Immunology 1983 **50** 625

Induction of IgE-isotype specific tolerance by passive antigenic stimulation of the respiratory mucosa

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Accepted for publication 6 July 1983

Summary. Repeated exposure of high IgE-responder BN rats to an aerosol of ovalbumin (OVA) induced tolerance which was specific both for the antigen and the IgE antibody class. Radiotracer studies with ^{125}I-OVA indicated that inhaled antigen doses in the low microgram range were tolerogenic, the bulk of the inhaled antigen being distributed 2:1 between the gastro-intestinal and respiratory tracts. Subsequent gastric intubation studies showed that the gastric component did not contribute to tolerance induction, suggesting that antigenic stimulation of the respiratory mucosa was central to this process.

INTRODUCTION

While IgE synthesis in response to passive antigenic stimulation of the respiratory mucosa is a well recognized clinical phenomenon, there is a paucity of information on the mechanism(s) which control it.

Abbreviations: OVA, ovalbumin; AH, aluminium hydroxide; AH-OVA, ovalbumin plus aluminium hydroxide; PBS, phosphate-buffered saline; i.p., intraperitoneal; i.g., intragastric; PCA, passive cutaneous anaphylaxis; HA, haemagglutination; SEM, standard error of the mean.

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There are few relevant studies on experimental antigen inhalation, the majority of which rely on the concurrent administration of adjuvants to trigger IgE responses (Van Hout & Johnson, 1972; Ishii *et al.*, 1973; O'Donnell & Mitchell, 1978; Bazin & Pauwels, 1982). The use of artificial stimulants in this situation appears to mask a potentially important endogenous regulatory mechanism, which has only recently been revealed in experiments which involve the passive exposure of animals to antigen aerosols (Holt, Batty & Turner, 1981a; Quetsch & Richerson, 1977).

These studies have shown that aerosol exposure of immunologically naive mice and guinea-pigs (in the absence of adjuvant) results, not in sensitization, but in suppression of IgE responsiveness to the antigen inhaled. This state of induced tolerance was apparent following parenteral challenge of the aerosol-exposed animals, it was confined to the IgE antibody class (Holt *et al.*, 1981a; Holt & Leivers, 1982), it was mediated by isotype-specific regulatory T cells (Holt & Leivers, 1982) and was also demonstrable employing allergens such as ragweed (Fox & Siraganian, 1981). In contrast, preliminary experiments with low IgE-responder rats demonstrated that both IgE and IgG were suppressed by an aerosol regime identical to that which induced IgE-specific tolerance in mice (Holt & Leivers, 1982). The present study extends these observations to high IgE-responder rats, and in particular focusses upon the relative importance of gut versus

lung-associated antigen in aerosol-exposed animals, in relation to tolerance induction.

MATERIALS AND METHODS

Animals

Conventionally raised rats of the Wistar (WAG) and Brown Norway (BN) inbred strains were obtained from the University of Western Australia and maintained on a diet of acidified water and OVA-free food pellets supplied *ad libitum*. All animals were aged between 2 and 4 months at the outset of experiments. Male BN rats were used exclusively for all experimental work whilst WAG rats of both sexes were employed for PCA reactions (see below).

Antigen exposure

Inhalation. The procedures for aerosol administration have been described previously (Holt *et al.*, 1981a). Briefly, rats were exposed for 7 min once weekly, in a plexiglass chamber (30 cm cubed), to aerosolized OVA (Grade V, Sigma Chemical Company, U.S.A.) in PBS. OVA solutions of 1·0% (w/v) were used (referred to as 1% OVA aerosol), except where otherwise stated. The aerosol was generated employing an Inspiron Mini-Neb Nebulizer (C.R. Bard Inc.) operated at 10·0 p.s.i. by medical grade air. This nebulizer generates an aerosol with a mean droplet size between 3 and 5 μm.

Other routes. Parenteral challenge of rats via the intraperitoneal (i.p.) route with OVA employed 100 μg OVA plus 10·0 mg aluminium hydroxide (AH; Amphojel, Wyeth Pharmaceuticals, Australia) in 0·5 ml PBS (AH-OVA).

Intragastric (i.g.) challenge was performed under light anaesthesia. The tip of a 23-cm (2·1 mm outside diameter) polyethylene tube (Dural Plastics, Australia) was introduced into the stomach perorally, and 0·5 ml of OVA solution inoculated by means of an attached syringe. Control animals received 0·5 ml PBS in the same manner.

Determination of anti-OVA antibody titres

Passive cutaneous anaphylaxis (PCA) titrations. The levels of circulating anti-OVA homocytotropic (IgE) antibody were assayed by PCA (Ovary, 1964). Briefly, serum samples were diluted in PBS, and 50-μl aliquots inoculated intradermally into the shaved backs of male or female WAG rats at marked sites. Forty-eight hours later the rats were challenged intravenously with 4·0 mg OVA in 0·9 ml 0·5% Evans Blue (Gurr). Thirty minutes later the rats were killed and the relevant skin areas examined. The reciprocal of the highest serum dilution giving a blue spot greater than 5 mm in diameter was taken as the PCA titre.

Haemagglutinating antibody. The levels of circulating anti-OVA IgG antibody were determined using the haemagglutination (HA) assay as previously described (Holt *et al.*, 1981a; Price & Bell, 1975), but with some slight modifications. All serum samples were pre-incubated for 1 hr at 20° in 0·1 M 2-mercaptoethanol (2-Me) then doubly diluted in PBS containing 0·01 M 2-Me using V-bottomed microtitre plates (Cooke Engineering Co., U.S.A.). OVA-coated sheep erythrocytes were then added and the reciprocal of the highest dilution exhibiting obvious agglutination after 2 hr at 4° was taken as the HA titre. This procedure has been shown to remove 2-Me sensitive (IgM) antibody and give antibody titres comparable with those using an OVA-specific enzyme-linked immunosorbent assay employing Fc-specific anti-rat IgG antisera (unpublished results).

Radiotracer studies

BN rats were exposed for 7 min to aerosolized ^{125}I-OVA (containing $< 1\%$ free iodine), as a 1% solution in PBS. At varying times after exposure, the rats were killed, their internal organs dissected out, and the amount of radiation in the latter determined in a Packard Gamma Spectrometer. Figure for blood content were calculated by assuming a total blood volume of 13 ml per animal.

Statistical analysis

All data are presented as the geometric mean ($\pm$SEM) of individual HA or PCA titres. A two-tailed Student's *t*-test was used to determine the significance of difference between experimental groups. For such calculations, where no antibody was detectable, the sera were designated a value of 1·0. If all animals in a group had undetectable serum antibody, however, the means were recorded as less than the lowest dilution tested (e.g. < 10).

RESULTS

Weekly exposure of BN rats to a 1·0% OVA aerosol induced a transient anti-OVA IgE response in the

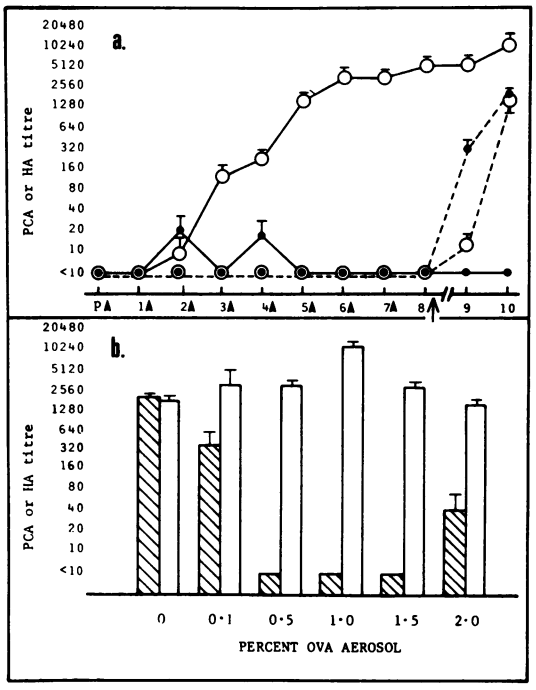

Figure 1. Tolerance induction by repeated exposure to aerosolized antigen: variations in antigen concentration in aerosol.

(a) Two groups of five BN rats were pre-bled (P), then exposed to an aerosol of 1·0% OVA (*solid lines*), or PBS (*broken lines*), once weekly for 8 consecutive weeks (▲). The animals were bled 5 days after each exposure, corresponding to numbers 1–8. One week after bleed 8 (12 days after the final exposure), all animals were challenged i.p. with AH-OVA (↑), then bled 7 and 14 days later (bleeds 9 and 10). Serum IgE (●) and IgG (○) titres were determined, each point representing the arithmetic mean (+SEM) of five single determinations.

(b) Following the protocol outlined above, groups of five BN rats were exposed to 8, weekly aerosols of either PBS, or 0·1, 0·5, 1·0, 1·5 or 2·0% OVA solutions. Twelve days after the final exposure, all animals were challenged i.p. with AH-OVA and their IgE (▨) and IgG (□) titres determined 14 days later. Each bar represents the arithmetic mean (+SEM) of five single determinations.

majority of animals, between the second and fourth weeks. Anti-OVA IgG levels, however, continued to increase over the course of aerosolization (Fig. 1a). Following parenteral challenge with AH-OVA, the aerosol-exposed rats demonstrated increasing IgG levels but markedly suppressed anti-OVA IgE titres compared to control animals (exposed to PBS aerosol). As shown previously with OVA-exposed mice (Holt *et al.*, 1981a), suppression of IgE synthesis in aerosol exposed rats appeared antigen-specific, as parenteral challenge with an irrelevant antigen (Ascaris protein) produced normal responses (data not shown).

When the concentration of OVA employed in the weekly aerosol exposure was varied (Fig. 1b), subsequent IgE responsiveness was significantly suppressed in each case (0·5–2·0%, $P<0·001$; 0·1%, $P<0·01$), whilst IgG responses were either not significantly different to control levels or higher (1·0%, $P<0·02$). Subsequent experiments in this study were restricted to the use of 0·5 or 1·0% OVA.

Table 1 examines the fate of inhaled ^{125}I-OVA in BN rats exposed to a 1·0% OVA aerosol. An average of 65·6 μg ^{125}I-OVA was recovered from each animal. At 30 min after exposure, approximately 23 μg of the OVA (equivalent to 30% of the recovered antigen load) was associated with the respiratory tract, the bulk of the remainder being found in the gastrointestinal tract.

After 3 hr, the total antigen load had not altered significantly although the percentage associated with the respiratory tract declined slightly over the period, perhaps reflecting slow clearance to the gut.

The recent literature indicates that antigen exposure via the gut is an effective means of tolerance induction (Chiller, Titus & Etlinger, 1979). As up to 65% of the whole bodyload of inhaled OVA becomes associated with the gastrointestinal tract within 30 min after aerosol exposure (Table 1), it was conceivable that this component played an important role in the subsequent induction of tolerance. In the experiments of Fig. 2, comparisons are drawn between animals repeatedly exposed to aerosolized 0·5% OVA (total body load 35 μg OVA, estimated from Table 1), those receiving 23 μg OVA (viz., 65% of the latter dosage) via i.g. intubation, and those receiving 12 μg OVA i.p. (the remaining 35% of the dosage).

It is evident that this level of i.g. OVA exposure does not induce tolerance, a finding consistent with other observations indicating the necessity of milligram doses of this and other antigens for tolerogenesis via the gut (Miller & Hanson, 1979; Thomas & Parrott, 1974). In contrast, the smaller i.p. dosage stimulated a steadily increasing IgG response, and evoked a 'persistent' IgE response, comparable to that previously observed in the mouse (Vaz, Vaz & Levine, 1971), which was not boostable by parenteral challenge. Additionally, simultaneous administration of both the i.p. and i.g. antigen doses to animals, produced results identical to those observed with i.p. antigen alone (not shown).

Table 1. Distribution of ^{125}I-OVA in organs of BN rats after a 1% ^{125}I-OVA aerosol exposure

Time after aerosol exposure	^{125}I-OVA (μg) recovered in: Lung	Trachea/ larynx	Stomach + oesophagus	Small intestine	Large intestine	Spleen	Kidney	Liver	Blood	Total ^{125}I-OVA recovered (μg)
30 min	22	0·25	32·6	13·1	0·6	0·03	0·69	1·37	1·6	72
60 min	21·8	1·76	12·3	20·6	1·7	0·1	1·5	2·5	9·8	72
105 min	12·5	1·2	12·7	16·7	1·5	0·05	1·4	1·9	7·7	56
150 min	9·9	2·6	18·0	11·7	5·3	0·11	1·1	2·2	3·2	54
180 min	15·9	6·7	18·4	7·3	10·7	0·25	6·5	2·6	6·0	74
Mean	16·4	2·5	18·8	13·8	4·0	0·1	2·2	2·1	5·6	65·6
% total	(25·0)	(3·8)	(28·7)	(21·0)	(6·1)	(0·2)	(3·4)	(3·2)	(8·6)	(100)

Five BN rats weighing between 210 and 250 grams were exposed for 7 min to an aerosolized 1·0% OVA solution of ^{125}I-OVA, then killed at the times shown. Organs were excised and counted in a Gamma spectrometer. Data shown are ^{125}I-OVA (μg) recovered per organ. Data in parentheses reflect percent of total ^{125}I-OVA recovered.

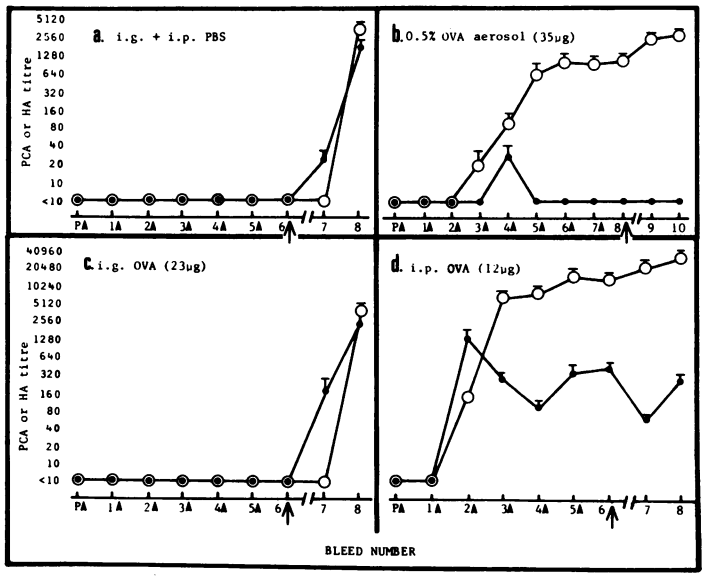

Figure 2. Lack of tolerance in rats following multiple low dose intragastric and intraperitoneal OVA administration. Four groups of five BN rats were repeatedly exposed (↑) to an aerosol of 0·5% OVA in PBS (b), or to a portion of the latter dosage via a single route as shown in (c) and (d). All animals, together with PBS-exposed controls (a), were challenged i.p. with AH-OVA (↑) as per Fig. 1a and bled for determination of IgE (●) and IgG (○) anti-OVA antibody 7 and 14 days later. Each point represents the arithmetic mean (+SEM) of five single determinations.

In the course of these studies, no overt animal discomfort was observed following antigen exposure. Only trasnsient IgE responses occurred as a direct result of aerosol exposure, followed by an inability to mount further IgE responses to the same antigen. This phenomenon is clearly distinct from the asthma-like syndrome observed in pre-sensitized rats exposed to protein aerosols (Piechuta *et al.*, 1979; Carswell & Oliver, 1978).

DISCUSSION

Exposure of BN rats to low levels of OVA by aerosol induces transient IgE synthesis, accompanied by steadily increasing IgG levels. After completion of the aerosol exposure regime, parenteral challenge of these animals with OVA revealed a state of selective unresponsiveness, apparently restricted to the IgE antibody class. The dose–response experiments of Fig. 1 indicate that OVA levels in the aerosol as low as 0·1% w/v were effective in this regard, suggesting (by extrapolation from Table 1) that inhaled OVA dosages as low as 6 μg OVA per exposure were tolerogenic via this route. It is likely that even smaller doses could be used successfully with additional aerosol exposures, in the light of evidence that the effectiveness of tolerance induction in this model is also directly related to exposure frequency (unpublished observations).

It is clear from earlier studies in the rat (Van Hout & Johnson, 1972), rabbit (Willoughby & Willoughby, 1977) and mouse (Holt *et al.*, 1981a), that a large proportion of inhaled antigen is rapidly translocated to the gastrointestinal tract, and the results of Table 1 indicate comparable antigen distribution in the present model. In view of the well established tolerogenic effects of i.g. administration of antigen (Chiller *et al.*, 1979), the possible contribution of gut-associated OVA in aerosol-exposed rats was examined directly.

The experiments of Fig. 2 unequivocally demonstrate that the levels of OVA reaching the gut following aerosol exposure in this model do not induce tolerance. In addition this dose of antigen does not trigger IgE and IgG production during administration, nor does it prime the animals for a secondary response following i.p. challenge (see Fig. 2c).

In preliminary experiments involving tracheal intubation, attempts to administer antigen exclusively to the lung without subsequent translocation to the gut were unsuccessful. Therefore, as a compromise, an OVA dosage equivalent to the respiratory tract component was administered to rats i.p. This procedure triggered significant response in both the IgE and IgG classes (Fig. 2d). The IgE response was unusual in that it became cyclical and persistent, comparable to that reported previously in the mouse (Vaz *et al.*, 1971; Holt *et al.*, 1981b). The IgE response in these rats was not boostable by AH-OVA parenteral challenge, indicating that the i.p. regime indeed induced some form of selective unresponsiveness. The presence of persistent IgE synthesis in the same animals, however, suggests a situation qualitatively different to that induced by exposure to the antigen aerosol.

It would appear, therefore, that repeated low-grade antigenic stimulation of the respiratory mucosa represents an extremely effective means of selectively suppressing IgE-responsiveness. This model provides a plausible mechanism for regulation of over-production of IgE in response to allergen deposition on mucosal surfaces, consistent with that hypothesized earlier by Jarrett (1977).

Additionally, we have demonstrated that the high-IgE-responder BN strain rat behaves in a similar fashion to mice (Holt *et al.*, 1981a; Fox & Siraganian, 1981) in that tolerance induced by aerosol exposure appears to be IgE-specific. This is in contrast to a previous report from these laboratories (Holt & Leivers, 1982) which demonstrated that, under identical conditions of antigen exposure, animals from two lower IgE-responding rat strains tolerized with respect to both IgE and IgG. This phenomenon is currently under investigation as it may provide fresh insight into the mechanisms underlying the differential sensitivities of the IgE and IgG isotypes to immunological control.

ACKNOWLEDGMENTS

This work was supported by the Princess Margaret Children's Medical Research Foundation and the Asthma Foundation of W.A. J. S. is supported by a Commonwealth Postgraduate Research Award. This is Publication No. 165 of the Clinical Immunology Research Unit of the Princess Margaret Children's Medical Research Foundation.

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