Aerosol-induced Immunoglobulin (Ig)-E Unresponsiveness to Ovalbumin Does Not Require CD8\(^+\) or T Cell Receptor (TCR)-\(\gamma/\delta\)\(^+\) T Cells or Interferon (IFN)-\(\gamma\) in a Murine Model of Allergen Sensitization

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Summary

Mice exposed for 20 min daily to aerosolized ovalbumin (OVA) for 10 d at concentrations from 1 to 0.01% OVA made greatly reduced immunoglobulin (Ig)-E responses to subsequent immunogenic OVA challenges, given either intraperitoneally or by aerosol. This IgE-specific unresponsiveness lasted for at least four months. However, these aerosol-treated mice were primed for larger OVA-specific IgG1 and IgG2a responses. The specific reduction in IgE responses was not due to preferential induction of a T helper (Th)-1 response as aerosol OVA–primed mice made greatly reduced Th2 and no detectable Th1 response after rechallenge in vitro. Consistent with this, the increase in circulating eosinophils observed in control Th2–primed mice was absent in aerosol OVA–treated animals. Interferon (IFN)-\(\gamma\) was not required for this unresponsiveness, as IFN-\(\gamma\) knockout mice and anti–IFN-\(\gamma\) antibody-treated wild-type mice had greatly reduced levels of IgE similar to wild-type controls. CD8\(^+\) T cells played a relatively small role as IgE responses were reduced to about the same extent in \(\beta_2\) microglobulin-deficient, or in anti-CD8–treated wild-type mice as in normal mice after aerosol OVA treatment. Similarly, T cell receptor (TCR)-\(\gamma/\delta\) T cells were not required for maximal inhibition of the IgE response. These results demonstrate that exposure to inhaled protein antigens can induce a state of unresponsiveness of CD4\(^+\) T cells that results in a prolonged loss of IgE and eosinophil responses to subsequent challenges. This T cell unresponsiveness was shown not to require CD8\(^+\) or TCR-\(\gamma/\delta\) T cells or IFN-\(\gamma\).

The respiratory tract is in constant contact with airborne particles from the environment. Immune responses are mounted to most inhaled pathogens but normally not to abundant nonpathogenic antigens such as pollen and animal danders. This differential responsiveness is due, in part, to a series of specific and nonspecific barriers including the filtration in the nose, tight junctions between epithelial cells, secretory antibodies, and pulmonary macrophages in the lower respiratory tract (reviewed in reference 1). Despite the presence of these barriers, sensitization against nonpathogenic antigens can occur, often with features of a Th2 response (2).

Th2 responses are mediated by CD4\(^+\) cells that secrete cytokines such as IL-4, IL-5, IL-10, and IL-13 that are known to play a central role in allergic responses (3–5). IL-4 (and IL-13 in humans) regulates the production of IgE (6–8) and IL-5 is responsible for the growth, differentiation, and activation of eosinophils (reviewed in reference 9). Elevated levels of eosinophils are an indicator of allergy and increased numbers correlate well with the severity of an allergic asthmatic condition (10, 11). A second major subset of T helper cells, designated Th1, secretes IL-2, TNF, and IFN-\(\gamma\), mediates delayed-type hypersensitivity responses, and is inhibitory for Th2 responses (12). IFN-\(\gamma\) is the Th1 cytokine responsible for the inhibition of IL-4–mediated IgE responses both in vitro and in vivo (13). More recently, it was found that IFN-\(\gamma\) from CD8\(^+\) cells may play a role in the natural immune response to inhaled soluble protein antigens (14). Exposure of naive rats to repeated doses of aerosolized OVA induced MHC class I–restricted, OVA-specific IFN-\(\gamma\)–producing CD8\(^+\) T cells that could suppress the IgE response to OVA. This suppression was transferable with a small subset of antigen responsive CD8\(^+\), TCR-\(\gamma/\delta\) T cells (15, 16).

This study was undertaken to analyze the effects of aerosol exposure of mice to OVA on subsequent immunogenic...
challenges with this antigen. To directly address whether IFN-γ and specific subsets of IFN-γ producing cells are required to confer a state of IgE unresponsiveness, in vivo experiments were carried out using mice deficient in IFN-γ, CD8, and γδ cells. OVA-specific immunoglobulins, cytokines, and blood eosinophils from these mice were compared with wild-type controls.

Materials and Methods

Animals. BALB/cAnN mice were obtained from Simonen Laboratories (Gilroy, CA) and Taconic Farms Inc. (Germantown, NY). C57BL/6J mice and C57BL/6J-Tg Ifngtm1Unc were obtained from The Jackson Laboratories (Bar Harbor, ME). BALB/c-Ifngm1 mice were obtained as heterozygotes and inbred to produce homozygous mutant mice. All animals were raised free of common mouse pathogens condition at the DNAX Research Institute animal facility. They were 6-8 wk old at the start of each experiment. Female mice were used for most experiments. However, comparison within some experiments using both male and female mice revealed no sex-related differences.

Aerosol and Intraperitoneal Antigen Exposure. The first experiment was a dose titration to determine the amount of inhaled OVA necessary to cause maximal reduction in the OVA-specific IgE response. Naive mice (5 mice/group) were exposed to different concentrations of aerosolized OVA in PBS for 10 d (beginning at day 0). Aerosolization was performed for 20 min using a Passport aerosol compresor (Invacare Corporation, Elyria, OH) connected to a box 3 ft in size that served as the deposition chamber for the mice. Control mice were exposed to aerosolized PBS from days 0 through 9. At day 27, all groups were challenged intraperitoneally (IP) with 10 μg OVA (grade V, A-5503; Sigma Chemical Co., St. Louis, MO) in 2 mg of aluminum hydroxide (AL) gel (OVA/AL IP). On day 120 all mice were exposed to 1% aerosolized OVA. For all other experiments, mice were typically divided into three groups. In one group, mice were sensitized with 10 μg OVA/AL IP. A second group was exposed to 1% aerosolized OVA daily from day 0 to 9 while a third group was exposed to aerosolized PBS. All groups received 10 μg OVA/AL IP on day 21 and 1% aerosolized OVA for 20 min on day 35. For experiments using TCR−δ−/− mice, OVA/AL IP challenge was carried out on day 19 and the final exposure to 1% aerosolized OVA was performed on day 44. Mice were bled from tail veins and serum stored at −70°C until assayed for antibody.

In Vivo Antibody Treatment. Mice were injected intraperitoneally with the monoclonal antibody 2.43 (0.5 mg/mouse) for the depletion of CD8 cells. For depletion of CD4 cells, each mouse was injected with 0.5 mg of GK 1.5. Isotype control antibody was GL113 that was given at 2 mg/mouse.

Mice were depleted of IFN-γ by injection of 1 mg of rat IgG anti-mouse IFN-γ (XMG-1.2). This dose of anti–IFN-γ has been shown to be effective in many experiments, and, in one instance, was able to neutralize both endogenous IFN-γ and 150 μg of recombinant IFN-γ over a 1-wk time course (17). The anti-CD8 depletion was verified by flow cytometry of spleen cells from mice treated 48 h earlier (not shown).

Quantification of Eosinophils in the Peripheral Blood. Eosinophils were quantified by dilution of heparinized blood in Discombe’s fluid as previously described (18). This cell suspension was placed on a hemacytometer and eosinophils counted with a light microscope.

Cytokine ELISA. Sandwich ELISAs were done to measure IL-3, IL-5, IL-10, and IFN-γ as described by Abrams (19). In brief, ELISA plates were coated with the appropriate anticytokine antibodies and incubated at 4°C overnight. After incubation, plates were blocked for 30 min at room temperature by adding 150 μl of 20% FCS PBS containing 0.04% Tween 20 (FCS-PBST; Sigma Chemical Co.) to each well. Supernatants from in vitro restimulations of lung cells were diluted in 5% FCS RPMI 1640 and added to each well at a volume of 50 μl/well. Plates were incubated overnight at 4°C then washed and the second-step antibody that was diluted in FCS-PBST was added at 50 μl/well. Plates were incubated for 1 h then washed and the enzyme conjugate, diluted in FCS-PBST, was added to each well. Plates remained at room temperature for 1 h after which they were washed and 100 μl/well of substrate containing 1 mg/ml 2′,2′-azinobis(3-ethylbenzthiazolinesulfonic acid (Sigma Chemical Co.), 0.003% H2O2 in 0.1 M Na2HPO4 and 0.05 M citric acid was added. After the substrate was developed, the reaction was stopped by applying 0.05 ml of 0.2 M citric acid solution to each well. The plates were read on an ELISA reader (Molecular Devices Corp., Sunnyvale, CA) at wavelengths λ1 and λ2, where λ1 equaled 405 and λ2 was 490.

Analysis of OVA-Specific Serum IgE. OVA-specific serum IgE was determined using a two-step sandwich ELISA as described by van Hateren and colleagues (20). The coating antibody was a monoclonal anti-IgE antibody called EM 95. The second step was a digoxigenin-coupled ovalbumin (DIG-OVA) that was prepared from a kit containing digoxigenin-3-0-methylcarbonyl-e-amono caproic acid N-hydroxy-succinimide ester (Boehringer Mannheim GmbH, Mannheim, Germany). In brief, plates were coated with 2 μg/ml of EM 95 and incubated overnight at 4°C. The serum samples were added and the DIG-OVA was added to the wells. The antibody fragment 2a conjugated to peroxidase (Boehringer Mannheim GmbH, Mannheim, Germany) was added. After 1 h of incubation, 0.1 ml of substrate as described above was added to each well.

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Abbreviations used in this paper: AL, aluminum hydroxide; βm−/−, β2 microglobulin knock-out mice; DIG, digoxigenin; IFN-γ−/−, IFN-γ gene knock-out mice; IP, intraperitoneally; NIP, nitroiodophenyl; T, T cell receptor δ chain; TcR−δ−/−, T cell receptor δ knock-out mice.
with the piston of a 10-ml plastic syringe. The suspended cells were washed three times in Hanks BSS solution. The cells were stimulated in 1-ml cultures. The culture medium consisted of RPMI 1640 (JRH Biosciences, Lenexa, KS) with 10% heat-inactivated FCS (Sigma Chemical Co.), 0.05 mM 2-ME (Sigma Chemical Co.), 2 mM L-glutamine (JRH Biosciences), and penicillin/streptomycin (GIBCO BRL, Gaithersburg, MD). Lung cells were stimulated at 5 \times 10^6 cells/ml in culture medium containing 0.25 mg/ml OVA. The supernatant was harvested at 72 h.

Measurement of OVA in the Lungs of Aerosolized Mice. Immediately after a single OVA exposure, lungs were removed and a cell suspension was prepared as above. The supernatant from the centrifugation was retained for quantification of OVA by an OVA-specific ELISA. Plates were coated with 5 \mu g/ml of rabbit anti-chicken egg albumin (Sigma Chemical Co.) in PBS and incubated overnight. After incubation, plates were blocked by adding FCS PBST. Plates were washed and samples were titrated on the test wells and incubated for a minimum of 3 h. Plates were washed and 1 \mu g/ml of biotin-labeled rabbit anti-chicken egg albumin was added to each test well. After 1 h of incubation horse-radish peroxidase-conjugated streptavidin was added to each well. After incubation for 1 h, substrate was added and plates developed as described for antibody ELISA.

Analysis of IL-4. IL-4 from cultured supernatant was detected by a bioassay using the IL-4-dependent CT.4S cell line (donated by Dr. William Paul, National Institutes of Health, Bethesda, MD). Cells were counted, washed, and resuspended in culture medium at a concentration of 1 \times 10^5 cells/ml of medium. Standard and supernatants to be tested were titered on to 96-well flat bottom plates at 50 \mu l/well. The range of the standard was 100 pg/ml to 0.78 pg/ml. The culture medium containing the CT.4S cells was added to the wells at a concentration of 50 \mu l per well. Plates were incubated for 72 h at 37°C in a humidified atmosphere containing 5% CO2. At the end of the incubation period, 10 \mu l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma Chemical Co.) was added to each well and the plates were incubated for 4 h at 37°C. After incubation, 150 \mu l of 0.04 N HCL in isopropanol was added to each well and the absorbance were measured from an ELISA reader at wavelengths 570 and 650.

Statistics. Levels of antibodies, eosinophils, and cytokines were calculated as mean and SEM. The two-tailed \( P \) values were calculated according to the Mann-Whitney test. A value of \( P < 0.05 \) was considered significant.

Results

OVA-specific IgE Antibodies Were Reduced but IgG1 and IgG2a Were Elevated in Mice Sensitized with Aerosolized OVA. A dose titration experiment was carried out to determine the amount of inhaled OVA necessary to cause maximal reduction in the OVA-specific IgE response. Naive mice were exposed to various concentrations of aerosolized OVA for 10 d, (beginning at day 0). At day 27, all groups were challenged with an intraperitoneal injection of 10 \mu g OVA/AL, a challenge previously shown to elicit strong primary and secondary IgE responses. OVA-specific IgE responses were much lower in all groups exposed to aerosolized OVA as compared with the control group exposed to aerosolized PBS (Fig. 1). Maximal reduction of the IgE response was observed in groups exposed to 1% down to 0.01% OVA. There was a 30-fold decrease in OVA-specific IgE in these groups when compared with the PBS control group (\( P < 0.02 \)). Partial reduction of IgE...
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was observed with 0.001% OVA, the lowest concentration tested. To determine the persistence of the IgE unresponsiveness, all mice were challenged again on day 120, this time with 1% aerosolized OVA. The OVA-specific IgE responses in all aerosol exposure groups remained substantially below the responses in the control group (Fig. 1).

To estimate the amounts of OVA actually delivered to the lungs, naive mice were exposed to varying doses of aerosolized OVA for 20 min and their lungs removed immediately for assay of OVA. Mice given a 20-min aerosol exposure to 1% OVA, the highest concentrations used in these experiments, received a dose of 3.35 ± 0.4 μg of OVA to their lungs (Fig. 2). Each 10-fold reduction of OVA concentration resulted in a 10-fold reduction in the amount of OVA delivered to the lungs although the OVA concentration in 0.001% OVA-treated mice was below the threshold of detection in the ELISA. The clearance of OVA from the lungs was rapid. Mice exposed to 1% OVA cleared 86% of the antigen within 17 h and 99.5% by 48 h (data not shown).

The effects of the initial aerosol exposures on the OVA-specific IgG1 and IgG2a responses to subsequent challenge with OVA/AL IP differed markedly from the effects on the IgE response (Fig. 1). Aerosol exposure led to a dose-dependent priming for both isotypes and all doses tested primed the mice for higher responses than the PBS control. After the second challenge (Fig. 1 day 131) OVA-specific IgG1 and IgG2a responses remained higher in all groups previously exposed to aerosolized OVA but the priming was less apparent, as even the PBS control group now represented a secondary antibody response. In summary, the 10-d course of aerosol exposure to a wide range of OVA concentrations led to enhanced IgG1 and IgG2a responses to subsequent immunogenic challenges with OVA, but simultaneously induced a profound and long lasting absence of OVA-specific IgE responses to the same challenges. These results demonstrated that primary exposure to an aerosolized protein can lead to an unresponsiveness to subsequent immunogenic challenges that is quite specific for the IgE isotype. We have recently found that five aerosol exposures, on alternate days, resulted in a comparable loss of the OVA-specific IgE response (data not shown).

Depletion or Absence of CD81 T Cells Had a Minimal Effect on IgE-specific Unresponsiveness. The IgE-specific suppression induced by aerosolized OVA that was described by Holt and colleagues could be transferred by CD81 T cells in both the rat (15) and the mouse (16). To determine whether CD81 T cells were required for the response in our studies, aerosol- and PBS-treated mice were depleted of CD81 T cells by injection of anti-CD8, either during the aerosol exposures (day 1 and 7) or just before the second challenge (day 7) or the second challenge (day 131). Mice treated with antibody to CD8, either on day 1 or day 7, showed no loss of the OVA-specific IgE response compared to PBS-treated controls (Table 1). These results were similar to those observed in our previous studies (15, 16).

Table 1. OVA-specific IgE and IgG1 in BALB/c Mice After Depletion of CD4 or CD8 and Exposure to OVA

| Antibody administered (day) | Primary exposure | IgE | IgG1 |
|-----------------------------|------------------|-----|------|
| GL113 (−1,+7)               | 1% Aerosolized OVA day 0–9 | 11 ± 2 | 6 ± 2 | 142 ± 39 | 424 ± 147 |
| Anti-CD4 (−1,+7)            | 1% Aerosolized OVA day 0–9 | <1.56 | <1.56 | <0.23 | <0.23 |
| Anti-CD8 (−1,+7)            | 1% Aerosolized OVA day 0–9 | 15 ± 7 | <1.56 | 180 ± 25 | 244 ± 60 |
| Anti-CD8 (20, 34)           | 1% Aerosolized OVA day 0–9 | 7 ± 3 | 33 ± 22 | 771 ± 447 | 1,321 ± 700 |
| Anti-CD4 (−1,+7)            | PBS               | <1.56 | <1.56 | <0.23 | <0.23 |
| Anti-CD8 (−1,+7)            | PBS               | <1.56 | 263 ± 40 | <0.23 | 259 ± 67 |
| Anti-CD8 (20, 34)           | PBS               | <1.56 | 244 ± 60 | <0.23 | 192 ± 36 |
| GL113 (−1,+7)               | PBS               | <1.56 | 169 ± 61 | <0.23 | 383 ± 73 |

2° 10 μg OVA/AL IP on day 21. 3° 1% Aerosolized OVA on day 35.
ondary and tertiary OVA challenges (day 20 and 34). For this, and all subsequent experiments, a standardized schedule of aerosol exposure on days 0 through 9, followed by intraperitoneal challenge with OVA/AL on day 21 and a single aerosol OVA challenge on day 35 was used (see Table 2).

Administration of anti-CD8 on day 1 and day 7 did not prevent aerosol OVA-induced IgE unresponsiveness (Table 1), after the secondary challenge (15 ± 7 compared with 11 ± 2 ng/ml anti-CD8 and control antibody, respectively). The slightly higher IgE response in anti-CD8-treated mice was transient as there was no elevation of OVA-specific IgE when these mice were reexposed to aerosolized OVA. However, in vivo depletion of CD8 cells in the PBS control groups led to a slight enhancement of the OVA-specific IgE response. To determine the importance of CD8 T cells after the IgE unresponsiveness was established, mice were treated with anti-CD8 1 d before both the 2nd and 3rd OVA exposures. The OVA-specific IgE of these mice was slightly higher than other aerosol-treated groups, but was still significantly lower than the PBS exposed, control antibody-treated group (33 ± 22 compared with 169 ± 61, P = 0.05, n = 5). As expected, treatment of mice with anti-CD4 resulted in the ablation of both the OVA-specific IgE and IgG1 responses.

To confirm that the OVA-specific IgE unresponsiveness in anti-CD8-treated, aerosol OVA-exposed mice was not due to small numbers of residual CD8+ T cells, experiments were carried out using BALB/c mice that lacked CD8 T cell due to the disruption of the β2m gene. In these experiments, an additional control group of mice injected with 10 μg OVA/AL IP at day 0 was added. All groups received a secondary challenge of OVA/AL IP on day 21 and a tertiary challenge of 1% aerosolized OVA on day 35. BALB/c β2m−/− mice exposed to aerosolized OVA had a sevenfold reduction in OVA-specific IgE when compared with the PBS control β2m−/− group (14 ± 10 vs. 96 ± 25, respectively, Table 2). However, in most experiments, the reduction in IgE responses was not quite as complete in β2m−/− mice as in the aerosolized OVA-treated wild-type group (14 ±10 vs. 6 ± 2, respectively) at day 7 after the 3rd challenge. Comparison of IgG1 responses between aerosol OVA- and OVA/AL IP-primed mice shows that aerosol priming, although significant, was not as effective as priming by OVA/AL injection. In summary, CD8+ T cells did not appear to be required for the loss of IgE responsiveness after aerosol OVA exposure.

IFN-γ W as Not Required for Aerosol OVA-induced IgE Unresponsiveness. IFN-γ produced either by CD8+ T cells or CD4+ Th1 cells has the potential to preferentially inhibit IgE responses. To determine whether IFN-γ was involved in OVA-specific IgE unresponsiveness in mice exposed to aerosolized OVA, experiments were done with BALB/c IFN-γ−/− mice (Table 2). The experimental protocol was as described for the β2m−/− mice. IgE-specific unresponsiveness after aerosolization was as complete in IFN-γ−/− as in wild-type controls. The OVA-specific IgE responses in control groups were similar to those in wild-type mice, but IgG1 responses were generally higher in IFN-γ−/− mice. In similar experiments, anti–IFN-γ antibody treatment of aerosol OVA primed BALB/c mice had no effect on the IgE unresponsiveness (data not shown). These results demonstrated that the specific loss of IgE responses was independent of IFN-γ and suggested that the aerosol exposure to OVA did not induce a dominant Th1-like response.

TCR-γδ T Cells Were Not Required for Aerosol OVA-induced IgE Unresponsiveness. It has been reported that T cells bearing TCR-γδ are the principal mediators of transferable IgE-specific suppression to OVA in mice (16) and rats (15). To try to confirm this, TCR-γδ−/− and wild-type mice were primed with aerosolized OVA or PBS or OVA/AL

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### Table 2. OVA-specific IgE and IgG1 in Wild-type, β2m−/− and IFN-γ−/− Mice After Exposure to OVA

| Mice       | Primary exposure | IgE | IgG1 |
|------------|------------------|-----|------|
|            |                  | Day 7 after 2° | Day 7 after 3° | Day 7 after 2° | Day 7 after 3° |
| IFN-γ−/−   | 1% Aerosolized OVA day 0-9 | 7 ± 3 | <1.56 | 120 ± 34 | 249 ± 99 |
| β2m−/−     | 1% Aerosolized OVA day 0-9 | 14 ± 9 | 14 ± 10 | 317 ± 241 | 187 ± 102 |
| Wild-type  | 1% Aerosolized OVA day 0-9 | 11 ± 5 | 6 ± 2 | 142 ± 39 | 424 ± 147 |
| IFN-γ−/−   | PBS              | <1.56 | 166 ± 52 | <0.23 | 82 ± 19 |
| β2m−/−     | PBS              | <1.56 | 96 ± 25 | <0.23 | 84 ± 39 |
| Wild-type  | PBS              | <1.56 | 169 ± 61 | <0.23 | 383 ± 73 |
| IFN-γ−/−   | 10 μg OVA/AL IP day 0 | 516 ± 61 | 555 ± 264 | 3,073 ± 705 | 10,122 ± 1607 |
| β2m−/−     | 10 μg OVA/AL IP day 0 | 337 ± 63 | 417 ± 73 | 2,768 ± 1466 | 2,077 ± 338 |
| Wild-type  | 10 μg OVA/AL IP day 0 | 379 ± 100 | 446 ± 150 | 4,141 ± 2216 | 3,351 ± 554 |

2° 10 μg OVA/AL IP on day 21. 3° 1% Aerosolized OVA on day 35.
IP and rechallenged twice, as in previous experiments. The ability of aerosol OVA priming to induce subsequent IgE unresponsiveness to OVA was not impaired in mice with no TCR-δ T cells. Indeed, the deletion of this population had no significant effect on the tertiary (Table 3) or secondary (not shown) IgE or IgG1 responses with any of the priming conditions. The threefold difference in IgG1 responses in the PBS primed groups in the experiment shown in Table 3 was not a consistent finding.

Blood Eosinophilia Was Reduced in Aerosolized OVA-sensitized Mice. To determine whether the eosinophil response that usually accompanies a strong Th2 response was also reduced in mice initially exposed to aerosolized OVA, eosinophils were counted at day 4 after the tertiary exposure to aerosolized OVA. In wild-type, IFN-γ−/−, and β2m−/− mice rendered unresponsive with aerosol OVA, however, eosinophils were not significantly elevated, as compared with unimmunized mice of the same genotype. Indeed, the slight elevation of eosinophils in some of the IgE unresponsive groups over the unexposed controls may be due to the presence of alum from the IP injection as there was no elevation in groups after the initial 10-d exposure to aerosolized OVA (data not shown). Injection of alum alone into naïve mice can cause elevation of blood eosinophils (unpublished observation). Thus, mice primed by aerosolized OVA are deficient in eosinophil responses, as well as IgE responses.

Cytokine Profiles in the Lungs of Mice After Allergen Sensitization. To determine whether Th2 responses were reduced and/or Th1 responses induced in aerosol OVA-exposed mice, cytokine production was measured after the tertiary exposure to aerosolized OVA. Time course experiments have shown that the peak cytokine recall response from lung cells in vitro occurred 5–8 d after OVA/AL-sensitized mice are exposed to aerosolized OVA (22). For these experiments, lungs were removed and restimulated in vitro on day 7 after the tertiary exposure. Pooled cells from the homogenized lungs and associated lymph nodes were used for in vitro restimulation. We have seen no substantial differences in the cytokine response in the lungs when compared with the associated lymph nodes (our unpublished observa-

Table 3. IgE and IgG1 Anti-OVA Response in Wild-type and TCR-δ−/− Mice After Exposure to OVA

| Mice | Primary exposure | IgE Day 7 after 3° | IgG1 Day 7 after 3° |
|------|------------------|-------------------|-------------------|
|      | ng/ml            | µg/ml             | ng/ml            | µg/ml             |
| Wild-type | 1% Aerosolized OVA day 0-9 | 15 ± 8 | 142 ± 61 |
| TCR-δ−/− | 1% Aerosolized OVA day 0-9 | 5 ± 0.5 | 310 ± 213 |
| Wild-type | PBS | 188 ± 132 | 233 ± 65 |
| TCR-δ−/− | PBS | 144 ± 73 | 694 ± 153 |
| Wild-type | 10 µg OVA/AL, day 0 | 600 ± 275 | 1,268 ± 413 |
| TCR-δ−/− | 10 µg OVA/AL, day 0 | 713 ± 412 | 1,283 ± 419 |

2° 10 µg OVA/AL IP on day 19. 3° 1% Aerosolized OVA on day 44.

Figure 3. Blood eosinophils in OVA-sensitized mice. □, Wild-type; ■, β2m−/−, and ■, IFN-γ−/− BALB/c mice were bled from the tail vein on day 4 after the tertiary exposure and the eosinophils were counted in Discombe’s fluid on a hemacytometer (18). Data represent the mean (± SEM) eosinophil counts per milliliter in groups of five mice.
Discussion

The nature of the initial CD4⁺ T cell response to an airborne antigen is central in determining whether or not an allergic response is made to subsequent exposures to the same antigen. However, relatively few studies have investigated the nature of the primary T cell response to aerosolized protein antigens. Substantially more is known about the allergic responses to aerosol antigens in experimental animals primed initially for a Th2 response by an intraperitoneal or subcutaneous route. In this study, the IgE, eosinophil, and CD4⁺ T cell responses of mice primed by exposure to repeated doses of aerosolized OVA were compared with optimally Th2-primed and unprimed mice. We have confirmed the observations of Holt and coworkers (14, 23) that animals repeatedly exposed to low levels of aerosolized OVA are unable to produce IgE antibodies to subsequent immunogenic challenges to OVA, but produce normal secondary IgG responses to such challenges. However, the results of investigation into the nature of the T h response that accompanies this isotype-specific unresponsiveness have led us to somewhat different conclusions about the mechanism of this isotype-specific unresponsiveness.

Intraperitoneal injection of 10 μg OVA/AL induced OVA-specific IgE in naive BALB/c mice, but not in mice exposed previously for 10 d to aerosolized OVA. This IgE unresponsiveness was induced, in a dose-dependent manner, by a wide range of aerosol OVA concentrations (0.001–1%). These same doses did not suppress OVA-specific IgG1 and IgG2a responses, but rather primed them, again in a dose-dependent manner. Thus, mice primed with 1% OVA made the highest amount of OVA-specific IgG1 and IgG2a and the lowest amount of OVA-specific IgE response after the IP challenge. Moreover, the IgE-specific unresponsiveness persisted with subsequent OVA challenges, either single aerosol exposures or OVA/AL IP injections (data not shown), and was observed as late as four months after the initial aerosol exposures.

The actual doses of OVA delivered to the respiratory tract were estimated by measuring the amounts of OVA recoverable in soluble form at the end of the exposure period. They varied proportionally with the concentrations of OVA used, from 3.5 μg to <1.6 ng/dose. The most consistent and complete inhibition of IgE responses was obtained with the 3.5-μg dose and this was chosen for subsequent studies. This dose would not be expected to give either the high dose or low dose tolerance seen in many classical studies of immunological tolerance in mice (24–26). Indeed, others have shown that 1–10 μg OVA given intraperitoneally every other day, without adjuvant, for 10–14 d produces a strong Th2 response with high levels of OVA-specific IgE (27).

Similar experiments have been performed to study regulation of the immune response to aerosol antigens in inbred BALB/c mice. Mice primed by OVA/AL IP or PBS mounted a strong Th2 response as indicated by the significant levels of IL-4, IL-5, and IL-10 and undetectable levels of IFN-γ (Table 4). IFN-γ−/− and β2m-deficient mice from the PBS control group made similarly elevated levels of IL-4, IL-5, and IL-10 (data not shown). Cytokines were generally undetectable in mice primed by passive aerosolization with OVA/AL IP except for very low levels of IL-4. Furthermore, IFN-γ was not detected in any of the aerosol OVA–treated groups. A similar pattern of cytokine response was observed from restimulated spleen cells. Spleens from the PBS control group that received subsequent challenges with OVA/AL IP made a strong Th2 cytokine response whereas those primed by repeated exposure to aerosolized OVA remained virtually unresponsive (unpublished observation). Thus, aerosol-sensitized mice showed no evidence of preferential induction of a Th1 response to OVA, rather the Th helper response in these mice was largely absent in comparison to the control groups.

**Table 4. Cytokine Production After In Vitro Restimulation of Lung Cells from Aerosol-exposed Wild-type, β2m−/−, and IFN-γ−/− BALB/c Mice**

| Mice              | Primary exposure | IL-3 | IL-4 | IL-5 | IL-10 | IFN-γ |
|-------------------|------------------|------|------|------|-------|-------|
| β2M−/−            | 1% Aerosolized OVA day 0–9 | <0.156 | 21 ± 1.9 | <0.156 | <0.625 | <0.156 |
| IFN-γ−/−          | 1% Aerosolized OVA day 0–9 | <0.156 | 21 ± 2.0 | <0.156 | <0.625 | <0.156 |
| Wild-type         | 1% Aerosolized OVA day 0–9 | <0.156 | 23 ± 2.4 | <0.156 | <0.625 | <0.156 |
| Wild-type         | PBS              | <0.156 | 3.52 ± 0.73 | 8.80 ± 2.60 | <0.156 |
| β2M−/−            | 10 μg OVA/AL IP day 0 | <0.156 | 425 ± 82 | 12.90 ± 1.1 | 34.10 ± 5.4 | <0.156 |
| IFN-γ−/−          | 10 μg OVA/AL IP day 0 | <0.156 | 119 ± 24 | 5.22 ± 1.27 | 8.00 ± 2.98 | <0.156 |
| Wild-type         | 10 μg OVA/AL IP day 0 | <0.156 | 583 ± 240 | 10.08 ± 2.6 | 18.30 ± 4.2 | <0.156 |

2° 10 μg OVA/AL IP on day 22, 3° 1% Aerosolized OVA on day 36. Restimulation was performed on day 7 after 3° in vivo antigen rechallenged using 5 × 10⁶ unseparated lung cells/ml plus 0.25 mg/ml OVA.
treated control mice. In PBS and OVA/AL IP controls, significantly different from those in wild-type mice. Thus, solized OVA was tested in two ways. Depletion of CD8 responses seen in mice exposed to repeated doses of aero-
sitization or during the secondary and tertiary challenges had very little effect on the degree of inhibition of the IgE response. This result was confirmed with β2m-deficient mice that lack CD8+ T cells. In most experiments, aerosol OVA-treated BALB/c β2m–/– mice did not make IgE responses higher than wild-type controls. In a minority of experiments, the inhibition of IgE was not as complete in the β2m–/– mice, but was well below the levels in PBS-treated control mice. In PBS and OVA/AL IP controls, BALB/c β2m–/– mice gave IgE responses that were not significantly different from those in wild-type mice. Thus, it appears that CD8+ T cells do not have a major role in aerosol-induced IgE unresponsiveness to soluble protein antigens. In contrast, Holt and coworkers have shown that as few as 1 × 10^6 CD8+ T cells from aerosol OVA sensi-
tized mice could adoptively transfer an active IgE-specific suppression in both rats (15) and mice (16) challenged with OVA/AL IP. Similarly, it has been shown that cells from OVA-exposed mice reconstituted with OVA-sensitized CD8+ T cells, made reduced levels of IgE and IgG1 responses when stimulated in vitro (30). It should be noted that our experiments do not rule out the possibility that CD8+ T cells could transfer an IgE-specific suppression, but simply show that such suppression does not require CD8+ T cells in the primary host.

Likewise it has been suggested that TCR-γδ+ T cells are the principal mediators of IgE-specific suppression in this model. In adoptive transfer experiments, as few as 3 × 10^4 TCR-γδ+ cells from aerosol OVA-primed mice sup-
pressed OVA-specific IgE from mice primed by OVA/ AL(16). Our finding that mice deficient in TCR-γδ+ T cells have the same degree of IgE-specific unresponsiveness as wild-type controls after aerosol priming and intraperito-
neal challenge with OVA argues that such cells are not re-
necessary for this unresponsiveness. Again, our results do not preclude that TCR-γδ+ T cells can also mediate this un-
responsiveness.

A reasonable hypothesis to explain the highly specific in-
hibition of the production of IgE, but no other isotype
would be that repeated aerosol exposure induced Th1-
biased T cell response and that IFN-γ was the specific in-
hibitor of IgE production. Th1 cells in vitro can provide help for all Ig isotypes except IgE (13). In vivo, there are many demonstrations of the IgE inhibitory effects of IFN-γ (31–33), and the ability of Th1 responses to suppress IgE production (34–36). However, the results presented here make clear that IFN-γ is not required for maximum IgE unresponsiveness, either during the initial aerosol sensitization or during subsequent immunogenic challenges. Mice rendered either temporarily deficient in IFN-γ by treatment with a neutralizing antibody, or congenitally deficient by disruption of the IFN-γ gene, developed quite normal IgE responses in control groups and developed IgE unre-
ponsiveness identical to control mice when exposed to aerosolized OVA. Furthermore preliminary studies show that treatment of mice with neutralizing antibodies to IL-
12, a cytokine required for optimum production of IFN-γ (37), was also ineffective in preventing this state of IgE-spe-
cific unresponsiveness (data not shown).

Direct examination of the OVA-specific T cell recall re-
response in vivo revealed that aerosol OVA-primed mice did not develop significant CD4+ T cell responses of either a Th1 or Th2 type in the lungs, after subsequent challenges with OVA under conditions that stimulate strong, highly polarized Th2 responses in control groups. Thus it appears that specific loss of IgE responses in aerosol OVA tolerized mice reflects a more fundamental unresponsiveness in the entire OVA-specific CD4+ T cell population. Whether this T cell unresponsiveness is intrinsic to the OVA-specific T cells or reflects the action of a separate regulatory popu-
lation, has not yet been determined by us. However, previous experiments with this model in both rats and mice sug-
gest that an active suppression is involved (15, 16).

Although this low dose, aerosol-induced IgE and Th2 unreponsiveness is not due to preferential induction of an OVA-specific Th1 response, a Th1 can lead to a pattern of unresponsiveness similar to that described here. Using several high (100 μg) intranasal doses of OVA to induce toler-
ance, van Haleren et al. (38) have shown induction of a Th1-like response to OVA that inhibited subsequent IgE responses via IFN-γ. This unresponsiveness was specific to IgE and could be transferred with CD4+ T cells. Similar results were observed by Hoyne et al. (39) when mice were given three intranasal doses (100 μg each) of peptides derived from house dust mite allergen. Before the develop-
ment of tolerance, there was a rapid and transient activation of CD4+ T cells that secreted Th1 cytokines when restim-
ulated in vitro.

Similar analyses have been done to study the unrespon-
siveness that occurs after oral antigen ingestion. Although some investigators have suggested that γδ T cells regu-
late the induction of oral tolerance others have shown that CD4+ T cells are required for its induction (41–42). Investi-
gators have shown that the feeding of mice transgenic for OVA-specific TCR with high doses of OVA can inhibit airway eosinophilic inflammation induced by intratrache-
ally administered OVA. This inhibitory effect can be adoptively transferred by splenic CD4+ T cells demonstrating that it is an active mechanism. The suppression of eosinophils can also be blocked by anti-TGF-β but not by anti-IFN-γ (43). Marth and colleagues have shown that when OVA-specific TCR transgenic mice were fed high doses of OVA, it led to the generation of T cells from Peyer’s patches that produced substantial amounts of IFN-γ that resulted in systemic unresponsiveness. However, administration of anti-IL-12 to these animals led to an increase in TGF-β. These investigators later postulated that low dose oral tolerance may result from insufficient Th1 cytokines but increase production of TGF-β (44, 45). Thus, a similar mechanism may be occurring in this study. Therefore, if this is an active suppression, mediated by CD4+ T cells, it may reflect similar activity of TGF-β-secreting CD4+ T cells (termed Th3; reference 46) or a CD4+ regulatory T cell subset similar to that recently described by Groux and colleagues (47).

Consistent with the absence of a significant Th2 response, primary exposure to aerosolized OVA also led to an almost complete lack of an eosinophil response to subsequent OVA challenges. Blood eosinophils were significantly elevated after challenges in PBS- and OVA/AL-primed mice. This absence of significant blood eosinophilia in aerosol OVA-primed mice confirmed the absence of a detectable IL-5 response.

In summary, the data presented above demonstrate a potentially important mechanism that limits the induction of Th2-mediated allergic responses to frequently encountered airborne antigens. Although IFN-γ, TCR-γ/δ+, and CD8+ T cells have been suggested by others to be important parts of the IgE-specific unresponsiveness, our results suggest a loss of both Th1 and Th2 CD4+ T cell responses occurs that is not mediated by TCR-γ/δ+, CD8+, or cells IFN-γ. Much about the mechanisms for regulation of a host response to inhaled antigens remains to be defined. A knowledge of this mechanism will help us understand the deregulation of the immune response that ultimately results in pulmonary allergic disorders.

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We acknowledge Dr. Gabriele Grunig for technical assistance and expert advice in the preparation of this manuscript and Drs. Amy Beebe, Stephen Hurst, Douglas Robinson, and Thierry von der Weid for constructive comments on the manuscript. We also thank Dr. Derry R. Oopenian of The Jackson Laboratories for the original breeding pairs of BALB/c β2m−/− mice.

This research was supported in part by the University of California, Tobacco Related Disease Research Program. The DNAX Research Institute is supported by the Schering Plough Corporation.

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Received for publication 10 September 1997 and in revised form 26 November 1997.
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