The guinea pig model of diisocyanate sensitization

I. Immunologic studies

S. Edith Chen, Ph.D., and I. Leonard Bernstein, M.D. *Cincinnati, Ohio*

Two strains of guinea pigs were parenterally immunized with well-characterized diisocyanate-protein conjugates. Hapten-specific IgE antibodies were detected in the sera of English short-hair strain guinea pigs immunized with either toluene diisocyanate–human serum albumin (TDI-HSA) or hexamethylene diisocyanate–HSA (HDI-HSA) when these sera were analyzed by the 168 hr passive cutaneous anaphylaxis (PCA) technique followed by intravenous challenges with conjugates of respective ligands coupled to an unrelated carrier protein, transferrin. IgG_1 antibodies and precipitating antibodies were demonstrated in Hartley strain guinea pigs immunized with TDI/HDI-HSA conjugates. The hapten specificity of these antibodies was proved by PCA inhibition experiments and antibody absorption experiments. In the precipitating antibody system, this was further confirmed by immunoelectrophoretic analysis. Cross-reactivity between HDI and TDI was not observed in the PCA experiments. However, apparent cross-reactivity in the double gel diffusion experiments was due to new antigenic determinants formed by isocyanates after conjugation with proteins. It was therefore apparent that immune responses of guinea pigs immunized with protein conjugates of bifunctional isocyanates were heterogeneous and involved multiple specificities for hapten, carrier protein, and new antigenic determinants. It was postulated that the complex nature of the immune response generated by diisocyanate compounds in the guinea pig may also serve as a more appropriate model of isocyanate-induced human sensitivity reactions, which are known to involve diverse immunologic and nonimmunologic mechanisms. (J ALLERGY CLIN IMMUNOL 70: 383, 1982.)

TDI and HDI are volatile diisocyanates widely used in the polyurethane foam industry.[1, 2] When accidental spills occur, these chemicals act as mucous membrane and respiratory irritants.[3] They have also been reported to induce hypersensitivity pneumonitis and allergic dermatitis.[4–6]

In 1973, the National Institute of Occupational Safety and Health revealed that 5% of 40,000 workers exposed to allowable threshold concentrations of volatile isocyanates developed chronic respiratory symptoms and that 10% of these affected workers experienced an asthmatic syndrome. The long latent period after initial exposure, the brisk recurrence after occupational or experimental provocation to minimal doses of diisocyanates, and the occasional association of eosinophilia had suggested a sensitization process in this subgroup of symptomatic workers. Possible immunologic mechanisms of diisocyanate-induced asthma have been investigated in exposed workers

Abbreviations used

TDI:	Toluene diisocyanate
HDI:	Hexamethylene diisocyanate
TR:	Human transferrin
GPA:	Guinea pig serum albumin
CFA:	Complete Freund's adjuvant
HSA:	Human serum albumin
HDA:	Hexamethylenediamine
PCA:	Passive cutaneous anaphylaxis
NAD:	New antigenic determinant

From the Department of Medicine, Division of Immunology, University of Cincinnati Medical Center, Cincinnati, Ohio.

This work was supported in part by grant OH 733-01 from the National Institute of Occupational Safety and Health.
Received for publication March 5, 1982.
Accepted for publication June 30, 1982.

Reprint requests to: I. Leonard Bernstein, M.D., University of Cincinnati Medical Center, 231 Bethesda Ave., Cincinnati, OH 45267.

0091-6749/82/110383+10$01.00/0

TABLE I. Quantitative analysis of isocyanate content in TDI/HDI-protein adducts

Conjugates	Isocyanate concentration (μmole isocyanate/μmole protein)
TDI-HSA	17.11
TDI-GPA*	74.01
TDI-TR	32.58
HDI-HSA	15.8
HDI-GPA*	23.89
HDI-TR	13.22

*Calculated on the basis of 67,000 as the molecular weight of GPA.

and laboratory animals.[7-12] A variety of immune responses were observed under several experimental conditions, but in some instances these data were inconsistent and not always reproducible. Although such controversial results could be attributed to the variability of hapten-protein conjugates prepared by different investigators, the possible role of immunopathologic sensitivity as the cause of TDI asthma appeared to be inconclusive. Other investigators proposed that obstructive airways disorders after exposure to TDI could be due to possible reflex, pharmacologic, or beta-adrenergic blockade effects of TDI.[13-14]

To clarify some of the controversial aspects of immunoreactivity ascribed to diisocyanates, the objective of this study was to examine the immunologic sequelae in several guinea pig strains after parenteral immunization with well-characterized diisocyanate-protein conjugates. Although the amount of autopolymerization and protein cross-linking in such bifunctional protein complexes could be variable, this laboratory had previously developed a standard preparatory technique that yielded protein conjugates with stable ratios (3.5:1) of bis- to monoureido derivatives.[15] This improved technology made it possible to develop an experimental animal model using the same chemicals to which workers are exposed in the workplace.

MATERIALS AND METHODS

Animals

Hartley strain guinea pigs weighing 300 to 350 gm were purchased from Perfection Breeders, Douglasville, Pa. Female English short-hair strain guinea pigs weighing 300 to 350 gm were purchased from Camm Research Institute, Wayne, N.J. All animals were acclimated to animal facilities for at least 1 wk before use.

Chemicals

TDI (Mondur TD-80, 80% 2,4-TDI; 20% 2,6-TDI) and HDI (Mondur HX, 100%) were obtained from the Mobay Chemical Co., Pittsburgh, Pa. HSA was purchased from Cutter Laboratories, Berkley, Calif. TR and GPA were obtained from the Sigma Chemical Co., St. Louis, Mo. CFA was obtained from Difco Laboratories, Inc., Detroit, Mich., and agarose from Marine Coloids, Inc., Rockland, Me.

Antigens

Diisocyanate antigens were prepared by coupling TDI/HDI to HSA/GPA or TR as previously reported.[12, 15] In brief, TDI/HDI (1 ml) was added dropwise to chilled phosphate-buffered saline solutions (pH 7.4) of HSA (5 mg/ml), GPA (2 mg/ml), or TR (2.5 mg/ml) by rapid stirring at room temperature for 20 min. Each reaction was interrupted by the addition of an equal volume of 2M ammonium carbonate and centrifuged at 250 × g for 15 min to remove unreacted or polymerized diisocyanates. The supernatant was dialyzed extensively against 0.1M ammonium carbonate (3×) and normal saline (3×). Aliquots of the conjugates were then stored at −20° C until used. Carrier proteins used as controls in all experiments were subjected to the same conditions of conjugation except that TDI/HDI was not added. Each conjugate was assayed for total protein content by a modified Biuret method.[16] Quantitative estimation of mono- or bisureido TDI derivatives, as well as total TDI bound to protein, were obtained by a modified Gutmann assay.[15] The total amount of HDA in the HDI-protein conjugates was determined by a gas chromatographic method after acid hydrolysis.[17] Aliquots (1 ml) of HDI-protein conjugates were hydrolyzed for 144 hr with 1 ml of 6N HCL at 110° C in vacuum. The hydrolyzed samples were dried in a Speed-Vac concentrator, redissolved in 0.5 ml of 0.1N aqueous NaOH and 5 μl of this solution were injected into a gas chromatographic system. Specific conditions of the gas chromatographic analysis were: column temperature, 170° C; injector temperature, 300° C; detector temperature, 320° C; carrier gas, He at column pressure of 12 psi; stainless steel column, 0.32 cm in diameter and 1 m in length, containing 25% Apiezon M plus 10% KOH coated on Chromosorb W-A.W. (80/100 mesh). Duplicate samples were analyzed and their peak areas were measured. The concentrations of HDA produced from hydrolysis of various HDI-protein conjugates were determined by comparison with a standard calibration curve of HDA. The ligand substitution of various TDI/HDI-protein conjugates are presented in Table I.

Immunization of guinea pigs

Groups of six English short-hair strain guinea pigs were immunized intraperitoneally for IgE antibody production with 10 μg TDI/HDI-HSA conjugates mixed with 1 mg of aluminum hydroxide (alum) every 30 days for a total of eight doses. Control guinea pigs received mixtures of normal saline and alum. Blood samples were drawn from all animals 7 days after their last booster injections. Serum from each guinea pig was stored individually in small aliquots at −20° C until used.

Groups of six female Hartley strain guinea pigs were immunized for IgG antibody production. Conjugates of TDI/HDI-HSA (0.5 mg) were emulsified with equal vol-

TABLE II. PCA reactions* of English short-hair strain guinea pigs immunized with protein conjugates of bifunctional isocyanates or saline

Homocytotropic antibodies vs:	Animal No.	I.V. Challenge antigens: TDI-TR Rec 1	TDI-TR Rec 2	TDI-TR G.M.†	TR Rec 1	TR Rec 2	TR G.M.
TDI-HSA	1	160	640	320	Neg‡	Neg	Neg
	2	1280	2560	1810	Neg	Neg	Neg
	3	160	160	160	Neg	Neg	Neg
	4	40	80	57	Neg	Neg	Neg
	5	5	10	7	Neg	Neg	Neg
	6	80	640	226	Neg	Neg	Neg
		HDI-TR			**TR**		
		Rec 1	**Rec 2**	**G.M.**	**Rec 1**	**Rec 2**	**G.M.**
HDI-HSA	1	640	1280	905	20	40	28
	2	640	320	453	Neg	Neg	Neg
	3	1280	640	905	Neg	Neg	Neg
	4	80	40	57	Neg	Neg	Neg
	5	640	1280	905	Neg	Neg	Neg
	6	320	640	453	Neg	Neg	Neg
		TDI-HDI-TR			**TR**		
		Rec 1	**Rec 2**	**G.M.**	**Rec 1**	**Rec 2**	**G.M.**
Saline	1-6	Neg	Neg	Neg	Neg	Neg	Neg

Rec = recipient animal; I.V. = intravenous.
*Expressed as RPCA titers.
†Geometric mean of the two RPCA titers.
‡Negative reaction: <5.

umes of CFA before their injection subcutaneously into four different sites on the shaved backs of guinea pigs. Two weeks later the animals were reinjected intradermally with 0.1 mg conjugates without adjuvant. This procedure was repeated weekly for 4 additional weeks. Blood samples were drawn 1 wk after the last immunization, and the sera were pooled, stored at −20° C until used, and designated as primary sera. One month after the first blood sampling, the animals were again immunized in the same way as on day 1. This procedure was repeated every 30 days for a total of two doses. Blood samples were taken again 1 wk after the second immunization. These sera were pooled and stored at −20° C and designated as secondary sera. Control groups of guinea pigs were treated according to the same schedules as the experimental groups.

Detection of homocytotropic antibodies

PCA was performed by intradermal injections (0.1 ml) of secondary antisera and successive twofold dilutions in normal saline into the backs of freshly shaved Hartley strain guinea pigs. PCA dose-response assays of each serum sample were performed in at least two animals. As many as 12 sites per animal were used. For detection of IgG_1 antibodies, a passive sensitization period of 48 hr was used, while analysis of IgE antibody formation required a passive sensitization interval of 168 hr. The animals were then challenged intravenously through cephalic veins with a mixture of appropriate diisocyanates coupled to an unrelated carrier protein, TR, and Evans blue dye. In all experiments, 0.5 ml of various conjugates (0.5 mg) was mixed with 0.5 ml of 1% Evans blue in normal saline. The reaction was read 20 min after challenge, and a blue spot of 5 mm or more in diameter was recorded in a positive PCA test.

The hapten specificity of antibodies generated against TDI/HDI-HSA conjugates was confirmed by additional PCA inhibition experiments. Antisera (IgG_1) derived from Hartley strain guinea pigs were incubated with equal volumes of TR conjugates containing respective homologous diisocyanates (TDI or HDI), TR alone, HSA alone, or normal saline at 37° C for 4 hr. Reaction mixtures were then centrifuged at 450 × g for 20 min. The supernatant was carefully removed and PCA tests were performed as described above.

Hapten-specific cross-reactivity of the diisocyanate PCA responses was assessed by comparing the effects of intravenous challenge with conjugates containing homologous and heterologous ligands. The results were confirmed by inhibition experiments as described above, except that respective

TABLE III. PCA reactions* of Hartley strain guinea pigs immunized with protein conjugates of bifunctional isocyanates or saline

Pooled homocytotropic antibody vs:	I.V. Challenge antigens					
	TDI-TR			TR		
	Rec 1	Rec 2	G.M.†	Rec 1	Rec 2	G.M.
TDI-HSA	640	1280	905	Neg‡	Neg	Neg
	HDI-TR			TR		
	Rec 1	Rec 2	G.M.	Rec 1	Rec 2	G.M.
HDI-HSA	2560	1280	1810	Neg	Neg	Neg
	TDI/HDI-TR			TR		
	Rec 1	Rec 2	G.M.	Rec 1	Rec 2	G.M.
Saline	Neg	Neg	Neg	Neg	Neg	Neg

Rec = recipient animal; I.V. = intravenous.
*Expressed as RPCA titers.
†Geometric mean of the 2 RPCA titers.
‡Negative reaction: <5.

antisera were absorbed with TR conjugates bound to heterologous haptens.

Immunodiffusion

The double diffusion technique was used to analyze for precipitating antibodies.[18] In brief, a clean microscope slide (75 by 38 mm) was coated with 3 ml of 1% agarose in normal saline containing 0.1% sodium azide. Antigens and antisera were placed in designated wells (3 mm in diameter). Diffusion was performed for 48 hr at room temperature in a humidity chamber. The plates were washed, dried, and stained with Schwartz amido black. For precipitin inhibition studies, equal volumes of antisera and inhibitory reagents were mixed, incubated at 37° C for 2 hr, and centrifuged at 450 × g for 20 min. The supernatants were then placed into appropriate wells.

Immunoelectrophoresis

Immunoelectrophoresis was performed using 80 by 100 mm glass plates coated with 12 ml of 1% agarose in 0.05M borate buffer, pH 8.4. Electrophoresis was performed for 90 min with a current of 5 mAmp per slide.

RESULTS

IgE antibody responses to diisocyanate conjugates in English short-hair strain guinea pigs

IgE antibodies were detected in sera of English short-hair strain guinea pigs immunized with TDI-HSA or HDI-HSA when these sera were analyzed by the 168 hr PCA technique and intravenous challenges with conjugates of respective ligands coupled to an unrelated carrier protein. Results of individual animal PCA responses to intravenous challenges with TDI/HDI-TR and TR alone are summarized in Table II and expressed as the reciprocals of PCA titers (RPCA). Significant anti-hapten IgE antibody responses to TDI and HDI are apparent in both TDI-HSA (geometric mean ranging from 7 to 1810) and HDI-HSA (geometric mean ranging from 57 to 905) immunized animals. One of six HDI-HSA antisera gave a low geometric mean RPCA titer (28) after intravenous challenge with TR alone. A simple explanation for this cannot be offered, but inasmuch as this reaction to the unrelated carrier protein was an isolated finding and only comprised 3% of the geometric mean RPCA titer obtained by the HDI-TR conjugate in the same animal, it was not considered to be immunologically relevant. Not included in Table II were negative results after challenge with TR conjugates of heterologous haptens. PCA assays of sera from control groups of animals were also negative.

Homocytotropic IgG_1 antibody responses to diisocyanate conjugates in Hartley strain guinea pigs

As shown in Table III, hapten-specific IgG_1 antibodies were also detected in Hartley strain guinea pigs immunized with TDI/HDI-HSA conjugates. Pooled sera from TDI/HSA immunized Hartley strain guinea pigs demonstrated RPCA titers of 640 to 1280 when challenged with TDI-TR and negative responses after challenge with TR alone. Similarly, analysis of

TABLE IV. Inhibition of PCA reactions* induced by IgG_1 homocytotropic antibodies

IgG_1 homocytotropic antibody	Inhibitors	I.V. Challenge antigen					
		TDI-TR			HSA		
		Rec 1	Rec 2	G.M.†	Rec 1	Rec 2	G.M.
Anti-TDI-HSA	Saline	640	160	320	320	160	226
	TDI-TR	Neg‡	Neg	Neg	—	—	—
	HDI-TR	640	320	452	—	—	—
	TR	640	160	320	—	—	—
	HSA	640	320	452	Neg	Neg	Neg
		HDI-TR			HSA		
		Rec 1	Rec 2	G.M.	Rec 1	Rec 2	G.M.
Anti-HDI-HSA	Saline	1280	640	905	2560	2560	2560
	TDI-TR	40	320	113	—	—	—
	HDI-TR	Neg	Neg	Neg	—	—	—
	TR	1280	320	640	—	—	—
	HSA	1280	640	905	80	Neg	<20

Rec = recipient animal; I.V. = intravenous.
*Expressed as RPCA titers.
†Geometric mean of the two RPCA titers.
‡Negative reaction: <5.

sera from HDI-HSA–immunized animals revealed RPCA titers of 1280 to 2560 after intravenous challenge with HDI-TR and negative responses after challenge with the unrelated carrier protein. No PCA responses were obtained after challenge with heterologous ligands coupled to TR (Table V). PCA results in control groups of animals were negative.

PCA inhibition experiments

Hapten specificity of IgG_1 antisera was further confirmed by a series of PCA inhibition experiments shown in Table IV. Incubation of TDI-HSA antisera with TDI-TR at 37° C for 4 hr completely removed TDI-specific antibodies. However, incubation of the same antisera with the unrelated carrier protein, TR, did not remove antibody (RPCA titer of 320) as compared to the PCA response after challenge with supernatant derived from antisera incubated with normal saline (RPCA titer of 320). Inhibition experiments utilizing antisera derived from HDI-HSA–immunized animals demonstrated similar results. Thus preincubation of these antisera with HDI-TR removed HDI-specific antibodies while incubation with TR alone did not. The presence of hapten-specific antibodies in both TDI and HDI-HSA antisera systems was also confirmed by undiminished PCA responses even after the respective antisera were preincubated with homologous HSA carrier proteins. Also supported by the data shown in Table IV is the fact that incubation of TDI/ HDI-antisera with HSA carrier protein at 37° C for 4 hr completely removed HSA carrier-specific antibodies except for a small residual titer (RPCA of 80) obtained in one of the recipient animals treated with HSA-absorbed HDI-HSA antiserum. Nevertheless, these carrier protein–absorbed antisera still demonstrated TDI- and HDI-specific reactions with geometric mean RPCA titers of 452 and 905, respectively. There were no reactions after these sera were challenged with heterologous hapten conjugates. These results clearly demonstrate the validity of TDI/HDI-TR conjugates for detection of TID/HDI-specific antibodies present in antisera generated by immunization with TDI/ HDI-HSA conjugates. The finding that both carrier protein–absorbed TDI-antisera showed a slightly higher mean geometric RPCA titer (452) than antisera incubated with normal saline (320) may reflect either the biologic variability of the PCA technique or an increased recognition of hapten-specific antibodies by TDI-TR after removal of specific antibodies directed against determinants in the carrier protein.

Immunodiffusion experiments

Precipitating antibody responses were evaluated by the double gel diffusion technique. Pooled sera from TDI/HDI-HSA immunized Hartley strain guinea pigs were tested against various diisocyanate-conjugated antigens. Typical results are illustrated in Fig. 1. Antibodies generated in response to TDI-HSA developed

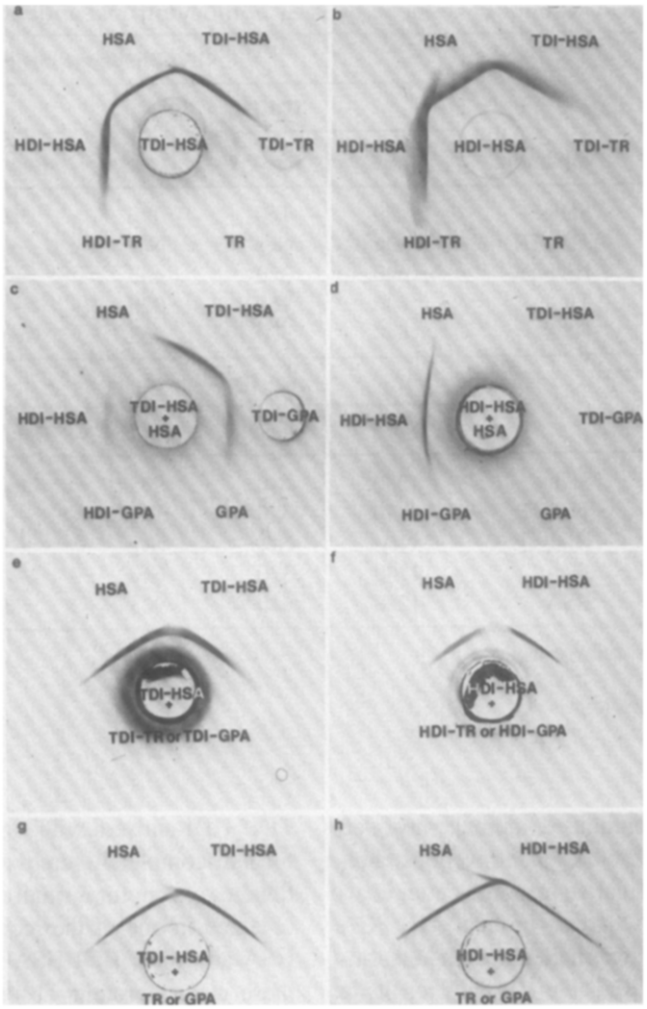

FIG. 1. Analysis of precipitating antibodies by the double gel diffusion technique. Double gel diffusion was performed in agarose with antigens placed in the surrounding wells and guinea pig antisera in the center wells. Diffusion was performed for 48 hr at room temperature. Plates were washed, dried, and stained with Schwartz amido black. For inhibition studies, equal volumes of antisera and inhibitory antigens were incubated at 37° C for 2 hr and centrifuged at 450 × g for 20 min, after which supernatants or upper parts of reactant mixtures were placed into the wells. Concentrations of antigens and antisera were selected for optimum reactions in each experiment. Faint precipitin lines that were observed between TDI-HSA–absorbed antiserum and TDI-TR **(a)**, TDI-HSA–absorbed antiserum and HDI-HSA **(c)**, HDI-HSA–absorbed antiserum and HDI-GPA and TDI-HSA **(d)** did not photograph well. Diffusion patterns similar to those shown in **g** and **h** were obtained when the central wells contained unabsorbed antisera (TDI/HDA-HSA reactants).

precipitin lines against conjugates of TDI-TR but not against TR alone (Fig. 1, *a*). Similar reactions were observed with HDI-HSA antiserum except for lack of reaction with HDI-TR (Fig. 1, *b*). TDI/HDI-HSA–specific antibodies revealed precipitin lines against both TDI/HDI-HSA (Fig. 1, *a* and *b*). Spur formation was observed between precipitin reactions elicited by TDI/HDI antisera reacted with TDI/HDI-HSA and HSA. Preincubation of various antisera with HSA eliminated the precipitin line between respective antisera and HSA but not TDI/HDI-HSA reactants (Fig. 1, *c* and *d*). Also noteworthy were the reactions between HSA-absorbed TDI/HDI antisera and TDI/HDI-GPA but not GPA alone (Fig. 1, *c* and *d*). Spur formation between TDI/HDI-HSA and HSA was removed when antisera were incubated with TDI/HDI-TR or TDI/HDI-GPA (1, *e* and *f*) but not when they were incubated with TR or GPA alone (Fig. 1, *g* and *h*). These observations suggested that some of the antigenic determinants in TDI/HDI-HSA were responsible for the production of hapten-specific antisera.

Immunoelectrophoresis

Immunoelectrophoresis revealed that TDI/HDI-HSA antisera precipitated with both TDI/HDI-HSA and HSA antigens. However, the greater anodic migrations of the single TDI/HDI-HSA bands indicated that TDI/HDI-HSA conjugates do not contain any free or unconjugated HSA carrier protein (Fig. 2). Thus reactions of respective antisera and HSA (Fig. 1, *a* and *b*) most likely denote the presence of HSA-carrier antigenic determinants within the TDI/HDI-HSA conjugates.

Cross-reactivity experiments

Negative PCA responses were obtained with TDI-HSA–specific IgE or IgG_1 antisera when HDI-TR was used as a challenging antigen (Table V). Similar results were observed when HDI-HSA–specific antisera were challenged with TDI-TR. Absence of cross-reactivity between TDI and HDI was also suggested by inhibition experiments previously listed in Table IV. Although such experiments were difficult to interpret in absolute terms because of twofold to fourfold variations of IgG_1 titers between different recipient animals, global assessment revealed no significant inhibition of TDI-HSA antisera by HDI-TR. However, incubation of HDI-HSA with TDI-TR produced a marked inhibition of the IgG_1 titer in one recipient animal (RPCA titer of 40) and a slight reduction of RPCA (320) in the other animal of this pair. Although increased sensitivity of inhibition experiments could account for possible cross-reactivity in one animal of this recipient pair, it is more likely that this apparent discrepancy between direct (Table V) and indirect (Table IV) cross-reaction experiments merely reflects the biologic variability inherent in the two PCA methods.

Double gel diffusion of TDI-HSA antisera with HDI-GPA or HDI-TR conjugates revealed no precipitin reactions. However, there were definite precipitin reactions between TDI-HSA antisera and the HDI-HSA conjugates (Fig. 1, *a*). Likewise, HDI-HSA–specific antisera also reacted only with the TDI-HSA conjugate and not with TDI-GPA or TDI-TR (Fig. 1, *b*). This cross-reactivity between TDI/HDI-HSA antisera and respective heterologous conjugates was most likely due to a determinant in the HSA carrier protein moiety or to a NAD but not to respective TDI/HDI haptens. To further clarify these relationships, cross-reactivity was also examined after absorbing HSA antibodies from the various antisera. Fig. 1, *c*, shows that TDI-HSA antisera absorbed with HSA reacted with HDI-HSA but not to HSA alone. Since haptenic cross-reactivity between TDI and HDI was not detected in the homocytotropic antibody experiments (Tables II and III), this finding suggests

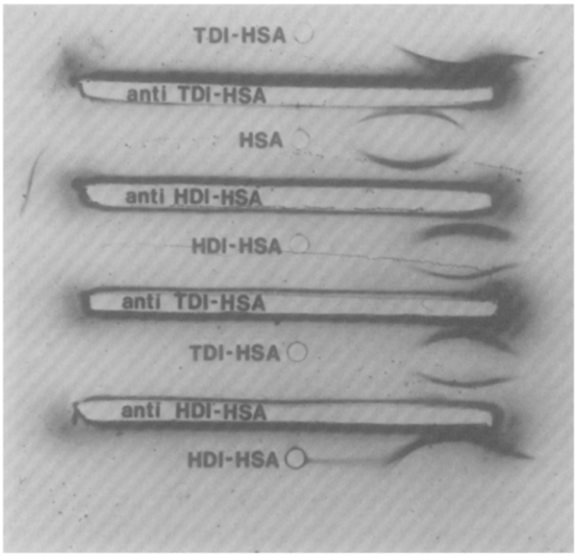

FIG. 2. Immunoelectrophoresis of diisocyanate-HSA conjugates in agarose. Electrophoresis was performed for 90 min in 0.05M sodium borate buffer, pH 8.4, with a current of 5 mAmp per slide. Symbols of all reactants are described in the text.

that there is a NAD in the HDI-HSA conjugate that is responsible for cross-reactivity between TDI-HSA antiserum and HDI-HSA antigen. Similar results were obtained in experiments with HDI-HSA–specific antiserum in which the NAD and the TDI-HSA conjugate appeared to be responsible for cross-reactivity between HDI-HSA antiserum and TDI-HSA conjugate (Fig. 1, *d*).

DISCUSSION

These results demonstrate that volatile, bifunctional isocyanate compounds are immunoreactive in susceptible strains of guinea pigs. In these studies, both homocytotropic (IgG_1 and IgE) and precipitating antibodies were produced under the appropriate conditions of parenteral immunization. Specific IgE responses to bifunctional isocyanate conjugates were observed in all English short-hair strain animals. The uniform appearance of IgE reactions in the current investigations was noteworthy because only half of the immunized animals developed this type of homocytotropic antibody in several previous specific IgE antibody systems.[19, 20] Moreover, the absolute titers of specific IgE antibody obtained in the current study were several orders of magnitude higher than those observed after respiratory immunization of guinea pigs with monofunctional and bifunctional isocyanate compounds.[20, 21] The qualitative and quantitative aspects of IgG_1 homocytotropic antibodies obtained in these experiments were similar to the IgE results. Both IgE and IgG_1 PCA titers induced by HDI conjugates

TABLE V. Absence of haptenic (TDI vs HDI) cross-reactivity in homocytotropic antibody systems*

	I.V. Challenge antigens					
	TDI-TR			HDI-TR		
Homocytotropic antibody	Rec 1	Rec 2	G.M.†	Rec 1	Rec 2	G.M.
IgE anti–TDI-HSA	160	160	160	Neg‡	Neg	Neg
IgE anti–HDI-HSA	Neg	Neg	Neg	1280	640	905
IgG_1 anti–TDI-HSA	640	1280	905	Neg	Neg	Neg
IgG_1 anti–HDI-HSA	Neg	Neg	Neg	2560	1280	1810

Rec = recipient animal; I.V. = intravenous.
*Expressed as RPCA titers.
†Geometric mean of the two RPCA titers.
‡Negative reaction: <5.

were higher than those produced after immunization with TDI conjugates (Tables II and III). These results are consistent with those of a previous report which revealed that HDI exhibited a stronger sensitization potential than TDI when applied to the skin of experimental animals.[22]

Hapten specificity of IgE and IgG_1 homocytotropic antibody responses was detected by the use of respective isocyanate ligands coupled to an unrelated carrier protein, TR (Tables II and III). Hapten specificity was further confirmed by PCA inhibition experiments of IgG_1 homocytotropic antibody (Table IV).

The precipitating antibody results provided additional data concerning the complexity of immune responses induced by TDI/HDI-HSA conjugates. In addition to the expected antibody responses elicited by carrier protein (HSA), TDI and HDI, by virtue of their high degree of reactivity, appeared to alter the native structure of carrier protein to the extent that at least two other antigenic determinants could be demonstrated in these conjugates: (1) haptenic (TDI/HDI) and (2) a NAD, which includes parts of both hapten and carrier protein. In these experiments the hapten determinant was clearly demonstrated by: (1) reactions between precipitating antisera and TDI-TR conjugates but not TR alone in double gel diffusion experiments, and (2) the disappearance of spurs between TDI/HDI-HSA and HSA precipitin lines when antisera were absorbed with TR or GPA conjugates of TDI/HDI, but not with TR or GPA alone.

The NAD was shown by: (1) absence of precipitin lines between TDI-HSA antisera and TR and GPA conjugates of HDI, and (2) persistence of reactions between TDI-HSA antisera and HDI-HSA conjugates (and vice versa) after HSA antibodies had been absorbed from the respective antisera. Given the lack of evidence for haptenic cross-reactivity between TDI and HDI in both homocytotropic and precipitating antibody systems, it appears most likely that the basis of cross-reactions between TDI/HDI-HSA antisera and HDI/TDI-HSA conjugates is due to NADs. The carrier protein determinant was detected by: (1) reactions of antisera with both unconjugated HSA and TDI/HDI-HSA conjugates that did not contain any free or uncombined HSA (Fig. 2), and (2) disappearance of precipitins between TDI/HDI-HSA antisera and HSA after absorption of antisera with HSA.

It is apparent that the immune response of guinea pigs immunized with protein conjugates of bifunctional isocyanates is heterogeneous and involves multiple specificities for hapten, carrier protein, and NAD(s). The demonstration of hapten specificity in these antisera required a conjugate prepared with an unrelated carrier protein as the challenging antigen. In this study, TR conjugates proved to be ideal reagents for this purpose. Although haptenic cross-reactivity between TDI and HDI was not demonstrable in this study, cross-reactions between TDI/HDI-HSA antisera and HDI/TDI-HSA conjugates were observed. It was deduced that these reactions were probably due to NADs produced by isocyanate interactions with one or several $-NH_2$, -COOH, -SH, or -OH radicals in the protein carrier molecule. Since the kinetics of isocyanate-protein interaction primarily favor the ureido linkage via epsilon amino groups, it is postulated that an essential component of the NAD should contain ureido moieties. Indeed, recent clinical data suggest that ureido conjugated monoisocyanate reagents manifest greater specificity than amido monofunctional conjugates in the detection of specific IgE antibodies of TDI-sensitive workers.[23] Cross-reactivity between NADs may also explain cross-reactive bronchospastic responses[24] and leukocyte inhibitory factor reactions[27] to HDI in TDI-sensitive workers never exposed to HDI.

In recent years, several animal models have been utilized to explore the immunogenic and allergenic

potentials of isocyanate compounds.[20, 21, 25, 26] It is now clearly established that a variety of immunologic responses occur in several species after sensitization with monoisocyanate or diisocyanate compounds by several routes. Some of these experiments were limited to the immunologic effects of monoisocyanate compounds (*p*-tolyl and hexyl) to eliminate the chemical complexity inherent in the production of bifunctional isocyanate protein conjugates.[20, 25] In the guinea pig, both precipitating and passive cutaneous anaphylactic antibodies were induced by immunization with monoisocyanates, but the titers of homocytotropic antibodies were uniformly low in the individual animals. Moreover, small quantities of IgE antibodies were only detected in about half of the immunized animals. Respiratory sensitization of guinea pigs with TDI has also been attempted.[21, 26] A minor proportion (one fifth) of sensitized animals demonstrated precipitating antibodies, about one third of the animals developed PCA antibodies, and only one positive specific IgE response was noted in these studies.

Despite the complexity of bifunctional isocyanate-protein interactions, we elected to use these reagents in previous and current investigations because workers are exposed to diisocyanates in the workplace.[12, 15, 27] Under preparatory conditions previously documented in this laboratory, bifunctional protein conjugates with stable mono- to bisureido ratios were found to be suitable and reproducible immunizing agents in both mice[12] and guinea pigs. The guinea pig model may be preferable because the immunogenic and allergenic sequelae induced by diisocyanates can be correlated sequentially with in vivo physiologic responses. In addition, the high absolute titers of antibodies (both precipitating and homocytotropic) obtained in this current investigation should provide an excellent standard reference system for evaluating the relative potencies of isocyanate conjugates to be used for future animal and clinical studies. The complex nature of the immune response generated by diisocyanate compounds in the guinea pig may also serve as a more appropriate model of isocyanate-induced human sensitivity reactions known to involve diverse immunologic and nonimmunologic effects.[11, 13, 14, 27, 28] It should be realized, however, that large amounts of carrier-specific antibody inherent in this model would not be expected to occur after alterations of cell protein by human exposure to TDI and/or HDI.

We appreciate the professional expertise of Dr. R. L. Sandridge, who performed the hexamethylenediamine gas chromatographic analysis of the HDI-protein conjugates, and the secretarial assistance of Ms. Jan Shulman.

REFERENCES

1. Williamson KS: Studies of diisocyanate workers (I). Transactions of the Association of Industrial Medical Officers **14**:81, 1964.
2. Munn A: Hazards of isocyanates. Ann Occup Hyg **8**:163, 1965.
3. Hamilton A, Hardy L: Industrial toxicology, ed. 3. Littleton, Mass., 1974, Publishing Service Group, Inc., pp. 331-334.
4. Charles J, Bernstein A, Jones B: Hypersensitivity pneumonitis after exposure to isocyanates. Thorax **31**:127, 1976.
5. Kabe J: Bronchial asthma and asthma-like dyspnea. Jpn J Allergol **20**:444, 1971.
6. Emmett EA: Allergic contact dermatitis in polyurethane plastic moulders. J Occup Health **18**:802, 1976.
7. Scheel LD, Killens R, Josephson A: Immunochemical aspects of toluene diisocyanate (TDI) toxicity. Am Ind Hyg Assoc J **25**:179, 1964.
8. Avery SB, Stetson DM, Pan PM, Mathews KP: Immunological investigation of individuals with toluene diisocyanate asthma. Clin Exp Immunol **4**:585, 1969.
9. Bruckner HC, Avery SB, Stetson DM, Dodson UN, Ronayne JJ: Clinical and immunologic appraisal of workers exposed to diisocyanates. Arch Environ Health **16**:619, 1968.
10. Butcher BT, Salvaggio JE, Weill H, Ziskind MM: Toluene diisocyanate (TDI) pulmonary disease—immunologic and inhalation challenge studies. J ALLERGY CLIN IMMUNOL **58**:89, 1976.
11. Karol MH, Ioset HH, Alarie YC: Tolyl-specific IgE antibodies in workers with hypersensitivity to toluene diisocyanate. Am Ind Hyg Assoc J **39**:454, 1978.
12. Tse CS, Chen SE, Bernstein IL: Induction of murine reaginic antibodies by toluene diisocyanate. An animal model of immediate hypersensitivity reactions to isocyanates. Am Rev Respir Dis **120**:829, 1979.
13. Davies RJ, Butcher BT, O'Neil CE, Salvaggio JE: The in vitro effect of toluene diisocyanate on lymphocyte cyclic adenosine monophosphate production of isoproterenol, prostaglandin and histamine. A possible mode of action. J ALLERGY CLIN IMMUNOL **60**:223, 1977.
14. Butcher BT, Karr RM, O'Neil CE, Wilson MR, Dharmarajan V, Salvaggio JE, Weill H: Inhalation challenge and pharmacologic studies of toluene diisocyanate (TDI)-sensitive workers. J ALLERGY CLIN IMMUNOL **64**:146, 1979.
15. Tse CS, Pesce AJ: Chemical characterization of isocyanate-protein conjugates. Toxicol Appl Pharmacol **51**:39, 1979.
16. Goa J: A micro Biuret method for protein determination: determination of total protein in cerebrospinal fluid. Scand J Clin Lab Invest **5**:218, 1953.
17. Sandridge RL: Laboratory methods. New Martinsville, W. Va., 1978, Mobay Chemical Corp.
18. Munoz J: Precipitation analysis by diffusion in cells, *in* Williams CA, Chase MW, editors: Methods in immunology and immunochemistry, vol. 3. New York, 1971, Academic Press, Inc., p. 146.
19. Ovary Z, Kaplan B, Kojima S: Characteristics of guinea pig IgE. Int Arch Allergy Appl Immunol **51**:416, 1976.
20. Karol MH, Hauth BA, Alarie Y: Pulmonary hypersensitivity to hexyl isocyanate-ovalbumin aerosol in guinea pigs. Toxicol Appl Pharmacol **51**:73, 1979.
21. Karol MH, Dixon C, Brady M, Alarie Y: Immunologic sensitization and pulmonary hypersensitivity by repeated inhalation of aromatic isocyanates. Toxicol Appl Pharmacol **53**:260, 1980.

22. Kondrat'ev GG, Mustaev RK: Allergenic properties of some chemical substances used in synthetic rubber production. Trans Leningrad Sanit Gig Med Inst **93:**154, 1971.
23. Karol MH, Alarie Y: Antigens which detect IgE antibodies in workers sensitive to toluene diisocyanate. Clin Allergy **10:** 101, 1980.
24. O'Brien IM, Harries MG, Burge PS, Pepys J: Toluene diisocyanate-induced asthma. I. Reactions to TDI, MDI, HDI and histamine. Clin Allergy **9:**1, 1979.
25. Karol MH, Ioset HH, Riley EJ, Alarie YC: Hapten-specific respiratory hypersensitivity in guinea pigs. Am Ind Hyg Assoc J **39:**546, 1978.
26. Karol MH: Study of guinea pig and human antibodies to toluene diisocyanate. Am Rev Respir Dis **122:**965, 1980.
27. Gallagher JS, Tse CST, Brooks SM, Bernstein IL: Diverse profiles of immunoreactivity in toluene diisocyanate (TDI) asthma. J Occup Med **23:**610, 1981.
28. Butcher BT, O'Neil CE, Reed MA, Salvaggio JE: Radioallergosorbent testing of toluene diisocyanate-reactive individuals using *p*-tolyl isocyanate antigen. J ALLERGY CLIN IMMUNOL **66:**213, 1980.