

## RAST-detection of specific IgE in diphenylmethane diisocyanate exposed workers: considerations in performance of the test

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Some parameters which might affect RAST results, i.e. incubation length, degree of ligand substitution, and total IgE levels, were examined in a RAST system employing diphenylmethane diisocyanate - human serum albumin (MDI-HSA) conjugates as antigens, both in exposed symptomatic and in non-exposed subjects. The reaction equilibrium was reached after 9 and 18 h, respectively, in the first and the second step of the test. A marked correlation with the degree of ligand substitution was observed, the highest sensitivity being achieved with the lowest (2.9) MDI/HSA molar ratio examined. Higher (up to 50) degrees of substitution resulted in progressively lower levels of radioactivity bound and in a loss of specificity, as confirmed by RAST inhibition experiments. At high levels of ligand substitution, high titers of total serum IgE affected RAST results.

*Key words:* antibody specificity; diphenylmethane diisocyanate; haptens; IgE; isocyanate sensitivity; RAST; standardization.

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Adverse respiratory reactions to the inhalation of diisocyanate compounds, with features suggesting the involvement of the immune system, have been extensively reported (13), and the presence of specific antibodies of the IgE class has been ascertained in part of the affected subjects. However, although most data were obtained by different authors employing the RAST technique, the results are often conflicting, and the relevance of such antibodies in the pathogenesis of isocyanate-induced disorders still remains questionable (2, 5, 7, 9, 14, 15, 19). Different criteria in sampling the subjects may partly explain such discrepancies; however, the preparation of the antigens and the performance of the test itself may represent other sources of variability. On this basis we examined the relevance of the following parameters on isocyanate RAST results: length of incubation time,

molar ratio of the hapten-carrier conjugate employed as antigen, and effect of total serum IgE related to each conjugate tested, in six symptomatic subjects exposed to MDI and in 15 non-exposed controls either with normal or elevated serum IgE. Our results indicate an optimum incubation time of 9 and 18 h for the first and the second part of the test, respectively, while the highest sensitivity and specificity are obtained employing conjugates with a low degree of ligand substitution.

### MATERIAL AND METHODS

#### *Subjects*

Patient serum samples were obtained from six exposed workers with diagnosis of MDI-asthma based on symptoms and positivity to specific

bronchial provocation test (20): all subjects had detectable levels of specific IgE when previously RAST-tested with a low isocyanate-carrier molar ratio conjugate; total IgE levels ranged from 13 to 959 U/ml. Control sera were obtained from seven non-exposed healthy individuals with normal ( $< 200$  U/ml) serum IgE levels and eight non-exposed subjects with elevated IgE, ranging from 397 to 15000 U/ml: two of them, with IgE levels  $< 1000$  U/ml, were asymptomatic, while the others suffered asthmatic symptoms due to grass pollens, *Dermatophagoides*, or food allergens.

#### *Preparation of conjugates*

MDI (a generous gift of Dr. A. Cossi, Stabilimento Petrolchimico Montedison, Porto Marghera, Italy) was conjugated to human serum albumin (HSA) according to Scheel et al. (17), slightly modified: MDI, dissolved in dioxane to give 8, 16, 32, 64 or 128 mol isocyanate/mol HSA (conjugates A, B, C, D and E, respectively) was added to a solution of 1% HSA in 0.1 M pH 9.4 borate buffer at  $0^{\circ}\text{C}$  over a period of 30 min. The reaction mixture was brought to equilibrium by stirring at  $0^{\circ}\text{C}$  and exhaustively dialysed against phosphate-buffered saline; finally, precipitates were removed by centrifuging at  $0^{\circ}\text{C}$  30 min at 16000 *g* and filtering through a 0.45  $\mu\text{m}$  Millipore filter. The degree of substitution in isocyanate-HSA conjugates was determined by the modified methods of Marcali (11) and Gutmann (12). The control solution consisted of 1% HSA taken through each of the above steps without the addition of MDI. The MDI-HSA conjugates and the control solution, at a concentration of 20 mg/ml, were coupled to filter paper discs according to Ceska & Lundkvist (3) and used in a RAST procedure for the determination of specific IgE. Two batches of MDI-HSA conjugates were used throughout this study, yielding comparable results.

#### *Anti-MDI IgE determination*

The procedure has been described in detail elsewhere (15). Briefly, 100  $\mu\text{l}$  serum were added

either to antigen-or HSA-coated disc and incubated for 9 h on a rotator at room temperature; after washing, 50  $\mu\text{l}$  anti-IgE  $^{125}\text{I}$  (Phadebas RAST Reagent) were added and the mixture was incubated for 18 h on a rotator, washed and counted on a gamma counter; in some experiments incubation times were varied. Specific IgE was evaluated as net bound radioactivity % (NBR), from the formula:  $\text{NBR} = \text{test bound radioactivity \%} - \text{blank bound radioactivity \%}$ ; NBR values  $> 0.9$  (mean + 4 SD of a control group of 50 non-exposed subjects tested with conjugate A) were considered positive for specific antibodies.

#### *Total IgE quantitation*

Total IgE were determined by the PRIST procedure (Phadebas PRIST kit, Pharmacia Diagnostics). Serum IgE normal range, determined in a population of 60 healthy subjects, was 0-200 U/ml.

#### *RAST inhibition experiments*

RAST inhibition experiments were performed modifying the technique employed by Karol (10): 50  $\mu\text{l}$  serum were incubated 2 h at  $37^{\circ}\text{C}$  with 50  $\mu\text{l}$  of a 8 mg/ml solution of MDI-HSA, dioxane-treated HSA or the unrelated control protein chymotrypsinogen A in IgE-free horse serum (PRIST diluent, Pharmacia Diagnostics). Control tubes contained serum plus 50  $\mu\text{l}$  diluent. After addition of an antigen-coated disc to the tube, samples were incubated for 9 h at room temperature. Subsequently, discs were treated according to the described RAST procedure and the percent inhibition was calculated.

## RESULTS

#### *Variation in incubation time*

The effect of different time intervals (1-24 h) on both incubation steps of RAST was investigated in a RAST positive patient and in a non-ex-

posed control (both with normal IgE levels). Conjugate A was employed in these experiments. Fig. 1 reports the results obtained varying the first step length and maintaining the second incubation constant (18 h): it appears that in the positive case the reaction is completed in 10–12 h, while 76% of maximum bound radioactivity (BR) is reached after 3 h; incubation time does not affect the control serum BR nor the radioactivity bound to HSA alone. When the first incubation length is fixed at 10 h, the second step reaches its equilibrium after 16–18 h in the exposed subject, while no time-related variation is observed in the control serum.

### Ligand substitution

Chemical analysis of the degree of substitution in MDI-HSA conjugates gave the results reported in Table 1. The two colorimetric methods employed gave comparable results: identical or slightly different values were found with conjugates A and B; 11.8%, 9.2% and 13.5% differences were observed with compounds C, D and E, respectively. Moreover, a linear relationship between moles of isocyanate added and substituted is present up to 64 mol MDI added, while results obtained with conju-

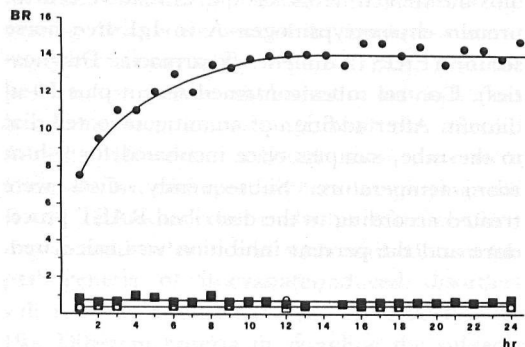


Fig. 1. Relationship between bound radioactivity (BR) and length of incubation in a RAST positive (●, ■) and a RAST negative (○, □) subject. The first step was varied and the second step kept constant (18 h); conjugate A was employed as antigen.

●, ○ : MDI-HSA coated discs  
■, □ : dioxane-treated HSA-coated discs

Table 1.

Comparison between degrees of substitution in different MDI-HSA conjugates, determined by two colorimetric methods.

| Conjugate | mol MDI<br>added/<br>mol HSA | Ligand substitution |                     | Mean<br>ligand<br>substitution |
|-----------|------------------------------|---------------------|---------------------|--------------------------------|
|           |                              | Gutmann<br>modified | Marcali<br>modified |                                |
| A         | 8                            | 2.9                 | 2.9                 | 2.9                            |
| B         | 16                           | 8.9                 | 8.7                 | 8.8                            |
| C         | 32                           | 13.4                | 15.2                | 14.3                           |
| D         | 64                           | 31.7                | 34.9                | 33.3                           |
| E         | 128                          | 46.3                | 53.5                | 49.9                           |

gate E (50 mol substituted out of 128 mol added) show a saturation of ligand sites.

### Effect of varying ligand substitution

The standard conjugate A (2.9 mol MDI/mol HSA) was compared with four conjugates with higher degrees of ligand substitution (up to 50 mol MDI/mol HSA), performing RAST on six patients with a diagnosis of MDI asthma, and

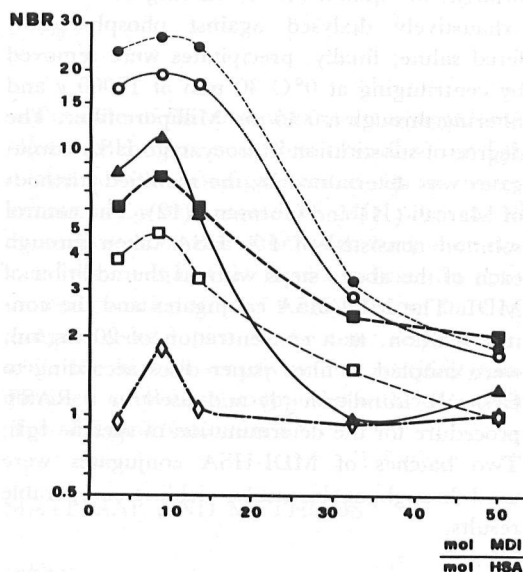


Fig. 2. RAST assay performed on six patients with MDI-asthma, employing MDI-HSA conjugates (A, B, C, D, E) at various degrees of ligand substitution.

NBR = net bound radioactivity %

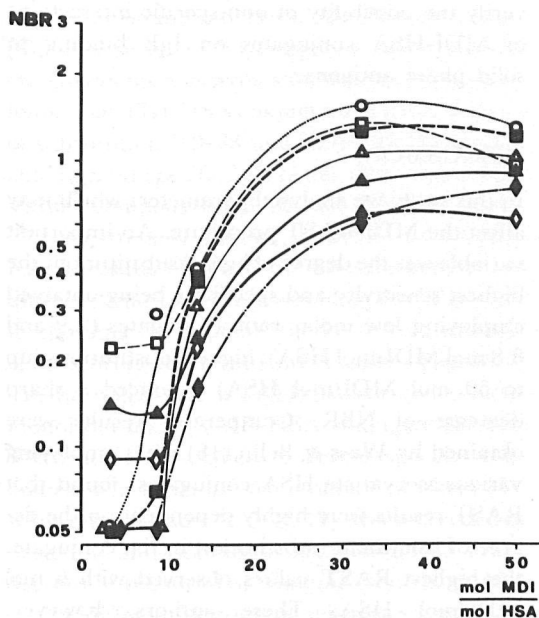


Fig. 3. RAST assay performed on seven non-exposed subjects with normal IgE levels (< 200 U/ml). Same conditions as in Fig. 2.

with detectable levels of specific IgE when conjugate A was employed. The results, summarized in Fig. 2, show that the highest NBR levels were reached with conjugate B, and the lowest with D and E, while A and C gave similar intermediate values. Parallel experiments were performed on non-exposed subjects, either with normal (Fig. 3) or elevated (Fig. 4) total serum IgE; unlike exposed patients, in both control groups, the lowest NBR were found with conjugate A and the highest with D and E compounds. A dependence of NBR values on IgE levels was observed for all the conjugates tested, particularly those with higher ligand substitution; in conjugate A, however, NBR levels were below the cutoff level of 0.9 up to 6200 U/ml of total serum IgE.

#### RAST inhibition

RAST inhibition experiments were performed in three exposed asthmatic patients and in four non-exposed subjects, two with normal and

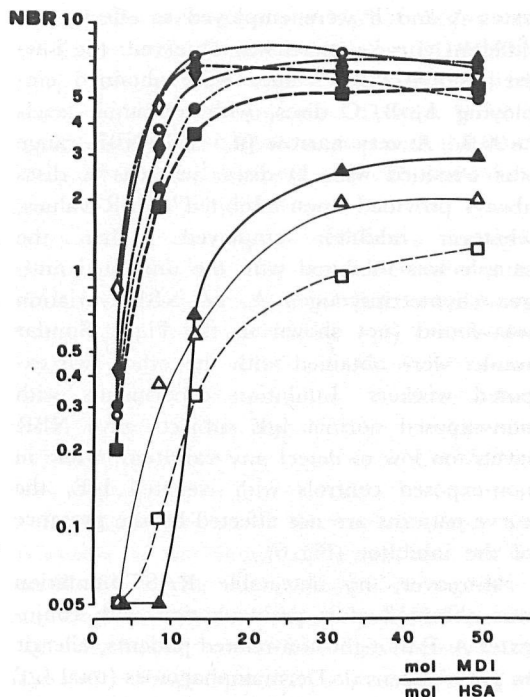


Fig. 4. RAST assay performed on eight non-exposed subjects with elevated IgE:

□-□ : 397 U/ml; △-△ : 729 U/ml; ▲-▲ : 1000 U/ml; ○-○ : 1300 U/ml; ■-■ : 1800 U/ml; ●-● : 3500 U/ml; ◆-◆ : 6200 U/ml; ◇-◇ : 15000 U/ml.

Same conditions as in Fig. 2.

two with elevated IgE. Test sera were inhibited with MDI-HSA conjugates (A-E), dioxane-treated HSA or the unrelated protein chymotrypsinogen A. After preincubation with the inhibitor, samples were tested with conjugate (A to E)- or HSA-coated discs with the usual procedure. Fig. 5 illustrates the results obtained with one of the exposed subjects, expressed as residual NBR. The non-inhibited curve and the one obtained with the carrier as inhibitor gave the same pattern: the carrier-mediated inhibition was less than 8% and was detectable with A, B, C, D discs. An analogous pattern was observed also when conjugates D and E were employed as effectors: both compounds gave similar NBR values, with an inhibition level of about 30%, in the test performed with conjugates A, B and C. In contrast, when low degree substitution conju-

gates A and B were employed as effectors, a different curve pattern was observed: the lowest residual NBR values were obtained employing A, B, C discs, with inhibition levels > 90%. A very narrow (0.5–1.3) NBR range was obtained with D discs, whereas E discs always provided “non-inhibited” NBR values, whatever inhibitor employed. When the sample was inhibited with the unrelated antigen chymotripsynogen A, no NBR variation was found (not shown in the Fig.). Similar results were obtained with the other two exposed workers. Inhibition experiments with non-exposed normal IgE subjects gave NBR levels too low to detect any variation, while in non-exposed controls with elevated IgE, the curve patterns are not affected by the presence of the inhibitor (Fig. 6).

Moreover, no detectable RAST-inhibition was observed after preincubation with conjugates A–E in eight non-related patients, allergic to grass pollens or Dermatophagoides (total IgE ranging from 21 to 3300 U/ml and radioactivity bound in allergen-specific RAST test ranging between 3.3% and 38%) examined in order to

verify the possibility of non-specific interactions of MDI-HSA conjugates on IgE binding to solid phase antigens.

## DISCUSSION

In this study we analysed parameters which may affect the MDI-RAST procedure. An important variable was the degree of ligand substitution, the highest sensitivity and specificity being obtained employing low molar ratio conjugates (2.9 and 8.8 mol MDI/mol HSA); higher substitutions (up to 50 mol MDI/mol HSA) produced a sharp decrease of NBR. Comparable results were obtained by Wass & Belin (18) who, employing various isocyanate-HSA conjugates, found that RAST results were highly dependent on the degree of isocyanate substitution in the conjugate, the highest RAST values observed with 6 mol MDI/mol HSA. These authors, however, obtained a final haptenization level of 8 mol MDI/mol HSA, whereas in our study a linear substitution relationship was present up to 33 mol

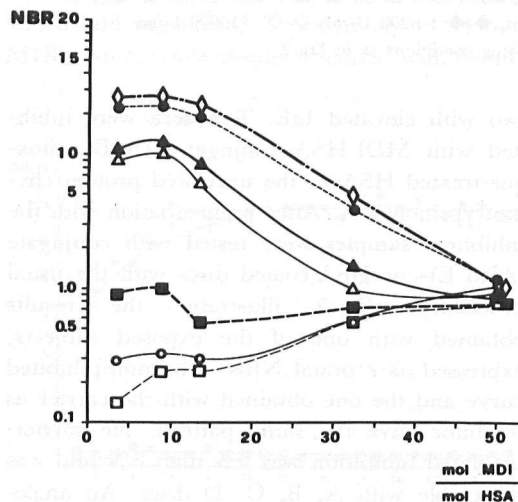


Fig. 5. RAST inhibition experiments in a MDI-positive patient (total IgE: 13 U/ml). Test performed with conjugates at various degrees of ligand substitution (A, B, C, D, E). NBR = net bound radioactivity %.  $\diamond$ - $\diamond$  = non inhibited;  $\bullet$ - $\bullet$  = inhibited with dioxane-treated carrier (HSA);  $\circ$ - $\circ$ ,  $\square$ - $\square$ ,  $\blacksquare$ - $\blacksquare$ ,  $\triangle$ - $\triangle$  = inhibited with conjugates A, B, C, D and E, respectively.

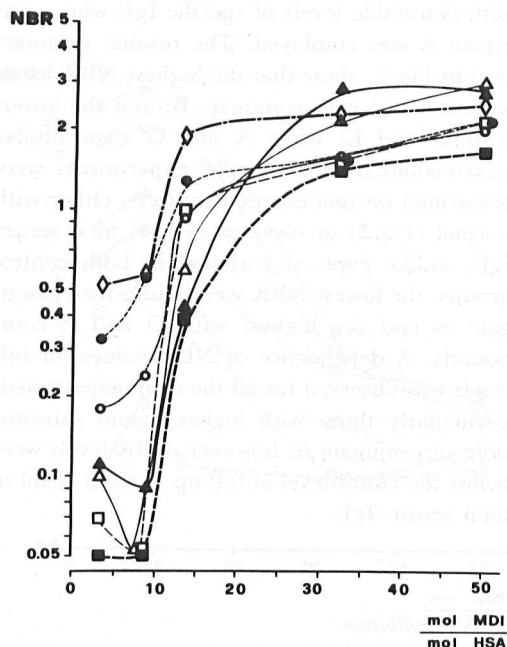


Fig. 6. RAST inhibition experiments in a non-exposed subject with elevated IgE (1500 U/ml). Same conditions and symbols as in Fig. 5.

isocyanate bound/mol HSA. Also Dewair & Baur (6), in a series of TDI-exposed subjects, even employing more heavily substituted compounds, found that TDI-HSA conjugates at lower degrees of substitution (18–28 mol TDI/mol HSA) were able to bind specific IgE better than highly substituted compounds (43–50 mol TDI/mol HSA).

It is extremely difficult, however, to compare RAST results obtained by different authors, as different criteria either in sampling the subjects or in choosing the haptens (mono- or bifunctional) or in scoring the results are usually adopted. It can be noted that a highly substituted p-tolyl-monoisocyanate (TMI)-HSA conjugate (57 mol TMI/mol HSA) failed to reveal specific antibodies in a series of symptomatic workers (5), while other authors (2, 7, 9, 14) who succeeded in detecting anti-isocyanate IgE, employed lower degrees of substitution, ranging from 10 to 40.

The different degrees of substitution may account for these discrepancies in IgE binding: either antibodies directed to the carrier (1, 7) may fail to recognize the protein denaturated by a heavy substitution, or the isocyanate residues may be sterically hidden to the hapten-directed antibodies (4), because of the chemical structure variation.

Moreover, the correlation observed between total IgE levels and NBR depended on the degree of ligand substitution. At the lowest substitution employed (2.9 mol MDI/mol HSA), NBR values did not exceed the cutoff limit up to 6200 U/ml of total IgE, while using conjugates with moderate substitutions (8.8 and 14.3), IgE levels > 200 U/ml did affect the result. At the highest substitutions examined (33.3 and 49.9) the aspecific effect is prevalent, even at normal IgE concentrations. The marked dependence of RAST values on total serum IgE observed by other authors (5, 10), in RAST systems employing TMI-HSA conjugates, might be due to the high substitution level; in fact, Karol and co-workers (8), in a previous study, where lower (10 mol TMI/mol HSA) ligand substitution conjugates were employed, did not find a correlation between high titers of specific antibodies and high serum IgE levels.

RAST inhibition results confirm sensitivity and specificity dependence on the degree of

ligand substitution, as the highest degrees of inhibition were obtained with low MDI/HSA ratio conjugates, while experiments where highly substituted conjugates D and E were used as inhibitors, yielded curves with the same "aspecific" pattern as observed with non-inhibited control sera.

As regards the length of incubation, the equilibrium in the first step is reached after 9 h, while a 3-h incubation yielded 76% of the maximum NBR. This observation may account for negative RAST results obtained by some authors who employed a 3-h first step incubation (2, 19); for the second step, a longer incubation time (18 h), as is usual for RAST (16), was necessary for optimal results.

In conclusion, our observations stress the relevance of the degree of ligand substitution on RAST results when MDI-HSA conjugates are employed for the detection of MDI-specific IgE antibodies, the highest sensitivity and specificity being achieved at a low degree of substitution.

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