

Differential roles for CD4 and CD8 T cells after diisocyanate sensitization: Genetic control of T_H2-induced lung inflammation

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Background: Exposure to diisocyanates is a major cause of occupational asthma. We previously developed a novel mouse model of diisocyanate-induced asthma involving epicutaneous sensitization to hexamethylene diisocyanate (HDI) that demonstrates many features of the human disease, including airway eosinophilia and mucus hypersecretion.

Objective: To determine what factors are critical for the development of HDI-induced airway inflammation, we investigated the strain distribution of this response and the roles of CD4⁺ and CD8⁺ T cells.

Methods: Mice were epicutaneously exposed to HDI and then challenged with HDI, either by means of inhalation to induce airway inflammation or on the ear to induce contact hypersensitivity (CHS). Lymph node cytokine production and serum antibodies were also measured.

Results: Induction of airway eosinophilia was highly dependent on the mouse strain used, with C57BL/6, A/J, CBA, C3H, and C57BL/10 mice all having significantly fewer eosinophils than BALB/c mice. HDI-specific antibodies and lymph node IL-5 and IL-13 production were also diminished in non-BALB/c strains. In contrast, CHS to HDI developed in all strains tested. Studies in mice deficient in either CD4⁺ or CD8⁺ T cells revealed that CD4⁺ T cells were critical for HDI-induced airway eosinophilia, whereas CD8⁺ T cells were the major effector cells in CHS.

Conclusion: The data suggest that, in contrast to CHS, induction of T_H2 responses after epicutaneous exposure to diisocyanates is strongly genetically influenced. Furthermore, the lung inflammatory response to inhaled HDI appears to depend primarily on effective generation of these CD4⁺ T_H2 responses, as is the case in atopic asthma. (*J Allergy Clin Immunol* 2003;111:1087-94.)

Key words: Asthma, diisocyanate, skin, airway, eosinophils, contact hypersensitivity

Abbreviations used

BAL: Bronchoalveolar lavage
CHS: Contact hypersensitivity
HDI: Hexamethylene diisocyanate
LN: Lymph node
OVA: Ovalbumin

Diisocyanates are a group of highly reactive, low-molecular-weight compounds used in the manufacture of polyurethanes. Diisocyanate-induced asthma is currently the most commonly reported form of chemically induced occupational asthma.¹⁻⁵ Approximately 250,000 workers in the United States are exposed annually, with 5% to 15% having reactive airway disease.¹

There are many similarities between diisocyanate-induced asthma and atopic asthma, such as airway hyperactivity, the presence of eosinophilic lung infiltrates, and mucus hypersecretion in airways.⁶⁻⁹ Certain distinctions, however, are also apparent. For instance, atopy does not appear to be a risk factor for development of diisocyanate-induced asthma, and IgE levels are increased in a minority of patients.¹⁰⁻¹³ In fact, levels of diisocyanate-specific antibodies in exposed workers generally do not correlate with development of reactive airway disease.^{12,14,15} Furthermore, although strong evidence exists for a T_H2-based cause of atopic asthma, the immunopathogenesis of diisocyanate-induced asthma remains unclear. Some studies suggest a role for both T_H1 and T_H2 CD4 T cells, whereas others implicate CD8 T cells as important effector cells in this form of asthma.¹⁶⁻²⁰

Additional questions exist regarding the types of exposure that result in the development of diisocyanate-induced asthma. Recent attention has been drawn to the possibility that in addition to inhalational exposure to diisocyanates, skin exposure in the workplace might also contribute to sensitization of workers.^{15,21} Of note, significant exposure of the skin to hexamethylene diisocyanate (HDI) in paints has been demonstrated in auto-body shops.²² Thus much remains unanswered with regard to both the cause of and risk factors for the development of diisocyanate-induced asthma.

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For this reason, we had previously established a mouse model of diisocyanate-induced asthma involving epicutaneous sensitization of mice to HDI.²³ In this model BALB/c mice had contact hypersensitivity (CHS) and HDI-specific antibodies, as well as vigorous inflammatory responses in the lung, after inhaled antigen challenge. These lung inflammatory responses were characterized by high numbers of eosinophils and mucus hypersecretion, which is similar to conditions in human disease.

In the current study we sought to determine what factors were critical for the development of HDI-induced lung inflammation. We show that airway eosinophilia after inhaled HDI challenge is highly dependent on the mouse strain tested, with robust responses being seen in BALB/c mice but not in several other strains. In contrast, CHS to HDI developed universally in all strains tested. Non-BALB/c strains also showed decreased cytokine production in skin-draining lymph nodes (LNs) and decreased HDI-specific antibodies in serum. Using mice genetically deficient in either CD4⁺ or CD8⁺ T cells, we demonstrate that CD4⁺ T cells are critical for HDI-induced airway eosinophilia, whereas CD8⁺ T cells are the major effector cells in CHS to HDI. Taken together, these data indicate that the type of response generated by exposure to diisocyanates might be genetically influenced and that a T_H2 response must occur for development of airway disease similar to that seen in patients with atopic asthma.

METHODS

Animals and epicutaneous sensitization

BALB/cAnNCr, C57BL/6NCr, A/JCr, CBAJ/Cr, C3H/HeJCr, and C57BL/10ScNCr mice were purchased from The National Cancer Institute (Frederick, Md). C.129P2(B6)- β 2m^{tm1Unc} (β 2m^{-/-}) and control (BALB/cJ) mice were purchased from The Jackson Laboratory (Bar Harbor, Me). BALB/c MHC class II transactivator deficient (CIITA^{-/-}) mice were kindly provided by C.-H. Chang.²⁴ Female mice (9–12 weeks of age) were epicutaneously exposed to HDI (0.1%; Sigma Chemical Co, St Louis, Mo) or acetone-olive oil alone on days 0 and 7, as previously described.²³

Contact hypersensitivity

Ten microliters of HDI (0.1%) was applied to the animals' ears on day 12. Ear thickness was measured with engineer's calipers before and then 6, 24, 48, and 72 hours after challenge. Data shown are from representative experiments (1 of 3 for BALB/c mice, 2 for C57BL/6 mice, 1 for A/J mice, or 1 for CBA mice; mean $\pm$ SEM of 4–5 mice per group in each experiment).

Lung inflammatory response

HDI was conjugated to mouse serum albumin (MSA, Sigma) according to published methods.²⁵ Mice were administered 50 μ L of HDI-MSA (2.0 mg/mL) in PBS by means of intranasal droplet on days 14, 15, 18, and 19 and killed with CO₂ asphyxiation on day 21, and bronchoalveolar lavage (BAL) was performed, all as previously described.^{23,26} Differential counts were performed on 200 cells per sample. Data shown are the means $\pm$ SEM of individual mice. The numbers of mice represented for each HDI- and acetone-olive oil-exposed group, respectively, are as follows: BALB/c, n = 23 and 24; C57BL/6, n = 20 and 20; A/J, n = 15 and 13; CBA, n = 16 and 14; C3H, n = 16 and 14; and C57BL/10, n = 21 and 17.

LN cytokine production

Skin-draining LNs were harvested from mice 4 days after the second epicutaneous exposure to HDI (day 11). Cells pooled from 2 to 3 mice per group were cultured (4×10^6 /mL) with antigen-presenting cells (syngeneic T-depleted splenocytes, 4×10^6 /mL) and HDI-MSA (100 μ g/mL) in Click/5% FCS at 37°C for 48 hours. Cytokines in supernatants were detected by using commercially available ELISA kits (IFN- γ , IL-4, or IL-5: Endogen, Woburn, Mass; IL-13: R&D Systems, Minneapolis, Minn). Sensitivities were as follows: IFN- γ , 1.95 ng/mL; IL-4, 12.5 pg/mL; IL-5, 31.25 pg/mL; and IL-13, 31.25 pg/mL.

CD4 and CD8 cell enrichment

CD4⁺ and CD8⁺ T cells were enriched from LNs by means of immunomagnetic negative selection with antibodies against CD32/CD16, B220, MHC class II I-A, natural killer (DX5), and either CD8 or CD4 cells, followed by incubation with anti-mouse and anti-rat Ig-coated magnetic beads (Polysciences, Warrington, Pa).²⁷ Enriched populations were 92% to 96% for CD4⁺ cells and 81% to 84% for CD8⁺ cells, respectively. Highly purified CD8⁺ cells were obtained by means of flow cytometric negative selection with FITC-labeled antibodies against CD32/CD16, B220, MHC class II I-A, natural killer (DX5), and CD4 cells and by using a FACS Vantage SE cell sorter (BD Biosciences, Mountain View, Calif). The resultant population was 98% CD8⁺ cells.

Serum antibodies

Serum was collected at animal death on day 21. HDI-specific IgG1 and IgG2a levels were measured by means of ELISA, as previously described.²³ All samples were tested with 2 horseradish peroxidase-labeled rat anti-mouse IgG2a secondary antibodies (R19-15; Pharmingen, San Diego, Calif; LO-MG2a-9; Biosource International, Camarillo, Calif) because of differences in the affinity of these antibodies for IgG2a of different allotypes (ie, different strains). Data are reported as the reciprocal of the highest dilution that still yielded a positive optical density reading of greater than background and negative control values. Data obtained by using the anti-IgG2a that yielded the highest titer for each strain are presented (R19-15 for BALB/c, A/J, and CBA mice and LO-MG2a-9 for C57BL/6 mice). Data shown are the means $\pm$ SEM of individual mice (BALB/c, n = 21; C57BL/6, n = 10; A/J, n = 11; and CBA, n = 11).

Statistical analyses

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

RESULTS

Strain distribution of HDI-induced airway eosinophilia versus CHS

We have previously reported that both CHS and eosinophilic lung inflammation develop in HDI-sensitized BALB/c mice after inhaled antigen challenge.²³ Different mouse strains were evaluated in the present study to determine whether these HDI-induced responses are genetically influenced. As before, HDI-sensitized BALB/c mice had significantly high numbers of eosinophils recovered by means of BAL after inhaled antigen challenge compared with numbers seen in sham-sensitized control animals (Fig 1, A). In comparison with BALB/c mice, all other strains tested, including C57BL/6, A/J, CBA, C3H, and C57BL/10, had significantly fewer airway eosinophils

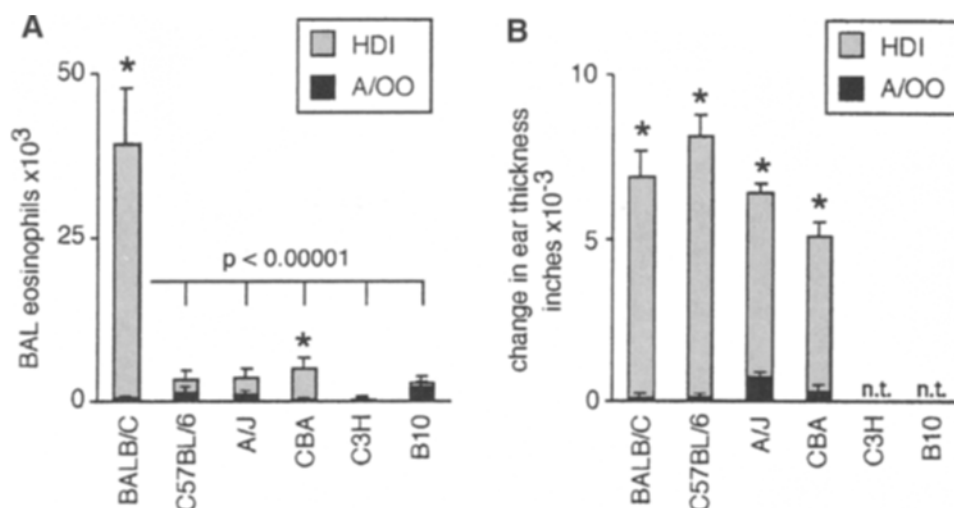


FIG 1. Strain distribution of HDI-induced airway eosinophilia versus CHS. **A**, HDI-sensitized or unsensitized (acetone-olive oil) mice were challenged with inhaled HDI-MSA. **B**, Ears of sensitized or unsensitized mice were challenged with HDI; peak swelling at 24 hours is shown. Data are reported as means $\pm$ SEM of individual mice. * $P \leq .05$, acetone-olive oil group versus HDI group. n.t., Not tested.

(Fig 1, A). Although HDI-sensitized CBA mice did have significantly higher BAL eosinophil numbers than sham-sensitized CBA control animals, their numbers were still significantly lower than those seen in BALB/c mice ($P < .00001$). Similar to the diminished eosinophilia, there was no increase in total BAL cell number or histologic evidence of lung inflammation in any strain other than BALB/c mice (data not shown).

In contrast to lung inflammation, CHS was not limited to BALB/c mice. After challenge of HDI-sensitized mice on the ear, significant swelling, similar to that seen in BALB/c mice, developed in all strains tested, including C57BL/6, A/J, and CBA mice (Fig 1, B). Thus although airway eosinophilia in HDI-sensitized mice was highly strain dependent, CHS was similar in all strains tested, arguing against a failure of these strains to respond to HDI altogether. One explanation for these data could be that the effector cell involved in CHS to HDI was distinct from that mediating airway eosinophilia and that only generation of the latter was impaired in non-BALB/c strains.

LN cytokine production in HDI-sensitized mice

Previous studies showed that generation of airway eosinophilia in HDI-sensitized BALB/c mice was dependent on the T_H2 cytokines IL-4 and IL-13.²³ The impaired ability of several strains to generate airway eosinophilia in the present study therefore would be consistent with decreased T_H2 generation. However, it was also possible that these low-responder strains simply had a defect in recruitment of inflammatory cells to the lung. Skin-draining LNs were isolated after HDI exposure of the high-responder BALB/c mice, as well as the low-responder C57BL/6, A/J, and CBA mice, for evaluation of cytokine production to investigate whether these mice had impaired

T_H2 responses. As shown in Fig 2, LN cells from BALB/c mice produced both T_H2 (IL-4, IL-5, and IL-13) and T_H1 (IFN- γ) cytokines on restimulation in culture. In contrast, LN cells from low-responder strains produced markedly reduced levels of IL-5 and IL-13 compared with those seen in BALB/c mice (Fig 2). These differences were statistically significant ($P \leq .05$) for all strains, with the exception of IL-13 production in CBA mice ($P = .06$). IL-4 and IFN- γ were present in cultures of LNs from BALB/c, A/J, and CBA mice at similar low levels, whereas LN cultures from C57BL/6 mice had no detectable IL-4 or IFN- γ (Fig 2). Thus compared with BALB/c mice, there appeared to be less T_H2 priming in low-responder strains, with decreased production of IL-5 and IL-13 in LNs.

Both CD4 and CD8 cells were enriched from LN cells before restimulation in vitro to confirm that IL-5 and IL-13 produced by LN cells from HDI-sensitized mice was coming from CD4⁺ cells. As shown in Fig 3, A, equivalent or higher levels of IL-4, IL-5, and IL-13 were produced by CD4-enriched cells compared with whole LN cells, whereas IFN- γ levels were decreased. In contrast, CD8-enriched cells produced less IL-5 and IL-13 compared with whole LN cells and no detectable IL-4 (Fig 3, A). Because the CD8-enriched population in these studies was only 81% to 84% CD8⁺, it was not clear whether the low levels of IL-5 and IL-13 in these cultures was due to production by a CD8⁺ cell or a contaminating cell population. Therefore CD8 T cells were highly purified (98%) by means of flow cytometric cell sorting. As shown in Fig 3, B, low levels of IL-5 and IL-13 were still detectable in CD8-purified cultures. However, it seems unlikely that these low levels could be contributing to airway eosinophilia in HDI-sensitized mice, and studies indicating that CD8 cells are not required for this response support this notion.

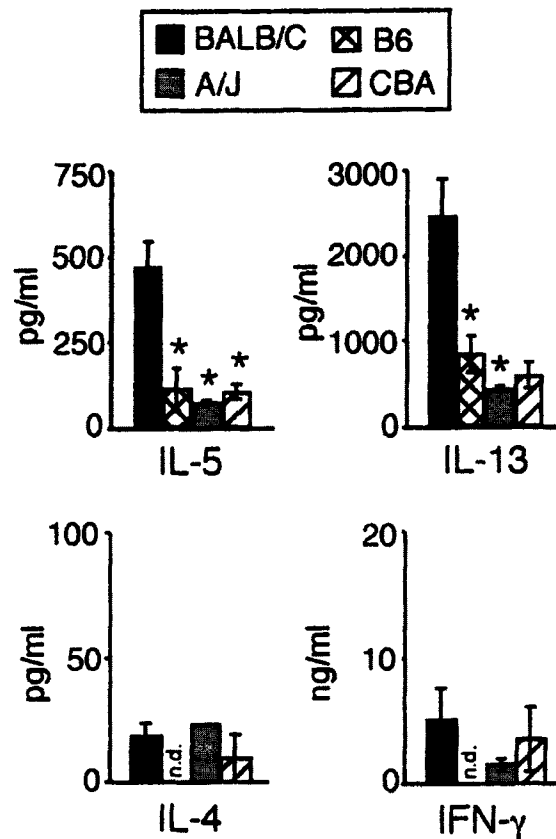


FIG 2. Strain distribution of skin-draining LN cytokine production. LN cells from HDI-exposed mice were restimulated in vitro with HDI-MSA. Cytokine levels were measured in supernatants after 48 hours. Data shown are the means $\pm$ SEM from 2 to 4 experiments for each group. * $P \leq .05$, difference from BALB/c mice. n.d., None detected.

Serum antibodies in HDI-sensitized mice

A comparison of HDI-specific antibody levels in serum was also consistent with decreased CD4 responses in HDI-sensitized C57BL/6, A/J, and CBA mice. We have reported previously that HDI-specific antibodies are only detectable after sensitization with a higher dose of HDI (1.0%).²³ Therefore for these experiments, mice were exposed to 1.0% HDI on days 0 and 7, followed by inhaled antigen challenge. Consistent with earlier findings, HDI-sensitized BALB/c mice had high levels of both HDI-specific IgG1 (T_H2 associated) and IgG2a (T_H1 associated) in serum (Fig 4). C57BL/6, A/J, and CBA mice, however, all had significantly lower levels of HDI-specific IgG1. There was also no detectable IgG2a in the sera of either C57BL/6 or A/J mice and markedly reduced levels in CBA mice (Fig 4).

Airway eosinophilia, but not CHS, is reduced in HDI-sensitized CIITA^{-/-} mice

Taken together, the above results suggested that impaired airway eosinophilic responses after HDI sensitization and challenge in low-responder strains reflected

decreased CD4⁺ T-cell priming. The preservation of HDI-induced CHS in these strains could indicate dependence on a different effector cell population, the generation of which was not strain dependent. To directly assess the role of CD4⁺ T cells in HDI-induced inflammatory responses, we exposed CIITA^{-/-} mice, which lack functional CD4⁺ T cells,²⁴ epicutaneously to HDI as before. As shown in Fig 5, A, the number of BAL eosinophils in HDI-sensitized CIITA^{-/-} mice after inhaled antigen challenge was significantly lower than that from wild-type BALB/c mice. In contrast, CHS responses in CIITA^{-/-} mice were similar to, if not slightly higher than, those of wild-type mice (Fig 5, B). Thus CD4⁺ T cells were not required for generation of CHS to HDI but were important for airway eosinophilia.

CHS, but not airway eosinophilia, is reduced in HDI-sensitized β_2 -microglobulin-deficient ($\beta_2m^{-/-}$) mice

Again, the data reported above indicated that the ability of low-responder strains to have CHS but not robust airway inflammatory responses after HDI sensitization

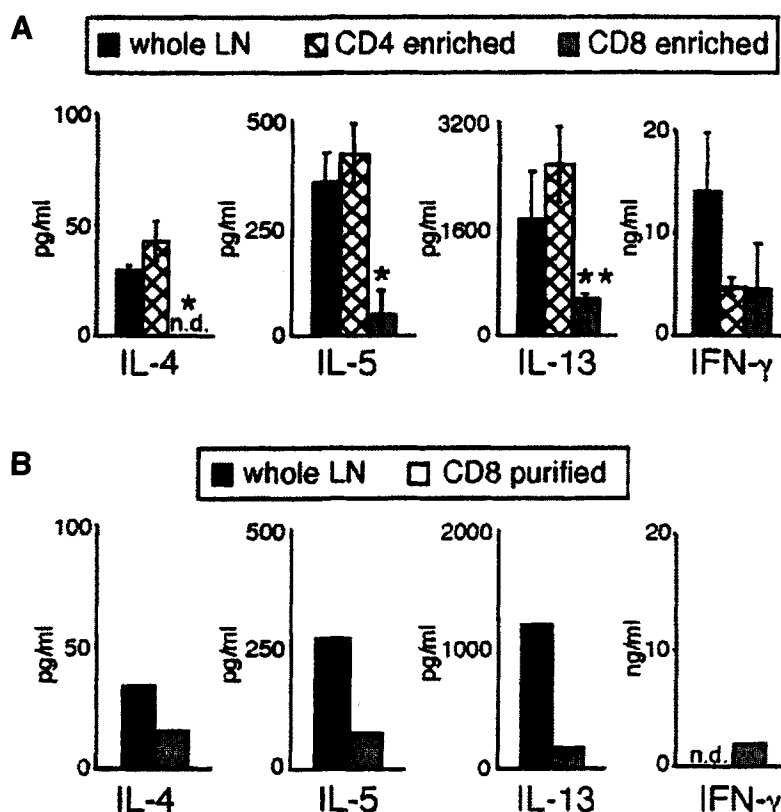


FIG 3. Cytokine production by CD4⁺ and CD8⁺ LN cells. **A**, LN cells from HDI-exposed BALB/c mice were enriched for CD4 or CD8 cells by means of immunomagnetic negative selection. Data shown are the means $\pm$ SEM from 3 experiments. * $P \leq .05$ and ** $P = .06$, difference between CD4- and CD8-enriched groups. *n.d.*, None detected. **B**, CD8 cells were highly purified by means of flow cytometric negative selection (cell sorting). Cytokine levels were measured in culture supernatants 48 hours after restimulation with HDI-MSA. *n.d.*, None detected.

could be explained by different capacities to mount effective CD8 versus CD4 T-cell responses to HDI. Both CD4⁺ and CD8⁺ effector T cells have been shown to be important in the CHS response to various haptens.²⁸⁻³⁰ In addition, although most mouse models demonstrate a clear role for CD4⁺ T cells in eosinophilic lung inflammation, CD8⁺ cells have been reported to contribute in some models.^{31,32} Thus to determine the role of CD8⁺ T cells in HDI-induced inflammatory responses, we exposed $\beta 2m^{-/-}$ mice, which lack functional CD8⁺ T cells,³³ epicutaneously to HDI. As shown in Fig 6, A, $\beta 2m^{-/-}$ mice had no deficit in airway inflammatory responses, with similar numbers of BAL eosinophils in $\beta 2m^{-/-}$ and wild-type mice after inhaled antigen challenge. In contrast, $\beta 2m^{-/-}$ mice had significantly less ear swelling after HDI challenge than wild-type mice ($P < .001$; Fig 6, B). Thus CD8⁺ T cells contribute to CHS to HDI but are not required for HDI-induced airway eosinophilia.

DISCUSSION

Using our recently described mouse model of diisocyanate-induced asthma, we show that the airway

eosinophilic response in HDI-exposed mice is highly dependent on mouse strain. Specifically, although BALB/c mice had high numbers of airway eosinophils after challenge with inhaled HDI, C57BL/6, A/J, CBA, C3H, and C57BL/10 mice all had significantly fewer numbers of BAL eosinophils. This was despite development of CHS to HDI in several of these non-BALB/c strains. Taken together, these findings suggested that distinct effector cell mechanisms were likely responsible for these two responses.

Because our previous studies had shown that HDI-induced airway eosinophilia was dependent on IL-4 and IL-13 but not IFN- γ , it seemed likely that these responses were mediated by T_H2 effector cells, as is the case in several models of atopic asthma.^{34,35} However, CD8⁺ T cells have also been reported to mediate airway eosinophilia in some mouse models.^{31,32} The results reported here with mice deficient in either CD4⁺ or CD8⁺ T cells clearly show that CD4⁺, and not CD8⁺, T cells mediate the airway eosinophilic response in HDI-sensitized mice. Thus the impaired ability of non-BALB/c strains to show airway eosinophilia after HDI sensitization might reflect decreased CD4⁺ T_H2 effector cell priming in these mice.

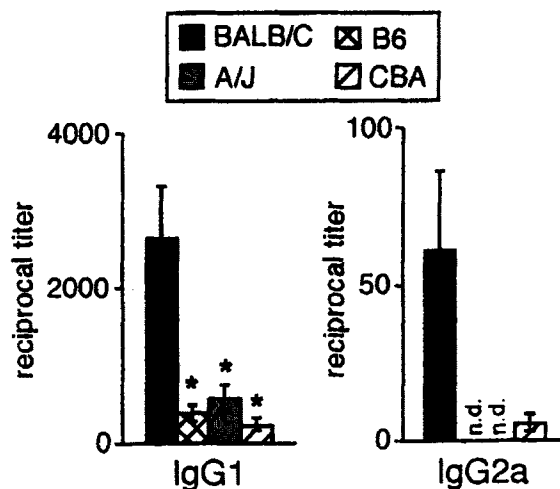


FIG 4. Strain distribution of HDI-specific antibody responses. Mice were epicutaneously exposed to HDI (1.0%) on days 0 and 7 and then challenged with inhaled HDI-MSA. Serum was obtained at the time of death for measurement of antibodies by means of ELISA. Data are reported as means $\pm$ SEM of individual mice. * $P \leq .05$, difference from BALB/c mice. n.d., None detected.

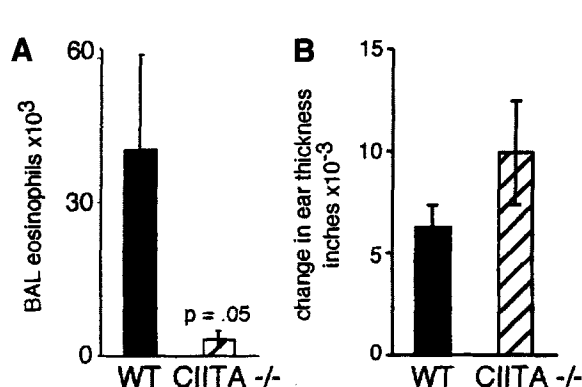


FIG 5. HDI-induced airway eosinophilia and CHS in *CIITA*^{-/-} mice. **A**, HDI-sensitized mice were challenged with inhaled HDI-MSA. Data are pooled from 2 experiments and shown as means $\pm$ SEM of 9 mice per group. **B**, Ears of HDI-sensitized mice were challenged with HDI, and peak swelling at 24 hours is shown. Data are reported as means $\pm$ SEM of 5 mice per group.

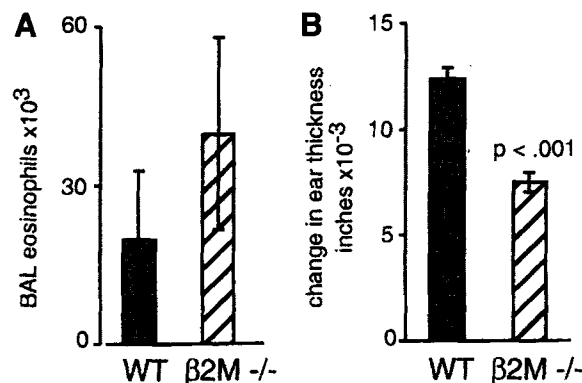


FIG 6. HDI-induced airway eosinophilia and CHS in $\beta 2m^{-/-}$ mice. **A**, HDI-sensitized mice were challenged with inhaled HDI-MSA. Data are reported as means $\pm$ SEM of 5 mice per group. **B**, Ears of HDI-sensitized mice were challenged with HDI, and peak swelling at 24 hours is shown. Data are reported as means $\pm$ SEM of 5 mice per group.

Reduced serum antibodies and decreased T_H2 cytokine production in skin-draining LNs of these low-responder strains is consistent with this model.

As stated above, the discordance between HDI-induced airway inflammation and CHS responses in certain strains suggested that distinct effector cell mechanisms were operating in each situation. This was verified in studies involving *CIITA* or $\beta 2m$ -deficient mice. Given that HDI-induced airway eosinophilia was largely dependent on $CD4^+ T_H2$ cells, it was not surprising that $CD8^+$ T cells were major effector cells in the CHS response to HDI.

The limited ability of several mouse strains to generate significant T_H2 responses and subsequent airway eosinophilia after HDI sensitization is a unique finding with regard to models of allergic airway disease. Although C57BL/6 mice have been shown to be T_H1 biased (and BALB/c T_H2 biased) in certain systems, such as *Leishmania* species infection, this does not hold true for airway inflammation. C57BL/6 mice are characterized as nonresponders with regard to airway hyperresponsiveness, but this is not the case for airway eosinophilia.^{36,37} In fact, there are reports of C57BL/6 mice showing

greater airway eosinophilia than BALB/c mice in direct comparisons.³⁸ Moreover, we have reported that C57BL/6 mice respond with robust airway eosinophilic responses, similar to those of BALB/c mice, when the skin is used as the site of sensitization to the protein antigen ovalbumin (OVA).^{26,39} Indeed, all strains tested thus far in this OVA model, including A/J, C3H, and FVB, are capable of generating these responses (unpublished observations). In addition, A/J mice, which have been characterized as high responders both for airway hyperresponsiveness and eosinophilic inflammation in other models,³⁶ also did not generate HDI-induced airway eosinophilia in the present study. Thus the results presented here are distinct from those previously reported with regard to airway inflammatory responses in different strains of mice. Taken together, the data suggest that the ability to generate airway inflammation after HDI exposure is under different and perhaps tighter genetic control than the ability to mount these responses after exposure to other antigens. This highlights an important distinction between this mouse model of asthma and others, underscoring the importance of using a variety of animal models in investigation of human disease.

The distinction between the strain distribution of HDI-induced airway eosinophilia and that observed in models of atopic asthma (eg, OVA induced) is intriguing, possibly indicating that development of these two types of responses is under different genetic control. Of note, atopy has not been shown to be a risk factor for development of diisocyanate-induced asthma,¹⁰⁻¹³ also suggesting distinct genetic control of human atopic and diisocyanate-induced asthma. In human subjects certain MHC alleles have been linked to an increased risk for developing diisocyanate-induced asthma.⁴⁰⁻⁴² Studies are currently underway to determine whether genetic control of HDI-induced airway responses described in the present study can be explained by differences in MHC haplotypes alone or if other factors might also play a role.

Taken together, the data indicate that the type of response induced after exposure to diisocyanates might be genetically influenced and that generation of an HDI-specific CD4⁺ T-cell response must occur for development of airway disease. Further dissection of the mechanisms underlying HDI-induced inflammatory responses in this mouse model should contribute to our understanding of the pathophysiology of diisocyanate-induced asthma.

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