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Characterization of histamine releasing factors in diisocyanate-induced occupational asthma

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Abstract

Immunologic mechanisms contributing to diisocyanate-induced occupational asthma (OA) are poorly defined. There is a relatively low incidence of diisocyanate-specific IgE antibody responses. The frequent occurrence of delayed onset asthmatic responses in workers with diisocyanate asthma suggests a role for cellular immune mechanisms. We have shown in vitro production of antigen-specific mononuclear cell-derived histamine releasing factors (HRF) by peripheral blood mononuclear cells (PBMCs) of workers with OA. Monocyte chemoattractant protein-1 (MCP-1) and RANTES (acronym for "regulated on activation normal T expressed and secreted") are chemokines found in PBMC supernatants that express HRF activity. Diisocyanate-exposed workers were tested for diisocyanate antigen-stimulated enhancement of HRF, MCP-1, and RANTES production in supernatants of PBMCs and for serum specific IgE and IgG antibody levels to diisocyanate antigens bound to human serum albumin (HSA). PBMCs of workers with diisocyanate OA showed significantly increased production of antigen-specific HRF activity and MCP-1 (>300 ng/ml) compared to diisocyanate-exposed asymptomatic workers ($P < 0.05$). Antigen-stimulated enhancement of MCP-1 mRNA was demonstrated by reverse-transcription PCR. RANTES mRNA and chemokine secretion (<1 ng/ml) was also demonstrated in PBMCs, but did not show antigen enhancement in OA workers. Hapten specificity for the diisocyanate chemical to which a patient had been exposed was demonstrated for HRF enhancement and for IgG antibody reactions, but not for IgE reactions. HRF production was demonstrated in PBMC subpopulations, including lymphocytes and purified T cells. OA subjects showed increased $CD8^+$ cells by immunofluorescence (mean $CD4^+$:$CD8^+ = 1.2 \pm 0.2$). The results suggest that diisocyanate antigen enhancement of HRF and MCP-1 production are stimulated by hapten-specific T cell reactions. Since a weak association has been found between IgE antibody synthesis and induction of diisocyanate OA, the role of T cell cytokines and chemokines in the pathogenesis of OA requires further investigation.

Keywords: Histamine releasing factors; Chemokine; Occupational asthma; Diisocyanate; MCP-1; RANTES

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1. Introduction

Histamine releasing factors (HRF) were first described as substances causing histamine release from basophils, that were generated by human peripheral blood mononuclear cells (PBMCs) after stimulation by Concanavalin A or streptococcal SK/SD antigen (Thueson et al., 1979). Subsequent studies suggested a role for HRF as soluble mediators in the late allergic reaction (LAR), that occurs hours after the early phase of an IgE-mediated reaction; since the LAR is characterized by an inflammatory cellular exudate (Lichtenstein, 1988; Kaplin et al., 1991). Increased production of PBMC-derived HRF was also implicated as an in vitro correlate of the clinical status of allergic asthma (Alam et al., 1987; Kuna et al., 1989), allergic rhinitis (Brunet et al., 1992) and atopic dermatitis (Sampson et al., 1989).

HRF activity was found to be expressed by known cytokines (GM-CSF, IL-3, IL-8) that were less potent as basophil activators compared to PBMC-derived HRF. The molecular structure of HRF remained elusive until investigations revealed that prodigious HRF activity is associated with low molecular weight proteins of the β- or C-C chemokine subfamily, which have chemotactic and activating properties for monocytes, eosinophils, basophils, and lymphocytes. Chemokine synthesis can be induced in immunologically active cells (lymphocytes, monocytes) and non-immunologically active cells (epithelial, endothelial, smooth muscle, fibroblasts, platelets), but their mechanism of action is highly selective for subsets of inflammatory cell types that express chemokine-specific receptors (Oppenheim et al., 1991; Baggiolini et al., 1994). Basophil activating chemokines are believed to account for at least 50% of HRF activity in PBMC supernatants. These include monocyte chemoattractant proteins (MCP-1, MCP-3), RANTES, and MIP-1α. MCP-1 has high potency for stimulation of basophil exocytosis similar to that of anti-IgE (Alam et al., 1992; Kuna et al., 1992; Bischoff et al., 1992), is a major chemoattractant for monocytes, basophils, $CD4^+$ and $CD8^+$ T cells, but is non-chemotactic or activating for eosinophils, since they do not express MCP-1 receptors (Baggiolini et al., 1994, Loetscher et al., 1994). RANTES is a relatively weak exocytotic stimulus for basophils, but is a chemoattractant for basophils, eosinophils, monocytes and $CD4^+$ memory T cells (Schall et al., 1990). MCP-3 expresses the combined activities of both MCP-1 (Alam et al., 1994b) and RANTES (Dahinden et al., 1994). MIP-1α is also a weak basophil stimulator, but has potent activity for mast cell exocytosis (Alam et al., 1994a), and preferentially attracts activated $CD8^+$ T cells (Taub et al., 1993).

Diisocyanate-induced occupational asthma (OA) is generally believed to be immunologically mediated, but cannot be readily explained as IgE-mediated allergic asthma since most patients do not produce diisocyanate-specific IgE antibody. An unusual feature of diisocyanate OA is the high frequency of late onset asthmatic reactions following bronchial challenge, that are similar to late phase allergic reactions, yet often occur in the absence of an early asthmatic reaction (Bernstein and Bernstein, 1993).

Basophils, eosinophils, monocytes, and T cells have all been implicated as effector cells of bronchial asthma and the LAR. HRF cytokines and chemokines have receptor-specific chemoattractant and activating activities that are independent of humoral antibody mechanisms. To evaluate cell-mediated immune mechanisms, we initiated investigations of the incidence, immunologic specificity, and cellular sources of HRF in diisocyanate OA. Our original studies demonstrated diisocyanate antigen enhancement of HRF in PBMC supernatants of workers with diisocyanate-induced asthma. The presence of antigen-specific HRF enhancement that we measured by the HRF bioassay was significantly associated with confirmed diisocyanate-induced asthma (Herd and Bernstein, 1994). In this paper, we summarize our findings in an extended group of 32 diisocyanate-exposed workers. We also studied MCP-1 and RANTES chemokine secretion as well as mRNA production by diisocyanate-HSA antigen-stimulated PBMCs in some of these subjects.

2. Materials and methods

2.1. Reagents

RPMI 1640, Hank's Balanced Salt Solution, glutamine, penicillin-streptomycin, HEPES, and phytohemagglutinin (M form) were obtained from GIBCO Laboratories (Grand Island, NY); fetal bovine serum (FBS) from HyClone Laboratories, Inc. (Logan, UT); Dextran (200–300 average kDa), bovine serum albumin (Fraction V, crystalline), mouse monoclonals: anti-CD3-FITC, clone UCHT-1, anti-human CD7, clone 3A1, anti-human CD8, clone UCHT-4, and FITC-goat anti-mouse IgG, FITC-rabbit anti-goat IgG, goat anti-human IgA, goat anti-human IgG, goat anti-human IgM, alkaline phosphatase-goat anti-human IgG, sodium pyruvate from Sigma Chemicals Co. (St. Louis, MO); goat anti-human IgE and alkaline phosphatase-rabbit anti-goat IgG from Kierkegard and Perry Laboratories (Gaithersburg, MD); human serum albumin (American Red Cross-tested) from Baxter Healthcare Corporation (Glendale, CA); Spectrapor dialysis tubing from Spectrum Medical Industries (Los Angeles, CA); Cedarlane Low-Tox-H rabbit complement and anti-human B cells (anti-mouse IAk alloantiserum), FITC-labeled, from Cedarlane Laboratories, Ltd, Accurate Chemical and Scientific Corp. (Westbury, NY); LeucoPREP cell separation tubes, tissue culture plates, plastic petri dishes from Becton Dickinson and Company (Lincoln Park, NJ); histamine RIA immunoassay kits from Amac, Inc. (Westbrook, ME); RNAzol™ guanidinium thiocyanate reagent from Biotecx Laboratories, Inc. (Houston, TX); human rRANTES, human rMCP-1, polyclonal rabbit anti-RANTES, monoclonal mouse anti-RANTES, polyclonal rabbit anti-MCP-1 were purchased from R & D Systems (Minneapolis, MN); goat anti-rabbit (H+L), human and mouse adsorbed, from Southern Biotechnology Associates, Inc. (Birmingham, AL); the Superscript Preamplification System from Life Technologies, Inc. (Gaithersburg, MD); *Taq* polymerase from Promega Corp. (Madison, WI); primers for MCP-1 were synthesized by Operon, Inc. (Alameda, CA); primers for RANTES were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX).

2.2. Subjects

Thirty-seven subjects were tested for in vitro HRF production. Information on the study population is provided in Table 1. Nineteen workers presented with work-related asthmatic symptoms associated with diisocyanate exposure (group 1; 18 males, 1 female). OA was objectively confirmed in 16 group 1 workers by physiologic means. Seven workers underwent specific bronchial provocation testing (SBPT) in the laboratory using a single blind challenge method with TDI (< 20 ppb) (Moller et al., 1986). Workplace challenges were carried out by monitoring of peak expiratory flow rates (PEFR) every 2–3 h (5–6 measurements/day) under careful supervision during waking hours while there was active exposure to diisocyanates and for variable periods away from the workplace (e.g. 2 week while at work and for 2 week while away from the workplace) or by measuring cross shift changes in forced expiratory volume at one second (FEV_1) during active diisocyanate exposure. The PEFR studies were considered to be significant if there was greater than 15% variability in the amplitude percentage of the mean (highest reading − lowest reading/mean for 24 h) on 2 out of 10 days at work with no changes on days away from work (Bernstein et al., 1993) or significant decreases in FEV_1 ($\geq 20\%$ from pre-exposure baseline) over the workshift. Asymptomatic exposed workers consisted of thirteen MDI foam operation factory workers (group 2; 3 females, 10 males). Normal control subjects were nonatopics and reported no known exposure to diisocyanates (group 3; 3 males, 2 females).

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

2.3. Diisocyanate antigens

Hexamethylene diisocyanate (HDI)-, methylene diphenyl diisocyanate (MDI)-, and toluene

Table 1
Description of diisocyanate-exposed workers and control subjects

Worker	Exposure			Clinical diagnosis	Bronchial challenge	AB[c]	HRF[d]	MCP-1[e]
	Chemical	Duration (months)[a]	Cessation (months)[b]					
Group 1: symptomatic workers								
1	MDI	336	38	+History OA	– (lab)[f]	IgE IgG	–	NT
2	MDI,TDI	84	0	+History OA	– (lab)	IgE IgG	–	NT
3	MDI,TDI	48	19	OA	+(lab) LAR[g]	IgG	+	+
4	MDI,TDI	33	0	OA	+(lab) LAR	–	+	NT
5	MDI	24	23	+History OA	– (lab)	IgG	–	NT
6	TDI	Not known		OA	+(lab) LAR	IgG	+	NT
7[h]	HDI	17	8	OA (urticaria)	+(lab)	IgE IgG	+	NT
8	MDI	25	10	OA	+(work)[i]	–	+	+
9	HDI,MDI	100	32	OA	+(work)	–	–	+
10[h]	HDI	60	1	OA	+(work)	IgE IgG	+	–
11[h]	HDI	240	19	OA	+(work)	–	–	+
12	HDI	39	0	OA	+(work)	–	+	NT
13	HDI	12	11	OA	+(work)	IgE IgG	–	+
14[h]	HDI,MDI	60	20	OA	+(work)	IgE	+	+
15	MDI,TDI	33	3	OA	+(work)	IgG	+	NT
16	HDI	12	11	OA	+(work)	IgE IgG	NT	NT
17[h]	HDI	36	0	+History OA	NT	–	+	+
18	MDI	24	0.5	+History OA	NT	–	+	NT
19	HDI	48	2	+History OA	NT	–	+	NT
Group 2: asymptomatic exposed workers								
20–32	MDI	variable	0	No symptoms	NT	2/13 IgE 1/13 IgG	–	–
Group 3: normal controls								
33–37	None			No symptoms	NT	–	–	–

NT, not tested; '–' = negative; '+' = positive.

[a]Total time of known exposure.

[b]Time elapsed since cessation of known exposure prior to clinical assessment.

[c]Antibody reactions were considered positive when OD readings were greater than 3 standard deviations above the mean of negative controls in the ELISA test.

[d]HRF activity in PBMC supernatant was considered positive when diisocyanate antigen-enhanced histamine release was at least 7% greater than spontaneous release in the basophil histamine release test.

[e]MCP-1 was considered positive when diisocyanate antigen stimulation produced at least a two-fold increase in chemokine concentration in PBMC supernatant.

[f]Single blind controlled laboratory challenge with TDI (< 20 ppb).

[g]Late airways reaction only, suggestive of isolated late phase response.

[h]Skin test positive.

[i]Workplace challenges consisted of PEFR measurements during periods of worksite exposure compared to periods away from work or by measuring cross shift changes in FEV_1.

diisocyanate (TDI)-conjugated human serum albumin (HSA) antigens (HDI-HSA, MDI-HSA, TDI-HSA) were prepared (Tse and Pesce, 1979) and characterized (Gallagher et al., 1981) as previously reported. In brief, isocyanate reagents (Eastman Kodak Co., Rochester, NY), either 2.44 g TDI (tolulene-2,4-diisocyanate), 3.50 g MDI (methylene-di-*p*-phenyl diisocyanate), or 0.21 g HDI (hexamethylene diisocyanate), were mixed with 0.9 g HSA (human serum albumin, USP, Baxter Healthcare Corp., Glendale, CA) in a final volume of 92 ml phosphate-saline buffer (0.11 M sodium phosphate, 0.15 M NaCl, pH 7.4) and stirred under a chemical fume hood for variable periods of 15 min, 30 min, 1 h, and 8 h at room temperature (24 ± 1°C). Reactions were stopped by addition of 92 ml 2 M ammonium carbonate and centrifugation at 3000 × *g* for 20 min. The supernatants, consisting of soluble isocyanate-conjugated protein, were exhaustively dialyzed against four changes of 40 vols PBS (0.01M sodium phosphate, 0.14 M NaCl, pH 7.4), and sterilized by 0.2 μm membrane filtration. Conjugates were characterized for the isocyanate/protein mol ratio by quantitative spectrophotometric analyses with appropriate standard solutions, using a modified Gutman assay (Modesto and Pesce, 1973) for determination of TDI and MDI, a gas chromatographic method for amine bound HDI (hexamethylene diamine) using 25% Apiezon L plus 10% KOH coated on Chromosorb W-H.P. (60/80 mesh) (Sandridge, 1978) after acid hydrolysis of HDI-HSA (144 h, 6 N HCl, 110°C under vacuum), and the Bio-Rad Protein Assay (Bio-Rad Chemical Division, Richmond, CA) for HSA. Conjugates containing an average of 2–13 mol isocyanate per mol of protein were selected for study.

2.4. Isolation and characterization of PBMCs and subpopulations

Venous blood (90–120 ml) was collected at a final concentration of 0.01 M EDTA. Mononuclear cells were purified by layering 20–30 ml of anticoagulated blood onto 50 ml LeukoPREP tubes (Becton-Dickinson), containing 1.077 density gradient medium, and centrifuging at 2500 × *g* for 30 min. The upper layer consisting of plasma was saved for antibody tests, and mononuclear cells were collected from the interface. Cells were washed twice with Hank's Balanced Salt Solution (HBSS) by low speed centrifugation (200 × *g*) to deplete platelets.

Purified cell subpopulations were prepared as previously described (Herd and Bernstein, 1994). PBMCs, monocytes (plastic adherent cells), T cells, and total lymphocytes were analyzed by immunofluorescence reactions with fluorescein-labeled anti-CD3, anti-CD7, and anti-human immunoglobulins; and with anti-CD8 by indirect assay, using FITC-anti-mouse immunoglobulins as the second antibody. Relative cell numbers were determined as the percent of cells stained. $CD4^+$ cells were determined by subtraction of $CD8^+$ cells from $CD3^+$ cells.

2.5. Measurement of HRF

Methods used for generation and assay of diisocyanate antigen-induced HRF in PBMC supernatants have been described (Herd and Bernstein, 1994). Briefly, 5×10^6 cells in 1.0 ml complete medium (RPMI with 4 mM glutamine, 2 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% FBS) were added to wells of a 24-well cell culture plate and incubated with 50 μg of diisocyanate-conjugated HSA antigen (HDI-HSA, MDI-HSA, TDI-HSA, or HSA carrier alone) in 0.1 ml HBSS for 72 h, 37°C, 5% CO_2. Supernatants were removed and dialyzed (MW 3500 cutoff dialysis tubing) vs. 100 vols HBS (0.15 M NaCl, 10 mM HEPES, pH 7.4).

HRF activity was assayed by demonstrating increased basophil histamine release (HR) when normal donor basophils were stimulated with PBMC supernatants. Venous blood from a single non-atopic donor was collected with 10 mM EDTA anticoagulant, and red cells were sedimented with Dextran 200. Peripheral blood leukocytes (PBLs) were collected, centrifuged, and washed twice with HBS, 10 mM EDTA, 0.125% HSA by low speed centrifugation (200 × *g*) to remove platelets. Then, 0.3 ml of HRF supernatant was added to 1×10^6 PBLs in 0.1 ml HBS containing 2 mM calcium, 1 mM

magnesium, and 0.125% HSA. Cells were incubated for 1 h, 37°, in a shaking water bath, centrifuged and supernatants were collected for histamine analysis. Complete histamine release was achieved by boiling cells for 10 min. Histamine was quantitated by competitive inhibition using an enzyme linked immunosorbent assay (ELISA) (histamine EIA kit, Amac, Inc.). HRF activity was determined as % HR from the formula: [(experimental HR − spontaneous HR) (100)/complete HR − spontaneous HR].

Antigen enhancement of HRF by HDI-HSA, MDI-HSA, TDI-HSA, or HSA carrier alone was determined by subtraction of spontaneous HRF activity (in medium control PBMC supernatant) from antigen-stimulated HRF activity. HRF enhancement was considered positive when the test supernatant minus spontaneous release was $\geqslant 7\%$, based on the observation that HRF enhancement in normal control subjects never exceeded 7% HR. Hapten-specific (i.e. diisocyanate-hapten enhancement) HRF activity was determined by subtraction of HSA-stimulated HRF activity from diisocyanate-HSA antigen-stimulated HRF activity.

2.6. Quantitation of secreted MCP-1 and RANTES

Chemokine levels were measured in PBMC supernatants, from diisocyanate-exposed subjects, before and after challenge of PBMCs with diisocyanate-HSA antigen, using an HSA conjugate prepared with the diisocyanate chemical to which the subject had been exposed. MCP-1 was quantitated by competitive inhibition ELISA (Hornbeck, 1991), using microtiter plates coated with human rMCP-1 as the standard antigen and rabbit polyclonal anti-MCP-1 at 2 μg/ml, followed by goat anti-rabbit IgG, alkaline phosphatase-conjugated. RANTES was quantitated by antibody sandwich ELISA (Hornbeck, 1991), using microtiter plates coated with mouse monoclonal anti-RANTES (2 μg/ml), human rRANTES as the standard antigen, polyclonal rabbit anti-RANTES as the sandwich antibody, and goat anti-rabbit IgG, human and mouse adsorbed, alkaline phosphatase-conjugated for indirect assay. Data were analyzed for antigen enhancement of chemokine synthesis by subtraction of spontaneous chemokine production from antigen-stimulated chemokine production by PBMCs.

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total PBMC cellular RNA was extracted and isolated by a modification of the single-step acidic guanidinium method using RNAzol® B (Biotecx Laboratories, Inc.) and procedures recommended in the manufacturer's protocol. First strand cDNA was generated by reverse transcription using aliquots of 5 μg RNA and the Superscript Preamplification System kit (Life Technologies) according to the manufacturer's instructions. PCR amplification was performed using 5′ and 3′ primer sets by methods that have been described (Alam et al., 1994). Nucleotide sequences for the primers used were MCP-1 primers: 5′-GATCTCAGTGCAGAGGCTCG -3′; 5′-TGCTTGTCCAGGTGGTCCAT-3′ and RANTES primers: 5′-GCTGTCATCCTCAT-TGCTAC-3′; 5′-TCTCCATCCTAGCTCATC-TC-3′ PCR products were separated by 3% agarose gel electrophoresis (Nusieve 3:1) and identified by molecular size (171 bp for MCP-1; 260 bp for RANTES) using φX174/*Hae*III molecular weight markers, and cDNA standards for MCP-1 and RANTES. Semi-quantitative PCR was carried out by amplification of MCP-1 cDNA in the presence of 0.12 μCi ^{32}P-labelled dCTP/25 μl reaction mixture. X-ray film (Kodak XAR-5 film) was exposed to the gel for 3 h and developed. The MCP-1 gel bands, identified from the autoradiogram, were then cut out and counted for ^{32}P incorporation. Mitogen and antigen enhancement of MCP-1 mRNA synthesis was analyzed by quantitation of ^{32}P dCTP incorporation in MCP-1 PCR products derived from PBMCs cultured 18 h in medium containing PHA or HDI-HSA stimulator (50 μg/ml/10^6 cells). Percent increase due to stimulation was determined from the formula: [(counts/min from stimulated cells − counts/min from non-stimulated cells) (100)/counts/min from non-stimulated cells (i.e. cultured in medium only)].

2.8. Serum antibody

IgE and IgG antibody levels to HDI-HSA, MDI-HSA, TDI-HSA, and HSA were determined by isotype-specific indirect ELISA tests (Liss et al., 1988; Sarlo et al., 1990). A monitored well assay procedure was used, in which all reactions were terminated with 1 N NaOH when a standard positive control serum achieved an $OD_{410\,nm}$ of 0.6. Sera producing an OD reading ≥ 0.1 and a reaction greater than three standard deviations above the mean of sera from six non-exposed control subjects were considered positive.

2.10. Data analysis

Data are expressed as mean ± S.E.M. Group comparisons were made using the Mann Whitney test. The Wilcoxon signed-ranks test was used for paired samples within groups. Fisher's exact test was used to analyze categoric data of subject populations. A P value ≤ 0.05 was considered significant.

3. Results

3.1. HRF activity in symptomatic subject populations

Clinical characteristics of workers with a history compatible with occupational asthma are shown in Table 1. The mean values for HRF in group 1 subjects were greater than for asymptomatic exposed or non-exposed control subjects after stimulation of PBMCs with phytohemagglutinin (PHA), and diisocyanate antigens (Table 2). Antigens used were human serum albumin conjugates of hexamethylene diisocyanate (HDI-HSA), methylene diphenyl diisocyanate (MDI-HSA), and 2,4-toluene diisocyanate (TDI-HSA). Twelve of 18 symptomatic workers showed HRF enhancement when PBMCs were stimulated with HSA antigen conjugate prepared using the diisocyanate chemical(s) to which the worker had been exposed (Fig. 1). HRF enhancement by antigen was not observed in 13 MDI-exposed asymptomatic control workers or in five normal control subjects. Data analysis showed a significant association ($P < 0.05$) of HRF with a clinical diagnosis of OA confirmed by a positive laboratory or workplace challenge (Table 3). HRF was also associated with a positive history of OA ($P < 0.0001$). Specific IgE antibody production was not shown to be significantly associated with OA. Specific IgG antibody production was associated with a positive history of OA ($P < 0.0001$). No association was found between HRF and antibody production of either isotype.

Table 2
HRF Activity in PBMC supernatants of diisocyanate-exposed and control subjects[a]

	Group 1 (OA, $n = 19$)		Group 2 (asymptomatic/exposed, $n = 13$)			Group 3 (normal controls, $n = 5$)		
	Mean	S.E.M.	Mean	S.E.M.	P[b]	Mean	S.E.M.	P[b]
Spontaneous	18.0	3.74	13.1	1.30	NS	10.2	1.93	NS
PHA	30.3	4.59	25.3	3.90	NS	22.5	9.80	NS
HSA	21.1	3.68	11.9	2.26	NS	11.7	2.39	NS
HDI-HSA	23.7	4.21	NT	NT				
MDI-HSA	30.6	5.31	11.4	1.13	<0.01	11.5	2.25	< 0.05
TDI-HSA	31.1	6.24	NT			NT		
DIISO-HSA	33.8	5.29	11.4	1.13	<0.01	NA		

NT, not tested; NA, not applicable; NS, not significant.

[a]Statistical data of total HRF activity in PBMC supernatants after culture of cells in medium alone (spontaneous production) or with mitogen (PHA), human serum albumin (HSA) or diisocyanate-HSA-conjugated antigens (MDI-HSA, TDI-HSA, HDI-HSA, or Diiso-HSA, i.e. diisocyanate antigen(s) to which the subject had been exposed), at a dose of 50 μg/ml/10^6 cells. HRF activity is expressed as the mean = HR ± S.E.M., by PBLs treated with HRF supernatant.

[b]Significance of difference in means, versus group 1 (Mann-Whitney test, two-tailed P values).

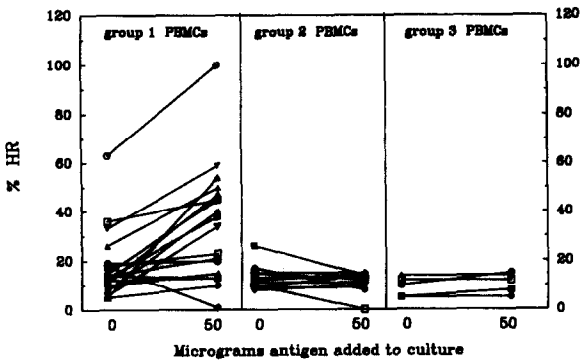

Fig. 1. Effects of antigen on the generation of HRF activity by PBMCs from 36 subjects. Results are shown for group 1 (symptomatic) and group 2 (exposed, asymptomatic) responses to 0 and 50 μg diisocyanate-HSA antigen (HDI-HSA, MDI-HSA, or TDI-HSA) selected for the diisocyanate to which each worker was exposed in the workplace. If a worker was exposed to more than one diisocyanate, the workplace-relevant diisocyanate antigen producing the greatest HRF response is given. MDI-HSA results are shown for group 3 (non-exposed controls).

3.2. Specificity of the HRF response for diisocyanate exposure

HRF data for the 12 workers showing antigen-enhancement of HRF was analyzed to determine whether there was specificity for the diisocyanate to which the worker had been exposed. Diisocyanate hapten-specific enhancement was determined by subtraction of HSA-stimulated HRF activity from HRF activity stimulated by diisocyanate-HSA antigens. HRF hapten-specific enhancement was determined for the diisocyanate(s) to which the worker had been exposed and compared to hapten enhancement by the diisocyanate(s) to which the worker had not been exposed. Fig. 2 shows that HRF enhancement by diisocyanate-HSA conjugates and hapten-specific enhancement of HRF were both significantly greater when PBMCs were stimulated in vitro with the diisocyanate chemicals to which the worker had been exposed.

Specificity for diisocyanate exposure chemicals was also demonstrated in the IgG (but not in the IgE) humoral immune response to diisocyanate antigens. In workers producing antibody, the mean values for IgG antibodies reacting with the diisocyanate chemical to which workers had been exposed were greater than the mean values for antibodies reacting with diisocyanates to which workers had not been exposed (Fig. 3).

3.3. Antigen enhancement of PBMC-derived HRF chemokines

MCP-1, RANTES, and HRF were measured in PBMC supernatants from eight subjects with diisocyanate-induced asthma and from seven

Table 3
Associations of specific challenge and OA history studies with in vitro specific production of IgE, IgG and antigen-enhanced HRF[a]

Sample	n[b]	Categories	In vitro assays								
			HRF[c]			IgE			IgG		
			+	−	P	+	−	P	+	−	P
Subjects in group 1	15 (16)	Challenge positive	9	3	0.044	5	8	0.550	5	3	0.509
		Challenge negative	0	3		2	1		3	0	
Subjects in groups 1–3	36 (37)	Positive history OA	12	6	<0.0001	7	12	0.249	8	3	0.001
		Negative history OA	0	18		2	11		1	8	
Subjects in groups 1–3	36	HRF positive	12	0	NA	3	9	1.000	5	2	0.124
		HRF negative	0	24		5	19		4	9	

NA, not applicable; '−' = negative; '+' = positive.
[a]Fisher's exact test.
[b]Antibody data includes one worker in group 1 that was not tested for HRF.
[c]Diisocyanate-HSA enhancement of spontaneous HRF production.

Fig. 2. Specificity of antigen-stimulated HRF production for workplace relevant diisocyanates in 12 workers showing HRF enhancement by antigen. (A) Complete antigen: the mean values were obtained after subtraction of spontaneous HRF activity from diisocyanate-HSA- (50 μg) stimulated HRF activity. (B) Hapten component: the mean values were obtained after subtraction of HSA- (50 μg) stimulated HRF activity from diisocyanate-HSA- (50 μg) stimulated HRF. The amount of HRF stimulated by antigen conjugates containing the diisocyanate to which each subject was exposed (workplace-relevant) was compared to the amount of HRF produced to the diisocyanate(s) to which subjects were not exposed (non-relevant) by the Wilcoxon signs test and the level of significance is given as the two-tailed P value.

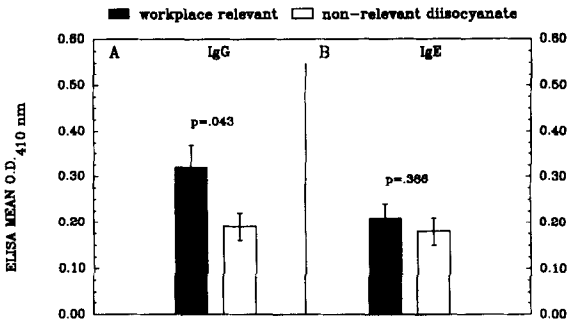

Fig. 3. Specificity of antibodies for workplace-relevant diisocyanates in, (A) 11 group 1 workers showing IgG antibody, and (B) seven group 1 workers producing IgE antibodies. The mean antibody levels were determined from optical density readings of ELISA test results. Antibody levels to the diisocyanate chemical to which each worker had been exposed (workplace-relevant diisocyanate) were compared to antibody levels to diisocyanate chemicals to which the worker had not been exposed (workplace non-relevant diisocyanate) by the Wilcoxon signs test and the level of significance is given as the two-tailed P value.

asymptomatic controls from groups 1 and 2, respectively. Symptomatic subjects showed higher mean production of antigen-stimulated MCP-1 (311 ng/ml) and HRF (>30% HR) than asymptomatic controls (Fig. 4). RANTES production was similar in OA subjects (1 ng/ml) and asymptomatic controls.

PBMCs of subject no. 13 with HDI-induced OA and a control subject (no. 35) were tested for mRNA for MCP-1 and RANTES by RT-PCR analysis (Fig. 5). PCR products, detected in ethidium bromide-stained gels, showed that mRNA for RANTES was present in PBMCs obtained at the time of cell isolation (T0) but was substantially decreased after 4 h of culture (T4). MCP-1 mRNA in PBMCs tested prior to culture was not detected in stained gels (T0), but was readily detected after 4 h culture. Enhancement of chemokine mRNA synthesis by mitogen or antigen, compared to spontaneous synthesis (Sp) by cells in medium alone, was not apparent for either subject by analysis of ethidium bromide-stained gels. However, quantitation of MCP-1 ^{32}P-labeled PCR products from 18-h cultures showed greater synthesis of antigen stimulated MCP-1 mRNA in the asthmatic subject, compared to the normal control subject (Table 4). HDI-HSA stimulation of PBMCs produced a 38% increase in MCP-1 mRNA from the asthmatic subject, compared to an 18% increase in mRNA from the normal subject.

3.4. Cellular origins of diisocyanate-induced HRF synthesis in PBMCs

HRF production by purified PBMC subpopulations was determined in four subjects, consisting of three workers (nos. 8, 10, 12) with MDI- or HDI-induced OA and one asymptomatic MDI-exposed subject (no. 20). Characteristics of total PBMCs and the purified cell populations, determined by flow cytometry and reactions with fluorescent antibody reagents are shown in Table 5. B cells were demonstrated in purified lymphocytes as cells bearing surface immunoglobulin markers (sIg^+). Purified T cells from the asymptomatic subject showed a normal $CD4^+$:$CD8^+$ ratio (2.2), while low $CD4^+$:$CD8^+$ ratios (1.0–1.4) were found in each of the OA patients, suggesting that $CD8^+$ (suppressor/cytotoxic) cells were increased.

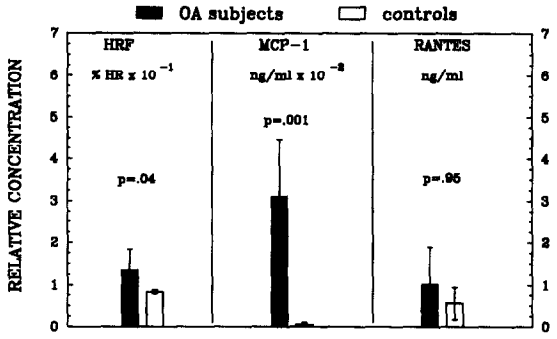

Fig. 4. Enhancement of HRF and chemokine production from PBMCs by diisocyanate-HSA conjugated antigens derived by subtraction of spontaneous production from diisocyanate-HSA- stimulated production. (A) HRF activity, measured as = HR in the basophil histamine release assay, and reduced by a factor of 10. (B) MCP-1 measured in ng/ml and reduced by a factor of 100. (C) RANTES measured in ng/ml. The mean values for eight patients with diisocyanate-induced asthma are compared to seven asymptomatic controls by the Mann-Whitney test and the level of significance is given as the two-tailed P values.

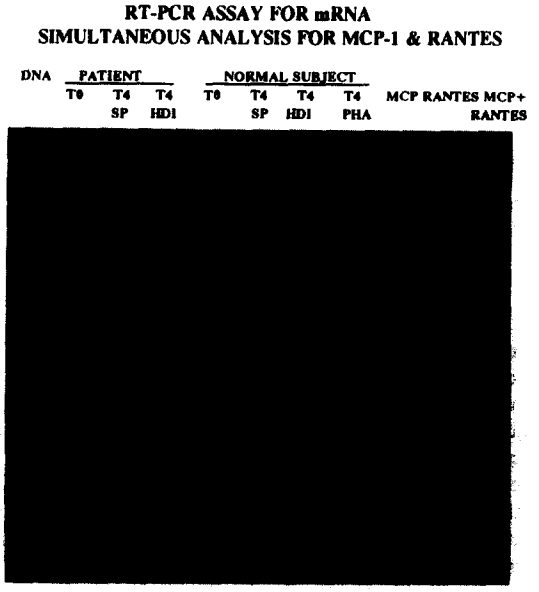

Fig. 5. Simultaneous analysis for MCP-1 and Rantes mRNA expression in PBMCs from a patient with diisocyanate-induced OA and a normal subject. PBMCs were tested prior to culture (T0) and after 4 h of culture (T4) in medium alone (RPMI, 5% FBS), or in medium containing mitogen (PHA) or antigen (HDI-HSA). mRNA was reverse-transcribed, and cDNA was amplified by PCR, using primers for MCP-1 and RANTES. The ethidium bromide-stained gel of PCR products shows that RANTES and MCP-1 mRNA were present in freshly isolated PBMCs and that cultured cells showed increased MCP-1 mRNA and decreased RANTES mRNA. Positive controls for the amplified products of MCP-1 (171 base pairs) and RANTES (260 base pairs) are shown.

The mean HRF response of the PBMC subpopulations is shown in Fig. 6. The results showed increased production of HRF by monocytes, lymphocytes, and T-cells. Purified T cells (B cell depleted) incubated in medium alone showed high spontaneous release of HRF. Reduced amounts of HRF were detected in diisocyanate antigen-stimulated T cell cultures, suggesting that T cell spontaneous release of HRF was downregulated by antigenic stimulation. Spontaneous release of T cell HRF was also reduced by PHA stimulation (data not shown). Spontaneous release of HRF did not occur in cultures of the whole lymphocyte preparations from which the T cells had been purified. It appears likely that non-T cells or cytokines generated in the lymphocyte cultures produced inhibition of spontaneous production of T cell HRF. Antigen specific enhancement of HRF was clearly demonstrated in whole lymphocyte cultures. Antigen enhancement of HRF was observed in only one monocyte culture, from subject no. 12 (results not shown). This monocyte preparation may have contained adherent B cells, since 10% of the cells showed immunofluorescent staining for surface immunoglobulin (Table 5). Alternatively, the sIg^+ reaction could have been due to cytophilic antibodies bound to monocyte Fc receptors, since subject no. 12 was an antibody-producing subject. T cell recognition of diisocyanate antigen was strongly suggested by the decreased production of spontaneous HRF in T cell cultures exposed to antigen, as well as by antigen enhancement of lymphocyte HRF in non-antibody-producing subjects (nos. 8 and 10).

4. Discussion

These studies have demonstrated that peripheral blood mononuclear cells (PBMCs) of

Table 4
PCR analysis of antigen and mitogen enhancement of MCP-1 mRNA synthesis in PBMCs of subject with HDI-induced OA

Subject/group	PBMC stimulator	Counts/min in PCR ^{32}P-MCP-1	% Increase in MCP-1
No. 35/group 3	Medium control	7027	0
	PHA	9424	34.1
	HDI-HSA	8347	18.8
No. 13/group 1	Medium control	9203	0
	PHA	13536	47.1
	HDI-HSA	12722	38.2

Peripheral blood mononuclear cells (PBMCs) (10^6/ml) were cultured in medium alone (RPMI, 5% FBS), or in medium containing mitogen (PHA) or antigen (HDI-HSA) for 18 h. MCP-1 mRNA was reverse-transcribed, and cDNA was amplified by the polymerase chain reaction (PCR) in the presence of ^{32}P-dCTP. ^{32}P-MCP-1-amplified DNA formed a single band in agarose gel electrophoresis. MCP-1 gel bands were cut out and ^{32}P incorporation was measured as counts/min minus background. Percent increase in MCP-1 due to mitogen or antigen enhancement was determined from MCP-1 DNA using the formula: [(counts/min from stimulated PBMCs − counts/min from medium control PBMCs) × 100]/counts/min from medium control PBMCs.

workers with confirmed diisocyanate-induced occupational asthma can be stimulated in vitro with diisocyanate-HSA antigens to produce basophil-activating histamine releasing factors (HRF) and monocyte chemoattractant protein 1 (MCP-1). Our previous studies of 19 subjects, that had been exposed to hexamethylene diisocyanate (HDI), methylene diphenyl diisocyanate (MDI), or toluene diisocyanate (TDI), showed significant antigen enhancement of HRF production in workers with clinical histories consistent with diisocyanate-induced occu-

Table 5
Characteristics of PBMCs and purified cell subpopulations

		Percentage of cells tested			
Subject no.		20	8	10	12
PBMC profile[a]					
Lymphocytes		78.6	74.7	85.2	77.5
$CD3^+$		70.0	80.0	79.0	77
$CD7^+$		81.0	72.0	78.0	73
Monocytes		16.2	19.9	13.5	18.8
Granulocytes		5.2	5.4	1.3	3.7
Purified subpopulations[b]					
Lymphocytes	sIg^+	<1	<1	7.0	5.0
T cells	$CD3^+$	>99	>99	>99	>99
	sIg^+	<1	<1	<1	<1
	$CD4^+$:$CD8^+$[c]	2.2	1.4	1.2	1.0
Monocytes	sIg^+	<1	<1	<1	10.0

[a]Peripheral blood mononuclear cells (PBMCs) were purified by density gradient sedimentation. The cellular profile of PBMC preparations (lymphocytes, monocytes, granulocytes) was determined by the use of a flow cytometer.

[b]Monocytes were purified by adherence to plastic tissue culture dishes. Non-adherent cells (lymphocytes) were treated with antisera to immunoglobulins plus complement to obtain B cell-depleted lymphocytes (T cells). Specific immunofluorescence reactions were used to detect T cell surface antigens (CD3, CD7, CD8) and B cell surface immunoglobulin (sIg^+).

[c]The $CD4^+$:$CD8^+$ ratio was determined as: $CD3^+$ cells minus $CD8^+$ (equal to $CD4^+$ cells) divided by $CD8^+$ cells.

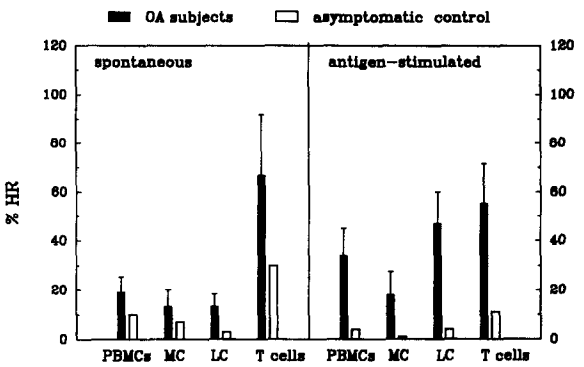

Fig. 6. HRF production (% HR) by unfractionated PBMCs and monocytes (MC), lymphocytes (LC), and T cells purified from PBMCs of four diisocyanate exposed workers. Cell supernatants were tested for HRF after 18-h incubation either in medium alone (spontaneous) or in medium containing diisocyanate-HSA antigen (antigen-stimulated). Mean HRF production by cells from three subjects with diisocyanate-induced OA is compared to HRF production by cells from an asymptomatic control.

pational asthma (OA), compared to exposed asymptomatic workers or non-exposed controls. In workers with a positive history of OA, antigen-specific HRF enhancement was shown to be associated with a positive response to specific bronchial provocation testing or to workplace challenge (Herd and Bernstein, 1994). In this paper, our previous findings have been confirmed in a larger group of 32 workers.

Immunological factors that contribute to diisocyanate-induced asthma have not been defined. The disease develops in about 5% of exposed workers, and atopy has not been shown to be a risk factor (Bernstein and Bernstein, 1993). Most cases of diisocyanate-induced OA are not mediated by IgE antibodies (Cartier et al., 1989). Despite the finding that 37% of workers in our symptomatic study population produced IgE antibodies and 58% produced IgG antibodies, there was no association between antibody production and confirmed OA.

Previous studies in sensitized workers have demonstrated humoral cross-reactions between different diisocyanate antigens (Baur, 1983). Interestingly, we found that HRF responses in individual workers were elicited by antigen conjugates containing occupationally relevant diisocyanates (i.e. determined to be present at the subject's worksite) as well as non-relevant diisocyanates. However data analysis showed that the magnitude of the mean HRF response to occupationally relevant diisocyanates was significantly greater than the HRF response to non-relevant diisocyanates, to which workers were not exposed. The hapten-specific component of the HRF response also showed greater specificity for occupationally relevant diisocyanates. Four of the symptomatic workers (nos. 3, 7, 8, 14) in this study showed antigen specific HRF responses after they had been removed from work exposure for 8–20 months. Thus, the HRF response in diisocyanate-induced asthma shows memory and specificity indicative of a cell-mediated immune mechanism.

Our studies of the cellular sources of HRF showed that lymphocytes were the most active producers of antigen-stimulated HRF. Purified T cells showed high spontaneous release, suggesting in vivo activation. No spontaneous release occurred in cultures of the whole lymphocyte preparations from which the T cells were purified. It is therefore possible that T cell production of HRF was inhibited or down-regulated by other cells in the whole lymphocyte preparations. Stimulation of purified T cells with antigen also had the effect of down-regulating HRF production. High spontaneous production of HRF by T cells has not previously been reported. In studies of purified cell populations from normal subjects or ragweed-sensitive asthmatics, monocytes and B cells were found to produce both spontaneous HRF and allergen-induced HRF, while T cells produced HRF only after stimulation with mitogen or SK/SD, a delayed hypersensitivity recall antigen (Goetzl et al., 1984; Alam et al., 1989; Turner et al., 1991). Of the chemically characterized cytokines with HRF activity that are known to be produced by lymphocytes, T and B lymphocytes have been reported to produce GM-CSF (Zupo et al., 1992) and MIP-1α, while T lymphocytes also produce RANTES and IL 8 (Baggiolini et al., 1994). We did not find that purified monocytes were a major source of antigen-stimulated HRF.

Quantitation of MCP-1 and RANTES showed diisocyanate antigen stimulation of MCP-1 secretion by PBMCs of subjects with diisocyanate-induced asthma, compared to asymptomatic control subjects. RANTES was present at 100-fold lower concentrations than MCP-1 in supernatants of PBMC cultures, and was not increased in PBMCs of symptomatic subjects, compared to asymptomatic subjects. Our limited investigations of chemokine mRNA synthesis in two subjects showed that RANTES mRNA was present at relatively high levels in freshly isolated PBMCs, compared to cultured PBMCs, and did not appear to be increased by specific antigen stimulation. MCP-1 mRNA appeared to be increased in cultured cells, compared to freshly isolated cells, and to show diisocyanate-specific stimulation in PBMCs of a symptomatic subject. The lower amount of HDI-HSA stimulation of MCP-1 mRNA that was found in normal human PBMCs is possibly due to the HSA carrier protein. Some normal and symptomatic subjects show a low level of HSA enhancement of HRF. MCP-1 is a major product of monocytes, and is not produced by lymphocytes (Baggiolini et al., 1994). Therefore, antigen-specific stimulation of MCP-1 in PBMC cultures would require either activation of monocytes by lymphokines, or cytophilic antibodies bound to Fc receptors.

Our observations suggested increased production of $CD8^+$ (suppressor/cytotoxic) T cells in the peripheral blood of diisocyanate OA subjects, as has previously been reported by others (Finotto et al., 1991). This finding is consistent with the chemokine profile that we demonstrated in PBMC supernatants. MCP-1 has chemoattractant activity for both $CD4^+$ and $CD8^+$ cells (Loetscher et al., 1994), while RANTES has been shown to be selectively chemotactic for $CD4^+$ memory T cells (Schall et al., 1990). Our studies do not rule out the possibility that other cytokines and chemokines, e.g. MIP-1α, which selectively recruits activated $CD8^+$ cells (Taub et al., 1993), may also contribute to HRF activity.

Increased numbers of $CD8^+$ T cells in diisocyanate-induced asthma may be relevant to the low incidence of IgE antibody synthesis in symptomatic workers. Cloned T cells derived from bronchial mucosa of diisocyanate asthmatics have been predominantly $CD8^+$ cells (82%) all showing production of IFN-γ (Maestrelli et al., 1994), which is known to suppress IgE antibody production in vivo (Finkelman et al., 1988) and to also increase MCP-1 gene expression (Gruss et al., 1994). Studies have suggested that the inhalation pathway of antigen exposure preferentially induces $CD8^+$ cells and $CD4^+$ Th-1 cells and results in selective suppression of IgE antibody (McMenamin and Holt, 1993).

The possibility that the induction of diisocyanate asthma is associated with MHC class I restricted T cell reactions requires further investigation. In animal models, hapten-specific T cells have been difficult to demonstrate, and it is generally assumed that T cells recognize epitopes of haptenated proteins in the context of a peptide/MHC ligand (Nalefshi and Rao, 1993). In addition, $CD8^+$ T cells generally have been shown to recognize endogenously synthesized antigens, while $CD4^+$ T cells recognize exogenous antigens (Brodsky and Guagliardi, 1991). However, $CD8^+$ cells with specificity for exogenous antigens have been demonstrated (Rock et al., 1993; Hisatsune et al., 1995). Whether hapten presentation by antigen presenting cells requires intracellular processing is currently unresolved, since peptide digests of haptenated proteins have shown T cell stimulation after binding to MHC class I molecules of glutaraldehyde-fixed cells (Ortmann et al., 1992).

HRF and related chemokines could contribute to the immunopathogenesis of diisocyanate-induced OA through their chemoattractant specificity for subsets of inflammatory cell types. Bronchial asthma is characterized by cellular infiltration of monocytes, T lymphocytes, and eosinophils into the bronchial mucosa (Azzawi et al., 1990; Poston et al., 1992). MCP-1 specifically attracts and activates monocytes, and has been shown to be present in the subepithelium of asthmatic airways at increased levels, compared to normal subjects (Sousa et al., 1994). RANTES is selective for eosinophils (Ebisawa et al., 1994) and has been found in the BAL fluid of asthmatics at higher than normal levels (Alam et al., 1994c). A comparative study of the bronchial

mucosa in diisocyanate-induced asthma and in atopic (extrinsic) asthma did not show any distinctive differences in the patterns of inflammatory cell infiltrates (Bentley et al., 1992). In bronchial biopsies, the basement membrane of asthmatics, compared to normals, showed significant increases in cell numbers of eosinophils and IL-2R$^+$ T cells, but not in numbers of CD3$^+$, CD4$^+$, or CD8$^+$ T cells, neutrophils, or macrophages. Other studies have shown increased numbers of total mononuclear cells, degranulated eosinophils and mast cells in the lamina propria, beneath the basement membrane, in subjects with diisocyanate OA, compared to normals (Saetta et al., 1992). It seems clear that eosinophils are major effector cells of diisocyanate-induced asthma. Our failure to induce antigen-stimulated RANTES secretion in cultured PBMCs does not preclude that this chemokine is produced locally in the airways or that other cytokines and mediators may be important in stimulation of eosinophilic migration and activation as a response to injury.

In conclusion, the HRF bioassay appears to detect a cell-mediated immune response to the diisocyanate hapten in subjects with diisocyanate-induced asthma. At least some of the long-lasting effects of diisocyanate-induced OA are likely to be explained as specific T cell sensitization to the diisocyanate hapten after chemical binding to an endogenous carrier protein.

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