

Inhibition of specific IgE responses in mice by pre-exposure to inhaled antigen

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Summary. Exposure of mice to aerosolized ovalbumin (OA) once weekly for 5 min, or once weekly to 10 μ g OA in PBS intranasally, elicited transient IgE responses which declined by the seventh week. When these animals were challenged intraperitoneally (i.p.) with soluble or alum-precipitated OA, their subsequent IgE responses were markedly suppressed relative to controls. In contrast, i.p. challenge provoked haemagglutinating antibody (HA) responses to OA in the same animals which were considerably more vigorous than in controls. Adoptive transfer experiments employing splenocytes from mice repeatedly exposed to OA via the respiratory tract revealed the presence of suppressor cells active against OA-specific IgE but not HA responses. Radiotracer studies employing ^{125} I-OA, administered intranasally and by aerosol, indicated that much of the antigen rapidly became associated with the gut.

INTRODUCTION

The past decade has witnessed an unprecedented increase in our understanding of the complex network of control mechanisms which regulate IgE synthesis in

experimental animals. This progress stems largely from experimental models which depend upon a restricted range of adjuvant-dependent immunization regimes for the preferential induction of IgE synthesis, particularly those involving the parenteral administration of antigen in conjunction with aluminium hydroxide or *Bordetella pertussis* (reviewed by Tada, 1975; Ishizaka, 1976). There is a clear need to translate these findings to models which more closely approximate the triggering of IgE responses under natural conditions, i.e. wherein the antigenic stimulus is not accompanied by adjuvant, and the portal of entry of the antigen is 'normal'.

The latter approach has proved fruitful in regard to regulation of IgE responses to antigens administered by the oral route. The early observations of Wells & Osborne (1911) and Chase (1946) on the modulation of systemic responses resulting from antigenic stimulation via the gut have recently attracted the attention of a number of workers. As a result we have an emerging picture of this organ as a potentially important site of tolerance induction (Andre, Bazin & Heremans, 1973; Thomas & Parrott, 1974), particularly in relation to IgE antibody synthesis (David, 1977; Hanson, Vaz, Maia, Hornbrook, Lynch & Roy, 1977; Ngan and Kind, 1978; Hanson, Vaz, Rawlings & Lynch, 1979).

Despite the obvious importance of the respiratory tract as a portal of entry for antigens which provoke immediate hypersensitivity reactions, interest in antigen inhalation as a mode of induction of specific IgE responses has not paralleled that seen in regard to

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oral ingestion of antigen. The only reported experiments involving IgE responses to passively inhaled antigen are those of Van Hout & Johnson (1972) in the rat, while the intranasal route has apparently been examined in only two recent studies employing mice (Gerbrandy & Bienenstock, 1976; Mitchell 1976). Significantly, all three of the aforementioned studies involved the administration of antigen in conjunction with artificial adjuvants.

The present study represents a preliminary step aimed at redressing this imbalance. We report below on a simple model for the chronic exposure of mice to protein antigen via the respiratory tract, wherein exposure conditions may be readily standardized, and trauma to the animals is minimized. Initial experiments indicate that antigen entering via this route readily evokes haemagglutinating antibody responses while at the same time tolerizing with respect to IgE.

MATERIALS AND METHODS

Animals

Female mice of the Balb/c and C3H strains were used throughout. WAG rats of both sexes were employed for PCA reactions (see below).

Chemicals

Ovalbumin (OA; Grade V) and Ovomucoid (OM) were obtained from the Sigma Chemical Company, U.S.A. The aluminium hydroxide (AH) employed was Amphojel, from Wyeth Pharmaceuticals, N.S.W., Australia.

Antigen exposure via the respiratory tract

Inhalation. Mice were exposed for 5 min once weekly, in plexiglass chamber (30 cm cubed), to aerosolized 1% OA in phosphate buffered saline (PBS). The aerosol was generated employing an Inspiron Mini-Neb Nebulizer (C.R. Bard Inc.) run at 10.0 p.s.i., and was piped into the vessel via 30 cm of wide-bore plastic tubing. This nebulizer generates aerosols with a mean droplet size between 3 and 5 μ m (manufacturer's specifications). However, due to the relatively large distance between the nebulizer and the animals, a significant degree of droplet association was deemed likely within the aerosol. As the latter would mitigate against deep penetration of antigen into the respiratory tree, a preliminary experiment (Table 1) was performed employing a more complex exposure system,

wherein the distance from the nebulizer to the mice was minimized (Table 1).

Intranasal. Mice were lightly anaesthetized with ether, and inoculated once weekly by the intranasal route with 10 μ g OA in 25 μ l PBS. The antigen was delivered directly to the nostrils as drops from an Oxford pipette.

Determination of anti-OA antibody titres

Homocytotropic antibody. IgE antibody titres were determined via the passive cutaneous anaphylaxis (PCA) reaction. For determination of PCA titres, serum samples (prepared from tail-vein blood) were diluted in PBS, and 50 μ l aliquots inoculated intradermally into the shaved backs of female WAG rats at marked sites. Twenty-four hours later the rats were challenged intravenously (i.v.) with 2.0 mg OA in 0.5 ml 1% Evans Blue (in PBS). Thirty minutes later the rats were killed and the relevant skin areas excised for examination of the lesions. The reciprocal of the highest serum dilution giving a 5 mm diameter flare or 'blueing reaction' was taken as the PCA titre.

Haemagglutinating antibody. Ovalbumin was coupled to sheep erythrocytes (SRBC) employing the glutaraldehyde method of Avrameus, Taudou & Cluïlan (1969). Haemagglutinating antibody (HA) to OA was titred in V-bottomed microtitre plates; serum samples were serially diluted in 25 μ l PBS, prior to the addition of 25 μ l of 1% OA-SRBC. The reciprocal of the highest dilution exhibiting obvious agglutination after 45 min incubation at 37° was taken as the HA titre.

Adoptive transfer

Spleen cells from OA-primed or control Balb/c mice were prepared by a modification of the method of Shortman, Williams & Adams (1972), which included the use of a low ionic strength medium (LISM) for the aggregation of damaged cells (Van Boehmer & Shortman, 1973). The solutions employed here were adjusted to mouse osmolality (333 mosmole/kg), and all procedures were carried out at 4°. Briefly, spleens were minced finely in chilled mouse-buffered balanced salt solution (MBBSS) and sieved. The cells were centrifuged and resuspended in LISM. The cell suspensions were filtered through absorbent cotton-wool columns and treated with ammonium chloride (0.184 M) to remove erythrocytes. The suspension was underlaid with 1.0 ml foetal calf serum (FCS) and centrifuged at 400 g for 7 min; cell ghosts, dead cells and

Table 1. IgE and HA antibody synthesis in Balb/c mice in response to repeated antigenic exposure via the respiratory tract

Day	Route of OA exposure		Experimental groups			Unexposed controls
	Oronasal	Intraperitoneal	I	II	III	
0	+		—	—	—	—
7	+		0 (0)	0 (0)	0 (0)	—
14	+		20 (8–32)	10 (64)	20 (8–128)	—
21	+		10 (8)	10 (64)	50 (4–256)	—
28	+		10 (8)	20 (8–32)	70 (8–256)	—
35	+		20 (4–8)	30 (8–16)	90 (8–64)	—
42	+		0 (0)	20 (4–16)	70 (4–16)	—
49	+		0 (0)	20 (8–16)	90 (4–32)	—
			<i>40</i>	<i>80</i>	<i>320</i>	
56		+	20 (8)	20 (16–32)	80 (8–32)	0 (0)
63			10 (32)	10 (32)	60 (32–128)	66 (8–16)
70		+	—	—	—	—
77			0 (0)	10 (64)	60 (32–64)	100 (320–1280)
			<i>160</i>	<i>640</i>	<i>1280</i>	<i>0</i>

Groups of ten Balb/c mice were exposed oronasally once weekly to OA by aerosol (I) or intranasally (III) as described in the text. A second group (II) were aerosolized under special conditions aimed at minimizing droplet association prior to inhalation: these mice were held in perspex restrainers attached via O-rings to a 250 ml chamber, with the nebulizer attached directly to the base—the conditions of restraint maintained the noses of the mice within the chamber, and thus in direct contact with the aerosol stream. The data shown reflect the percentage of animals within each group which were serum PCA-positive, the range of titres for each group shown in parentheses. Figures in italics are mean HA titres determined on pooled samples.

debris remained at the interface. The pelleted cells were resuspended in MBSS, and viable cell counts performed. The cells were resuspended in MBBSS containing 20% FCS, at a concentration of 2.5×10^8 viable cells/ml (viability was always > 95%).

Recipient female Balb/c mice were X-irradiated (600 R) 24 h before the transfer was effected. The latter comprised the i.v. inoculation (tail vein) of 5.0×10^7 – 10^8 spleen cells, in 0.2–0.4 ml MBBSS + 20% FCS, as detailed in the text. Recipients were bled from the tail vein 7 and 14 days after the adoptive transfer.

Radiotracer studies

Balb/c mice were exposed for 5 min to aerosolized ^{125}I -OA, as a 1% solution in PBS, as described in Methods. The ^{125}I -OA was prepared by the method of McConahey & Dixon (1966); it was dialysed to remove unbound ^{125}I prior to use. In parallel, other Balb/c mice received $10 \mu\text{g}$ ^{125}I -OA in $25 \mu\text{l}$ PBS intranasally. At varying times after exposure the mice were killed, and internal organs dissected out, and the

amount of radiation in the latter determined in a Packard Gamma Spectrometer.

RESULTS

IgE synthesis in mice during repeated antigenic exposure via the respiratory tract

As detailed in Table 1, groups of ten Balb/c mice were exposed once weekly for 5 min, to aerosolized OA (Groups I and II), or to $10 \mu\text{g}$ OA in PBS via the intranasal route (Group III). This procedure was repeated on eight occasions, all animals being bled 4 h prior to each exposure for the determination of anti-OA antibody levels. Animals in all three groups exhibited transient IgE responses, individuals generally exhibiting peak levels between the third–fifth exposure, after which PCA titres declined. HA antibody was not detected until the day 28 bleed, at which time a small proportion of animals in Groups II and III exhibited HA titres of 10–20 (data not shown). Subsequent data on HA titres was obtained on pooled

sera from each group—these indicated a slow rise up to day 49 (titres shown in Table 1).

On day 56, all three groups (plus unexposed controls) were challenged intraperitoneally (i.p.) with 10 μ g OA in PBS. Antibody titres determined on individual tail-blood samples collected 7 days later indicated only minor fluctuations in PCA titres, while pooled sera from Groups I–III exhibited small but consistent increases in HA titres.

All animals were rechallenged i.p. 1 week later (day 70) with 10 μ g OA, and bled after a further 7 days. Individual animals from Groups I, II and III again failed to mount significant IgE responses, whereas HA titres determined in pooled sera from these groups showed further rises. The control animals mounted vigorous IgE responses following this secondary i.p. challenge, but remained HA-negative.

These data indicated no major differences between animals exposed to OA via the two aerosol protocols (Groups I and II in Table 1), and consequently the simpler system (detailed in Methods) was adopted thereafter.

Subsequent experiments compared the effects of repeated OA exposure in high-responder Balb/c *v.* moderate-responder C3H (Holt & Batty, 1980). In these studies the exposure period was shortened (six exposures performed at weekly intervals), and in the parenteral challenge with antigen which followed the repeated respiratory tract exposures, comparisons were drawn between the effects of minimal *v.* maximal antigenic stimulation.

Table 2 examines the anti-OA responses of these latter animals to minimal antigenic challenge, i.e. OA

given i.p. without adjuvant. These data are not as clear cut as those of Table 1. In these experiments, the initial i.p. challenge (day 0 in Table 2) elicited a detectable IgE response in all pre-exposed groups. With the exception of Group B (C3H), the latter was transient, and had declined by day 14, at which time a second i.p. challenge was performed. The control groups exhibited vigorous IgE responses to secondary challenge in comparison to the pre-exposed animals. In contrast, the exposed mice exhibited vigorous HA-antibody responses, whereas the controls remained HA-negative. The capacity of these animals to respond to parenteral challenge with OA thus differs subtly from those in Table 1. In the latter experiments the pre-exposed mice were refractory to both i.p. challenges, whereas in Table 2 diminished OA-specific IgE responsiveness was found only after the second i.p. dose of OA. These differences may reflect variations in the pre-exposure periods employed (7 *v.* 5 weeks).

Figures 1 and 2 depict the PCA and HA-antibody responses of mice pre-exposed to OA via the respiratory tract, following systemic challenge under conditions of maximal restimulation with the antigen, viz. employing AH as adjuvant (AH-OA). These mice represent the second half of the experiments in Table 2. The results obtained with this challenge regime are considerably easier to interpret than those above, and clearly indicate a severe depression in the capacity of animals from the pre-exposed groups to mount anti-OA IgE responses, but not HA responses. Challenge of OA-aerosolized and control mice with the irrelevant antigen OM produced comparable anti-OM PCA responses in both groups.

Table 2. PCA and HA responses in pre-exposed mice following challenge with soluble antigen

Day	C3H Mice			Balb/c Mice		
	Aerosol	Intranasal	Controls	Aerosol	Intranasal	Controls
0→	8 (16)	32 (64)	—	4 (4)	16 (64)	—
4	64 (66)	16 (316)	<4 (4)	307 (197)	51 (214)	<4 (8)
10	16 (162)	64 (350)	<4 (<4)	64 (239)	112 (449)	<16 (<4)
14→	<16 (176)	256 (>1400)	16 (<4)	26 (162)	8 (273)	<16 (<4)
20	45 (700)	67 (700)	400 (<4)	128 (700)	64 (700)	1024 (<2)

Groups of C3H and Balb/c mice were exposed on six occasions (at 7-day intervals) to OA intranasally or by aerosol. One week after the final exposure, together with unexposed syngeneic controls, the mice were challenged i.p. with 10 μ g OA. All mice were bled on days 4, 10 and 14, and rechallenged with the same antigen dose. Figures shown represent mean PCA titres within the groups. Figures in parentheses represent mean HA titres. → i.p. challenge with 10 μ g OA in PBS.

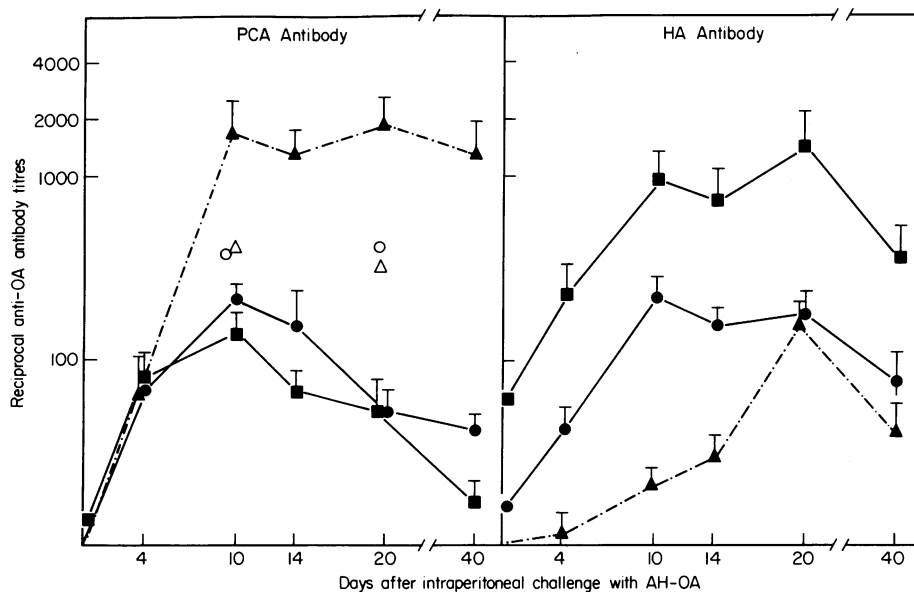


Figure 1. PCA and HA titres following AH-OA challenge of C3H mice pre-exposed intranasally or by aerosol to OA. C3H mice, pre-exposed to OA via aerosol (●) or intranasally (■), together with unexposed controls (▲), were challenged intraperitoneally with 10 μ g OA plus 4 mg AH (AH-OA). Serum PCA and HA titres were determined at intervals thereafter; data shown are mean $\pm$ SE of reciprocal titres. In other experiments, OA-aerosolized (○) and control (△) mice were challenged intraperitoneally with 10 μ g OM plus 4 mg AH. Serum anti-OM PCA titres were determined on days 10 and 20, employing pooled samples from the groups.

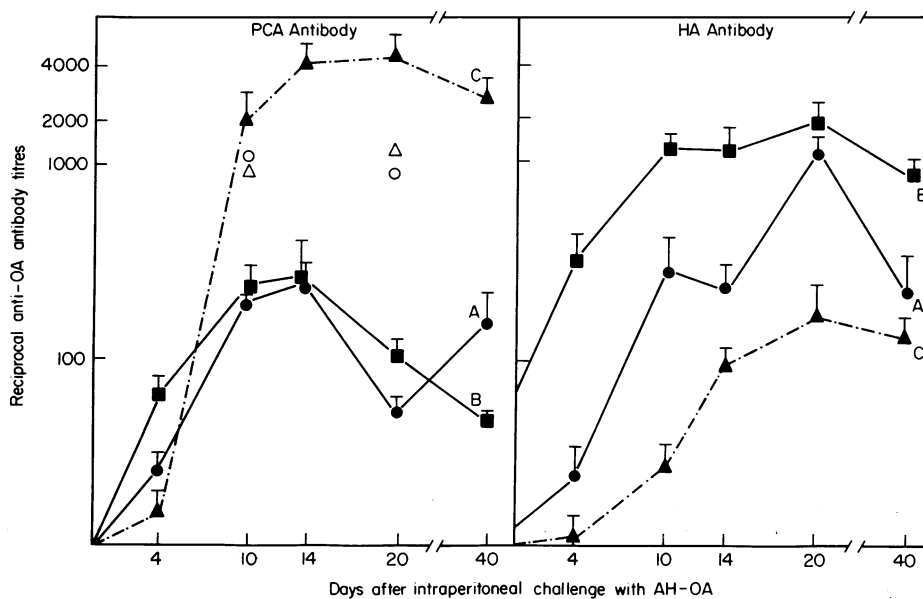


Figure 2. PCA and HA titres following AH-OA challenge of Balb/c mice pre-exposed intranasally or by aerosol to OA. See Fig. 1 for details. The mice were killed after the day 40 bleed, and spleen cells prepared for the adoptive transfer experiments of Table 3.

Adoptive transfer.

The experiment of Table 3 aimed to elucidate the mechanism underlying the apparent suppression of IgE-responsiveness in pre-exposed animals. This experiment employed an adoptive transfer system wherein the recipients of AH-OA-primed spleen cells from normal mice develop persistent OA-specific IgE responses in the absence of further antigenic restimulation (Peeters & Carter, 1978). Spleen cells were prepared from the Balb/c mice designated as Groups A, B and C in Fig. 2; spleen cells were also prepared from syngeneic mice which had not previously encountered OA antigen (designated normal controls in the Table). After i.v. transfer of the spleen cells to X-irradiated syngeneic mice in various combinations (Table 3), examination of ensuing antibody responses revealed high PCA and HA titres in recipient animals, with the exception of those which had received cells from the A/B pool—PCA titres in the latter were depressed by approximately 90%.

Radiotracer studies

Table 4 examines the fate of ^{125}I -OA in Balb/c mice following aerosol and intranasal administration, sampled within the 2 h following exposure. We recovered 10–12 μg OA from animals exposed for 5 min to aerosolized OA, of which an average of 13% was associated with the respiratory tract, the bulk of the remainder being found in the gastrointestinal tract. Of the 10 μg ^{125}I -OA administered intranasally, an average of 3.63 μg was recovered. The distribution within these animals was more variable than that following aerosol exposure. However, the gut was again a major repository of radiolabel, but in this latter group consistently more ^{125}I -OA was found associated with the lung (15%–60% of the total recovered) compared with the aerosolized group (1.5–3.1%).

DISCUSSION

This study represents the first demonstration of specific IgE synthesis in normal (non-adjuvanted) animals, in response to antigen passively inhaled under essentially 'natural' conditions. However, repeated exposure to the same antigen produced more complex results, which are summarized below:

(1) Repeated exposure of mice to OA via the respiratory tract initiates OA-specific IgE synthesis; however, the response is transient, and continued exposure via

the respiratory tract results in eventual diminution of IgE titres (Table 1);

(2) Corresponding HA antibody levels in these animals rise with continuing respiratory tract exposure (Table 1);

(3) Mice repeatedly exposed to OA via the respiratory tract develop tolerance to this antigen, as evidenced by profoundly diminished IgE-responsiveness to subsequent systemic challenge with AH-OA (see particularly Figs 1 and 2);

(4) The choice of the parenteral challenge regime clearly influences the ease with which tolerance is demonstrable in this system (Table 2 compared with Figs 1 and 2);

(5) The tolerance which develops following repeated respiratory tract exposure to OA is specific for IgE antibody, as the pre-exposed mice exhibited anamnestic HA-antibody responses following systemic challenge (Figs 1 and 2);

(6) The conditions of respiratory tract antigen exposure are not critical to tolerance induction in this system, neither is the 'IgE-responder-status' (to the particular antigen employed) of the mouse strains.

Preliminary studies on the nature of the tolerance resulting from repeated respiratory tract exposure to OA implied a cellular basis for the phenomenon (Table 3). Employing a system recently described for the adoptive transfer of ongoing persistent OA-specific IgE and IgG responses (Peeters & Carter, 1978), we were able to abrogate the adoptive transfer of an ongoing IgE response with spleen cells from the exposed animals (Table 3).

Data obtained from examination of the fate of OA administered via the respiratory tract (Table 4) merit special comment. It is clear from these experiments that a high proportion of both passively inhaled and intranasally administered antigen is rapidly transported to the gut, probably via the combined effects of irritant-induced coughing, muco-ciliary transport, and the direct swallowing of antigen deposited in the nares and the oropharynx. This distribution closely parallels that described by Van Hout & Johnson (1972), employing similar exposure conditions in the rat. While the distribution noted in the intranasally exposed group may in part result from the 'unnatural' mode of administration of the antigen, this is less likely for the aerosol-exposed group, wherein the exposure was essentially atraumatic. Rapid transport to the gut may indeed represent one of the most important natural routes of clearance for inhaled antigen deposited on mucosal surfaces in the upper airways, as

Table 3. Suppression of adoptive transfer of IgE response by spleen cells from mice exposed to antigen via the respiratory tract

Spleen cells transferred	Anti-OA antibody titres in recipients			
	Day 7		Day 14	
	PCA	HA	PCA	HA
5.0×10^7 from C	1024 ± 0	933 ± 238	2048 ± 1043	$1400 \pm$
5.0×10^7 from C	2389 ± 920	2123 ± 725	2048 ± 1043	2373 ± 488
5.0×10^7 from normal controls				
5.0×10^7 from C	256 ± 0	233 ± 59	256 ± 0	1867 ± 476
5.0×10^7 from A/B				

Spleen cells were prepared from normal control Balb/c mice, plus the animals from Groups A, B and C killed after the day 40 bleed in Fig. 2. Cells from Groups A and B were pooled (A/B above). The spleen cells were inoculated i.v. into X-irradiated syngeneic recipients in the combinations shown, and PCA and HA titres determined in the recipients on days 7 and 14. Data shown are reciprocal mean titres $\pm$ SE.

Table 4. Distribution of ^{125}I -OA in organs of Balb/c mice after aerosol or intranasal exposure

Animals	^{125}I -OA (μg) recovered in						Total ^{125}I -OA recovered (μg)
	Blood	Lung	Upper resp. tract	Oesophagus + stomach	Intestines	Spleen & kidneys	
80 min post-aerosol exposure	n.d.	0.18	0.74	9.33	1.44	0.17	11.86
98 min "	2.52	0.30	1.61	3.99	3.42	0.72	12.56
120 min "	2.23	0.32	1.45	3.66	2.24	0.42	10.32
145 min "	2.13	0.28	1.13	4.85	1.67	0.30	10.36
$\bar{X}$	2.29	0.27	1.23	5.46	2.19	0.40	11.28
(% total)	(20.3)	(2.3)	(10.9)	(48.4)	(19.4)	(3.5)	(100)
73 mins post-intra-nasal exposure	n.d.	0.29	0.27	0.66	0.65	0.14	2.01
96 min "	0.27	0.93	0.27	2.05	0.35	0.09	3.96
117 min "	0.33	3.16	0.17	0.82	0.37	0.08	4.93
$\bar{X}$	0.30	1.46	0.24	1.18	0.46	0.10	3.63
(% total)	(8.3)	(40.2)	(6.6)	(32.5)	(12.7)	(2.8)	(100)

Mice were exposed for 5 min to aerosolized ^{125}I -OA or intranasally to $10 \mu\text{g}$ ^{125}I -OA in $25 \mu\text{l}$ PBS, and killed at the times shown. Organs were excised and counted in a Gamma Spectrometer. Data shown are ^{125}I -OA (μg) recovered per organ. Data in parentheses reflect percent of total ^{125}I -OA recovered.

suggested by this and numerous earlier studies (e.g. see Willoughby & Willoughby, 1977).

These data suggest a partial explanation for the tolerizing effect of OA in this model. The repetitive feeding of a variety of antigens to animals, including OA, has been shown to produce tolerance (David, 1977; Hanson *et al.*, 1979) which is associated with the presence of suppressor cells in central lymphoid organs (Ngan & Kind, 1978). Consequently, it may be postulated that the portion of inhaled OA which was transported to the gut was responsible for the tolerance observed here. However, it would be premature to accept this thesis without further data, given firstly that earlier studies with OA indicated the necessity for *milligram* levels of the antigen to tolerize with respect of IgE (Vaz, Maia, Hanson & Lynch, 1977; Hanson *et al.*, 1977; Ngan & Kind, 1978), and secondly that tolerance induced in this fashion was manifested equally against IgG and IgE responses (Vaz *et al.*, 1977; Ngan & Kind, 1978). These data contrast sharply with those herein, which indicate the development of essentially IgE-class-specific tolerance to *microgram* levels of OA administered via the respiratory tract. It is also of interest to note that Hanson *et al.* (1979) have shown that the tolerizing effects of antigen administered orally is markedly diminished by concurrent antigen exposure via another route. This observation is difficult to reconcile with our present data which indicate that OA distributed between the respiratory and gastrointestinal tracts was still ultimately tolerogenic.

Despite the difficulties of interpretation, we are encouraged by the results of the present study to pursue this experimental approach in more detail. In particular, we plan to examine the nature of the suppressor cell population induced by respiratory tract exposure to antigen employing appropriate antisera, and the distribution of the latter in exposed animals. It is also planned to examine the relative contribution of lung *v.* gut antigen in this model, employing appropriate intratracheal *v.* oesophageal intubation techniques. It is hoped that these experiments will permit evaluation of the significance of this phenomenon in relation to the normal regulation of IgE responses to ubiquitous airborne allergens.

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