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Down-regulation of immune responses to inhaled antigen: studies on the mechanism of induced suppression

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Summary. Repeated exposure of rats to an aerosol of ovalbumin (OVA) or its dinitrophenylated derivative (DNP-OVA) induced carrier-specific tolerance to subsequent challenge with the same haptenated antigen. Following parenteral challenge with DNP-OVA, both anti-DNP and anti-OVA IgE titres were reduced relative to controls, whereas anti-DNP responses following challenge with DNP-*Ascaris* were normal. Stimulation of tolerant rats with OVA, together with the polyclonal B-cell mitogen LPS, restored their capacity to respond to the antigen.

In contrast to WAG rats, which have previously been shown to develop equivalent tolerance in the IgE an IgG antibody classes (Sedgwick & Holt, 1984), BN rats exposed to an OVA aerosol developed high serum titres of anti-OVA IgG. Following parenteral challenge with DNP-OVA, however, anti-DNP IgG responses in the BNs were markedly reduced relative to unexposed controls, while anti-OVA IgG titres were maintained at a high level.

Further strain-dependent differences in T-cell function in tolerized rats appeared in *in vivo* assays of DTH reactivity and in *in vitro* antigen-driven lymphocyte proliferation. Both BN and WAG rats displayed diminished *in vitro* responses, whereas DTH reactions were only suppressed in the latter strain.

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INTRODUCTION

It has been established that repeated brief exposure of rats and mice to doses of aerosolized antigen within the mg–ng range induces a state of immunological tolerance which is antigen specific, and preferentially directed towards the IgE isotype (Holt, Batty & Turner, 1981; Holt & Leivers, 1982; Sedgwick & Holt, 1983a; 1984). By analogy to tolerance induced by antigen feeding, the induction of tolerance to inhaled antigen is accompanied by the appearance of IgE-isotype specific suppressor cells in central lymphoid organs (Holt *et al.*, 1981; Sedgwick & Holt, 1984) which, in the mouse, carry the Thy 1.2 surface marker (Holt & Leivers, 1982), and in the rat are MRC $OX8^+$, $W3/13^+$ and $W3/25^-$ (Sedgwick & Holt, 1985), a surface phenotype characteristic of the T suppressor/cytotoxic subset in this species (Brideau *et al.*, 1980).

It was recently demonstrated (Sedgwick & Holt, 1985) that these suppressor cells arose initially in the lymph nodes draining the respiratory tract (RTLN), and after further aerosol exposures could be found in the spleen, mesenteric lymph nodes and thymus. The major source of suppressor cells in the RTLN were the superficial cervical nodes draining the upper respira-

tory tract (Sedgwick & Holt, 1985). Earlier radiotracer experiments indicated that inhaled antigen in these animals rapidly partitioned between the lungs and the gut (Holt *et al.*, 1981; Sedgwick & Holt, 1983a). However, the demonstration that repeated intragastric administration of equivalent amounts of antigen to naïve animals was not tolerogenic (Sedgwick & Holt, 1983a), is consistent with a primary role for the RTLN in this process.

In the mouse, IgE-isotype specific tolerance was observed (Holt *et al.*, 1981). However, the isotype specificity of tolerance in the rat was not absolute. Indeed, the low IgE responder WAG strain demonstrated tolerance in both the IgE and IgG isotypes (Holt & Leivers, 1982), while animals of the high IgE-responder BN strain developed high serum titres of specific IgG during exposure to aerosolized antigen, associated with the appearance of radiation-resistant plasma cells in the spleen and bone marrow (Holt *et al.*, 1984). BN rats moreover displayed transient IgE responses followed by stable and long-lived tolerance in this isotype (Sedgwick & Holt, 1983a; 1984).

Secondary challenge of IgE-tolerant BN rats, however, unmasked unresponsiveness in the IgG class also, and dose response experiments with WAG and BN rats revealed that susceptibility to tolerance induction was related to both IgE-responder phenotype and isotype (Sedgwick & Holt, 1984). Thus, rats of the high IgE-responder BN strain required 1000 times more antigen than the low-responder WAG to induce tolerance in this system, the IgE isotype in both strains being considerably more sensitive to suppression than IgG (Sedgwick & Holt, 1984).

This report, employing hapten-carrier immunogens, examines the cellular target for suppression in this model and demonstrates the carrier specificity of the suppressor mechanisms which serve to limit the production of both IgE and IgG following OVA aerosol exposure. Additionally, suppression in the cell-mediated compartment of the immune response, notably of DTH and antigen-induced *in vitro* T-cell proliferation, is also demonstrated.

MATERIALS AND METHODS

Animals

SPF rats of the Brown Norway (BN) and Wistar (WAG) inbred strains were obtained from the Animal Resource Centre, Murdoch University, Western Australia. They were maintained on a diet of acidified water and OVA-free food pellets supplied *ad libitum*. All animals were aged between 8 and 12 weeks at the outset of experiments. WAG or Sprague Dawley rats of both sexes were employed for PCA reactions (see below).

Antigens

Ovalbumin (OVA) and bovine serum albumin (BSA) were from Sigma Chemical Co., St Louis, MO; *Ascaris* (ASC) extracts were prepared as previously described (Sedgwick & Holt, 1984). Dinitrophenyl (DNP)-conjugates of OVA ($DNP_{2\cdot9}$ OVA and $DNP_{4\cdot3}$ OVA), BSA ($DNP_{25\cdot1}$ BSA), and ASC ($1\cdot8 \times 10^{-7}$ mol DNP per mg ASC protein) were prepared as described by Eisen (1964). $DNP_{2\cdot9}$ OVA was used for parenteral challenge of rats while $DNP_{4\cdot3}$ OVA was employed for aerosolization.

E. coli (strain 026:B6) lipopolysaccharide (LPS), was obtained from Difco, Detroit, MI. Dose response experiments established that 50 μg LPS intravenously (i.v.) was well tolerated by the animals used in these experiments. Freund's complete adjuvant (FCA) and Tuberculin purified protein derivative (PPD) were both obtained from Commonwealth Serum Laboratories, Melbourne, Australia. Rabbit anti-rat IgG (γ-chain specific) and sheep anti-rabbit IgG-alkaline phosphatase conjugate were as described previously (Sedgwick & Holt, 1983b).

Antigen exposure and immunization

The procedures for OVA-aerosol administration have been described in detail in previous publications (Holt *et al.*, 1981; Sedgwick & Holt, 1983a). In these experiments, an aerosol derived from 1·0% OVA (w/v) in PBS was employed, except where otherwise stated. Parenteral challenge of rats was in the majority of cases via the intraperitoneal (i.p.) route employing 100 μg of either OVA, DNP-OVA or DNP-ASC plus 10·0 mg aluminium hydroxide (AH; Amphojel, Wyeth Pharmaceuticals, Sydney, Australia) in 0·5 ml PBS.

Determination of serum IgE and IgG titres

Levels of circulating anti-OVA, ASC or DNP homocytotropic (IgE) antibody were assayed by the PCA method of Ovary (1964) as detailed previously (Sedgwick & Holt, 1983a). DNP-BSA was used as challenging antigen for determination of anti-DNP IgE. Anti-OVA, ASC or DNP IgG antibodies were assayed using the ELISA technique essentially as outlined by Voller, Bidwell & Bartlett (1976).

Briefly, 96-well plastic microtitre plates (Linbro, Flow Labs, McLean, VA) were coated with 100 μg/ml of antigen (OVA, ASC or DNP-BSA) in alkaline-carbonate buffer, pH 9·6. Serum samples were diluted

out, together with an appropriate standard serum followed later by anti-rat IgG and anti-rabbit alkaline phosphatase conjugate; p-nitrophenyl phosphate (Sigma Chemical Co.) was finally added as substrate and the optical density at 405 nm measured. Dilutions of each standard serum were assigned a consistent but arbitrary number of ELISA units and a standard curve generated. Values for tests were computed from these standard curves.

Delayed-type hypersensitivity

Examination of the ability of aerosol exposed rats to mount a delayed-type hypersensitivity (DTH) response was carried out as follows.

Rats were sensitized by injecting 200 μg of OVA in FCA (100 μl OVA in PBS+100 μl FCA per rat) subcutaneously (s.c.) in the base of the tail and both hind foot-pads. Ten days later, DTH was elicited by challenging the animals in the ear with either 50 μg OVA or 460 units of PPD in 20 μl PBS.

The contralateral ear serving as control was injected with PBS alone. Twenty-four hours post-challenge, ear thickness was measured with a micrometre screw gauge, and the extent of swelling calculated by subtracting the thickness of the PBS-injected ear from that of the antigen-challenged ear.

In vitro *antigen-driven blastogenesis*

Aerosolized or control rats were sensitized by s.c. injection with OVA plus FCA as described above. Fifteen days later, popliteal and para-aortic lymph nodes were removed and cells resuspended in RPMI (Gibco, Grand Island, NY) containing 5×10^{-5} M 2-mercaptoethanol and 5·0% (v/v) fresh syngeneic (BN or WAG) rat serum as described previously (Holt, Warner & Mayrhofer, 1981).

Lymphoid cells (4×10^5 per well in 200 μl RPMI) were dispensed into flat-bottomed 96-well microtest plates (Falcon, Cockeysville, MD; Becton-Dickinson, Oxnard, CA) and 20 μg OVA or 35 μg PPD in PBS added in 10 μl volumes to appropriate wells; these antigen concentrations were established as optimal for *in vitro* stimulation in preliminary experiments. Control wells received 10 μl PBS alone. After 72 hr incubation at 37° in a humidified atmosphere containing 5% CO_2, each well received 0·5 microcuries [^{3}H]thymidine (Amersham, Australia Pty. Ltd, Melbourne, Australia). The cultures were processed 16 hr later in a cell harvester (Skatron, Lierbyen, Norway) and [^{3}H]DNA synthesis determined by liquid scintillation spectrometry.

Statistical analysis

Serum titres are expressed as $\log_2$ of the geometric mean of individual reciprocal PCA titres or ELISA units ± 1 SD. Student's *t*-test was performed on log transformed data to determine the significance of difference between experimental groups. DTH measurements were not log transformed prior to statistical analysis by Student's *t*-test.

RESULTS AND DISCUSSION

Status of T helper cell function in rats pre-exposed to OVA aerosol

T helper cell function in BN rats tolerized by aerosol exposure to OVA was examined by challenging the animals parenterally with a haptenated form of the antigen administered with aluminium hydroxide (Table 1).

It was apparent that the capacity of these OVA-tolerant rats (see data in column 2) to mount IgE responses to DNP was a reflection of their ability to respond to the carrier employed. Thus, there was a significant depression of anti-DNP IgE responses following challenge with DNP-OVA (cf. Groups A and B), but not when challenged with DNP-ASC (cf. Groups C and D). Similar data were obtained in experiments involving exposure of BN rats to an aerosol where DNP was a component of the tolerogen (Table 2). Collectively, these results point to carrier responsiveness at the level of the T helper cell as the prime target for aerosol induced suppression of the IgE response.

The IgG response in these same animals presented a more complex picture. Experiments equivalent to those of Table 1 examining anti-DNP IgG responses in tolerized rats of the low-responder WAG strain yielded comparable evidence of diminished IgG responses resulting from depressed carrier reactivity (data not shown). In contrast, as noted above, OVA-aerosol exposure of BN rats triggers progressively increasing antigen-specific IgG synthesis which stabilizes after cessation of exposure to yield a 'persistent' antibody response of several months duration (Sedgwick & Holt, 1984; Holt *et al.*, 1984). However, the IgG response in BNs is not significantly boosted by repeated parenteral challenge with antigen (Sedgwick & Holt, 1984). Indeed, the majority of post-challenge IgG in these animals is due to antibody already present at the time of challenge (see Sedgwick & Holt, 1984; also legend † and data column 5 of Table 1).

Table 1. Helper T-cell function in rats exposed to an OVA aerosol

Group	Aerosol treatment	Challenge antigen	IgE* DNP	IgE* OVA	IgE* ASC	IgG* DNP	IgG* OVA	IgG* ASC
A	–	DNP-OVA	12·0±1·4	10·7±1·3	—	16·3±0·7	11·6±1·4	—
B	+	DNP-OVA	7·5±1·3‡	3·7±3·2‡	—	12·8±0·3‡	15·2±1·6†	—
C	–	DNP-ASC	11·5±2·4	—	8·0±2·0	17·0±0·4	—	11·6±0·6
D	+	DNP-ASC	11·2±0·9	—	8·2±1·5	16·8±0·4	—	12·4±0·5

Two groups of BN rats ($n=4$) were exposed for 7 min, once weekly for 6 weeks to a 1·0% OVA aerosol. Seven days after the final exposure these animals, together with unexposed controls, were challenged i.p. with either AH/DNP-OVA or AH/DNP-ASC and bled 10 and 21 days later for determination of serum IgE and IgG antibody. Peak responses (day 10) are shown here; Day 21 data were qualitatively similar.

* Anti-DNP, OVA or ASC IgE or IgG are shown here as the geometric mean of individual $\log_2$ PCA titres or ELISA units (±1 SD).

† Anti-OVA IgG titres in aerosol exposed rats prior to challenge ranged from 12·3 to 18·3 ELISA units.

‡ Significantly less than control ($P<0·002$–$0·01$).

Table 2. Helper T-cell and B-cell function in rats exposed to an aerosol of DNP-OVA

Group	Aerosol treatment	Challenge antigen	IgE* DNP	IgE* OVA	IgE* ASC	IgG* DNP	IgG* OVA	IgG* ASC
A	–	DNP-OVA	12·0±1·4	10·8±1·3	—	16·3±0·7	11·7±1·4	—
B	+	DNP-OVA	3·5±2·4†	6·0±2·8†	—	14·1±0·8†	14·1±0·7	—
C	–	DNP-ASC	11·3±2·2	—	8·0±2·0	17·0±0·4	—	10·9±1·4
D	+	DNP-ASC	10·0±0·8	—	7·8±0·9	16·8±0·4	—	9·1±0·5

Two groups of BN rats ($n=4$) were exposed for 7 min, once weekly for 6 weeks, to a 1·0% DNP-OVA aerosol. See Table 1 for details of parenteral challenge and bleed schedule.

* Anti-DNP, OVA or ASC IgE or IgG are shown as the geometric mean of individual $\log_2$ PCA titres or ELISA units (±1 SD).

† Significantly less than control ($P<0·001$–$0·05$).

The use of hapten-carrier conjugates in the present experiments has significantly clarified this picture. It is evident that BN rats exposed to the carrier OVA, alone or conjugated to DNP, are unable to provide normal levels of T-cell help for anti-DNP IgG responses when DNP is presented on the same carrier (Tables 1 and 2, data column 4). Thus, despite the maintenance of relatively high levels of ongoing anti-OVA IgG production in these rats as a result of exposure to the OVA-aerosol, the induction of further *de novo* responses (i.e. primary anti-DNP IgG responses, or secondary anti-OVA IgG responses) against the same carrier appears to be under covert suppression.

An apparent parallel to these observations is the phenomenon of epitope-specific regulation defined by Herzenberg, Tokuhisa & Hayakawa (1983). They have shown that priming with a carrier, and subsequent secondary challenge with a hapten (DNP) conjugated to the same carrier, results in epitope (hapten)-specific IgG suppression without a parallel reduction in the ongoing anti-carrier IgG response. Suppressor T cells with specificity for DNP and not the carrier have been isolated (Tagawa *et al.*, 1984), and similar cells may function in this system.

Two alternative possibilities are suggested by recent studies from this laboratory. Firstly, we have described the development of long-lived radioresistant IgG-secreting cells in aerosolized rats. These cells were refractory to downregulation by antigen-specific suppressor T cells which were capable of inhibiting the

induction of primary antibody responses (Holt *et al.*, 1984). Conceivably, both cell populations could co-exist in these rats, leading to a situation of apparently stable IgG antibody production concomitant with active suppression of any further T helper cell activation. Alternatively, the presence of high levels of anti-carrier IgG antibody in these animals could inhibit further carrier-specific T helper cell activation by feedback inhibition, and earlier serum transfer experiments indeed produced evidence of suppression of this isotype (Sedgwick & Holt, 1984). Experiments are in progress to test these possibilities.

Antigen-specific B-cell function in aerosol-exposed rats

LPS is a potent B-cell mitogen capable of initiating both mitosis and conversion of antigen-stimulated B cells to antibody producing cells (Hoffman *et al.*, 1977), and as such has been used to confirm the anergic state of B lymphocytes in induced immunological tolerance (Nossal & Pike, 1980; Fernandez & Moller, 1978) and to bypass functionally suppressed T helper cells in tolerant animals by direct stimulation of B lymphocytes (Titus & Chiller, 1981; Chiller & Weigle, 1973; Schleimer *et al.*, 1982). The latter approach was adopted with OVA-aerosol exposed rats to determine the functional status of the OVA-specific B-cell pool in these animals (Table 3).

BN rats were exposed once weekly for 6 weeks to an OVA-aerosol, then challenged i.p. employing AH-OVA, with LPS administered i.v. to a proportion of the animals 3 hr later. Administration of the polyclonal B-cell mitogen with the challenge antigen produced a significant restoration of the IgE response in OVA-aerosol exposed animals, without significantly affecting the corresponding IgG response.

Table 3. Co-stimulation of tolerant rats with antigen together with a polyclonal B-cell mitogen

Group	Aerosol treatment	Challenge antigen	IgE*	IgG*
A	–	OVA	12·4±0·9	14·7±0·7
B	+	OVA	2·0±2·0†	14·5±0·2
C	+	OVA+LPS	9·0±1·0†	15·8±0·6
D	–	OVA+LPS	12·3±0·5	15·2±0·6

Two groups of four BN rats were exposed to an OVA aerosol as in Table 1. Seven days after the final exposure, these animals, together with unexposed controls, were immunized i.p. with AH-OVA and 3 h later iv. with 50 μg LPS or an equivalent volume of PBS.

* Peak levels of anti-OVA IgE or IgG at day 10 post-challenge are shown as the geometric mean of individual $\log_2$ PCA titres or ELISA units (±1 SD).

† B < A, $P < 0{\cdot}001$; C < D, $P < 0{\cdot}001$; C > B, $P < 0{\cdot}001$.

The mechanism by which LPS exerts its enhancing effect in tolerant animals is not completely understood. The B lymphocyte is potentially a major target, as it has been estimated that up to one in every three splenic B cells responds to LPS (Andersson, Coutinho & Melchers, 1977). However, a number of lines of evidence suggest that LPS may also stimulate some T lymphocytes (Scibienski & Gershwin, 1977; Uchiyama & Jacobs, 1978; Newburger, Hamaoka & Katz, 1974), secondarily perhaps to macrophage activation and subsequent IL-1 production (Hoffman *et al.*, 1979). However, given the low frequency (3%) of putatively LPS-responsive T cells (Vogel, Hilfiker & Caulfield, 1983) in lymphoid organs and reported failure of attempts to detect antigen-specific T-cell stimulation by LPS administration *in vivo* to tolerized animals (Titus & Chiller, 1981), it is reasonable to suggest that the mechanism by which LPS reversed suppression in the present model involved T-cell-independent stimulation of antigen-reactive IgE B cells.

Status of other T-cell functions in aerosolized rats

Previous reports on tolerance induction to inhaled antigen have focussed upon the humoral compartment of the immune response. The experiments below examined two other manifestations of T-cell reactivity, namely the capacity of lymphocytes to participate in antigen induced proliferation *in vitro* and the expression of DTH responsiveness *in vivo*. Additionally, high and low IgE-responder rat strains were assessed in parallel to determine whether the genetically-determined differences previously observed in humoral responsiveness (Sedgwick & Holt, 1984) extended to these other immune functions.

Control animals and those exposed to OVA aerosols were injected s.c. with OVA-FCA and *in vitro* lymphoproliferation (Table 4) or *in vivo* DTH (Table 5) was assessed as detailed in Methods. Reactivity to a control antigen PPD, present in the mycobacterial component of FCA but not in the aerosol, was tested in parallel.

Both BN and WAG rats which had been exposed to an OVA aerosol were severely compromised in their capacity to respond to OVA *in vitro* where control values for [^{3}H]DNA synthesis were in each case 100–

Table 4. T-cell proliferation *in vitro*

Group	Strain	Aerosol treatment	Challenge antigen *in vivo*	Challenge antigen *in vitro*	Log_2 DPM*
A	BN	–	OVA in FCA	OVA	18·0 ± 0·9
B	BN	+	OVA in FCA	OVA	10·6 ± 0·5†
C	BN	–	OVA in FCA	PPD	17·4 ± 0·2
D	BN	+	OVA in FCA	PPD	16·5 ± 0·2‡
E	BN	–	OVA in FCA	OVA	16·8 ± 0·6
F	WAG	+	OVA in FCA	OVA	10·6 ± 0·3†
G	WAG	–	OVA in FCA	PPD	16·6 ± 0·2
H	WAG	+	OVA in FCA	PPD	15·2 ± 0·8‡

Groups of BN and WAG rats ($n = 4$) were exposed to an OVA aerosol as in Table 1. Fifteen days after the final exposure, these animals, together with unexposed controls, were challenged s.c. in the base of the tail and both hind foot pads with 200 μg OVA in FCA. The *in vitro* proliferative capacity of their LN cells following antigenic challenge with either OVA or PPD was determined, as described in the Materials and Methods.

* [^{3}H]DNA synthesis of triplicate cultures, shown as the mean of individual log_2 disintegration per minute (DPM) values for each culture, after subtraction of background (PBS-stimulated) proliferation values (± 1 SD).

† Significantly less than controls ($P < 0·001$).

‡ Significantly less than controls ($P < 0·05$).

200 times those of cells from tolerant animals (Table 4). Antigenic specificity in this instance was not absolute as the proliferative capacity of cells from aerosolized animals to stimulation *in vitro* with the control antigen PPD was also reduced, albeit minimally.

In contrast to the data in Table 4, DTH responses to OVA (Table 5) were shown to be normal in aerosol-exposed BN rats, but significantly suppressed in the WAG strain ($P < 0·001$ compared to control animals), and in this case complete antigen specificity was observed. While the absolute loss of antigen-specific

Table 5. DTH Responses in aerosol exposed rats

Group	Strain	Aerosol treatment	Challenge antigen	Ear tested for:	Increase in ear thickness*
A	BN	–	OVA in FCA	OVA	42·3 ± 13·5
B	BN	+	OVA in FCA	OVA	39·9 ± 14·4
C	BN	–	OVA in FCA	PPD	18·1 ± 5·3
D	BN	+	OVA in FCA	PPD	29·8 ± 4·1†
E	WAG	–	OVA in FCA	OVA	40·8 ± 9·9
F	WAG	+	OVA in FCA	OVA	9·1 ± 3·8‡
G	WAG	–	OVA in FCA	PPD	19·1 ± 5·2
H	WAG	+	OVA in FCA	PPD	23·1 ± 7·5

Groups of BN and WAG rats ($n = 4$) were exposed to an OVA aerosol as in Table 1. One week after the final exposure, these animals, together with unexposed controls, were challenged s.c. in the base of the tail and in both hind foot pads with 200 μg OVA in FCA. DTH reactions were elicited and measured according to the protocol described in the Materials and Methods.

* Geometric mean of individual values ± 1 SD (mm × 10²).

† This increase was not a consistent finding in follow-up experiments.

‡ Significantly less than control ($P < 0·001$).

T-cell function in aerosol-exposed WAG rats reflects this strain's exquisite sensitivity to tolerance induction (see Introduction and Sedgwick & Holt, 1984), the significance of the differences between the performance of cells from the BN strain in the various functional assays is not clear. It is conceivable that these assays measure the activity of distinct T-cell populations, such that a loss of function may be manifested in one while the other remains normal. Alternatively, the respective effector cell populations may be differentially sensitive to the same suppressor signals. In this context, recent work with orally tolerized mice has demonstrated similar variations in the sensitivity of different components of the immune response to tolerance induction, where DTH was more readily suppressed than IgG antibody production (Miller & Hanson, 1979; Mowat *et al.*, 1982).

In conclusion, the basic cellular mechanisms associated with the induction of tolerance in the IgE antibody class in aerosol-exposed rats appear consistent with the published literature concerning the immunoregulation of IgE in this species (Tada, 1978; Ishizaka, 1984). Loss of T helper rather than B-cell function clearly appears to be the dominant factor in suppression of IgE in this model, in keeping with the earlier demonstration of MRC $OX8^+$ antigen-specific suppressor T cells in aerosolized animals (Sedgwick & Holt, 1985). The precise nature of the cell(s) operative in regulation of other aspects of the immune response to inhaled antigen and the basis for variations in their activity in animals of different genetic backgrounds, awaits clarification. In this context, it would appear logical to explore further the apparent similarities between tolerance induction to inhaled antigen, and the more well defined process which results from antigen feeding.

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