

Transfer of Immediate Hypersensitivity and Airway Hyperresponsiveness by IgE-positive B Cells

GIDEON LACK, AKIHIRO OSHIBA, KATHERINE L. BRADLEY, JOAN E. LOADER, DAVID AMRAN, GARY L. LARSEN, and ERWIN W. GELFAND

Divisions of Basic Sciences and Pulmonary Medicine, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado

The role of allergen-specific sIgE⁺ B cells in the development of airway hyperresponsiveness to electrical field stimulation was examined in a murine model of allergic sensitization. Ovalbumin (OVA)-specific B cells (OVA⁺) were isolated from mice that were sensitized to aerosolized OVA. The OVA⁺ B cell population was shown to be distinct from the remaining, non-OVA-responsive B cells (OVA⁻). There was a high frequency of sIgE⁺ B cells and a low frequency of sIgG⁺ B cells in the OVA⁺ population compared with the OVA⁻ population, where the ratio was reversed. Although both populations produced immunoglobulin *in vitro*, only the OVA⁺ cells secreted anti-OVA antibodies. Transfer of 10⁶ OVA⁺ B cells or as few as 5 × 10⁴ OVA⁺/sIgE⁺ B cells was able to transfer the capability for anti-OVA IgE synthesis and cutaneous reactivity to OVA in naive recipients. Exposure to OVA via the airways in addition to transfer of OVA⁺ B cells was necessary for development of airway hyperresponsiveness, whereas recipients challenged with an irrelevant allergen, ragweed, had normal airway function. Transfer of up to 10⁷ OVA⁻ B cells failed to induce production of anti-OVA IgE. Despite production of polyclonal IgE, recipients of OVA⁻ B cells did not develop airway hyperresponsiveness after OVA challenge. We conclude that both allergen-specific IgE production and local challenge via the airways with specific allergen are necessary to change airway function in this model. **Lack G, Oshiba A, Bradley KL, Loader JE, Amran D, Larsen GL, Gelfand EW. Transfer of immediate hypersensitivity and airway hyperresponsiveness by IgE-positive B cells.**

AM J RESPIR CRIT CARE MED 1995;152:1765-73.

Although IgE is central to the development of allergic responses, its precise role in the pathogenesis of airway hyperresponsiveness is unclear. *In vitro* work on animal and human tracheal smooth muscle certainly suggests that IgE may be causally implicated in changing airway function (1, 2). IgE affects the migration and function of various effector cells such as basophils, mast cells, macrophages, and eosinophils by stimulating mediator release from these cells (3-11). The discovery of the low-affinity IgE receptor on cells such as lymphocytes potentially allows for further interactions between IgE and the cellular and humoral immune systems (12, 13).

We developed a murine model of allergic sensitization to ovalbumin (OVA) via the airways to better characterize interactions between IgE, inflammatory cells, and the immune compartments involved in the pathogenesis of increased airway responsiveness (AR). We showed that Balb-C mice exposed to nebulized ovalbumin (OVA) in the absence of adjuvant developed anti-OVA IgE in the serum (14), cutaneous reactivity to OVA (type I hypersensitivity) (15), and increased AR to electrical field stimulation (EFS) (15, 16). These mice also develop increased AR as mea-

sured by body plethysmography in response to challenge with intravenous methacholine (14). The alteration in AR is thought to reflect a change in neural control within the airway with enhanced release of acetylcholine from neural terminals (17). SJL mice that failed to develop anti-OVA IgE and cutaneous reactivity to OVA did not develop airway hyperresponsiveness after sensitization via the airways (16). Thus, despite different manipulations of this model, we have failed to observe a dissociation between airway hyperresponsiveness and anti-OVA IgE following sensitization through the airways; an increased AR was always accompanied by a rise in anti-OVA IgE in the serum (16). These findings were also observed following treatment protocols such as with interferon- γ ; administration of nebulized interferon- γ decreased not only anti-OVA IgE but also resulted in normalization of AR (18). However, these findings do not necessarily imply a causal link between allergen-specific IgE and airway hyperresponsiveness because other immunological changes that occur after airways sensitization could potentially account for alteration of airway function. Furthermore, in an adaptation of this model, animals sensitized to OVA via the cutaneous route produced anti-OVA IgE in the serum but failed to develop increased AR unless challenged with OVA via the airways (19). This indicates that there are other factors beyond the presence of allergen-specific IgE that contribute to the development of airway hyperresponsiveness. In addition to the rise in anti-OVA IgE, we have observed an increase in polyclonal IgE in the serum after sensitization of OVA (18), expansion of the peribronchial lymph nodes (14), and a selective expansion of the V β 8.1/8.2 T cells in these lymph nodes (10). In transfer experiments, we confirmed that

(Received in original form February 10, 1995 and in revised form April 21, 1995)

Supported by grants P01-HL36577 (E. W. Gelfand and G. Larsen) and AI-29704 (E. W. Gelfand) from the National Institutes of Health.

Correspondence and requests for reprints should be addressed to Dr. Erwin W. Gelfand, M.D., National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, CO 80206.

Am J Respir Crit Care Med Vol 152. pp 1765-1773, 1995

it is this population of V β 8.1/8.2 T cells that plays a major role in supporting IgE production (21). Irrespective of the precise immunologic mechanism(s) that underlies alteration in airway function, it appears that allergen challenge via the airways is necessary for the development of increased AR. Even mice that develop anti-OVA IgE in the serum in response to cutaneous sensitization fail to develop increased AR unless challenged with aerosolized OVA (19).

To better define the role of antigen-specific B cells in the induction of increased AR, transfer experiments were performed using OVA-specific B cells (OVA⁺ B cells) from sensitized mice, adapting a technique recently developed to isolate human antigen-specific B cells (22). We show that the production of allergen-specific IgE rather than polyclonal IgE, as well as local and specific allergen challenge to the airways, were necessary conditions for the alteration of airway function.

METHODS

Animals

Female Balb-C mice between 8–12 wk of age were obtained from Jackson Laboratories (Bar Harbor, ME). The animals were maintained on OVA-free diets.

Immunization

Groups of 8 to 12 mice were immunized by ultrasonic nebulization of 1% ovalbumin (chicken ovalbumin, grade V, Sigma, St. Louis, MO) diluted in sterile phosphate-buffered saline (PBS) (14). Nebulization of ragweed was carried out using a 7 ml (500 μ g/ml) solution of ragweed extract in PBS (23). As defined by laser nephelometry (24), more than 90% of the OVA and ragweed particles had a diameter in the 1–5 μ range.

Immunization was carried out by exposing the mice to nebulized antigen in a chamber for 20 min each day over a 10-d period. Previous work with Balb-C mice had shown this was a reliable immunization schedule for the production of allergen-specific IgE (14, 15). Age-matched control animals were exposed to PBS alone using the same protocol. On day 12, two days after the last exposure, serum was obtained from the animals and testing was carried out in a blinded fashion so that the investigator was unaware of the sensitization and treatment status of the animals.

Preparation and Isolation of Lymphocyte Subpopulations

Single-cell suspensions were prepared from spleens of immunized and control mice two days after completing the sensitization protocol by dispersing small tissue pieces through stainless steel mesh. Mononuclear cells (MNC) were isolated by density gradient centrifugation (Lymphocyte Separation Medium; Organos Teknika, Durham, NC) for 15 min at 1,500 rpm at room temperature. Cells from the interface were washed three times in PBS and then counted in a Coulter Counter (Coulter Electronics, Hialeah, FL). Splenic MNC were composed of the following lymphocyte subsets as determined by flow cytometry: B cells, $45 \pm 3.6\%$, CD4⁺ cells, $41 \pm 4.2\%$, and CD8⁺ cells, $15 \pm 4.9\%$. OVA⁺ B cells were purified from splenic MNC 2 d after completion of the sensitization protocol by positive immunoselection using OVA-coated magnetic beads. OVA⁺ B cells were isolated as previously described (22, 24–26). Tosylactivated immunomagnetic beads (Dynabeads M-450 Tosylactivated; Dynal, Oslo, Norway) were coated with OVA (150 μ g/ml) over 24 h. The beads were washed with PBS containing 0.1% bovine serum albumin. Splenic MNC were mixed with antigen-coated beads in a cell:bead ratio of 1:3 and placed in an ice bath for 15 min. The rosettes and free beads were separated by means of a magnetic field (MPC-1; Dynal, Great Neck, NY). The rosetted cells, containing the antigen-specific (OVA⁺) cells were washed, yielding a purified B cell population; 97% of cells were stained with B220, a marker of murine B cells. Trypan blue staining revealed greater than 95% viability for these cells. Suspensions of the OVA⁺ rosetting cells were incubated for 1 h in 0.5 ml of medium at 37° C, and then vigorously pipetted and placed on a vortex mixer to detach the bound beads from the cells. The unbound cells were isolated from beads by use of the magnetic field. The viability of the B cells was more than 95% after the detachment procedure and yielded a population of > 97% cells stained positively with B220 antibody.

The residual or non-rosetting OVA⁺ B cells were purified by positive immunoselection using beads coated with anti-kappa antibody and were more than 98% positive for B220. The remaining cell population consisted of more than 98% CD3⁺ T cells. From this population CD8 cells were isolated by positive immunoselection, yielding a negatively selected population of cells that was 92% positive for CD4. In a second approach to isolate B cells free of T cells, CD4 and CD8 cells were positively selected at the beginning, and then OVA⁺ B cells were positively selected from the remaining B cells. There were no differences in any of the results after either isolation procedure of OVA⁺ B cells. To isolate OVA-specific sIgE⁺ and sIgE⁺ cells, OVA⁺ B cells were incubated with beads coated with monoclonal rat anti-mouse IgE (provided by Dr. F. Finkelman, Bethesda, MD), and the positively selected cells were detached as described previously.

Flow Cytometric Analysis

The levels of expression of CD4, CD8, and B220 were monitored on freshly prepared MNC and lymphocyte subpopulations as previously described. Anti-CD4 and anti-CD8 antibodies were FITC-conjugated. An anti-B220 biotinylated antibody (provided by Drs. P. Marrack and J. Kappler, Denver, CO) was used. Cell surface Ig isotype staining was performed using rat antibodies against different mouse isotypes. Polyclonal FITC-labeled goat anti-mouse IgE and unconjugated rat anti-mouse IgG (both from the Binding Site, Birmingham, UK) and unconjugated rat anti-mouse IgM (provided by Dr. J. Cambier, National Jewish Center, Denver, CO) were used. Indirect staining was performed with a FITC-labeled goat anti-rat Ig antibody (Southern Biotechnology Associates, Birmingham, AL). The stained cells were washed with PBS and analyzed on a flow cytometer (Epic Profile II, Coulter). Surface Ig staining was confirmed by fluorescent microscopy after fixing the cells in formalin and mounting them on slides.

Immunoglobulin Production in Co-culture Experiments

OVA⁺ or OVA⁺ B cells (1×10^6) were plated in 24-well flat-bottom tissue culture plates together with 1×10^6 CD4 cells and IL-4 (100 U/ml; provided by Dr. P. Trotta, Schering-Plough, Bloomfield, NJ) in a final volume of 1 ml. The culture was carried out in complete tissue culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 5 mM 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum [Hyclone Sterile Systems, Logan, UT]) for 7 d in a 95% air/5% CO₂ humidified atmosphere at 37° C. At the end of the culture period cell-free tissue culture supernatants were harvested and stored at –20° C until further analysis for anti-OVA antibody and total immunoglobulin levels.

Lymphocyte Transfer Protocol

Desired numbers of lymphocytes (between 5×10^4 and 10^7 per recipient) were resuspended in sterile PBS in a final volume not exceeding 0.25 ml. The cell suspension was injected into the tail vein of age- and sex-matched syngeneic recipients. The recipients were 8- to 12-wk-old Balb-C female mice that had not been exposed to nebulized OVA. Immediately after lymphocyte transfer (day 1 of the transfer protocol), the recipient mice were exposed to nebulized PBS, OVA, or ragweed for 20 min and then rechallenged with the same allergen on day 7 by nebulization. A single challenge in preliminary experiments was found to be not as effective. Control mice received 0.25 cc of PBS intravenously and were challenged on days 1 and 7 with nebulized OVA or ragweed. Testing was carried out on day 8 of the protocol, and the animals were then killed.

Determination of Anti-OVA Antibody Levels

Anti-OVA IgE and IgG antibody levels in serum and culture supernatants were measured by ELISA using either alkaline phosphatase conjugated monoclonal rat anti-mouse IgE (Pharmingen Corp., San Diego, CA) or goat anti-mouse IgG (Sigma) (22, 25). Anti-OVA IgG subclass levels were measured using goat anti-mouse IgG₁, -2a, -2b, and 3 (Fisher Scientific). Plates were read in a microplate autoreader (BioRad Inc., Pleasanton, CA) at 410 nm. Analysis of ELISA data was performed with the Microplate Manager software for the MacIntosh computer (BioRad Inc.). The specificity of the ELISA was determined by preincubation of standard serum and serum samples from experimental animals with 100 μ g/ml OVA. This procedure completely blocked activity in the ELISA while preincubation with an irrelevant antigen (BSA, 100 μ g/ml) had

no effect on the measurement of specific antibody titers. Addition of 1,000-fold excess anti-OVA IgG did not influence the titers of anti-OVA IgE.

Measurement of Total IgE

Serum and culture supernatants were analyzed for total IgE production by ELISA as described (15, 20). ELISA plates were coated with polyclonal sheep anti-mouse IgE (The Binding Site, San Diego, CA), diluted to a concentration of 3 mg/ml in 0.1% NaHCO₃ buffer pH 8.2. Sensitivity of this assay was determined to be 300 picograms of IgE.

Determination of Immediate Hypersensitivity Skin Test Responses to OVA

Antigen-specific immediate cutaneous reactivity was analyzed by skin testing as described (23). After shaving the abdomen, 20 μ l of a test solution was injected intradermally with a 30-gauge needle from a tuberculin syringe. The test solutions were OVA (500 μ g/ml) diluted in PBS, PBS solution as the negative control, compound 48/80 (Sigma) as the positive control, and HSA and BSA as protein control solutions. Distances of at least 1 cm were kept between the sites of injections to avoid confluence of the solutions. In the case of a positive reaction, a wheal (minimum of 3 mm) developed actively at the site of injection and reached a maximum between 15 to 20 min after injection of the test solution. In contrast, in the case of a negative reaction, no wheal development was observed. Skin test responses were evaluated by an investigator who was unaware of the treatment status of the animals.

Assessment of Airway Smooth Muscle Responsiveness

Airway smooth muscle responsiveness was assessed *in vitro* as recently described (16, 21). Mouse tracheal smooth muscle segments were prepared and placed in Krebs-Henseleit baths suspended by triangular supports, transducing the force of contractions. Electrical field stimulation (EFS) was delivered by a Grass S44 stimulator (Grass SIV 5) using 8V, 2 ms pulse duration and 0.5 to 40 Hz frequencies. Each stimulation was maintained until peak contractile responses were obtained. A frequency of 40 Hz was established earlier to result in maximal contractions. ES50, the frequency that caused 50% of maximal contractions, was calculated from linear plots. All tissues were assessed in terms of the *in vitro* responsiveness by one investigator who was blinded to the immunologic and treatment status of each airway.

Statistical Analysis

Groups were compared by ANOVA. Total serum IgE was compared using a Student's *t* test. To isolate sufficient OVA⁺ B cells, a minimum of 20 mice were used for primary sensitization. For the transfer experiments, analysis was performed using the data from a minimum of three separate experiments (each experiment used a minimum of 3 recipient mice) unless otherwise stated. Values for all measurements are expressed as the mean $\pm$ standard error.

RESULTS

Isolation and Characterization of Selected Lymphocyte Populations

The spleens of 20 to 28 OVA-sensitized mice were pooled in each experiment, and OVA-specific B cells were isolated using OVA-coated magnetic beads. Splenic MNC, OVA⁺ and OVA⁻ B cells were stained for surface markers and analyzed by flow cytometry. B cells comprised between 40 and 50% of the MNC by surface marker analysis. The results are summarized in Table 1. OVA⁺ B cells comprised 2.1 $\pm$ 0.4% of the total B cell population from the spleens of OVA-sensitized mice. In other experiments we first isolated the total B cells by coating beads with anti-CD4 and anti-CD8 antibodies and then isolated the OVA⁺ B cells, achieving a highly purified B cell population (99% B220⁺) with no T-cell contamination. There were no differences in any of the results using either means for isolating B cells. Both OVA⁺ and OVA⁻ populations differed significantly in their surface immunoglobulin phenotype as measured by immunofluorescence (Table 1). Staining for other surface isotypes was negative in the popula-

TABLE 1
FLOW CYTOMETRIC ANALYSIS OF OVA⁺
AND OVA⁻ B-CELL POPULATIONS

	% Positive			
	OVA ⁻ B Cells	OVA ⁺ B Cells	sIgE ⁺ OVA ⁺ B Cells	sIgE ⁻ OVA ⁺ B Cells
B220	98.2 $\pm$ 2.4	97 $\pm$ 1.3	99.2 $\pm$ 1.3	98.4 $\pm$ 1.8
CD4	2.5 $\pm$ 0.4	3 $\pm$ 0.3	< 1	2.8 $\pm$ 0.36
CD8	1.4 $\pm$ 0.3	1.1 $\pm$ 0.2	< 1	0.9 $\pm$ 0.27
sIgM	78 $\pm$ 9.8	70 $\pm$ 6.3	< 1	83.9 $\pm$ 0.6
sIgG	15 $\pm$ 3.1	0.9 $\pm$ 0.5	< 1	1.1 $\pm$ 0.1
sIgE	2.3 $\pm$ 1.2	13.3 $\pm$ 4.1	> 98	0.7 $\pm$ 0.6

Splenic MNC from OVA-sensitized mice were separated into OVA⁺ and OVA⁻ B cells using magnetic beads. The OVA⁺ B cells were further separated into OVA⁺/sIgE⁺ and OVA⁺/sIgE⁻ B cells by positive selection of surface IgE⁺ B cells with magnetic beads. The resultant four populations were stained for CD4, CD8, B220, sIgM, sIgG, and sIgE and were analyzed by flow cytometry. Expressed is the mean percentage $\pm$ SE of cells stained positively for these markers in the OVA⁺ and OVA⁻ populations. The results of three separate experiments are shown.

tion of OVA⁺/sIgE⁺ B cells and the frequency of sIgE⁺ cells in the negatively selected residual OVA⁺/IgE⁻ population was < 1%, indicating a greater than 10-fold reduction in frequency of sIgE⁺ cells in this population of cells.

In vitro Anti-OVA Ig Production Occurs Exclusively in OVA⁺ Population

To examine the antigen specificity of the OVA⁺ and OVA⁻ B cells, we analyzed immunoglobulin synthesis in these populations. OVA⁺ or OVA⁻ B cells were co-cultured with CD4⁺ T cells in the presence of IL-4 for 7 d. The supernates were then analyzed for the presence of anti-OVA IgE, anti-OVA IgG₁, and polyclonal IgE. Supernates from OVA⁺ B cells secreted high levels of anti-OVA IgE (> 5,000 EU/ml), whereas OVA⁻ B cells in the presence of CD4 cells plus IL-4 did not produce any anti-OVA IgE (Figure 1a). Anti-OVA IgG₁ was also detected in the supernates of the OVA⁺ cultures but not in supernates from cultures of OVA⁻ cells (Figure 1b). However, the OVA⁻ B cells secreted polyclonal IgE in response to IL-4 (Figure 2), indicating that the selection procedure for this population had not impaired their ability to synthesize IgE.

Passive Transfer of OVA⁺ B Cells Causes Anti-OVA Ig Production in Recipients

We next examined whether OVA⁺ B cells were capable of transferring the ability to produce anti-OVA IgE *in vivo*. Recipients of 10⁶ OVA⁺ B cells developed high titers of serum anti-OVA IgE on day 8 that were approximately double the antibody levels in the OVA-sensitized donors (Figure 3). Unlike the donor animals, the recipients of OVA⁺ B cells also developed high levels of anti-OVA IgG₁ (Figure 4); IgG_{2a}, IgG_{2b}, and IgG₃ were not detected (not shown). Recipients of OVA⁺ cells challenged with ragweed (RGW) on days 1 and 7 developed serum titers of anti-OVA IgE and IgG₁ comparable to the titers in recipients of OVA⁺ cells challenged with OVA (Figures 3 and 4). Even in the absence of an allergen challenge, recipient mice exposed to PBS on days 1 and 7 developed similar levels of anti-OVA IgE (3,293.3 $\pm$ 252.4 EU/ml) and IgG₁ (1,086 $\pm$ 169 EU/ml). Control mice that received 10⁶ OVA⁻ B cells did not produce significant amounts of anti-OVA IgE or anti-OVA IgG₁ in their serum even after the two exposures to OVA by aerosolization. Similarly, mice that received up to 10⁷ OVA⁻ B cells failed to produce anti-OVA IgE or anti-OVA IgG₁ in their serum, confirming the virtual absence of any OVA⁺ B cells in the OVA⁻ population.

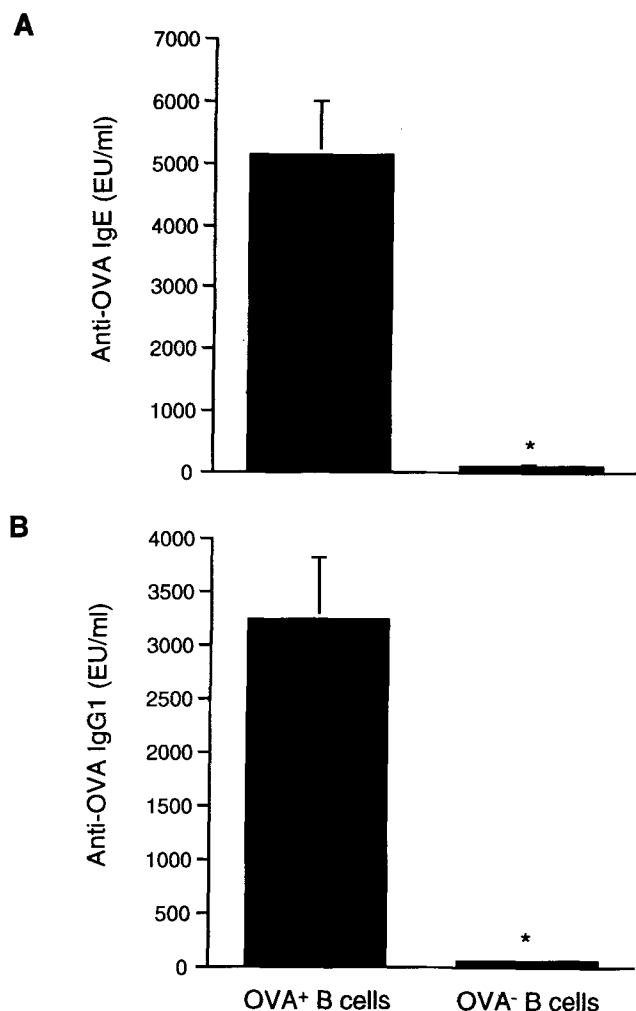


Figure 1. Production of allergen specific IgE and IgG₁ antibody. OVA⁺ and OVA⁻ B cells were isolated from splenic MNC of OVA-sensitized mice, 2 d after sensitization. OVA⁺ B cells (1×10^6) or OVA⁻ B cells (1×10^6) were cocultured with 10^6 CD4⁺ lymphocytes in the presence of IL-4 (100 U/ml) in 24-well plates. After 7 d, the supernatants were collected and anti-OVA IgE (A) and anti-OVA IgG₁ (B) levels determined by ELISA. Shown are data from three separate experiments expressed as mean $\pm$ SE using cells pooled from a minimum of 20 mice in each experiment (* $p < 0.01$ compared with OVA⁺ B cells). EU = ELISA units.

Transfer of OVA⁺ or OVA⁻ B Cells Results in Increased Total Serum IgE

Donor mice sensitized to OVA developed increased total serum IgE levels compared with PBS control mice. The transfer of 10^6 OVA⁺ B cells caused a comparable increase in serum IgE in the recipients (Figure 5). Although the transfer of 10^6 OVA⁻ B cells did not significantly alter total serum IgE, the transfer of 10^7 such cells caused an increase in serum IgE levels (Figure 5). The specific allergen challenge (OVA or RGW) did not affect the levels of serum IgE measured in these transfer experiments (data not shown).

Passive Transfer of OVA⁺ B Cells Causes Cutaneous Reactivity to OVA

Intradermal skin testing to OVA on day 8 in recipients of OVA⁺ B cells resulted in large wheals, more than twice the diameter of the wheals observed in the primary OVA-sensitized donor mice

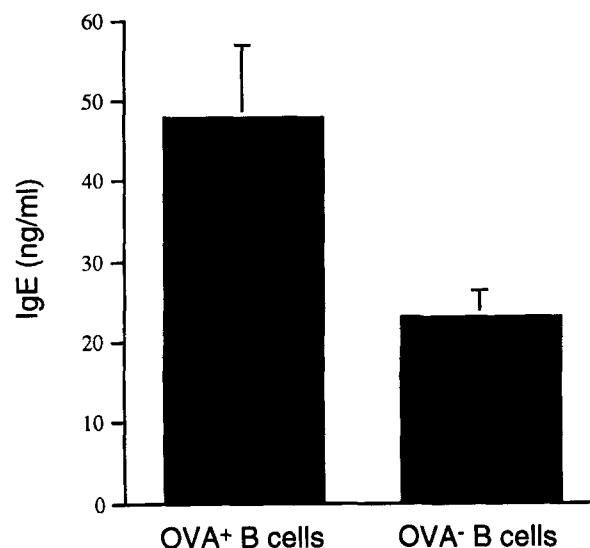


Figure 2. Production of polyclonal IgE. Supernatants of OVA⁺ (1×10^6) and OVA⁻ B cells (1×10^6) cocultured with CD4⁺ cells (1×10^6) in the presence of IL-4 (100 U/ml) were harvested on day 7, and total IgE was determined by ELISA. Analysis was done on the same supernatants in which anti-OVA IgE/IgG₁ was determined, as depicted in Figure 1. Shown are data from three separate experiments expressed as mean $\pm$ SE using cells pooled from a minimum of 20 mice in each experiment.

(Table 2). The diameter of the wheals in these recipients was comparable to that observed in recipients of OVA⁺ B cells that were challenged with nebulized ragweed, indicating that the challenge with OVA on days 1 and 7 did not contribute to the development of cutaneous reactivity. In the absence of an allergen challenge, mice exposed to PBS on days 1 and 7 developed cutaneous reactivity comparable to the recipients challenged with OVA or ragweed. The recipients of OVA⁻ B cells did not develop cutaneous reactivity to OVA even when challenged with OVA on days 1 and 7. Mice in all treatment groups retained equivalent reactivity to compound 48/80.

Transfer of OVA⁺ B Cells Is Associated with Increased AR

The transfer of 10^6 OVA⁺ B cells resulted in a decrease in ES50 values in recipient mice when measured on day 8 and subsequent to allergen challenge on days 1 and 7. The ES50 values in these mice were statistically equivalent to the ES50 values in (primary) OVA-sensitized donor mice (Figure 6). The transfer of 10^6 OVA⁻ B cells with OVA challenges on days 1 and 7 resulted in normal ES50 levels, equivalent to measurements in PBS-treated mice. Even the transfer of 10^7 OVA⁻ B cells, which resulted in elevations of total serum IgE, did not lead to an increase in AR.

Allergen-specific Challenge via the Airways Is Necessary for Development of Increased AR

The necessity for allergen-specific challenge in the development of AR was tested. A group of recipient mice received OVA⁺ B cells and were challenged on days 1 and 7 with aerosolized ragweed antigen. However, in contrast to OVA-challenged mice, which developed a mean of ES50 of 2.1 ± 0.3 Hz, ES50 levels in the ragweed-challenged mice remained (normal) at 4 ± 0.4 Hz (Figure 6). Mice challenged with PBS on days 1 and 7 retained normal airway function with a mean ES50 level of 4.1 ± 0.4 Hz. Therefore, in addition to the transfer of OVA⁺ B cells, aerosol challenge with specific antigen was required for the development of increased AR.

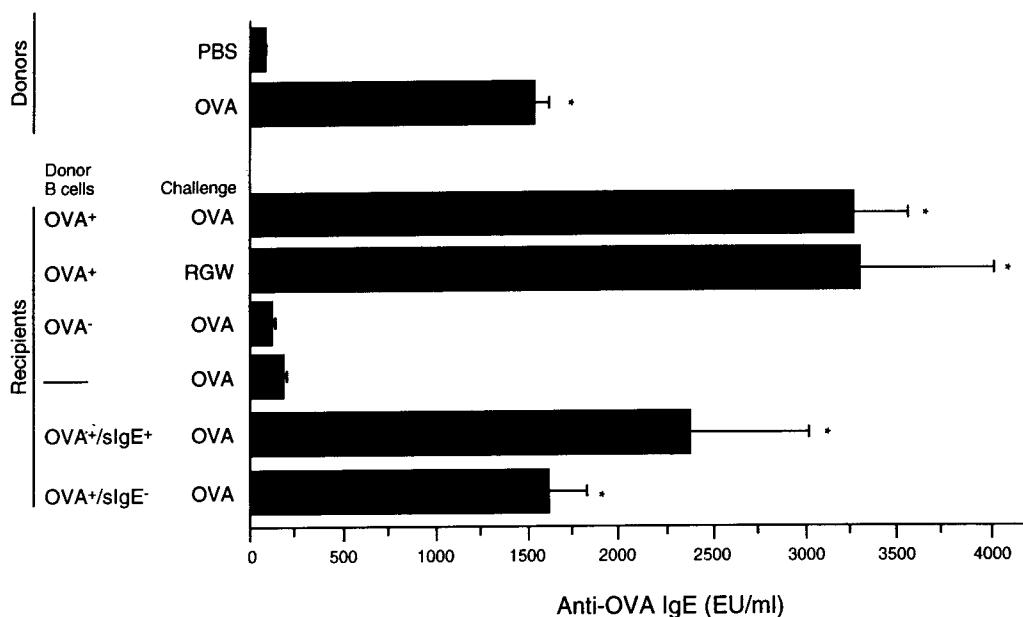


Figure 3. Transfer of anti-OVA IgE production. Donor mice were sensitized to nebulized OVA daily over 10 d, while control mice were exposed to PBS. Two days later serum was collected and the animals were killed. B cells were isolated as described in METHODS and injected into naive recipients, that were then either challenged with nebulized OVA or ragweed on days 1 and 7 of the protocol. The source of the B cells and the challenge received are indicated: 10^6 OVA⁺ B cells/OVA challenge, $n = 10$; 10^6 OVA⁺ B cells/RGW challenge, $n = 6$; 10^7 OVA⁻ B cells/OVA challenge, $n = 9$; no B cells/OVA challenge, $n = 6$; 5×10^4 OVA⁺/sIgE⁺ B cells/OVA challenge, $n = 7$; 5×10^5 OVA⁺/sIgE⁻ B cells/OVA challenge, $n = 7$. Recipients were killed on day 8, and serum was analyzed for anti-OVA IgE. Expressed are the mean values for anti-OVA IgE $\pm$ SE. * $p < 0.001$ compared with the group with no cells transferred. Results for the OVA⁺ B cell/OVA challenge and OVA⁻ B cell/OVA challenge were from three separate experiments. Results for other groups are derived from two experiments.

Surface IgE⁺/OVA⁺ Cells Transfer OVA Sensitization

To determine whether the IgE⁺ committed population of OVA⁺ B cells was responsible for transferring OVA sensitization, OVA⁺/sIgE⁺ cells (5×10^4) were isolated from OVA⁺ B cells and transferred into naive recipients followed by OVA challenges on days 1 and 7. These animals developed anti-OVA IgE in their serum (Figure 3) but not IgG₁ (Figure 4), cutaneous responses to OVA (Table 2) and increased AR (Figure 6). Antibodies to OVA of other immunoglobulin isotypes were not detected in the serum of animals receiving OVA⁺/sIgE⁺ cells. As shown previously in experiments with transfer of nonselected OVA⁺ B cells, specific challenge with OVA was not required for the transfer of anti-OVA IgE production or cutaneous reactivity by the OVA⁺/sIgE⁺ B cells, but was necessary for the development of increased AR (data not shown).

Surface IgE⁻/OVA⁺ Cells Transfer OVA Sensitization

Surface IgE⁻/OVA⁺ cells were also transferred into naive recipients. Whereas transfer of 5×10^4 OVA⁺/sIgE⁻ B cells failed to transfer consistent responses, 5×10^5 OVA⁺/sIgE⁻ B cells were capable of doing so. Following OVA challenges as described previously, these animals developed anti-OVA IgE in their serum (Figure 3) as well as IgG₁ (Figure 4). Similarly, they developed cutaneous reactivity to OVA (Table 2) and altered AR (Figure 6).

DISCUSSION

A technique for selecting antigen-specific B cells with antigen-coated beads was applied to a murine model of allergic sensitization. It allowed us to study *in vitro* the functions of a highly

purified population of allergen-specific B cells and to further study *in vivo* the effects of such cells on allergic sensitization. We showed that OVA-specific B cells specifically transfer allergic sensitization to OVA in recipients. Recipient mice that received OVA⁺ B cells, OVA⁺/sIgE⁺ B cells, or OVA⁺/sIgE⁻ developed anti-OVA IgE in the serum and cutaneous reactivity to OVA. However, in addition to B-cell transfer, aerosol challenge with OVA was required for the development of increased AR as measured by EFS.

The specificity of the OVA⁺ B cells was confirmed by demonstrating that this population of cells was capable of producing high levels of anti-OVA IgE (and IgG₁) both *in vitro* and *in vivo*. The failure of the OVA⁻ B-cell population to produce anti-OVA IgE was not due to differences in any experimental selection process but provides support for the efficiency of depleting B cells capable of producing anti-OVA antibody. Indeed, these cells were capable of producing polyclonal IgE *in vitro* as well as *in vivo*. It is possible that the OVA⁺ B cells were positively selected on the basis of cytophilic rather than secretory antibody to OVA. This is unlikely since one would expect cytophilic immunoglobulin to bind through Fc receptors to all B cells rather than to a small subset of B cells. As well, cytophilic anti-OVA IgE could bind to a small subset of nonspecifically activated CD23⁺ B cells. This was not the case in our experiments because the recipients of OVA⁺/sIgE⁻ B cells developed anti-OVA IgE in the serum that could only have occurred as a result of isotype switching to ϵ and the subsequent production of anti-OVA IgE. The purity of both the OVA⁺ and OVA⁻ populations was demonstrated by flow cytometry, indicating that the results of our cell transfer experiments were attributable to OVA-specific B cells without a re-

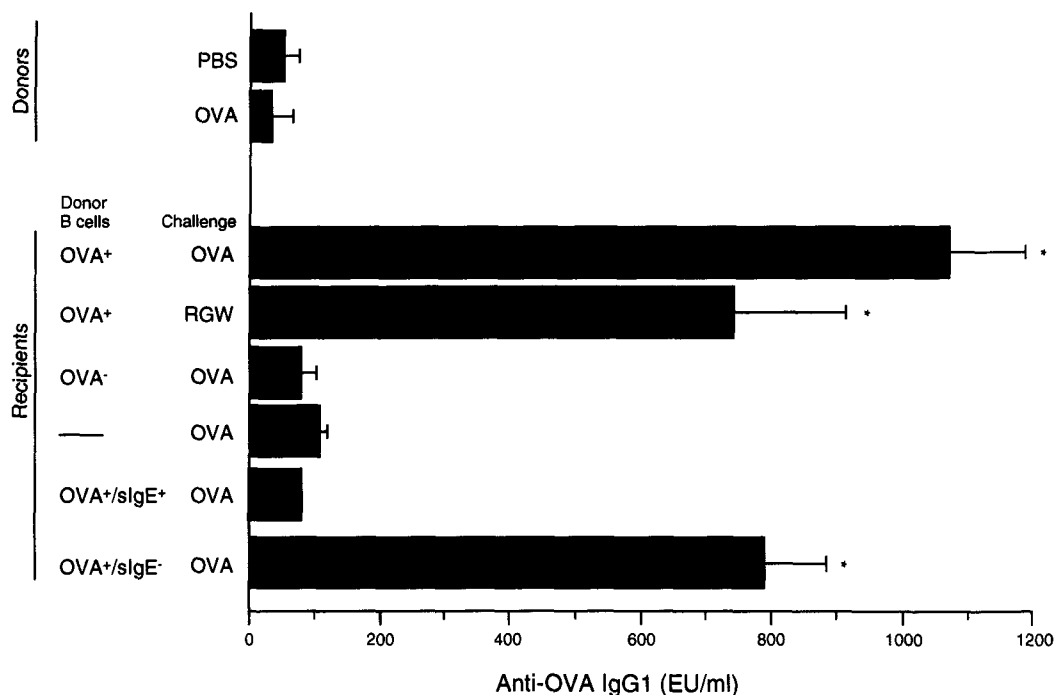


Figure 4. Transfer of anti-OVA IgG₁ production. OVA⁺ and OVA⁻ B-cell populations were transferred exactly as described in Figure 3 and into the same recipients described in Figure 3. Serum was obtained at the same time points, and anti-OVA IgG₁ was determined by ELISA. Expressed are the mean values for each group $\pm$ SE. * $p < 0.001$ compared with the group with no cells transferred.

quirement for OVA-specific T cells. Interestingly, a much higher frequency of OVA⁺ B cells was committed to IgE production compared to the OVA⁻ population, as demonstrated by analysis of surface IgE. The frequency of sIgE⁺ B cells in the OVA⁺ population was $13.3 \pm 4.1\%$ compared to $2.3 \pm 1.2\%$ OVA⁻ B cells.

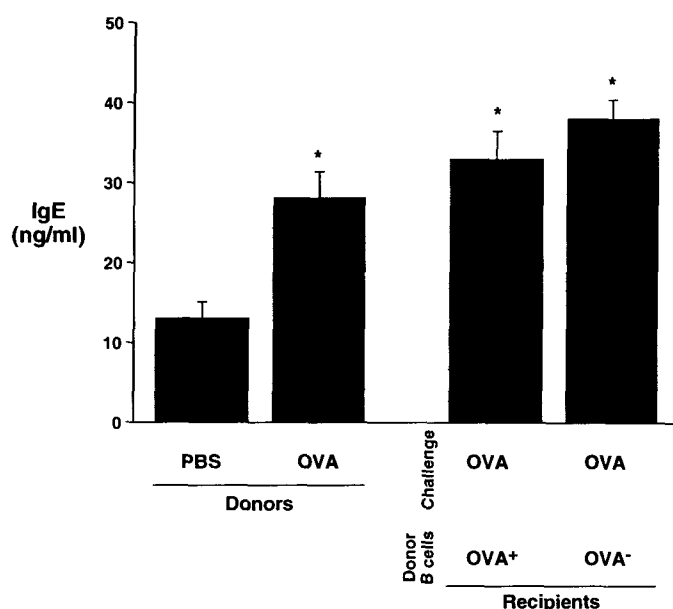


Figure 5. Both OVA⁺ and OVA⁻ B cells transfer the capacity to produce IgE. Serum was obtained from the same animals described in Figure 3 and analyzed for total IgE by ELISA. Recipients received either 10^6 OVA⁺ B cells or 10^7 OVA⁻ B cells. Expressed are the mean values $\pm$ SE. * $p < 0.001$ compared with the group with no cells transferred.

sIgM⁺ cells predominated in both groups. These results imply that the inhalation of OVA induces a class switch from IgM to IgE. One cannot, however, rule out the possibility that the sensitization process per se directly induced an expansion of a small subset of pre-existing OVA⁺/IgE-committed B cells.

The transfer of 10^6 OVA⁺ B cells resulted in OVA-specific IgE and IgG₁ production in naive recipients. Interestingly, the primary sensitized donors failed to produce anti-OVA IgG₁, and yet OVA⁺ B cells derived from these mice transferred the capacity to produce anti-OVA IgG₁ in the recipient mice. If indeed isotype switching in the mouse from IgM to IgE occurs sequentially through IgG₁, as several reports suggest (27–31), then one would expect to see the appearance of anti-OVA IgG₁ before or coinciding with the appearance of IgE. Our data suggest that the trans-

TABLE 2
TRANSFER OF IMMEDIATE CUTANEOUS REACTIVITY

		Mean Wheal Diameter (mm)	Number of Positive Responders to OVA
Donors			
PBS		0 $\pm$ 0	0/10
OVA		3.8 $\pm$ 0.2*	9/10
Recipients			
Donor B Cells	Challenge		
OVA ⁺	OVA	7.7 $\pm$ 0.5*	10/10
OVA ⁺	RGW	8.0 $\pm$ 0.4*	6/6
OVA ⁻	OVA	0.5 $\pm$ 0.3	0/9
—	OVA	0.2 $\pm$ 0.4	0/5
OVA ⁺ /sIgE ⁺	OVA	6.0 $\pm$ 0.5*	7/7
OVA ⁺ /sIgE ⁻	OVA	4.7 $\pm$ 0.5*	6/7

Cutaneous reactivity to OVA was determined in the same mice described in Figure 3. Following intradermal injection of OVA, the development and size of a wheal response was assessed 15 minutes later.

* $p < 0.001$ for wheal diameter compared to PBS or no cell transfer group.

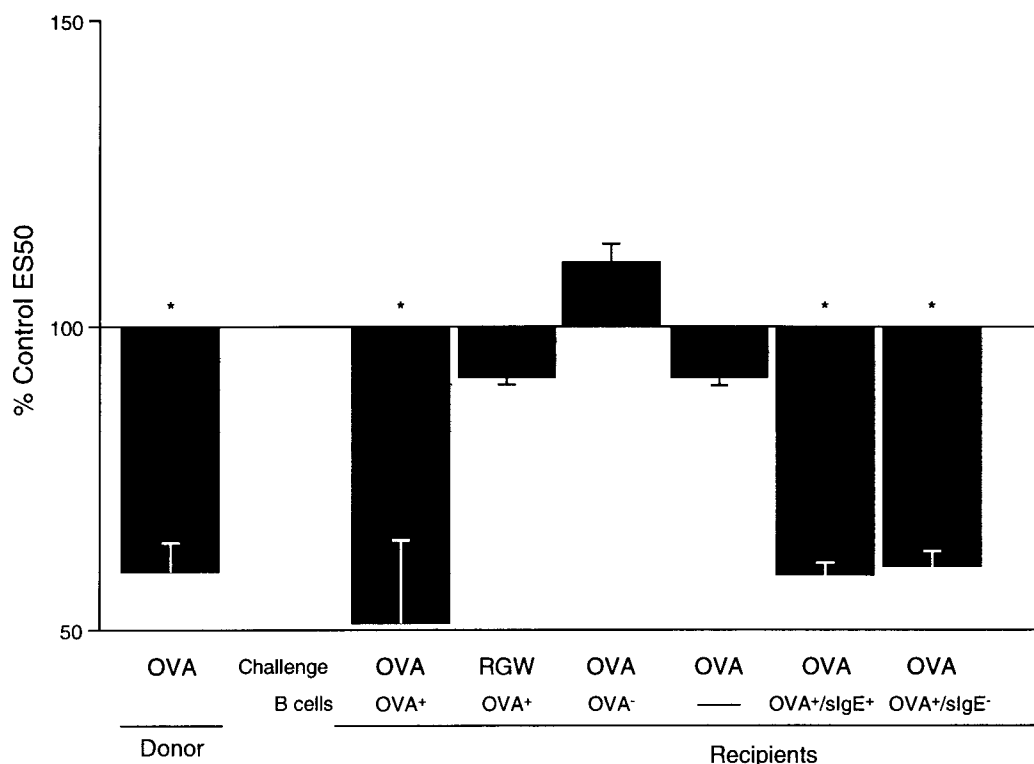


Figure 6. OVA⁺ B cells transfer the capacity for increased airway responsiveness. Electrical field stimulation was performed on trachea smooth muscle preparations as described from the same animals as in Figure 3. Recipients of B cells were analyzed on day 8 of the transfer protocol after challenge with either OVA or RGW (see Figure 3 for identification of groups). Expressed is the % control ES50 where 100% represents the value obtained in control mice exposed to PBS inhalation (ES50 4.0 ± 0.2 Hz). * $p < 0.001$ compared with PBS- treated control animals.

ferred OVA⁺ B cells undergo isotype switching to IgG₁ during or after the transfer, by which time commitment to IgE has already occurred. Indeed, at the time of the transfer, the OVA⁺ B cells consisted of sIgE⁺ and largely sIgM⁺ cells with < 1% staining for sIgG.

In recipient mice, the anti-OVA IgE levels were approximately twice the levels measured in primary mice sensitized to OVA. In keeping with these findings, immediate cutaneous responses to OVA in the recipients, which we know to be due to anti-OVA IgE (19), were almost twice the diameter observed in the donor mice. These responses appeared solely dependent on the transferred cells, irrespective of the challenge, since mice that received OVA⁺ B cells but were challenged with ragweed developed cutaneous reactivity to OVA and serum anti-OVA IgE levels comparable to the recipients of OVA⁺ B cells challenged with OVA. This implies that further allergen-specific challenge, at least via the airways, was not required for the induction of OVA-specific IgE synthesis by transferred OVA⁺ B cells. Under normal circumstances, activation and expansion of B cells and isotype switching follows antigen presentation to the B cells. Surface immunoglobulin staining of the OVA⁺ B cell population demonstrated that a high proportion of this population was already committed to IgE production. Therefore, allergen exposure (at least via the airways) played little role in augmenting the response and was presumably no longer necessary as isotype switching had already occurred. In addition, the OVA⁺ B cells are likely to have been activated before transfer since they originated from sensitized mice exposed to OVA and were likely stimulated by the cross-linking of surface immunoglobulin during the antigen-coated bead selection process. This may explain why further allergen

challenge of the recipients may not have influenced the activity of the OVA⁺ B-cell population.

To determine whether the population of B cells that transferred immediate hypersensitivity to OVA was indeed sIgE⁺, we purified a subpopulation of OVA⁺/sIgE⁺ B cells. The transfer of as few as 5×10^4 cells was sufficient to induce OVA-specific IgE (but not IgG₁) in the serum and cutaneous reactivity to OVA. Importantly, this highly purified population contained no T cells and therefore further excludes the possibility that the transfer of sensitization was mediated or influenced by a small subset of allergen-specific T cells. Interestingly, recipients of OVA⁺/sIgE⁻ B (5×10^5) cells were able to develop OVA-specific IgE as well as IgG₁ responses. Since this population of B cells contained < 1% sIgE⁺ B cells and 1% sIgG⁺ B cells, these data suggest that this population of largely sIgM⁺ B cells was in the process of isotype switching to IgE and IgG₁ at the time of transfer. When compared to the findings with OVA⁺/sIgE⁺ B cells, the data are not explainable by contamination of this population of OVA⁺/sIgE⁻ cells by small numbers (< 1%) of sIgE⁺ B cells. Since the levels of IgG₁-specific antibody in these animals was similar to OVA⁺ B cell recipients, the development of immediate hypersensitivity and increased AR may have been contributed to by IgG₁ as well.

The etiology of airway hyperresponsiveness is thought to be dependent on the route, dosage, and frequency of allergen exposure as well as the atopic response and capacity to produce IgE, especially allergen-specific IgE. In our model, the combination of systemic anti-OVA IgE production and local challenge to the airways resulted in airway hyperresponsiveness. The necessity for challenge with appropriate or specific allergen for the induction of airway hyperresponsiveness was also demonstrated.

We settled on two challenges in this study, the first at the time of B-cell transfer and the second on day 7, closer to the time of assessment of AR. Preliminary data indicated that unless challenged within a few days of measuring ES50, AR reverts to normal. After the transfer of OVA⁺ B cells, the two challenges with OVA induced an increase in AR in the recipients. In contrast, challenge with an irrelevant aeroallergen, ragweed, failed to induce such airway hyperresponsiveness despite comparable levels of serum anti-OVA IgE antibody levels and immediate cutaneous reactivity as in OVA-challenged mice. Similarly, in the OVA⁺/sIgE⁺ and OVA⁺/sIgE⁻ B-cell transfer experiments, specific OVA challenge via the airways was required for the development of airway hyperresponsiveness. These results highlight the different requirements for development of immediate hypersensitivity and the manifestations of airway hyperresponsiveness. The requirement for specific allergen would suggest that type I hypersensitivity underlies the changes in airway function. However, whether the changes are due to mast-cell degranulation or subsequent influx of inflammatory cells needs to be further characterized. The participation of B cells in the development of altered AR, independent of their role in IgE synthesis, is currently being investigated in passive transfer experiments.

The development of increased AR required both specific airway challenge with OVA and the induction of anti-OVA IgE in the serum, while development of cutaneous reactivity to OVA occurred independently of allergen challenge. These findings confirm and extend our previous observations that the induction of OVA-specific IgE, though necessary, is in itself not sufficient to cause an increase in AR. In a model of cutaneous sensitization to OVA (19), we were able to show that despite systemic anti-OVA IgE production and cutaneous reactivity to OVA, mice developed airway hyperresponsiveness only if they received a local OVA challenge to the airways. The presence of polyclonal IgE in the serum did not contribute to increased AR because mice that received 10⁷ OVA⁻ B cells had normal ES50 values after two OVA challenges, despite serum IgE levels that were higher than in the OVA⁺ B-cell recipients. These are consistent with our previous findings that IFN- γ treatment of OVA-sensitized mice resulted in lowered anti-OVA IgE production together with a normalization of airway responsiveness despite raised total serum IgE (18), suggesting that polyclonal IgE is not causally implicated in airway hyperresponsiveness.

Overall, these data strongly support an IgE-mediated allergic mechanism in the induction of airway hyperresponsiveness in Balb-C mice sensitized with aerosolized allergen. SJL mice that are relatively deficient in IL-4 and fail to mount an anti-OVA IgE response do not develop airway hyperresponsiveness (16). The combination of allergen-specific IgE and local allergen challenge plausibly leads to early and late phase allergic reactions with inflammation and altered neural control of the airways. Using the same model, we have recently shown that acetylcholine release was greater during electrical stimulation of tracheas from sensitized mice that mounted an allergen-specific IgE response compared with release from the tracheas of nonimmunized mice (17). Associated with the increase in acetylcholine release in immune mice was loss of muscarinic autoreceptor function. This suggests that repeated exposure to allergen through the airways with development of an IgE-responsive state can lead to altered neural control of airway function with increased release of acetylcholine.

We have shown in this murine model of allergic hypersensitivity that specific allergen and allergen-specific B cells committed to IgE production are central to the induction of immediate hypersensitivity and increased AR. The isolation of OVA⁺ B cells, OVA⁻ B cells, OVA⁺/sIgE⁺, and OVA⁺/sIgE⁻ cells allowed us to directly evaluate the role of allergen-specific versus polyclonal

IgE production on airway function in the context of an environmental challenge. Such manipulations will permit us to further study the *in vivo* interactions in local lymphoid tissues between allergen-specific B cells, allergen, and reactive T cells and, as a result, better define the immunopathology of allergic disease and airway hyperresponsiveness.

Acknowledgment: The writers are grateful to Dr. J. Greenstein, ImmuLogic (Waltham, MA) for providing ragweed antigen, Dr. F. Finkelman (Bethesda, MD) for providing anti-IgE antibody, Drs. P. Marrack and J. Kappler (Denver, CO) for their gift of anti-B220 antibody, and Dr. Paul Trotta (Bloomfield, NJ) for the gift of IL-4. The writers also thank Debbie Remley for her help in preparation of the manuscript.

References

1. Norel, X., I. Haye-Legrand, C. Labat, J. Benveniste, and C. Brink. 1991. Antigen-induced contraction of human isolated lung preparations passively sensitized with monoclonal IgE: effects of indomethacin. *Int. Arch. Allergy Appl. Immunol.* 96:368-375.
2. Haye-Legrand, I., X. Norel, C. Labat, J. Benveniste, and C. Brink. 1988. Antigenic contraction of guinea pig tracheal preparations passively sensitized with monoclonal IgE: pharmacological modulation. *Int. Arch. Allergy Appl. Immunol.* 87:342-348.
3. Atkins, P. C., M. Norman, H. Weiner, and B. Zweiman. 1977. Release of neutrophil chemotactic activity during immediate hypersensitivity reactions in humans. *Ann. Intern. Med.* 86:415-418.
4. Lee, T. H., M. J. Brown, L. Nagy, R. Causon, M. J. Walport, and A. B. Kay. 1982. Exercise-induced release of histamine and neutrophil chemotactic factor in atopic asthmatics. *J. Allergy Clin. Immunol.* 70:73-81.
5. Lee, T. H., T. Nagakura, N. Papageorgiou, Y. Iikura, and A. B. Kay. 1983. Exercise-induced late asthmatic reactions with neutrophil chemotactic activity. *N. Engl. J. Med.* 308:1502-1505.
6. Nagy, L., T. H. Lee, and A. B. Kay. 1982. Neutrophil chemotactic activity in antigen-induced late asthmatic reactions. *N. Engl. J. Med.* 306:497-501.
7. Gleich, G. J. 1989. The eosinophil and bronchial asthma: current understanding. *J. Allergy Clin. Immunol.* 89:422-436.
8. Ishizaka, T. 1981. Analysis of triggering events in mast cells for immunoglobulin E-mediated histamine release. *J. Allergy Clin. Immunol.* 67:90-96.
9. MacGlashan, D. W., R. P. Schleimer, S. P. Peters, E. S. Schulman, G. K. Adams, H. H. Newball, and L. M. Lichtenstein. 1982. Generation of leukotrienes by purified human lung mast cells. *J. Clin. Invest.* 70:747-751.
10. Schleimer, R. P., D. J. MacGlashan, E. S. Schulman, S. P. Peters, G. Adams, N. J. Adkinson, and L. M. Lichtenstein. 1983. Human mast cells and basophils—structure, function, pharmacology, and biochemistry. *Clin. Rev. Allergy* 1:327-341.
11. Tomioka, M., S. Ida, S. Yuriko, T. Ishihara, and T. Takishima. 1984. Mast cells in bronchoalveolar lumens of patients with bronchial asthma. *Am. Rev. Respir. Dis.* 129:1000-1005.
12. Kintner, C., and B. Sugden. 1981. Identification of antigenic determinants unique to the surfaces of cells transformed by Epstein-Barr virus. *Nature* 294:458-460.
13. Gonzalez-Molina, A., and H. L. Spiegelberg. 1975. Binding of IgE myeloma proteins to human cultured lymphoblastoid cells. *J. Immunol.* 117:1838-1845.
14. Renz, H., H. R. Smith, 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.* 89:1127-1138.
15. Saloga, J., H. Renz, G. Lack, K. Bradley, J. Greenstein, G. Larsen, and E. W. Gelfand. 1992. Development and transfer of immediate cutaneous hypersensitivity in mice exposed to aerosolized antigen. *J. Clin. Invest.* 91:133-140.
16. 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.* 89:747-752.
17. 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. (Lung Cell Mol. Physiol.)* 266:L263-L270.
18. 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

- interferon- γ decreases IgE production and normalizes airways function in a murine model of allergen sensitization. *J. Immunol.* 152: 2546-2554.
19. Saloga, J., H. Renz, G. L. Larsen, and E. W. Gelfand. 1994. Increased airways responsiveness in mice depends on local challenge with antigen. *Am. Rev. Respir. Dis.* 149:65-70.
 20. 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. U.S.A.* 89:6438-6442.
 21. Renz, H., K. L. Bradley, J. Saloga, J. Loader, G. L. Larsen, and E. W. Gelfand. 1993. T cells expressing specific V β elements regulated immunoglobulin E production and airways responsiveness in vivo. *J. Exp. Med.* 177:1175-1180.
 22. Oshiba, A., and J. Yata. 1991. Increase of ovalbumin (OVA)-specific B cells in the peripheral blood of egg-allergic patients. *J. Allergy Clin. Immunol.* 87:729-736.
 23. Renz, H., J. Saloga, K. L. Bradley, J. E. Loader, J. L. Greenstein, G. L. Larsen, and E. W. Gelfand. 1993. Specific V β T cell subsets mediate the immediate hypersensitivity response to ragweed allergen. *J. Immunol.* 151:1907-1917.
 24. Nustad, K., L. Johansen, J. Ugelstad, T. Ellingsen, and A. Berge. 1984. Hydrophilic monodispersed particles as solid-phase material in immunoassays: comparison of shell-and-core particles with compact particles. *Eur. Surg. Res.* 2:80-87.
 25. Egeland, T., A. Hovdenes, and T. Lea. 1988. Positive selection of antigen-specific B lymphocytes by means of immunomagnetic particles. *Scand. J. Immunol.* 27:439-444.
 26. Lea, T., E. Smeland, S. Funderud, F. Vartdal, C. Davies, K. Bieske, and J. Ugelstad. 1986. Characterization of human mononuclear cells after positive selection with immunomagnetic particles. *Scand. J. Immunol.* 23:509-519.
 27. Snapper, C. M., F. D. Finkelman, D. Stefany, D. H. Conrad, and W. E. Paul. 1988. IL-4 induces co-expression of intrinsic membrane IgG $_1$ and IgE by murine B cells stimulated with lipopolysaccharide. *J. Immunol.* 141:489-498.
 28. Yoshida, K., M. Matsuoka, S. Usuda, A. Mori, K. Ishizaka, and H. Sakano. 1990. Immunoglobulin switch circular DNA in the mouse infected with *Nippostrongylus brasiliensis*: evidence for successive class switching from μ to ϵ via γ 1. *Proc. Natl. Acad. Sci. U.S.A.* 87:7829-7833.
 29. Mills, F. C., G. Thyonephritis, F. D. Finkelman, and E. E. Max. 1992. Ig μ - ϵ isotype switch in IL-4-treated human B lymphoblastoid cells. *J. Immunol.* 14:1075-1085.
 30. Siebenkotten, G., C. Esser, M. Wabl, and A. Radbruch. 1992. The murine IgG $_1$ /IgE class switch program. *Eur. J. Immunol.* 22:1827-1834.
 31. Mandler, R., F. D. Finkelman, A. D. Levine, and C. M. Snapper. 1993. IL-4 induction of IgE class switching by lipopolysaccharide-activated murine B cells occurs predominantly through sequential switching. *J. Immunol.* 150:407-418.