0.5 ml of 0.5 N AcOH containing 0.072 M $NaNO_2$ at room temperature for 16 h. The product was applied to a column of 0.5 ml of AG 50W-X12 (H^+) and 0.5 ml of AG 3 (OH^-) and eluted with water. The eluate was evaporated to dryness.

Results

Anomeric Configuration of the Terminal Gal1-3Gal Linkages of Oligosaccharides from Bovine C1q

Radioactive complex type oligosaccharides of bovine C1q: Gal1-3Galβ1-4GlcNAcβ1-2Manα1-6[Gal1-3Galβ1-4GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4[Fucα1-6]GlcNAc$_{OT}$ (N-1) and Gal1-3Galβ1-4GlcNAcβ1-2Manα1-6[Galβ1-4GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4[Fucα1-6]GlcNAc$_{OT}$ (A-N), were obtained as described previously [6]. N-1 and A-N were eluted from a Bio-Gel P-4 (-400 mesh) column at the positions of 16.4 and 15.4 glucose units, respectively Fig. 1A and B).

Both oligosaccharides ($3.0\text{-}3.3 \times 10^4$ cpm) were incubated with 0.25 units of coffee bean α-galactosidase in the presence of 1 mg/ml of γ-D-galactonolactone. This inhibitor was added in order to protect the oligosaccharides from the action of β-galactosidase, if any, in the α-galactosidase preparation, although it decreased also the α-galactosidase activity to 55%. Analysis of the radioactive products from N-1 (Fig. 1C) and A-N (data not shown) by the Bio-Gel P-4 column chromatography revealed that both of these radioac-

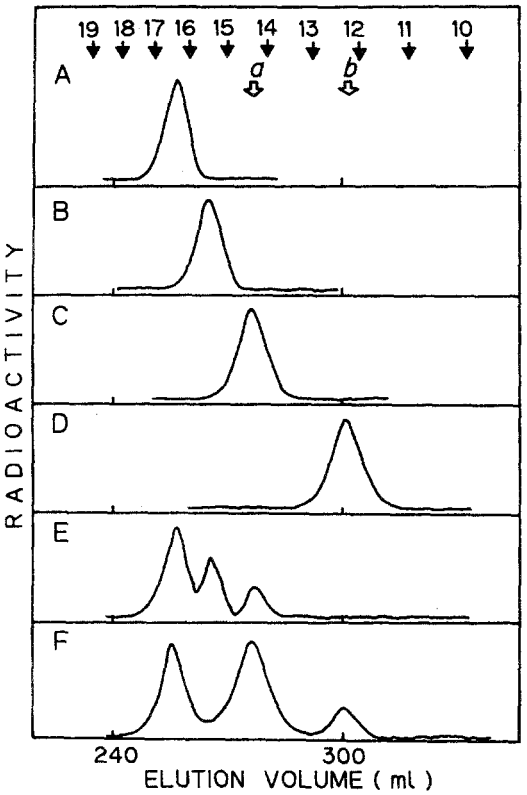

Figure 1. Gel permeation chromatograms of exoglycosidase digestion products of radioactive oligosaccharides N-1 and A-N obtained from bovine C1q. The radioactive oligosaccharides were applied to a column (2 × 200 cm) of Bio-Gel P-4 and the radioactivity in each tube (2 ml/tube) was determined by liquid scintillation spectrometry. A, oligosaccharide N-1; B, oligosaccharide A-N; C, oligosaccharide N-1 incubated with coffee bean α-galactosidase; D, oligosaccharide N-1 incubated with 2 units of jack bean β-galactosidase for 42 h; E, oligosaccharide N-1 incubated with 2 units of jack bean β-galactosidase in the presence of 20 mg/ml of γ-D-galactonolactone for 42 h; F, oligosaccharide N-1 incubated with 0.5 units of jack bean β-galactosidase for 23 h.

The black arrows indicate the elution positions of glucose oligomers added as internal standards and the numbers refer to the number of glucose units. The white arrows indicate the elution positions of authentic oligosaccharides; *a*, Galβ1-4GlcNAcβ1-2Manα1-6[Galβ1-4GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4[Fucα1-6]GlcNAc$_{OT}$; *b*, GlcNAcβ1-2Manα1-6[GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4[Fucα1-6]GlcNAc$_{OT}$.

tive oligosaccharides were converted to the same decasaccharide with the same mobility as authentic Galβ1-4GlcNAcβ1-2Manα1-6[Galβ1-4GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4[Fucα1-6]GlcNAc$_{OT}$. The decrease in size of the two oligosaccharides indicated that two and one galactose residues were released from oligosaccharides N-1 and A-N, respectively. Under the same conditions, the authentic radioactive oligosaccharide Galβ1-4GlcNAcβ1-2Manα1-6[Galβ1-4GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4[Fucα1-6]-GlcNAc$_{OT}$ (3.4 × 10^4 cpm) was not converted to any smaller oligosaccharide at all (data not shown). Therefore, it was concluded that oligosaccharides N-1 and A-N contain two

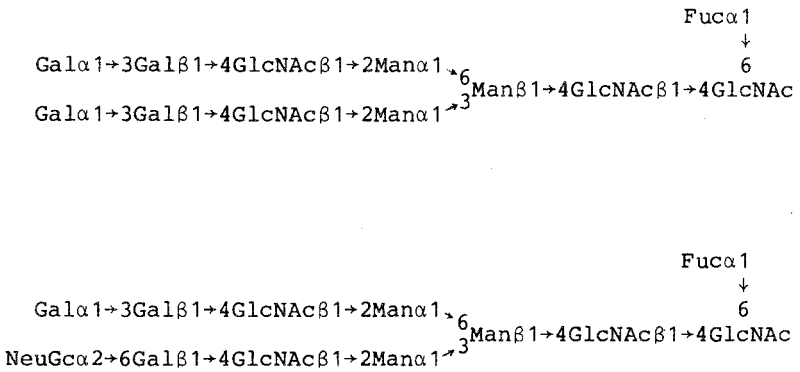

Figure 2. Revised structures of the asparagine-linked sugar chains of bovine C1q.

and one α-galactosyl residues, respectively, as non-reducing terminal sugars. The products digested with α-galactosidase were not susceptible to jack bean β-*N*-acetylhexosaminidase, but they were converted to $Man_3GlcNAc(Fuc)GlcNAc_{OT}$ by sequential digestion with diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase with the release of two galactose and two *N*-acetylglucosamine residues, respectively (data not shown). Based on the results obtained here together with the previous study [6], the structures of the asparagine-linked sugar chains of bovine C1q should be revised as shown in Fig. 2.

In order to find out the reason why we were previously misled to conclude that the Gal1-3Gal linkage was beta, we re-examined the experiments performed previously. The oligosaccharide N-1 was digested with two units of jack bean β-galactosidase and the product was then analyzed by Bio-Gel P-4 chromatography. As shown in Fig. 1D, the oligosaccharide was eluted at the same position as authentic GlcNAcβ1-2Manα1-6[GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4[Fucα1-6]$GlcNAc_{OT}$, indicating that all four galactose residues were released by this digestion. This result was identical to that obtained in the previous study [6]. Therefore, we carefully examined the possible contamination of α-galactosidase activity in the jack bean β-galactosidase preparation. By using *p*-nitrophenyl α-D-galactoside as a substrate, 0.24 mU of α-galactosidase activity in 2 units of β-galactosidase were detected. This raised the possibility that the contaminating α-galactosidase might be responsible for the hydrolysis of peripheral Gal1-3Gal linkages if the anomeric configuration is alpha. Therefore, the radioactive oligosaccharide N-1 was incubated with 2 units of jack bean β-galactosidase in the presence of 20 mg/ml of γ-D-galactonolactone, a known inhibitor of β-galactosidase. As shown in Fig. 1E, two galactose residues were the maximum amount released from oligosaccharide N-1, as in the case of digestion of the same oligosaccharide by coffee bean α-galactosidase (Fig. 1C), and the radioactive product with the smallest size was

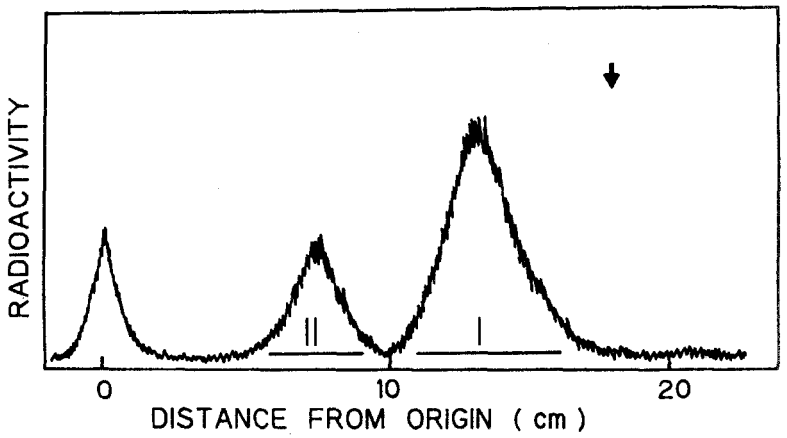

Figure 3. Paper chromatography of the deamination products. The product, released from galactose-labeled oligosaccharides of calf thymocyte plasma membrane glycoproteins by the hydrazinolysis-nitrous acid deamination method, were subjected to paper chromatography using solvent II for 14 h. The radioactivity was monitored by a radiochromatogram scanner. The arrow indicates the position where lactose migrated.

eluted at the same position as authentic Galβ1-4GlcNAcβ1-2Manα1-6[Galβ1-4GlcNAcβ 1-2Manα1-3]Manβ1-4GlcNAcβ1-4[Fucα1-6]GlcNAc$_{OT}$. These results indicated that the anomeric configurations of the non-reducing terminal galactose residues of oligosaccharide N-1 are alpha, and that jack bean α-galactosidase can easily hydrolyze the peripheral Galα(1-3)-linkage of natural substrates. Even 0.06 milliunits of α-galactosidase, present as a contaminant in 0.5 units of jack bean β-galactosidase, could act partially during 23 h incubation (Fig. 1F). Thus, the data indicated that jack bean α-galactosidase works on the natural substrate far more efficiently than on the synthetic substrate.

Isolation of a Trisaccharide Containing the Gal1-3Gal Sequence from the Oligosaccharides Obtained from Calf Thymocyte Plasma Membrane Glycoproteins

As already reported in the previous papers [2, 3], calf thymocyte plasma membrane glycoproteins have been found to contain a variety of asparagine-linked sugar chains with the Gal1-3Galβ1-4GlcNAc sequence at the non-reducing termini. Therefore, we designed an experiment to obtain, and analyze the structure of, the trisaccharide moiety of the oligosaccharides. For this purpose, we applied the hydrazinolysis-nitrous acid deamination method which was developed by Strecker *et al.* [16], since this method leads to the specific cleavage of *N*-acetylglucosaminyl linkages resulting in the release of the trisaccharide moiety as an oligosaccharide.

The asparagine-linked sugar chains were released as oligosaccharides by hydrazinolysis of calf thymocyte plasma membranes as described in detail in the Materials and Methods section, and then desialylated. The oligosaccharide mixture thus obtained,

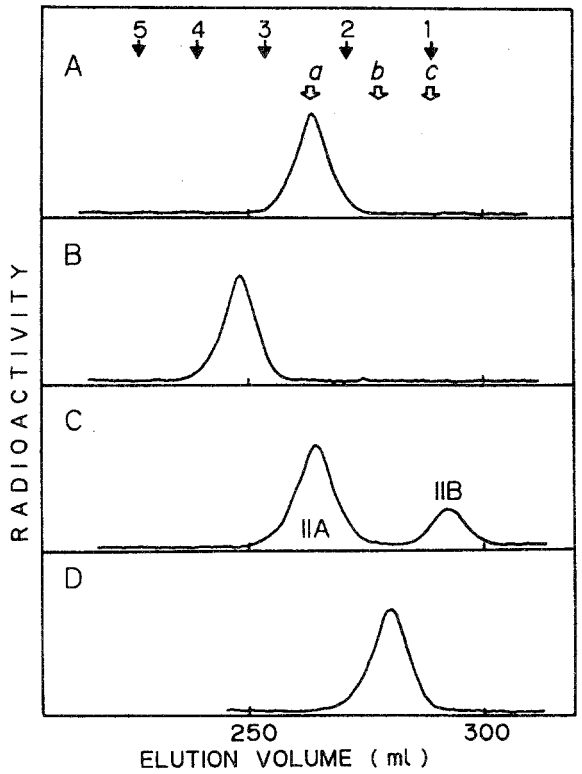

Figure 4. Sequential exoglycosidase digestion of the deamination products. A column (2.0 × 100 cm) of Bio-Gel P-4 was used for chromatography. A and B, fragments I and II shown in Fig. 3 after reduction with NaB^3H_4, respectively; C, the radioactive peak in B digested with coffee bean α-galactosidase under the same condition as in Fig. 1C; D, the radioactive peak IIA in C digested with 1.5 units of jack bean β-galactosidase. The black arrows are the same as in Fig. 1. Arrows *a*, *b* and *c* indicate the elution positions of authentic Gal-(2,5-anhydro-Man_{OT});2,5-anhydro-Man_{OT} and galactose, respectively.

which contained neutral and asialo-oligosaccharides, was incubated with galactose oxidase followed by NaB^3H_4 reduction to label the non-reducing terminal galactose residues in their outer chain moieties. The labeled oligosaccharides were then subjected to the hydrazinolysis-nitrous acid deamination procedure. When the products were examined by paper chromatography, two radioactive fragments I (72%) and II (16%) were detected (Fig. 3). The radioactive peak at the origin (12%) consisted of incomplete degradation products (data not shown). The fragments I and II, recovered from the paper, had sizes of 2.0 and 3.1 glucose units, respectively, upon Bio-Gel P-4 column chromatography (data not shown). These results indicated that the fragments I and II correspond to [^{3}H]Gal-(2,5-anhydro-Man) and [^{3}H]Gal-Gal-(2,5-anhydro-Man). Therefore, a half portion of each of the remaining fragments was labeled with NaB^3H_4 and

another portion was reduced with $NaBH_4$. After paper chromatography using solvent II, di- and trisaccharides double-labeled at the reducing and non-reducing termini were extracted from the paper.

Glycosidase Digestion of the Trisaccharide Fragment

The double-labeled fragments I and II, obtained as described in the previous section, were eluted as 2.6 and 3.7 glucose units, respectively upon Bio-Gel P-4 column chromatography (Figs. 4A and 4B), which were larger than the unreduced counterparts by 0.6 glucose units, as reported previously [19]. When fragment II was digested with coffee bean α-galactosidase under the same condition as used in the case of C1q, two radioactive products IIA and IIB were obtained (Fig. 4C). Product IIA had the same mobility as the double-labeled fragment I, which was eluted in the identical position as authentic Gal-(2,5-anhydro-Man_{OT}), and IIB had the same mobility as galactose. This result indicated that an α-linked galactose residue was removed from the non-reducing terminal of the trisaccharide, and that the previous assignment should be withdrawn. Further digestion with jack bean β-galactosidase converted IIA to radioactive 2,5-anhydromannitol (Fig. 4D). The double-labeled fragment I was completely resistant to α-galactosidase treatment, but was cleaved to radioactive 2,5-anhydromannitol and galactose by jack bean β-galactosidase digestion (data not shown). Based on the results so far described, together with the results of previous studies [2, 3], all the structures of the trisaccharide sequences found in the outer chain moieties of asparagine-linked sugar chains of calf thymocyte plasma membrane glycoproteins should be revised to Galα1-3Galβ1-4GlcNAc.

Discussion

It has been reported that α-galactosidase, present in the crude extract of jack bean meal, is inactivated by heat treatment at 60°C for 30 min [24]. However, this treatment could not perform the complete removal of the α-galactosidase. Further purification procedures using ion-exchange column chromatography [17] were also effective, but the final preparation of β-galactosidase thus purified was still contaminated with 0.012% of α-galactosidase activity as determined by a synthetic substrate as shown in this paper. Van den Eijnden *et al.* [9] have also shown the contamination of α-galactosidase activity (0.02%) in a commercial jack bean β-galactosidase. The finding that even a trace amount of α-galactosidase can easily act on glycopeptide substrates [9] and oligosaccharide substrates (this study) raises a serious problem, because this enzyme has been widely used for the structural studies of sugar chains. Thus, the parallel use of coffee bean α-galactosidase is essential for the correct assignment of the anomeric configuration of galactosyl linkages in sugar chains by jack bean β-galactosidase.

Recently, the α(1-3)-galactosyltransferase responsible for the formation of the Galα1-3Galβ1-4)GlcNAc sequence has been purified from calf thymus and characterized [10]. In that study, α-galactosylation has been shown to compete with sialylation of the galactose residue in the Galβ1-4GlcNAc sequence by α(2-6)- and α(2-3)-sialyltransferases. Therefore, the presence of α-galactosyl residues decreases the content of anionic charge of glycoproteins. This may be very important for the conformation and function of certain glycoproteins, such as C1q. Human and bovine C1q differ

in that the human subcomponent has a disialyl bi-antennary sugar chain without the Galα(1-3)-linkage [15], whereas bovine C1q does not contain a disialyl sugar chain but instead a monosialyl bi-antennary sugar chain in which one of the outer chains is α-galactosylated. This may account for the observation that bovine C1q, but not human C1q, was unable to aggregate latex particles coated with human IgG, even though bovine and human C1q are similar in their amino acid composition and molecular structure and the former could be substituted for human C1q when active C1 was reconstituted with human C1r and C1s [25].

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