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# Anaphylactic and non-anaphylactic murine IgG1 differ in their ability to bind to mast cells: relevance of proper glycosylation of the molecule

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## Abstract

We have previously shown that murine IgG1 antibodies comprise two functionally distinct types regarding their ability to induce mast cell degranulation. In this work, we identified two IgG1-producing hybridomas, both with the same antigenic specificity (anti-DNP), but different *in vivo* anaphylactic activities. Whereas one of them secretes the anaphylactic IgG1 antibody, as assessed by passive cutaneous anaphylaxis, the other produces the non-anaphylactic IgG1 molecule. The evaluation of the ability of both types of IgG1 to bind to and activate a mouse mast cell line revealed that the anaphylactic IgG1 has a higher binding capacity and releases more β-hexosaminidase from mast cells than the non-anaphylactic IgG1. Aglycosylated IgG1 obtained by treatment of the anaphylactic IgG1-producing hybridoma line with an inhibitor of *N*-glycosylation failed to elicit anaphylaxis. In addition, a goat anti-mouse IgG1 antibody reacted less with this aglycosylated IgG1 than with the glycosylated form. These results suggest that the anaphylactic activity of IgG1 antibodies is closely related to their structural conformation and the proper *N*-glycosylation of these molecules. Finally, the difference in the anaphylactic property between the two types of IgG1 seems to be primarily due to binding to the mast cell surface.

**Abbreviations:** PCA = passive cutaneous anaphylaxis; mAb = monoclonal antibody

## Introduction

The humoral immune response against several antigens is mediated by distinct antibody isotypes which exert their effector functions through the constant regions of the heavy chain. More specifically, the CH2 domain of the Fc portion of the molecule is responsible for the expression of interaction sites involved in the mechanisms of antigen clearance and destruction. In most immune mechanisms, such as complement activation, antibody-dependent cell-mediated cytotoxicity and macrophage activation, it was shown that the integrity of this region as well as the presence of the *N*-linked carbohydrate chain in this portion are essential to sustain the functional activity of

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these molecules (Lund et al., 1996; Jefferis et al., 1998).

The CH3 domain is involved in other biological functions, such as the ability of IgE to induce mast cell degranulation and elicit anaphylactic reactions (Ishizaka, 1989; Nissim et al., 1991). Mast cells and basophils are the main cells involved in the anaphylactic reactions through the exocytosis of their granule contents, synthesis and release of lipid mediators (Ishizaka & Ishizaka, 1984; Serafin et al., 1987; Galli, 1993). In mice, it is known that these cells are also activated by the binding of IgG1 antibodies to low affinity Fcγ receptors (FcγRIII) expressed on the cell surface, followed by cross-linking with multivalent antigens (Miyajima et al., 1997; Hazenbos et al., 1998). Recently, we identified two distinct types of murine IgG1 antibodies according to their ability to induce anaphylactic reactions, one that displays anaphylactic activity and another that is unable to elicit passive cutaneous anaphylaxis (PCA). The production of these antibodies is regulated by IL-4 and IL-12, respectively, and can be modulated by different adjuvants (Faquim-Mauro et al., 1999; Faquim-Mauro & Macedo, 2000). In this report, we describe two distinct IgG1-producing hybridomas that secrete antibodies with the same specificity (anti-DNP), but different anaphylactic activities *in vivo*. This functional difference could be due to their mast cell binding or activating capacity. Therefore, these antibodies were studied *in vitro* regarding their ability to bind to a mouse mast cell line and to activate these cells, evaluated by the release of the granular enzyme β-hexosaminidase. Furthermore, an aglycosylated IgG1 obtained by the treatment of the IgG1 anaphylactic-producing hybridoma line with the *N*-glycosylation inhibitor tunicamycin was tested for its anaphylactic activity, in order to demonstrate the role of the carbohydrate chains associated to the glycoprotein in the elicitation of PCA reactions.

## Materials and methods

### Production of monoclonal IgG1 antibodies

Two IgG1-producing hybridoma cell lines (U7.6 and B8405-H5) were cultured in RPMI 1640 plus 0.1 mM L-glutamine, 0.5 mM 2-mercaptoethanol and 10% heat-inactivated fetal calf serum (FCS). The monoclonal antibodies (mAb) were purified by affinity chromatography using a protein G-Sepharose column (Amersham-Pharmacia, Sweden) and their total concentrations were estimated by the Lowry method (Lowry et al., 1951) and by ELISA. The light chains of both were of the κ isotype. The degree of aggregation of the two mAbs was checked by gel filtration, using a Superdex HR200 10/30 column (Amersham-Pharmacia), previously equilibrated with 0.01 M phosphate buffer, pH 7.2. Proteins were size-fractionated by HPLC (Ackta Explorer) at a constant flow rate of 0.5 ml/min and samples monitored by measuring the optical density at 280 nm. Part of the U7.6 mAb was biotinylated with EZ-Link™ Sulfo-NHS-biotin (Pierce, Rockford, IL), according to product instructions.

### Production of aglycosylated IgG1

An aglycosylated IgG1 antibody was produced by the U7.6 cell line using $2 \times 10^6$ cells/ml in RPMI medium containing 2 μg/ml of tunicamycin, as described by Leatherbarrow and collaborators (1985). The cells were incubated in this medium at 37 °C/5% $CO_2$, for 4 hours, which was a sufficient time for all the remaining glycosylated IgG1 to be secreted (Leatherbarrow et al., 1985). The cells were then centrifuged at 1 500 rpm, for 10 minutes, the supernatant was removed, and the cells were washed twice with a fresh medium containing tunicamycin, resuspended at $2 \times 10^6$ cells/ml in a new RPMI medium containing 2 μg/ml of tunicamycin and cultured for another 3 days. After this time, the supernatant containing the aglycosylated IgG1 was collected and the antibodies were purified by affinity chromatography in a protein G-Sepharose column. The total amount of antibody was calculated by the Lowry method.

### Analysis of antibodies by SDS-PAGE

In order to verify the efficiency of the tunicamycin treatment, the antibodies were analyzed by SDS-PAGE following standard protocols. The aglycosylated or glycosylated antibody samples were reduced with 2-mercaptoethanol and applied on a 9% discontinuous polyacrylamide gel containing SDS. Reduced samples of anaphylactic and non-anaphylactic IgG1 mAb were also compared by SDS-PAGE. The protein bands were visualised by silver staining (Sigma Chemical Co.).

### Quantification of IgG1 antibodies by ELISA

Monoclonal IgG1 antibodies were quantified using 96 well-plates coated with a goat anti-mouse Ig antibody and biotinylated, goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL). The reactions were developed with streptavidin-peroxidase conjugate, *o*-phenylenediamine and

$H_2O_2$ (Sigma Chemical Co.); the plates were read at 450 nm on an automated ELISA reader (Dynatech, Chantilly, VA). The total concentration of IgG1 antibodies was determined referring to a standard curve of mouse IgG1.

### Titration of IgG1 antibodies by PCA

Titration of IgG1 mAb was performed by PCA reactions in mice as described by Ovary (1958). BALB/c mice were shaved on the back and three intradermal injections (50 μl) of different concentrations of IgG1 (anaphylactic or non-anaphylactic; glycosylated or aglycosylated) were made in each side of the dorsal skin. The concentrations of either type of IgG1 varied from 5000 to 4.875 μg/site. After 2 h, mice were challenged i.v. with 0.5 ml of 0.25% Evans blue solution containing 250 μg of DNP-bovine serum albumin (DNP-BSA). All tests were made in triplicate and the results express the antibody concentration that gave or not a lesion of at least 5 mm in diameter. The PCA protocol (023/99) was approved by the Biomedical Sciences Institute/ USP – Ethical Committee for Animal Research.

### Detection of Fcγ RII/ Fcγ RIII on mast cell line PT18 surface

The expression of Fcγ RII/III on mast cell line PT18 was evaluated using a rat anti-mouse Fcγ receptor mAb (2.4G2). For this, $1 \times 10^6$ cells were incubated for 30 minutes with different concentrations of 2.4G2 (2, 1 and 0.5 μg/$10^6$ cells) in phosphate buffered saline (PBS) containing 0.1% sodium azide (PBS-Az) and 5% FCS, at 4 °C. After this incubation, the cells were washed with the same buffer and incubated with 1 μg/$10^6$ cells of a FITC-conjugated, goat anti-rat IgG antibody (Amersham-Pharmacia, Sweden), for 30 minutes, at 4 °C. Then, the cells were washed and resuspended in PBS-Az with 1% paraformaldehyde before being subjected to flow cytometry analysis. The results represent the average percentage of labeled cells in duplicate samples.

### IgG1 binding to Fcγ receptors on mast cells

To compare the binding capacity of anaphylactic and non-anaphylactic IgG1 to Fcγ receptors on a mouse mast cell line PT18, $1 \times 10^6$ cells were incubated with different concentrations (4 to 0.5 μg) of each type of IgG1 for 2, 1 hour or 30 minutes in RPMI 1640 plus 0.1 mM L-glutamine and 0.5 mM 2-mercaptoethanol and 10% FCS. After this time, the cells were washed twice and incubated with biotinylated, goat anti-mouse IgG1 antibody (Southern Biotechnology Associates, Birmingham, AL) in PBS-Az containing 5% FCS, for 30 minutes, at 4 °C. The cells were washed, resuspended in streptavidin-FITC (Amersham-Pharmacia, Sweden) and incubated for 30 minutes, at 4 °C. After the incubation, the cells were centrifuged and resuspended in PBS-Az with 1% paraformaldehyde. The cells were analyzed by flow cytometry with a FACScalibur (Becton Dickinson). The results represent the average percentage of labeled cells in duplicate samples.

A competition assay was performed incubating the cells firstly with 16 μg of the biotinylated anaphylactic IgG1 for 1 hour, followed by incubation with 32 or 16 μg of anaphylactic or non-anaphylactic IgG1 for 1 hour more, at 37 °C. After this time, the cells were washed and incubated with streptavidin-FITC for 30 minutes, at 4 °C. The cells were centrifuged and resuspended in PBS-Az with 1% paraformaldehyde. Flow cytometric analyses of duplicate samples were performed with a FACScalibur. The results express the percentage of inhibition of maximum mast cell labeling in the absence of added competitor. Every experiment was repeated at least three times.

### Measurement of β-hexosaminidase activity

The release of the granular enzyme β-hexosaminidase was measured according to Ortega and collaborators (1991) in the supernatants obtained from PT18 mast cells. For this, $4 \times 10^6$ mast cells (200 μl) were incubated in the presence of 160, 80 or 40 μg of the anaphylactic or non-anaphylactic IgG1 for 30 minutes in Tyrode's buffer followed by challenge with 10 μl of DNP-BSA (20 μg) or goat anti-mouse Ig antibody (5 μg) for 15 minutes. Afterwards, the cells were centrifuged and the supernatants collected. The cells incubated only with the Tyrode's buffer were lysed with 0.5% Triton X-100 (200 μl) (Sigma Chemical Co.) 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 these samples, 90 μl of the substrate solution containing 1.3 mg/ml of *p*-nitrophenyl-*N*-acethyl-β-D-glucosamine (Sigma Chemical Co.) in 0.1 M citrate, pH 4.5, were added and the plates incubated for 12 hours at 37 °C. The reactions were stopped by addition of 100 μl of 0.2 M glycine solution, pH 10.7, and the optical density determined at 405 nm in an ELISA reader (Dynatech MR5000, Chantilly, VA). The extent of secretion was expressed as the net percentage of the total β-hexosaminidase activity present in the cells, after subtracting the activity in the supernatant of

unstimulated cells. The results represent the mean of quadruplicate tests ± standard deviation (SD).

### Statistical analysis

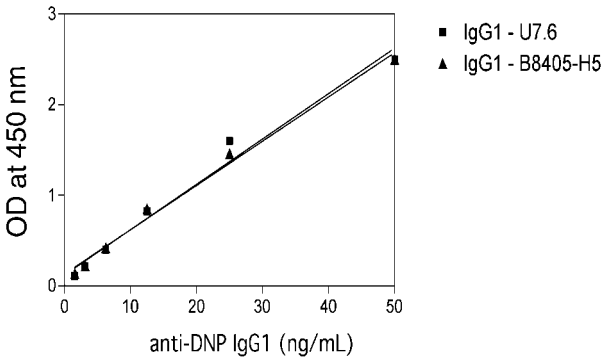
The results obtained from the β-hexosaminidase activity assay were submitted to one-way ANOVA analysis, followed by the Tukey's multiple comparison test (Zar, 1984).

## Results

### Evaluation of antigenic specificity and anaphylactic activity of two monoclonal IgG1 antibodies

The antigenic specificity of two distinct monoclonal antibodies was investigated by ELISA using plates coated with DNP-BSA (10 μg/ml) and developed with a biotinylated, goat anti-mouse IgG1 and streptavidin-peroxidase. Fig. 1A shows that both antibodies were able to react with DNP-BSA at the same extent and were equally recognized by the anti-IgG1 antibody, leading to identical titration curves. Different concentrations of either IgG1 antibodies were also used to coat the ELISA plate. The titration curves obtained after the addition of the anti-mouse IgG1 antibody were coincident too (data not shown). The anaphylactic activity of the two antibodies was determined by PCA reactions, elicited in mice sensitized with different concentrations of IgG1. We can observe in Fig. 1B that whereas IgG1 antibody from clone U7.6 had the anaphylactic activity, IgG1 antibody from clone B8405-H5 was unable to induce a positive reaction even at a 250-fold higher concentration. The two types of mAb did not differ in their degree of aggregation that could explain the difference in anaphylactic activity (data not shown).

**A Antigenic specificity**




**B Biological activity**

| | anti-DNP PCA reaction | |
|---|---|---|
| IgG1- U7.6 | positive | (19.5 ng/site) |
| IgG1- B8405-H5 | negative | (5000 ng/site) |

**Fig. 1.** (A) Detection of anti-DNP IgG1 antibodies by sandwich-ELISA using a DNP-BSA-coated plate, biotinylated goat anti-mouse IgG1 and streptavidin-peroxidase conjugate. (B) anti-DNP PCA reactions elicited in mice previously sensitized with different concentrations of either IgG1 mAb. The lowest and highest antibody concentrations intradermally injected are shown in parentheses.

### Binding capacity of anaphylactic and non-anaphylactic IgG1 antibodies to FcγR on mast cells

The inability of B8405-H5 mAb to induce PCA could be due to an intrinsic deficiency in binding to FcγRIII on mast cell surface. Alternatively, it could be the result of a defect in transmitting an activation signal to the mast cells. Therefore, we investigated the binding and activating properties of the anaphylactic and non-anaphylactic IgG1 molecules.

The mast cell line PT18 was checked for the presence of Fcγ receptors using a rat anti-mouse FcγRII/III mAb, followed by FITC-conjugated, goat anti-rat IgG antibody. The flow cytometric analysis showed that 98.4% of the PT18 mast cells expressed high levels of these receptors on their surface.

Experiments were then performed incubating the PT18 mast cells with different concentrations of either type of IgG1 antibody. Fig. 2 shows that a higher percentage of mast cells was labeled when these cells were incubated for 1 hour with the anaphylactic IgG1 compared to the cells incubated with the non-anaphylactic IgG1, at the same conditions. Importantly, the difference in cell labeling was greater at lower concentrations of antibody (4–5-fold). These results were also obtained when the cells were incubated for 2 hours or 30 minutes (data not shown).

Another experiment was delineated incubating the PT18 mast cells with the anaphylactic IgG1, previously biotinylated, followed by the anaphylactic or non-anaphylactic IgG1. The competition assay (Table 1) showed that only the anaphylactic IgG1 was able to displace the biotinylated IgG1 previously bound to the mast cells. While the addition of 32 and 16 μg of the anaphylactic IgG1 inhibited 43% and 40% of mast cell labeling, respectively, 32 μg of the non-anaphylactic IgG1 produced only 12% of inhibition and 16 μg had no effect at all on the binding of the biotinylated anaphylactic IgG1.

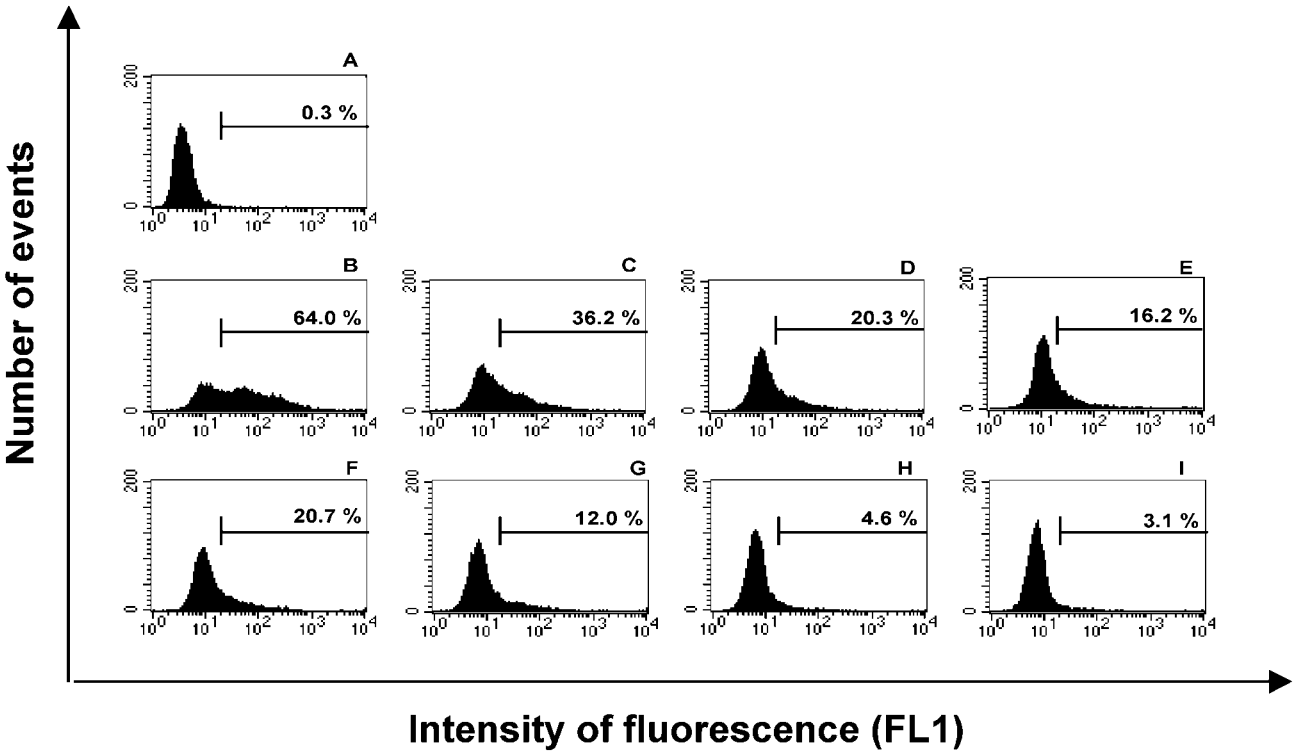



**Fig. 2.** Analysis by flow cytometry of PT18 mast cells incubated with anaphylactic or non-anaphylactic IgG1, followed by a biotinylated, goat anti-mouse IgG1 and streptavidin-FITC: (A) PT18 cells incubated only with the second antibody and streptavidin-FITC; (B, C, D, E) Percentage of labeled PT18 cells obtained after incubation with 4, 2, 1 or 0.5 μg of anaphylactic IgG1, respectively; (F, G, H, I) Percentage of labeled PT18 cells obtained after incubation with the same concentrations of non-anaphylactic. The example is representative of three different experiments.

**Table 1.** Displacement of biotinylated, anaphylactic IgG1 from mast cell surface by anaphylactic or non-anaphylactic IgG1

| Cells[a] | IgG1$^+$ PT18 cells (%)[b] | Inhibition (%)[c] |
|---|---|---|
| biotinylated IgG1 (16 μg) | 97.3 | – |
| biotinylated IgG1 + anaphylactic IgG1 (32 μg) | 55.7 | 43 |
| biotinylated IgG1 + anaphylactic IgG1 (16 μg) | 57.9 | 40 |
| biotinylated IgG1 + non-anaphylactic IgG1 (32 μg) | 85.3 | 12 |
| biotinylated IgG1 + non-anaphylactic IgG1 (16 μg) | 97.2 | 0 |

[a] $1 \times 10^6$ PT18 mast cells incubated with biotinylated, anaphylactic IgG1 only, for 1 hour, or followed by incubation with different concentrations of anaphylactic or non-anaphylactic IgG1 (1 hour) and streptavidin-FITC (30 minutes).
[b] Percentage of labeled PT18 mast cells, analyzed by flow cytometry.
[c] Percentage of inhibition of mast cell labeling, calculated as described under Materials and Methods.

### In vitro mast cell degranulation induced by the anaphylactic and non-anaphylactic IgG1

The mast cell activating property of the two types of IgG1 was tested through their ability to degranulate the PT18 cell and release its β-hexosaminidase content. Fig. 3 shows a significantly higher percentage of β-hexosaminidase release from cells incubated with 160 or 80 μg of anaphylactic IgG1 compared to the cells incubated at the same conditions with the non-anaphylactic IgG1, independently of the type of challenge. There was no significant difference when PT18 cells were incubated with 40 μg of anaphylactic or non-anaphylactic IgG1.

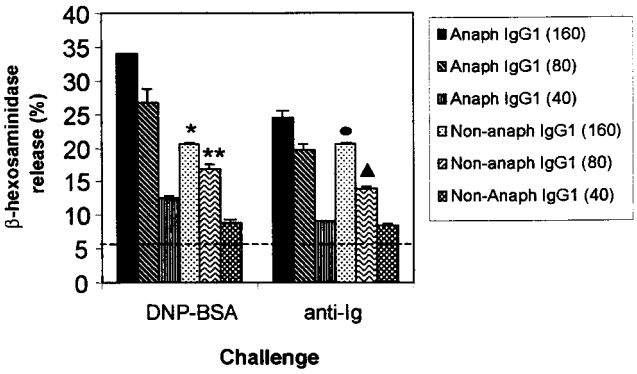



**Fig. 3.** Release of the granular enzyme β-hexosaminidase from PT18 mast cell incubated with 160, 80 or 40 μg of anaphylactic or non-anaphylactic anti-DNP IgG1 and challenged with DNP-BSA or a goat anti-mouse Ig antibody. The results are expressed as the net percentage of the total β-hexosaminidase activity present in the cells, after subtracting the activity in the supernatant of unstimulated cells (mean of quadruplicate tests ± SD). $*p < 0.05$ compared to Anaph IgG1 (160) challenged with DNP-BSA; $**p < 0.05$ compared to Anaph IgG1 (80) challenged with DNP-BSA; ● $p < 0.05$ compared to Anaph IgG1 (160) challenged with anti-Ig; ▲ $p < 0.05$ compared to Anaph IgG1 (80) challenged with anti-Ig.

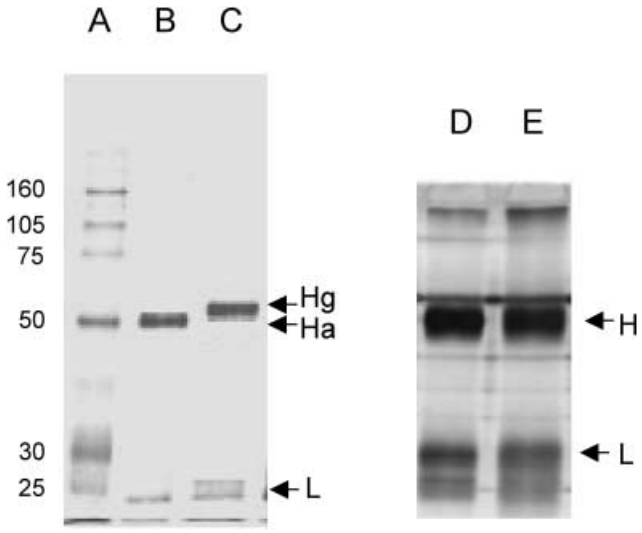



**Fig. 4.** SDS-PAGE (9% polyacrylamide) of aglycosylated (B) and glycosylated (C) IgG1 antibodies, obtained from cultures of U7.6 cells in the presence or absence of tunicamycin, for 72 hours. Anaphylactic (U7.6) and non-anaphylactic (B8405-H5) IgG1 mAb were also compared by SDS-PAGE (D and E, respectively). The samples were reduced with 2-mercaptoethanol and the gels were stained with silver. Hg, glycosylated heavy chain; Ha, aglycosylated heavy chain; L, light chain. (A) molecular weight standards (kD).

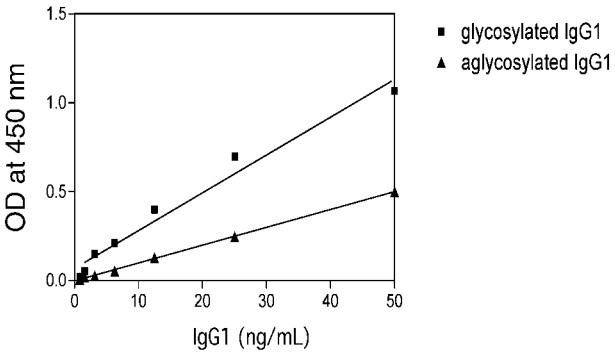
## Effect of tunicamycin treatment on the anaphylactic activity of U7.6 IgG1

Since there is only one constant region gene for the IgG1 isotype among the murine immunoglobulin genes (Coffman et al., 1993), the difference between the mast cell binding capacity of the two types of IgG1 molecules is likely to be due to a post-translational modification. To test this hypothesis, we treated the U7.6 hybridoma cells with tunicamycin, an inhibitor of *N*-glycosylation.

The SDS-polyacrylamide gel profile of glycosylated and aglycosylated IgG1 in Fig. 4B, C shows two distinct positions for the migration of their respective H-chain, with the latter having the expected lower apparent molecular weight for an aglycosylated chain, indicating that the treatment with tunicamycin was effective in the inhibition of *N*-glycosylation of the heavy chain. Anaphylactic and non-anaphylactic IgG1 mAb heavy and light chains, on the other hand, display similar migration patterns (Fig. 4D, E).

An ELISA performed with plates coated with the glycosylated or aglycosylated IgG1 and developed with biotinylated, goat anti-mouse IgG1 showed distinct curves obtained with the same concentrations of IgG1 antibodies, demonstrating that the absence of glycosylation of the heavy chain also inhibited the recognition of the aglycosylated antibody by the goat anti-mouse IgG1 antibody (Fig. 5A). Therefore, labeling of mast cells with aglycosylated

**A IgG1 recognition**




**B Biological activity**

anti-DNP PCA reaction

| | | |
|---|---|---|
| Glycosylated IgG1 | positive | (19.5 ng/site) |
| Aglycosylated IgG1 | negative | (5000 ng/site) |

**Fig. 5.** (A) Detection by ELISA of glycosylated or aglycosylated IgG1 monoclonal antibodies, produced by U7.6 cells treated or not with tunicamycin, for 72 hours. ELISA plates were coated with either type of IgG1, followed by biotinylated, goat anti-mouse IgG1 and streptavidin-peroxidase conjugate. (B) anti-DNP PCA reactions elicited in mice previously sensitized with different concentrations of either IgG1 mAb. The lowest and highest antibody concentrations intradermally injected are shown in parentheses.

vs. glycosylated IgG1, using the protocol mentioned above, could not be performed.

The aglycosylated IgG1 was, however, evaluated regarding its anaphylactic activity by PCA reaction. The anaphylactic activity was abolished when the glycosylation process was inhibited, suggesting that the aglycosylated IgG1 could not bind to FcγRIII and elicit a PCA reaction (Fig. 5B).

## Discussion

The aim of the present study was to examine the difference in mast cell binding and activating properties of the anaphylactic and non-anaphylactic murine IgG1 antibodies, previously described by us in immunization protocols using three different antigens (Faquim-Mauro et al., 1999). For the sake of purity, we identified two IgG1-secreting hybridomas that produced the two types of IgG1 with the same antigenic specificity. One of them was unable to elicit PCA reaction even if its concentration was increased by more than 250-fold.

It is known that immune effector functions are mediated by the interaction between IgG and distinct Fcγ receptors. Three classes of FcγR were identified in murine leukocytes which bind with different affinities to subclasses of IgG (Ravetch & Kinet, 1991; Ravetch, 1997). Hazenbos and collaborators (1996), using FcγRIII-deficient mice, showed that this is the main receptor involved in triggering passive cutaneous anaphylaxis and complement-independent Arthus reaction, induced by polyclonal IgG antibodies. Furthermore, using these same mice and a panel of mouse mAb of different IgG subclasses, it was shown that FcγRIII is required for various IgG1-mediated functions such as phagocytosis of IgG1-coated erythrocytes by macrophages *in vivo* and *in vitro*, trapping of immune complexes by follicular dendritic cells, lysis of tumor target cells by whole blood and passive cutaneous anaphylaxis (Hazenbos et al., 1998).

Therefore, to test the mast cell binding capacity of the two IgG1 mAb, we used the PT18 cell line that expressed high levels of FcγRII/III, detected by the rat 2.4G2 mAb, which recognizes an epitope of the extracellular domain of FcγRII/III (Weinshank et al., 1988). When the binding of the two IgG1 mAb was revealed by a second, biotinylated, anti-IgG1 Ab plus streptavidin-FITC, we observed that 4 – 5-fold less labeled mast cells were detected with the non-anaphylactic mAb compared with the anaphylactic one. This result was not due to a less efficient binding of the second antibody to the non-anaphylactic mAb, as demonstrated by the identical titration curves obtained with the two types of mAb by ELISA (Fig. 1A, data not shown). Furthermore, using a competition assay, only the anaphylactic, and not the non-anaphylactic, mAb was able to displace a previously bound, biotin-conjugated, anaphylactic IgG1 from the mast cell surface. These results indicate that the difference in the anaphylactic property of the two IgG1 mAb is primarily due to binding to the FcγRIII. The difference in the activating capacity that was also detected (Fig. 3) may be a consequence of the former binding capacity.

It is difficult at this point to establish a direct correlation between the *in vivo* (PCA reaction) and *in vitro* (binding to and degranulation of PT18 cells) results. The reasons for this might be the following: first, the mast cell line may not accurately reflect the behavior of skin mast cells; second, the proportion of antibody molecules per mast cell is hard to compare under *in vivo* and *in vitro* conditions; and third and most important, a threshold of antibody binding seems to be required *in vivo* in order to elicit full activation of mast cells and release of all the mediators involved in PCA. Even so, the *in vitro* results confirmed that anaphylactic and non-anaphylactic IgG1 antibodies are different regarding the initial events of this biological reaction.

The binding of IgG subclasses to FcγRs includes multiple sites, mainly within the CH2 domain which also contains the glycosylation site (Jefferis et al., 1998). Glycosylation of IgG-Fc is essential for the recognition by FcγRs, although the carbohydrate moieties are in the periphery of the FcγRIII-Fc interface (Sondermann et al., 2000). In order to study the participation of the carbohydrate moieties in the elicitation of anaphylaxis, we produced an aglycosylated form of the anaphylactic mAb using the *N*-glycosylation inhibitor tunicamycin in the culture medium. The PCA reaction performed with this antibody showed, for the first time to our knowledge, that the anaphylactic activity of the murine IgG1 was lost. The aglycosylated antibody was also unable to degranulate PT18 cells after addition of antigen, indicating that it could not bind to mast cells *in vitro* (data not shown). In addition, an ELISA performed with the aglycosylated IgG1 showed that an anti-IgG1 antibody did not recognize this form as it did the glycosylated form, suggesting alterations in the protein conformation. These results altogether indicate that the anaphylactic activity of IgG1 is closely related to its structural feature and the *N*-glycosylation of the molecule.

However, they do not mean that the non-anaphylactic type of IgG1 is devoid of carbohydrate chains, since the SDS-PAGE profile of anaphylactic and non-

anaphylactic IgG1 antibodies were similar (Fig. 4D, E). The recognition of the non-anaphylactic IgG1 by the anti-IgG1 antibody was also like that of the glycosylated, and not of the aglycosylated, IgG1. Furthermore, the non-anaphylactic IgG1 had the ability to degranulate mast cells *in vitro*, although not so efficiently as the anaphylactic one, while the aglycosylated IgG1 had not.

Indeed, all IgG molecules have a single *N*-linked biantennary complex oligosaccharide at Asn297, which is buried between the CH2 domains, forming extensive contacts with amino acid residues within CH2 (Wright & Morrison, 1997). Heterogeneous processing of the core oligosaccharide structure can result in multiple glycoforms even among monoclonal antibodies (Patel et al., 1992; Lund et al., 1993). The structure of the Fc-attached oligosaccharide can also influence the biological activity of the antibody. For example, Mimura and collaborators (2000) reported that the FcγRI and complement activating capacity of some truncated glycoforms were reduced compared with the intact IgG1 anti-MHC class II antibody.

Therefore, it is quite possible that the non-anaphylactic IgG1 is a different glycoform that impairs the FcγRIII binding ability of this type of antibody and consequently the capacity to elicit PCA reactions. Some of our preliminary results show that, in contrast to the anaphylactic IgG1, the non-anaphylactic IgG1 is not susceptible to *N*-glycanase F. Experiments to elucidate the oligosaccharide profile of the two types of IgG1 are now underway.

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