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Suppression of IgE responses by antigen inhalation: studies on the role of genetic and environmental factors

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SUMMARY

Repeated inhalation of low levels of ovalbumin (OVA) by mice or rats preferentially induces tolerance in the IgE antibody class, and this process may represent an important protective mechanism that normally prevents allergic sensitization to air-borne antigens. Dose–response experiments involving exposure of a number of inbred rat strains to graded doses of aerosolized OVA confirmed the inverse relationship between sensitivity to tolerogenesis and IgE-responder phenotype. These experiments additionally demonstrated that F_1 hybrids derived from low × high responder crosses co-inherited high sensitivity to tolerance induction, together with the low IgE-responder phenotype. Sensitivity to tolerance induction in low versus high IgE-responder strains was found to be independent of the route of administration of OVA, indicating that the relevant genetically determined control mechanism(s) operated systemically. However, pre-exposure of animals to a variety of exogenous agents, noteably inhaled irritants (NO_2 and histamine), the inflammatory adjuvants pertussigen and aluminium hydroxide injected at sites that stimulate the regional lymph nodes draining the respiratory tract, or a single subcutaneous injection of the reticuloendothelial system stimulator oestradiol, were shown to partially abrogate this natural tolerance process and promote allergic sensitization.

INTRODUCTION

Recent studies from this laboratory have shown that antigenic stimulation of the respiratory mucosa by passive inhalation of antigen aerosols induces systemic immunological tolerance that is antigen-specific and is preferentially expressed against the IgE antibody isotype (Holt, Batty & Turner, 1981; Holt & Leivers, 1982; Sedgwick & Holt, 1983, 1984).

Tolerance induction in this model is associated with the development of IgE isotype-specific suppressor cells in the regional lymph nodes draining the upper respiratory tract and the oronasal cavity (Sedgwick & Holt, 1985). In the mouse these cells are Thy 1.2^+ T lymphocyts (Holt & Leivers, 1982), while in the rat they express the $W3/23^+$, MRC $OX8^+$ surface phenotype (Sedgwick & Holt, 1985) characteristic of the suppressor/cytotoxic subset in this species (Brideau *et al.*, 1980).

Sensitivity to tolerance induction appears to be related to IgE-responder phenotype. This conclusion stems from comparative dose-response experiments with high and low IgE-responder rat strains. These studies revealed 1000-fold differences in sensitivity to tolerogenesis, high IgE-responders requiring repeated microgram antigen doses, in contrast to low IgE-responders which become tolerant in response to inhalation of nanogram amounts of antigen (Sedgwick & Holt, 1984).

The present experiments extend these observations to low versus high IgE-responder mice, and additionally document a range of exogenous environmental factors that modulate the tolerance induction process.

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MATERIALS AND METHODS

Animals

Inbred adult rats of the BN, WAG and Lou/M strains and mice of the BALB/c and SJL strains were used in these experiments. All animals were supplied SPF from the Animal Resource Centre, Murdoch University, and maintained under barrier conditions. Sprague–Dawley rats were used as serum recipients in bioassays for IgE (see below). SPF animals were used in all but one series of experiments, which employed conventional (Conv) Lou/M rats supplied by the University of Western Australia Preclinical Animal Breeding Unit.

Antigen exposure and immunization

The antigen employed in these experiments was ovalbumin (OVA; Grade V, Sigma Chemicals, St Louis, MO). Aerosols were generated from a solution of OVA dissolved in phosphate-buffered saline (PBS) at 1% w/v (except where specified) and

piped into a 30-cm³ plexiglass exposure chamber as detailed previously (Holt *et al.*, 1981). Exposure periods lasted 7 min at a frequency of one per week. For parenteral immunization, OVA was administered together with the adjuvant aluminium hydroxide (AH, Wyeth Pharmaceuticals, Paramatta, Australia); for rats AH-OVA doses of 10·0 mg/100 μg were used, and for mice the doses were 4·0 mg/10μg.

In some experiments, OVA was administered in drinking water or by intravenous (i.v.) inoculation via the tail vein; relevant dosages are shown in the figure legends.

Administration of inflammatory stimulants

A variety of agents with known irritant activity were systematically tested for the capacity to modulate the tolerance induction process. Preliminary dose–response experiments were initially performed to determine the respective working ranges for these agents; data below are only considered over the active dose ranges.

Pertussigen (PN), purified from the culture supernatant of the Tohoma 1 strain of *Bordetella pertussis* (Arai & Munoz, 1981), was generously provided by Dr J. Munoz. Lyophilized PN was dissolved in alanine/Tris buffer, and 1·0 μg in 1·0 ml buffer was administered to rats subcutaneously (s.c.) in the head region, or intraperitoneally (i.p.). Freund's complete adjuvant (FCA) was emulsified with an equal volume of PBS; rats received 0·5 ml via the i.p. route. AH suspended in PBS was administered at doses of 10 mg per rat, either i.p. or distributed between three s.c. sites in the head region above the jaw line.

Oestradiol (the benzoate salt; Sigma Chemicals) was suspended in sterile olive oil and inoculated s.c. in the backs of mice as per Mowat & Parrott (1983), at doses of 2·0 mg in 0·2 ml oil.

Histamine was administered via aerosol to some animals directly prior to antigen exposure. The same nebulizer and inhalation chamber that were used for antigen exposures were employed here; a solution of 10·0 mg/ml histamine in PBS was aerosolized for 7 min, and then replaced by 1% OVA in PBS for the antigen exposure. In experiments involving NO_2 exposure, the gas was diluted in medical air to 50 p.p.m. and piped at approximately 2 l/min into the inhalation chamber; animals were exposed to NO_2 for 30 min prior to antigen exposure.

Determination of serum IgE and IgG titres

Levels of circulating anti-OVA IgG were determined by the haemagglutination (HA) assay, as detailed in Holt *et al.* (1981). For IgE determinations, the passive cutaneous anaphylaxis (PCA) bioassay of Ovary (1964) was used; PCA recipients were Sprague–Dawley rats, and latent periods of 24 and 48 hr were used for mouse and rat sera, respectively, in order to exclude contributions from anaphylactic IgG. Except where specified, bleed times of Day 10 and Day 21 post-challenge were employed for rats and mice, respectively, being the peak of the primary reponse as determined in preliminary experiments. Data are expressed as PCA or HA units, 1 unit = $\log_2$ reciprocal serum titre.

Induction of immediate hypersensitivity in vivo

Animals were challenged i.v. with 1·0 mg OVA in PBS, and the severity of resultant anaphylactic shock reactions was graded on the basis of recovery times, as previously described (Turner, Fisher & Holt, 1982).

RESULTS

IgE-responder phenotype of test animals

Primary IgE responses in the strains employed in this study are illustrated in Fig. 1. These responses were elicited under optimal conditions of parenteral stimulation, as determined in earlier experiments. With the exception of the BN, all rat strains were within the low-responder range, manifesting primary IgE titres < 7 PCA units, which did not persist beyond 20 days. The high-responder BN rats, in contrast, displayed persistent primary IgE responses of up to 14 PCA units, which did not decline over a 60-day observation period. Comparable results were obtained with high IgE-responder BALB/c mice, in contrast to the low and transient IgE responses in the SJL strain.

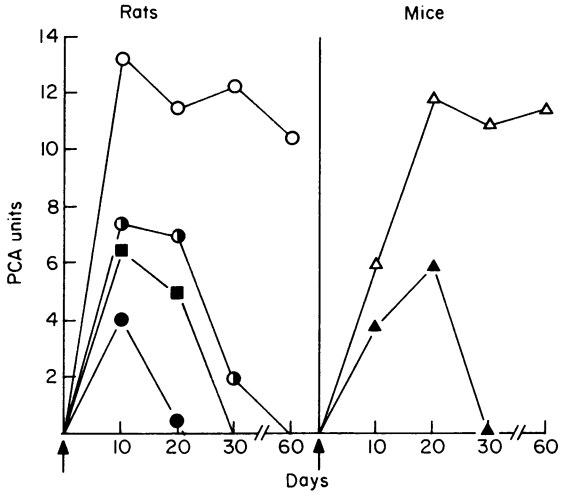

Figure 1. IgE-responder phenotype of animals employed in this study. Rats and mice were inoculated i.p. with 100 or 10 μg OVA, respectively, together with AH adjuvant on Day 0 (designated by arrow). Data shown are mean PCA titres derived from groups of six animals. (○) BN rats; (●) WAG rats; (◑) BN × WAG (F_1) rats; (■) Lou/M rats; (△) BALB/c mice; (▲) SJL mice.

Tolerance induction via different routes

The experiments of Fig. 2a confirm our earlier observations on the relationship between IgE-responder phenotype and susceptibility to tolerance induction. In this study, groups of high-responder (BN) and low-responder (WAG) rats were repeatedly exposed to aerosols derived from OVA solutions of 1·0–0·0001% w/v. After six exposures, the rats were challenged i.p. with OVA, and peak primary IgE responses determined. The results of this trial, confirmed in a follow-up study (not shown), indicate that the high IgE-responder animals require 100–1000-fold more antigen than their low-responder counterparts to induce tolerance. The dose–response curve for F_1 hybrids derived from these strains was comparable to that of the WAG parent.

In Panel 2b, comparative dose–response experiments (similar to those in 2a) were performed to determine the relative threshold for tolerance induction in these strains via routes other than antigen inhalation. It can be seen that the WAG

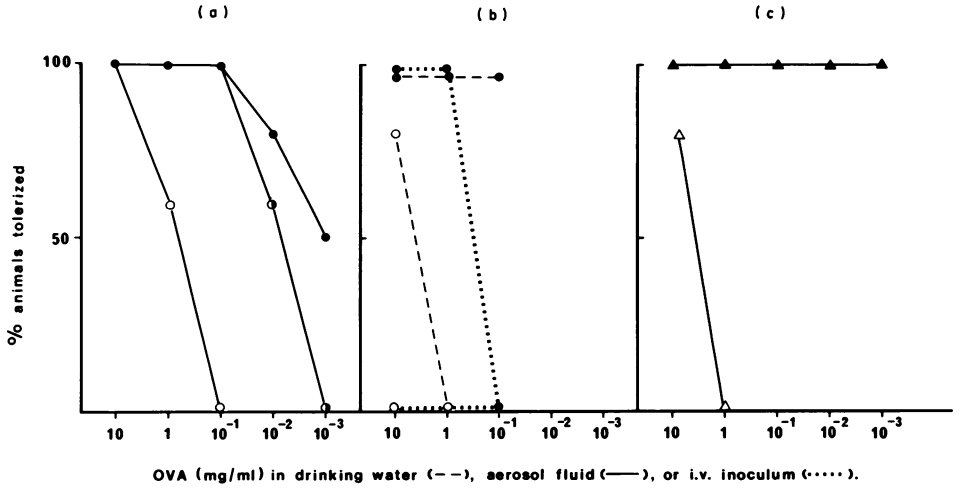

Figure 2. Variations in sensitivity to tolerance induction. Groups of six to eight animals were repeatedly exposed to OVA, either by aerosol (once weekly for 6 weeks; Panels a and c), in drinking water (continuously for 1 week; Panel b), or intravenously (3 doses, 2 days apart; Panel b), prior to i.p. challenge with AH-OVA 5 days after the final exposure, together with unexposed, age-matched syngeneic controls. The IgE titres for individual OVA-exposed animals were compared to group means for their respective unexposed controls, and scored as 'tolerant' if their titres were reduced ≥ four-fold (i.e. ≤25% of control). (○) BN rats; (●) WAG rats; (◑) BN × WAG (f_1) rats; (■) Lou/M rats; (△) BALB/c mice; (▲) SJL mice.

strain required 100–1000-fold less OVA for tolerance induction, relative to the high-responder BNs, regardless of the route of administration.

The experiments of Panel 2c extend these observations to mice and clearly demonstrate that the low IgE-responder SJL strain requires at least 1000-fold less inhaled OVA than high responder BALB/c mice to induce tolerance in the IgE antibody class.

Effect of air-borne irritants

The experiments of Fig. 3 examine the immunological effects of exposure of high IgE-responder animals to air-borne irritants, at or around the time of antigen inhalation.

BN rats and BALB/c mice were exposed once weekly to aerosols of PBS (controls) or tolerogenic doses (1% w/v) of aerosolized OVA, some animals receiving pre-exposure to 50 p.p.m. NO_2. Subsequent parenteral challenge confirmed that OVA exposure alone abrogated IgE responsiveness in these animals (group median titres ≤ 1 PCA unit), while pre-exposure to NO_2 consistently effected a reversal of this tolerance process (group medians approximately 5 PCA units). Comparable results were obtained in several experiments. Data obtained from pre-exposure to histamine aerosols were more variable; in the experiment illustrated here, 2/6 animals failed to develop tolerance as a result of histamine exposure—in two follow-up experiments, figures of 0/6 and 1/6 were obtained.

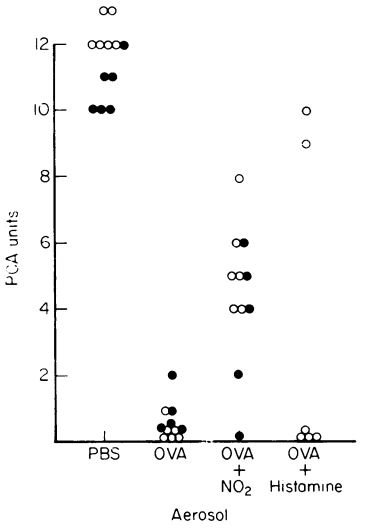

Figure 3. Effect of pre-exposure to aerosols containing NO_2 or histamine, on induction of tolerance to inhaled antigen. BN rats (○) and BALB/c mice (●) were pre-exposed weekly to 50 p.p.m. NO_2 or an aerosol of 10 mg/ml histamine, prior to inhalation of a 1% OVA aerosol. Data shown are individual peak primary PCA titres following subsequent parenteral challenge with AH-OVA, compared to unexposed controls. Preliminary experiments (not shown) established that NO_2 or histamine administrated via this protocol did not *per se* affect IgE responses.

Effect of inflammatory agents administered parenterally

In Table 1 high IgE-responder BN rats were exposed to an aerosol of OVA (Group 1) or PBS (Group 7) once weekly for 6 weeks. They were bled 5 days after the third and sixth exposure, and then challenged i.p. with AH-OVA and finally bled 10 days later. Some of the animals were pretreated with adjuvant(s) (Groups 2–6), as indicated in the legend.

As shown in an earlier study (Sedgwick & Holt, 1983), BN rats developed transient antigen-specific IgE responses during the course of aerosol exposure, which spontaneously switched off by the sixth exposure week (Group 1), and subsequent parenteral challenge with AH-OVA failed to recall the IgE response (compare Groups 1 and 7). Pretreatment of the animals with the adjuvants PN or AH (Groups 2, 3, 5, and 6)

Table 1. Stimulation of respiratory tract regional lymph nodes during aerosol exposure by administration of inflammatory adjuvants: effect on tolerogenesis in BN rats

Exposure groups	Sample time		
	Week 3 of exposure	Week 6 of exposure	Post-challenge
(1) OVA aerosol	2·9±0·2 (8·7±0·3)	<1 (11·3±0·3)	1·2±0·4 (13·5±0·2)
(2) OVA aerosol+AH (i.p.) Day 0	4·0±1·1 (12·6±1·3)*	2·1±0·6 (12·9±0·7)	<1 (14·9±0·4)
(3) OVA aerosol+PN (i.p.) Day 0	6·3±0·6* (12·3±0·4)*	4·3±1·5* (11·5±0·5)	7·5±2·5* (14·3±1·1)
(4) OVA aerosol+FCA (i.p.) Day 0	1·5±0·8 (6·2±1·1)	<1 (9·8±1·3)	<1 (9·8±2·8)
(5) OVA aerosol+AH (s.c; head) Day 0	2·5±1·6 (6·8±1·3)	5·0±0·4* (10·3±0·8)	1·8±0·9 (12·3±0·9)
(6) OVA aerosol+PN (s.c; head) Day 0	6·3±0·2* (10·5±0·3)*	3·8±1·3* (13·9±0·4)*	6·8±2·2* (14·6±0·6)
(7) PBS aerosol	<1 <1	<1 <1	10·3±0·3* (8·3±0·3)

Data are mean PCA titres±SE (HA titres in parentheses) from groups of six BN rats. Doses of adjuvants were as follows: PN, 1·0 μg; FCA, 0·5 ml; and AH, 10 mg.

*Significantly ($P<0·01$–$0·001$) greater than the OVA-aerosol group, by variance analysis.

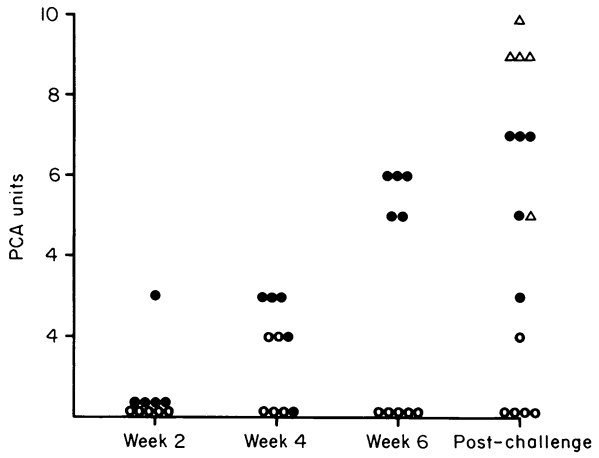

Figure 4. Effect of oestradiol administration on tolerance induction. BALB/c mice were given a single s.c. inoculation of PBS (○) or 2·0 mg oestradiol (●), prior to the commencement of a 6-week 1% OVA-aerosol exposure regime. Data shown are individual IgE titres exposure, and after subsequent parenteral challenge with AH-OVA, compared to unexposed syngeneic controls (△).

increased the magnitude of the transient IgE response, and in the case of PN (Groups 3 and 6) prevented the development of tolerance. These agents also stimulated the development of specific IgG responses in the aerosol-exposed rats. In contrast, the adjuvant FCA exhibited no stimulatory effects on OVA-specific antibody responses.

Oestradiol

Groups of high IgE-responder BALB/c mice were exposed once weekly to an aerosol of 1·0% OVA, bled fortnightly, and then challenged i.p. with AH-OVA. IgE titres were determined 21 days later.

In the experiment shown in Fig. 4, the OVA-exposed group displayed transient IgE responses at Week 4 of exposure, which switched off by Week 6, and subsequent parenteral challenge of these mice revealed tolerance. Pretreatment of animals with oestradiol abrogated tolerance induction, and instead promoted the development of OVA-specific IgE responses.

Gut flora

In the early stages of these experiments a change in our animal suppliers necessitated a switch to the exclusive use of SPF (as opposed to Conv) rats.

The SPFs are maintained under strict barrier conditions and, in contrast to the Conv animals, have an essentially Gram-positive bacterial flora in the gut. A chance observation with Lou/M rats, reported in Table 2 and confirmed in subsequent experiments (not shown), suggests that the state of the gut flora

Table 2. Tolerance induction in SPF and Conv Lou/M rats

Bacteriological status	Aerosol	Serum titres	
		IgE	IgG
Conv	OVA	2·3±0·3*	3·1±1·2*
	PBS	6·9±0·4	12·2±1·7
SPF	OVA	1·4±0·2*	11·9±2·7
	PBS	7·7±0·9	11·3±0·9

Data are mean PCA or HA titres (±SE) from groups of six rats at 10(IgE) or 21(IgG) days after i.p. challenge.
*Significantly less than controls, $P<0·01$–$0·001$.

in these animals may influence their susceptibility to tolerance induction in this system.

In the experiment illustrated, two groups of age-matched male Lou/M rats raised under SPF or Conv conditions were repeatedly exposed to aerosols comprising PBS or OVA in PBS, prior to parenteral challenge with AH-OVA. In the SPF animals, exposure to the aerosol selectively suppressed responses in the IgE antibody class, whereas an identical aerosol exposure protocol with Conv rats of this strain resulted in suppression of both the IgE and IgG isotypes. Subsequent experiments comparing SPF and Conv high IgE-responder BN rats (not shown) indicated that the state of the host bacterial flora in this strain was without detectable effect upon sensitivity to tolerance induction.

Immediate hypersensitivity *in vivo*

During the course of these experiments, groups of animals exhibiting serum IgE titres between 1 and 10 PCA units were

challenged i.v. with OVA, and the duration of their anaphylactic shock reactions was recorded. No reactions or equivocal reactions were recorded in mice and rats with titres ≤ 3 PCA units. All animals with titres ≥ 4 PCA units exhibited shock reactions of at least 20 min duration (data not shown), whch is consistent with previous observations (Turner *et al.*, 1982).

DISCUSSION

Earlier dose–response experiments indicated that low-IgE-responder WAG rats required 1000-fold less inhaled antigen than high responder BNs for the induction of tolerance in the IgE antibody class (Sedgwick & Holt, 1984). The finding is confirmed in Fig. 2a, and a similar phenomenon is shown to occur in mice (Fig. 2c). In addition, the relativity between high and low IgE-responders in regard to sensitivity to tolerance induction is clearly maintained, regardless of the route of antigen administration (Fig. 2b). The data of Fig. 2a also indicate that high susceptibility to this form of tolerance induction is inherited in F_1 hybrid rats together with low IgE-responder phenotype. Collectively, these observations suggest that genetic factors, operative systemically, primarily determine the sensitivity of the IgE system to inhaled antigen.

Notwithstanding these data, a variety of environmental factors, including some that function locally in the respiratory tract and others that operate systemically, appear to be capable of modulating these genetic regulatory mechanisms. The data of Fig. 3 demonstrate that pre-exposure of high IgE-responder rats and mice to NO_2 immediately prior to antigen inhalation abrogates tolerance induction and promotes the subsequent development of IgE responses. These latter responses were (median) 5 log PCA units, compared to < 1 unit in the groups exposed to OVA alone. However, IgE responses in non-OVA-exposed controls were 12 PCA units, which indicates that IgE production was substantially reduced even in the presence of NO_2. This degree of reduction was nevertheless insufficient to protect the animals against allergic sensitization, as serum levels of OVA-specific IgE equivalent to 4 PCA units render animals sensitive to i.v. challenge with the antigen (see Results).

In preliminary experiments we noted that pre-exposure of OVA-immune animals to levels of NO_2 as low as 25 p.p.m., immediately prior to challenge with a 1% OVA aerosol, markedly increased the magnitude of ensuing secondary IgE and IgG anti-OVA responses. It has also been reported earlier that NO_2 in this dose range promotes widening of trans-epithelial junctions in the respiratory tract, thus stimulating antigen uptake (Ranga, Kleinman & Collins, 1980). Consequently, it appears likely that the effects of NO_2 on antibody production in this system may involve quantitative (and perhaps qualitative) changes in antigen processing following deposition on the respiratory mucosa.

The mechanism(s) underlying the variable stimulatory effects of histamine aerosols on allergic sensitization in this model remain to be elucidated, and may involve altered antigen absorption/transport resulting from increased vascular permeability, or alternatively may reflect changes in the activity of regulatory T cells bearing histamine receptors (e.g. see Rocklin, 1977). It has yet to be established whether histamine released endogenously in the respiratory tract, e.g. via local mast cell activation, exerts similar effects.

It has been noted (Katz, 1980; Ishizaka, 1984) that certain inflammatory adjuvants such as PN and AH, but not FCA, selectively stimulate IgE responses, and the experiments reported in Table 1 indicate that pretreatment of animals with these agents modulates the tolerogenic activity of inhaled antigen. Thus, pre-inoculation with PN or AH (but not FCA) increases the magnitude of the (normally transient) IgE response that occurs during the early phase of OVA-aerosol exposure, and in the case of PN prevents the induction of tolerance in the IgE class. The adjuvants employed in Table 1 were inoculated either s.c. in the head region (lymphatic drainage to the superficial cervical nodes, which also drain the oronasal cavity—see Tilney, 1971), or i.p. (drainage to parathymic and posterior mediastinal nodes which also service the lung), and all three agents induced overt hypertrophy of respective regional lymph nodes. These nodes have previously been identified as the initial sites of activation of IgE isotype-specific suppressor T cells during tolerogenesis in this model (Sedgwick & Holt, 1985).

PN may function at two different levels in this system. Firstly, parenteral administration of this agent increases vascular permeability in a variety of tissues, and its efficiency as an adjuvant in experiments involving breakdown of tolerance mechanisms has been linked to this activity (Linthicum, Munoz & Blaskett, 1982). Secondly, it has been postulated to stimulate IgE T-helper cells via mechanism(s) that involve the elaboration of soluble factors from macrophages (Hirashima, Yodoi & Ishizaka, 1981; Ishizaka, 1984). The mode of action of AH in this system may also involve macrophages, either via stimulating the release of macrophage-derived factors analogous to those stimulated by PN, which act upon IgE-regulatory T cells (Ishizaka, 1984), or via alterations in antigen processing (Nakano, 1976).

Support for the notion that changes in macrophage function may affect tolerance induction in this system is provided by the data of Fig. 4, which demonstrate that inoculation of oestradiol also prevents tolerance induction in this model. These experiments were based upon the ability of oestradiol to stimulate reticuloendothelial system (RES) function (Slijvic, Clark & Warr, 1975), and employed a dose regime shown previously (Mowat & Parrott, 1983) to abrogate tolerance induction in the same strain of mice (BALB/c) to fed OVA.

However, this hormone has also been shown to exert direct effects upon lymphocytes (Slijivic *et al.* 1975; Cohn, 1979; Kittas & Henry, 1980). While Mowat & Parrott (1983) have provided evidence that suggests that these latter effects are not central to oestradiol-mediated changes in oral tolerogenesis, such a possibility must still be considered in the context of the present model. We plan further experiments with more selective RES-modifying agents to probe this question further.

The final environmental factor considered in this study was the status of the host gastrointestinal (GIT) microflora. Table 2 demonstrates that the acquisition of a Conv flora influences the selectivity of tolerance in low–moderate IgE-responder rats. A precedent for this finding exists in the oral tolerance literature (e.g. see Wannenmuehler *et al.*, 1982), wherein lipopolysaccharide (LPS) from Gram-negative organisms, which comprise the bulk of Conv GIT microflora, has been implicated as an important factor in the maturation of mucosal regulatory (in particular, suppressor) T cells. Experiments are in progress in our laboratory to test the effects of endogenous LPS on the maturation of suppressor T cells, which are involved in the

induction of tolerance to antigens impinging on the respiratory mucosa.

In summary, the natural response to passive inhalation of antigen is the induction of immunological tolerance, particularly in the IgE antibody class, and this may represent an important mechanism that normally protects against allergic sensitization to non-pathogenic antigens. Sensitivity to this form of tolerance induction is controlled by genetic factors that operate systemically via mechanism(s) which appear closely related to those that determine overall IgE-responder phenotype. A variety of agents that trigger inflammatory responses at the level of respiratory mucosa, or which are capable of modifying immune/inflammatory cell function(s) either systemically or in the regional lymph nodes draining the respiratory tract, can modulate this natural process. These include chemical irritants such as NO_2 and AH, endogenous pharmacological agents such as histamine and oestradiol, and bacterial products from pathogens such as *Bordetella pertussis* as well as from the natural commensal flora of the GIT. Elucidation of the mechanism(s) underlying the stimulatory activity of these agents may provide insight into 'risk factors' associated with the expression of the allergic phenotype.

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