Antigen-specific Stimulation of Histamine Releasing Factors in Diisocyanate-induced Occupational Asthma

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Diisocyanate-induced asthma differs from occupational asthma (OA) caused by protein allergens in that specific IgE antibody responses are rarely identified. To investigate the immunopathogenesis of diisocyanate asthma, diisocyanate-exposed workers were evaluated for *in vitro* production of antigen-specific mononuclear cell-derived histamine releasing factor (HRF). The mean HRF response to diisocyanate-HSA antigens was significantly greater in patients with OA than in diisocyanate-exposed asymptomatic subjects ($p < 0.05$). No association was found between HRF and diisocyanate-specific antibodies. Analysis of HRF production by subpopulations of peripheral blood mononuclear cells (PBMC) showed that lymphocytes and adherent cells were major sources of both spontaneous and antigen-stimulated HRF. The results suggest that antigen-specific HRF produced by PBMCs are an important biomarker for diisocyanate-induced asthma. This is the first report of hapten-specific stimulation of PBMCs resulting in HRF production. **Herd ZL, Bernstein DI. Antigen-specific stimulation of histamine releasing factors in diisocyanate-induced occupational asthma. Am J Respir Crit Care Med 1994;150:988-94.**

Diisocyanates are low-molecular-weight chemicals that are spontaneously reactive with proteins and are common causes of occupational asthma (OA) (1, 2). Toluene diisocyanate (TDI) and methylene diphenyldiisocyanate (MDI) are used as activators for the manufacture of polyurethanes. Hexamethylene diisocyanate (HDI) is used as a hardening agent in spray paints. Diverse patterns of bronchospastic responses elicited by diisocyanates include early asthmatic responses (EAR), isolated late asthmatic responses (LAR), and dual-phase responses (EAR followed by a LAR). Isolated LARs occur in about 40% of workers with diisocyanate asthma (3).

Despite evidence that diisocyanate asthma is immunologically mediated in some workers, underlying immune mechanisms have not been completely defined. Serum-specific IgE responses to diisocyanate–human serum albumin (HSA) conjugate antigens (MDI-HSA and TDI-HSA) have been detected in only 10 to 30% of workers with OA (1). Clinical features of diisocyanate OA that support allergic mechanisms include a latency period of exposure before the onset of asthmatic symptoms; development of OA in a small number (5%) of exposed workers; and elicitation of early and late asthmatic responses by subirritant levels of ambient diisocyanates. Cellular immune mechanisms could be important, as demonstrated by the finding of activated T cells in bronchial biopsies of workers with diisocyanate-induced OA (4) and elevations in peripheral $CD8^+$ T cells following inhalation of TDI (5). Recently, Siracusa and colleagues demonstrated spontaneous production of tumor necrosis factor by peripheral blood mononuclear cells (PBMCs) of workers with TDI asthma 48 to 72 h after TDI inhalation challenge (6).

Histamine releasing factor (HRF) was initially isolated from PBMCs of normal individuals after *in vitro* coincubation with concanavalin A (Con A) or streptokinase/streptodornase (SK/SD) streptococcal antigens (7, 8). Subsequently, HRF synthesis has been associated with a variety of cell sources, including T and B lymphocytes (9–11), pulmonary macrophages (12, 13), platelets, and neutrophils (7). Molecules with potent HRF activities have been characterized as low-molecular-weight cytokines belonging to the intercrine family (14–16). HRF-related molecules with HRF inhibitory activity have also been demonstrated (17, 18).

In this study, we report the production of diisocyanate-stimulated HRF by PBMCs of workers with diisocyanate asthma. These studies suggest that diisocyanate hapten-specific HRF is a relevant cytokine in the pathogenesis of OA and a potentially useful biomarker of diisocyanate asthma.

METHODS

Reagents

RPMI 1640, Hanks' balanced salt solution (HBSS), glutamine, penicillin-streptomycin, HEPES, and phytohemagglutinin, M form (PHA) were obtained from GIBCO Laboratories (Grand Island, NY), fetal bovine serum (FBS) from Hyclone Laboratories, Inc. (Logan, UT), bovine serum albumin (Fraction V, crystalline), calcium ionophore A23187, concanavalin A, dextran (200 to 300 average kD), methyl α-D-mannopyranoside, p-nitrophenylphosphate, sodium pyruvate, mouse monoclonals (anti-CD3-FITC, clone UCHT-1, antihuman CD8, clone UCHT-4, antihuman CD7, and clone 3A1), and other immunochemicals (alkaline phosphatase goat antihuman IgG, FITC-goat antimouse IgG, FITC-rabbit antigoat IgG, goat antihuman IgA, goat antihuman IgG, and goat antihuman IgM) from Sigma Chemical Co. (St. Louis, MO), goat antihuman IgE and alkaline phosphatase rabbit antigoat IgG from Kierkegaard & Perry Laboratories (Gaithersburg, MD), human serum albumin (American Red Cross tested) from Baxter Healthcare Corporation (Glendale, CA), Spectrapor® dialysis tubing from

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Spectrum, Medical Industries (Los Angeles, CA), Low-Tox-H® rabbit complement (Cedarlane Laboratories, Ltd., Accurate Chemical & Scientific Corp., Westbury, NY), LeucoPREP® cell separation tubes, tissue culture plates, and plastic petri dishes from Becton Dickinson and Company (Lincoln Park, NJ), histamine radioimmunoassay (RIA) kits from Amac, Inc. (Westbrook, ME), and Immulon® 4 ELISA plates from Dynatech Laboratories, Inc. (Chantilly, VA).

Subjects

A group of 25 subjects was tested for *in vitro* HRF production (Table 1): 11 workers presented with work-related asthmatic symptoms associated with diisocyanate exposure (10 males and one female). To confirm diisocyanate asthma, these workers underwent either a single-blind controlled laboratory challenge (3) to TDI (< 20 ppb) or a workplace challenge. Workplace challenges were carried out by monitoring of peak expiratory flow (PEF) rates every 2 h (five to six measurements per day) for 2 wk before, during, and after work while there was active exposure to diisocyanates and for 2 wk while away from the workplace. Patterns consistent with work-related asthma were determined by visual inspection of PEF records by two experienced physicians. This approach has been demonstrated to have good sensitivity and specificity for diagnosis of occupational asthma when specific inhalation challenge in the laboratory is used as the gold standard (19). For specific laboratory inhalation challenge, a 20% fall in lung function (FEV_1) during active exposure compared with preexposure baseline along with no response on placebo control days was considered a positive response. Eight workers were assigned to Group 1 in whom OA was confirmed (Table 1). Group 2 consisted of 3 subjects with histories of OA and in whom the diagnosis was not confirmed by physiologic means. Nine were assigned to Group 3 asymptomatic MDI-exposed foam workers. The 5 control subjects who had no prior exposure to diisocyanates included two non-atopic subjects (Group 4) and three subjects (Group 5) with either allergic rhinitis or asthma caused by nondiisocyanate antigens (phthalic anhydride [n = 1] or short ragweed [n = 2]).

Study protocols were reviewed and approved by the University of Cincinnati Institutional Review Board. Before participation, informed written consent was obtained from each subject.

Isolation of PBMCs and Subpopulations

Venous blood (90 to 120 ml) was collected in 10 mM EDTA. Mononuclear cells were purified by density-gradient centrifugation using LeucoPREP (Becton-Dickinson Laboratories). Cells were washed three times with HBSS using low-speed centrifugation (200 × *g*) to deplete platelets. PBMCs were processed to obtain total lymphocytes and B cell-depleted lymphocytes (T cells). PBMCs at 5 × 10^6/ml in RPMI were plated in 100 mm plastic petri dishes (10 ml/plate) and incubated for 2 h at 37° C. Nonadherent

TABLE 1

DESCRIPTION OF DIISOCYANATE-EXPOSED WORKERS AND CONTROL SUBJECTS*

		Exposure						
Worker	Chemical	Time (*mo*)	Cessation (*mo*)	Clinical Diagnosis	Bronchial Method	Skin Test	Ab	HRF
Group 1								
1	MDI, TDI	48	1		+, LAR§ (lab)	–	IgG	+
2	MDI	33	0	OA	+, LAR (lab)	–	–	+
	HDI	11	0					
3	TDI	unknown		OA	+, LAR (lab)	–	IgG	+
4	HDI	17	8	OA	+, (lab)	+	IgE IgG	+
5	MDI, TDI	33	3	OA	+, (work)	–	IgG	+
6	MDI	25	10	OA	+, (work)	–	–	–
7	HDI	60	1	OA	+, (work)	+	IgE IgG	+
8	HDI	39	0	OA	+, (work)	–	–	+
Group 2								
9	MDI	336	38	Positive history of OA	–, (lab)	–	IgE IgG	–
10	MDI, TDI	84	0	Positive history of OA	–, (lab)	–	IgE IgG	–
11	MDI	24	23	Positive history of OA	–, (lab)	–	IgG	–
Group 3								
12-20	MDI	Variable	0	No symptoms	ND	ND	2/9 IgE 1/9 IgG	–
Group 4								
21, 22	None	None		No symptoms	ND	ND	–	–
Group 5								
23	PA†	10	39	OA	+, (work)	+	IgE	+
24, 25	RW‡	Unknown		Allergic asthma	ND	+	IgE	+

Definition of abbreviation: ND = not done.

* Total time of known exposure and time elapsed since cessation of known exposure before clinical assessment. Single-blind controlled laboratory challenge with TDI (< 20 ppb). Antibody (Ab) reactions were considered positive when OD readings were greater than 2 SD above the mean of negative control subjects by ELISA. An HRF response was considered positive when diisocyanate-HSA stimulation of PBMCs resulted in at least 7% enhancement: a 7% increase over spontaneous release as measured by % HR in the basophil histamine release test.

† Exposure to phthalic anhydride; HRF test results given for PA-HSA.

‡ Ragweed-sensitive atopic subject; HRF test results given for ragweed.

§ Late airways reaction only, suggestive of isolated late-phase response.

cells were obtained from the supernatant and one-half of the cells used as the source of total lymphocytes. Loosely adherent cells were removed by washing with HBSS and discarded. Adherent cells were removed with a cell scraper and collected in RPMI. T cells consisted of the remaining cells treated with goat antihuman IgG, IgA, and IgM and complement to deplete B cells. Purified cell subpopulations were analyzed by immunofluorescence with fluorescein-labeled anti-CD3 and antihuman immunoglobulins and with anti-CD7 and anti-CD8 by indirect assay using FITC-antimouse immunoglobulins as the second antibody.

Measurement of HRF

Generation of and assay for diisocyanate-induced HRF were performed using modified methods originally described by Thueson and colleagues (8). Cells were suspended in complete RPMI (RPMI with 4 mM glutamine, 2 mM sodium pyruvate, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 5% FBS) at a cell concentration of 5×10^6/ml. Cell suspension (1 ml) was added to wells of a 24-well cell culture plate, and cells were treated with 0.1 ml HRF stimulator reagents at three concentrations (5, 50, and 500 μg/ml) in HBSS. After 48 h incubation at 37° C in 5% CO_2, supernatants were removed and dialyzed against 100 vol HBS (0.15 M NaCl and 10 mM HEPES, pH 7.4) with a membrane that excluded molecules less than 3,500 D.

HRF activity was assayed by testing cell culture supernatants for basophil histamine release with basophils obtained from a single nonatopic donor. Venous blood was collected with 10 mM EDTA. After sedimentation with dextran 200, peripheral blood leukocytes (PBLs) were collected, centrifuged, and washed twice with HBSS, 10 mM EDTA, and 0.125% HSA by low-speed centrifugation (200 × *g*) to deplete platelets and resuspended at a cell concentration of 1×10^7/ml in HBS containing 2 mM calcium, 1 mM magnesium, and 0.125% HSA. HRF supernatant (0.3 ml) was added to 0.1 ml PBLs. Methyl α-D-mannopyranoside (0.1 M) was added to Con A-derived supernatants to inhibit direct histamine release effects of this mitogen. To verify responsiveness, donor basophils were challenged with rabbit antihuman IgE and the calcium ionophore, A23187. Basophils from a nonatopic donor were selected for all test assays because they had a high histamine content (450 nM/10^7 cells/ml) and exhibited histamine release to antihuman IgE (33 to 67% HR) and calcium ionophore (85 to 100% HR). Finally, the donor basophils had a low level of spontaneous release and did not show histamine release when challenged directly with PHA or diisocyanate-HSA antigens. Cells were incubated for 1 h at 37° C and centrifuged and supernatants collected for histamine analysis. Controls included mononuclear cell supernatant dialysate in culture media (spontaneous basophil histamine release), supernatant from basophils boiled for 10 min (complete release of total basophil histamine), antihuman IgE (1:300 dilution), and calcium ionophore (100 μg/ml). Histamine was quantitated by competitive inhibition radioimmunoassay (histamine RIA kit, Amac, Inc.). HRF activity expressed as % HR was derived from the following formula:

$$\%\ HR = \frac{\text{experimental HR - spontaneous HR}}{\text{complete HR - spontaneous HR}} 100$$

Antigen enhancement of HRF activity was determined by subtraction of spontaneous HRF activity from antigen-stimulated (for example, MDI-HSA) HRF activity. Hapten-specific or diisocyanate-hapten enhancement of HRF activity was determined by subtraction of HSA-stimulated HRF activity from diisocyanate-HSA antigen-stimulated HRF activity. HRF activity was considered significant when the test supernatant minus spontaneous histamine release ≥ 7%.

HRF Dose-response Studies

Basophil responsiveness to HRF-containing supernatants from PBMCs of some subjects was analyzed by determining % HR in the standard histamine release assay after basophils were exposed to increasing dilutions of HRF-containing cell supernatants. Augmentation of histamine release was assessed as an increased basophil response to diluted HRF.

Serum-specific Antibody Studies

HDI-HSA, MDI-HSA, TDI-HSA, and HSA (Baxter Healthcare) were prepared at 5 mg/ml of protein in 0.9% saline and sterilized with a 0.22 μm Millipore membrane filter. Conjugates were prepared and characterized as previously described (20), with an average 3:1 hapten to carrier molar ratio. Specific IgE and IgG to MDI-HSA antigens were performed using a previously described ELISA method (21, 22). Plates were coated with 0.1 ml of 10 μg/ml of antigen. All reactions were terminated with 1 N NaOH when a positive control serum showed an optical density (OD) at 405 nm of 0.6. Sera at a 1:10 dilution producing an OD reading ≥ 0.1 and a reaction greater than 2 standard deviations (SD) above the mean of six nonexposed control subjects were considered positive.

Data Analysis

HRF activity is expressed as percentage of histamine release (mean ± standard error of the mean, SEM). Group HRF activities were compared by the Mann-Whitney test. Fisher's exact test was used to analyze categoric data of subject populations. A p value ≤ 0.05 was considered significant.

RESULTS

Clinical characteristics of the study groups are shown in Table 1. The duration of diisocyanate exposure ranged from 17 mo to 28 yr. Of 11 diisocyanate-exposed workers with suspected OA, 2 had positive skin prick tests to diisocyanate-HSA antigens.

Mononuclear Cell-derived HRF Activity

HRF activity was assayed in PBMCs of subjects with confirmed diisocyanate asthma and incubated with varying dilutions of HDI-HSA, MDI-HSA, TDI-HSA, HSA, Con A, PHA, or medium alone. The mean PBMC-derived HRF activities obtained with varying doses of antigens and mitogens are shown in Table 2 for diisocyanate-exposed Groups 1, 2 and 3 and in Table 3 for nonexposed Groups 4 and 5. Mean spontaneous production of HRF was greater in Group 1 compared with Groups 2, 3, 4, and 5 but differences were not significant. Con A stimulation of HRF production showed

TABLE 2

HRF ACTIVITY IN PBMC SUPERNATANTS OF DIISOCYANATE-EXPOSED SUBJECTS*

	Group 1 OA	Group 2 Symptomatic Non-OA	Group 3 Exposed Asymptomatic	p Value
Spontaneous	25.6 ± 8.1	17.0 ± 1.5	11.2 ± 0.9	NS
Con A 50	30.9 ± 11.7	45.3 ± 27.7	18.2 ± 0.8	NS
Con A 0.5	28.0 ± 8.9	18.7 ± 11.9	11.4 ± 1.6	NS
PHA 50	35 ± 6.1	9.3 ± 2.1	17.6 ± 1.8	NS
PHA 0.5	21.5 ± 5.8	9.0 ± 1.5	11.0 ± 1.6	NS
HSA 50	18.9 ± 6.6	9.3 ± 5.2	9.1 ± 1.6	NS
HSA 5	22.0 ± 5.2	10.0 ± 2.1	NT	
HSA 0.5	17.8 ± 4.8	7.0 ± 3.5	9.6 ± 1.8	NS
MDI 50	39.9 ± 10.9	6.7 ± 3.4	10.7 ± 1.6	0.03
MDI 5	44.0 ± 9.0	10.3 ± 5.7	8.3 ± 2.0	0.03
MDI 0.5	23.4 ± 5.7	8.6 ± 4.6	9.5 ± 1.8	0.04
TDI 50	40.8 ± 12.3	14.0 ± 5.9	NT	
TDI 5	26.4 ± 8.7	5.3 ± 5.3	NT	
TDI 0.5	26.2 ± 8.0	8.3 ± 4.6	NT	
HDI 50	25.4 ± 7.2	13.0 ± 2.4	NT	
HDI 5	20.3 ± 9.3	19.0 ± 1.4	NT	
HDI 0.5	19.2 ± 6.8	7.5 ± 2.8	NT	
Diiso 50	43.0 ± 10.4	11.3 ± 4.4	10.7 ± 1.6	0.014
Diiso 5	38.1 ± 6.8	10.3 ± 5.8	8.3 ± 2.0	0.004
Diiso 0.5	32.6 ± 6.6	10.0 ± 4.3	9.5 ± 1.8	0.006

Definition of abbreviations: NT = not tested; NS = not significant ($p > 0.05$).

* Statistical data for total HRF activity in PBMC supernatants after culture of cells in medium alone (spontaneous production) or with mitogens (Con A or PHA) or antigens (MDI-HSA, TDI-HSA, HDI-HSA, or diiso-HSA = diisocyanate antigen(s) to which the subject had been exposed) at a dose of 50, 5, or 0.5 μg. HRF activity is expressed as the mean % HR ± SEM by PBLs treated with HRF supernatant. Significance of difference in means of Group 3 versus Group 1 is indicated.

TABLE 3

HRF ACTIVITY IN PBMC SUPERNATANTS OF NON-DIISOCYANATE-EXPOSED SUBJECTS*

	Group 4 Normal Controls†	p Value	Group 5 Atopic Control
Spontaneous	7.5 ± 0.9	NS	16.3 ± 5.6
Con A 50	30.9 ± 12.6	NS	53.0 ± 23.6
Con A 0.5	8.6 ± 2.0	NS	19.3 ± 10.4
PHA 50	16.8 ± 3.8	0.04	60.7 ± 20.2
PHA 0.5	7.7 ± 2.3	NS	22.3 ± 12.8
HSA 50	7.0 ± 2.8	0.04	18.0 ± 14.6
HSA 5	4.3 ± 1.5	NS	NT
HSA 0.5	NT		NT
MDI 50	5.1 ± 1.5	0.02	8.0 (n = 1‡)
MDI 5	3.8 ± 1.5	NS	5.0 (n = 1‡)
MDI 0.5	NT		NT
TDI 50	NT		7.0 (n = 1‡)
TDI 5	NT		0.0 (n = 1‡)
		Phthalic	Ragweed
Ag 50		35.0	39.0
Ag 5		35.0	29.0
Ag 0.5		40.0	23.0

Definition of abbreviations: NT = not tested; NS = not significant ($p > 0.05$).

* Statistical data for total HRF activity in PBMC supernatants after culture of cells in medium alone (spontaneous production) or with mitogens (Con A or PHA) or antigens (MDI-HSA, TDI-HSA, HDI-HSA, or antigen [Ag], either phthalic anhydride or ragweed) to which the subject had been exposed) at a dose of 50, 5, or 0.5 μg as indicated. HRF activity is expressed as the mean % histamine release ± SEM, by PBLs treated with HRF supernatant. Significance of difference in means of Group 4 versus Group 1 is indicated.

† Mean of seven independent experiments for two subjects.

‡ Ragweed-sensitive Subject 25.

high individual variability and no significant differences between groups.

Diisocyanate-HSA Antigen-specific Stimulation of HRF Production

In Figure 1, the antigen HRF activity is shown for individual diisocyanate-exposed subjects (Groups 1 through 3) in response to three dilutions of diisocyanate-HSA antigen of the specific diisocyanate to which each individual was exposed (HDI, MDI, or TDI). In individuals exposed to more than one diisocyanate, results are shown for the diisocyanate conjugate eliciting the maximal HRF response. In Group 1, positive HRF responses (≥ 7% increase above spontaneous levels) were seen in seven of eight subjects with confirmed OA. In subjects in both Groups 2 and 3, no positive antigen-stimulated HRF responses were detected.

As shown in Table 2, significantly greater mean HRF was observed in Group 1 compared with the asymptomatic exposed Group 3 for all concentrations of MDI-HSA tested ($p \leq 0.04$), as well as for the specific diisocyanate antigens to which subjects were exposed in the workplace ($p \leq 0.014$). Although mean MDI-HSA and diisocyanate antigen-induced HRF was also greater in Group 1 than in Groups 2, 4, or 5, the differences between the latter groups achieved statistical significance only with Group 4 ($p = 0.02$).

As shown in Table 3, three allergic subjects showed specific HRF enhancement to specific allergens to which cutaneous sensitivity was demonstrated. A ragweed-sensitive subject did not exhibit MDI-HSA- or TDI-HSA-induced stimulation of HRF. Mean specific antigen enhancement of HRF for Groups 1 through 4, calculated as diisocyanate-HSA minus spontaneous HRF, which was significantly greater in Group 1 than in Groups 2, 3, and 4 at the highest antigen dose (50 μg), is shown in Figure 2. At 5 μg, there were significant differences between Groups 1 and 3 and between Groups 1 and 4; at 0.5 μg, a significant difference remained between Groups 1 and 3.

Specific Antibodies, HRF, and Clinical Symptoms

Specific IgE and IgG antibody data are summarized in Table 1. Workers were tested for antibodies reactive with HDI-HSA, MDI-HSA, and TDI-HSA and unconjugated HSA. Anti-diisocyanate antibody was absent from sera of nonexposed individuals. IgE antibodies were present in two of nine asymptomatic MDI-exposed workers (Group 3) and four of 11 workers with OA symptoms (Groups 1 and 2). Specific IgG was present in one of nine asymptomatic subjects (Group 3) and in eight of 11 with OA symptoms (Groups 1 and 2).

As shown in Table 4, specific antibody status and HRF were compared with OA diagnosed by history alone and with OA diagnoses confirmed objectively. No significant association was found between the presence of specific antibodies and antigen-enhanced HRF production. An association was found between the presence of diisocyanate-HSA–enhanced HRF and confirmed OA (Table 4; $p < 0.05$, chi-square). In contrast to HRF, no significant association was found between antibody status (IgE and/or

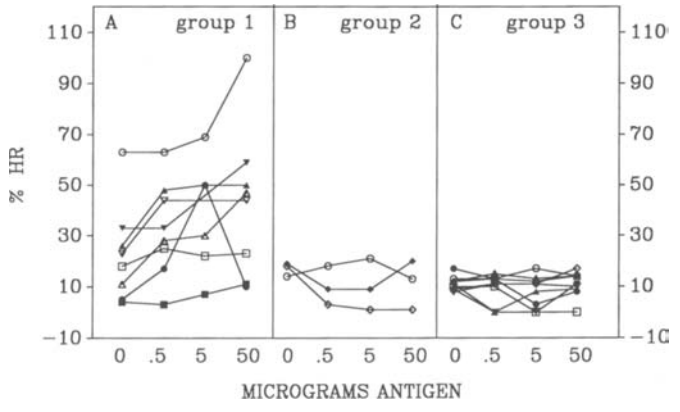

Figure 1. Effects of antigen dose on the generation of HRF activity by PBMCs from 20 exposed subjects in response to 0, 0.5, 5, or 50 μg diisocyanate-HSA antigens (HDI-HSA, MDI-HSA, and TDI-HSA) specific for the diisocyanate to which each worker was exposed in the workplace.

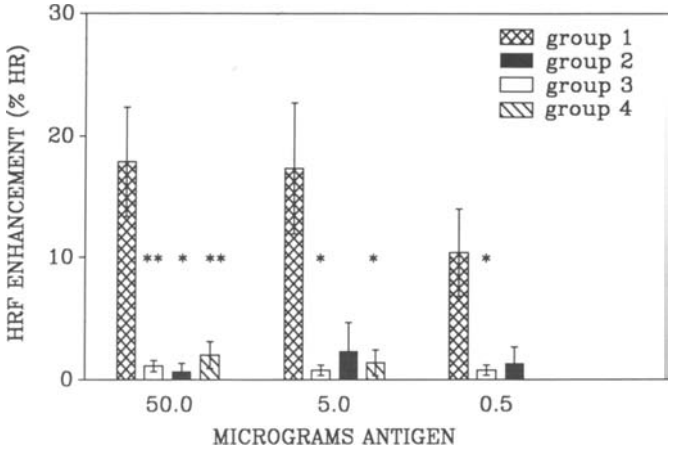

Figure 2. Enhancement of HRF production from PBMCs by diisocyanate-HSA–conjugated antigens derived by subtraction of spontaneous HRF production from diisocyanate-HSA–stimulated HRF activity. The level of significance for differences in groups are shown as $*p < 0.05$, $**p < 0.01$.

TABLE 4

ASSOCIATIONS OF SPECIFIC CHALLENGE AND OA HISTORY STUDIES WITH *IN VITRO* SPECIFIC IgE, IgG, AND ANTIGEN-ENHANCED HRF PRODUCTION*

			In Vitro Assays								
			HRF†			IgE			IgG		
Sample	N	Categories	+	−	p	+	−	p	+	−	p
Subjects in Groups 1 and 2	11	Confirmed OA	7	1	< 0.05	2	6	> 0.05	5	3	> 0.05
		Unconfirmed OA	0	3		2	1		3	0	
Subjects in groups 1, 2, and 3	20	Positive history OA	7	4	< 0.01	4	7	> 0.05	8	3	< 0.01
		Negative history OA	0	9		2	9		1	8	
Subjects in Groups 1, 2, and 3	20	HRF positive	7	0		2	5	> 0.05	5	2	> 0.05
		HRF negative	0	13		4	9		4	9	

* Fisher's test.
† Diisocyanate-HSA enhancement of spontaneous HRF production.

IgG isotypes) of the subject population and confirmed occupational asthma ($p > 0.05$). Among all diisocyanate-exposed subjects (Groups 1, 2, and 3), a significant association ($p < 0.01$) was found between a positive history of OA and diisocyanate-enhanced HRF. There was no significant association between antigen enhancement of HRF and antibody status. A significant association was found between IgG but not IgE antidiisocyanate antibody and a positive history of OA ($p < 0.01$).

Cellular Sources of Diisocyanate-stimulated HRF

Cellular sources of HRF production by PBMC subpopulations were investigated in Workers 6, 7, and 8 with confirmed OA and in one asymptomatic exposed worker, Subject 20 (Table 5). In these four workers, mean total lymphocytes were 79 ± 4.5% of total PBMCs, 76.5 ± 4.5% being $CD3^+$, and monocytes represented a mean 17.2 ± 2.9%. A mean 7% of PBMCs bore immunoglobulin receptors. Purified T cells contained > 99% $CD3^+$ and < 1% IgM, IgA, or IgG receptors. The mean CD4/CD8 ratio for the four workers was 1.45 ± 0.5. Purified T cells from two workers with confirmed OA (Subjects 7 and 8) exhibited spontaneous HRF activity of 100%. Coculture of purified T cells with Con A, PHA, or diisocyanate antigens resulted in reduced HRF responses in the same individuals.

The dose response of normal donor basophils to varying dilutions of HRF derived from PBMCs of Workers 6 and 7 are shown in Figures 3 and 4, respectively, and compared with Con A-stimulated HRF supernatants pooled from three normal donors (Figure 3D). The basophil response to the pooled Con A-stimulated HRF showed augmented histamine release by diluted HRF supernatants derived from diisocyanate-HSA culture above those derived from spontaneous cultures (Figure 3D). Maximum histamine release (90%) occurred at an HRF dilution of 1:100 and was suboptimal at higher or lower concentrations.

In Figure 3B and C, a dose-related change in HRF activity was elicited from adherent cells and T lymphocytes of Worker 6 in response to increasing HRF supernatant concentrations from MDI-HSA cultures; the greatest HRF responses occurred at or near a 1:5 dilution of HRF. In Worker 7 with IgE-mediated sensitization to HDI-HSA (Figure 4A through D) a general increase in HRF activity was observed in PBMC, purified adherent T cell, and total lymphocyte subpopulations with increasing concentrations of supernatants obtained from HDI-HSA cultures.

TABLE 5

HRF PRODUCTION BY PBMC SUBPOPULATIONS*

	HRF Activity (% HR)												
	Control Subject 20			MDI-OA, Ab- Subject 6			HDI-OA, Ab^- Subject 8			HDI-OA, Ab^+ Subject 7			
	MC	MP	T	MC	MP	T	MC	LC	T	MC	MP	LC	T
Spontaneous	10	7	30	11	17	0	33	12	100	23	0	0	100
Stimulator													
Con A	0	23	11	11	20	17	23	10	80	33	33	99	80
PHA	23	17	67	40	77	51	60	60	44	10	33	80	60
HSA	0	6	11	7	7	11	23	44	60	33	10	44	80
MDI-HSA	4	0	11	11	17	17	23	33	80	44	44	44	60
HDI-HSA	NT	NT	NT	NT	NT	NT	33	13	80	44	13	60	60
TDI-HSA	NT	NT	NT	NT	NT	NT	59	23	80	NT	NT	NT	NT

Definition of abbreviations: MC = total purified PBMCs; MP = plastic adherent cells containing macrophages; LC = nonadherent cells (lymphocytes) recovered from plating to obtain macrophages; T = nonadherent cells enriched for T cells by B cell cytotoxic treatment of cells with antiimmunoglobulins plus complement; NT = not tested.

* Cells were obtained from subjects 20, asymptomatic MDI-exposed worker; 6, confirmed OA related to MDI exposure without detectable anti-MDI serum antibody; 8, symptomatic, confirmed occupational asthma without antidiisocyanate serum antibody; 7, confirmed OA related to HDI exposure, with IgE and IgG serum antibodies showing specificity for HDI. Results are shown for cells stimulated with 50 μg antigen or mitogen.

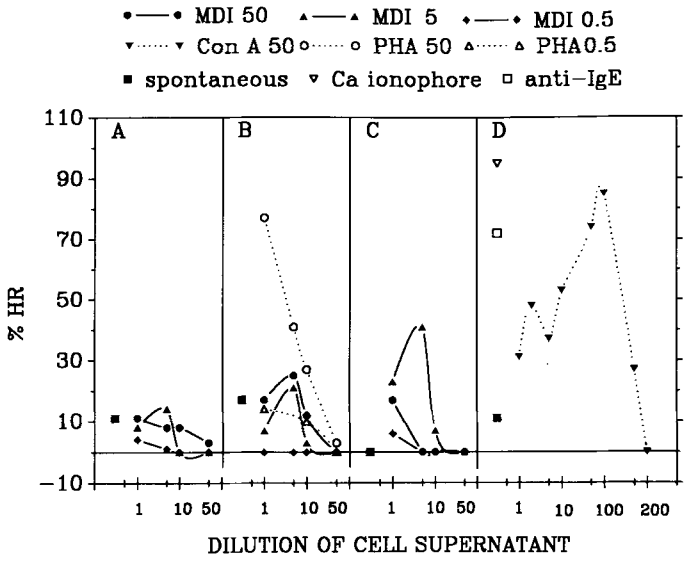

Figure 3. Comparison of HRF dose-dependent effects on basophil histamine release for HRF produced by PBMC cell subpopulations from a non–antibody-producing patient (Subject 6) with MDI-induced OA. Results shown are HRF activity in response to neat (1), 1:5 (5), 1:10 (10), 1:50 (50), and 1:100 (100) dilutions. (*A*) Spontaneous and MDI-HSA-stimulated HRF from unfractionated PBMCs. (*B*) Spontaneous and MDI-HSA-stimulated HRF from adherent cells (monocytes or macrophages). (*C*) Spontaneous and MDI-HSA-stimulated HRF from patient T cells. (*D*) Basophil histamine release in response to dilutions of three pooled supernatants containing Con A-stimulated HRF. Basophil response to anti-IgE and calcium ionphore are shown, as is spontaneous release of pooled normal PBMCs.

The significance of the T cell response was not evaluable in Subjects 7 and 8 because spontaneous T cell HRF was 100% (Table 5). In both Subjects 6 and 8, diisocyanate-specific antigen enhancement of HRF by purified T cells was apparent, as evidenced by the greater HRF response to MDI-HSA (Subject 6) or TDI-HSA (Subject 8) than to unconjugated HSA (Table 5). Antigen enhancement of 40% for T cell-derived HRF from Subject 6 is apparent by comparison of antigen-stimulated release to spontaneous release in Figure 3C. A similar comparison of the data in Figure 3A and B shows that there was no antigen-enhanced HRF in supernatants from PBMCs or adherent cells of Worker 6.

Data in Table 5 show that Worker 7 with HDI allergic asthma did not exhibit diisocyanate-hapten enhancement of HRF by T cell cultures, because antigen-stimulated HRF activity (60% HR) was less than HSA-stimulated HRF (80% HR) (Table 5). However, unfractionated total lymphocytes and PBMCs from Worker 7 exhibited HDI hapten enhancement of HRF.

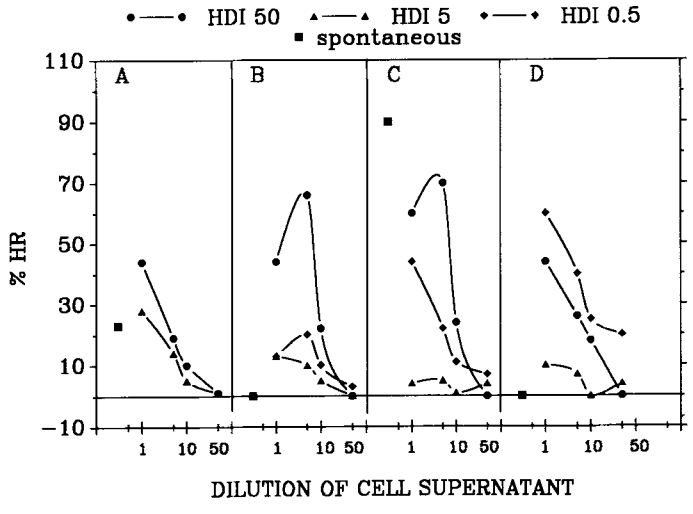

Figure 4. Comparison of HRF dose-dependent effects on basophil histamine release for HRF produced by PBMC cell subpopulations from a worker with HDI-induced OA (Subject 7) producing IgE and IgG antidiisocyanate antibodies. Results shown at HRF dilutions of neat (1), 1:5 (5), 1:10 (10), 1:50 (50), and 1:100 (100). (*A*) Spontaneous and HDI-HSA-stimulated HRF from unfractionated PBMCs. (*B*) Spontaneous and HDI-HSA-stimulated HRF from plastic adherent cells (monocytes or macrophages). (*C*) Spontaneous and HDI-HSA-stimulated HRF from T cells. (*D*) Spontaneous and HDI-HSA-stimulated HRF from total lymphocytes (nonadherent cells).

DISCUSSION

This study has demonstrated that diisocyanate-HSA antigens stimulated the *in vitro* synthesis of HRF in workers with diisocyanate asthma. *In vitro* production of HRF has been described after specific stimulation with protein allergens, including dust mite, grass, and ragweed allergens (8, 9, 23) and in response to SK/SD antigens (7–9). *In vitro* production of HRF after stimulation with haptenated or chemically modified proteins has not previously been reported.

Diisocyanate-enhanced HRF production was found in both antibody and non–antibody-producing subjects. Diisocyanate enhancement of HRF was significantly associated with a diagnosis of confirmed OA. Diisocyanate-HSA–induced HRF activity exceeded spontaneous HRF activity (attributable to media alone), as well as that produced by the HSA protein carrier alone. This latter finding demonstrated that there was significant HRF activity that could be attributed to the diisocyanate haptenic determinants. Specific diisocyanate-HSA enhancement of HRF was not observed in the unexposed or asymptomatic diisocyanate-exposed control groups. Diisocyanate-specific HRF was not detected in a group of three workers with suspected OA in whom the diagnosis was not confirmed based on specific inhalation tests or by monitoring of lung function in the workplace.

Antigen specificity of the HRF response was also demonstrated in two short ragweed-sensitized and one phthalic anhydride (PA)-sensitive OA control subject who showed HRF stimulation in response to ragweed antigen and PA-HSA, respectively.

No association was found between the presence of diisocyanate serum-specific antibodies and HRF, nor between the presence of antibody and confirmed OA. This finding is consistent with a recent study of 243 MDI-exposed workers in which OA was confirmed in three workers who did not exhibit elevated specific antibody levels (24). In this study, OA appeared to correlate better with HRF than with specific antibody status. In contrast, Cartier and colleagues suggested that the presence of elevated specific IgG (25) is significantly associated with diisocyanate asthma confirmed by a positive specific inhalation challenge. Antibodies were found in exposed asymptomatic as well as symptomatic workers and therefore lacked specificity for diagnosing occupational asthma. Our data suggest that specific immune responses involving cellular rather than humoral immune reactions may be more relevant to understanding the pathogenesis of diisocyanate-induced asthma.

Studies of purified cell subpopulations confirmed in a single worker (Subject 6) that T cells produced diisocyanate-specific HRF. Because of the high spontaneous release by T cells of two other patients with OA, one of whom also had specific IgE antibodies to HDI-HSA, antigen-specific HRF responses of T cells were not evaluable. Interestingly, previous studies of PBMC-derived HRF identified that B cells rather than T cells spontaneously produce

HRF (11), although T cells have been identified as the primary source of mitogen-stimulated (Con A and PHA) HRF (9–11) and SK/SD-stimulated HRF (26). None of these studies reported spontaneous production of HRF by purified T cells as observed in Workers 7 and 8. In these individuals, antigen-induced HRF was less than that spontaneously produced by T cells and was suggestive of an inhibitory effect. A possible interpretation of this phenomenon is that T cell activation by antigen induced an inhibitor of spontaneous synthesis and concomitantly enhanced HRF production in a typical dose-response fashion (18).

In the antibody-producing patient, purified adherent cells or macrophages also appeared to contribute to antigen-enhanced HRF synthesis. In contrast to T cells, total lymphocytes and adherent cells from the antibody-producing patient showed no spontaneous release and a dose-related HRF response to antigen. This suggested that antigen-driven HRF in this patient was being produced primarily by adherent cells and B cells. Thus, it is apparent from these limited studies that diisocyanate-induced HRF is produced by a diversity of cells and that sources of this cytokine are variable from worker to worker.

A variety of cytokines have been demonstrated to possess histamine releasing activity (6). These include MCAF, RANTES, and interleukin-3, which are members of the intercrine family of cytokines (14, 15). Of these, MCAF is produced by monocytes; RANTES is produced by T cells (27). The molecular and biologic characteristics of diisocyanate-induced HRF(s) remain to be defined. Because of the observation that HRF activity was demonstrated in all experiments with nonatopic donor basophils, it is unlikely that this particular HRF is IgE dependent (28). It is unknown whether our HRF could have other biologic effects that could augment asthmatic responses. One intercrine, RANTES, possesses HRF activity (15) as well as chemotactic properties for eosinophils (29).

In conclusion, HRF was detected in subjects with confirmed OA after specific stimulation of PBMC with appropriate diisocyanate antigens. In selected workers, lymphocytes as well as adherent cells were identified as sources of HRF cytokines. The demonstration of diisocyanate-specific HRF suggests that cellular immune mechanisms play an important role in the pathogenesis of diisocyanate asthma. In the future, such cytokines could serve as useful biomarkers of OA.

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