RECOGNITION OF IgG BY Fc RECEPTOR AND COMPLEMENT: EFFECTS OF GLYCOSIDASE DIGESTION

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SUMMARY By extensive glycosidase digestion, most of carbohydrates were removed from IgG antibody against sheep erythrocytes without impairing hemagglutinating activity. The sugar-depleted IgG significantly lost the activities in antibody-dependent cell-mediated cytotoxicity, rosette formation and complement-dependent hemolysis. The results indicated the requirement of the carbohydrate moieties in recognition by Fc receptor and complement. Furthermore differential glycosidase digestion suggested that N-acetylglucosamine or mannose was the key sugar residue required for the maintenance of the activities.

While carbohydrates are attached to Fc-region of IgG (1), their roles in immunological reactions have not yet been clarified. Williams et al. observed that glycosidase digestion decreased the activity of an anti-bacterial antibody in complement fixation and opsonization (2). However, the identical procedure did not change the activities of other antibodies (2). The main obstacle in such research has been the inability to remove most of the sugars from intact IgG (2). So far, galactose and sialic acid have been sufficiently removed, but not N-acetylglucosamine (2, 3). In this communication, we will present a satisfactory procedure to accomplish the sugar-depletion. The application of this digestion method enabled us to propose that GlcNAc-Man region of IgG carbohydrates is required for the recognition by Fc receptor and complement.

MATERIALS AND METHODS

Analytical Methods. Neutral sugars were determined by phenol $-H_2SO_4$ reaction (4) using mannose as a standard. Sialic acid was determined by Warren's method (5) after hydrolysis by 0.1 N H_2SO_4

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at 80°C for 30 min, using N-acetylneuraminic acid as a standard. N-Acetylglucosamine was determined by Morgan-Elson reaction (6). Protein was determined by the method of Lowry et al. (7) using bovine IgG (Sigma) as a standard. Thin layer chromatography was performed on a glass plate coated with silica gel using N-propanol/ethyl acetate/H_2O (7/2/1, by volume) as a solvent. Neutral sugars on the plates were detected by anisaldehyde-H_2SO_4 reagent (8).

IgG. IgG was purified from rabbit antiserum against sheep erythrocytes by ammonium sulfate precipitation (50%) followed by DEAE-cellulose column chromatography. Content of neutral sugars and sialic acid of the IgG was 9.6 and 0.92 μg per mg of protein, respectively.

Glycosidases. Glycosidases from *Diplococcus pneumoniae* (9, 10) were purified essentially as described previously (10), except that DEAE-Sephadex was replaced with DEAE-cellulose and negative adsorption through Con A-Sepharose was employed. The last step was efficient in removing impurities of glycoprotein nature which interfered with the analysis of the products of enzymatic hydrolysis. These glycosidases were concluded to be free from contaminating proteases, since they released no acid soluble material from amino acid labeled IgG. Glycosidases were assayed as described in the respective references (9, 10), and one unit of the enzymes was defined as the amount of enzymes hydrolyzing 1 μmole of substrates per min.

Glycosidase Digestion. The purified IgG (430 μg) was incubated with 7.2 milliunits of neuraminidase, 62 milliunits of β-galactosidase, 117 milliunits of β-N-acetylglucosaminidase and 29 milliunits of endo-β-N-acetylglucosaminidase D in 0.2 ml of 0.04 M citrate-phosphate buffer, pH 6.0 containing 6 μg penicillin and 10 μg streptomycin at 37°C for 15 hr. The enzymatically treated IgG was then assayed for the possible change in their activities.

When the degree of sugar release was to be analyzed, antibiotics were replaced with a few drops of toluene, and the scale of the reaction mixture was increased 20-fold. After the incubation, 5 volumes of ethanol was added, and sugars released in the supernatant were determined. This value was corrected by the value of the control run, in which the enzyme and the substrate were incubated separately and were mixed at the time of the termination of the reaction.

RESULTS AND DISCUSSION

Purified IgG from rabbit antiserum against sheep erythrocytes was extensively digested with glycosidases from *Diplococcus pneumoniae*. This treatment removed 8.0 μg of neutral sugars from 1 mg of IgG. In other words, 83% of total neutral sugars were removed. Thin layer chromatography of the reaction mixture indicated that the products containing neutral sugars were galactose and $(Man)_3GlcNAc$. Similarly, 90% of total sialic acids were removed by the enzyme treatment. Release of free N-acetyl-

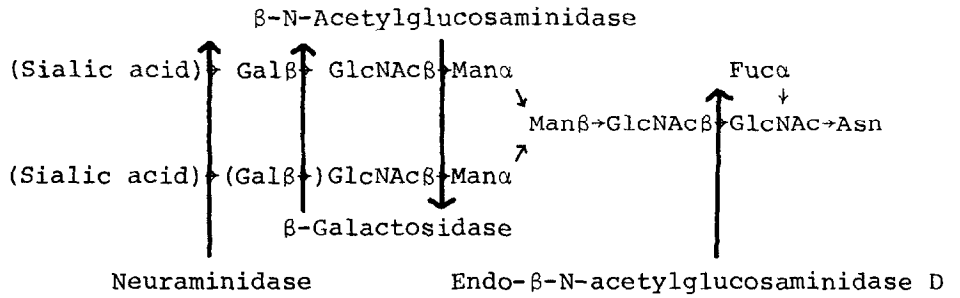

Fig. 1. Removal of sugars from intact IgG by glycosidase digestion.

Enzymatic hydrolysis proceeds in a sequential manner. Even for the action of endo-β-N-acetylglucosaminidase D, removal of the N-acetylglucosaminyl residue covering the key α-mannosyl residue was necessary (12, 13).

glucosamine after the digestion was 3.8 moles per mole of IgG, namely 95% of the expected value when we consider that one mole of IgG usually contains 4 moles of N-acetylglucosamine external to mannosyl residues (11). Therefore, we concluded that most of carbohydrates were removed from intact IgG except fucose and N-acetylglucosamine involved in protein-carbohydrate linkage (Fig. 1). From the analysis by Sephadex G-200 column chromatography and by SDS disc gel electrophoresis, we also concluded that no fragmentation of the peptide moiety occurred during the incubation. Hemagglutination activity of the IgG antibody was unchanged after the digesting, indicating that the antigen-combining capability was not affected by the sugar-depletion.

We found that the sugar-depleted IgG significantly decreased the activity in rosette formation, ADCC[1] and complement-dependent hemolysis (Table I, Exp. 1, 2). Glycosidases themselves did not inhibit the immunological reactions (Exp. 3). In case of ADCC, activity of the sugar-depleted IgG decreased to 20% of that of

[1] ADCC : Antibody-dependent cell-mediated cytotoxicity

TABLE I

Effects of Glycosidase Digestion on Activities of IgG

Treated IgG[1)]	Activities in Rosette Formation[a)]	ADCC[b)]	Complement -dependent Hemolysis[c)]
1. Control IgG[2)]	37.6	55.2	100
2. Sugar-depleted IgG	7.1	25.3	21.6
3. Enzyme Control[3)]	30.3	49.3	118
4. IgG digested with neuraminidase and β-galactosidase	33.3	52.2	94.1
5. IgG digested with all the glycosidases in the presence of N-acetylglucosamino-lactone (16 mM)	33.7	51.2	106

1) The minimum dose of IgG required for complete hemagglutination was identical in all the cases and was 4.4 μg. Hemagglutination assay was carried out in 0.1 ml of Dulbecco's phosphate buffered saline containing 0.5% sheep erythrocytes.

2) IgG was incubated with 0.1% bovine serum albumin instead of glycosidases. This treatment did not decrease the hemagglutinating activity.

3) IgG and glycosidases were mixed, and then directly used for immunological assays.

a) Human monocytes were adhered to 16 mm Linbro disposable tissue culture trays as described by Ciccimarra et al. (14). Then, 0.25 ml of Hanks' solution containing 0.8% sheep erythrocytes coated by 30 μg of IgG preparation was added to the monolayer. After 5 min, the monolayer was washed three times by 1 ml Hanks' solution, and was observed by Nikon inverted phase contrast microscopy. Results were expressed by per cent of rosettes (monocytes with red cells) per total number of monocytes.

b) The reaction was performed in 0.21 ml of RPMI 1640 medium (15) with 10% fetal calf serum. The reaction mixture contained 1×10^6 ^{51}Cr-labeled sheep erythrocytes, 5×10^6 spleen cells from BALB/c mice and 0.11 μg of IgG which had been centrifuged at 105,000 g for 1.5 hr before use to remove aggregated IgG. The reaction mixture was placed in Falcon 3040 Tissue Culture Plate, and was incubated at 37°C in 5% CO_2 in air and 100% humidity for 18 hr. After centrifugation, the supernatant was counted for the released ^{51}Cr. Results were expressed by the per cent chromium release which was calculated from the formula (ER-SR)/(MR-SR) x 100. ER is experimental release, SR is spontaneous release in the presence of effector cells without IgG and MR is maximum release obtained by freezing and thawing. SR was about 10% of the incorporated count, MR was 90%. The dose-response curve of the assay is shown in Fig. 2.

c) Assay was performed as described by Lachmann et al. (16). Results were expressed by relative activities as compared to that of control IgG.

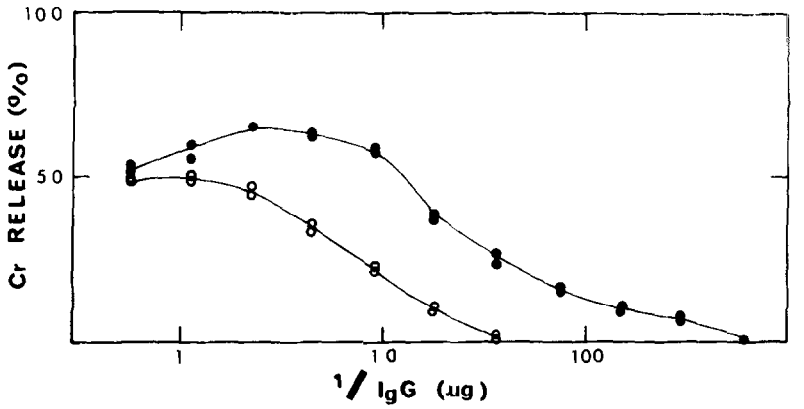

Fig. 2. Dose-response curve of intact and sugar-depleted IgG in antibody-dependent cell-mediated cytotoxicity (ADCC). Assay was done in duplicate as described in the legand of Table I.
o —— o: The sugar-depleted IgG
● —— ●: The control IgG (see legand of Table I)

undigested IgG as shown in the dose-response curve (Fig. 2). Degree of inactivation in other reactions was also similar (Table I, Exp. 1, 2). In both ADCC and rosette formation, Fc-region of IgG sensitizing the erythrocytes is recognized by Fc receptor (17), which is either on the surface of monocytes (in case of rosette formation) or on the surface of cytotoxic lymphoid cells (in case of ADCC). Thus, the above results indicate that sugar chains of IgG are required for the recognition of IgG by Fc receptor and by complement.

We observed that digestion with neuraminidase and β-galactosidase was not sufficient to cause the inactivations (Table I, Exp. 4). Furthermore, N-acetylglucosamino-1-5-lactone, a specific inhibitor of β-N-acetylglucosaminidase, inhibited the inactivations (Exp. 5). Therefore, either β-N-acetylglucosaminidase or endo-β-N-acetylglucosaminidase D was responsible for the inactivation (Fig. 1). Thus the sugar residue required for the maintenance of the activities is proposed to be either N-acetylglucosamine or

TABLE II

Effects of Sugars on Activities of IgG

Sugars	Activities in		
	Rosette Formation	ADCC	complement -dependent Hemolysis
None	22.5	55.1	100
N-Acetylglucosamine	10.3	32.9	73.6
N-Acetylgalactosamine	14.0	N.D.	61.0
Mannose	21.0	48.2	76.7
Galactose	18.1	48.4	77.9

The concentration of sugars in the reaction mixture was 100 mM in case of rosette formation and complement-dependent hemolysis, and 20 mM in case of ADCC. Assays were performed as described in the legand of Table I.

N.D.: Experiment was not performed.

mannose. Since β-N-acetylglucosaminidase from the source was contaminated with trace amounts of endo-β-N-acetylglucosaminidase D, we could not tell whether removal of the external N-acetylglucosamine was sufficient to cause the inactivations.

Since glycosidase digestion simultaneously impaired the three recognition phenomena, namely recognition by Fc receptor on two different cells and also by complement, we suggest that the inactivations occurred by a similar mechanism. One possibility is that in addition to the peptide sequence (14), carbohydrates are also recognized by Fc receptor and complement. The observation that sugars, especially N-acetylglucosamine inhibited the three immunological reactions (Table II) may support this possibility. The sugars did not inhibit the hemagglutination reaction at the concentration of 0.1 M. The other possibility is that carbohyd-

rates are required to maintain the specific three dimentional structure of Fc region which is essential for the recognition. In any event it appears that the carbohydrate of IgG is important in the efficient performance of various immunological functions.

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