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Hevein, an allergenic lectin from rubber latex, activates human neutrophils' oxidative burst

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Hevein is an N-acetyl-D-glucosamine (GlcNAc) specific lectin that has been hypothesized to participate in the IgE-mediated allergic reactions in patients with latex allergy. In this work we assessed the specificity and biological effect of hevein purified from rubber latex on human leukocytes, using epifluorescence microscopy and flow cytometry. Purified human granulocytes were stimulated *in vitro* with hevein, and production of oxidative radicals was measured by reduction of nitroblue tetrazolium formazan. Histochemical staining and flow cytometry showed that hevein recognizes specifically monocytes (CD14+) and neutrophils (CD16+), but not lymphoid cells. Hevein induced oxidative response in purified granulocytes; this effect was 1.3–1.5-fold higher than the effect observed with the lectin WGA (wheat germ agglutinin), or other lectins with different sugar specificity. The induced reactions and cellular recognition by hevein were inhibited with GlcNAc and its oligomers; as well as by glycoproteins containing tri-and tetra-antennary N-glycosydically linked glycans. Our findings suggest that neutrophils are the main target for latex hevein; this lectin induces production of oxidative radicals, which seem to play an important role in tissue damage during latex allergy.

Keywords: hevein, lectins, latex, allergy, atopic dermatitis, oxidative burst, neutrophils, N-acetyl-D-glucosamine

Introduction

In recent years, with the enormous increase in the use of latex gloves in hospitals, latex sensitization has become a potentially serious health problem, particularly for health care workers [1]. Latex-sensitive individuals usually have atopic predisposition and are at greater risk from latex allergy than non-atopic ones [2]. Several latex components have been identified that might be involved in the generation of immediate hypersensitivity reactions in latex-sensitive individuals [3]. Prohevein and hevein are major latex (*Hevea brasiliensis*) allergen products, recognized by IgE antibodies in the sera of latex allergic adults and children [4,5]. Hevein is a protein domain with the main IgE-binding epitope [6] and this domain is also present in the N-terminal end of prohevein [4,6]. Structural and thermodynamic studies have been performed to demonstrate that hevein binds specifically to GlcNAc residues and saccharidic structures containing GlcNAc, such as chitin [7–9].

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Diverse studies have suggested that hevein is a very conserved domain, which confers the chitin-binding property to several plant proteins, such as the class I endochitinases [10]. In addition, these investigations have shown that hevein is responsible for the observed serologic cross-reactivity between latex allergy and fruit allergies; moreover, immunization with these allergens, as therapy, has not been successful, suggesting the participation of cellular groups independent from T-cell regulation in this allergy [11,12]. The wheat germ agglutinin (WGA) possesses in its structure four hevein domains and is a GlcNAc specific lectin [13]; recent findings indicate that it is a potent activator of oxidative burst in human neutrophils [14]. Considering that there is increasing evidence of neutrophil participation in the allergic process [15], in this work we studied the specificity of hevein for human peripheral blood leukocytes to better understand the mechanisms of damage involved in latex allergy.

Material and methods

Reagents

Histopaque (density 1.119), red blood cell lysis buffer, RPMI-1640 culture medium, bovine serum albumin (BSA) fraction V, all sugars used in this study, wheat germ agglutinin (WGA),

Lotus tetragonobulus agglutinin (LTA, specific for L-fucose), Concanavalin A (Con A, Man/Glc specific), phorbol 12-myristate 13-acetate (PMA), Zymosan-A, fluorescein isothiocyanate (FITC), nitroblue tetrazolium (NBT) grade III crystalline, sodium azide, dimethyl sulfoxide, and octyl phenoxy polyethoxyethanol (Triton X-100) were purchased from Sigma Chemical Co. (St Louis, MO, USA); R-phycoerythrin (RPE)-conjugated mouse anti-human CD14 and CD16 monoclonal antibodies were from Serotec Ltd (Oxford, England); methanol, and salts were acquired from J.T. Baker, Inc. (Phillipsburg, NJ, USA). The lectins from *Amaranthus leucocarpus* (ALL, specific for GalNAc), peanut agglutinin (PNA, specific for Galβ1, 3GalNAc) and the erythroagglutinin from *Phaseolus coccineus* (Alubia, specific for N-acetyllactosamine) were purified by affinity chromatography [16–18].

Hevein

Hevein was purified from the bottom fraction of fresh ultracentrifuged rubber latex (*Hevea brasiliensis*, clone GV-42) by gel filtration and then by anionic exchange chromatography on a TSK-DEAE-35W column [19]. Hevein-containing fractions were collected, dialyzed against water, concentrated using a YM-1 membrane in an AMICON 8 MC system. Purity of the sample was confirmed by mass spectrometry (MS) on an Esquire ion trap mass spectrometer (Bruker-Pflanzen Analytical, GmbH) as described by Jensen *et al.* [20]. The nanoelectrospray mass spectra gave a sharp peak with a mass of 4720.1, indicating the homogeneity of the sample. Hevein in 0.1 M carbonate buffer, pH 8.5, was conjugated to FITC according to [21]. After 2 h of incubation at room temperature [$22 \pm 3^{\circ}$C), FITC-hevein was dialyzed against phosphate buffered saline (PBS: 0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.2). Final sample concentration was adjusted to 1 mg/ml, which was fractionated in aliquots and stored at -20°C until use.

Leukocytes

Heparinized human peripheral blood was obtained from healthy adult volunteers without atopic antecedents. Leukocytes were isolated by incubating peripheral blood in cold red blood cell lysis buffer, for 10 min. The treated cells were washed twice with PBS, centrifuged at $400 \times$ g for 10 min at 4°C and the supernatant was removed. The cell pellet was suspended in RPMI-1640 culture medium at a concentration of 10^7 cells/ml. Cell viability ($>90\%$) was determined by trypan blue dye exclusion.

Granulocytes

The cells were isolated from heparinized human peripheral blood by Histopaque density gradient centrifugation as described previously [22]. The granulocytes were washed by centrifugation and the cellular pellet was suspended at a cell concentration of 4×10^6 cells/ml of RPMI-1640 culture medium. Cell population purity was assessed by flow cytometry.

Histochemical staining

Leukocytes were suspended in 5 µl of culture medium, placed on glass slides and fixed in cold acetone/chloroform (v/v) for 10 min, at 4°C. Fixed cells were washed extensively in PBS, incubated for 15 min with 10% normal human blood serum and washed for 15 min in PBS supplemented with 0.2% BSA and 0.01% Triton X-100 (PBS-BSA-T) before staining. The cells were incubated for 30 min at room temperature [$22 \pm 3^{\circ}$C) in a humidified atmosphere with a 10 µl aliquot of FITC-hevein. The slides were washed thrice in PBS-BSA-T under gentle shaking at room temperature, and finally the cells were washed with PBS/glycerol (v/v) and observed through a Standard 25 ICS Zeiss microscope equipped with an epifluorescence condenser (Carl Zeiss Co., Germany). Specificity was determined by adding 200 mM GlcNAc/30 µl PBS to fixed cells before adding the FITC-hevein, under these conditions treated cells showed negative staining. Double stains were performed with the cells first stained with FITC-hevein, as described above, and then incubated with 5 µl of RPE-mouse anti-human CD14 or CD16 monoclonal antibodies (diluted 1 : 20) in PBS supplemented with 0.2% BSA and 0.2% sodium azide (PBS-BSA-A). After 30 min of incubation at room temperature, the slides were washed thrice in PBS-BSA-T, as described above, and observed by epifluorescence microscopy.

Flow cytometry

The hevein-positive leukocytes were determined by Flow Cytometry Analysis in a FACS Excalibur, Becton Dickinson Co. (Mountain View, CA, USA). Suspended leukocytes [10^6 in PBS) were incubated separately in FITC-hevein at concentrations of 0.1 µg to 10 µg, for 15 min at 4°C. Double stains were also performed through 30 min of incubation with FITC-hevein and, simultaneously, with 10 µl of RPE-mouse anti-human CD16 monoclonal antibodies (diluted 1:100) in PBS-BSA-A. After incubation at room temperature, stained leukocytes were washed twice in PBS-BSA-A. The cells were suspended in 1 ml of FACS flow solution and analyzed through flow cytometry, 10,000 cells were counted. The number of hevein positive leukocytes was determined in three different experiments using FITC-hevein. Specificity of each lectin was demonstrated through inhibiting the cell-lectin interaction by adding 200 µl of 200 mM of specific carbohydrates for each lectin in PBS to the lectin-leukocytes suspension, and analyzed through flow cytometry.

Granulocytes oxidative burst

Oxidative burst was tested in Ficoll-purified granulocytes, using an NBT-Zymosan solution, as follows 0.2% NBT in isotonic saline solution was filtered through 0.45-µm pore Millipore membrane (Molsheim, France) and incubated with 0.1%

Zymosan-A during 2 h at 37°C in water bath under gentle shaking. The assay was performed according to [23]. In brief, 100 µl suspended granulocytes (4×10^5 cells) and 100 µl culture medium were placed into each well of a 96-well polystyrene microtiter plate (Nunc Inc, Roskilde, Denmark) and incubated for 30 min at 37°C in a 5% CO_2 atmosphere to form a monolayer of adherent cells; 100 µl NBT-Zymosan per well were added. Hevein, PMA (0.5 mg/ml), or each lectin at different concentrations in 5 µl/culture medium, was added to each well individually and incubated during 60 min at 37°C in 5% CO_2 atmosphere. The supernatant was then removed and wells were washed two times thoroughly with 200 µl 70% methanol and dried at room temperature to achieve total evaporation of methanol. Then, 120 µl 2 M KOH/well was added and incubated for 60 minutes more under gentle shaking followed by the addition of 140 µl/well dimethyl sulfoxide. The content of the wells was mixed immediately and optical density from the developed color was read at 630 nm on an automatic ELISA reader (Labsystems, Multiskan MS, Finland), using as a blank a well without cells but containing Zymosan-NBT solution, and revealed as indicated. Control experiments were performed using 5 µl BSA (1 mg/ml). The specificity of the effect induced by each lectin was confirmed by using lectins previously incubated for 30 min with 200 mM of each specific carbohydrate. Mann-Whitney U-test determined significant differences.

Hevein sugar specificity

The sugar specificity of hevein was determined by inhibition of the interaction of FITC-hevein with granulocytes, this assay was achieved by incubating the lectin with different carbohydrates and glycoproteins for 30 min at room temperature, before adding it to the granulocytes suspension. Fetuin, transferrin, and α_1-acid glycoprotein were desialylated by incubating at 100°C for 1 h in the presence of 0.02 N sulfuric acid [24] and desalted on a Bio-Gel P-2 column (2×60 cm) equilibrated with water. Inhibition was determined through cytometry comparing the effect of carbohydrates and glycoproteins on the fluorescence intensity of FITC-hevein.

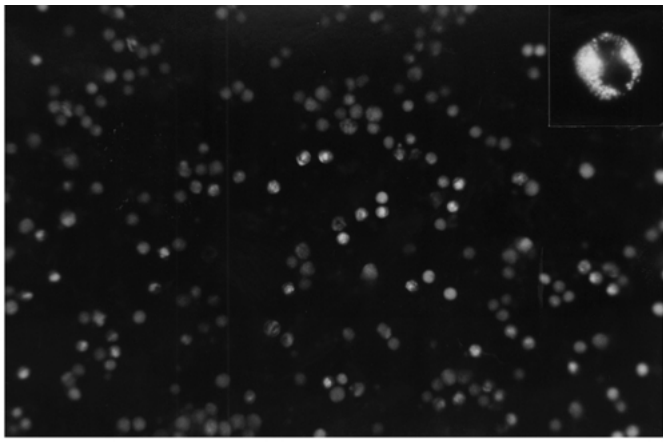

Figure 1. Specificity of hevein for human neutrophils and monocytes. Leukocytes were fixed on glass slides with cold acetone/chloroform and incubated with FITC-hevein for 30 min at room temperature (22 ± 3°C). Cells were observed through a Standard 25 ICS Zeiss microscope equipped with an epifluorescence condenser. In the figure, neutrophils show higher fluorescence intensity, monocytes somewhat lower, whereas lymphocytes display no fluorescence (40×). Insert: a FITC-hevein stained neutrophil (100×). Representative figure from one experiment out of four.

Results

Histochemical analysis

As indicated in Figure 1, 90 ± 2% of the stained leukocytes with FITC-hevein were granulocytes and CD16+, and 5 ± 1% of monocytes (CD14+ cells) were also stained; lymphocytes were not recognized. Prior incubation of leukocytes with GlcNAc inhibited binding of the lectin to the stained cells. When 200 mM GlcNAc was added to the recognized cells, significant diminution in cellular fluorescence was observed. Double staining assays showed that those cells that bound hevein were also stained by anti-CD16 antibodies, indicating that they were neutrophils.

Flow cytometry analysis

The results of flow cytometry of leukocytes incubated with FITC-hevein were analyzed by plotting cell size against cell granularity, showing three well-defined cell subpopulations. Hevein recognized 79 ± 3% of granulocytes or monocytes, whereas < 1% recognition was observed in lymphocytes. Binding of FITC-hevein to granulocytes was observed even at concentrations as low as 0.1 µg/10^6 cells; however, optimal concentration of hevein was identified at 5 µg (Figure 2A–E). No higher fluorescence intensity was recorded if the cells were incubated with more than 5 µg FITC-hevein. Double fluorescence analysis showed that 75% of hevein-positive granulocytes were also CD16+ cells, confirming that they correspond to neutrophil polymorphonuclear cells (Figure 3). All the monocytes recognized by FITC-hevein were also CD14+.

Granulocytes oxidative burst

Granulocytes were stimulated with 5 µg hevein per 4×10^5 cells, as optimal dose. Although hevein and PMA produced 1.3–1.5 times more oxidative response than granulocytes stimulated with WGA, our results indicate that hevein and WGA are more powerful stimulators of oxidative burst of granulocytes than the other lectins tested (Table 1). ALL and PNA showed no significant effect on granulocytes oxidative burst; although ConA, LTA and Alubia lectins increased the oxidative burst, their effect was lower than that of hevein ($p < 0.05$).

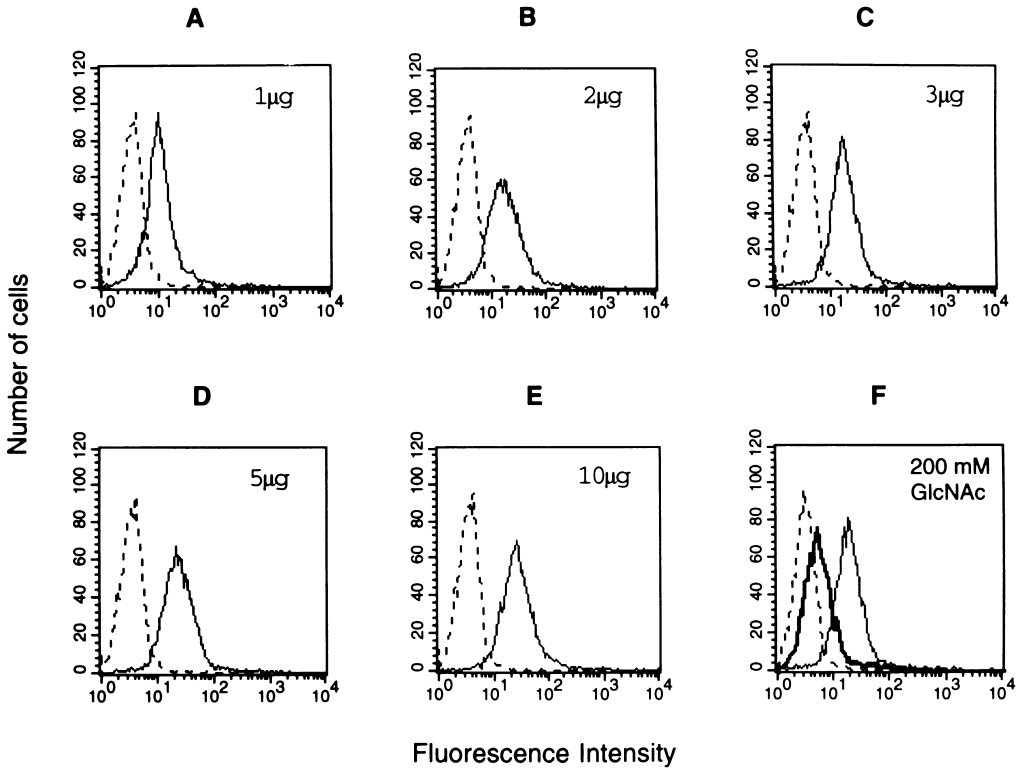

Figure 2. Flow cytometric analysis of human peripheral blood granulocytes stained with several concentrations of FITC-hevein. (**A–E**) The increase of fluorescence intensity was measured as a function of the added hevein concentration. (**F**) Fluorescence inhibition of FITC-hevein treated cells by 200 mM GlcNAc. Dotted line indicates the background level; thin line indicates the fluorescence level of the hevein positive cells; thick line indicates fluorescence inhibition. Representative results from one experiment out of four.

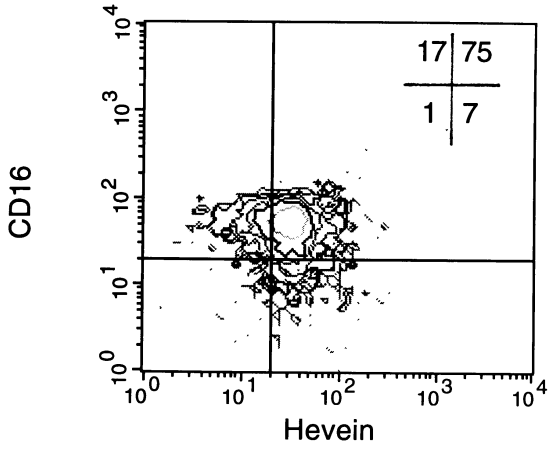

Figure 3. Two-color fluorescence analysis of human peripheral blood polymorphonuclear cells stained with both FITC-hevein and RPE-conjugated anti-CD16 mAb. In the panel, the numbers indicate the percentage of cells in the respective quadrant. Representative results from one experiment out of four.

Table 1. Nitroblue tetrazolium (NBT) reduction by human peripheral blood granulocytes

Stimulation	*Concentration* µg/4 × 10^5 cells	*nmoles NBT*
BSA	18	1.7 ± 0.7
PMA	25	5.9 ± 0.8*
WGA	9	4.7 ± 1.1*
WGA + GlcNAc	9	2.4 ± 0.4
PNA	9	1.6 ± 0.5
PNA + Gal	9	1.5 ± 0.2
Con A	6	3.4 ± 0.6*
Con A + αMM	6	2.0 ± 0.3
ALL	8	1.7 ± 0.5
ALL + GalNAc	8	1.7 ± 0.4
Alubia	7	2.8 ± 0.7
Alubia + N-acetyllac	7	2.0 ± 0.2
UEA	6	3.1 ± 0.4
UEA + L-fuc	6	2.1 ± 0.3
Hevein*	5	6.9 ± 1.0*
Hevein + GlcNAc	5	2.9 ± 0.6

Numerical values represent the mean ± SD of four experiments. *Significant difference ($p < 0.05$) determined by Mann-Whitney U-test observed when the effect was compared with BSA treated cells. Phorbol myristate acetate (PMA) was used as a control of positive simulation. Sugars were tested at 200 mM concentration. αMM = α-methyl-mannoside

Specificity of hevein interaction

The sugar specificity of FITC-hevein was tested by inhibiting its interaction with CD16+ granulocytes, and determining the fluorescence intensity through cytometry. The addition of

inhibitors to FITC-hevein-cells indicated that GlcNAc and its oligomers, N,N′-diacetyl-chitobiose, N,N′,N′-triacetyl-chitotriose, inhibited the hevein-granulocytes interaction; N,N′,N′-triacetyl-chitotriose was a two-fold more powerful inhibitor than GlcNAc and N,N′-diacetyl-chitobiose. Cell recognition was also inhibited by pre-incubation of the lectin with GlcNAc. Other monosaccharides, such as D-glucose, D-galactose, D-mannose, L and D-fucose, Neu5Ac, and N-acetyl-D-galactosamine were ineffective to inhibit hevein interaction. These results agree with previous ones [7–9]. Binding inhibition of FITC-hevein resulted in significant diminution of fluorescence intensity as observed in the thick line of the granulocytes' histogram in Figure 2F. Glycoproteins, such as fetuin, asialo-fetuin, and α_1-acid glycoprotein were the most powerful inhibitors of the hevein cellular interaction and, in minor proportion, transferrin. Elimination of sialic acid by mild-hydrolysis diminished the inhibitory effect of these glycoproteins, hen ovalbumin and pig stomach mucin were ineffective to inhibit hevein interaction with granulocytes (Table 2).

Discussion

We determined the cellular specificity of hevein for human neutrophils. Hevein is the main allergen recognized by IgE antibodies in the sera of adults and children with latex hypersensitivity [4,5]. Hevein is a 4.7 kDa protein with lectin activity that recognizes GlcNAc as a central structure on an oligosaccharide [7–9]. Hevein domains have been demonstrated in other lectins with specificity for GlcNAc, such as WGA [13]. WGA is able to activate the NADPH oxidase enzyme complex, stimulating the respiratory burst from human neutrophils [14]. We found that hevein recognizes specifically neutrophil polymorphonuclear cells (CD16+) and in minor extent monocytes (CD14+), but is unable to interact with lymphocytes. This binding property was dependent on the specific recognition of GlcNAc receptors, as demonstrated by reversal of FITC-hevein binding to previously recognized cells by treatment with GlcNAc. Hevein recognition of neutrophils can be explained by the fact that neutrophils have high density of non-reducing terminal GlcNAc residues [25]. Hevein recognized this cellular group, but also activated their respiratory burst. Hevein and WGA showed higher capacity to induce oxidative burst than lectins with specificity for GalNAc (ALL), Fuc (LTA), Gal/GalNAc (PNA), and for oligomannosidic (ConA) or N-acetyllactosaminic structures (Alubia erythroagglutinin). The WGA-induced effect on granulocytes was lower than that of hevein, suggesting that, indeed, density of non-reducing terminal GlcNAc residues plays a relevant role in neutrophils; these results were confirmed by the fact that hevein effect on granulocytes' activity was inhibited more by $(GlcNAc)_3$ oligomers than by GlcNAc. Glycoproteins, which characteristically possess sialylated bi- and tri-antennary N-glycosidically linked glycans of the N-acetyllactosaminic type and N,N′-diacetylchitobiose core, such as fetuin [24–27], α_1-acid glycoprotein [28], and in minor proportion transferrin [29], were better inhibitors than sugars. The presence of the N,N′-diacetylchitobiose core in desialylated inhibitors might explain why those glycoproteins still inhibited the hevein effect. Hen ovalbumin, which possesses oligomannosidic and hybrid-type structures and lacks Neu5Ac residues [30], and an O-glycosidically linked protein, such as porcine stomach mucin [30], did not inhibit the activity of hevein. Recent works have indicated that hevein lectin binds $(GlcNAc)_{2–4}$ oligomers with high affinity; however, a significant increase in binding affinity was observed for $(GlcNAc)_5$, suggesting that hevein possesses an extended binding site involved in lectin-sugar contact [31]. Neu5Ac showed no capacity to inhibit the hevein interaction; however, this sugar seemed to participate in the interaction of hevein with glycoproteins. It could be possible that the 4-OH and 5-NHAc on the Neu5Ac ring are lined up with the same group of

Table 2. Effect of sugars and glycoproteins on FITC-hevein binding to CD16+ cells

Compound	*Concentration (mM)*	*Relative Fluorescence Intensity*	*Inhibition (%)*
Control (Hevein)	–	$10^{3.6}$	0
GlcNAc	200	$10^{2.0}$	42
N,N′-diacetyl-chitobiose	200	$10^{1.3}$	50
N,N′,N′′-triacetyl-chitotriose	200	$10^{0.8}$	76
Fetuin	0.0002	$10^{0.5}$	87
Asialofetuin	0.001	$10^{1.3}$	75
Transferrin (human)	0.001	$10^{1.7}$	22
Asialotransferrin	0.005	$10^{3.2}$	4.7
α_1-acid-glycoprotein	0.0008	$10^{1.4}$	72
Asialoα_1-acid-glycoprotein	0.001	$10^{2.0}$	64
Ovalbumin	0.005	$10^{3.3}$	4.1
Mucin pig stomach	0.005	$10^{3.5}$	1.2

Other sugars that showed no effect on hevein activity at 200 mM concentration were: Neu5Ac, Gal, Man, GalNAc, Glc, L-Fuc and D-Fuc. Results represent the average of four independent determinations. Standard deviation of the mean was never $\pm$ than 10%

GlcNAc (3-OH and 2-NHAc) in the oligosaccharide sequence [32]. Although studies on the mechanisms that induce the oxidative burst in granulocytes by hevein are in progress, previous reports suggested that the oxidative burst induced by stimulation of the GlcNAc-containing receptor for WGA was dependent on microfilaments and serine protease functions [33] similarly to PMA (a membrane perturbating agent).

Hevein interacts with specific GlcNAc-containing receptors in neutrophils, which could be expressed on the surface of these cells in a particular activation or maturation state. It is important to consider that recent findings indicate that neutrophils from atopic individuals are more reactive than neutrophils from non-atopic ones [34]. Allergic dermatitis is usually considered a hypersensitivity process mediated by IgE and regulated by CD4+ T cells [35]; however, immunization with latex allergens, as therapy, has not been successful, suggesting the participation of cellular groups independent from the T-cell regulation in this allergy [36]. Taken together, our results (specific interaction with neutrophils and the production of oxidative radicals) strongly suggest that hevein could contribute to tissue damage during the initial stage of the latex hypersensitivity or in atopic disorders.

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