

Sialic Acid Residues Are Essential for the Anaphylactic Activity of Murine IgG1 Antibodies¹

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Glycosylation of the Ab molecule is essential for maintaining the functional structure of Fc region and consequently for Ab-mediated effector functions, such as binding to cells or complement system activation. Alterations in the composition of the sugar moiety can dramatically influence Ab activity; however, it is not completely clear how differences in the *N*-linked oligosaccharide structure impact the biological function of Abs. We have described that murine IgG1 Abs can be separated according to their ability to elicit in vivo anaphylaxis in a fraction of anaphylactic and other of non-anaphylactic molecules. Furthermore, we showed that the *N*-linked oligosaccharide chain is essential for the structural conformation of the anaphylactic IgG1, the binding to FcγRIII on mast cells, and, consequently, for the ability to mediate anaphylactic reactions. In this study, we evaluated the contribution of individual sugar residues to this biological function. Differences in the glycan composition were observed when we analyzed oligosaccharide chains from anaphylactic or non-anaphylactic IgG1, mainly the presence of more sialic acid and fucose residues in anaphylactic molecules. Interestingly, the enzymatic removal of terminal sialic acid residues in anaphylactic IgG1 resulted in loss of the ability to trigger mast cell degranulation and in vivo anaphylactic reaction, similarly to the deglycosylated IgG1 Ab. In contrast, fucose removal did not affect the anaphylactic function. Therefore, we demonstrated that the ability of murine IgG1 Abs to mediate anaphylaxis is directly dependent on the amount of sialic acid residues associated to the oligosaccharide chain attached to the Fc region of these molecules. *The Journal of Immunology*, 2008, 181: 8308–8314.

Abs are functional molecules that mediate Ag/pathogen elimination through Ag-Ab complex formation, followed by activation of immune defense mechanisms. After the Ag/pathogen recognition, distinct Ab isotypes are able to interact with different cells via FcR expressed on the cell surface and then promote enhancement of phagocytosis and macrophage activation, Ag presentation, Ab production, and Ab-dependent cellular cytotoxicity, and also mediate complement activation by the classical pathway (1–3).

The ability of Abs to bind and activate cells via FcR and C1q component of the complement system is directly related to the integrity of C_H2 and C_H3 domains of the Fc region of IgG and IgE molecules, respectively. Furthermore, it was shown that *N*-linked oligosaccharides attached to the Fc region are essential for maintaining the conformational structure and functional activity of the Ab molecule (4–6). The attached carbohydrate structure varies according to the Ab isotype and can include high mannose, multiply branched, as well as biantennary complex chains. Studies on the glycosylation pattern of different subclasses of Abs showed

large heterogeneity among the structures attached to them, indicating that in some cases certain structures appear to be associated with aberrant Ab function (7–12). IgG Abs from patients with rheumatoid arthritis present a higher proportion of oligosaccharides structures ending in *N*-acetylglucosamine than from normal ones (13, 14). More recently, several aberrant patterns of glycans have been detected in circulating IgA from patients with IgA nephropathy, including increased *N*-acetylglucosamine residue content and decreased galactose content on *O*-linked glycans in the hinge region of H chains, suggesting that the carbohydrate chain can be involved in the IgA reactivity in this disease (15, 16). By contrast, general approaches to remove or inhibit oligosaccharide attachment revealed profound depression of certain Ab functions (5, 11, 17). However, it is not completely clear to date how differences in the *N*-linked oligosaccharide structures impact the biological activity of Abs.

Among the Ab isotypes, IgE is known to induce mast cell degranulation and elicit anaphylactic reactions (18, 19). Clustering of FcεRI-bound IgE by multivalent allergens stimulates cellular activation and release of lipid mediators and cytokines involved in the development and maintenance of the allergic process (20). In rodent models, Ag cross-linking of IgG1 bound to FcγRIII on mast cells and basophils is able to elicit anaphylactic reactions in a similar way to those induced through IgE-FcεRI (21–23).

However, we have previously demonstrated that only a fraction of murine IgG1 Abs has the ability to induce anaphylaxis. Anaphylactic and non-anaphylactic IgG1 Abs are regulated by distinct cytokines, and their synthesis can be modulated by adjuvants (24, 25). In addition, using two murine IgG1 mAbs with the same Ag specificity (anti-DNP), one being anaphylactic and the other non-anaphylactic, we have also shown that the ability to mediate anaphylaxis is directly related to their binding to FcγRIII expressed on mast cells and that the *N*-linked oligosaccharide chains are essential for the proper structural conformation that allows this binding (26). In the present report, we studied the structure of the *N*-linked

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oligosaccharide chains associated to these two types of IgG1 and the contribution of individual sugar residues to the biological difference that they display. Although the same amino acid sequence in the C_H2 and C_H3 domains of the H chain was found in both IgG1 Abs, lectin-binding assays showed differences in the glycan composition of their Fc regions. The analysis of the oligosaccharide chains by high pH anion exchange chromatography (HPAEC-PAD)³ revealed minimal sialic acid and fucose residues in the carbohydrate chain of non-anaphylactic IgG1 compared with anaphylactic IgG1 Ab. The enzymatic removal of the carbohydrate chain or of the terminal sialic acid residues in anaphylactic IgG1 resulted in loss of the Ab ability to trigger mast cell degranulation and in vivo anaphylactic reaction. In contrast, fucose removal did not have this effect. These results demonstrate that the ability of the murine IgG1 Ab to mediate anaphylaxis is directly dependent on the amount of sialic acid residues associated to the oligosaccharide chain attached to the Fc region of the molecule.

Materials and Methods

Production of IgG1 mAbs

Two IgG1-producing hybridoma cell lines (U7.6 and B84405-H5) were cultured in RPMI 1640 plus 0.1 mM L-glutamine, 0.5 mM 2-ME, and 10% heat-inactivated FCS. The mAb were purified from each supernatant sample by protein G-Sepharose 4 Fast Flow affinity chromatography, as described by the manufacturer's manual (GE Healthcare Life Science), and their total concentrations were estimated by the Bradford method (27).

Obtaining IgG1 Fab and Fc fragments by papain digestion

To obtain Fab and Fc fragments, IgG1 samples were digested by solid-phase papain (Sigma-Aldrich), as described by Coulter and Harris (28). Briefly, 3.2 mg of cysteine hydrochloride, 1.6 mg of EDTA, and 0.25 mg of solid-phase papain in a final volume of 1 ml were added to 1 mg of anaphylactic or nonanaphylactic IgG1 in PBS. The samples were incubated at 37°C for 4 h under shaking. The digested samples were isolated from the solid phase (papain-agarose) by centrifugation at 1200 × g for 5 min. The IgG1 Fab or Fc fragments were purified by protein A-agarose affinity chromatography (Sigma-Aldrich), according to manufacturer's instructions. The fractions were collected, dialyzed against PBS, and concentrated by ultrafiltration system (Millipore), and the protein contents were estimated by Bradford method (27). The purity of the Fab and Fc fragments was checked by 12% SDS-PAGE stained with Coomassie blue.

Lectin-binding affinity of anaphylactic and nonanaphylactic IgG1 by ELISA

The binding affinity of intact anaphylactic or non-anaphylactic IgG1, IgG1 Fab, or Fc fragments, for different lectins was evaluated by an ELISA, using 96-well-plates (Costar) coated with 1 µg/ml *Canavalia ensiformis*, *Limulus polyphemus*, *Sambucus nigra* (SNA), *Datura stramonium*, *Ulex europaeus* I, *Bauhinia purpurea*, or *Erythrina cristagalli* (Sigma-Aldrich) diluted in PBS (pH 7.2), and incubated overnight at 4°C. After this, the plates were washed with PBST and blocked with PBST plus gelatin 3% for 3 h at 37°C. The plates were washed three times, and 500 ng of each sample of IgG1 was added, followed by 1 h of incubation at 37°C. The IgG1 binding to the different lectins was developed using a biotinylated goat anti-mouse IgG1 (Southern Biotechnology Associates), followed by streptavidin-peroxidase (Sigma-Aldrich). For IgG1 Fab and Fc detection, a peroxidase-labeled goat anti-mouse κ L chain (Sigma-Aldrich) or a biotinylated goat anti-mouse IgG1 (Southern Biotechnology Associates) plus streptavidin-peroxidase was used, respectively. The reactions were developed by the addition of o-phenylenediamine and 30% hydrogen peroxide (Sigma-Aldrich) diluted in 0.1 M sodium citrate buffer. Reactions were stopped after 15 min with sulfuric acid (2%) and were read at 492 nm on an automated ELISA reader (LabSystems Multiskan EX).

Analysis of the anaphylactic activity of IgG1 Abs by passive cutaneous anaphylactic (PCA)

The anaphylactic activity of different IgG1 samples was evaluated by PCA reactions in mice, as described by Ovary (29). BALB/c mice were shaved on the back, and three intradermal injections (50 µl) of different concentrations of anaphylactic, nonanaphylactic, desialylated, or defucosylated IgG1 Abs were made in each side of dorsal skin. The concentrations of each sample varied from 50 to 5000 ng/site. After 2 h, the mice were challenged i.v. with 0.5 ml of 0.25% Evans blue solution containing 250 µg of DNP-BSA. All tests were made in triplicate, and the results express the Ab concentration that gave or did not give a lesion of at least 5 mm in diameter. The PCA protocol (308/06) was approved by Butantan Institute (and by Biomedical Sciences Institute/University of São Paulo) Ethical Committee for Animal Research.

IgG1 binding to FcγR on mast cells

Samples of IgG1 Ab treated with neuraminidase (described below) were tested for their ability to bind to mast cells, according to the protocol described by Faquim-Mauro et al. (26). Thus, 1 × 10⁶ mast cells (PT18) were incubated with 40 or 20 ng of desialylated IgG1 (from neuraminidase treatment) or nonanaphylactic IgG1 Abs for 30 min at 4°C. As positive control, PT18 mast cells were incubated with the same concentrations of anaphylactic IgG1 Ab. Afterward, the cells were washed twice (PBS plus 0.1% BSA) and incubated with biotinylated, goat anti-mouse IgG1 Ab (Southern Biotechnology Associates) in PBS-azide containing 5% FCS, for 30 min at 4°C. The cells were washed, resuspended in streptavidin FITC (GE Healthcare Life Science), incubated for 30 min more at 4°C, centrifuged, resuspended in PBS-azide with 1% paraformaldehyde, and analyzed by flow cytometry with FACSCalibur (BD Biosciences). The results represent the average percentage of labeled cells in duplicate samples.

Measurement of β-glucuronidase released by mast cells

The ability of desialylated IgG1 Ab to induce mast cell degranulation was investigated in vitro using the protocol of quantification of the granular enzyme β-glucuronidase in the supernatants from PT18 mast cells, according to Ortega et al. (30). Mast cells (8 × 10⁵) were incubated in the presence of 40 ng of desialylated IgG1, anaphylactic, or nonanaphylactic IgG1 Abs for 30 min in sodium PIPES buffer at 37°C/5% CO₂. The cells were washed with Na-PIPES buffer and challenged with 5 ng of DNP-BSA for 15 min. Afterward, the cells were centrifuged and the supernatants were collected. The cells incubated only with Na-PIPES buffer were lysed with 0.1 M acetate buffer (pH 4.5; 200 µl) and agitated gently to evaluate the total enzyme content. From each experimental sample to be assayed, four aliquots (10 µl) of the supernatant were taken to separate microwell plates. To each sample, 20 µl of the substrate solution (0.01 M phenolphthalein glucuronic acid; Sigma-Aldrich) in 70 µl of 0.1 M sodium acetate buffer (pH 4.5) was added, and the plates were incubated for 12 h at 37°C. The reactions were stopped by addition of 100 µl of 0.2 M glycine solution (pH 10.7), and the OD was determined at 570 nm in an ELISA reader (LabSystems Multiskan EX). The extent of secretion was expressed as the net percentage of the total β-glucuronidase activity in the supernatant of unstimulated cells. The results represent the mean of quadruplicate tests ± SD.

Enzymatic modification of IgG1 anaphylactic carbohydrate chain residues

IgG1 deglycosylation. The N-linked oligosaccharides chains from anaphylactic or nonanaphylactic IgG1 were removed by N-glycosidase F (PNGase) (New England Biolabs) enzymatic treatment. Samples containing 240 µg of each type of IgG1 Ab were resuspended in 20 mM sodium phosphate buffer (pH 7.5) and treated with 120 mU enzyme PNGase F for 18 h at 37°C. The reaction was stopped by the addition of 0.2% of SDS solution according to Verbet (31). The samples were dialyzed against PBS and stored at -20°C.

IgG1 desialylation. Samples of anaphylactic IgG1 Abs were dialyzed against 50 mM sodium acetate buffer (pH 5.0) and subsequently subjected to enzymatic cleavage according to protocols established by the supplier (New England Biolabs). The Ab samples were incubated overnight at 37°C with a neuraminidase enzyme specific for α2-3-, α2-6-, and α2-8-linked N-acetyl-neuraminic acid residues (from *Clostridium perfringens*; New England Biolabs) using 1 µl of enzyme per 12 µg of IgG1 Ab. The efficiency of the treatment was tested by affinity chromatography using a Sepharose cross-linked to SNA lectin column (Vector Laboratories).

³ Abbreviations used in this paper: HPAEC-PAD, high pH anion exchange chromatography; PCA, passive cutaneous anaphylactic; PNGase F, N-glycosidase F; SNA, *Sambucus nigra*.

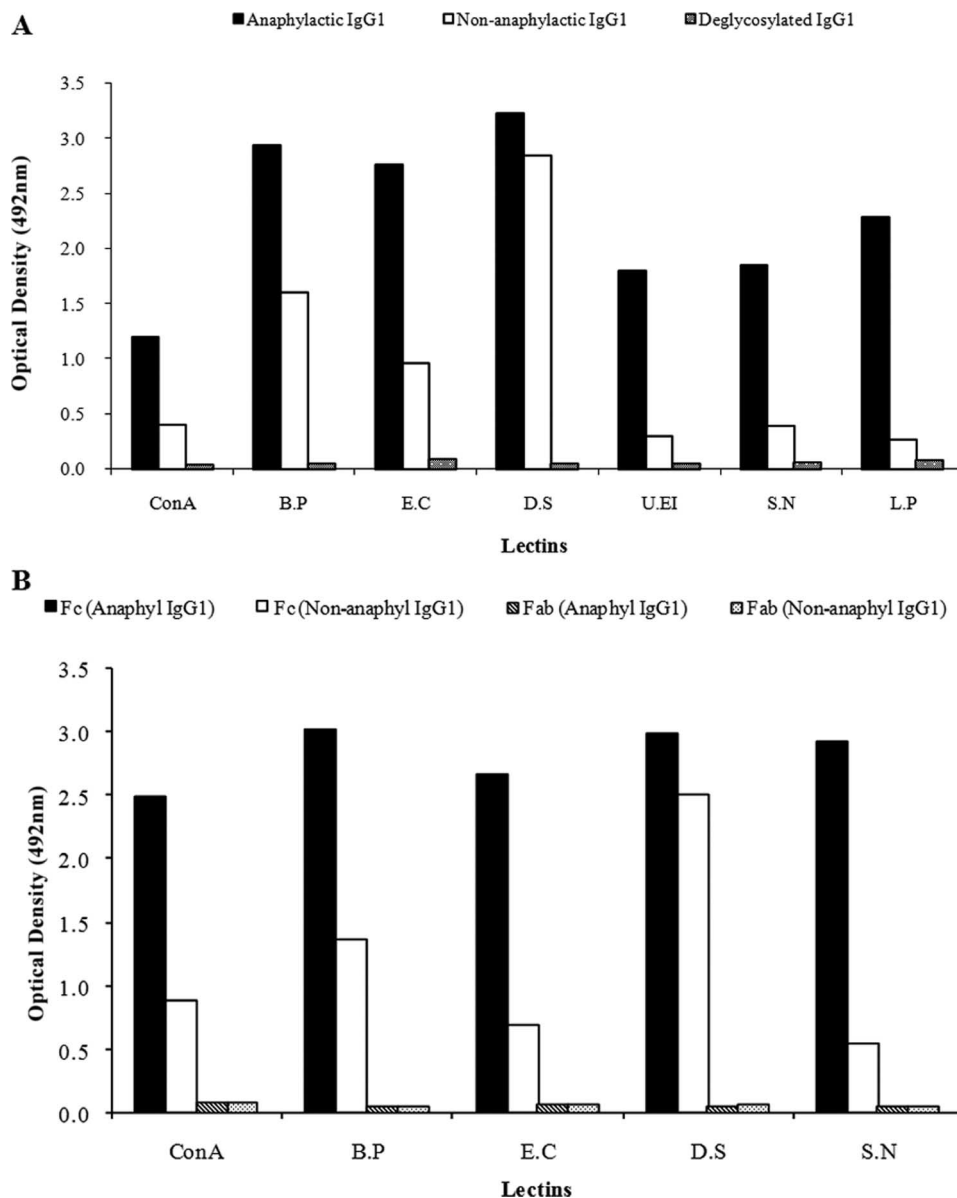


FIGURE 1. Lectin affinity of anaphylactic IgG1 (■), nonanaphylactic IgG1 (□), and deglycosylated IgG1 (▨) by ELISA. Samples of 500 ng of each type of IgG1 (A) or IgG1 Fab and Fc fragments (B) were incubated in plates previously covered with distinct lectins, followed by incubation with biotinylated anti-mouse IgG1 Ab plus streptavidin-peroxidase or peroxidase-labeled anti-mouse κ L chain. The following lectins were used: *C. ensiformis* (Con A), *B. purpurea* (B.P), *E. cristagalli* (E.C), *D. stramonium* (D.S), *U. europaeus I* (U.EI), *S. nigra* (S.N), and *L. polyphemus* (L.P).

IgG1 defucosylation. Anaphylactic IgG1 Abs in 50 mM sodium acetate (pH 5) were treated with α -fucosidase (from *Xanthomonas manihotis*; New England Biolabs), in the proportion of 12 μ g of the Ab to 1 μ l of the enzyme, overnight at 37°C. To analyze the efficiency of the enzymatic treatment, the samples were tested for their affinity to *U. europaeus I*-agarose column specific for α -L-fucose residues attached to glycoprotein.

Analysis of the N-linked oligosaccharide chains from IgG1 Abs

Preparation of the samples and enzymatic digestion. Anaphylactic and nonanaphylactic IgG1 Abs in PBS were dialyzed against Milli-Q water cutoff 12,000 for 24 h, samples were lyophilized (SC-110A Speed Vac Plus; Thermo Savant), and glycoproteins were dissolved in 0.5% SDS and 1% 2-mercaptoethanol, and heated for 2 min at 100°C. Digestion buffer (500 mM sodium phosphate (pH 7.5) and 1% Nonidet P-40) and PNGase F enzyme (New England Biolabs) were added, and the samples were incubated for 18 h at 37°C. The oligosaccharides were separated from the protein by Ultrafree-MC filters (Millipore; 5 kDa) and analyzed by HPAEC-PAD.

HPAEC-PAD analysis

The N-linked oligosaccharide chains from IgG1 Abs were analyzed by HPAEC-PAD in a DX-3000 Dionex BioLC system with pulsed amperometric detection (HPAEC-PAD; Dionex). Separation of carbohydrates was conducted on a CarboPac PA-200 column (25 $\times$ 0.4 cm) with a PA-200 precolumn and a 20- μ l injection loop. The following conditions were used:

1) 100 mM NaOH, followed by a gradient elution 0–5 mM sodium acetate during 50 min; the flow rate was 0.5 ml/min⁻¹; 2) 100 mM NaOH, followed by a gradient elution with 2–15 mM sodium acetate during 60 min; the flow rate was 0.46 ml $\cdot$ min⁻¹.

Mild acid hydrolysis

Samples were hydrolyzed with 0.01 M HCl for 30 min at 100°C and further freeze dried.

Affinity chromatography

***U. europaeus I*-agarose column.** The gel beads were packed in 1-ml column and washed with 0.1 M phosphate saline buffer (PBS; pH 7.4). The column was loaded with 0.4 ml of anaphylactic IgG1 Ab or defucosylated IgG1. The efflux was clamped, and the column was incubated for 1 h at room temperature. Elution was performed with PBS and 0.1 M L-fucose (Sigma-Aldrich) in PBS. Fractions of 1.0 ml were collected, and protein was monitored by absorbance at 280 nm.

***S. nigra*-agarose column.** Agarose-bound SNA lectin (Vector Laboratories) was used for evaluation of sialic acid containing IgG1. For this, IgG1 mAb (1.0 mg) diluted in TBS (pH 7.5; 4 ml) was applied to a SNA resin column (5 ml). After incubation for 10 min at room temperature, the SNA column was washed with TBS (5 ml), and the flow-through fraction was collected and defined as the non-SNA-binding fraction. After additional washing with TBS (5 ml), the bound glycoprotein was eluted with

0.5 M lactose (Sigma-Aldrich) in TBS (4 ml), followed by the same amount of 0.5 M lactose in 0.2 M acetic acid (4 ml).

Statistical analysis

The results obtained from the β -glucuronidase activity assay were submitted to one-way ANOVA analysis, followed by Tukey's multiple comparison test (32).

Results

Analysis of the binding of anaphylactic and nonanaphylactic IgG1 Abs to distinct lectins

The ability of lectins to bind specifically monosaccharidic or oligosaccharidic sequences is frequently used to explore and characterize carbohydrate chains linked to glycoproteins (33, 34). Therefore, we performed an ELISA using plates coated with different lectins, followed by incubation with the IgG1 Abs, IgG1 Fab, or Fc fragments to analyze and compare the structures of oligosaccharide chains of both types of IgG1.

The interaction with lectins specific for α -D-mannose residues (*C. ensiformis*), β -N-acetylgalactosamine residues (*B. purpurea*), β -D-galactose and β -D-N-acetylglucosamine residues (*E. crista-gali*), β -N-acetylglucosamine residues (*D. stramonium*), L-fucose residues (*U. europaeus I*), and sialic acid residues (*S. nigra* and *L. polyphemus* lectins) was analyzed. Interestingly, both types of IgG1 bound to the lectins (Fig. 1A) as well as their Fc fragments (Fig. 1B), indicating that complex type oligosaccharides were present in both Abs and only attached to the Fc region. However, differences in the degree of interaction of anaphylactic and non-anaphylactic Abs with almost all the lectins tested were observed, especially with *S. nigra*, *L. polyphemus*, and *U. europaeus I* lectins, pointing out a possible different content of sialic acid and fucose residues between anaphylactic and non-anaphylactic Abs. The specificity of the assay was confirmed by using a sample of deglycosylated IgG1 Ab obtained after treatment of anaphylactic IgG1 Ab with PNGase F enzyme.

Analysis of the oligosaccharide chains attached to anaphylactic IgG1 and non-anaphylactic IgG1 by affinity chromatography and HPAEC-PAD

To confirm the differences in the sialic acid and fucose content bound to the carbohydrate chains of anaphylactic and non-anaphylactic IgG1, samples of both Abs were subjected to immobilized lectin affinity chromatography. First, a *S. nigra* lectin column that shows specificity for sialic acid attached to α 2-6 Gal/GalNAc containing chains was performed (Fig. 2). The profile obtained clearly shows that the anaphylactic IgG1 Ab presents high binding affinity for the lectin, being only desorbed by addition of the appropriate competitive sugar. On the contrary, the non-anaphylactic IgG1 did not bind to the *S. nigra* lectin and was eluted at the void volume with the equilibrating buffer. As expected, the desialylated anaphylactic Abs did not bind to the lectin column (Fig. 2).

In parallel, samples of the anaphylactic and non-anaphylactic IgG1 were subjected to an enzymatic deglycosylation, and oligosaccharides were separated from the protein moiety and were analyzed by HPAEC-PAD using conditions in which monosialylated, disialylated, and trisialylated oligosaccharides are separated (Fig. 3). The profile obtained clearly shows that anaphylactic Abs present peaks migrating as mono- and disialylated standard oligosaccharides (Fig. 3A), whereas the non-anaphylactic sample only showed a minor peak corresponding to a disialylated chain (Fig. 3B). Neuraminidase digestion of the oligosaccharide sample obtained from anaphylactic Abs, followed by analysis by HPAEC-PAD under the same conditions, unambiguously showed the disappearance of the whole pattern of oligosaccharides (Fig. 3C).

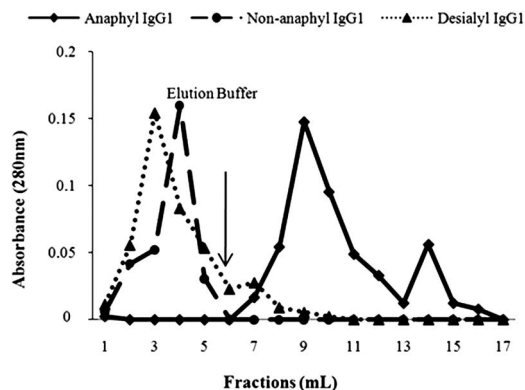


FIGURE 2. Representative profile of anaphylactic IgG1 (◆), non-anaphylactic IgG1 (●), and desialylated IgG1 (▲) isolated by affinity chromatography on immobilized *S. nigra* lectin column. Samples of each type of IgG1 (0.8 mg) were applied to an agarose-bound SNA lectin column. The arrow indicates when the elution with TBS solution containing the 0.2 M lactose as competitor sugar began.

When the fucose residues attached to the oligosaccharide chains were analyzed using an *U. europaeus I* lectin column, it was observed that the anaphylactic IgG1 bound to the column and could only be released by elution with the competitive monosaccharide. In contrast, the non-anaphylactic IgG1 Ab was completely eluted in the equilibrating buffer. In the same way, defucosylated anaphylactic Abs did not bind to the lectin column (Fig. 4).

These results clearly confirm that anaphylactic IgG1 Abs have differences in the oligosaccharide chains, mainly concerning sialic acid and fucose residues, with the non-anaphylactic ones.

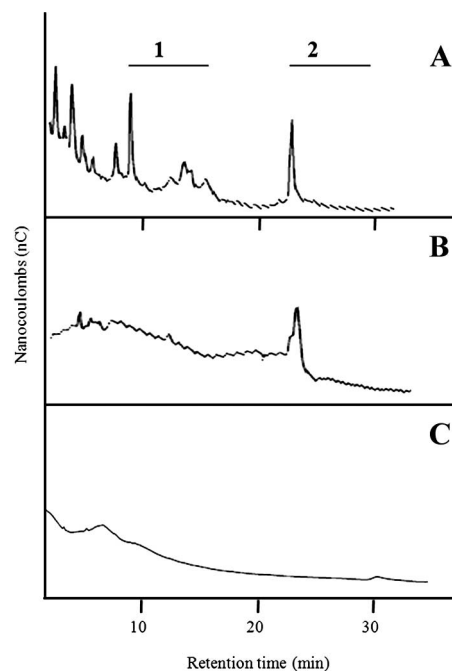


FIGURE 3. HPAEC-PAD analysis of the N-linked oligosaccharide chains released by PNGase F treatment from anaphylactic, nonanaphylactic, and desialylated IgG1 Abs. Samples of anaphylactic (A), nonanaphylactic (B), or desialylated IgG1 Abs (C) were treated with PNGase F to release the carbohydrate chains, and these products were analyzed under acidic conditions for acidic glycans. The indication 1 and 2 represent the monosialylated and disialylated chains, respectively, by comparison with the elution of standard oligosaccharides obtained from fetuin.

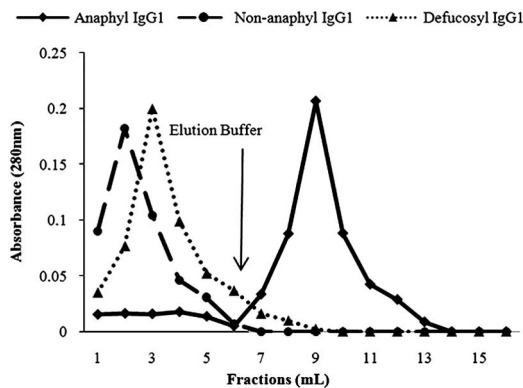


FIGURE 4. Representative profile of anaphylactic, nonanaphylactic, and defucosylated IgG1 Abs submitted to agarose-bound *U. europaeus* I lectin chromatography. Samples (150 μ g) of anaphylactic ($\blacklozenge$), nonanaphylactic IgG1 ($\bullet$), or defucosylated IgG1 ($\blacktriangle$) were applied to an agarose-bound *U. europaeus* I column. The arrow indicates when elution with 0.1 M L-fucose in PBS (pH 7.4) began.

Involvement of sialic acid residues in the ability of IgG1 to mediate anaphylaxis

Taking into account the differences in sialic acid and fucose contents between the oligosaccharide chains of both types of IgG1 molecules and to evaluate the potential role of these monosaccharides in the biological activity of the anaphylactic IgG1, PCA assays were performed using desialylated and defucosylated Abs.

Samples of anaphylactic IgG1 were treated with neuraminidase or fucosidase to completely remove the sialic acid or fucose residues attached to the sugar chains. Afterward, the samples were analyzed for their ability to elicit PCA reactions. Samples of untreated anaphylactic and non-anaphylactic IgG1 were also tested as positive and negative controls, respectively.

Table I shows that anaphylactic IgG1 at a concentration of 50 ng/site elicited positive PCA reaction, whereas non-anaphylactic IgG1 Ab was unable to mediate anaphylactic reaction, as previously described (26). Interestingly, desialylated IgG1 Ab was also unable to induce positive PCA reaction after antigenic challenge, even when higher concentrations of this sample (100 times more) were tested. In contrast, the defucosylated IgG1 elicited PCA reactions in the same way as untreated anaphylactic IgG1.

In all cases, the efficient removal of sialic acid and fucose from IgG1 Ab samples was confirmed by affinity chromatography in *U. europaeus* I lectin column and *S. nigra* lectin column, and the integrity of the desialylated IgG1 Ab was verified by Western blot (data not shown).

Altogether, these results indicate a strong participation of the sialic acid residues in the ability of IgG1 to mediate anaphylaxis.

Table I. PCA reactions^a

Antibody	Anti-DNP PCA Reaction
Anaphylactic IgG1	Positive (50 ng/site)
Deglycosylated IgG1	Negative (50-5,000 ng/site)
Desialylated IgG1	Negative (50-5,000 ng/site)
Defucosylated	Positive (50 ng/site)
Nonanaphylactic IgG1	Negative (50-5,000 ng/site)

^a Anti-DNP PCA reactions elicited in mice previously sensitized with different concentrations of either type of IgG1 Ab. The lowest and highest Ab concentrations intradermally injected are shown in parentheses.

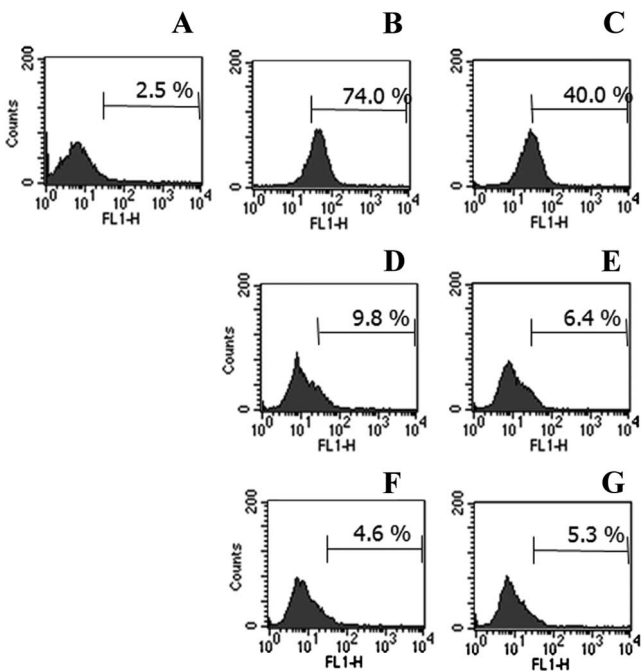


FIGURE 5. Analysis by flow cytometry of PT18 mast cells incubated with anaphylactic, non-anaphylactic, or desialylated IgG1 Abs, followed by a biotinylated goat anti-mouse IgG1 and streptavidin FITC: A, mast cells incubated only with the secondary Ab and streptavidin FITC; B and C, percentage of labeled mast cells incubated with 40 or 20 ng of anaphylactic IgG1, respectively; D and E, percentage of labeled mast cells incubated with the same concentrations of non-anaphylactic IgG1; and F and G, percentage of labeled mast cells incubated with 40 or 20 ng of desialylated IgG1. The histograms are representative of three different experiments.

Analysis of the binding to Fc γ R on mast cells and consequent cellular degranulation

Murine IgE and IgG1 Abs are able to elicit anaphylaxis through binding to specific FcRs on mast cells and basophil surfaces that after Ag contact are activated, degranulate, and secrete mediators involved in the reaction (23, 35, 36). Therefore, the inability of desialylated IgG1 Abs to elicit PCA reactions could be due to a deficiency in binding to mast cells or a defect in transmitting an activation signal. To investigate this topic, we incubated a cell line (PT18), which expresses Fc ϵ R1 and Fc γ RII/III as a typical mast/

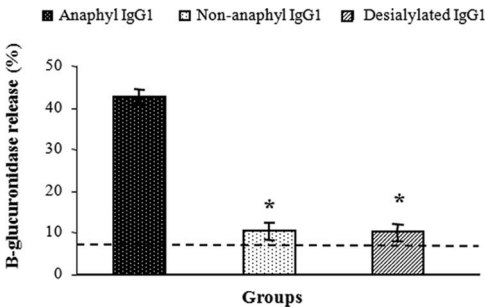


FIGURE 6. Release of granular β -glucuronidase enzyme from PT18 mast cells incubated with anti-DNP anaphylactic, nonanaphylactic, or desialylated IgG1 Abs and challenged with DNP-BSA. The cells were incubated for 30 min with 40 ng of anaphylactic, nonanaphylactic, or desialylated IgG1 Abs. Then, they were challenged with DNP-BSA, and the released β -glucuronidase was evaluated. The results are expressed as the net percentage of the total β -glucuronidase activity present in the cells, after subtracting the activity in the supernatant of unstimulated cells (mean of quadruplicate tests $\pm$ SD). *, $p < 0.05$ compared with anaphylactic IgG1.

basophil cell (26, 37), with two concentrations of desialylated IgG1, and performed a flow cytometry analysis (Fig. 5). Anaphylactic and non-anaphylactic IgG1 Abs were also used as positive and negative controls, respectively. The results showed that whereas the percentage of labeled mast cells when incubated with the anaphylactic IgG1 was 74–40% (Fig. 5, *B* and *C*), the percentage of labeled mast cells when incubation was performed with desialylated IgG1 Ab was only 4.6–5.3% (Fig. 5, *D* and *E*). Similarly, a low percentage of labeled mast cells was obtained when incubated with both concentrations of the non-anaphylactic IgG1 (9.8–6.4%; Fig. 5, *F* and *G*).

In addition, the ability to induce cellular degranulation was evaluated by measuring the amount of released β -glucuronidase enzyme (Fig. 6). The results showed that the percentage of released β -glucuronidase was significantly lower when cells were incubated with the desialylated IgG1 Ab than when cells were incubated with the nontreated anaphylactic IgG1. Moreover, a low percentage of released β -glucuronidase was also observed in mast cells incubated with the nonanaphylactic IgG1.

These results show that the inability of desialylated IgG1 Ab to elicit *in vivo* anaphylaxis is directly related to its low binding to Fc γ RIII receptors on mast cells, and, consequently, low activation of these cells.

Discussion

We have previously described that murine IgG1 Abs can be separated in two distinct populations according to their ability to elicit anaphylactic reactions. The anaphylactic and the non-anaphylactic population differ in regard to their induction by Th2 and Th1 cytokines, respectively (24). Moreover, the carbohydrate chain found in the Fc region of the anaphylactic IgG1 Ab is crucial for its biological activity, because deglycosylated molecules behave like the non-anaphylactic IgG1 (26). In this study, we demonstrated that the ability of murine IgG1 Abs to elicit anaphylaxis is directly dependent on the sialic acid content of the oligosaccharide chain attached to these molecules. These sialic acid residues are essential for the binding of the Fc region of IgG1 to Fc γ RIII on mast cell surface that results in cellular activation and degranulation after cross-linking of Ab molecules by anti-IgG1 Abs or Ag.

The impact of glycosylation, one of the most important post-translational modifications in the structure and biological properties of proteins, including Abs, has been well documented (6, 9, 38, 39). Several reports have clearly shown that glycosylation is essential for optimal IgG Ab-mediated immune responses, and that the carbohydrate composition can also influence its effector function (4, 12, 40, 41). For IgG, the major serum Ig, the abundant oligosaccharide chain attached at Asn²⁹⁷ is of the complex biantennary type containing mainly *N*-acetylglucosamine, mannose, galactose, and variable contents of fucose and terminal sialic acid residues. Over 30 different *N*-linked core oligosaccharide structures were detected for serum polyclonal IgG (39, 42, 43).

Analysis of oligosaccharidic chains from anaphylactic and non-anaphylactic IgG1 by lectin binding, affinity chromatography, and HPAEC-PAD showed that both IgG1 Abs present *N*-linked complex type chains composed by *N*-acetylglucosamine and mannose, but with different contents of galactose, fucose, and sialic acid residues attached to Fc region, suggesting that one or all of these sugar residues could be important for the anaphylactic activity. The type of the oligosaccharide chain attached to proteins is influenced by many parameters, including the galactosylation on the mannose-*N*-acetylglucosamine arm that is essential for sialylation (44, 45). Sialic acid is usually the last glycosyl group transferred to glycoconjugates, and therefore, has potential for changing the interaction of proteins with cell surface components (46, 47).

The relevance of sialic acid present on the polysaccharide chains attached to Abs to their effector functions was confirmed by Kaneko et al. (42), who demonstrated that differential sialylation of human or mouse IgG Abs determines the capacity of these molecules to mediate pro- or anti-inflammatory activities. These authors showed in a murine model of arthritis that the *i.v.* administration of human IgG is efficient in the treatment of the disease and this protection is mediated by the sialylated IgG fraction. The protective activity of sialylated IgG Abs was mediated by the enhancement of the inhibitory Fc γ RIIB on effector macrophages, as described for unfractionated *i.v.* administration of human IgG.

For anaphylaxis, only a few reports described the essential role of *N*-glycosylation of murine IgE Abs to their ability to bind to and induce mast cell degranulation and consequently to mediate anaphylactic reaction (48, 49). In this work, we demonstrated for the first time a direct correlation between the presence of high sialic acid content in the oligosaccharide structure of IgG1 Fc region and its anaphylactic activity, because the non-anaphylactic IgG1 oligosaccharide chain contained much less sialic acid residues than the anaphylactic IgG1 chain. In addition, the desialylated anaphylactic IgG1 lost its ability to induce PCA reactions. The loss of anaphylactic activity was not due to Ab degradation by neuraminidase treatment, as confirmed by SDS-PAGE analysis (data not shown). Besides Fc γ RI and Fc γ RIII, murine mast cells also express Fc γ RII, which plays a negative role in the activation process, Fc γ RII-deficient mice being highly sensitive to IgG-triggered degranulation mediated by Fc γ RIII (50). The inability of non-anaphylactic IgG1 and desialylated IgG1 to mediate anaphylaxis might, therefore, be due to binding to Fc γ RII and down-regulation of the anaphylactic reaction. This hypothesis, however, was discarded, because our flow cytometry results showed that the low content or lack of sialic acid residues resulted in reduction of Ab binding to PT18 cells, and less cell activation and degranulation, as measured by β -glucuronidase enzyme release. Moreover, the possibility that removal of sialic acid would affect the affinity of the Fab arm of IgG1 Ab for Ag was also discarded, because desialylated IgG1 recognized Ag in the same way as anaphylactic and non-anaphylactic IgG1 in ELISA (data not shown).

Recently, Shield et al. (51) studying the effect of galactosylation and fucosylation on the binding affinity of human IgG to Fc γ Rs reported that IgG1 binding to Fc γ RI is insensitive to the presence of fucose and galactose; however, for the binding to Fc γ RIIA, fucose residues are rather important. In contrast, for human IgG1 binding to Fc γ RIIIA, the lack of fucose results in a higher Ab-binding capacity to this receptor, and thus enhances Ab-dependent cytotoxicity by NK cells. According to our observations in PCA reactions, fucose does not seem also to participate in binding of murine IgG1 to Fc γ RIIIs on mast cells, because its removal did not have any effect on the anaphylactic activity of IgG1. Therefore, we did not pursue this approach any further.

It is well known that the final structure of an oligosaccharide chain is dependent upon the action of cellular glycosyltransferases and exoglycosidases. Nishiura et al. (52) found hypogalactosylated IgG paraproteins and reduced galactosyltransferase activity in bone marrow cells from patients with multiple myeloma. In contrast, other studies showed high levels of oversialylated IgG Abs in the serum from patients with multiple myeloma and increased sialyltransferase activity in their peripheral mononuclear cells (53–55). Therefore, the high sialylation observed in the anaphylactic IgG1 might be due to increased glycosyltransferase, specially sialyltransferase, activity mediated by distinct enzymes during its synthesis.

In conclusion, the present data confirm our previous hypothesis that the structural difference between the anaphylactic- and the

non-anaphylactic-type IgG1 relies on the glycosylation pattern of the molecule. In addition, they also clearly demonstrate that the degree of sialylation of the murine IgG1 Ab determines its ability to mediate anaphylaxis. Studies to elucidate the regulation of sialyltransferase activities under different experimental conditions are now underway.

Disclosures

The authors have no financial conflict of interest.

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