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IgE antibody suppression following aerosol exposure to antigens

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Summary. Exposure of mice to aerosolized antigens induced a low level IgG1 response but not detectable IgE antibodies. Subsequent intraperitoneal immunization of these mice demonstrated immunoglobulin class-specific IgE suppression. Low concentrations of nebulized antigen induced IgE suppression which was antigen specific and persisted on subsequent secondary and tertiary injections. Although a single aerosolized antigen exposure significantly decreased the IgE response, maximal suppression was observed when the mice were exposed to nebulized antigen once weekly for at least 6 weeks. The suppression was not observed until 3 weeks following nebulizer exposure. Mice exposed once weekly to nebulized antigen for 6 weeks and then rested for 2 months before intraperitoneal immunization still demonstrated suppression. However, animals first immunized intraperitoneally and then exposed to nebulized antigen produced normal secondary IgE and IgG1 responses. These results suggest that antigen exposure by aerosol may profoundly alter the IgE response.

INTRODUCTION

Periodic exposure to small amounts of antigen produces an atopic state in genetically susceptible individuals. It is believed that such sensitization to pollens and other allergens occurs by aerosol exposure and results in the presence of circulating antibodies of the IgE class which mediate the resultant allergic hypersensitivity state. The present experiments studied the development of a sensitized state in mice by measuring IgE antibody levels following aerosol exposure to protein allergens.

The route of antigen exposure is known to influence the resulting immune response. Injections of antigen can be immunogenic or tolerogenic depending on such factors as dosage, route of administration, use of adjuvant, age of animals or chemical modification of antigen. Aerosolized antigen exposure in rats results in IgE production if adjuvant is administered concurrently by another route (Van Hout & Johnson, 1972; Barboriak, Knoblock, Hensley, Gombas & Fink, 1976). In the rabbit, an IgG and IgM antibody response is observed following nebulized antigen exposure (Willoughby & Willoughby, 1977). In contrast to these findings in rats and rabbits, we report here that exposure of mice to aerosolized antigen results not in sensitization but in specific suppression of the IgE response to protein antigens.

MATERIALS AND METHODS

Animals

Female mice of the different inbred strains, 8–10 weeks old, were obtained from the Jackson Laboratory, Bar Harbor, Maine. For passive cutaneous anaphylactic (PCA) reactions, NIH bred, general-purpose female mice, 5–7 weeks old, were utilized.

Antigens

Ovalbumin, 5x recrystallized, was from Sigma Chemi-

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cal Co., St Louis, Mo. Short ragweed pollen was purchased from Hollister-Stier Laboratories, Spokane, WA.; 1 gm of the pollen was extracted overnight with 1 litre of distilled water, the mixture was centrifuged at 12,000 *g* for 30 min and the supernatant used for aerosolization. Ragweed utilized for intravenous and intraperitoneal injection was lyophylized, phenol free, giant and short ragweed purchased from Greer Laboratories, Lenoir, N.C.

Antigen administration

Intraperitoneal (i.p.). Ovalbumin or ragweed was dissolved in Tris buffered saline (pH 8·2, 0·1 M NaCl, 0·05 M Tris), mixed with alum (2 mg/ml) and prepared so an appropriate dose was contained in 0·5 ml.

Oral. Ovalbumin was prepared as a stock solution of 2 mg/ml in normal saline. Appropriate dilutions were prepared in distilled water and animals were given free access to the water bottle during a 24-hour period. The approximate oral intake of a 3-month-old laboratory mouse is 2–3 ml in a 24-hour period.

Aerosol. Appropriate concentrations of ovalbumin in distilled water were placed in the chamber of an ultrasonic nebulizer (DeVilbiss Model 35B, Somerset, Pa) which atomized the solution to droplets of 1–5 μm diameter. The nebulizer was adjusted to deliver a volume of 100 ml in a 6-hour period. Mice were exposed to nebulized antigen in groups of four in filter topped cages. Following treatment, animals were transferred to cages with clean bedding and water.

Serum

Mice were bled from the retro-orbital plexus into heparinized Natelson capillary tubes (Sherwood Medical Industries, St Louis, Mo.). After centrifugation, the plasma from mice within each group were pooled and stored at $-20°$. These are referred to as sera in the text.

Passive cutaneous anaphylaxis (PCA) assay

Serum IgE and IgG1 levels were determined by PCA reactions in NIH general purpose mice. Serial dilutions of sera were made in phosphate buffered saline (pH 7·4) containing 0·03% human serum albumin and 0·03 ml was injected intradermally. Following a latent period (3 hr for IgG1 and 72 hr for IgE) 300–500 μg of the appropriate antigen was injected intravenously with 2% Evans blue solution. After 20 min, the mice were killed and the diameter of the blue reaction was measured directly on the undersurface of the skin. All serum dilutions were tested in four mice. Titres are reported as the reciprocal of the dilution giving a measurement of 5 mm. A greater than two-fold difference between titres was considered significant. In general, a 1:10 dilution was the lowest tested; no response at this dilution was recorded as $< 1:5$.

ELISA

Modified, automated, enzyme-linked immunoadsorbent assays (ELISA; Engvall & Perlmann, 1972) were carried out to determine serum antibody levels and to test for the presence of free antigen or immune complexes (EIA PR-50, Gilford Instrument, Oberlin, Ohio). Cuvettes were sensitized by overnight incubation at 4° with 100 μg/ml ovalbumin for the detection of specific antibody. Dilutions of serum from nebulizer-exposed mice and appropriate controls of affinity-purified mouse anti-ovalbumin antibody were incubated in cuvettes for 90 min at 37°. After washing, a rabbit anti-mouse immunoglobulin antibody was added for 1 hr at room temperature. The second antibody was a goat anti-rabbit immunoglobulin antibody conjugated to alkaline phosphatase. After 1 hr at room temperature, the substrate, p-nitrophenyl phosphate, 1 mg/ml, was added. Optical density at 405 nm was determined at sixty min. This assay could detect concentrations of 5 ng/ml of specific ovalbumin antibody.

To determine the concentration of ovalbumin or immune complexes, cuvettes were sensitized with an affinity-purified rabbit anti-ovalbumin antibody, 10 μg/ml. Dilutions of ovalbumin, preformed immune aggregates and serum from nebulizer treated mice were added to the cuvettes and incubated for 90 min at 37°. An affinity-purified mouse anti-ovalbumin antibody was then added, followed by a rabbit anti-mouse immunoglobulin antibody conjugated to alkaline phosphatase. The sensitivity of the ELISA for ovalbumin as free antigen was 1 ng/ml. The control immune aggregates had been formed with ovalbumin mixed overnight with mouse anti-ovalbumin antibody at concentrations which ranged from antigen excess (3 μg antibody + 10 μg antigen) to antibody excess (3 μg antibody + 0·1 μg antigen). The assay could detect each of the preformed immune complexes at dilutions of 1:100.

RESULTS

Inability to induce a primary IgE response with aerosolized antigen

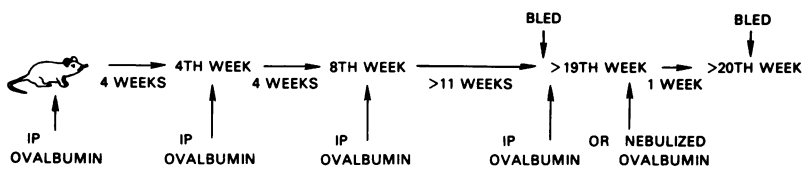
Attempts were made to sensitize mice by treatment

consisting of twelve once-weekly exposures to 100 ml of either 1, 10, 100 or 1000 μg/ml ovalbumin nebulized over a 6-hour period. Mice were bled weekly immediately before treatment and 3 weeks following the final exposure. Mice failed to produce detectable levels of antigen-specific IgE at any time during this course of primary nebulizer exposure (72-hr PCA reaction negative with undiluted serum). Following 5 weeks of nebulization, low levels of IgG1 antibody (3-hr PCA reaction) were first noted only in mice exposed to 1000 μg ovalbumin (titres of 1:20). This low level persisted throughout nebulizer treatment but did not increase with continued exposure during the next 7 weeks. This titre is lower than the levels of IgG1 observed following immunization of mice with adjuvant by the intraperitoneal or subcutaneous routes. The mice did produce antibody following exposure to nebulized ovalbumin at lower concentrations. Utilizing a sensitive ELISA, serum from animals nebulized six times at weekly intervals with 100 μg/ml ovalbumin and then bled 21 days following the final exposure was found to contain 40–50 μg/ml of anti-ovalbumin antibody.

Aerosol generation of ovalbumin by the ultrasonic nebulizer did not result in a change of antigenicity of the molecule. Antigen collected by condensation of nebulized mist showed only lines of identity with fresh ovalbumin solutions by radial double diffusion against a rabbit anti-ovalbumin serum. Immunization of mice with recollected nebulized ovalbumin or untreated ovalbumin, both in alum, gave similar IgE titres (data not shown). Nebulization of mice previously immunized intraperitoneally was found to be as effective in boosting circulating IgG1 and IgE antibody levels as an intraperitoneal injection with 1 μg ovalbumin in 2 mg alum (Fig. 1). It is clear that animals were capable of absorbing and recognizing the antigen and that nebulizer exposure was capable of inducing a secondary response in animals initially immunized by another route.

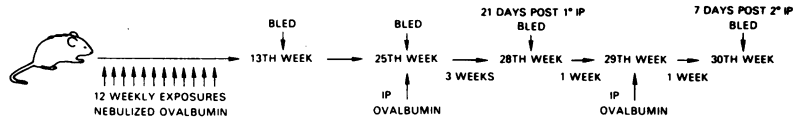
Suppression of IgE response by aerosolized antigen

Suppression of IgE responses was noted when mice exposed to aerosolized antigen were then immunized with the same antigen in alum (Fig. 2). Ovalbumin was administered by nebulizer once weekly for 12 weeks to six inbred strains of mice. After 13 weeks the animals were injected intraperitoneally with 1 μg ovalbumin in 2 mg alum. These injections were repeated 4 weeks later. Three weeks after the first injection the mice were bled (21 days post primary injection). These sera and those obtained 1 week after the second intraperitoneal injections (7 days post secondary injection) were assayed for IgE antibody levels. Both sets of sera showed a significant decrease in IgE anti-ovalbumin antibody levels. Suppression was observed in all six F_1 strains tested following a second intraperitoneal injection.



	RECIPROCAL PCA TITRES					
	IgG_1			IgE		
Strain	Pre-Nebulizer	7 Days Post-Nebulizer	7 Days Post-IP	Pre-Nebulizer	7 Days Post-Nebulizer	7 Days Post-IP
$AKD2F_1$	600	3,000	1,200	20	200	400
$B6AF_1$	300	400	1,200	40	200	160
$B6D2F_1$	600	1,000	800	200	300	200
CAF_1	600	400	1,200	80	400	400
$C3D2F_1$	400	150	400	20	100	160
LAF_1	400	1,200	800	60	400	400

Figure 1. Effect of nebulizer *vs.* intraperitoneal antigen exposure on IgG1 and IgE responses in immunized mice. Mice were immunized intraperitoneally with 0·3 mg ovalbumin in 2 mg alum at time 0, 4 and 8 weeks. At more than 11 weeks after the final injection, the mice were bled. Groups were divided and either; (a) exposed to 100 ml nebulized ovalbumin, 100 μg/ml, 6 hours per day, for 5 consecutive days; or (b) injected intraperitoneally with 0·3 μg ovalbumin in 2 mg alum. Seven days following this injection or the final nebulizer exposure the mice were bled. Further experiments demonstrated that 2 consecutive days of aerosolized antigen exposure were as effective as 5 days (data not shown).



Strain	RECIPROCAL IgE PCA TITRES			
	21 Days Post-Primary IP Injection		7 Days Post-Secondary IP Injection	
	Control	Nebulizer	Control	Nebulizer
$AKD2F_1$	< 5	< 5	40	< 5
$B6AF_1$	10	< 5	100	20
$B6D2F_1$	160	< 5	800	< 10
CAF_1	10	< 5	80	< 5
$C3D2F_1$	40	< 5	40	< 5
LAF_1	80	< 5	200	10

Figure 2. Alterations in IgE response due to aerosolized antigen exposure. Groups of 4 mice were exposed to 100 ml nebulized ovalbumin, 100 μg/ml, in a 6-hour period, once weekly for 12 consecutive weeks. After a 13-week rest, the animals were bled and then given intraperitoneal injections of 1 μg ovalbumin in 2 mg alum. This was repeated 4 weeks later.

An enzyme-linked immunoadsorbent assay was used to test for the presence of free ovalbumin in the serum of mice exposed to nebulized antigen. Serum was obtained 21 days after the last of six weekly nebulized ovalbumin exposures (100 μg/ml). No free ovalbumin was found at any serum concentration tested. The assay was capable of detecting ovalbumin at a concentration of 1 ng/ml.

This assay was also used to test for the presence of ovalbumin–anti-ovalbumin immune complexes which might be present in serum from nebulizer-treated mice. Nebulized-ovalbumin-exposed mouse serum obtained 21 days after the sixth and final nebulizer treatment (100 μg/ml) had no detectable immune complexes when tested at concentrations from 1:10 to $1:1 \times 10^6$.

Conditions for IgE suppression

The effect of concentration of aerosolized antigen solutions on suppression. Varying concentrations of ovalbumin solutions were administered by nebulizer once a week for 13 consecutive weeks and after a 3-week rest period the mice were injected with 1 μg ovalbumin in 2 mg alum (Table I). All aerosolized

Table 1. Antibody responses to immunization in mice previously exposed to oral or aerosolized antigen

Ovalbumin concentration (μg/ml)	Reciprocal PCA titres—7 days post secondary i.p. injection			
	Aerosolized antigen exposure		Oral antigen exposure	
	IgG1	IgE	IgG1	IgE
0	1600	200	1600	400
1	400	40	800	400
10	1600	40	400	400
100	400	5	400	200
1000	20	10	10	10

Mice were exposed by nebulizer to 100 ml ovalbumin solutions for a 6-hour period once a week for 13 consecutive weeks or ovalbumin in drinking water for a 24-hour period once a week for 22 consecutive weeks. Three and 7 weeks following the final exposure, mice were immunized intraperitoneally with 1 μg ovalbumin in 2 mg alum.

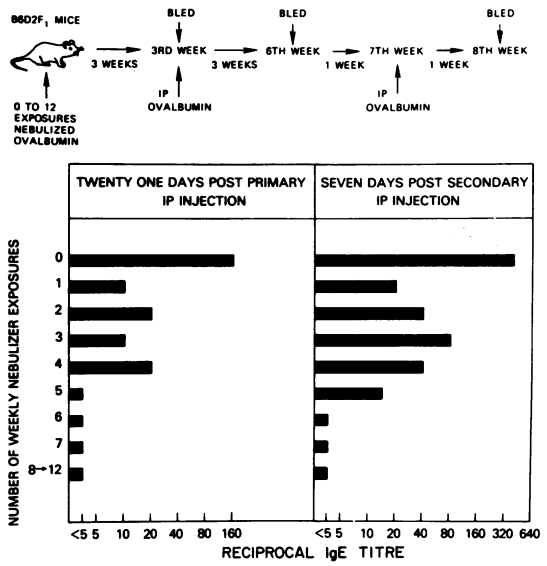


Figure 3. Effects of number of aerosolized antigen exposures on IgE response. B6D2F$_1$ mice were exposed to 100 ml nebulized ovalbumin, 100 μg/ml, in a 6-hour period once a week. Three and 7 weeks following the last nebulizer exposure, mice were injected intraperitoneally with 1 μg ovalbumin in 2 mg alum. The geometric mean titre of IgG1 of all groups at 21 days post primary intraperitoneal injection was 237 (range 160–800) with a control titre of 200. At 7 days post secondary intraperitoneal injection the geometric mean IgG1 titre was 351 (range 100–800) with a control titre of 1600.

antigen concentrations produced a significant reduction of IgE antibody levels following intraperitoneal injection. A significant suppression of IgG1 antibody levels was noted at the highest ovalbumin concentration following the secondary but not the primary immunization. All further experiments were carried out at antigen concentrations of 100 μg/ml.

Effect of the number of exposures to aerosolized antigen. Groups of 4 B6D2F$_1$ mice were exposed once-weekly to 100 ml nebulized ovalbumin, 100 μg/ml, from one to twelve times (Fig. 3). Three weeks following the final aerosol exposure, the mice were injected intraperitoneally with 1 μg ovalbumin in 2 mg alum and bled 21 days later. A single exposure produced a significant reduction in IgE antibody levels. Five exposures reduced the specific IgE to levels undetectable by our assay system. There was no significant alteration of IgG1 responses (data not shown). The IgE suppression was still present following a second immunization 4 weeks after the first injection (Fig. 3). Similar suppression of IgE responses has been demonstrated following aerosolization of antigen in the following strains of mice: A/J, C3H/HeJ, DBA/1, C57BL/6, BALB/c, NZB, AKD2F$_1$, B6AF$_1$, CAF$_1$ LAF$_1$ and C3D2F$_1$.

Effect of interval between aerosolized antigen exposure and i.p. immunization. A latent period was necessary following nebulization for the appearance of IgE suppression. Mice were injected intraperitoneally at varying times after a single 100 ml nebulizer exposure of 100 μg/ml ovalbumin solution. Introduction of antigen by intraperitoneal injection earlier than 21 days following initial nebulizer exposure resulted in normal IgE responses. However, when mice had received at least three weekly exposures to nebulized antigen, suppression was observed even when the intraperitoneal injections immediately followed the last nebulization (i.e. no rest period was required). When intraperitoneal injection preceded or was coincident with initial nebulizer exposure no suppression was noted.

Persistence of IgE suppression. The duration of IgE suppression by aerosolized antigen was dependent on a number of factors. Animals given intraperitoneal injections 2 months following the last of six weekly nebulizations (100 μg/ml, 100 ml, once weekly) showed a significant reduction in IgE when compared with controls (72-hr PCA titres of 5 *vs.* 160). However, a 3-month rest before intraperitoneal injection allowed a return of normal IgE response to ovalbu-

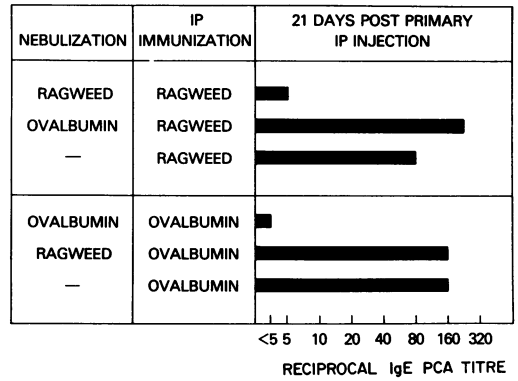


Figure 4. Specificity of nebulizer-induced suppression. B6D2F1 mice were exposed to 100 ml nebulized solutions of ovalbumin (100 μg/ml) or ragweed (1000 μg/ml), for a 6-hour period, once weekly for 6 consecutive weeks. Three weeks following the final nebulizer exposure, groups of four mice were injected intraperitoneally with either 1 μg ovalbumin or 10 μg ragweed, both in 2 mg alum. Animals were bled 21 days following this intraperitoneal injection.

min. Mice exposed for 12 weeks to a higher ovalbumin concentration (1000 μg/ml) showed suppression after a rest period of 13 weeks (see Fig. 2). However, animals which were suppressed by the injection of antigen in alum 3 weeks following a course of nebulization retained their IgE suppression for 18 months when injected at intervals of several months. Therefore, antigen concentration, number of nebulizer exposures and time of antigen–adjuvant injection play important roles in the duration of suppression of the IgE response.

Specificity of IgE suppression by nebulization

The nebulizer-induced suppression was antigen specific. Groups of mice were exposed for 6 weeks to ovalbumin (100 μg/ml) or ragweed (1000 μg/ml). Animals were then given intraperitoneal injections (1 μg ovalbumin or 10 μg ragweed in 2 mg alum) and serum was collected 3 weeks later (Fig. 4). Animals injected with the same antigen as that used for nebulization demonstrated IgE suppression. Normal IgE responses were found to intraperitoneal injections given to animals nebulized with a different antigen. In all cases, IgG1 responses did not deviate from control levels (data not shown).

Effects of alternate routes of immunization

Since nebulized antigen could be deposited in the nasopharynx and then swallowed, we compared the effects of oral and aerosol immunization (Table 1). The addition of antigen once weekly to the drinking water failed to induce a detectable primary IgE or IgG1 response even after 22 weeks (data not shown). Only high concentrations of antigen administered orally (in this experiment 1 mg/ml) produced a significant suppression of both IgE and IgG1 responses to subsequent intraperitoneal injection of ovalbumin in alum. In contrast, aerosolized antigen doses as low as 1 and 10 μg/ml were effective in suppressing IgE responses. Orally-induced antibody suppression therefore differs substantially from nebulizer-induced suppression in that the former requires much higher concentrations of antigen to evoke IgE suppression and is not immunoglobulin class specific.

DISCUSSION

The present experiments demonstrate that aerosolized antigen exposure induces suppression of IgE responses to subsequent immunization with that antigen. Maximal suppression was observed when mice were exposed to nebulized antigen once weekly for at least 6 weeks. However, even one aerosolized antigen exposure was sufficient to significantly decrease the IgE response to subsequent intraperitoneal immunizations. This observed suppression was both antigen and IgE immunoglobulin class specific. Mice were very sensitive to the effect of nebulized antigen as even minute doses of antigen were effective in decreasing IgE responses.

A 3-week interval between initial nebulization and intraperitoneal immunization was required to demonstrate the IgE suppression. This would suggest that

some maturational event must occur. Following a full course of aerosol antigen exposure, suppression could be elicited for a 2–3-month period. However, if mice were boosted intraperitoneally with the antigen 3 weeks after the last nebulization, the IgE suppression persisted for up to 18 months. The intraperitoneal challenge therefore seemed to extend the tolerant state.

These experiments demonstrate that exposure of mice to nebulized antigens did not induce an IgE response. This was surprising since this is assumed to be the route by which individuals are sensitized to pollens or other airborne particles. Since a number of different strains were used in these experiments, it would be difficult to assume that they are all genetically resistant to nebulized antigens. Also, the exposure of intraperitoneally immunized mice to nebulized antigen caused a boost in IgE and IgG1 responses. This indicates that nebulized antigen is absorbed and is immunogenic. Furthermore, the mice used were capable of mounting an IgE response to an injected antigen. Whether other maturational, genetic or antigen presentation factors might be involved is unclear from these experiments. In contrast to the present experiments, rats exposed to aerosolized ovalbumin for short periods and given adjuvant intraperitoneally produced IgE responses (Van Hout & Johnson, 1972; Barboriak *et al.*, 1976). Adjuvant was essential for the induction of an IgE response.

Nebulized antigen exposure differs from the effect of introduction of antigen via the gastrointestinal tract. It has been known for some time that oral administration of antigens or haptens results in immunological unresponsiveness to subsequent immunization (Andre, Heremans, Vaerman & Cambiasco, 1975; David, 1977; Vaz, Maia, Hanson & Lynch, 1977; Ngan & Kind, 1978; Chalon, Milne & Vaerman, 1979; Hanson, Vaz, Maia, & Lynch, 1979). Similarly, in the present experiments oral feeding of ovalbumin induced tolerance to this antigen. This tolerance was not class specific and required high doses of antigen (ovalbumin at 100 μg/ml). In contrast, aerosolized antigen produced class-specific suppression and was effective at an antigen concentration three orders of magnitude lower. Therefore, the suppression following nebulized antigen cannot be explained on the basis of gastrointestinal exposure to swallowed antigen.

The present experiments demonstrate disassociation between IgE and IgG1 antibody suppression. The data from a number of studies (Ishizaka, 1976) suggest there are distinct T-helper and suppressor cells for the regulation of IgE and IgG B cells which could explain our observation. The lack of an IgE response could be due to suppression by humoral antibodies, unresponsiveness of the IgE B cells or due to suppression by T cells (Tada, 1975; Ishizaka, 1976). It is well known that antibody formation is regulated by feedback inhibition by antibodies against the same antigen. In mice, passive administration of anti-ovalbumin antibody before intraperitoneal injection suppressed both the IgE and IgG primary responses (Ishizaka & Okudaira, 1972). Antibodies have been shown to play a role in the suppression induced by orally-administered antigen (Andre *et al.*, 1975; Chalon *et al.*, 1979). Unlike the effect observed with nebulized antigen, passive antibody-mediated suppression is antigen, but not immunoglobulin class specific.

Serum factors, besides antibody, can also control the IgE response. The serum from low IgE-responder mice (e.g. SJL) primed with Freund's complete adjuvant contains a molecule which suppresses the IgE response (Katz, 1978). As this serum factor is not antigen-specific it is unlikely that it plays a role in suppressing the IgE response following aerosolized antigen. Unresponsiveness of the IgE B cells can be induced by coupling of haptens or antigens to non-immunogenic carriers (Lee & Sehon, 1978). However, this is quite different on a phenomenological level from the suppression observed with nebulized antigens. T-cell suppressor systems also have been described which are effective in controlling IgE production (Takatsu & Ishizaka, 1976; Watanabe, Kojima, Shen & Ovary, 1977). In preliminary experiments, we have been unable to to transfer aerosol antigen-induced IgE suppression with spleen cells (Fox & Siraganian, unpublished observations). Therefore it is unclear whether the aerosolized antigen-induced suppression is due to T cells. The understanding of the mechanisms for this suppression induced by aerosolized antigen could shed light on the normal controls of IgE production.

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