

Nebulized IFN- γ Inhibits the Development of Secondary Allergic Responses in Mice¹

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The effects of nebulized IFN- γ on primary and secondary IgE production and development of airway hyper-responsiveness (AHR) were investigated. BALB/c mice received primary exposure to aerosolized OVA daily for 10 days and developed anti-OVA IgE responses, immediate cutaneous reactivity to OVA, and altered airway function when assayed on day 12. After secondary exposure to OVA challenges on days 30 and 31, these mice developed an amplified IgE response, heightened cutaneous reactivity to OVA and AHR when measured on day 37. Administration of IFN- γ for 13 days, beginning 3 days prior to and during primary OVA sensitization, resulted in a decrease in anti-OVA IgE, increases in serum anti-OVA IgG2a levels, a decrease in cutaneous reactivity to OVA, and normal airway function when assessed on day 12 after primary sensitization. This treatment also prevented the development of secondary anti-OVA IgE responses and altered airway responsiveness but did not induce a secondary rise in anti-OVA IgG2a in the serum measured on day 37. Treatment with IFN- γ on days 26 to 30, well after primary responses were established but just prior to secondary OVA challenge, abolished the development of secondary anti-OVA IgE responses, resulted in an increase in anti-OVA IgG2a in the serum, and prevented the development of AHR. In vitro, CD4⁺ T cells obtained from OVA-sensitized mice treated with either "early" or "late" IFN- γ inhibited IgE production. Delivery of IFN- γ to the airways can prevent secondary allergen sensitization even after primary sensitization has been achieved and this effect is mediated by CD4⁺ T cells. *The Journal of Immunology*, 1996, 157: 1432–1439.

IFN- γ exhibits a multiplicity of biologic effects in part due to the widespread expression of the receptor on numerous cell types (1). Together with IL-4, it plays a central role in the regulation of both murine and human IgE production. Indeed, IFN- γ and IL-4 reciprocally regulate murine Ig isotype production: IL-4 stimulates the production of IgG₁ and IgE whereas IFN- γ inhibits the production of these isotypes and stimulates IgG2a production (2). Similarly, in humans, IFN- γ inhibits the effect of IL-4 on IgE production in culture systems using either purified B cells (3) or PBMC (4, 5). In an in vivo mouse model, IFN- γ has been shown to suppress polyclonal IgE production in response to activation of B cells with goat anti-mouse IgD (6). In a murine model of OVA sensitization via the airways, we showed that parenteral IFN- γ effectively suppressed polyclonal IgE production but did not affect allergen-specific IgE production (7). Furthermore, cutaneous reactivity to the inhaled allergen and development of airway hyper-responsiveness (AHR) was unaffected by this parenteral treatment. In contrast, nebulized IFN- γ was found to decrease anti-OVA IgE in the serum of sensitized mice and to reciprocally increase levels of anti-OVA IgG2a Ab production. There was a significant inverse correlation between the suppression of anti-OVA IgE and the induction of anti-OVA IgG2a. In parallel to

these changes, nebulized IFN- γ diminished cutaneous reactivity to OVA and prevented alterations in airway function that normally occur following OVA sensitization. In these previous studies, the timing of treatment was critical in that nebulized IFN- γ had to be administered prior to the 7th day of OVA sensitization in order to suppress anti-OVA IgE production. This requirement is consistent with in vitro observations that the early addition of IFN- γ to mononuclear cells incubated with IL-4 is necessary in order to inhibit IgE production (5). These data implied that IFN- γ may only be effective in inhibiting IgE production both in vitro and in vivo at an early stage, prior to heavy chain rearrangement in B cells; once the B cell is committed to IgE production, IFN- γ may no longer be effective in blocking IgE production.

These data raise the question of whether secondary IgE production can be inhibited by IFN- γ treatment after a primary allergic response has been established. This is an important issue when considering approaches to modify human allergic disease in which seasonal exposure to various allergens results in renewed allergic symptoms. If indeed secondary and subsequent IgE responses are derived largely from a pool of memory B cells committed to IgE production, it may be too late to intervene and modulate secondary IgE production. If, on the other hand, secondary IgE responses result from IgM-producing memory B cells that must undergo heavy chain rearrangement from μ to ϵ , there exist potential windows of opportunity to modulate secondary IgE responses. No data exist to distinguish between these two hypotheses in humans. However, there is evidence in the mouse that both polyclonal and specific secondary IgE responses are derived largely from IgM-producing memory B cells (8, 9). In mice reinfected with *Nippostrongylus braziliensis*, 65 to 90% of the secondary polyclonal IgE response remains IL-4-dependent (9). The small secondary IgE response that occurred in the anti-IL-4-treated mice was due to incomplete inhibition of IL-4 rather than due to IL-4-independent B cells precommitted to IgE; the further addition of anti-IL-4R Ab

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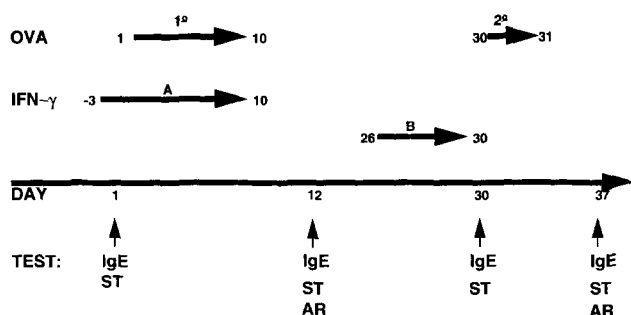


FIGURE 1. Experimental design. Mice received primary exposure (1°) to OVA for 10 days. On days 30 and 31 they received secondary (2°) OVA challenges. Serum was collected and skin testing (ST) to OVA was performed on days 1, 12, 30, and 37 as indicated. AR was measured either on day 12 following primary exposure or day 37 following secondary exposure. Mice were treated according to one of two protocols. In treatment protocol A, mice received daily nebulized IFN- γ for 3 days prior to primary OVA sensitization and then daily for 10 days during OVA sensitization (–3 to 10). In protocol B, mice received IFN- γ only on days 26 to 30 prior to secondary OVA challenge.

to the anti-IL-4-treated animals caused a 99% inhibition of the secondary IgE response (10).

In order to assess whether IFN- γ can affect secondary allergen-specific IgE responses, we extended our model of OVA sensitization. Mice treated with nebulized OVA for 10 days developed an anti-OVA IgE response, immediate cutaneous reactivity to OVA and increased airway responsiveness (AR)³ (11). By day 30 of this protocol, anti-OVA IgE responses and cutaneous reactivity to OVA were diminished and airway function returned to normal. Secondary challenge with nebulized OVA on days 30 and 31 resulted in an amplified, secondary anti-OVA IgE response measured on day 37, that was accompanied by renewed cutaneous reactivity to OVA and AHR. In the present study we examined B and T cell function following primary and secondary allergen challenge and treatment with IFN- γ .

Materials and Methods

Sensitization procedure

Virus-free BALB/c mice between 8 and 12 wk of age (The Jackson Laboratory, Bar Harbor, ME) were sensitized to OVA (chicken OVA, grade V; Sigma Chemical Co., St. Louis, MO) as described previously (11). Briefly, groups of mice were exposed to OVA (1% w/v diluted in PBS) for 20 min daily over a period of 10 consecutive days (days 1 to 10). They were rested and then rechallenged 20 days later for 20 min on 2 consecutive days (days 30 and 31). Groups of three to four mice were treated daily with nebulized IFN- γ (Genentech, San Francisco, CA; 250,000 U dissolved in 7 ml of PBS) (7) using two different protocols: for 13 days including 3 days of pretreatment and the entire 10-day period of primary OVA sensitization or, for 5 days on days 26 to 30 prior to secondary OVA challenge (see Fig. 1). Both the 13- and 5-day schedules of IFN- γ treatment were shown to effectively inhibit anti-OVA IgE production in preliminary studies. Serum was obtained for Ig determinations and immediate skin testing for OVA was performed on days 1, 12, 30, and 37.

Determination of serum Ig

Serum IgE and IgG anti-OVA Ab levels were measured by ELISA as described (11), using alkaline phosphatase-conjugated rat anti-mouse IgE, IgG, or IgG subclass mAbs (PharMingen, San Diego, CA). The serum Ab levels in individual samples were calculated by comparison with internal

standards. Addition of a 1000-fold excess anti-OVA IgG did not affect the ability to detect anti-OVA IgE. Heating the serum for 2 h at 56°C completely prevented the detection of anti-OVA IgE but did not prevent the detection of anti-OVA IgG. Analysis of ELISA data was performed with the microplate manager software for the Macintosh computer (Bio-Rad, Richmond, CA). Total serum IgE, IgG, and IgG subclasses were determined using the same method in ELISA plates coated with goat anti-mouse IgE or goat anti-mouse IgG Ab, and levels were calculated by comparison with known mouse IgE or IgG standards (PharMingen).

Intradermal skin testing

The mice were tested for immediate cutaneous hypersensitivity as described previously (11). The ventral surface of the mouse was carefully shaved, and 20 μ l of test solution were injected intradermally with a 30-gauge needle. Test solutions consisted of PBS and OVA at a concentration of 500 μ g/ml, pH 7. Resultant wheal reactions were assessed after 15 to 20 min and were scored as positive if the wheal diameter was greater than 3 mm in any direction. The evaluator was blinded to the sensitization status of the animals.

Determination of airway responsiveness

In vitro airways smooth muscle responsiveness was assessed by electrical field stimulation of tracheal smooth muscle preparations (12). Briefly, after killing the animal, tracheal smooth muscle segments of about 0.5 cm in length were cut and placed in tissue baths suspended by triangular supports transducing the force of contractions. Electrical field stimulations were delivered by a stimulator (Grass SIVS) using 8-V, 2-ms pulse durations. Stimuli were delivered successively at increasing electrical frequencies starting at 0.5 Hz and augmenting the frequency until a peak contractile response was obtained. Generally, the contractile response reached a plateau at 40 Hz. The electrical frequency that resulted in 50% of the maximal contraction (ES_{50}) was derived from linear plots. The evaluator was unaware of the sensitization status of the animals. Alterations in AR as measured by electrical field stimulation in sensitized animals reflect a change in acetylcholine release from neural terminals (13).

Preparation and isolation of MNC and lymphocyte subpopulations

Local draining peribronchial lymph nodes (PBLN) and spleens were removed from the different experimental groups of mice on day 12 after primary OVA exposure or on day 37 after secondary OVA challenge. The tissue was washed through stainless steel sieves, and single cell populations were obtained. Mononuclear cells (MNC) were purified by lymphoprep gradient centrifugation and washed three times with PBS. Cells were resuspended in complete tissue culture medium (RPMI 1640, Life Technologies, Grand Island, NY) supplemented with streptomycin (100 μ g/ml), penicillin (100 U/ml), L-glutamine (5 mmol/L), 2-ME (2 mmol/L), and 10% heat-inactivated FCS (Hyclone, Logan, UT). Two approaches were used to purify CD4⁺ T cells; in the first approach, B cells were eliminated by positive selection using beads coated with anti- κ Ab. From the residual cells that were more than 98% CD3⁺, CD8⁺ T cells were removed by positive selection (anti-CD8-coated magnetic beads) yielding a population of cells that was 92% positive for CD4. In a second approach, CD4⁺ T cells were positively selected achieving a population of >97% CD4⁺ T cells.

Measurement of TdR incorporation

For proliferation assays, 2×10^5 MNC/well were incubated in triplicate. Cells were cultured in the presence of medium alone or in the presence of OVA at different concentrations. Incorporation of [³H]TdR was measured after 5 days. During the last 4 h of culture, cells were pulsed with 1 μ Ci of [³H]TdR (ICN Biomedicals, Costa Mesa, CA; sp. act., 6.7 Ci/mmol/L), and DNA was harvested with a Cambridge cell harvester (Cambridge Technology, Watertown, MA). [³H]TdR incorporation was measured in a scintillation counter.

Flow cytometry

Levels of expression of CD4, CD8, V β , and B220 were assessed on freshly prepared MNC and lymphocyte subpopulations as previously described. Anti-CD4 and anti-CD8 Abs were FITC-conjugated. An anti-B220 biotinylated Ab was used to identify B cells. The following biotinylated anti-V β Abs were used: V β 2[B20.6]; V β 8.1+8.2[KJ16]; and V β 14[14-2]; (kindly provided by Drs. P. Marrack and J. Kappler, Denver, CO). Cell analysis was performed using an EPIC PROFILE II flow cytometer (Coulter, Hialeah, FL).

³ Abbreviations used in this paper: AR, airway responsiveness; AHR, airway hyper-responsiveness; EU, ELISA units; PBLN, peribronchial lymph nodes; MNC, mononuclear cells; ES_{50} , electrical frequency that resulted in 50% of the maximal contraction.

Measurement of Ig production in culture supernatants

MNC prepared from spleens of OVA-sensitized mice were suspended in complete tissue culture medium and cultured at a concentration of 1×10^6 cells/well in 24-well tissue culture plates. One million CD4⁺ T cells pooled from sensitized animals were added to the MNC and cultures contained medium alone, IL-4 (400 U/ml), or IL-4 plus anti-IFN- γ Ab (1 μ g/ml). At the end of a 14-day culture period, IgE and IgG levels were measured by ELISA as described (7).

Statistical analysis

Individual experiments consisted of three to four mice per treatment group. The data analyzed represent the results of three or more individual experiments. The two-tailed Student *t* test was used to determine significance between groups for airway responsiveness. Significance for skin test responses was calculated using the χ^2 test. Comparison of the treatment groups for Ab levels was performed by a one-way analysis of variance.

Results

Secondary challenge with OVA results in an amplified IgE response

In three different experiments, mice were sensitized to OVA on days 1 through 10, and then rechallenged on days 30 and 31. Anti-OVA Ig measurements were performed at different time points. As shown previously (7), at day 12 sensitized mice developed increased serum levels of anti-OVA IgE Abs (Fig. 2A); in control animals that received IFN- γ or PBS alone, no anti-OVA IgE was detectable at this time (data not shown). IgE Ab levels plateaued at 1520 ± 62 EU/ml on day 12 and decreased by 59% to 626 ± 48 EU/ml on day 30. Secondary OVA challenges on days 30 and 31 triggered a secondary anti-OVA IgE response when measured on day 37 that exceeded the primary response by 31% (1998.3 ± 78 EU/ml). Other anti-OVA Ig isotypes were not significantly produced during the early primary Ab response. Anti-OVA IgG1 began to increase only on day 18 and reached a plateau by day 25 (data not shown). Secondary OVA challenge on days 30 and 31 induced an increase in anti-OVA IgG1 as measured on day 37. Other isotypes (IgG2a, IgG2b, IgG3) remained undetectable following both the primary and secondary exposures to Ag (data not shown).

OVA sensitized mice lose their cutaneous reactivity to OVA but regain the response after secondary OVA challenge

Eleven out of fifteen mice developed cutaneous reactivity to OVA on day 12 after primary OVA exposure (Table I). The cutaneous response was specific for OVA sensitization; mice exposed to OVA did not develop cutaneous reactivity to PBS or an irrelevant allergen, ragweed. By day 30, only 4 out of 15 mice retained cutaneous reactivity to OVA. However, after secondary challenge with OVA on days 30 and 31, 12 out of 15 mice had cutaneous responses to OVA on day 37.

Secondary OVA challenge causes AHR

Primary OVA sensitization results in altered airway function as determined by the decrease in ES_{50} values compared to animals exposed to PBS (from 4.0 ± 0.2 Hz to 2.4 ± 0.2 Hz). By day 30, airway function reverted to normal (3.8 ± 0.2 Hz). However, secondary challenge with OVA on days 30 and 31 resulted in AHR as measured by a $55 \pm 4.4\%$ decrease in the ES_{50} compared to controls (Fig. 3). This protocol of secondary airway challenge on two successive days proved to be optimal for the development of increased AR when compared to a single airway challenge which did not consistently alter airway responsiveness. Control mice that received two exposures to nebulized OVA on two consecutive days without having been previously sensitized did not develop AHR (data not shown).

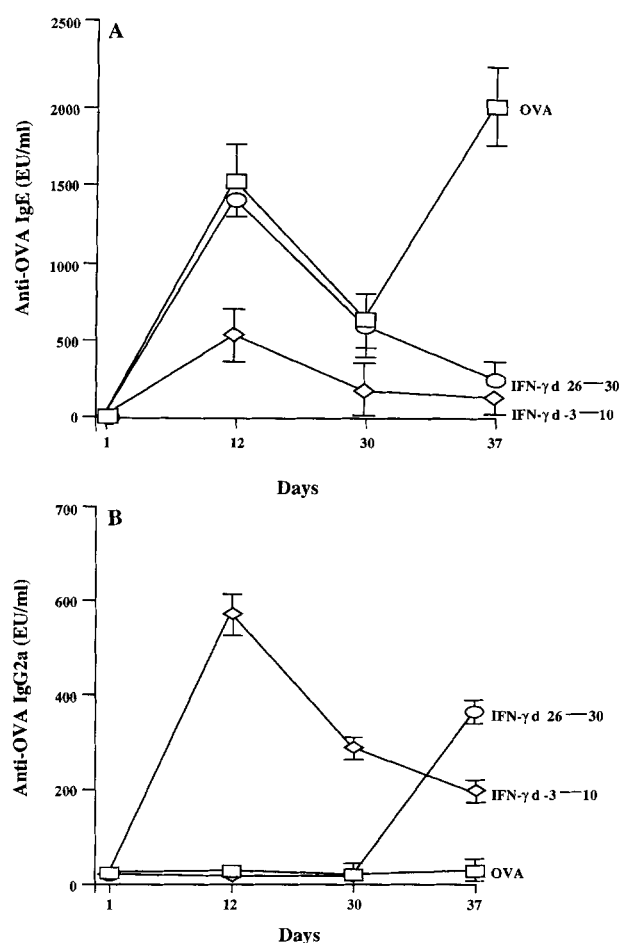


FIGURE 2. Nebulized IFN- γ prevents secondary IgE responses. Groups of mice were exposed to nebulized OVA over 10 days and rechallenged with nebulized OVA on days 30 and 31. In parallel, mice exposed to nebulized OVA were treated with nebulized IFN- γ on day -3 through day 10 or on days 26 to 30. Sera were obtained on days 1, 12, 30, and 37. Shown are the mean ($\pm$ SEM) for A) anti-OVA IgE, and B) anti-OVA IgG2a levels expressed as EU/milliliter from three separate experiments ($n = 12$ to 15 mice in each group). (* $p \leq 0.01$ compared with OVA treatment alone).

Early treatment with nebulized IFN- γ inhibits both primary and secondary anti-OVA IgE responses in the serum

We showed previously that treatment of mice with nebulized IFN- γ was most effective in the inhibition of IgE responses following primary OVA sensitization when administered daily for 3 days prior to OVA sensitization and then daily during the 10-day period of OVA exposure (7). We employed this treatment procedure and confirmed our previous results showing that this regimen of nebulized IFN- γ treatment inhibited anti-OVA IgE by more than 60% on day 12 (Fig. 2A). Mice treated in such a manner were rested and subsequently received a secondary OVA challenge on days 30 and 31. These mice showed no increase in secondary anti-OVA IgE measured on day 37, in contrast to untreated animals that received both primary sensitization and secondary OVA challenge. Indeed, on day 37 following secondary OVA challenge, serum anti-OVA IgE levels had fallen to 117.4 ± 13.6 EU/ml. In a similar number of IFN- γ treated animals not killed on day 37, serum anti-OVA IgE levels were no longer detectable on days 41 and 50 (data not shown). Thus, treatment with IFN- γ during primary sensitization suppressed the secondary allergen-specific IgE

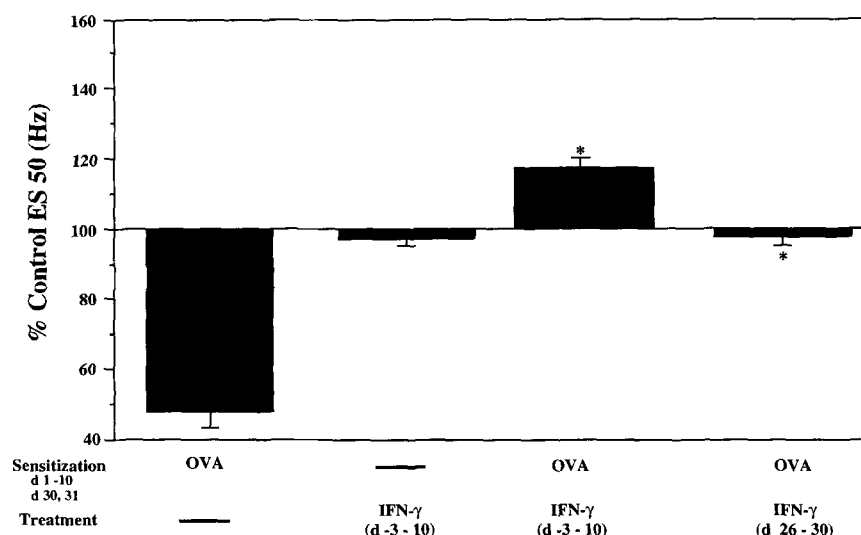
Table I. *IFN- γ inhibits immediate cutaneous reactivity after secondary sensitization^a*

Groups	Primary (Day 12)	Before Secondary (Day 30)	After Secondary (Day 37)
OVA 10 days	11/15 (73.3%)	4/15 (26.7%)	12/15 (80%)
IFN days 3 to 10	5/15 (33.3%) ^b	2/15 (13.3%)	1/15 (6.6%) ^b
IFN days 26 to 30	8/12 (62.5%)	3/12 (25%)	2/12 (12.5%) ^b

^a Cutaneous reactivity to OVA was determined sequentially in the same animals described in Figure 2 on days 12, 30, and 37. Expressed are the number and percentage of mice with positive skin tests to OVA.

^b $p < 0.001$ compared with OVA group.

FIGURE 3. Nebulized IFN- γ prevents the development of AHR following secondary OVA challenge. Groups of mice were treated as described in Figure 2. Additionally, a control group of mice received IFN- γ in the absence of OVA sensitization on day -3 through day 10. Airway responsiveness was assessed on day 37. Expressed along the y-axis is the percentage of inhibition of the normal ES_{50} ($\pm$ SEM) obtained in control PBS-treated mice which was 4.0 ± 0.2 Hz ($*p < 0.001$ compared with the OVA group).



response as well as inhibiting the primary IgE response. Parenteral IFN- γ not only failed to inhibit Ag-specific IgE responses on day 12, but this treatment also failed to inhibit secondary responses as well (data not shown).

Late treatment with nebulized IFN- γ eliminates anti-OVA IgE responses to secondary challenge even after normal primary sensitization

In order to determine whether treatment with IFN- γ affects secondary IgE responses after primary sensitization had been established, mice were sensitized to OVA on days 1 through 10 and were treated with nebulized IFN- γ for 5 days on days 26 to 30, prior to secondary challenge. Despite achieving normal primary anti-OVA IgE production, these mice failed to develop secondary anti-OVA IgE responses in the serum (Fig. 2A). There was no significant difference in the serum anti-OVA IgE levels in this group of mice on day 37 (post-secondary challenge) as compared with the levels measured in the mice that received early IFN- γ treatment, indicating that the late treatment protocol was as effective at suppressing secondary IgE responses as was the early treatment regimen. Administration of IFN- γ i.p. on days 26 to 30 failed to alter the secondary Ag-specific IgE responses (data not shown). This demonstrates that it is possible to inhibit the development of secondary IgE responses even when nebulized IFN- γ is administered to mice after they have developed a primary anti-OVA IgE response.

Both early and late treatments with nebulized IFN- γ inhibit the development of cutaneous reactivity to OVA after secondary OVA challenge

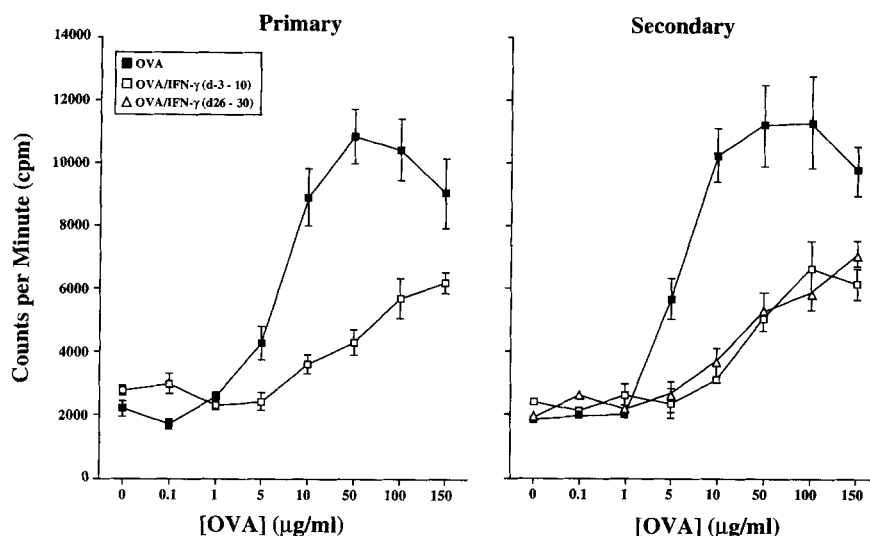
As demonstrated previously, nebulized IFN- γ treatment for the 13 days of primary sensitization decreased cutaneous responses to

OVA measured on day 12 (7). This treatment, however, did not interfere with the ability of mast cells to degranulate since intradermal injection with compound 48/80 produced significant wheals in the treated mice. Secondary OVA challenge on days 30 and 31 did not induce cutaneous responses to OVA in these IFN- γ -treated mice (only 1/15 positive responders), in contrast to untreated animals that received secondary OVA challenge and developed renewed cutaneous reactivity to OVA (Table I). Therefore, early treatment with IFN- γ prevented the subsequent development of cutaneous reactivity to OVA associated with secondary challenge. Treatment with IFN- γ during days 26 to 30, after primary sensitization, also prevented the development of cutaneous reactivity to OVA on day 37 following secondary OVA challenge. Only 2/12 mice had positive cutaneous responses on day 37 after secondary OVA challenge. Thus, even after primary sensitization to OVA and the development of cutaneous responses on day 12, subsequent treatment with IFN- γ was still able to prevent the development of cutaneous reactivity following secondary OVA challenge (Table I).

Both early and late treatments with nebulized IFN- γ prevent the development of AHR after secondary OVA challenge

We previously found that treatment with nebulized IFN- γ prevented the development of AHR after primary OVA sensitization (7). We examined the effects of IFN- γ on the development of AHR following secondary OVA challenge using the same 13 day pre-treatment protocol. We found that airway function was normal following secondary OVA challenge (ES_{50} 4.7 Hz $\pm$ 0.4), indicating that IFN- γ administered during primary sensitization prevented the development of AHR even after secondary OVA challenge (Fig. 3). In order to determine whether IFN- γ treatment

FIGURE 4. Nebulized IFN- γ inhibits T cell proliferation. One group of mice was treated with OVA or OVA and IFN- γ (day -3 through day 10) and killed on day 12 (primary). A second group of mice was sensitized to OVA through day 10 and again on days 30 and 31; one set of mice received IFN- γ on days -3 to 10, and the other IFN- γ from days 26 to 30. These animals were killed on day 37 (secondary). PBLN-derived MNC were stimulated in vitro with OVA and [^{3} H]thymidine uptake was assessed after 5 days of in vitro stimulation. Expressed are the counts per minute from a representative experiment (of three similar experiments) using PBLN pooled from three to four mice in each set, carried out in triplicate.



administered after primary sensitization induced alterations in airway function, mice that underwent primary sensitization to OVA, were treated with nebulized IFN- γ on days 26 to 30 and rechallenged with OVA on days 30 and 31. ES_{50} levels on day 37 were 4.4 ± 0.6 Hz, indicating that IFN- γ prevents the development of AHR in response to secondary challenge even if administered after the establishment of primary allergic sensitization (Fig. 3).

Early treatment with IFN- γ induces primary but not secondary anti-OVA IgG2a production, whereas late treatment with IFN- γ induces secondary IgG2a production

Nebulized IFN- γ given prior to and during primary OVA sensitization induces the production of anti-OVA IgG2a in the serum and IgE and IgG2a anti-OVA Ab levels are reciprocally regulated by IFN- γ (7). Figure 2B shows that this early regimen of IFN- γ treatment for 13 days induced a marked increase in serum IgG2a in the serum compared with OVA control animals in which IgG2a was undetectable. Secondary challenge with OVA on days 30 and 31 did not cause a secondary IgG2a response on day 37 following secondary OVA challenge in mice treated with IFN- γ for the 13 days. However, the 5-day treatment regimen with nebulized IFN- γ on days 26 to 30 induced an anti-OVA IgG2a response on day 37 in response to secondary challenge to OVA, despite the failure to detect this isotype in mice who underwent primary sensitization in the absence of IFN- γ treatment at that time.

Nebulized IFN- γ suppresses OVA-induced proliferation in PBLN-derived T lymphocytes

Proliferative responses to OVA were measured in PBLN-derived lymphocytes. Lymphocytes were prepared and cultured from mice on day 12 following primary OVA sensitization. In vivo sensitization with OVA resulted in a heightened proliferative response to in vitro OVA stimulation in PBLN-derived lymphocytes as compared with responses in the PBS-exposed group (Fig. 4). Analysis of cells on day 5 of culture showed the lymphoblast population to consist of greater than 98% CD3 $^+$ cells; $92 \pm 2.1\%$ stained for CD4 whereas $7.1 \pm 1.4\%$ stained for CD8. Peak proliferative responses for PBLN-derived lymphocytes were observed at OVA concentrations between 5 and 50 μ g/ml for lymphocytes from OVA-sensitized mice. PBLN-derived lymphocytes from PBS and IFN- γ control groups showed either minimal or no proliferation in response to OVA (data not shown). Thirteen days of nebulized IFN- γ resulted in a marked decline in the proliferative response to

OVA in lymphocytes from sensitized mice with complete suppression occurring in some experiments. In those experiments in which low levels of proliferation were observed in the IFN- γ treated group, the dose response curve was shifted to the right with peak proliferative responses occurring between 50 and 150 μ g/ml (Fig. 4).

Proliferative responses to OVA were also determined in mice that received secondary OVA challenge, and lymphocytes were prepared on day 37 of the protocol and cultured in the presence of Ag. At this time point, the degree of proliferation was comparable with that obtained in lymphocytes following primary sensitization. Lymphocyte proliferation remained suppressed in the group that had received nebulized IFN- γ during primary sensitization compared with the OVA-sensitized group that received no IFN- γ treatment. Treatment with IFN- γ on days 26 to 30 also resulted in suppression of the proliferative response to OVA, comparable with the suppression observed in the group that received early treatment. The lymphoblast composition in the IFN- γ -treated groups was similar to that seen in the OVA-sensitized mice and always consisted of greater than 90% CD4 $^+$ cells and less than 9% CD8 $^+$ cells.

CD4 $^+$ T cells from IFN- γ treated mice inhibit in vitro IgE production

Addition of CD4 $^+$ T cells from OVA-sensitized mice did not significantly affect IgE production by sensitized MNC whereas coculture with CD4 $^+$ T cells from OVA-sensitized mice that had been treated with early IFN- γ decreased IgE production (Table II). This inhibitory effect on IgE production occurred irrespective of whether the CD4 $^+$ T cells were prepared on day 12 after primary OVA exposure or on day 37 after secondary OVA challenge, but after the early treatment with IFN- γ . The inhibition of IgE production by CD4 $^+$ cells appeared to be due in part to the production of IFN- γ since the addition of anti-IFN- γ Ab (1 μ g/ml) partially reversed this inhibition. CD4 $^+$ T cells from OVA-sensitized mice that received late treatment with IFN- γ were also able to inhibit in vitro IgE production by MNC, indicating that both early and late treatment with IFN- γ resulted in CD4 $^+$ T cell-mediated inhibition of IgE production.

Discussion

We established a murine model of primary and secondary allergen exposure associated with immediate hypersensitivity responses and altered airway function. Whereas previous animal models that

Table II. *CD4⁺ T cells from IFN- γ treated mice inhibit IgE production^a*

CD4 ⁺ T Cells Added	IgE (ng/ml)	
	Anti-IFN Ab –	+
None	52 $\pm$ 9.1	61 $\pm$ 6.4
CD4 (OVA) day 12	53.9 $\pm$ 6.2	48.8 $\pm$ 2.6
CD4 (OVA/IFN early) day 12	20.3 $\pm$ 4.2 ^b	37 $\pm$ 5.7 ^c
CD4 (OVA/IFN early) day 37	16.2 $\pm$ 3.5 ^d	40.1 $\pm$ 4.3 ^c
CD4 (OVA) day 37	58.7 $\pm$ 9.3	64.3 $\pm$ 6.0
CD4 (OVA/IFN late) day 37	31.1 $\pm$ 3.2 ^b	48.0 $\pm$ 3.7 ^c

^a Spleen MNC (10⁶) were obtained from OVA-sensitized mice on day 12 of the protocol and cultured for 14 days in the presence of IL-4, in the presence or absence of anti-IFN- γ Ab (1 μ g/ml). Supernatants were collected and IgE production was assayed by ELISA. In co-culture experiments 10⁶ CD4⁺ spleen T cells were added to the MNC. These T cells were prepared on day 12 from OVA-sensitized or OVA/IFN-treated animals (early IFN treatment from day –3 to 10) or prepared on day 37 from mice exposed to primary and secondary challenge with OVA and treated with IFN early or late (day 26 to 30). Expressed is the mean IgE production $\pm$ SE from three separate experiments.

^b $p < 0.01$ compared with OVA-sensitized, non-interferon-treated control animals.

^c $p < 0.01$ compared with cultures in the absence of anti-interferon Ab.

^d $p < 0.001$ compared with OVA-sensitized, non-interferon treated control animals.

examined IgE regulation focused on polyclonal IgE production (2–6), we studied allergen-specific IgE responses to allergen sensitization via the airways. BALB/c mice sensitized to nebulized OVA for 10 days develop a predominant IgE response, cutaneous reactivity to OVA and AHR on day 12 (7). To analyze secondary allergic responses in this model, mice were rested from day 11 to day 29 following primary OVA sensitization; they then received a secondary OVA challenge on days 30 and 31 and by day 37 developed an amplified/secondary anti-OVA IgE response, renewed cutaneous reactivity to OVA, as well as AHR. We found that anti-OVA IgE titers during the primary response peaked by day 12, and by day 30, serum anti-OVA IgE levels were less than 50% of the peak response. The anti-OVA IgE response measured on day 37 following secondary OVA challenge was 131% of the anti-OVA IgE response that followed primary OVA sensitization. Having established the kinetics of primary and secondary IgE responses, we evaluated whether nebulized IFN- γ was capable of inhibiting secondary IgE responses to the same degree as primary IgE responses. Nebulized IFN- γ administered 3 days prior to OVA exposure and during the 10 days of primary OVA exposure, previously shown to inhibit anti-OVA IgE production and immediate hypersensitivity on day 12 (7), was also found to inhibit secondary anti-OVA IgE production, immediate cutaneous reactivity, and alterations in airway function (on day 37). In mice that had fully established primary anti-OVA IgE responses in the absence of IFN- γ , subsequent treatment with IFN- γ (days 26 to 30) prevented secondary anti-OVA IgE production and immediate hypersensitivity responses. These data indicate that IFN- γ is capable of inhibiting both primary and secondary allergen-specific IgE responses in mice and that the inhibition of the secondary response is possible even if treatment is initiated after primary allergic sensitization has been established.

Although it is known that IFN- γ inhibits IgE synthesis in vitro by inhibiting production of both germ-line and productive ϵ transcripts (14), it is not established in vivo whether IFN- γ works directly on B cells to inhibit isotype production or whether its effects are mediated through T cells (3, 14, 15). Our data provide

support for both mechanisms of action. Early treatment with IFN- γ during primary OVA sensitization significantly inhibited the primary anti-OVA IgE response on day 12. The de novo appearance of anti-OVA IgG2a in the serum following 13 days of IFN- γ treatment, which was not seen in untreated, OVA-sensitized mice, points to a role for IFN- γ in the regulation of isotype switching in B cells. We showed previously that the regulation of these two isotypes is reciprocally regulated by IFN- γ (7). These data argue for IFN- γ acting directly on B cells to inhibit isotype switching to IgE and inducing isotype switching to IgG2a. This also explains our findings (7) that late treatment with IFN- γ during the primary response (after day 6) is ineffective in inhibiting IgE synthesis, since by this stage Ig class switching has already occurred and anti-OVA IgE is detectable in the serum.

Additionally, we found that early treatment with IFN- γ for 13 days during primary sensitization inhibited secondary IgE responses even though no IFN- γ was administered at the time of secondary OVA challenge. This inhibition of secondary IgE responses cannot be explained by persistence of exogenous IFN- γ in the circulation or airways since the half-life of this cytokine is in the order of hours (16). Neither can the effect of early treatment with IFN- γ on inhibition of secondary IgE responses be explained in terms of altered B cell function. Although IFN- γ induced IgG2a during the primary response, this effect on B cell function was no longer apparent after secondary OVA challenge (in the absence of a second course of IFN- γ), which did not result in IgG2a production. Our data demonstrate that inhibition of the secondary IgE response following the early IFN- γ treatment regimen is due to a persistent effect of IFN- γ on T cells, which are necessary for IgE production by B cells. We found that 13 days of IFN- γ treatment significantly inhibited lymphocyte proliferation to OVA and that this inhibitory effect on T cells persisted through to day 37, even after secondary OVA challenge (Fig. 4). Furthermore CD4⁺ T cells obtained from these mice were able to inhibit IgE production by MNC, even when the CD4⁺ T cells were obtained on day 37 following secondary OVA challenge and 4 weeks after the last exposure to IFN- γ (Table II). This indicates that early IFN- γ treatment has a long-lived effect on CD4⁺ T cell-mediated inhibition of IgE production. We are currently studying the longevity of this effect of early IFN- γ treatment to determine whether tertiary and subsequent IgE responses are similarly inhibited.

Most importantly, we found that initiation of IFN- γ treatment after establishment of a normal primary IgE response prevented the development of secondary IgE responses. Nebulized IFN- γ given on days 26 to 30 prevented the secondary anti-OVA IgE response following OVA rechallenge. This favors the hypothesis that secondary IgE Ab responses are derived from IgM-expressing (or IgG-expressing) B cells (8). Despite the appearance of IgE during the primary response and the absence of IgG2a, late IFN- γ treatment modulated the secondary response to OVA-inducing IgG2a in the absence of IgE. Based on these data and the findings that IFN- γ does not prevent IgE production by IgE-committed B cells, we argue that there was a limited number of IgE-committed B cells in the secondary response and that the IgG2a response was derived from a population of IgM-producing memory B cells that underwent Ig isotype switching from μ to γ 2a in the presence of IFN- γ . Thus, late treatment with IFN- γ had the same effects on the Ab response to secondary OVA challenge that were observed with early IFN- γ treatment on the Ab response to primary OVA sensitization. In both situations, IFN- γ modulates the B cell response by inhibiting the switch to IgE and inducing the switch to IgG2a. Additionally, we found that late IFN- γ treatment resulted in T cell-mediated inhibition of in vitro IgE production.

Since we previously demonstrated that different T cell subsets in this model are critical in the regulation of anti-OVA IgE (17, 18), we examined the effects of IFN- γ on these populations. The V β 8.1/8.2⁺ T cells that are increased following OVA sensitization stimulate IgE production in vitro (17); in in vivo transfer experiments this population was also able to stimulate anti-OVA IgE production in naive mice that received a single OVA challenge (18). The effects were antagonized by the V β 2⁺ expressing T cells both in vitro and in co-transfer experiments. Additionally, we have shown that the V β 8.1/8.2 population shows a TH₂ pattern of cytokine production (19). However, following early or late IFN- γ treatment, the PBLN showed no change in the absolute numbers or relative proportions of the V β 2 and V β 8.1/8.2 T cell subsets when assayed on day 12 or day 37 (data not shown). Nonetheless, the data provide indirect support that IFN- γ treatment may have resulted in an alteration in the balance between IL-4 and IFN- γ production by the CD4⁺ T cells since in vitro inhibition of IgE production was reversed to a significant extent by anti-IFN- γ Ab.

Cutaneous reactivity in this model is due to mast cell degranulation in response to cross-linking of surface-bound IgE and has positively correlated with anti-OVA IgE in the serum (20). Both primary and secondary anti-OVA IgE responses were associated with cutaneous reactivity to OVA. IFN- γ treatment regimens that inhibited the primary and secondary IgE responses also prevented the development of cutaneous reactivity to OVA. IFN- γ may block cutaneous reactivity in ways other than by inhibiting IgE production. There is evidence that sensitization of murine mast cells with IgE and their release of serotonin (21, 22) is blocked in vitro by the addition of IFN- γ . However, IFN- γ did not alter overall cutaneous reactivity in our experiments as demonstrated by the normal responses to the mast cell degranulating agent, compound 48/80.

Nebulized IFN- γ prevented the development of AHR following both primary and secondary sensitization. Although there is no direct evidence that IgE causes AHR, we have observed that the production of anti-OVA IgE correlates with altered airway function, and treatment regimens that decrease anti-OVA IgE tend to interfere with the development of AHR. There is indeed a complexity of biologic interactions whereby IFN- γ may modulate allergic inflammation of the airways. It has numerous effects on T cell differentiation (23–26), eosinophil migration, and degranulation (27, 28), mast cell differentiation and degranulation (21, 22, 29) and on basophil function (30). The role of eosinophils in asthma has received increasing recognition and there are clinical data correlating circulating eosinophil numbers with severity of asthma (31, 32) as well as histologic data demonstrating the presence of eosinophils (33) and major basic protein (34) in the bronchial mucosa of patients who died with severe asthma. We now have evidence that OVA sensitization does indeed induce eosinophil infiltration of the trachea in BALB/c mice and correlates with AHR; treatment with IFN- γ which normalizes airway function inhibits eosinophil infiltration and decreases local IL-5 production (G. Lack, E. Hamelmann, and E. W. Gelfand, manuscript in preparation). Thus, IFN- γ has the potential to normalize airway function by blocking both the IgE-mediated limb of the response as well as modulating the response of T lymphocytes, cytokine production, and inflammatory effector cells.

We have established that mice sensitized to OVA that develop primary IgE responses can develop secondary IgE responses following OVA rechallenge. The secondary IgE response appears to be derived from memory B cells prior to switching to IgE (8, 9), providing a window of opportunity to modulate secondary IgE responses by preventing Ig isotype switching to IgE. In addition, the early administration of IFN- γ has long-lasting effects on the development of secondary hypersensitivity responses. Although it

is unclear how long these effects do persist, they appear mediated by CD4⁺ T cells, whose overall function has been modified by IFN- γ treatment. Even when treatment is initiated after primary allergic sensitization is established, IFN- γ remains capable of preventing secondary anti-OVA IgE production, cutaneous reactivity, and AHR following re-exposure to allergen. These important effects of IFN- γ delivered to the site of sensitization on the in vivo inhibition of allergen-specific IgE production thus result both from immediate effects on B cell function and more prolonged effects on T cell function. These data highlight the important role of this regulatory cytokine in murine IgE production and that organ-targeted delivery of nebulized IFN- γ may have therapeutic potential in allergic disease beyond initial encounters with allergen.

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References

- Valente, G., L. Ozmen, F. Novelli, M. Geuna, G. Palestro, G. Forni, and G. Garotta. 1992. Distribution of interferon- γ receptor in human tissues. *Eur. J. Immunol.* 22:2403.
- Snapper, C. M., and W. E. Paul. 1987. Interferon- γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 236:944.
- Thyphronitis, G., G. C. Tsokos, C. H. June, A. D. Levine, and F. D. Finkelman. 1989. IgE secretion by Epstein-Barr virus-infected purified human B lymphocytes is stimulated by interleukin 4 and suppressed by IFN- γ . *Proc. Natl. Acad. Sci. USA* 86:5580.
- Pene, J., F. Rousset, F. Briere, I. Chretien, J. Y. Bonnefoy, H. Spits, T. Yokota, N. Arai, K. I. Arai, J. Banachereau, and J. E. DeVries. 1988. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons α , γ and prostaglandin E₂. *Proc. Natl. Acad. Sci. USA* 85:6880.
- Chretien, I., J. Pene, F. Briere, R. De Waal-Malefijt, F. Rousset, and J. E. DeVries. 1990. Regulation of human IgE synthesis. I. Human IgE synthesis in vitro is determined by the reciprocal antagonistic effects of interleukin 4 and interferon- γ . *Eur. J. Immunol.* 20:243.
- Finkelman, F. D., I. M. Katona, T. R. Mosmann, and R. L. Coffman. 1988. IFN- γ regulates the isotypes of Ig secreted during in vivo humoral immune responses. *J. Immunol.* 140:1022.
- Lack, G., H. Renz, J. Saloga, K. L. Bradley, J. Loader, D. Y. M. Leung, G. Larsen, and E. W. Gelfand. 1994. Nebulized but not parenteral IFN- γ decreases IgE production and normalizes airways function in a murine model of allergen sensitization. *J. Immunol.* 152:2546.
- Finkelman, F. D., I. M. Katona, J. F. Urban, Jr., J. Holmes, J. O'hara, A. S. Tung, J. vG. Sample, and W. E. Paul. 1988. IL-4 is required to generate and sustain in vivo IgE responses. *J. Immunol.* 141:2335.
- Finkelman, F. D., J. Holmes, I. M. Katona, J. F. Urban, Jr., M. P. Beckmann, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann, and W. E. Paul. 1990. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol.* 8:303.
- Katona, I. M., J. F. Urban, Jr., S. Kang, W. E. Paul, and F. D. Finkelman. 1991. IL-4 requirements for the generation of secondary in vivo IgE responses. *J. Immunol.* 146:4215.
- Renz, H., H. Renz, J. E. Henson, B. S. Ray, C. G. Irvin, and E. W. Gelfand. 1992. Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *J. Allergy Clin. Immunol.* 89:1127.
- Larsen, G. L., H. Renz, J. E. Loader, K. L. Bradley, and E. W. Gelfand. 1992. Airway response to electrical field stimulation in sensitized inbred mice. *J. Clin. Invest.* 89:747.
- Larsen, G. L., T. M. Fame, H. Renz, J. E. Loader, J. Graves, M. Hill, and E. W. Gelfand. 1994. Increased acetylcholine release in tracheas from allergen-exposed IgE-immune mice. *Am. J. Physiol.* 266:L263.
- Gauchat, J. F., D. A. Leberman, R. L. Coffman, H. Gascan, and J. De Vries. 1990. Structure and expression of germ-line ϵ transcripts induced by interleukin 4 to switch to IgE production. *J. Exp. Med.* 172:463.
- Coffman, R. L., and J. Carty. 1986. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon- γ . *J. Immunol.* 136:949.
- Jaffe, H. A., R. Buhl, A. Mastrangeli, K. J. Holroyd, C. Saltini, D. Czerski, H. S. Jaffe, S. Kramer, S. Sherwin, and R. G. Crystal. 1991. Organ specific cytokine therapy: local activation of mononuclear phagocytes by delivery of an aerosol of recombinant interferon- γ to the human lung. *J. Clin. Invest.* 88:297.
- Renz, H., K. L. Bradley, P. Marrack, and E. W. Gelfand. 1992. T cells expressing variable elements of T-cell receptor β 8 and β 2 chain regulate murine IgE production. *Proc. Natl. Acad. Sci. USA* 89:6438.
- Renz, H., K. Bradley, J. Saloga, J. Loader, G. L. Larsen, and E. W. Gelfand. 1993. T cells expressing specific V β elements regulate immunoglobulin E production and airways responsiveness in vivo. *J. Exp. Med.* 177:1175.
- Renz, H., K. Bradley, and E. W. Gelfand. 1996. Production of IL-4 and IFN- γ by TCR-V β expressing T-cell subsets in allergen sensitized mice. *Am. J. Respir. Cell Mol. Biol.* 14:36.

20. Saloga, J., H. Renz, G. Lack, K. L. Bradley, J. L. Greenstein, G. Larsen, and E. W. Gelfand. 1993. Development and transfer of immediate cutaneous hypersensitivity in mice exposed to aerosolized antigen. *J. Clin. Invest.* 91:133.
21. Coleman, J. W., and A. G. Morris. 1991. Identification of a spleen cell-derived factor that inhibits sensitization of murine peritoneal mast cells as interferon- γ . *Int. Arch. Allergy Appl. Immunol.* 94:179.
22. Coleman, J. W., M. G. Buckely, M. R. Holliday, and A. G. Morris. 1991. Interferon- γ inhibits serotonin release from mouse peritoneal mast cells. *Eur. J. Immunol.* 21:2559.
23. Gajewski, T. F., and F. W. Fitch. 1988. Anti-proliferative effect of IFN- γ in immune regulation. I. IFN- γ inhibits the proliferation of Th2 but not Th1 murine CTL clones. *J. Immunol.* 140:4245.
24. Gajewski, T. F., J. Joyce, and F. W. Fitch. 1989. Anti-proliferative effect of IFN- γ in immune regulation. III. Differential selection of Th1 and Th2 murine helper T lymphocyte clones using recombinant IL-2 and recombinant IFN- γ . *J. Immunol.* 143:15.
25. Maggi, E., P. Parronchi, R. Manetti, C. Simonelli, M.-P. Piccinni, F. S. Rugiu, M. Carli, M. Ricci, and S. Romagnani. 1992. Reciprocal regulatory effects of IFN- γ and IL-4 on the in vitro development of human Th1 and Th2 clones. *J. Immunol.* 148:2142.
26. Swain, S. L., L. M. Bradley, M. Croft, S. Tonkonogy, G. Atkins, A. D. Weinberg, D. D. Duncan, S. M. Hedrick, R. W. Dutton, and G. Huston. 1991. Helper T cell subsets: phenotype, function, and the role of lymphokines in regulating their development. *Immunol. Rev.* 123:115.
27. Iwamoto, I., H. Nakajima, H. Endo, and S. Yoshida. 1993. Interferon- γ regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4⁺ T cells. *J. Exp. Med.* 177:573.
28. Fujisawa, T., R. Abu-Ghazaleh, H. Kita, C. J. Sanderson, and G. J. Gleich. 1990. Regulatory effect of cytokines on eosinophil degranulation. *J. Immunol.* 144:642.
29. Takagi, M., K. Kenichi, and T. Nakahata. 1990. Antiproliferative effect of IFN- γ on proliferation of mouse connective tissue-type mast cells. *J. Immunol.* 145:1880.
30. Schleimer, R. P., C. P. Derse, B. Friedman, S. Gillis, M. Plaut, L. M. Lichtenstein, and D. W. MacGlashan, Jr. 1989. Regulation of human basophil mediator release by cytokines. I. Interaction with anti-inflammatory steroids. *J. Immunol.* 143:1310.
31. Durham, S. R., and A. B. Kay. 1985. Eosinophils, bronchial hyperreactivity and late-phase asthmatic reactions. *Clin. Allergy* 15:411.
32. Bousquet, J., P. Chanet, J. Y. Lacoste, G. Barneon, N. Gavanian, J. Enander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, P. Godard, and F. B. Michel. 1990. Eosinophilic inflammation in asthma. *N. Engl. J. Med.* 323:1033.
33. Azzawi, M., P. W. Johnston, S. Majumdar, A. B. Kay, and P. K. Jeffery. 1992. T lymphocytes and activated eosinophils in airway mucosa in fatal asthma and cystic fibrosis. *Am. Rev. Respir. Dis.* 145:1477.
34. Filley, W. V., K. E. Holley, G. M. Kephart, and G. J. Gleich. 1982. Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma. *Lancet* 2:11.