

Nitrocellulose-Based RAST to Detect IgE Antibodies in Workers Hypersensitive to Diphenylmethane-4,4'-Diisocyanate

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RAST was performed using both nitrocellulose (NC) and paper discs to compare these solid supports for ability to detect IgE antibodies in sera from two patients with hypersensitivity to the industrial chemical, diphenylmethane-4,4'-diisocyanate (MDI). NC discs had distinct advantages in ease of coupling the hapten-conjugate antigen to discs, and in use of lesser amounts of antigen. With regard to sensitivity, NC discs were better able to distinguish the positive sera from 34 control sera including those with elevated levels of total IgE. In both RAST systems, inhibition studies indicated high specificity of antibodies for MDI, with no reactivity detected toward the serum albumin portion of the MDI-HSA conjugate antigen. However, only in the NC RAST did antibodies from both patients react with p-tolyl isocyanate inhibitor. Based on the above results, NC discs displayed several advantages in RAST and are recommended for routine serological assay of isocyanate-specific antibodies.

Key words: IgE radioimmunoassays; isocyanate sensitivity; nitrocellulose binding; RAST.

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The ability to detect allergen-specific IgE antibody has resulted in great progress in the diagnosis and treatment of many allergic diseases. The most widely used assay for detection of IgE antibody is RAST, a "sandwich" technique in which the allergen is covalently bound to a paper support (13). Although this system is sensitive to picogram amounts of antibody and is effective when used with a wide range of natural and synthetic antigens, it possesses certain drawbacks as follows: 1) Cyanogen bromide is used for covalent binding of antigens. This chemical is highly reactive, chemically unstable and toxic. 2) The procedure requires antigen solutions in concentrations of 0.5 % or greater. With chemical allergens, preparation of sufficient quantities of hapten-protein conjugates with uniform composition is frequently difficult. 3) Certain antigens do not react readily with cyanogen brom-

ide-activated sites on cellulose discs. For these reasons, alternative solid support systems were sought for RAST determination of isocyanate-specific IgE antibodies.

Nitrocellulose (NC) has been successfully employed in electrophoretic transfer of antigens from gels to solid supports. This material provides the basis of the Western blotting procedure, immunoblotting and dot immunoblotting (11). In addition, NC supports have been employed as an alternative to the PRIST assay for detecting total IgE (4) and to detect antigen-antibody complexes through immunogold staining (5).

The current study examined the use of NC as an alternative to paper discs in RAST for detection of antibodies to a chemical allergen. Since sensitivity and hapten specificity are of utmost importance in such immunoassays, these features were compared in the two RAST

procedures using sera obtained from two individuals with respiratory hypersensitivity to MDI.

Clinical aspects

Patient A was most recently employed as a laboratory technician. He had a history of exposure to aromatic isocyanates. His recent symptomatology was related to an MDI foaming operation. Initially, his symptomatology was most prevalent in the evening and included uncontrollable coughing, and difficulty in breathing. He later experienced severe asthmatic episodes at work when cutting polyurethane foam panels. Following his most severe attack, his total IgE level was 3663 IU/ml.

Patient B was a grain elevator operator/repairman who developed asthmatic reactions 90 min after cutting a polyurethane plate containing MDI. The clinical history of this patient has been described elsewhere (2). The patient had a total IgE level of 1760 IU/ml, and was strongly RAST positive for MDI using cellulose-based RAST assay.

Control sera were from 42 individuals working in an isocyanate production facility, none of whom gave evidence of isocyanate sensitivity. Two of the control patients had total IgE values greater than 1000 IU/ml.

MATERIAL AND METHODS

Antigens

Isocyanate-conjugate antigens were prepared by the dropwise addition of isocyanates to rapidly

stirred solutions of human serum albumin (HSA) in 0.05 M borate buffer at pH 9.4 (7). The isocyanates were diphenylmethane-4,4'-diisocyanate (MDI, Mondur MR, Mobay Chemical Corporation), toluene diisocyanate (TDI, Mondur TD₈₀, Mobay Chemical Corporation) and p-tolyl isocyanate (p-TMI, Eastman Chemicals). Both the amount of isocyanate added, and the length of reaction time were varied to achieve binding of 20–30 moles hapten per mole HSA as determined by spectrophotometric analysis (6).

Preparation of discs

Cellulose. Discs of 5 mm diameter were cut from Whatman #44 cellulose paper. Activation of discs using cyanogen bromide, reaction with antigen, and antibody assay were as described previously (2).

Nitrocellulose. Five mm diameter discs were cut from nitrocellulose paper (Trans-Blot nitrocellulose, 0.45 μ m pore size, Bio-Rad Laboratories). For coupling, antigen solutions (0.001–0.1 %) in 0.01 M Tris-buffered saline (TBS), pH 7.6, were incubated with discs in a water bath at ambient temperature for 2 h with gentle shaking. 2 ml of antigen solution were used to coat 25 discs. After incubation, the antigen solution was decanted and the remaining protein-binding sites on discs were blocked using the following procedure: An equal volume of 3 % bovine serum albumin (BSA, Sigma) in TBS, was added and discs were incubated for 1 h at room temperature with gentle agitation. After decanting, an equal volume of a 1 % BSA, 1 % rabbit serum in TBS, was added followed by incubation for an additional 1 h

Table 1

Comparison of methodology for RAST using nitrocellulose vs. paper discs

Procedure	Solid support	
	Nitrocellulose	Cellulose
Chemical activation of disc	None	Cyanogen bromide
Optimal antigen concentration	0.001–0.1 %	1 %
Binding of antigen to disc	2 h, 37°C	6 h, ambient temp.
Reaction of antigen with serum	2 h, 37°C	16 h, ambient temp.
Reaction with second antibody	16 h, ambient temp.	16 h, ambient temp.
Storage of discs	Dry at ambient temp.	In buffer at 10°C

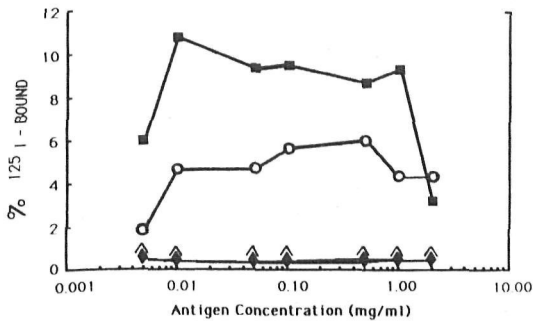


Fig. 1. Antigen concentrations effective in nitrocellulose RAST assay. Solutions of increasing MDI-HSA concentration were used to coat nitrocellulose discs. Discs were then reacted with 50 μ l of a 1:1 dilution of serum with 0.1 % BSA in TBS. Final incubation was with ^{125} I-anti-human IgE reagent.

Closed squares, serum from patient A; open circles, serum from patient B; closed and open diamonds, control patients (2) with elevated total IgE levels.

with agitation. Discs were air dried and stored at ambient temperature. When prepared in this manner, discs were stable for several months. Comparison of the paper and NC disc methodologies is presented in Table 1.

Nitrocellulose RAST

Nitrocellulose discs were rehydrated with TBS containing 0.1 % BSA. Single discs were placed in 10 $\times$ 75 mm test tubes. 50 μ l of serum dilution were added to each tube, then tubes were placed in a 37°C water bath for 2 h. Serum was removed and discs were washed five times with a solution of 1 % BSA in TBS. Bound IgE antibody was detected by addition of 50 μ l of ^{125} I-goat anti-human IgE (Vast, Ventrex Laboratories) followed by incubation of discs at ambient temperature for 16 h. Discs were washed five times then transferred to polypropylene tubes for determination of bound ^{125} I-reagent using a Packard Gamma Counter. Results were expressed as the percent of ^{125} I-reagent bound to the discs. Replicate determinations varied by less than 20 %.

Paper disc RAST

RAST using antigens covalently attached to paper discs was performed as described pre-

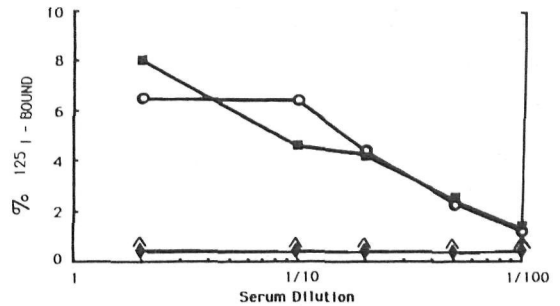


Fig. 2. Effect of serum dilution on nitrocellulose RAST titers. Sera were diluted with TBS-0.1 % BSA, then assayed for IgE antibody using nitrocellulose discs coated with 0.005 % MDI-HSA. Closed squares, serum from patient A; open circles, serum from patient B; closed and open diamonds, serum from two control patients as described in Fig. 1.

viously (2) and outlined in Table 1. The ^{125} I-reagent was identical to that used in NC RAST.

RAST inhibition assays

Specificity of both immunoassays was evaluated by inhibition studies using several isocyanate-HSA antigens as test inhibitors. Sera (25 μ l) were incubated with increasing amounts of MDI-HSA, TDI-HSA, or pTMI-HSA dissolved in 25 μ l assay buffer. Control tubes received serum and buffer. After incubation at 37°C for 2 h, an antigen-coated disc was added to each tube and the assays performed as described above.

The percent of inhibition was calculated as follows:

$$\% \text{ inhibition} = \left(1 - \frac{Ci - Cb}{Cs - Cb} \right) \times 100$$

Ci, cpm bound by serum in the presence of inhibitor; Cs, cpm bound by serum in the absence of inhibitor; Cb, non-specifically bound radioactivity.

Statistics

Least squares regression analysis was used to evaluate all RAST inhibition studies (3).

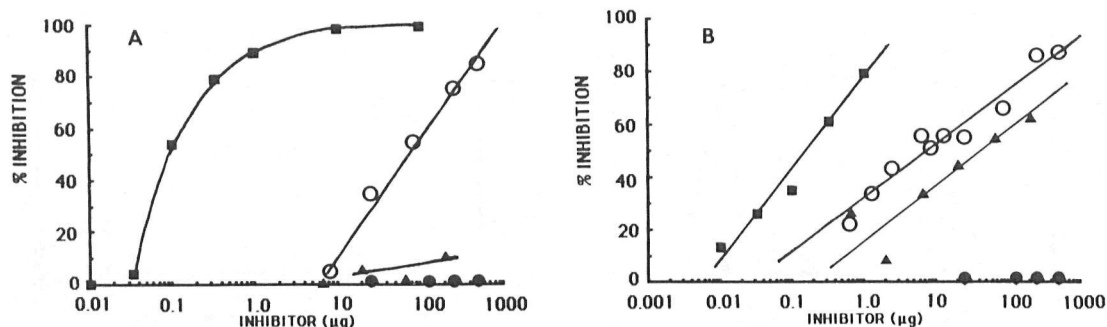


Fig. 3. Inhibition of nitrocellulose MDI-HSA RAST. Increasing amounts of antigen were added to 25 μ l serum dilution. After incubation at 37°C for 2 h NC discs, coated with 0.005% MDI-HSA, were added and the assay performed as described in the text. A) Patient A, serum assayed at 1:10 dilution; B) Patient B, serum assayed at 1:5 dilution. Inhibitors: MDI-HSA (squares), pTMI-HSA (open circles), TDI-HSA (triangles), HSA (closed circles).

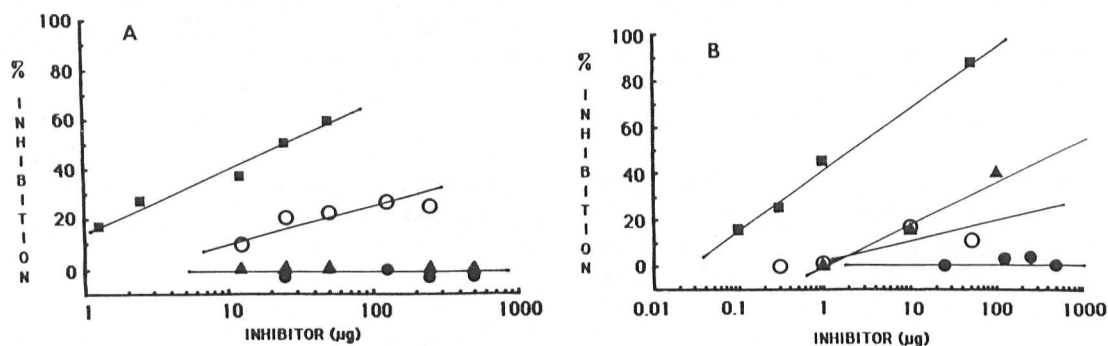


Fig. 4. Inhibition of cellulose MDI-HSA RAST. Assay was performed as described for Fig. 3 except for the use of cellulose discs to which MDI-HSA antigen was covalently bound. Symbols and sera as described in Fig. 3.

RESULTS

Antigen concentration

The effect of antigen concentration on the ability of NC discs to detect MDI-specific IgE antibody is presented in Fig. 1. Both experimental sera displayed maximum titers when discs were prepared using 0.01–1.00 mg/ml antigen solutions (0.001–0.1% w/v). The control sera gave essentially no binding to discs prepared using 0.005–2.0 mg/ml antigen solutions. By comparison, in cellulose RAST assays, maximum titers were obtained when antigen concentrations of 10–20 mg/ml were used for binding. The minimum effective concentration in the cellulose RAST assay was 5 mg/ml.

Serum dilutions

The percent of ^{125} I bound as a function of serum dilution is shown in Fig. 2. In both patients,

titers decreased with increased serum dilution. When diluted 1:100, sera from both patients still had titers greater than two times those of the control sera.

Specificity of binding

Inhibition assays were performed to assess the specificity of antibody binding in both NC and paper disc RAST assays. Results are shown in Figs. 3 and 4. The lines were generated using linear regression (3) except for the data relating inhibition by MDI-HSA in Fig. 3, which was best described by a polynomial function. Statistical analyses of the regression lines are given in Table 2. Using the regression equations, calculation was made of the amount of antigen-conjugate inhibitor required to effect 50% inhibition of each reaction. These data are given in Table 3.

In the NC system, MDI-HSA was the most

Table 2
Statistical analysis of RAST inhibition regression lines

Patient	Inhibitor	Nitrocellulose discs			Cellulose discs		
		Slope	Coefficient (R)	P	Slope	Coefficient (R)	P
A	MDI-HSA ¹	54.62	0.975	< 0.005	24.62	0.977	< 0.005
A	TDI-HSA	0.05	0.159	> 0.05	9.04	0.824	< 0.05
A	pTMI-HSA	44.03	0.995	< 0.001		no effect	
A	HSA		no effect			no effect	
B	MDI-HSA	33.40	0.986	< 0.005	28.21	0.998	< 0.005
B	TDI-HSA	25.84	0.972	< 0.01	4.78	0.798	< 0.05
B	pTMI-HSA	19.01	0.932	< 0.0001	19.00	0.984	< 0.05
B	HSA		no effect			no effect	

¹ Data best fit by a polynomial regression. All other lines generated using linear regression analysis (3).

potent inhibitor of reaction using serum from either patient A (Fig. 3A) or patient B (Fig. 3B). pTMI-HSA afforded some inhibition; TDI-HSA was an inhibitor only with serum from patient B. HSA did not show any inhibitory ability with either serum. In the cellulose-based RAST, MDI-HSA was also the most effective inhibitor. In this assay, too, some inhibition was noted with TDI-HSA using serum from patient B.

A difference between assays was observed with regard to the effect of pTMI-HSA as an inhibitor (see Table 3). This antigen inhibited binding by both sera only in the nitrocellulose RAST. It was completely ineffective in inhibiting antibody binding in the cellulose-based RAST.

A difference between the two patients was

noted with regard to specificity of antibody. Serum from patient B showed some reactivity (albeit limited) with TDI-HSA; that from patient A did not. This effect was seen in both the NC and cellulose RAST inhibition studies.

DISCUSSION

RAST assay for IgE antibody has become a standard tool to assist in clinical diagnosis of allergic sensitization. The purpose of the current study was to evaluate the use of NC discs in performing RAST assay, by comparing results with those obtained using cellulose discs.

Nitrocellulose discs offered several advantages over paper discs. NC assay appeared to be more sensitive. In comparing reaction of sensitized vs. control sera, 20–30-fold differences were noted in NC assay (6 % or greater binding with sensitized sera compared with 0.2 % binding by control sera, see Fig. 1). In the cellulose RAST, binding of 30 % by sensitized sera must be compared with binding of 5 % by control sera (2).

Another advantage of the NC system was its need for much smaller quantities of antigen. With NC discs, 0.005 % antigen solutions were optimum for disc coating, whereas with cellulose discs 1.0 % antigen solutions were needed. This difference is substantial when considering the difficulties routinely encountered in preparing and purifying effective isocyanate-protein conjugates (6). In addition, much less antigen was needed in the NC assay to perform

Table 3

Comparison of specificity of nitrocellulose and cellulose RAST assays

Inhibitor	µg Required for 50 % inhibition ¹			
	Patient A		Patient B	
	Nitro-cellulose	Cellulose	Nitro-cellulose	Cellulose
MDI-HSA	0.091	26	0.17	2.0
TDI-HSA	> 1000	> 1000	49	383 ²
pTMI-HSA	69	> 1000	7.7	> 1000
HSA	> 1000	> 1000	> 1000	> 1000

¹ The amount of inhibitor required to effect 50 % inhibition was determined from dose-response curves (see Figs. 3, 4).

² Data from reference 2.

inhibition studies. Such studies are essential when investigating chemical hypersensitivity where it is important to distinguish antibody specificity for hapten from carrier specificity, and to determine cross-reactivity of antibody for related chemical haptens.

A third advantage of the NC assay was the absence of need to activate discs prior to coating with antigen. Cyanogen bromide is used for activation in the conventional cellulose RAST. This is a highly irritating chemical which must be carefully handled and stored. Additionally, with cyanogen bromide activation of discs, the antigen must be dissolvable in aqueous 0.1 M sodium carbonate buffer (1, 9). By comparison, the use of nitrocellulose discs has the advantage that a wider range of solvents may be used to dissolve the antigen. Thus, it may be possible to make discs with antigens which dissolve poorly in aqueous solution (12). Moreover, with NC, it has been possible to apply several antigens to different sites on the discs (12), a modification not possible with cyanogen bromide-activated discs.

The specificity of RAST assays was compared using inhibition studies. Both assays were highly specific for MDI-HSA and required more than 150 times as much TDI-HSA to effect comparable inhibition (see data of patient B, Table 3). However, a difference was noted between RAST assays with regard to antibody specificity. pTMI-HSA was an effective inhibitor of NC RAST but not cellulose RAST (Table 3). This difference was observed with sera from both patients. The interactions between protein antigens and cyanogen bromide-activated cellulose involve covalent bonds. The interactions between proteins and nitrocellulose are of an absorptive nature. The observed specificity of antibodies may then have been influenced by the nature of the attachment of antigen to the solid support.

In comparing the two patients, it was apparent that antibodies from patient B showed some recognition, although limited, of TDI-HSA, whereas those from patient A did not. Whether this difference reflects the time or extent of prior exposure to TDI, or the route of exposure, is uncertain. However, both sera showed overwhelming specificity for MDI-HSA.

This study evaluated an alternative method for performing RAST, i.e., use of nitrocellulose rather than cellulose discs. The NC-based method exhibited both sensitivity and specificity, as well as distinct procedural advantages. No disadvantages were noted. Based on these results, use of nitrocellulose discs in RAST is recommended for evaluation of isocyanate-specific antibodies.

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