



Immunoglobulin-E reactivity to wine glycoproteins in heavy drinkers

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Abstract

N-glycans from plant and invertebrate allergens can induce extensive immunoglobulin-E (IgE) cross-reactivity in vitro. IgE antibodies against these N-glycans, also termed cross-reactive carbohydrate determinants or CCDs, are prevalent in alcohol drinkers. This study investigated the prevalence and biological significance of IgE antibodies to N-glycans from wine glycoproteins in heavy drinkers. A structured questionnaire, skin prick tests, serum IgE levels, IgE-immunoblotting to wine extracts, and basophil activation tests were used to characterize 20 heavy drinkers and 10 control subjects. Eleven heavy drinkers (55%) showed IgE binding to proteins in wine extracts. The proteins were identified by mass spectrometry as grape-derived vacuolar invertase and thaumatin-like protein. Immunoblot reactivity was closely associated with the presence of IgE to CCDs and was inhibited by preincubation with a glycoconjugate containing bromelain-type N-glycans. The same conjugate, CCD-bearing allergens, and wine extracts activated basophils in patients with high-titer CCD-specific IgE but not in healthy controls. There was no relationship between immunoblot reactivity and consumption of any specific type of wine. No patient reported symptoms of hypersensitivity to Hymenoptera venom, food, or wine. In conclusion, heavy drinkers frequently show IgE reactivity to the N-glycans of wine glycoproteins. Glycans and wine glycoprotein extracts can induce basophil activation in sensitized alcoholics. The clinical significance of these findings remains to be elucidated. © 2010 Elsevier Inc. All rights reserved.

Keywords: Immunoglobulin-E; Wine; Cross-reactive carbohydrate determinants; Glycans; Allergy; Basophil activation

Introduction

Immunoglobulin-E (IgE) is the molecule responsible for allergic hypersensitivity. Allergen-induced cross-linking of IgE on the surface of mast cells and basophils induces the release of mediators of allergic inflammation (Hamilton, 2010). Determination of IgE specific to allergens is routinely used for the diagnosis of allergic disease (Hamilton, 2010), and allergen-mediated basophil activation tests are performed in selected cases (De Week et al., 2008). The ability of an allergen to trigger the activation of basophils indicates that allergen-specific IgE has biological activity in that patient (De Week et al., 2008; Hamilton, 2010). Of note, in vitro IgE reactivity or basophil activation are not always accompanied by in vivo activity or clinical allergy.

Allergenic cross-reactivity should be taken into account when interpreting the results of allergy tests. Primary sensitization mediated by IgE against a particular allergen can induce reactivity to structurally related molecules (Aalberse et al., 2001). Along this line, many plant and invertebrate glycoproteins contain similarly fucosylated and/or xylosylated N-glycans, also known as cross-reactive carbohydrate determinants (CCDs) (Altmann, 2007; Malandain, 2005; Mari, 2002; van Ree, 2002). Because CCDs are ubiquitous in nature, CCD sensitization can induce widespread IgE reactivity, therefore limiting the specificity of in vitro tests for allergy (Altmann, 2007; Malandain, 2005; Mari, 2002; van Ree, 2002).

Previous studies revealed an increased rate of IgE sensitization to CCDs in alcohol drinkers (Coutinho et al., 2008; Gonzalez-Quintela et al., 2008; Vidal et al., 2009), although the mechanisms and biological significance of this are unknown. Alcohol (ethanol) is a powerful immunomodulator that may shift immunity toward Th2 responses (Heinz and

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Waltenbaugh, 2007; Linneberg et al., 2008; Szabo, 1999). In fact, alcohol consumption is associated with increased serum total IgE levels in both observational studies in humans (Gonzalez-Quintela et al., 1995, 2003; Hallgren and Lundin, 1983; Linneberg et al., 2003; Vidal et al., 1994) and experimental studies in rodents (Andrade et al., 2006; Heinz and Waltenbaugh, 2007; Linneberg et al., 2008). In theory, additional factors for CCD exposure and subsequent sensitization in alcohol drinkers could include the glycoprotein content of alcoholic beverages. Grapes and wines contain proteins that in rare cases can induce allergic sensitization (Pastorello et al., 2003; Vassilopoulou et al., 2007). Wine proteins are derived mainly from grapes (*Vitis vinifera*) (Cilindre et al., 2008; Ferreira et al., 2001; Yokotsuka and Singleton, 1997), although some may come from yeasts that induce wine fermentation (Dambrouck et al., 2003). Some wine proteins are glycosylated (Ferreira et al., 2001); interestingly, the glycan content of wine glycoproteins is higher in young wines than in older wines (Yokotsuka and Singleton, 1997). In addition, it has been suggested that Hymenoptera allergens may be present in wines (particularly in young wines) because honeybees or wasps may be crushed along with grapes during the initial steps of winemaking (Armentia et al., 2007). The presence of Hymenoptera allergens in wine could be relevant because exposure to Hymenoptera stings may be a factor for CCD sensitization (Aalberse et al., 1981; Kochuyt et al., 2005). Rural areas in northwestern Spain have a long tradition of viticulture, and consumption of young homemade wines is very common (Mateos et al., 2002). The present study was aimed to investigate the frequency, associated factors, and the biological significance of IgE antibodies to N-glycans (CCDs) of wine glycoproteins in heavy drinkers.

Methods

Participants

This study included 20 heavy drinkers consecutively admitted to the Internal Medicine Department of a University Hospital in Northwest Spain (2007–2008) because of alcohol withdrawal syndrome ($n = 9$), alcoholic hepatitis ($n = 7$), complications of advanced liver disease ($n = 3$), and cerebrovascular disease ($n = 1$). All patients were predominantly wine consumers, with 10 consuming primarily homemade young wines. Thirteen patients also consumed beer, and nine also included spirits. Ten healthy volunteers with an alcohol intake of less than 1 unit/d were included as controls. Patients and controls were comparable in epidemiological factors (Table 1). The study was approved by the Regional Ethics Committee. Written informed consent was obtained from all participants.

Questionnaire

A physician-administered questionnaire included tobacco consumption, residence, occupation, respiratory symptoms,

symptoms of food allergies, oral allergy syndrome, lifetime history of Hymenoptera stings, and Hymenoptera venom allergy. The average daily alcohol consumption was expressed in grams. For that purpose, we registered the number of drinks (glasses of wine, bottles of beer, and drinks of spirits) consumed per day. Validation of the standard drinking unit (SDU) system in Spain indicates that drinks of wine and beer are approximately equivalent to one SDU or 10 g of alcohol, and drinks of spirits are approximately equivalent to two SDUs or 20 g of alcohol (Gual et al., 1999).

Liver function tests

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), and bilirubin were assayed in an Advia analyzer (Siemens Medical Solutions Diagnostics, Munich, Germany). The upper normal limit for serum AST, ALT, and GGT is 25, 29, and 32 IU/L, respectively. The prothrombin time was expressed as the international normalized ratio.

Glycoconjugates

Neoglycoproteins (MUXF-BSA and MM-BSA) consisting of N-glycopeptides conjugated to bovine serum albumin (BSA) were provided by the Institute of Chemistry, Universitaet fuer Bodenkultur, Vienna, Austria. MUXF (the N-glycan from bromelain) has, like most CCDs, a backbone with two N-acetylglucosamines and a mannose. “M” indicates the presence of a terminal α -mannose, “U” indicates an unsubstituted 3-hydroxyl on the β -mannose, “X” indicates a xylose, and “F” indicates the presence of a core α 1,3-fucose. MM-BSA contains similar N-glycans with an additional mannose but lacking the xylose and fucose that are crucial for the antigenic properties of CCDs, as reviewed by Altmann (2007).

Serum IgE and inhibition studies

Total IgE was measured by chemiluminiscent immunoassay (Immulite-2000, Siemens Medical Solutions, Gwynedd, UK). Allergen-specific IgE was assayed using the UniCAP-250 system (Phadia, Uppsala, Sweden). Determinations included IgE to grapes (*V. vinifera*, f259), honeybee venom (*Apis mellifera*, i1), common wasp (yellow jacket) venom (*Vespula* spp., i3), grass pollen (*Lolium perenne*, g5), olive tree pollen (*Olea europaea*, t9), peanuts (*Arachis hypogaea*, f13), latex (*Hevea brasiliensis*, k82), and two CCD markers (MUXF [Ro214] and bromelain itself [*Ananas comosus*, k202]). The reportable range of the CAP method is 0.01–100 kU/L. As per the manufacturer's instructions, IgE levels greater than or equal to 0.1 kU/L were considered to be “positive” in some analyses.

CAP inhibition by CCD was performed in individuals with specific IgE levels greater than or equal to 0.35 kU/L (the classic threshold for positivity) using MUXF-BSA as inhibitor, as previously described (Gonzalez-Quintela et al.,

Table 1

Epidemiological and immunological characteristics of healthy controls and heavy drinkers stratified according to the results of IgE binding (immunoblotting) to the protein extract of a homemade young wine (No. 1)^a

Characteristics	Healthy controls (n = 10)	Total (n = 20)	Heavy drinkers (n = 20)	
			Negative IgE-immunoblotting to wine (n = 9)	Positive IgE-immunoblotting to wine (n = 11)
Age (y)	48 (32–51)	47 (37–69)	57 (46–62)	44 (37–69)*
Sex (male)	8 (80)	17 (85)	9 (100)	8 (73)
Residence (rural)	5 (50)	16 (80)	5 (56)	11 (100)*
Average alcohol consumption (g/d)	NA	150 (50–320)	150 (100–250)	150 (50–320)
Consumption of homemade wines	NA	10 (50)	4 (44)	6 (54)
Participation in grape harvesting	3 (30)	10 (50)	4 (44)	6 (54)
Smoking (current)	2 (20)	8 (40)	3 (33)	5 (45)
Hymenoptera stings (lifetime history) ^b	7 (70)	16 (84)	6 (67)	10 (100)
Liver function tests				
Serum AST (IU/L)	16 (10–27)	52 (21–247)**	54 (21–247)	49 (25–159)
Serum ALT (IU/L)	15 (10–41)	28 (16–171)**	34 (18–60)	26 (16–171)
Serum GGT (IU/L)	15 (6–60)	263 (30–1282)**	383 (47–1282)	195 (30–651)
Serum bilirubin (mg/dL)	0.8 (0.3–1.2)	1.75 (0.5–22.0) [†]	1.8 (0.9–12.8)	0.8 (0.5–22.0)
Prothrombin time (INR)	ND	1.12 (0.86–1.85)	1.18 (0.91–1.49)	1.07 (0.86–1.85)
SPT positivity ^c	5 (50)	4 (20)	1 (11)	3 (27)
Total serum IgE (IU/mL)	35 (4.8–801)	275 (24–656)**	332 (91–463)	224 (24–656)
Specific IgE positivity (≥0.1 kU/L)				
MUXF	0 (0)	9 (45) [†]	1 (11)	8 (73)*
Bromelain	0 (0)	9 (45) [†]	1 (11)	8 (73)*
Grape	0 (0)	8 (40) [†]	1 (11)	7 (64)*
Honeybee venom	2 (20)	12 (60)	2 (22)	10 (91) ^{††}
Wasp venom	1 (10)	10 (50) [†]	3 (33)	7 (64)
Grass pollen	4 (40)	11 (55)	2 (22)	9 (82)*
Olive tree pollen	1 (10)	12 (60) [†]	3 (33)	9 (82)
Peanuts	1 (10)	9 (45)	1 (11)	8 (73)*
Latex	1 (10)	9 (45)	1 (11)	8 (73)*

Data are presented as median and range (within parentheses) or as absolute numbers and percentages (within parentheses). IgE = immunoglobulin E; MUXF = the N-glycan from bromelain; NA = not applicable; ND = not determined; AST = aspartate aminotransferase; ALT = alanine aminotransferase; GGT = gammaglutamyltransferase; INR = international normalized ratio; SPT = skin prick test. * $P < .05$ (with respect to heavy drinkers with negative immunoblotting to wine); ** $P < .01$, [†] $P < .05$ (with respect to controls); ^{††} $P < .01$ (with respect to heavy drinkers with negative immunoblotting to wine).

^aImmunoblotting to wine was negative in all controls.

^bLifetime history of bee or wasp stings (data unavailable in one heavy drinker with acute cerebrovascular disease).

^cPositive skin prick test to mites or grass pollen.

2008). Briefly, 150 μ L of serum was preincubated overnight at 4°C with 150 μ L of PBS-buffered MUXF-BSA (100 μ g/mL), and CAP results were assessed. The results were expressed as a percentage, with a value greater than 50% considered indicative of extensive inhibition.

IgE immunoblotting and inhibition studies

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions (Laemmli, 1970), lyophilized protein extracts of alcoholic beverages (10 μ g/lane) were dissolved in sample buffer (0.05 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, and 0.1% bromophenol blue) and boiled at 100°C for 5 min before loading onto the gel. After separation by SDS-PAGE, protein bands were detected with Coomassie brilliant blue. The separated protein bands were electrophoretically transferred onto a polyvinylidene fluoride membrane (Towbin et al., 1979). After blocking,

membranes were incubated overnight at 4°C with 1-mL serum samples (diluted 1:10 or 1:20), incubated with 1:3,000 mouse antihuman IgE–horseradish peroxidase conjugate (Southern Biotech, Birmingham, AL), and detected by chemiluminescence (ECL-Plus; Amersham Pharmacia Biotech). For inhibition assays, serum samples were preincubated with the inhibitor (MUXF-BSA, honeybee venom or wasp venom [Vespa Lab, Spring Mills, PA], 50 μ g/mL) overnight at 4°C before immunoblotting as previously mentioned. The degree of inhibition was semi-quantitatively registered after visual inspection of gel lanes as 0 (no inhibition), 1 (weak inhibition), 2 (strong inhibition), or 3 (total inhibition). Commercial BSA was used as a negative control and wine extract was used as a positive control. Extracts of alcoholic beverages (Fig. 1A) included 10 wines (homemade and commercial, red and white, and of different quality and age), and two homemade spirits (aquavit, a hard liquor) obtained by distillation from grapes left over after winemaking.

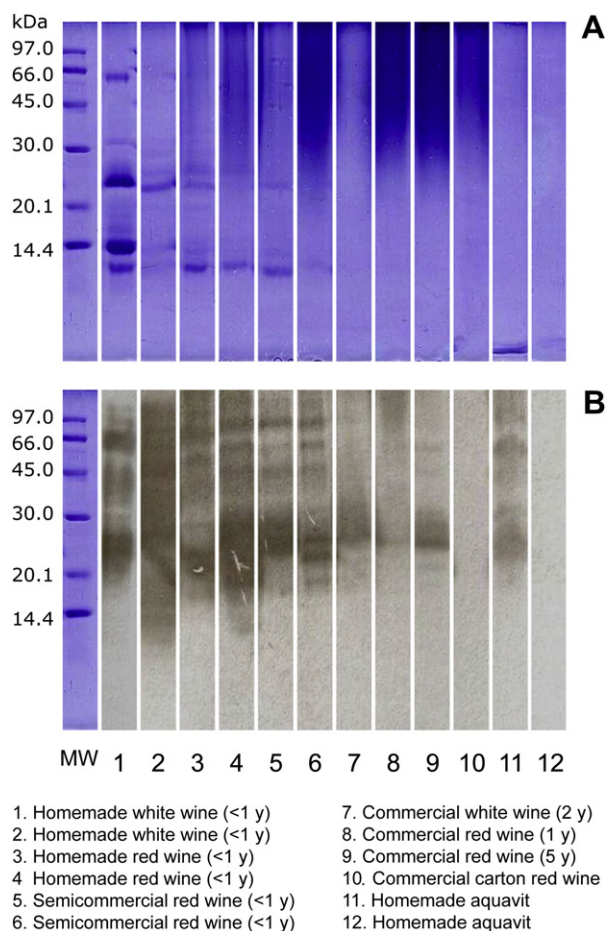


Fig. 1. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of alcoholic beverage extracts. The proteins are visualized with Coomassie brilliant blue staining. (B) Immunoblot using the serum of patient No. 11 to the same extracts. MW, molecular weight markers.

Proteomic analyses

To identify wine proteins, automatic in-gel trypsin digestion was performed as described previously (Lokhov et al., 2004), and the resulting tryptic peptide profiles were analyzed in a ABI-4800 matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF)/TOF mass spectrometer (Applied Biosystems, Framingham, MA) in positive ion reflector mode. Peptide mass fingerprint spectra were searched on the NCBI nonredundant protein database, using the Mascot program search tool (Matrix Science, London, UK, <http://matrixscience.com>) after selecting Viridiplantae green plants as taxonomy. The probability of identification was shown by the resulting MOWSE score (Shevchenko et al., 1996).

Flow cytometric cellular allergen stimulation tests

Allergen-specific basophil activation was evaluated by flow cytometric analysis of CD63 expression (Sanz et al., 2001). Briefly, cells from the layer on top of the erythrocytes were collected by centrifugation and suspended in HEPES calcium buffer (20 mM HEPES, 133 mM NaCl,

5 mM KCl, 7 mM CaCl₂, 3.5 mM MgCl₂, and 1 mg/mL BSA, pH 7.4) containing interleukin-3 (2 ng/mL) and 10 μ L of heparin (5000 UI/mL). Allergens were mixed with the patient's cell suspension and incubated at 37°C for 40 min. The reaction was stopped, and the basophils were double-labeled with anti-CD63 PE- and anti-IgE FITC-labeled antibodies (Caltag, Burlingame, CA) and incubated for 30 min at 4°C; finally, 4 mL of an erythrolytic reagent (Ortho Diagnostic System, Madrid, Spain) was added. Flow cytometric analysis of basophil surface markers was performed at 488 nm on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Flow cytometric cellular allergen stimulation test (FAST) responses were considered positive when the stimulation index (% activated basophils with the allergen/% activated basophils in basal conditions) was greater than or equal to 2% and the allergen-specific activation was greater than 15%. The allergens included MUXF-BSA (50, 5, and 0.5 μ g/mL), bromelain (5, 0.5, and 0.05 μ g/mL; Sigma, St. Louis, MI), honeybee venom (5, 0.5, and 0.05 μ g/mL; ALK-Abelló, Spain), yellow jacket venom (5, 0.5, and 0.05 μ g/mL; ALK-Abelló), grass pollen (80 and 20 μ g/mL; ALK-Abelló), and olive tree pollen (50 and 12.5 μ g/mL; ALK-Abelló). FAST using these allergens was available for 17 of the 20 heavy drinkers. Negative control using MM-BSA (50, 5, and 0.5 μ g/mL) and purified BSA (50, 5, and 0.5 μ g/mL; Sigma-Aldrich, St. Louis, MI) were performed in selected cases showing positive activation with MUXF-BSA. In addition, FAST using five different wine extracts (0.01, 0.1, and 1 mg/mL) was performed in five heavy drinkers and five controls.

Skin prick tests

The panel of skin prick tests (SPTs) included CCD markers (horseradish peroxidase and bromelain), the most prevalent aeroallergens in the area (mites [*Dermatophagoides pteronyssinus*] and grass pollen [*L. perenne*]), and CCD-bearing allergens that less frequently induce allergies in the area (olive tree pollen, peanut, and latex; ALK-Abelló). Wheals with a maximum diameter of greater than 3 mm were considered positive.

Statistical analyses

The Fisher's exact test and the Mann-Whitney test were used to compare categorical and numerical variables, respectively. Spearman's rank test was used to assess correlation.

Results

Identification of proteins and IgE immunoblotting in wine extracts

Extract of alcoholic beverages showed protein bands of similar molecular weight that tended to be more evident in young wines than in older wines or spirits (Fig. 1A). Thus, wine No. 1 was chosen for subsequent immunoblotting

studies. The most distinct protein bands in wine No. 1 were about 60, 24, and 14 kDa. These proteins were identified by MALDI-TOF/TOF as vacuolar invertase 1 (GIN-1, M_r 71786, accession number gi|1839578, protein MASCOT score 408), *V. vinifera* thaumatin-like protein (VVTL1 or Vit v TLP, M_r 24947, accession number gi|7406716, protein MASCOT score 452), and pathogen-related type-4 protein (PR-4, M_r 15674, accession number gi|3511147, protein MASCOT score 565), respectively.

IgE immunoblotting to wine extracts

Eleven patients (55%) showed IgE reactivity to wine proteins (Fig. 2). Wine protein reactivity was not detected

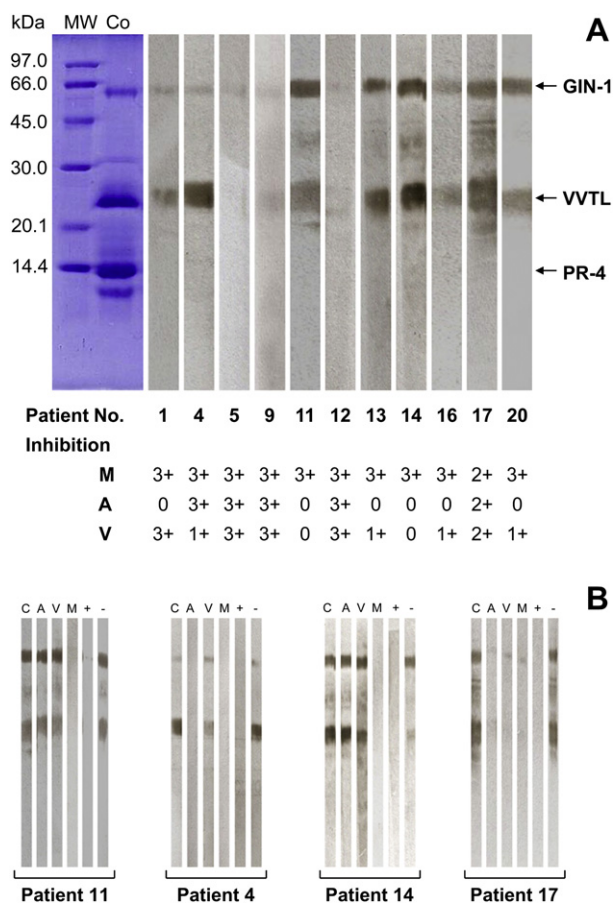


Fig. 2. (A) Immunoblot of proteins from a homemade young wine with sera from alcoholic patients. The 11 positive immunoblots are shown. Inhibition (0, none; 1+, weak; 2+, strong; 3+, complete) of immunoglobulin E binding with MUXF-BSA and Hymenoptera venoms is indicated below each lane. The position of identified wine proteins (GIN-1, vacuolar invertase; VVTL, *Vitis vinifera* thaumatin-like protein; and PR-4, pathogen-related type-4 protein) is indicated on the right. MW, molecular weight markers. Co, Coomassie brilliant blue staining. M, inhibition with MUXF (the N-glycan from bromelain) coupled to bovine serum albumin (BSA); A, inhibition with *Apis mellifera* (honeybee) venom; V, inhibition with *Vespula* spp (wasp) venom. (B) Representative examples of immunoblot inhibition. M, A, V: inhibition with MUXF-BSA, *A. mellifera* and *Vespula* spp, respectively, as previously mentioned; C, no inhibition; +, positive control of inhibition with wine extract; –, negative control of inhibition with BSA.

in any of the 10 healthy controls ($P = .004$). With only one exception (patient No. 5), the patients' sera showed reactivity to both the 60-kDa (vacuolar invertase) and the 24-kDa (thaumatin-like) proteins. No patient showed IgE reactivity to PR-4 at ~24-kDa. Serum from patient No. 11 was also tested for reactivity to proteins from all 12 alcoholic beverages. This serum showed IgE immunoreactivity to most wines (with the exception of the poorest-quality carton wine) and to one of two homemade aquavits (Fig. 1, bottom panel).

IgE reactivity to wine proteins was associated with younger age and living in a rural environment and tended to be associated with the patient's lifetime history of Hymenoptera stings. Consumption of homemade wines and participating in grape harvesting were not associated with reactivity. All patients had at least an abnormal liver test (serum AST, ALT, GGT, or bilirubin). Liver test abnormalities tended to be higher in patients without IgE reactivity to wine glycoproteins than in patients with it, although the differences were not statistically significant. Wine protein reactivity in heavy drinkers was unrelated to total IgE levels, which were higher in heavy drinkers than in healthy controls (Table 1).

Relationship of immunoblot reactivity with IgE to CCDs

Wine protein reactivity was closely related to positivity of IgE to CCDs and CCD-bearing allergens (grapes, latex, peanuts, pollens, and Hymenoptera venoms, Table 1). Nine patients (45%) had positive (≥ 0.1 kU/L) IgE titers to MUXF. The same nine patients also had IgE positivity to bromelain (correlation coefficient 0.83, $P < .001$). In cases with the highest CCD–IgE levels, reactivity to CCD-bearing allergens was extensively inhibited by preincubation with MUXF-BSA (Table 2). IgE to CCDs (MUXF or bromelain) was not detected in any of 10 healthy controls ($P = .01$, Table 1).

Preincubation with MUXF-BSA induced extensive inhibition of IgE reactivity to both the 60-kDa (vacuolar invertase) and 24-kDa (thaumatin-like) wine proteins in all patients (Fig. 2). Honeybee venom induced extensive inhibition of IgE binding to these protein bands in one case, wasp venom in one case, and both venoms in four cases. MM-BSA induced no inhibition of IgE reactivity (data not shown).

FAST with glycoproteins and wine extracts

Among the 17 patients with available test results, three patients (17%) showed positive basophil activation with MUXF-BSA, bromelain, honeybee venom, grass pollen, and olive tree pollen (Table 2). A similar pattern of basophil activation was observed neither in the 14 patients with lower level of CCD–IgE ($P = .001$) nor in the 10 healthy controls ($P = .003$). These three patients showed the highest levels of IgE to CCD markers and CCD-bearing allergens, strong positivity for wine proteins in immunoblotting, and extensive

Table 2

Epidemiological, clinical, and immunological characteristics of heavy drinkers with positive basophil activation tests with CCD-bearing allergens

Patient number	11	13	17
Gender/age (y)	Male/40	Female/45	Female/50
Reason for hospital admission	Alcohol withdrawal syndrome	Alcohol withdrawal syndrome	Alcoholic hepatitis
Alcohol consumption			
Average consumption (g/d)	200	200	50
Main alcoholic beverage	Wine	Wine	Wine
Consumption of homemade wines	Yes	No	No
Smoking (current)	Yes	No	Yes
Habitat	Rural	Rural	Rural
Hymenoptera stings (lifetime history)	Yes	Yes	Yes
Allergy symptoms ^a	No	No	No
Skin prick tests ^b	Negative	Negative	Negative
Total IgE (IU/mL)	656	131	361
Specific IgE (kU/L)			
MUXF	12.2	4.88	13.7
Bromelain	10.9	4.09	19.2
Honeybee venom	17.5	9.66	24.3
Wasp venom	10.0	3.78	20.0
Grass pollen	13.7	5.64	22.5
Olive tree pollen	12.8	5.10	21.1
Peanuts	12.7	5.82	23.0
Latex	14.3	5.35	25.9
Grapes	9.24	2.02	16.7
Inhibition of IgE with MUXF-BSA (%)			
Honeybee venom	80.9	62.9	90.0
Wasp venom	95.2	99.4	97.9
Grass pollen	89.7	91.8	95.0
Olive tree pollen	96.7	98.0	94.8
Grapes	96.3	99.0	99.6
Basophil activation (% of cells) ^c			
Basal response	1.4	3.6	6.9
Anti-IgE	86.4	60.0	85.7
MUXF-BSA	(+) 95.7	(+) 86.4	(+) 91.2
Bromelain	(+) 87.4	(+) 74.5	(+) 77.8
Honeybee venom	(+) 61.3	(+) 69.5	(+) 33.9
Wasp venom	(-) 5.0	(-) 4.5	(+) 87.3
Grass pollen	(+) 91.1	(+) 85.0	(+) 93.5
Olive pollen	(+) 95.5	(+) 85.0	(+) 79.0
Wine extracts	(+)	ND	(+)
Immunoblot to wine extract	(+)	(+)	(+)

CCD = cross-reactive carbohydrate determinants; IgE = immunoglobulin E; MUXF = the glycan part of bromelain; BSA = bovine serum albumin; ND = not determined; (+), positive; (–), negative.

^aSystemic reaction to hymenoptera sting, symptoms of rhinitis, asthma, or oral allergy syndrome.

^bSkin prick tests to mites, grass pollen, bromelain, horseradish peroxidase, olive tree pollen, latex, and peanut.

^cMaximum percentage of basophil activation observed with any of the concentrations used.

inhibition of IgE reactivity after preincubation with MUXF-BSA in both CAP and immunoblotting (Table 2). Basophil activation with wine extracts showed positive results in two of these patients with available determination (refer to following parts in this section). However, no activation was observed with MM-BSA (Fig. 3). An additional patient, No. 4, showed basophil activation with Hymenoptera venoms but not with CCDs. In that patient, the CCD–IgE was low level, and preincubation with MUXF-BSA induced no inhibition of IgE reactivity to Hymenoptera venoms.

Basophil activation tests with wine extracts were performed in five heavy drinkers. Wine extracts induced basophil activation in two patients who had positive immunoblotting results to wine glycoproteins and positive CCD–IgE

(Fig. 4). Basophil activation was observed with all five wine extracts tested, but activation tended to be higher with young wines. Basophil activation with wine extracts was negative in all controls and in three heavy drinkers with negative CCD–IgE.

SPTs and allergy symptoms

SPTs to common aeroallergens (grass pollen or mites) were positive in four patients and five controls (Table 1). SPTs to bromelain were negative in all patients, and only one CCD-sensitized patient (No. 12) had a positive SPT with horseradish peroxidase. SPTs to olive tree pollen, peanut, and latex were uniformly negative in patients with

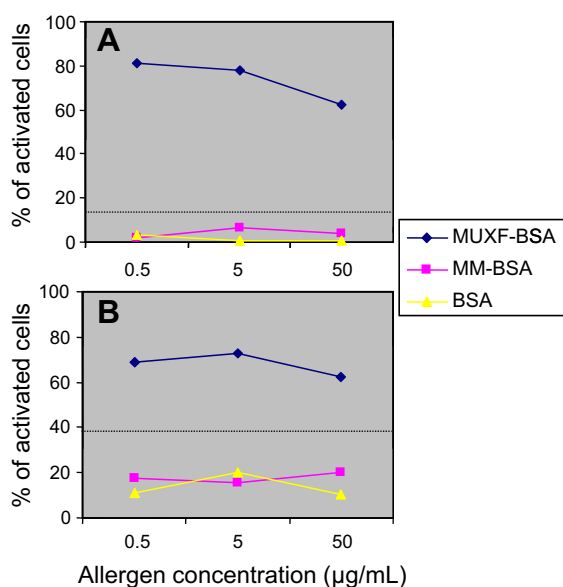


Fig. 3. Basophil activation (flow cytometric cellular allergen stimulation) in two heavy drinkers with positive results after stimulation with glycoconjugates (MUXF, the N-glycan from bromelain; MM, the same N-glycan lacking xylose and fucose), and purified bovine serum albumin (BSA). Dotted lines represent the threshold for positivity. (A) Patient No. 11. (B) Patient No. 13.

specific IgE positivity. No patient reported a history of Hymenoptera venom hypersensitivity, food allergy, oral allergy syndrome, or adverse reaction to alcoholic beverages.

Discussion

This study indicates that a sizable proportion of heavy drinkers admitted to the hospital show IgE reactivity to the N-glycans of wine glycoproteins. Furthermore, N-glycans and wine glycoprotein extracts can induce basophil activation in alcoholics who are highly sensitized to N-glycans (CCDs). IgE reactivity to wine glycoproteins is consistent with previous findings of increased CCD sensitization and subsequent IgE reactivity to glycoproteins in heavy drinkers (Gonzalez-Quintela et al., 2008) and in moderate drinkers from the same area (Coutinho et al., 2008) and from areas with different patterns of alcohol consumption (Linneberg et al., 2010). Here, more than half of heavy drinkers showed IgE binding to proteins that were identified by mass spectrometry as a vacuolar invertase and a thaumatin-like protein; these proteins are present in grapes (Sarry et al., 2004) and wine (Cilindre et al., 2008; Dambrouck et al., 2003; Ferreira et al., 2001; Yokotsuka and Singleton, 1997) and may be glycosylated (Cilindre et al., 2008; Dambrouck et al., 2005). IgE binding was inhibited by preincubation with a CCD and MUXF-BSA, indicating that glycans are involved in reactivity. Furthermore, preincubation with a similar conjugate lacking xylose and fucose (MM-BSA) did not inhibit reactivity, confirming that these sugar residues are important components of the epitope (Altmann, 2007; Malandain, 2005; Mari, 2002; van Ree, 2002). Although

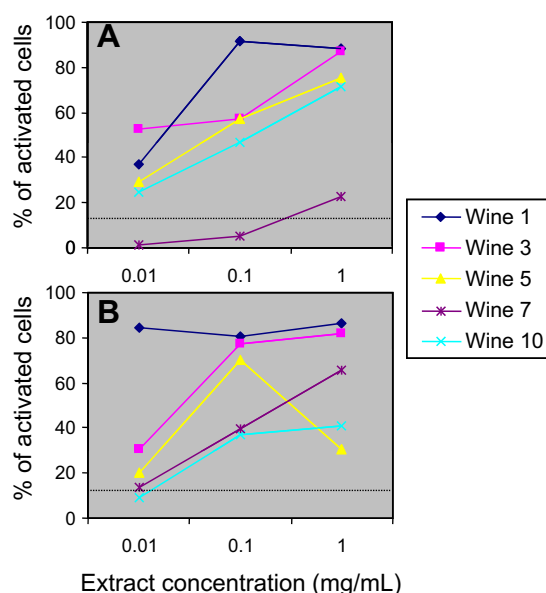


Fig. 4. Basophil activation (flow cytometric cellular allergen stimulation) in two heavy drinkers with positive results after stimulation with wine extracts (Wine 1, homemade white; Wine 3, homemade red; Wine 5, semi-commercial red; Wine 7, commercial white; Wine 10, commercial poor quality red wine). Dotted lines represent the threshold for positivity. (A) Patient No. 17. (B) Patient No. 11.

most experiments in this study were performed using a home-made wine, data from selected cases indicated that IgE binding extends to glycoproteins in commercial wines and grape-derived spirits. Furthermore, IgE binding to wine glycoproteins was not associated to the regular consumption of a particular type of beverage.

Most importantly, heavy drinkers with high CCD–IgE values showed basophil activation with CCD itself (MUXF-BSA) as well as with CCD-bearing allergens. This is consistent with previous case reports indicating that CCD-specific IgE may have biological activity in vitro in patients with symptomatic allergies as reviewed by Altmann (2007). In the present study, bromelain, pollen extracts, wine extracts, and Hymenoptera venoms activated basophils in a subset of otherwise asymptomatic heavy drinkers. The latter is of note because basophil activation tests have been proposed as a way to confirm allergy in patients with doubtful diagnosis (Ebo et al., 2007). Factors that may increase basophil activation (Christensen et al., 2008) include elevated IgE concentrations, which are commonly present in heavy drinkers (Gonzalez-Quintela et al., 1995, 2003; Hallgren and Lundin, 1983; Linneberg et al., 2003; Vidal et al., 1994), increased IgE affinity for the epitope, as shown for IgE to CCDs (Jin et al., 2008), and increased numbers of epitopes recognized by the IgE repertoire. Even bromelain (with only one MUXF-type N-glycan) elicited basophil activation in some heavy drinkers, but it should be noted that bromelain has enzymatic activity. However, it is unlikely that CCD-induced basophil activation represents an unspecific phenomenon

because it was observed with glycoconjugates such as MUXF-BSA but not with MM-BSA. Taken together, these results indicate that CCD interference on allergy in vitro tests should be specially considered in heavy drinkers.

From the clinical standpoint, our data support the notion that CCDs have low biological activity in vivo (Altmann, 2007; Malandain, 2005; Mari, 2002; Mari et al., 2008; van Ree, 2002). No patient with CCD sensitization reported food allergy symptoms after eating CCD-containing foods or when drinking alcoholic beverages. Furthermore, SPTs with CCD-bearing allergens were uniformly negative in patients with positive specific IgE to these allergens. Discrepancy between strong in vitro activity (basophil activation) and no apparent in vivo activity of IgE to CCDs indicates that mechanisms for tolerance should exist, but they remain elusive. Further studies in heavy drinkers, a specific subset of individuals with high prevalence of CCD sensitization, may offer an insight into more general questions about CCD tolerance in sensitized individuals. Finally, it could be speculated that some clinical consequences of CCD sensitization may be only evident in the long term. It is well known that alcoholic liver disease is partly induced by gut permeability for endotoxin, which triggers an innate inflammatory response within the liver via CD14-TLR4 engagement on Kupffer cells (Nagy, 2003). CCDs are widespread in nature, and in vivo activity of a given allergen in the gut mucosa could lead to enhanced intestinal permeability in IgE-sensitized patients (Heyman, 2005). Of note, we found no significant relationship between liver disease and reactivity to wine glycoproteins or CCDs among heavy drinkers.

Regarding the potential mechanisms of CCD sensitization in heavy drinkers, the relationship between alcohol, food (and beverage) glycoproteins, and Hymenoptera venom glycoproteins is intriguing. It should be noted that CCDs are the epitopes to which humans are most frequently exposed (Altmann, 2007). Notably, all patients with CCD sensitization and reactivity to wine glycoproteins in the present study lived in a rural environment. Higher pollen levels and a greater chance of Hymenoptera stings could contribute to CCD exposure in such environment. We found no clear relationship between a history of stings and either CCD sensitization or wine protein reactivity, but all sensitized patients reported being stung in the past. However, none of healthy controls with a history of Hymenoptera stings showed CCD reactivity. This suggests that alcohol consumption may modify the immunological effect of Hymenoptera stings. Among patients truly allergic to Hymenoptera venoms, alcohol consumption is associated with CCD reactivity (Carballada et al., in press). Along these lines, it has been suggested that young wines may contain Hymenoptera allergens (Armentia et al., 2007). However, in our experience, wine proteins with IgE reactivity in heavy drinkers are derived from grapes. Hymenoptera venoms (which mainly contain nonxylosylated glycans) (Altmann, 2007) inhibited IgE reactivity to wine proteins in some cases, but

inhibition was less consistent than that observed with MUXF-BSA. Interestingly, individuals with increased IgE levels are prone to develop specific IgE antibodies when exposed to a given allergen (Horner and Kawakami, 2009), and heavy drinkers frequently show increased total IgE levels, as already noted and confirmed in the present study. Taken together, these results may indicate that alcohol consumption enhances sensitization to CCDs in exposed individuals. Specific mechanisms favoring CCD exposure in heavy drinkers could include increased intestinal permeability (Parlesak et al., 2000) or decreased protein digestion (Andrade et al., 2006), a well-known factor for allergic sensitization (Untersmayr and Jensen-Jarolim, 2008). In fact, alcohol consumption has been found associated with asymptomatic sensitization to foods (Bakos et al., 2006) and with symptomatic food allergy (Serghini-Idrissi et al., 2001). In this scenario, glycoproteins in alcoholic beverages may represent a source for CCD sensitization or, alternatively, IgE reactivity to these glycoproteins may represent an epiphenomenon of sensitization to CCDs from another source. The precise role of alcohol and food/hymenoptera venom glycoprotein exposure on CCD sensitization in heavy drinkers should be addressed in further studies.

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