

Heterogeneity of IgE antibody response to reactive dye in sera from four different sensitized workers

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Summary

We studied RAST and RAST inhibition tests to black GR, the most frequent sensitizer among several reactive dyes in our previous study, in order to evaluate the specificity of IgE antibodies to hapten or new antigenic determinants and the crossreactivity between two reactive dyes, black GR and orange 3R, in sera from four different sensitized workers. RAST inhibition studies with black GR-human serum albumin (HSA) conjugate discs demonstrated that black GR-HSA conjugates were the most effective inhibitors. Orange 3R-HSA conjugates, unconjugated forms of black GR and orange 3R were weak inhibitors in two patients and non-inhibitory in one patient, whereas they caused strong dose-dependent inhibitions in one patient. These results suggested that the IgE response to black GR-HSA conjugates might be heterogenous and the cross-reactivity between two reactive dyes differed from one patient to another.

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Introduction

Reactive dyes have been increasingly used as colouring agents for textiles for 30 years. Several investigators including us demonstrated that exposure to reactive dyes could induce IgE-mediated respiratory symptoms [1–4]. In previous studies [1–4], many cases of occupational asthma associated with occupational exposure to reactive dyes were reported. It was possible to detect specific IgE antibodies to different reactive dye-HSA conjugates [1,3–5]. In our previous studies [1,4], black GR was the most frequent sensitizer among several reactive dyes.

The aim of this study was to characterize the specificity of IgE responses to both hapten and new antigenic determinants (NAD) formed by the interaction of black GR and human serum albumin (HSA) and the crossreactivity with another structurally similar reactive dye, orange 3R, in sera from four different sensitized workers.

Materials and methods

Subjects

Four workers employed in a dye industry located in Incheon, Korea, with high specific serum IgE binding to

black GR (more than 20% of bound radioactivity) were selected among the 309 employees. They had been exposed to 50 kinds of reactive dyes. Black GR and orange 3R were the most frequently handled dyes among them. Two asthmatic workers, one asymptomatic worker and one symptomatic worker without airway hyperresponsiveness were selected. Table 1 illustrates the clinical data of study subjects. Work-related respiratory symptoms were noted in three patients, but case 2 showed a negative result on the methacholine bronchial challenge test. Case 3 had no work-related symptoms. The bronchoprovocation test with unconjugated black GR solution showed an immediate asthmatic response in case 1. Case 4 had work-related respiratory symptoms and showed a positive result on the methacholine bronchial challenge test. The bronchoprovocation test with the inhalation of 1 mg/ml black GR-HSA conjugate solution revealed a dual asthmatic response, although he showed a negative result on the bronchoprovocation test with the unconjugated form of black GR. Sera from these four workers were collected and stored at -20°C .

Preparation of dye-HSA conjugates

Two kinds of reactive dyes (black GR and orange 3R) were presented by the dye industry. Their trade names

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Table 1. Clinical summary of study subjects

Workers	Sex/Age	Exposure duration to Black GR (months)	Atopy	Total IgE (IU/ml)	RAST (bound %)		Work-related symptoms
					Black GR-HSA	Orange 3R-HSA	
1	M/46	74	—	676	23	17	R
2	M/53	34	—	655	36	24	R + N
3	M/50	74	—	441	20	13	None
4	M/49	27	—	976	43	45	R + N

HSA, human serum albumin. ND, not done. Atopy, positive reactor ($\geq 2+$) to one or more allergens on skin prick test with seven common inhalant allergens. R, respiratory symptoms included cough and shortness of breath. N, nasal symptoms included sneezing, rhinorrhoea and nasal congestions.

(colour index number) were rifazol black GR(Bk-5) and rifazol brilliant orange 3R(0-16): their chemical structures are shown in Fig. 1. Dye-HSA conjugate solution was prepared according to our previously described method [1,4]. In brief, 0.1 mg of each two dyes was added to 2 mg/ml of HSA (Sigma Chemical Co., St Louis, Missouri, U.S.A.) solution dissolved in 100 mM Na_2CO_3 (pH 11.0) and incubated with stirring at 4°C for 18 hr, which was dialysed against 100 mM Na_2CO_3 at 4°C for 48 hr. A similar technique was used to prepare a solution of HSA alone without reactive dyes. These dye-HSA conjugate solutions were used in preparing RAST discs and inhibitors in RAST inhibition tests. For the other experiments, black GR and orange 3R were coupled to ovalbumin (OA, Sigma Chemical Co.) in a corresponding manner. The discs of nitrocellulose filter paper (NFP, Millipore, Cat No HAHY 08250, 0.45 μm , Millipore Co., Bedford, Massachusetts, U.S.A.) were immersed into this dye-HSA conjugate solution for 72 hr at 4°C. The binding to nitrocellulose paper might be due to hydrophobic interaction between conjugates and the paper. Then, the

discs were dried at room temperature and used as a solid phase in the subsequent RAST and RAST inhibition tests.

RAST assay

The dried discs which had been immersed in dye-HSA conjugate solutions were blocked with 10% newborn calf serum for 1 hr and incubated with 50 μl of each patient's serum for 6 hr at room temperature. Then, the discs were washed three times with 2.5 ml of 0.9% NaCl containing RAST washing solution additive (Pharmacia, Uppsala, Sweden). Fifty microlitres of ^{125}I -labelled polyclonal anti-human IgE (Pharmacia) was added and left for 18 hr at room temperature. The discs were washed again with washing solution and the bound radioactivity was measured using a gamma counter. All the assays were performed in duplicate. The results were expressed as per cent binding, defined as percentage of added counts per minute (c.p.m.) bound to the reactive dye-HSA conjugate discs. The bound radioactivities of all the control subjects

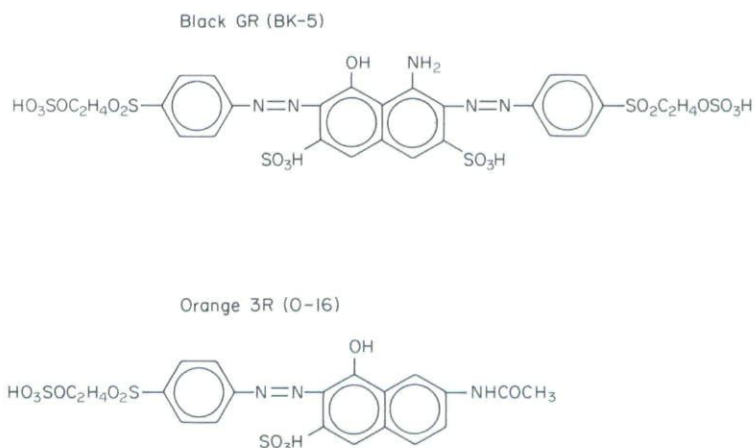


Fig. 1. Chemical structures of black GR (BK-5) and orange 3R (O-16).

were <1% (mostly <500 c.p.m.). The cut-off value of positive specific IgE binding was determined as 2%, which was derived from a twofold increase of 1%.

RAST inhibition tests

Competitive RAST inhibition was used to investigate the specificity of IgE binding to dye-HSA conjugate and the relationship between the two reactive dyes. Fifty microlitres of 1:4 or 1:10 diluted sera were incubated with four concentrations (from 0.0025 to 0.1 mg/ml) of reactive dye-HSA conjugates, dye-OA conjugates, unconjugated dyes and HSA alone for 1 hr at room temperature. Sera was diluted with 10% newborn calf serum. Inhibitors were diluted with phosphate buffered saline. The inhibited sera were then incubated with black GR-HSA conjugate discs for 6 hr at room temperature. After washing three times with 2.5 ml of 0.9% NaCl containing RAST washing additive, 50 μ l of ^{125} I-labelled polyclonal anti-human IgE (Pharmacia) was incubated with the discs for 18 hr at room temperature. After repeated washing steps, the bound radioactivities were determined using a gamma counter. The inhibition of specific IgE binding to allergen discs was estimated by comparing with control samples in which an equal volume of phosphate buffered saline (pH 7.5) was preincubated instead of inhibitors, and expressed as per cent inhibition as follows:

$$100 - \frac{\text{c.p.m. bound to dye-HSA disc with inhibitor}}{\text{c.p.m. bound to dye-HSA disc without inhibitors}} \times 100$$

Bronchoprovocation tests with reactive dyes

Bronchoprovocation tests were performed according to the previously described method [1,4] in two subjects using unconjugated black GR which was prepared by dissolving in 0.4% phenolized saline and black GR-HSA in one subject. The forced expiratory volume in 1 sec (FEV₁) and maximum mid-expiratory flow (MMEF) were measured with a spirometer before and 10 min after inhalation. The test solutions were delivered by a Vaponefrine nebulizer (Meiko Co., Japan) using a compressed air source. The patients were asked five times to breathe the nebulized aerosol up to their vital capacity. Serial increments in antigen concentration (0.01, 0.1, 1.0, 2.5 mg/ml) were given at 10 minute intervals until a 20% or greater decrease in FEV₁ was observed. FEV₁ and MMEF were measured frequently during the first hour, and hourly thereafter for 9 or 10 hr after the challenge.

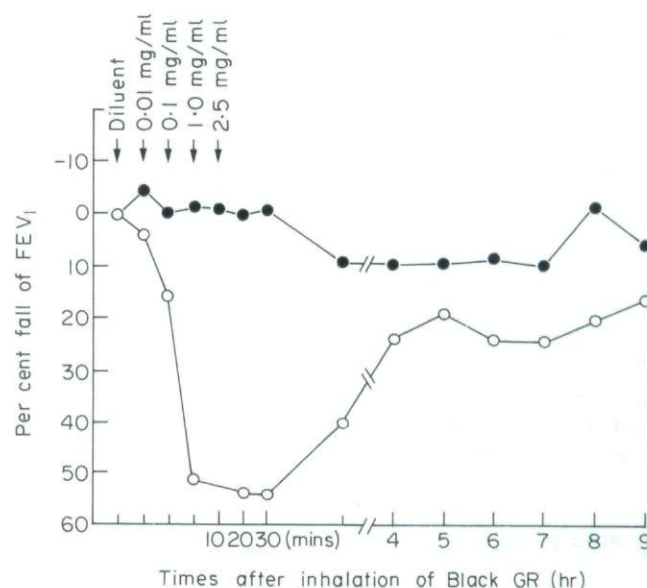


Fig. 2. Bronchoprovocation test result. ●, unconjugated black GR. ○, black GR-HSA conjugate.

Results

Bronchoprovocation test result

In case 4, there were no significant changes in FEV₁ after the inhalation of unconjugated black GR (until 2.5 mg/ml). After the inhalation of 1.0 mg/ml of black GR-HSA conjugate solution, FEV₁ fell more than 50% and severe dyspnoea developed, which continued until 9 hr after the inhalation (Fig. 2). Caution might be required to perform the bronchoprovocation test with reactive dye and reactive dye-HSA conjugate.

RAST inhibition tests

RAST inhibition studies were performed to determine the specificity and crossreactivity of structurally related allergenic determinants of two dye-HSA conjugates. Figure 3a, b, c, d illustrate the black GR-RAST inhibition results, RAST per cent bindings for the uninhibited discs ranged from 12 to 15%. With the serum of case 1 patient, it was found that IgE binding to black GR-HSA conjugate could be significantly inhibited by preincubation of the serum with black GR-HSA conjugates; orange 3R-HSA conjugate and unconjugated forms of black GR and orange 3R gave negligible inhibitions. HSA alone gave no inhibitions. As opposed to HSA conjugates, black GR-OA conjugates were non-inhibitory (Fig. 3a). With sera of case 2 and 3, black GR-HSA conjugates were the most effective inhibitors. Unconjugated forms of black GR and orange 3R-HSA conjugates showed some inhibitions. No inhibition was observed with black GR-OA conjugate,

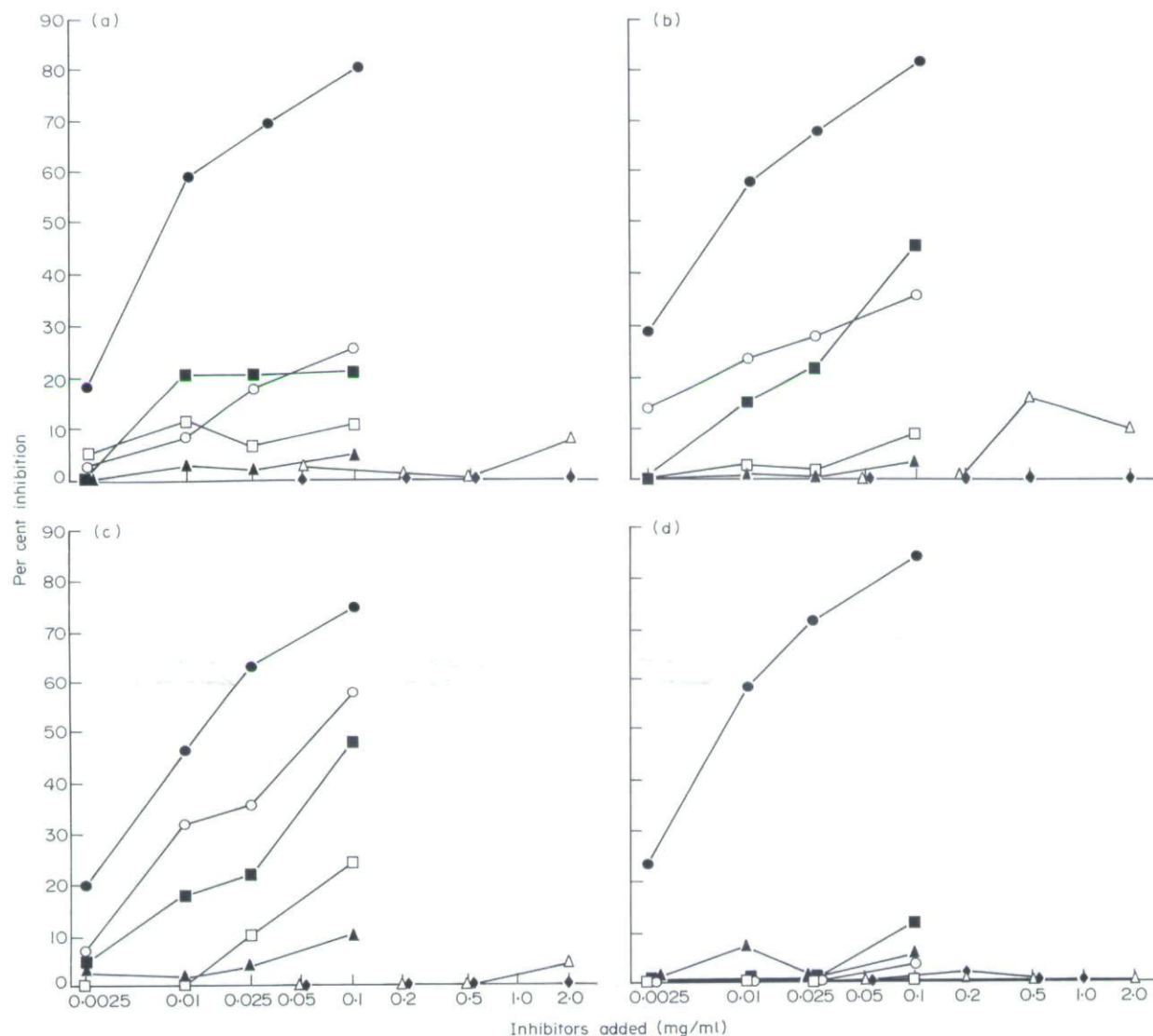


Fig. 3. Black GR-RAST inhibition results. (a-d) See text for details. ●, black GR-HSA conjugate. ○, free black GR. ■, orange 3R-HSA conjugate. □, free orange 3R. ▲, black GR-OA conjugate. △, HSA alone. ◆, OA alone.

OA alone or HSA alone as shown in Fig. 3b and 3c. With serum of patient 4, although the IgE bindings were inhibited up to 85% by black GR-HSA conjugates, the other three inhibitors and black GR-OA conjugate gave no inhibitions (Fig. 3d).

Discussion

Reactive dyes which covalently bind with organic macromolecules of textiles result in a faster staining than other dyes. Because these reactive dyes have the molecular weight ranging from 500 to 1000 Da (black GR, 883 Da; orange 3R, 565 Da), they can act as haptens and combine with a protein to elicit an immunologic response like

isocyanates or acid anhydride. We studied clinical and immunological findings in 309 dye-exposed workers including skin test and RAST to dye-HSA conjugates. The data revealed 13 cases of occupational asthma associated with exposure to several reactive dyes [4].

Wass and Belin [8] suggested that the immune response induced by isocyanate could involve both carrier and hapten; it might involve new antigenic determinants of the protein. Electrically charged hydrophilic groups, such as NH_3 , on the surface of a macromolecule may be the preferential site reacting with isocyanate. This may lead not only to binding of a hapten to the surface but also to a change of the three-dimensional configuration of the carrier. Presumably, the antigenic determinants that

evoke the immunological responses are formed by the interactions of the chemical hapten and autologous proteins. Baur [9] noted the complexity of immune responses induced by toluene diisocyanate (TDI) and hexamethylene diisocyanate (HDI)-HSA conjugates. TDI and HDI, by virtue of their high degree reactivity, appeared to alter the natural structure of carrier protein such that at least two different antigenic determinants, i.e. haptenic and NAD which included parts of both hapten and carrier proteins, could be demonstrated in these conjugates. In this study, the *in vivo* immunological responses to reactive dyes might be induced by hapten-protein conjugates as the previous studies suggested [1,4,5]. These reactive dye-HSA conjugates might develop two or more antigenic determinants.

The immunological reactivity to black GR-HSA conjugate showed individual difference in this study. All four workers showed high specific IgE levels to both black GR and orange 3R-HSA conjugates. One asthmatic worker (case 4) who showed dual asthmatic responses after inhalation of black GR-HSA conjugate solution but no response with the unconjugated form of black GR solution, might have had reagenic antibodies specific for NAD with hapten specificity. RAST inhibition of the serum of patients 2 and 3 suggested that they might develop IgE antibodies to both hapten and NAD. These results suggest that the immunological responses occurring after exposure to black GR might be heterogeneous and different from one patient to another.

The crossreactivity between two reactive dyes, black GR and orange 3R, was partial and varied from one patient to another. They were structurally similar; black GR had two *b*-sulphatoethyl sulphone groups as functional groups and orange 3R had one. It was evident that there was a substantial amount of crossreactivity of IgE responses between the two dyes and two populations of antibodies to both NAD and hapten might be elicited in sera from patients 2 and 3. As opposed to these results, there were only minimal inhibitions by orange 3R-HSA conjugates in that of patient 4. Gallagher *et al.* [10] suggested in their isocyanate study that the basis of crossreactivity between TDI and HDI conjugates was determined by new antigenic sites created by isocyanate protein conjugates. Our previous study [4] showed that strong dose-dependent inhibitions of IgE bindings on orange 3R-HSA conjugate discs were found with additions of both black GR-HSA conjugates and its unconjugated form as well as orange 3R-HSA conjugates and its unconjugated form, suggesting that haptenic and NAD

specific antibodies to orange 3R might crossreact with those of black GR.

The importance of the carrier had been noted by Baur [9] who found an almost complete lack of IgE binding of OA-bound TDI in the RAST. Our study confirmed that the carrier protein was of importance for the specific IgE responses. OA was not effective when it was used as an inhibitor in RAST inhibition tests in sera from case 2 and 3 as well as case 1 and 4. It might be caused by the chemical binding with a carrier leading to a change of the protein structure, as Wass and Belin [8] suggested in the isocyanate study.

In conclusion, the IgE responses to black GR-HSA conjugates might involve both hapten and NAD and vary between individuals.

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