

Diisocyanate antigen-enhanced production of monocyte chemoattractant protein-1, IL-8, and tumor necrosis factor- α by peripheral mononuclear cells of workers with occupational asthma

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Background: Previous studies have shown a significant association between confirmed diisocyanate-induced asthma (DOA) and in vitro production of diisocyanate antigen-stimulated histamine-releasing factors by PBMCs. Chemokines found in PBMC supernatants are known to express histamine-releasing factor activity.

Objective: PBMCs of diisocyanate-exposed workers were tested in vitro for diisocyanate antigen-specific enhancement of monocyte chemoattractant protein-1 (MCP-1), monocyte chemoattractant protein-3 (MCP-3), macrophage inflammatory protein-1 α , RANTES, IL-8, and T-cell cytokines that could play a regulatory role in chemokine synthesis (IL-4, IL-5, IFN- γ , and TNF- α).

Methods: Secretion of chemokines and cytokines was determined by quantitative immunochemical assays of PBMC supernatants. Synthesis of mRNA for β -chemokines was determined by reverse transcription-polymerase chain reaction. **Results:** PBMCs of workers with DOA showed significantly enhanced secretion for MCP-1 compared with diisocyanate-exposed asymptomatic workers ($P < .05$). In vitro induction of antigen-stimulated MCP-1 mRNA synthesis in cultured PBMCs was demonstrated by reverse-transcription polymerase chain reaction. Quantitation of cytokines in supernatants showed increased mean production of IL-8 and TNF- α . IFN- γ , IL-4, and IL-5 were not enhanced in subjects with DOA.

Conclusion: Antigen stimulation of MCP-1 and TNF- α suggest that diisocyanate-specific cellular immune reactions result in activation of macrophages, which may be important in the pathogenesis of DOA. (*J Allergy Clin Immunol* 1998;102:265-74.)

Key words: Chemokines, occupational asthma, diisocyanate, monocyte chemoattractant protein-1, TNF- α

The diisocyanate class of chemicals is the most common cause of occupational asthma (OA) in industrialized countries.¹ Immunologic mechanisms that contribute to

Abbreviations used

DOA:	Diisocyanate-induced occupational asthma
HDI:	Hexamethylene diisocyanate
HRF:	Histamine-releasing factor
HSA:	Human serum albumin
MCP:	Monocyte chemoattractant protein
MDI:	Methylene diphenyl diisocyanate
MIP:	Macrophage inflammatory protein
OA:	Occupational asthma
³² P-dCTP:	[α - ³² P]-deoxycytidine triphosphate
RANTES:	Regulated on activation, normal T cell expressed and secreted
RT-PCR:	Reverse transcription-polymerase chain reaction
TDI:	Toluene diisocyanate

diisocyanate-induced occupational asthma (DOA) are poorly defined. The disease develops in about 5% of exposed workers, although atopy has not been shown to be a risk factor.² Although diisocyanate-specific IgE antibodies are produced by a subset of workers with DOA, they are absent in more than two-thirds of workers with DOA.³

Evidence has accumulated to suggest that cellular immune and inflammatory processes are important in DOA. Asthmatic responses that are elicited in affected workers are specific for diisocyanate exposure. Isolated late-phase inflammatory responses are very common.² Activated T lymphocytes have been demonstrated in workers with DOA after diisocyanate inhalation challenge.^{4,5} Bronchial biopsy specimens show features that are typically indistinguishable from those of extrinsic asthma, with increased numbers of total mononuclear cells, degranulated eosinophils, and mast cells in the lamina propria in subjects with DOA compared with normal subjects.⁶ Previously, our laboratory demonstrated diisocyanate antigen-specific induction of histamine-releasing factor (HRF) synthesis by PBMCs from subjects with DOA. Diisocyanate-antigen enhancement of HRF was shown to be associated with confirmed DOA, but was absent in control subjects without OA.^{7,8}

Among the cytokines found in PBMC supernatants that could account for HRF activity, the β -chemokines or CC chemokines, which have chemotactic and activating properties for monocytes, eosinophils, basophils, and

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lymphocytes, are the most plausible.⁹ These include monocyte chemoattractant proteins (MCP-1, MCP-2, MCP-3, and MCP-4); regulated on activation, normal T cell expressed and secreted (RANTES); and macrophage inflammatory protein-1 α (MIP-1 α). IL-8, a CXC chemokine with neutrophil and eosinophil chemoattractant activity, has weak histamine-releasing potency for IL-3 primed basophils, but also inhibits histamine release (HR) by CC chemokines.¹⁰ In addition to their receptor-specific chemotactic and degranulating properties, chemokines are also capable of providing costimulatory signals for T-lymphocyte activation.^{11,12} Thus chemokines could play a pivotal role in the inflammatory response of DOA through receptor-specific chemoattractant and activating properties for characteristic cell types.¹⁰

We therefore investigated antigen-induced synthesis of proinflammatory chemokines and cytokines by diisocyanate antigen-stimulated PBMCs obtained from workers with DOA. Chemokines (MCP-1, MCP-3, MIP-1 α , RANTES, and IL-8) and cytokines that are considered to play a pivotal role in immunologic airway inflammatory responses (IL-4, IL-5, IFN- γ , and TNF- α) were evaluated.

METHODS

Reagents

RPMI 1640, HBSS, glutamine, penicillin-streptomycin, and HEPES were obtained from GIBCO Laboratories (Grand Island, NY); FBS was obtained from HyClone Laboratories, Inc (Logan, Utah); BSA (Fraction V, crystalline), alkaline phosphatase-goat anti-human IgG, alkaline phosphatase mouse monoclonal anti-rabbit IgG, and sodium pyruvate were obtained from Sigma Chemicals Co (St. Louis, Mo); goat anti-human IgE and alkaline phosphatase-rabbit anti-goat IgG were obtained from Kierkegaard & Perry Laboratories, (Gaithersburg, Md); human serum albumin (HSA) (American Red Cross tested) was obtained from Baxter Healthcare Corporation (Glendale, Calif); Spectrapor dialysis tubing was obtained from Spectrum Medical Industries (Los Angeles, Calif); LeucoPREP cell-separation tubes, tissue culture plates, and plastic petri dishes were obtained from Becton Dickinson and Company (Lincoln Park, NJ); histamine RIA immunoassay kits were obtained from Immunotech, Inc (Westbrook, Me); RNAzol guanidinium thiocyanate reagent was obtained from Biotecx Laboratories, Inc (Houston, Tex); human rRANTES, human rMCP-1, human rMCP-3, human MIP-1 α , human rIL-8, polyclonal neutralizing rabbit anti-human RANTES, monoclonal mouse anti-human RANTES, polyclonal neutralizing rabbit anti-human MCP-1, anti-human MCP-3, anti-human MIP-1 α , and anti-human IL-8 were purchased from R & D Systems (Minneapolis, Minn); human IL-4, IL-5, IFN- γ , TNF- α , and ELISA kits were purchased from Endogen, Inc (Cambridge, Mass); 100 bp DNA ladder-READY LOAD and Superscript Pre-amplification System were obtained from Life Technologies, Inc (Gaithersburg, Md); Taq polymerase was obtained from Promega Corp (Madison, Wis); primers for MCP-1, MCP-3, and MIP-1 α were synthesized by Operon, Inc (Alameda, Calif); primers for RANTES were synthesized by Genosys Biotechnologies, Inc (The Woodlands, Tex); and [α -³²P] deoxycytidine triphosphate (³²P-dCTP), specific activity 3000 Ci/mmol was purchased from NEN Life Science Products (Boston, Mass).

Subjects

Twenty subjects (divided into 3 groups) were tested for in vitro cytokines. Group 1 consisted of 8 workers with work-related asth-

matic symptoms associated with diisocyanate exposure in whom DOA was objectively confirmed either by specific bronchial provocation testing in the laboratory with a single-blind challenge method with toluene diisocyanate (TDI) (< 20 ppb)¹³ or by monitoring of PEFs or FEV₁ during diisocyanate exposure at work.⁸ Group 2 consisted of 4 asymptomatic diisocyanate-exposed factory workers. Group 3 consisted of 8 nonexposed control subjects.

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.

Diisocyanate antigens

Hexamethylene diisocyanate (HDI)-, methylene diphenyl diisocyanate (MDI)-, and TDI-conjugated (HSA) antigens (HDI-HSA, MDI-HSA, and TDI-HSA) were prepared and characterized as previously reported, with an average 3:1 hapten-to-carrier molar ratio.^{8,14} HSA (Baxter Healthcare) and diisocyanate-HSA antigen conjugates were prepared at 5 mg/mL in 0.9% saline and sterilized by passage through a 0.22- μ m membrane filter.

Production and assay of HRF

Venous blood (90 to 120 mL) was collected at a final concentration of 0.01 mol/L EDTA. Mononuclear cells were purified by layering 20 to 30 mL of anticoagulated blood onto 50 mL LeucoPREP tubes (Becton-Dickinson) containing 1.077 density gradient medium and centrifuging at 2500 *g* for 30 minutes. Cells were washed twice with HBSS by low-speed centrifugation (200 *g*) to deplete platelets.

Previously described methods were used for generation and assay of diisocyanate antigen-induced HRF in PBMC supernatants.⁷ Briefly, 5×10^6 cells in 1.0 mL complete medium (RPMI with 4 mmol/L glutamine, 2 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 5% FBS) were added to wells of a 24-well cell-culture plate and incubated with 50 μ g of diisocyanate-conjugated HSA antigen (HDI-HSA, MDI-HSA, TDI-HSA, or HSA carrier alone) in 0.1 mL HBSS for 18 hours at 37° C in an atmosphere of 5% CO₂. Supernatants were removed and dialyzed (MW 3500 cutoff dialysis tubing) against 100 volumes HBS (0.15 mol/L NaCl and 10 mmol/L HEPES, pH 7.4). HRF activity was assayed by demonstrating increased basophilic HR when normal donor basophils were stimulated with PBMC supernatants. 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 7% or greater on the basis of the observation that HRF enhancement in 12 control subjects never exceeded 7% HR.

Quantitation of secreted cytokines

Chemokine levels in PBMC supernatants were measured before and after in vitro culture of PBMCs in medium alone or with the diisocyanate chemical antigen (HSA conjugate) to which subjects had been exposed. MCP-1, MCP-3, MIP-1 α , and IL-8 were quantitated by competitive inhibition ELISA¹⁵ modified for indirect assay by using microtiter plates coated with 25 ng/mL human recombinant chemokine as the standard antigen and rabbit polyclonal anti-chemokine antibody at 2 μ g/mL followed by alkaline phosphatase-conjugated mouse monoclonal anti-rabbit IgG. The lower limit of detection of chemokines by competitive inhibition assay was less than 70 pg/mL. RANTES was quantitated by double-antibody sandwich ELISA¹⁵ modified for indirect assay by using microtiter plates coated with mouse monoclonal anti-RANTES (2 μ g/mL), human rRANTES as the standard antigen, polyclonal rabbit anti-RANTES (1 μ g/mL) as the sandwich antibody, and alkaline

TABLE I. Description of diisocyanate-exposed workers and control subjects

Worker nos.	Exposure			Clinical diagnosis	Bronchial challenge	AB‡	MCP-1§	IL-8§	TNF-α§
	Chemical	Duration (mos)*	Cessation (mos)†						
Group 1: Symptomatic workers									
1	MDI, TDI	48	19	OA	+(lab) , LAR¶	IgG	+	+	—
2	MDI	25	10	OA	+(work)#	—	+	+	+
3	HDI, MDI	100	32	OA	+(work)	—	+	+	+
4	HDI	60	1	OA	+(work)	IgE, IgG	—	+	+
5	HDI	240	19	OA	+(work)	—	+	+	—
6	HDI	12	11	OA	+(work)	IgE, IgG	+	+	+
7	HDI, MDI	60	20	OA	+(work)	IgE, IgG	+	—	—
8	HDI	36	0	OA	NT	—	+	+	—
Group 2: Asymptomatic exposed workers									
9-12	MDI	Variable	0	No symptoms	NT	—	—	+(n = 2), -(n = 2)	—
Group 3: Asymptomatic nonexposed control subjects									
13-20	None	NA	NA	No symptoms	NT	—	—	—	—

*Total time of known exposure.

†Time elapsed since cessation of known exposure before clinical assessment.

‡Antibody reactions were considered positive when OD readings were greater than 2 SD above the mean of negative controls in the ELISA test.

§Cytokines were considered positive when diisocyanate-antigen stimulation produced at least a 2-fold increase in cytokine concentration in PBMC supernatant.

||Single-blind controlled laboratory challenge with TDI (< 20 ppb).

¶Late airways reaction only (24% decrease from prechallenge baseline).

#Workplace challenges consisted of PEFr measurements or measurements of cross-shift changes in FEV₁ during periods of worksite exposure compared with periods away from work. A 20% decline in either parameter from preworkshift baseline during diisocyanate exposure with a decrease of less than 10% on nonexposure days was considered a positive challenge.

AB, Antibody; NT, not tested; NA, not applicable.

phosphatase-conjugated mouse monoclonal anti-rabbit IgG for indirect assay. The lower limit of detection of RANTES by the antibody sandwich assay was less than 10 pg/mL.

TNF-α, IL-4, IL-5, and IFN-γ were measured with human ELISA kits from Endogen, Inc (Cambridge, Mass), according to the manufacturer's instructions. The lower limit of detection of the assays were verified to be as follows: TNF-α, less than 5 pg/mL; IL-4, less than 2 pg/mL; IL-5, less than 2 pg/mL; and IFN-γ, less than 2 pg/mL.

Data were analyzed for antigen enhancement of chemokine and cytokine synthesis by subtraction of spontaneous production from antigen-stimulated production by PBMCs. An antigen-enhanced chemokine or cytokine response was defined as supernatant levels that were 3 SD above the mean level obtained for the asymptomatic control group.

Inhibition of HRF by antichemokine antibodies

To obtain adequate amounts of supernatant for inhibition studies, PBMCs from a patient with HDI-induced OA were prepared at 1×10^6 cells/mL in RPMI complete medium containing 20 U/mL rhIL-2 and 10 μg/mL HDI-HSA and cultured without refeeding for 8 days in an atmosphere of 5% CO₂. PBMC supernatant (0.1 mL) was mixed with 0.1 or 0.05 mL of 1 mg/mL polyclonal neutralizing antichemokine antibody (either anti-rMCP-1, anti-rMCP-3, anti-rRANTES, anti-rMIP-1α, or anti-rIL-8) and added to 1 mL of pooled peripheral blood leukocytes from 3 atopic donors. The

basophil HR test was performed as previously described and results were expressed as percent of HR.⁷ Percent inhibition was determined as follows: $100 \times (1 - \text{HR in presence of antibody} / \text{HR in absence of antibody})$. Neutralizing antibodies were purchased from R & D Systems (Minneapolis, Minn) as chemokine-specific antibodies, with the exception of anti-MCP-3, which showed cross-reactivity (< 6%) with MCP-1 or MCP-2.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total PBMC cellular RNA was extracted and isolated by a modification of the single-step acidic guanidinium method with RNAzol B (Biotecx Laboratories, Inc) and procedures recommended in the manufacturer's protocol. First-strand cDNA was generated by reverse transcription with 5-μg aliquots of RNA and the Superscript Preamplification System kit (Life Technologies), according to the manufacturer's instructions. PCR amplification was performed by using 5' and 3' primer sets by methods that have been described.¹⁶ PCR products were separated by 3% agarose gel electrophoresis (Nusieve 3:1) and identified by molecular size (570 bp for β-actin, 171 bp for MCP-1, 174 bp for MCP-3, 260 bp for RANTES, and 254 bp for MIP-1α) by using φX174/Hae III molecular weight markers and cDNA standards for MCP-1, MCP-3, MIP-1α, and RANTES. Semiquantitative PCR was carried out by amplification of cDNA in the presence of 1 μCi [α-³²P]-deoxycytidine triphosphate (³²P-dCTP)/25-μL reaction mixture. ³²P was identified from

TABLE II. Demographic data of subject population

Subject	Current asthma symptoms (+ or -)*	Sex	Age (yrs)	Drugs	FEV ₁
Group 1: Subjects with DOA					
1	+	M	58	Theophylline, albuterol	2.75
2	+	M	35	None	3.85
3	+	M	36	None	3.61
4	+	M	28	None	4.33
5	+	M	38	ICS, BD	2.83
6	+	M	52	None	4.05
7	+	M	27	None	3.82
8	+	M	33	None	3.14
Mean $\pm$ SD					3.51 $\pm$ 0.57
Group 2: Exposed asymptomatic subjects					
9	-	M	32	None	4.04
10	-	M	49	None	4.79
11	-	M	31	None	3.66
12	-	M	36	None	4.63
Mean $\pm$ SD					4.28 $\pm$ 0.52
Group 3: Nonexposed asymptomatic subjects					
13	-	M	34	None	4.34
14	-	M	38	None	4.34
15	-	M	42	None	2.39
16	-	M	44	None	4.59
17	-	F	40	None	2.56
18	-	F	37	None	3.52
19	-	M	50	BD	2.91
20	-	F	38	None	3.12
Mean $\pm$ SD					3.47 $\pm$ 0.86

ICS, Inhaled corticosteroid; BD, β_2 -agonist bronchodilator.*Asthma clinically active (+) or inactive (-) at time of evaluation; diagnosis given because of 12% to 15% improvement from baseline in FEV₁ after bronchodilators, methacholine challenge, or both.

x-ray film (Kodak XAR-5 film) that was exposed to the gel for 3 hours and developed. The gel bands were counted for ^{32}P incorporation. For determination of relative differences in mRNA samples, preliminary titration analysis and comparative kinetic analysis showed that 40 PCR cycles were optimal for obtaining a linear dose-response relationship between amounts of cDNA used for PCR and amplified DNA products. Results were normalized to represent equivalent cDNA loading on the basis of ^{32}P -dCTP incorporation in β -actin bands. Antigen enhancement of mRNA synthesis was analyzed by quantitation of ^{32}P -dCTP incorporation in PCR products derived from PBMCs cultured for 18 hours in medium containing diisocyanate-HSA antigen conjugate prepared with the diisocyanate chemical to which the subject had been exposed (either MDI, TDI, or HDI [50 $\mu\text{g}/\text{mL}/10^6$ cells]). Percent increase caused by stimulation was determined by means of the following formula: (cpm from stimulated cells - cpm from nonstimulated cells)/(100)/cpm from nonstimulated cells (ie, cultured in medium only).

Serum antibody

Serum-specific IgE and IgG levels to HDI-HSA, MDI-HSA, TDI-HSA, and HSA were determined by isotype-specific indirect ELISA tests.¹⁵ Reactions were terminated when a standard positive control serum achieved an OD_{410nm} of 0.6. Sera producing an OD

reading of 0.1 or greater and a reaction greater than 3 SD above the mean of sera from 6 nonexposed control subjects were considered positive.

Data analysis

Data are expressed as means $\pm$ SEM. Group comparisons were made by using the Mann-Whitney test. Fisher's exact test was used to analyze categorical data of subject populations. A *P* value of .05 or less was considered significant.

RESULTS

Antigen enhancement of HRF and chemokine synthesis

Clinical characteristics of workers with DOA and antibody and cytokine responses are shown in Tables I and II. MCP-1, MCP-3, IL-8, RANTES, and MIP-1 α were measured in PBMC supernatants from 8 subjects with DOA and 12 control subjects after culture with diisocyanate-HSA antigen. Cells from exposed subjects were challenged with a diisocyanate-HSA conjugate specific for the diisocyanate chemical to which the subject had been

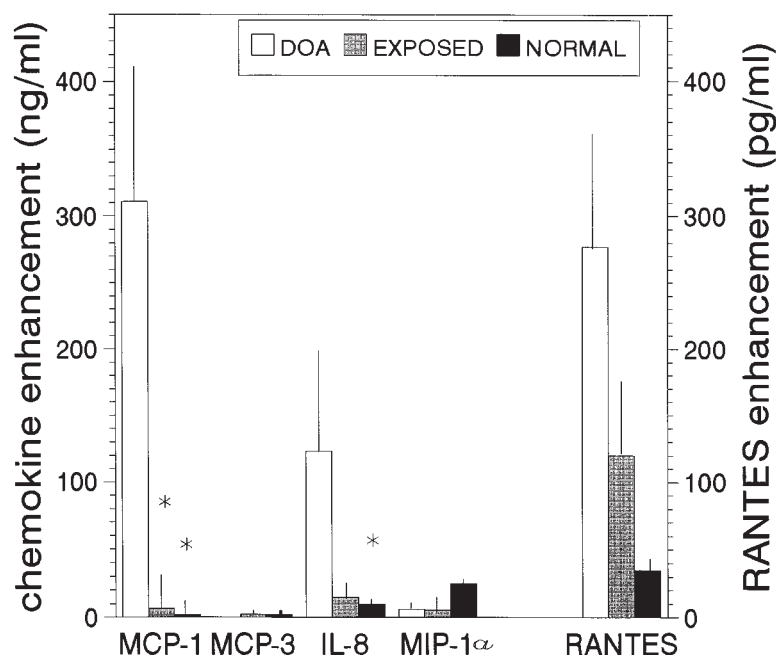


FIG. 1. Enhancement of MCP-1, MCP-3, MIP-1α, and RANTES secretion from PBMCs by in vitro culture with diisocyanate-HSA conjugated antigen for 18 hours. Enhancement is derived by subtraction of spontaneous production in media alone from diisocyanate-HSA-stimulated production. Mean values for 8 patients with DOA are compared with 4 asymptomatic diisocyanate-exposed workers and with 4 nonexposed normal subjects by Mann-Whitney test. Significant differences are indicated by asterisks ($P < .05$).

exposed. Cells from nonexposed control subjects were challenged with MDI-HSA. Data were analyzed for diisocyanate-HSA enhancement of chemokine production by PBMCs.

The DOA group showed greater total mean production of antigen-stimulated MCP-1 (345 ng/mL) and greater specific antigen enhancement (311 ng/mL) than asymptomatic exposed workers ($P < .05$) or nonexposed normal control subjects ($P < .001$) (Fig. 1). A positive association ($P < .05$, Fisher's test) was found between enhanced production of MCP-1 (ie, 3 SD above the mean of the control group) and confirmed DOA. Mean enhancement of HRF production was also significantly greater ($P < .05$) in the DOA group (16.25% HR) than in the exposed worker control group (0.7% HR). Mean IL-8 production in subjects with DOA was also significantly increased (128 ng/mL) above that of normal control subjects (10 ng/mL, $P < .05$) and increased above that of exposed worker control subjects (14.5 ng/mL), which did not show statistical significance because of the small sample size ($n = 4$). The group with DOA showed enhancement of MCP-3, MIP-1α, or RANTES that was similar to that of both asymptomatic control groups.

Mean total production of MCP-1 at 18 hours after stimulation with diisocyanate-HSA was remarkably high (345 ng/mL) compared with unstimulated cultures (23 ng/mL). The concentration of MCP-1 in PBMC supernatants increased in a time-dependent manner after in vitro exposure to mitogen or diisocyanate-HSA (Fig. 2). There was no such increase in medium alone, with unmodified HSA carrier protein, or with exposure to

HSA conjugated with an irrelevant hapten (ie, phthallic anhydride-HSA). Maximum production of antigen-stimulated MCP-1 was achieved at 24 to 48 hours and began to diminish at 72 hours after stimulation (results not shown). When cells were diluted from 5×10^6 /mL to 1×10^6 /mL and cultured in complete medium containing 20 U/mL IL-2 and 10 μg/mL diisocyanate-HSA antigen or PHA, production could be sustained or increased up to a period of at least 8 days. PBMC supernatant from an HDI-induced patient with OA was assayed at 8 days and found to contain 800 ng/mL MCP-1, 150 pg/mL RANTES, and showed 21% HR in the basophil HR test. This supernatant was used for HRF inhibition studies (see below).

Cells from group 1 and group 2 subjects were analyzed for expression of chemokine mRNA transcription at time 0 and after culture for 4 and 18 hours with antigen. Cellular RNA was extracted and reverse transcribed, and semiquantitative PCR was carried out in the presence of 0.12 μCi ^{32} P-labeled dCTP per 25 μL by using β-actin as an internal standard. PCR products showed that mRNA for MCP-3 was present at the time of cell isolation. RANTES was present at the time of cell isolation in 50% of the subjects tested but was substantially decreased after 4 hours (results not shown) or 18 hours of culture. MCP-3 and MIP-1α were increased. MCP-1 and MIP-1α mRNA were not detected in freshly isolated cells, but were detected after 4 and/or 18 hours of in vitro culture in both subject groups (Fig. 3). Enhancement of chemokine mRNA synthesis by antigen compared with spontaneous synthesis by cells in medium alone was not

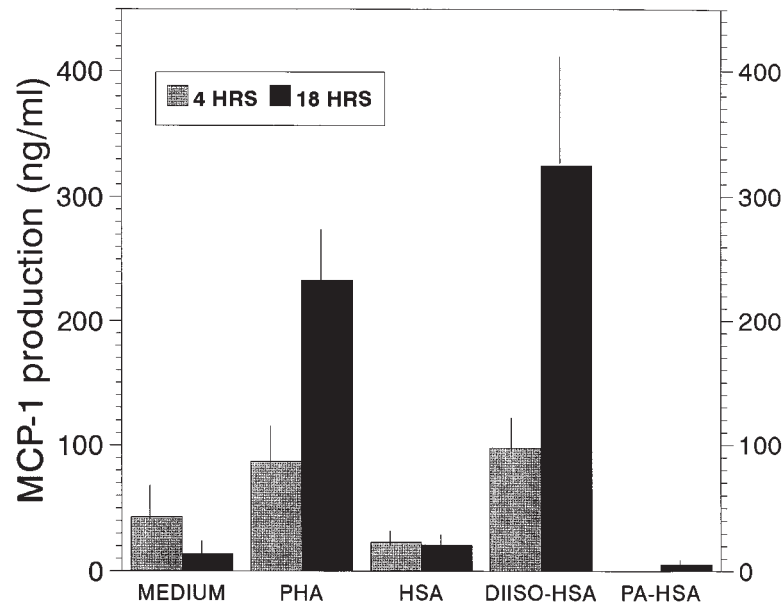


FIG. 2. Kinetics of synthesis of MCP-1 by PBMC cultures from 8 subjects with DOA. PBMC supernatants were tested after in vitro cell culture at 4 and 18 hours in medium alone (*Medium*), with PHA, with HSA, with diisocyanate-HSA conjugated antigen (*DIISO*), or with an irrelevant antigen conjugate consisting of phthallic anhydride conjugated to HSA (*PA-HSA*). Values are mean levels of total MCP-1 found in cell supernatants.

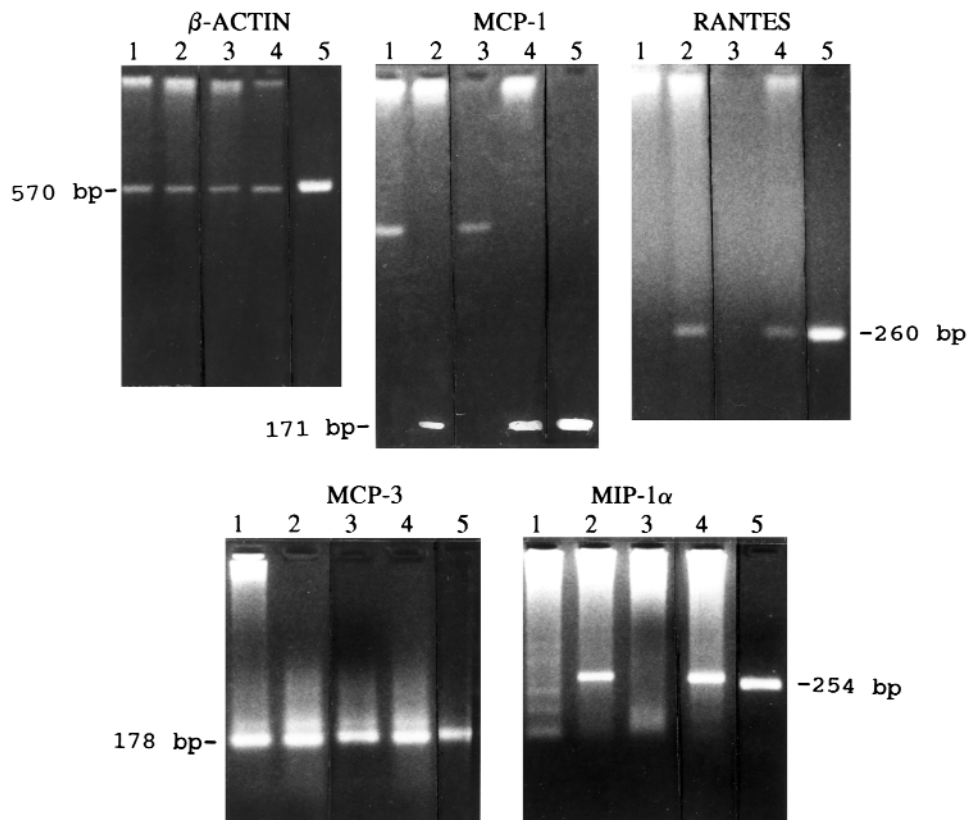


FIG. 3. Ethidium bromide-stained PCR products after RT-PCR analysis for β -actin, MCP-1, RANTES (*upper gels*), MCP-3, and MIP-1 α (*lower gels*) mRNA expression in PBMCs from asymptomatic control subject number 12 (*lanes 1 and 2*) and patient number 10 with DOA (*lanes 3 and 4*). Chemokine-positive control subjects are represented in *lane 5*. PBMCs were tested before culture (*lanes 1 and 3*) and after 18 hours of culture in medium containing HDI-HSA antigen (*lanes 2 and 4*). Total cellular RNA was reverse-transcribed, and cDNA was amplified by PCR with chemokine-specific primers.

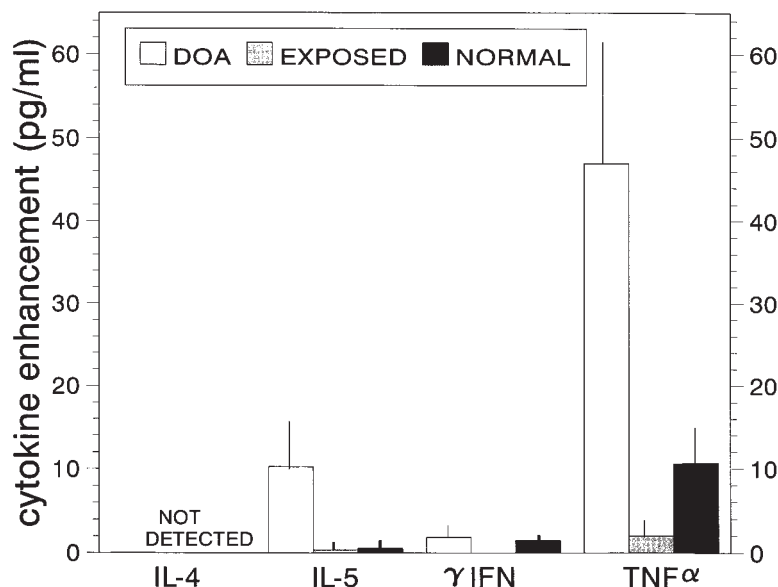


FIG. 4. Enhancement of IL-4, IL-5, IFN- γ , and TNF- α secretion from PBMCs by in vitro culture with diisocyanate-HSA-conjugated antigen for 18 hours. Enhancement is derived by subtraction of spontaneous production in media alone from diisocyanate-HSA-stimulated production. Mean values for 8 patients with diisocyanate-induced asthma are compared with 4 diisocyanate-exposed asymptomatic workers by the Mann-Whitney test.

apparent by inspection of ethidium bromide-stained gels. However, enhancement in mRNA synthesis was demonstrated after biosynthetic labeling with ^{32}P -dCTP. Electrophoretically separated chemokine PCR products, identified from autoradiograms by molecular size, were quantitated for ^{32}P incorporation. Chemokine mRNA data was expressed as the ratio of chemokine to β -actin PCR products, and the percent increase of chemokine mRNA caused by antigen was calculated. As shown in Table III, the DOA group showed significantly greater mean antigen-stimulated mRNA synthesis for MCP-1 than in the exposed but asymptomatic worker control group ($P < .05$) or in the normal control group ($P < .001$). Mean mRNA for MCP-3 and MIP-1 α was also greater than in the control group but did not reach statistical significance.

Quantitative measurements of IL-4, IL-5, IFN- γ , and TNF- α showed a mean increase in TNF- α production by PBMCs from members of the DOA group compared with the asymptomatic group (Fig. 4), which did not reach statistical significance ($P = .084$) because of the small number of control subjects in group 2 ($n = 4$). TNF- α was significantly increased ($P < .05$) when group 1 subjects were compared with all asymptomatic control subjects (groups 2 and 3). IL-5 and IFN- γ cytokine responses were noted in 3 of 8 subjects with DOA, but overall mean group values were not significantly increased above those of the asymptomatic group. No association was apparent between IL-5 and IgE antibody production. Two of the 4 subjects with DOA that produced IgG and IgE anti-diisocyanate antibodies showed IL-5 production. IL-4 synthesis was not detected in any subject tested.

A role for chemokines in the HRF activity of diisocyanate antigen-stimulated PBMC supernatants was

investigated by an antibody inhibition test. PBMC supernatants obtained from an 8-day culture of HDI-HSA antigen-stimulated PBMCs derived from a patient with HDI-induced DOA were tested for HRF activity in the presence and absence of chemokine-specific neutralizing antibodies. Inhibition of HRF activity was shown by anti-MCP-1, anti-MCP-3, anti-RANTES, and anti-MIP-1 α , but not by anti-IL-8 (Table IV). Inhibition results obtained by testing 2 concentrations of antibodies (100 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$) showed stronger inhibition by 100 $\mu\text{g/mL}$ anti-MCP-3 (73%) than by anti-MCP-1, which may be explained in part by the fact that the anti-MCP-3 antibody can cross-react with other MCP chemokines.

DISCUSSION

In this study workers with confirmed DOA exhibited enhanced in vitro diisocyanate-HSA antigen-stimulated synthesis of MCP-1, IL-8, and TNF- α . Diisocyanate-antigen enhancement of MCP-1 and IL-8 was demonstrated in 7 of 8 workers with confirmed OA. Of these subjects, 6 had been completely restricted from exposure to diisocyanate chemicals for longer than 10 months, indicating persistence of diisocyanate-responsive mononuclear cells. Diisocyanate-antigen specificity of MCP-1 production was demonstrated in that cells from patients with DOA cultured with either an irrelevant antigen conjugate (phthallic anhydride-HSA) or the HSA carrier protein alone failed to show enhanced synthesis of MCP-1. Only 3 of 8 MCP-1 producers with OA exhibited elevated serum-specific IgE to diisocyanate-HSA antigens, suggesting the existence of divergent mechanisms underlying in vitro antigen-induced cellular and specific IgE immune responses in workers with DOA.

TABLE III. RT-PCR analysis of antigen stimulation of mRNA in PBMCs by quantitation of ³²P-dCTP incorporation in PCR products

Subject group	Percent increase in mRNA (diisocyanate-HSA-stimulated minus medium control), mean ± SEM			
	MCP-1	MCP-3	MIP-1α	RANTES
Exposed control subjects without OA	6.3 ± 4.92	10.5 ± 7.11	13.9 ± 13.85	26.9 ± 10.97
Symptomatic subjects with OA	45.6 ± 14.29*	20.5 ± 14.05†	47.6 ± 20.35†	14.0 ± 6.30†

*P = .05.

†P = not significant.

TABLE IV. Antichemokine antibody inhibition of HRF activity in PBMCs of HDI-asthmatic patient*

Neutralizing antibody	Percent inhibition of basophil HR	
	100 µg/mL antibody	50 µg/mL antibody
None	0	0
Anti-MCP-1	25	38
Anti-MCP-3	72	33
Anti-RANTES	21	0
Anti-MIP-1α	33	8
Anti-IL-8	8	-8

*PBMC supernatant (0.1 mL, containing 80 ng of MCP-1 and 0.015 ng of RANTES) from HDI-challenged PBMCs was mixed with 0.1 mL or 0.05 mL of 1 mg/mL antichemokine antibody and added to 1 mL of peripheral blood lymphocytes from a normal basophil donor. After 1 hour in a 37° C shaking water bath, peripheral blood leucocytes were removed by centrifugation, and supernatants were analyzed for percent of HR. Percent inhibition was determined as 100 × (1-[HR in presence of antibody/HR in absence of antibody]).

Because many investigators have reported the absence of specific IgE responses to diisocyanate-HSA antigens in all but a minority of workers with DOA,³ alternative cellular immune mechanisms have been investigated. The presence of cellular immune mechanisms is supported by earlier reports of in vitro peripheral blood lymphocyte-proliferative responses to TDI-HSA^{17,18} and production of leukocyte migration inhibition factor by TDI-HSA-stimulated peripheral blood leukocytes in workers with DOA. Our laboratory has performed studies demonstrating a significant association between the presence of HRF activity in supernatants of a group of workers with confirmed DOA.^{7,8} In these studies the specificity of diisocyanate-enhanced HRF activity was shown by demonstrating that there was greater enhancement of HRF production after stimulation with antigens relevant to the workplace (as defined above) but not to irrelevant diisocyanate antigens. Because MCP-1 is the most potent HRF in stimulated PBMCs, it is likely that this latter observation would apply to MCP-1. Bernstein et al.¹⁸ recently reported selective increases in T-cell receptor Vβ 1 and Vβ 5 gene expression in peripheral lymphocytes from workers with DOA after in vitro incubation with diisocyanate antigen. The combined results of these studies suggest that in vitro cellular and cytokine responses to diisocyanate antigens could serve as biologic markers of

OA and that these responses possess immunologic memory and antigen specificity that are characteristic of cell-mediated immunity.

Production of MCP-1 was also stimulated by the mitogen PHA, as has previously been demonstrated by others.¹⁹ Although MCP-1 was measured here in PBMCs, it should be noted that there is mounting evidence indicating that MCP-1 is also an important cytokine mediator of airway inflammation. In addition to HRF effects, MCP-1 has been shown in an experimental model to stimulate transendothelial migration of T cells and was superior to other chemokines tested, including RANTES, IL-8, or MIP-1α.²⁰ In a transgenic murine model of allergic asthma, Lukacs et al.²¹ demonstrated maximal production of MCP-1, MIP-1α, and RANTES in the lungs 8 hours after allergen challenge. Neutralization with specific mAbs of MIP-1α and RANTES, but not MCP-1, decreased the percentages of peribronchial eosinophils. In contrast to MCP-3,¹⁶ RANTES,^{22,23} and MIP-1α,^{23,24} neither MCP-1 or IL-8 have potent chemoattractant activity for eosinophils, which are prominent effector cells in DOA.²⁵ It is possible that other β-chemokines that attract eosinophils such as eotaxin (also upregulated by TNF-α) might have been measurable in this system. However, reduction in total leukocyte counts (>50%), including CD4+ and CD8+ cells, and reduction in airway responsiveness to methacholine was achieved by neutralization of MCP-1, but not of MIP-1α and RANTES.²¹ Thus MCP-1 could function as an important chemoattractant signal in initiating airway inflammatory responses.

MCP-1 is a major product of monocytes and is not produced by lymphocytes.¹⁰ Therefore antigen stimulation of MCP-1 production by monocytes in PBMC cultures would require either activation of monocytes by lymphokines or antigen binding to cytophilic antibodies bound to Fc receptors.²⁶ The results of this study suggest that in vitro chemokine production is caused by protein synthesis rather than release of preformed chemokine. MCP-1 mRNA transcripts were not detected as a constitutive factor in cells before culture. This finding is consistent with a previous study showing that in vitro synthesis of MCP-1 production by normal human peripheral blood monocytes occurs after adherence to plastic or treatment with IFN-γ; MCP-1 is not produced by freshly isolated nonactivated human monocytes.²⁷ The observed increases could occur as a result of activation of an

inducible gene. Kinetic studies demonstrated that MCP-1 production was maximally increased during 18 hours of culture with diisocyanate-HSA. The observation that production of MCP-1 was increased after 4 hours appears to correspond to the kinetics of DOA in which the late-phase inflammatory asthmatic response is manifested at least 4 hours after diisocyanate exposure in the majority of affected workers.²⁸ Although local MCP-1 production has not been studied in DOA, it could have a putative pathogenetic role in elicitation of late asthmatic responses and may be particularly applicable to consequences and effects elicited by mononuclear cells infiltrating the airways.

There was preferential enhancement of both MCP-1 and IL-8 after in vitro diisocyanate-antigen stimulation and insignificant production of the β -chemokines MCP-3 and RANTES by 18-hour cell cultures. However, in antigen-stimulated cells cultured for 8 days, MCP-1, MCP-3, MIP-1 α , and RANTES were shown to be present in PBMC supernatants and to contribute to HRF activity. This finding is consistent with PCR results showing antigen-stimulated synthesis of mRNA for MCP-3 and MIP-1 α , as well as for MCP-1, at 18 hours after stimulation. Although 18-hour cultures of antigen-stimulated PBMCs showed an absence of secreted MCP-3 (0 ng/mL) and low levels of MIP-1 α (6.25 ng/mL) and RANTES (0.62 ng/mL) compared with MCP-1 (345 ng/mL) or IL-8 (128 ng/mL), the HRF inhibition results suggest that some HRF-active chemokines can appear as secreted proteins after a longer period of cell culture in the presence of a specific antigen.

No definite pattern of T_{H1} or T_{H2} cytokine profiles (ie, IFN- γ or IL-4 or IL-5) were produced in this culture system. TNF- α , which was increased in workers with DOA, could have a number of important pathogenetic roles in DOA. TNF- α is chemotactic for neutrophils and monocytes²⁹ and mediates recruitment of neutrophils and eosinophils during airway inflammation.³⁰ In animal models TNF- α caused increased bronchial responsiveness whether administered parenterally or by inhalation.^{31,32} It is relevant to this study that both MCP-1 and IL-8 synthesis can be induced in monocytes by a variety of stimuli, including TNF- α .¹⁰ Although unproven, it is possible that TNF- α may upregulate MCP-1 and IL-8 production by PBMCs after diisocyanate-HSA stimulation.

It should be emphasized that antigen-stimulated production of cytokines or chemokines by PBMCs would not be expected to define regulatory events in the airways of workers with OA.³³ There is evidence to suggest that inflammatory events relevant to DOA are not confined to the airways. Increased CD8⁺ T cells and eosinophils in the peripheral circulation have been documented during late-phase asthma after inhalation of diisocyanates.³⁴ Furthermore, TNF- α is produced by cultured PBMCs and is enhanced after TDI challenge.³⁵ Although these studies have not addressed possible mechanistic roles of MCP-1, IL-8, and TNF- α in the pathogenesis of DOA, it is possible that in vitro cellular assays of cytokine synthesis could serve as diagnostic biomarkers of OA. Studies are currently underway in larger cohorts of workers to

determine whether diisocyanate-antigen stimulated MCP-1 production is a valid diagnostic biomarker of DOA.

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