A novel mouse model of diisocyanate-induced asthma showing allergic-type inflammation in the lung after inhaled antigen challenge

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Background: Exposure to diisocyanates, a group of highly reactive, low-molecular-weight compounds, is a major cause of occupational asthma. In contrast to mouse models of atopic asthma, previous mouse models of diisocyanate-induced asthma have failed to show lung inflammation with characteristics of human disease.

Objective: Our goal was to establish a novel mouse model of diisocyanate-induced asthma in which lung inflammation reminiscent of that seen in human asthma is generated after inhaled antigen challenge.

Methods: BALB/c mice were epicutaneously sensitized to hexamethylene diisocyanate (HDI) and then challenged with an HDI-protein conjugate administered by means of an intranasal droplet.

Results: HDI sensitization resulted in development of contact hypersensitivity and HDI-specific antibody production. Most importantly, however, vigorous inflammatory responses with characteristics of human asthma were generated in the lung after inhaled HDI challenge. Challenge of sensitized, but not unsensitized, mice resulted in airway eosinophilia, mucus hypersecretion, and production of T_H1-type (IFN-γ) and T_H2-type (IL-4, IL-5, and IL-13) cytokines by lung inflammatory cells. Despite the mixed T_H1/T_H2 response induced by HDI sensitization, use of cytokine-deficient mice revealed that airway eosinophilia was mediated by T_H2 cytokines and not by IFN-γ.

Conclusion: We report a novel mouse model of diisocyanate-induced asthma that, in contrast to previous models, demonstrates antigen-induced lung inflammation with characteristics of human disease. This model will allow investigation of the immunopathogenesis of diisocyanate-induced asthma and should provide insight into this common form of occupational disease. (J Allergy Clin Immunol 2002;109:873-8.)

Key words:*Asthma, diisocyanate, skin, airway, lung, eosinophils, T_H2, IL-4, IL-13, IFN-γ*

Abbreviations used
BAL: Bronchoalveolar lavage
CHS: Contact hypersensitivity
HDI: Hexamethylene diisocyanate
OVA: Ovalbumin

Asthma is a clinical syndrome characterized by reversible airway obstruction, bronchial hyperresponsiveness, and airway inflammation. Although the causes of distinct variants might differ, airway inflammation is a constant feature thought to play a fundamental role in disease expression.[1-3] One important form of asthma results from exposure to diisocyanates, a group of highly reactive, low-molecular-weight compounds used in the manufacture of polyurethanes. Diisocyanates are one of the most commonly identified causes of occupational asthma in industrialized countries.[4-6]

Although numerous studies have been performed in mouse models of atopic asthma, few investigations of asthma resulting from sensitization to haptens, such as diisocyanates, have been performed in mice. This species offers numerous advantages with regard to the availability of reagents and genetically manipulated strains. Previously described mouse models of diisocyanate-induced asthma have shown increased airway reactivity but no lung inflammation representative of human asthma. In one case inflammation was reported but appeared irritant in nature, consisting primarily of neutrophils and being present in both sensitized and unsensitized animals.[7,8] A more recently described model showed mild inflammation in the trachea, but not in the lung, and no eosinophils.[9]

Our goal was to develop a mouse model of diisocyanate-induced asthma that demonstrated lung inflammation characteristic of human disease.[10-12] We report such a model here, involving sensitization to hexamethylene diisocyanate (HDI) through the skin, which resulted in both contact hypersensitivity (CHS) and HDI-specific antibody responses. Most importantly, however, challenge of sensitized, but not unsensitized, mice with inhaled HDI resulted in a strong inflammatory response in the lung and airway, with high numbers of eosinophils and mucus hypersecretion. Interestingly, HDI-sensitized mice generated a mixed T_H1/T_H2 response, as evidenced by serum antibody and cytokine profiles. This is in contrast to the strongly biased T_H2 response we have seen

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Supported by National Institutes of Health grants R01-HL65209 (K. Bottomly and C. Herrick), P50-HL56389 and K24-ES00355 (C. Redlich), and RO1-HL62622 (A. Wisnewski). C. Herrick was supported by a Dermatology Foundation Clinical Career Development Award.

Received for publication December 17, 2001; revised January 24, 2002; accepted for publication January 28, 2002.

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0091-6749/2002 $35.00 + 0 **1/86/123533**
doi:10.1067/mai.2002.123533

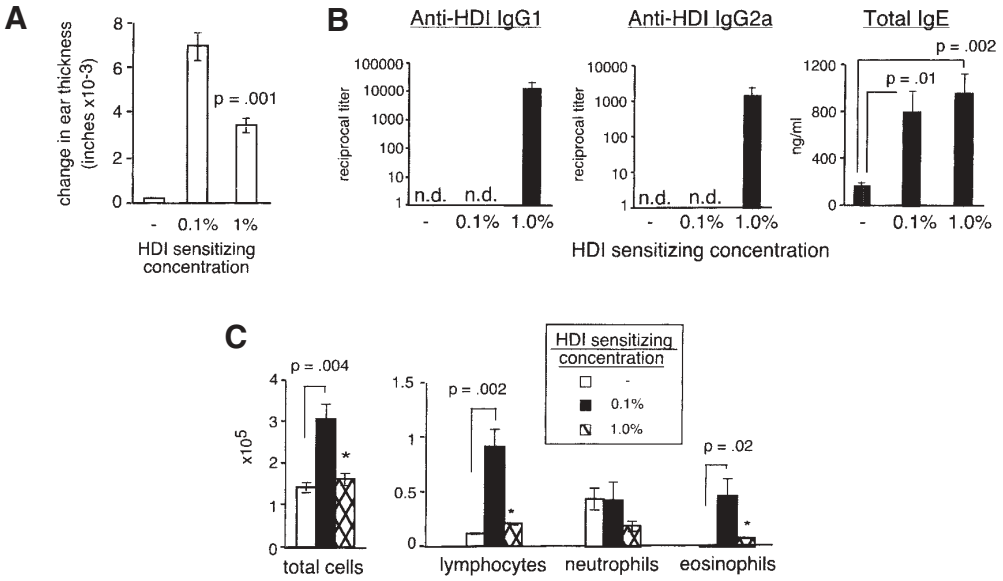

FIG 1. CHS, serum antibody, and airway inflammatory responses in HDI-sensitized mice. **A,** Ears of sensitized mice were challenged with HDI, and peak swelling at 24 hours is shown. Data are reported as means ± SEM of 4 to 5 mice per group (representative experiment, 1 of 3). **B** and **C,** HDI-sensitized or unsensitized mice were challenged with inhaled HDI-MSA. Antibody data are reported as means ± SEM of 8 mice per group (2 experiments pooled). BAL data are reported as means ± SEM of 5 mice per group (representative experiment, 1 of 4). *$P < .05$ for the 0.1% versus 1.0% HDI– sensitized groups.

after epicutaneous sensitization to the soluble protein ovalbumin (OVA).[13] Despite T_H1 activation, however, airway eosinophilia in HDI-sensitized mice was not dependent on IFN-γ but was mediated by T_H2 cytokines (IL-4 and IL-13). The model described here should contribute to our understanding of the pathogenesis of some of the less well-studied human asthma variants.

METHODS

Animals and epicutaneous sensitization

BALB/c mice were purchased from The National Cancer Institute (Frederick, Md). BALB/c-Il4^{tm2Nnt} (IL-4–/–) mice were purchased from The Jackson Laboratory (Bar Harbor, Me). BALB/c IL-13–/– mice were provided by A. McKenzie. IFN-γ receptor–deficient (IFN-γR–/–) mice were provided by J. Aguet and backcrossed onto BALB/c. Female mice 9 to 12 weeks of age were used in all experiments. Backs of mice were shaved with electric clippers 1 to 2 days before exposure to HDI (0.01%-10%) diluted in a 4:1 acetone/olive oil mixture or acetone/olive oil mixture alone (50 μL) on days 0 and 7.

Contact hypersensitivity

Ten microliters of HDI (0.1%) was applied to ears on day 12 after sensitization. Ear swelling was measured with engineer's calipers just before and then 6, 24, 48, and 72 hours after challenge.

Lung inflammatory response

1,6-Diisocyanatohexane (hexamethylene diisocyanate [HDI]; Sigma Chemical Co, St Louis, Mo) was conjugated to mouse serum albumin (MSA; Sigma), according to published methods.[14] Mice were lightly anesthetized with methoxyflurane; administered 50 μL of HDI-MSA (2.0 mg/mL) in PBS by means of an intranasal droplet on days 14, 15, 18, and 19; and killed by means of CO_2 asphyxiation on day 21. Bronchoalveolar lavage (BAL) was performed as previously described.[13] Differential counts were performed on 200 cells per sample. Paraffin-embedded lungs were sectioned at 5 μm and stained with hematoxylin and eosin or periodic acid–Schiff.

Isolation and culture of lung inflammatory cells

On day 21, lungs from 3 to 4 mice per group were pooled, and cells were isolated by means of enzymatic digestion, as previously described.[13] Cells were cultured (2×10^6/mL) with antigen-presenting cells (syngeneic T-depleted splenocytes, 2×10^6/mL) and HDI-MSA (100 μg/mL) in Click's/5% FCS at 37°C for 48 hours. Cytokines in culture supernatants were detected with commercially available ELISA kits (IFN-γ, IL-4, or IL-5, Endogen, Woburn, Mass; IL-13, R&D Systems, Minneapolis, Minn).

Antibody measurement by means of ELISA

Serum was collected at the time of death on day 21. HDI-specific ELISAs involved (1) coating with HDI-BSA (10 μg/mL) in 0.1 mol/L $NaHCO_3$ (pH 9.5) at 4°C overnight, (2) blocking with 3% milk in PBS for 1 hour at 37°C, (3) placing serum samples in 3% milk in PBS/0.05% Tween for 2 hours at 37°C, and (4) using horseradish peroxidase–labeled rat anti-mouse secondary antibodies for 1 hour at 37°C: either anti-IgG1 (A85-1; Pharmingen, San Diego, Calif) or anti-IgG2a (R19-15, Pharmingen). Data are reported as the reciprocal of the highest dilution that still yielded a positive OD reading greater than background and negative control readings. Specificity was demonstrated by the following: (1) HDI-specific antibodies were only detected in mice exposed to HDI or HDI-protein conjugates and not in mice exposed to carrier protein alone; (2) serum containing high titers (>1:5000) of an irrelevant antibody served as negative controls; and (3) no antibody was detected in HDI-exposed mice when ELISA plates were coated with BSA alone. Total serum IgE levels were measured as previously described.[13]

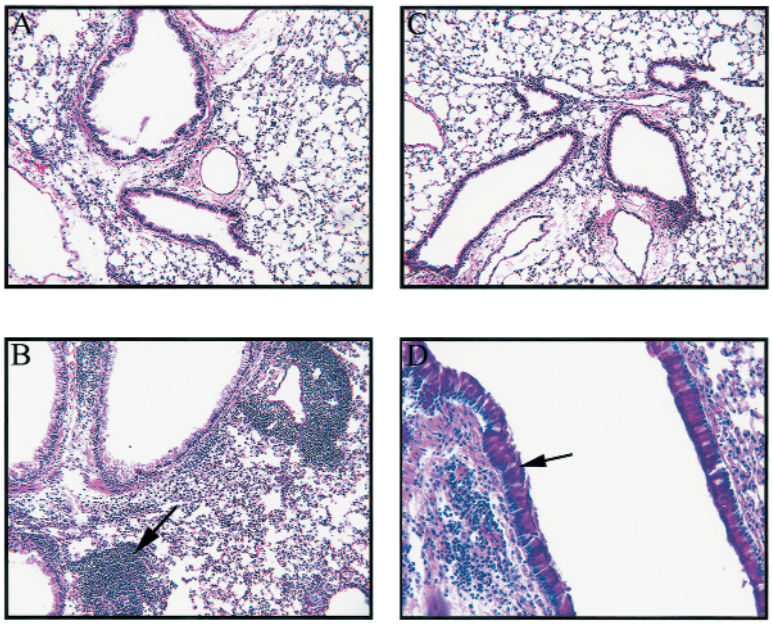

FIG 2. Lung inflammation in HDI-sensitized mice. Sensitized mice (**A,** 1.0% HDI; **B,** 0.1% HDI; **C,** vehicle alone) were challenged with inhaled HDI-MSA. Lungs were harvested on day 21 and stained with hematoxylin and eosin (original magnification 100×). **D** shows a periodic acid–Schiff stain of lung from a mouse sensitized with 0.1% HDI.

Statistical analyses

Statistical significance was determined with an unpaired Student *t* test or with analysis of covariance when data from more than one experiment were pooled.

RESULTS

CHS and antibody responses in HDI-sensitized mice

As shown in Fig 1, *A*, mice exposed to either 0.1% or 1.0% HDI on days 0 and 7 had significant ear-swelling responses after challenge on day 12. Mice exposed to the lower HDI concentration (0.1%), however, had significantly greater increases in ear thickness than mice painted with the higher HDI concentration (1.0%; $P = .001$). In contrast, HDI-specific antibodies were only detected in mice sensitized with the higher dose of HDI (1.0%), including both IgG1 and IgG2a (Fig 1, *B*). No HDI-specific antibodies were detected in mice sensitized with 0.1% HDI. Total serum IgE levels were significantly higher than those of control animals in mice sensitized with either dose of HDI (Fig 1, *B*).

Cellular composition of BAL fluid after inhalational challenge

A major concern with previous mouse models of diisocyanate-induced asthma has been the lack of lung inflammation, as seen in mouse models of atopic asthma. When inflammatory responses were reported, they appeared to be irritant in nature.[7,8] Our approach was to first conjugate HDI to a mouse self protein, MSA, to allow inhalation of HDI in a PBS-soluble, nonirritating form. HDI-sensitized mice were challenged by means of intranasal droplet application of HDI-MSA in PBS over a 6-day period. As shown in Fig 1, *C*, there were significantly higher numbers of cells in the BAL fluid of mice sensitized with the low dose of HDI (0.1%) compared with those in mice exposed to vehicle alone ($P = .004$). Increased numbers of both lymphocytes ($P = .002$) and eosinophils ($P = .02$) were present. In contrast, BAL cell numbers in mice sensitized with the higher concentration

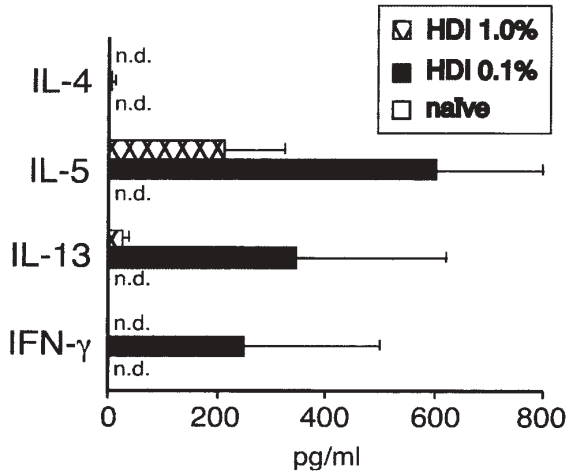

FIG 3. Cytokine production by lung inflammatory cells. HDI-sensitized mice were challenged with inhaled HDI-MSA and killed on day 21. Lungs from 3 to 4 mice per group were harvested and pooled, and inflammatory cells were isolated by means of enzymatic digestion. Cells were restimulated in vitro with HDI-MSA, and cytokines were measured in supernatants. Data shown are means ± SEM from 3 experiments. *nd,* None detected.

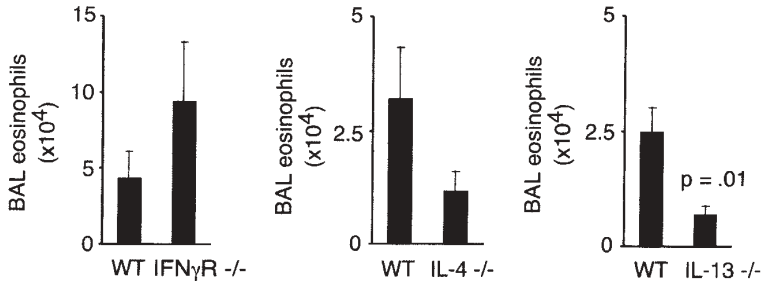

FIG 4. Airway eosinophilia in HDI-sensitized, cytokine-deficient mice. Wild-type *(WT)*, IFN-γR–/– **(A)**, IL-4–/– **(B)**, or IL-13–/– **(C)** mice were sensitized to HDI (0.1%) and challenged with inhaled HDI-MSA. Mice were killed, and BAL was performed. Data are reported as the number of eosinophils from individual mice (mean ± SEM of 8-9 mice per group pooled from 2 experiments for Fig 4, *A*; 14-15 mice per group from 3 experiments for Fig 4, *B*; and 10 mice per group from 2 experiments for Fig 4, *C*). Statistical significance between groups was determined by using analysis of covariance.

of HDI (1.0%) were not elevated compared with those in control animals. Of note, HDI-sensitized mice similarly exposed to intranasal MSA alone over 6 days had no increase in BAL cell numbers (data not shown). Thus as was the case for CHS, more vigorous airway inflammatory responses were generated in mice sensitized with the lower dose of HDI.

Lung inflammatory response in HDI-sensitized mice

As shown in Fig 2, intranasal HDI-MSA challenge led to marked inflammatory infiltrates composed of both lymphocytes and eosinophils in a predominantly perivascular and peribronchiolar distribution in the lungs of mice sensitized with the low dose of HDI (0.1%; Fig 2, *B*) but not in mice exposed either to 1.0% HDI or to vehicle alone (Fig 2, *A* and *C*). Periodic acid–Schiff stains also revealed strong induction of mucus secretion in the airways of mice sensitized with the low dose of HDI (0.1%; Fig 2, *D*) but not in mice sensitized with 1.0% HDI or vehicle alone (not shown). These results are consistent with the BAL findings reported above, confirming the absence of significant lung inflammation in mice exposed to the higher dose of HDI.

The density of inflammation in lung sections from HDI-sensitized mice appeared similar to what we have seen in epicutaneously OVA-sensitized mice.[13] This was in contrast to the generally lower BAL cell numbers in HDI-sensitized mice (Fig 1, *C*) compared with those in OVA-sensitized mice.[13,15] To better quantitate the lung inflammation, cells were isolated by means of enzymatic digestion. Equivalent numbers of cells were recovered from lungs of HDI- and OVA-sensitized mice. However, the ratio of lymphocytes to eosinophils was consistently higher in HDI-sensitized mice (data not shown). Lung cells from HDI-sensitized mice were restimulated in vitro with HDI-MSA to further characterize the type of T cells present, and secreted cytokines were measured in culture supernatants. As shown in Fig 3, both T_H2 (IL-4, IL-5, and IL-13) and T_H1 (IFN-γ) cytokines were produced, indicating a mixed response to HDI sensitization. This is in contrast to the markedly T_H2-skewed cytokine profile we have seen in OVA-sensitized mice.[13]

Airway eosinophilia in HDI-sensitized mice is mediated by T_H2 cytokines

Given the presence of a mixed response in HDI-sensitized mice, we sought to determine what role T_H1 and T_H2 cytokines played in the generation of airway inflammation. Therefore IFN-γR–/–, IL-4–/–, or IL-13–/– mice were sensitized to HDI (0.1%) and challenged with intranasal HDI-MSA, as before. As shown in Fig 4, *A*, IFN-γ was not required for induction of airway eosinophilia. In fact, HDI-sensitized IFN-γR–/– mice had higher numbers of eosinophils in BAL fluid than wild-type control animals, although this was not statistically significant. In contrast, both HDI-sensitized IL-4–/– and IL-13–/– mice had fewer eosinophils in BAL fluid than wild-type control animals (Fig 4, *B* and *C*, respectively). This difference was only statistically significant for the IL-13–/– mice. Thus despite the presence of both T_H1 and T_H2 cytokines, only T_H2 cytokines were important for airway eosinophilia.

DISCUSSION

In the present study we describe a novel mouse model of diisocyanate-induced asthma. To our knowledge, this is the first such model to demonstrate lung inflammation with characteristics of human asthma, including eosinophilia and mucus hypersecretion. The lung inflammation in our model was present specifically in sensitized animals. Scheerens et al[7,8] have reported increased airway reactivity after intratracheal challenge of toluene diisocyanate–sensitized mice. However, only nonspecific, primarily neutrophilic inflammation was seen in both sensitized and unsensitized mice, which is consistent with an irritant response. A more recently described model of toluene diisocyanate–induced airway reactivity again failed to show lung inflammation or airway

eosinophilia.[9] This new model of diisocyanate-induced asthma should aid in identifying potential distinctions from the more widely studied atopic asthma.

A possible explanation for our ability to induce lung inflammation was use of an HDI-MSA conjugate in PBS for challenge. This likely eliminated nonspecific irritant effects and allowed better delivery of HDI to the lower airways.[16] Another unique feature of our model was use of different doses of HDI for sensitization. Lung inflammation was only seen in mice sensitized with 0.1% HDI and not in mice sensitized with 1.0% HDI, the optimal dose for inducing antibody production.

The nature of the lung inflammation in our model was similar to that seen in patients with diisocyanate-induced asthma. Bronchial biopsy specimens from patients with diisocyanate-induced asthma show infiltrates with high numbers of lymphocytes and eosinophils,[10-12] as were evident in the lungs of HDI-sensitized mice. Furthermore, the presence of eosinophils in airway infiltrates, T_H2 cytokines, and antigen-specific IgE in the sera of some patients with diisocyanate-induced asthma,[17-20] supports a role for T_H2 activation in at least a subset of patients. Similar indicators of T_H2 activation were present in HDI-sensitized mice, with high numbers of airway eosinophils, mucus hypersecretion, T_H2 cytokines, and elevated serum IgE levels. T_H1 activation was also evident, given the presence of IFN-γ in lung cell cultures and IgG2a antibodies in serum. Interestingly, our group and others have reported that T cells from patients with HDI-induced asthma secrete high levels of IFN-γ in response to in vitro stimulation with HDI.[21-24] Thus it might be that occupational exposure to HDI leads to development of a mixed T_H1/T_H2 response similar to that seen in our mouse model. However, despite the mixed response after HDI sensitization, our studies with cytokine-deficient mice show that airway eosinophilia is mediated by T_H2 cytokines and not IFN-γ. An intriguing finding in the present study was the lack of correlation between the HDI-sensitizing dose optimal for either lung inflammation or CHS (0.1%) and the optimal dose for inducing antibody responses (1.0%). It is noteworthy that detection of diisocyanate-specific antibodies in workers frequently does not correlate with reactive airway disease.[19,20,25-27]

We noted certain distinctions between the model of HDI-induced asthma described here and our previously described model of atopic asthma involving epicutaneous sensitization to the soluble protein OVA.[13] For one, HDI exposure led to a mixed T_H1/T_H2 response, with production of both IgG1 and IgG2a and secretion of both T_H1 and T_H2 cytokines. In contrast, epicutaneous exposure to OVA results in a highly skewed T_H2 response, with no OVA-specific IgG2a and production of T_H2 cytokines only by lung cells.[13] Another interesting observation was that the total number of BAL cells in HDI-sensitized mice, including eosinophils, was generally less than that typically seen in OVA-sensitized mice.[13,15] This was despite equivalent cell numbers in the lungs. We and others have noted a similar situation in patients with diisocyanate-induced asthma, whose BAL cell numbers and percentage of eosinophils often appear less impressive than those of atopic asthmatic subjects (unpublished observations).[28-31] Further study of these 2 models of human asthma could provide insight into potential clinical differences between diisocyanate-induced and atopic asthma.

In summary, we have described a new mouse model of HDI-induced asthma that demonstrates many features of the human disease, most importantly characteristic lung inflammatory responses after airway challenge. This model should allow further dissection of the immunopathogenesis of diisocyanate-induced asthma and provide greatly needed improved strategies for early detection of sensitized workers.

We thank Ms Heather MacLeod for technical assistance and Dr Robert Homer for his input regarding the histologic appearance of lung inflammatory infiltrates.

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