

Heterogeneity of IgE epitopes of vinyl sulphone reactive dye: human serum albumin that react with IgE

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Summary

Background Vinyl sulphone reactive dye (vRD), which consists of vinyl sulphone reactive groups and a chromogen, can elicit IgE-mediated occupational asthma (OA) by haptenation. Human serum albumin (HSA) is known as the most reliable carrier protein for the vRD, the IgE epitopes of vRD-HSA are not well characterized. In this study we evaluated the epitope of vRD-HSA-specific IgE.

Methods Two vRD (Remazole Black-GR and Remazole Orange-3R), Procion Red-MX-5B, which has a dichlorotriazine reactive group, and vinyl sulphone (VS), were haptenated to HSA, respectively. vRD-HSA was denatured by heat or mercaptoethanol treatment and the allergenicities of denatured and non-denatured vRD-HSA were compared by ELISA and IgE immunoblotting using the sera of six vRD-OA patients. vRD-HSA-specific, Procion Red-MX-5B (pRD)-HSA-specific and VS-HSA-specific IgE were also measured with ELISA and the cross-reactivity between them was evaluated with ELISA inhibition.

Results Denaturation of vRD-HSA by heat affected its allergenicity markedly in five of six sera of RD-OA. When vRD was conjugated to the pre-heated HSA, its allergenicity also disappeared or was markedly attenuated compared with the vRD-HSA in five of six sera. Mercaptoethanol treatment markedly affected the allergenicity of the RD-HSA in all six RD-OA sera. Immunoblotting from non-denatured PAGE showed strong IgE affinity to vRD-HSA but immunoblotting from denatured SDS PAGE did not show IgE affinity. Among six RD-OA patients, five and four patients had pRD-HSA-specific and VS-HSA-specific IgE, respectively. However, the vRD-HSA-specific IgE was neither inhibited by pRD-HSA nor VS-HSA.

Conclusion We considered that the conformational structure of HSA would be critical for the IgE epitopes during the haptenation process and both of the chromogen and reactive groups of the vRD would contribute to the formation of IgE epitope. Our results also confirmed the heterogeneity of IgE epitopes in the RD-HSA complex.

Keywords: vinyl sulphone reactive dye, IgE epitopes, occupational asthma

Clinical and Experimental Allergy, Vol. 31, pp. 1779–1786. Submitted 30 January 2001; revised 21 March 2001; accepted 30 April 2001.

Introduction

Reactive dyes (RD) have molecular weights ranging from 500 to 1000 dalton and contain a chromogen and reactive functional groups designed to form irreversible covalent

bonds to the amino acid residues of cellulosic fibres. The vinyl sulphone RDs (vRD) have one or two vinyl sulphone reactive groups and the Procion RDs (pRD) have a dichlorotriazine functional group (Fig. 1). However, they can share similar chromogen components. RDs can easily covalently bind to soluble autologous proteins [1] and elicit occupational asthma (OA) in exposed workers [1–9]. The presence of specific IgE and positive skin responses to the

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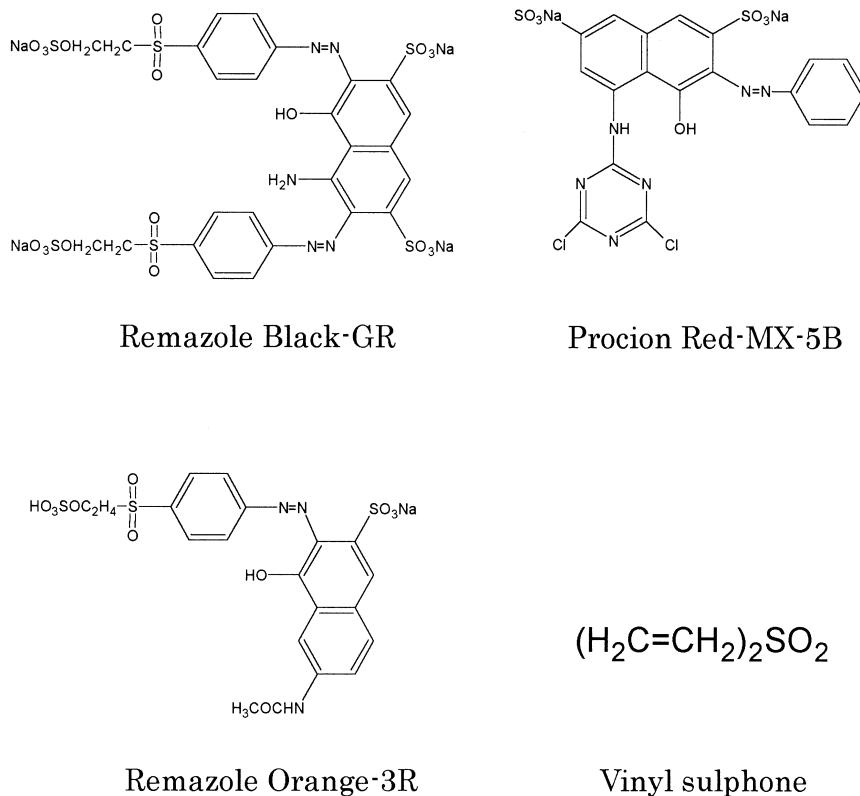


Fig. 1. Chemical structures of various RDs and vinyl sulphone.

RDs in these OA patients suggests that IgE-mediated immune responses may play an important role in the development of this disease [2–10].

However, the IgE epitopes of the RD-carrier protein complex have not been clarified, and knowledge of the IgE epitopes of RDs would be very useful for the standardization of the allergen for diagnosis and screening for RD-OA. Human serum albumin (HSA) is the most abundant protein in the blood and extravascular fluid and easily bound to variable ligands such as drugs, metallic ions and amino acids [11]. Studies with trimellitic anhydride also suggest that the major target protein in low molecular weight chemical hapten-protein complexes would be HSA [12], and it also has been identified as the most effective carrier protein for RDs [7]. In this study, we evaluated the epitopes of vRD-specific IgE using the vRD-HSA complex and found heterogeneity in the IgE epitopes of RD-HSA.

Methods

Subjects

We enrolled six RD-OA patients showing high levels of RD-HSA-specific IgE. Their clinical features and the levels of RD-HSA-specific IgE are summarized in Table 1. Four patients showed positive responses to bronchial provocation

tests with RDs and two patients were diagnosed by skin prick, ELISA and methacholine challenge tests.

Preparation of RD-HSA complexes

The following RD-HSA complexes were produced; Remazole Black-GR (Mw-991.8, Ewha Co., Incheon, Korea), -HSA (Mw-65K, Dongshin Pharmaceuticals, Seoul, Korea), Remazole Orange-3R (Mw-617.5, Ewha Co.) -HSA, and Procion Red MX-5B (Mw-615.3, Aldrich, Milwaukee, WI, USA) -HSA. In addition, the vinyl sulphone (Mw-118.2, Aldrich) reactive group was also haptenated to HSA. The vRDs and Procion RD have different reactive groups but have similar chromogens (Fig. 1). Remazole vRDs have vinyl sulphone reactive groups and Procion Red MX-5B has a dichlorotriazine reactive group. The RD-HSA complexes were prepared according to the previously described method [8]. Briefly, 1 mg of RDs and 1 μl of 97% vinyl sulphone were dissolved in 1 mL of 2% HSA in 0.1 M Na_2CO_3 (pH 11), respectively. The molar ratios of Remazol Black-GR : HSA, Remazole Orange-3R : HSA, Procion MX-5B : HSA and vinyl sulphone : HSA were 3.3 : 1, 5.3 : 1, 5.3 : 1 and 25 : 1, respectively. The solutions were incubated with stirring at 4 °C for 18 h and we used the RD-HSA complexes after dialysis in 0.1 M Na_2CO_3 at 4 °C.

Table 1. Clinical features and ELISA OD values of specific IgE in six RD-OA

Subjects	Age/ sex	Atopy to common allergens	tIgE (U/L)	PC ₂₀ M (mg/mL)	RD BPT	ELISA OD for specific IgE			
						HSA	Black-GR-HSA	Red-MX-5B-HSA	VS-HSA
1	49/M	Yes	660	2.38	early	0.000	0.547	0.009	0.073
2	52/M	Yes	675	3.52	early	0.012	0.272	0.450	0.014
3	38/M	Yes	979	ND	Dual	0.000	0.122	0.060	0.004
4	33/M	Yes	ND	6.7	ND	0.000	0.102	0.089	0.024
5	21/M	No	ND	14.1	ND	0.006	0.865	0.026	0.044
6	33/M	Yes	631	ND	Dual	0.001	0.656	0.031	0.271

tIgE = total IgE; BPT = bronchial provocation test; ND = not done. Cut-off value of Black-GR-HSA, Red-MX-5B-HSA, VS-HSA specific IgE, > 0.015.

For preparation of denatured Black-GR-HSA complex, the RD-HSA or unconjugated HSA was boiled in a water bath at 100 °C for 5 min. The boiled HSA was also conjugated with Remazole Black-GR using the methods described above. Black-GR-HSA complex was also denatured by incubating the complex with 2.9 mM 2-mecaptoethanol in a boiling water bath for 5 min

Measurement of RD-HSA-specific IgE

We measured RDs-HSA-specific IgE by ELISA. Polystyrene microplates (Costar, Cambridge, MA, USA) were coated with 50 µl of 1 : 100 diluted RD-HSA conjugates in 0.05 M carbonate buffer (pH 9.6) for 2 h at room temperature (20°C) and overnight at 4 °C. After washing with PBS-T, each well of a microplate was blocked by incubation with 200 µl of 1% bovine serum albumin (BSA) in PBS-T (137 mM NaCl, 1.8 mM KH₂PO₄, 10 mM Na₂HPO₄, 27 mM KCl, and 0.1% Tween-20, pH 7.4) for 1 h at room temperature. Fifty µl of each patient's sera were incubated in each well for 1 h at room temperature.

After washing three times with PBS-T, 50 µl of 1 : 1000 biotin-labelled goat anti-human IgE (Vector, Burlingame, CA, USA) were added to each well and incubated for 1 h. After washing, the wells were further incubated with 1 : 1000 vol/vol streptavidin-peroxidase (Sigma, St Louis, MO, USA) for 30 min and re-washed. The colourimetric reaction was developed with 50 µl of ABTS solution (25 mg of 2,2-azino-bis-3-ethylbenzthiazoline-sulfonic acid in 50 mM citrate phosphate buffer, 50 µl of 30% H₂O₂) for 5 min, and stopped by adding 50 µl of 2 N NaN₃. Optic density (OD) was measured at 410 nm using an automated ELISA spectrophotometer (Dynatec, Alexandria, CA, USA). The ELISA OD for specific IgE were determined by extraction of the OD of 0.1% BSA from the OD of RD-HSA. All assays were performed in duplicate.

ELISA inhibition of RDs-HSA-specific IgE

For ELISA inhibition, the non-diluted pooled sera with a high level of Black-GR-HSA-specific IgE (*n* = 4) were pre-incubated with various concentrations (1 ng/mL, 6.2 ng/mL, 32 ng/mL, 160 ng/mL, 0.8 µg/mL, 4 µg/mL and 20 µg/mL) of Black-GR-HSA, Red-MX-5B-HSA and VS-HSA overnight at 4 °C. The HSA-Black-GR-specific IgE in the pre-incubated sera was then measured using the method described above. The ELISA inhibition of Red-MX-5B-HAS-specific IgE was also performed with Black-GR-HSA, Red-MX-5B-HSA and VS-HSA.

Gel electrophoresis under denaturing and non-denaturing conditions

Denaturing SDS-PAGE was carried out using the method described by Laemmli [13]. vRD-HSA complexes were dissolved in the reducing sample buffer (12.5 mM Tris-HCl, 5% glycerol, 0.4% SDS, 2.9 mM 2-mecaptoethanol and 0.02% bromophenol blue) and 10 µg of vRD-HSA in each lane was then separated in 13% polyacrylamide gels with 0.1% SDS (Mighty Small electrophoresis unit; Hoeffer, San Francisco, CA, USA) at 50 V for 30 min and 180 V for 90 min. For non-denaturing PAGE, vRD-HSA complexes were dissolved in native sample buffer (62.5 mM Tris-HCl, 5% glycerol and 0.01% bromophenol blue) and 10 µg of vRD-HSA in each lane was separated in 8% polyacrylamide gel without SDS at 50 V for 30 min and 180 V for 90 min.

IgE immunoblotting

Transfer of the proteins in the gel onto nitrocellulose membranes (pore size – 0.45 µm; Amersham, Buckingham, UK) was performed on a Transpor TE 42 Unit

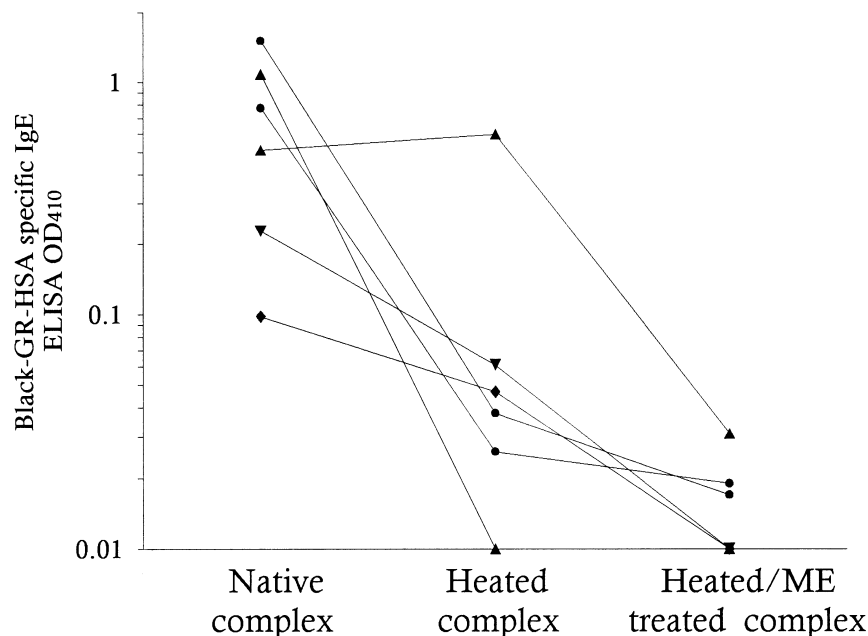


Fig. 2. Effects of denaturation on the allergenicity of vRD-HSA complex. ME = mercaptoethanol

(Hoeffer) in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3) at 350 mA for 2 h. Membranes were blocked by 5% non-fat dried milk in TBS-T (50 mM Tris with 0.1% Tween, pH 7.5) and then incubated with patient sera (1 : 10 diluted in TBS-T, 0.02% NaN₃) at 4 °C for 18 h and washed with TBS-T. Goat anti-human IgE conjugated with alkaline phosphatase (Sigma) was diluted 1 : 1000 in TBS-T before incubation with the blots for 60 min. After washing with TBS-T, the bound goat antibodies were detected with a BCIP/NBT system (Promega, Madison, WI, USA).

Results

Influence of denaturation by heat on the allergenicity of Black-GR-HSA

When the Black-GR-HSA was boiled for 5 min, the allergenicity of Black-GR-HSA was markedly affected in five of six RD-OA subjects but the degrees of attenuation of the allergenicity by heat were individually variable. The affinity of patient number 2's specific IgE was not changed whether the Black-GR-HSA was heated or not. However, mercaptoethanol treatment of vRD-HSA at heating condition affected its allergenicity markedly in all subjects (Fig. 2). PAGE was also performed under denaturing and non-denaturing conditions. PAGE gel showed 65 kDa and 130 kDa bands that may correspond to the monomer and dimer of RD-HSA. The density of 130 kDa was higher in Black-GR-HSA compared with the Orange 3R-HSA or unconjugated HSA (Fig. 3).

In IgE immunoblotting after PAGE, patients' pool sera

($n = 6$) were bound to the non-denatured vRD-HSA but not bound to the denatured vRD-HSA (Fig. 4). We also evaluated the allergenicity of the complex in which the vRD was haptenated to pre-heated HSA (vRD-heated HSA). The allergenicity of vRD-heated HSA was also markedly affected in five of six patients compared with the allergenicity of non-denatured vRD-HSA. The affinity of

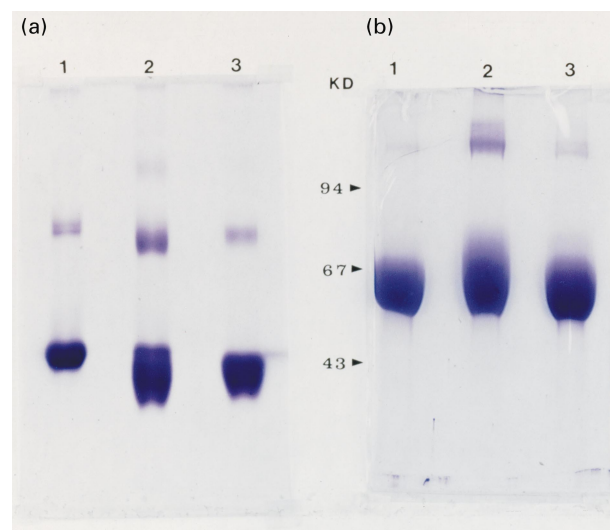


Fig. 3. Gel electrophoresis of vRD-HSA under non-denaturing (a) and denaturing conditions (b). Lane 1 = HSA control; lane 2 = Remazole Black-GR-HAS; lane 3 = Remazole Orange-3R-HSA. The gel was stained with Coomassie blue and the molecular marker was for denaturing SDS-PAGE gel.

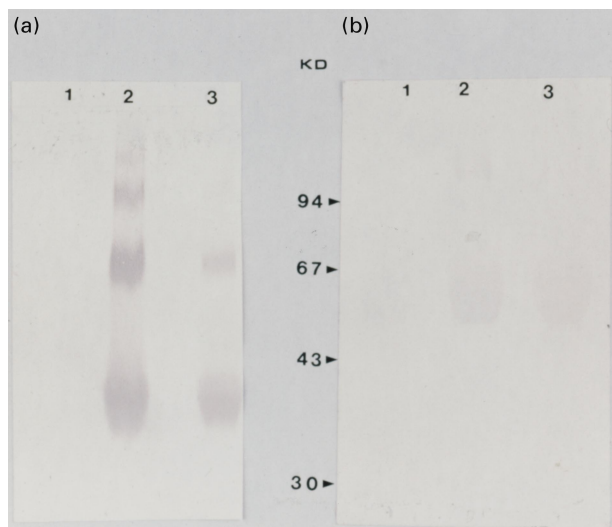


Fig. 4. IgE immunoblotting of vRD-HSA under non-denaturing (a) and denaturing conditions (b). The bands shown in lanes 2 and 3 of (b) are the colour of the RD-HSA itself and the molecular marker was for denaturing condition. Lane 1 = HSA control; lane 2 = Remazole Orange-3R-HSA; lane 3 = Black-GR-HSA.

patient number 2's specific IgE was not affected by haptenation of the Black-GR to the pre-heated HSA. However, in two patients (numbers 3 and 4), affinity of specific IgE was more affected by the conditions of coating antigens, such as heated Black-GR-HSA or Black-GR-preheated HSA complex (Fig. 5).

Cross-reactivity of vRD-HSA, pRD-HSA and VS-HSA

We measured Black-GR-HSA, and Procion Red-MX-5B-HSA, VS-HSA specific IgE. Although the reactive functional groups were different, the structure of the chromogens of Black-GR and Procion Red-MX-5B are

similar (Fig. 1). The cut-off values of RD-HSA-specific and VS-HSA-specific IgE were determined as the mean $\pm$ 2 SD of the 20 RD unexposed atopic patients' sera. The pRD-HSA-specific and VS-HSA-specific IgE were found in five and four patients, respectively, but the ELISA OD values of VS-HSA were lower than that of Black-GR-HSA (Table 1). Black-GR-HSA-specific IgE was dose-dependently inhibited by Black-GR-HSA but not by Red-MX-5B-HSA or VS-HSA (Fig. 6). Inhibition ELISA for Procion Red-MX-5B-HSA-specific IgE was carried out with two patients' sera (numbers 2 and 5). The Red-MX-5B-HSA-specific IgE was dose-dependently inhibited by Red-MX-5B-HSA but not inhibited (Fig. 7A) or very weakly inhibited (Fig. 7B) by Black-GR-HSA.

Discussion

In this study, we show that the allergenicity of RD-HSA is labile to denaturation and these features suggest that the conformational structures of the vRD-HSA complex are critical for the IgE epitopes. As the haptenation of vRDs to pre-denatured HSA did not have allergenicity in some RD-OA patients, our study also shows that the structure of HSA *per se* is important for the IgE epitopes of vRD-HSA. HSA consisted of three domains with 17 disulphide bonds and is easily denatured by heat [14]. HSA is easily bound to endogenous and exogenous compounds by non-covalent bonds and is well recognized as the depot or transport protein for many drugs and ligands [11]. In addition, it can also form covalent bonds with drugs such as penicillin or aspirin. Aspirin, for example, is covalently bound to lysine residues of HSA [15] and β -lactam antibiotics acetylate the ϵ -amino groups of the lysine residues and imidazole groups of histidine residues, causing drug allergy in some patients [16].

HSA is also widely used as the carrier protein for low

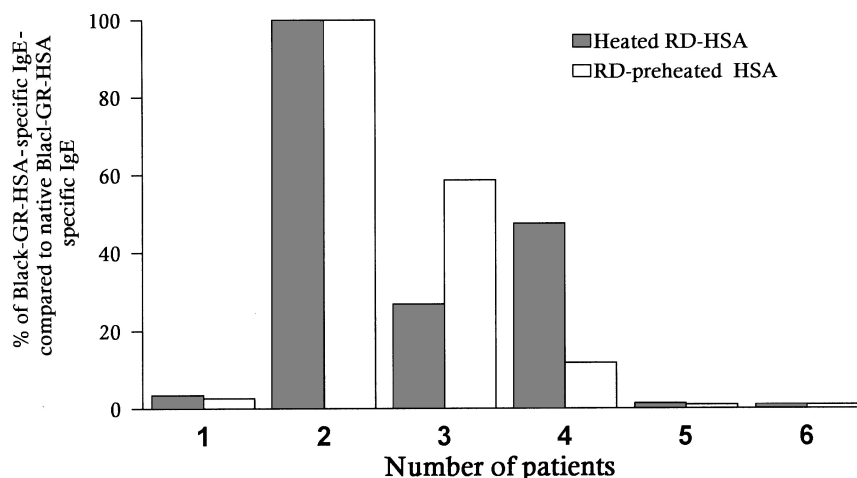


Fig. 5. Allergenicity of the heated Black-GR-HSA complex and the Black-GR-preheated HSA complex in relation to the allergenicity of non-denatured Black-GR-HSA.

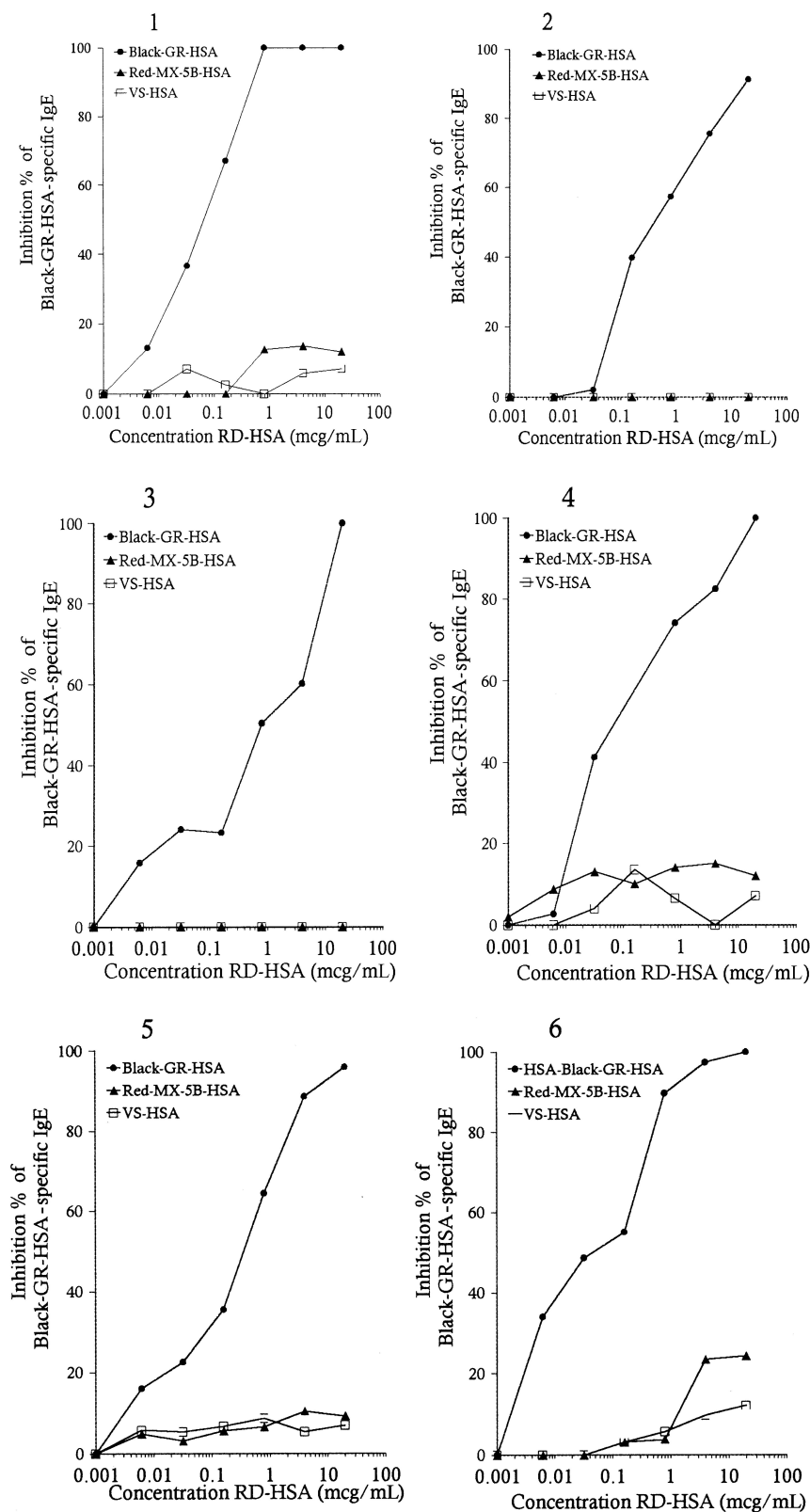


Fig. 6. Inhibition ELISA of Black-GR-HSA-specific IgE with Black-GR-HSA (●), Red-MX-5B-HSA (▲) and VS-HSA (□).

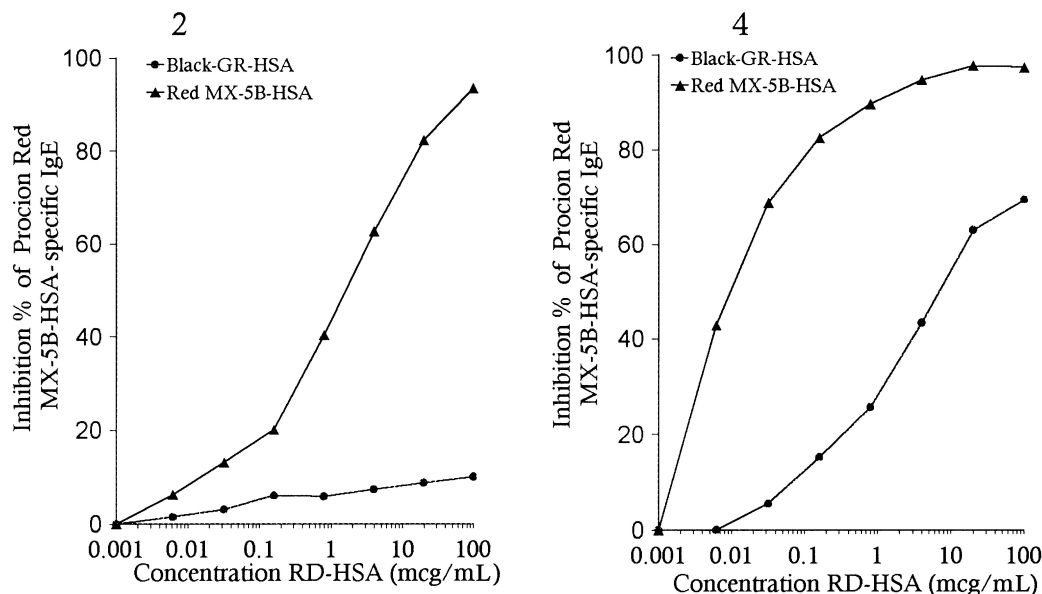


Fig. 7. Inhibition ELISA of Red-MX-5B-HSA-specific IgE with Red-MX-5B-HSA (▲) and Black-GR-HSA (●).

molecular weight occupational allergens such as trimellitic anhydride [12]. It is known that vRD can react with the cysteine, histidine, lysine, serine and tyrosine residues of fibre proteins [17]. As the HSA contains 59 lysines, 15 histidines and one free cysteinyl thiol group, we believe that various conformational structures of the RD-HSA complex might be possible. It is, for example, well known that covalent or non-covalent bonding with ligands can cause conformational changes in HSA [11,18]. The RDs easily haptenated to autologous proteins but the reactions were markedly influenced by pH, temperature, types of reactive groups and the molar ratios of RD and autologous proteins [4,19]. These factors may cause different RD-HSA conformations. In addition, the chemical structure of vRD would also be important for determining the conformational status of the vRD-HSA complexes. In SDS-PAGE, the 130 kDa band, which may be the dimer of the RD-HSA complex, of the Remazole Black-GR is more intense than that of the Remazole Orange-3R or unconjugated HSA. As the Black-GR has two vinyl sulphone reactive groups compared with the one reactive group of Orange 3R, we think that the Black-GR is more likely to form the polymer of the RD-HSA complexes.

The allergenicity of RD-HSA was markedly attenuated by heat, but the degree of the attenuation was quite different individually. These findings support the heterogeneity of IgE epitopes of RD-HSA. We also report upon the heterogeneous allergenicity of RDs, for example most patients showed positive responses upon inhaling RD alone, but some RD-OA patients showed positive responses after inhaling RD-HSA and not by inhaling unconjugated

RD [20]. This result may reflect the diverse features of IgE-epitopes of the RD-autologous proteins. VS-HSA-specific IgE (subjects number 1, 4, 5 and 6) and Red-MX-5B-HSA-specific IgE (subjects number 2, 3, 4, 5 and 6) were found in some patients but their IgE ELISA OD were lower than that of the Black-GR-HSA. Red-MX-5B has a different reactive group but has a chromogen which is similar to that of Black-GR. Jones *et al.* [21] showed negligible cross-reactivity between Procion RDs and its reactive binding group, cyanuric chloride, and they suggested that the major determinant of the IgE epitopes would be the chromogen. However, our ELISA inhibition tests showed that the Black-GR-specific IgE was not inhibited by Red-MX-5B-HSA or VS-HSA. The Remazole Black-GR and Procion Red-MX-5B dyes may bind to different amino acid residues of HSA during the haptenation process and, as a sequence, Procion Red-MX-5B may induce complexes that have different conformation from that of the Black-GR-HSA.

On the basis of these results, we believe that the reactive groups and chromogen components might contribute to the IgE epitope, and the neoantigenic determinant, which is formed during the haptenation process, is perhaps the more important consideration. Types of carrier proteins are very important for determining the allergenicity of RD-carrier protein. Wass *et al.* [7] showed the superior allergenicity of the RD-HSA compared with the other RD-carrier protein complexes, such as bovine serum albumin, porcine serum albumin, ovalbumin and immunoglobulins, and their results may also suggest the presence of different IgE epitopes according to the types of carrier proteins used. The

haptation process may occur very rapidly *in vivo*. The skin prick test with RD without carrier protein showed good diagnostic sensitivity, as was obtained with β -lactam antibiotics [9,10]. Luckzinska *et al.* [4] reported that unconjugated free RD could considerably inhibit RD-specific IgE in RAST and suggested the possibility of non-covalent binding of RD to various carrier proteins during incubation periods. Some RD-OA patients showed positive skin reactivity to RD alone but specific IgE to the RD-HSA was not detected with ELISA in their sera. On the contrary, some RD-OA patients had negative responses to skin prick test with RD alone but high titre of RD-HSA-specific IgE was detected with ELISA in their sera [10]. Thus, we thought that the skin prick test with RD alone and serological measurement of specific IgE with RD-HSA may have a complementary effect for screening for and diagnosis of RD OA.

With these results, we believe that the IgE epitopes of RD-HSA are heterogeneous and may cause difficulties in the standardization of RD agents for screening for and diagnosis of RD-OA.

Acknowledgement

This study was supported by the Yonsei University College of Medicine, Internal Medicine Research grant.

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