

ORIGINAL PAPER

Genetic selection for resistance or susceptibility to oral tolerance imparts correlation to both Immunoglobulin E level and mast cell number phenotypes with a profound impact on the atopic potential of the individual

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Summary

Background Immunological oral tolerance is being studied with great interest due to its therapeutic potential in allergy and autoimmunity processes, although the cellular and molecular mechanisms linking these different phenomena remain elusive. In the present study, two mouse lines with extreme phenotypes for susceptibility [TS Line] or resistance [TR Line] to oral tolerance and their [TS × TR]F₂ segregants were used in order to evaluate the impact of these traits on the atopic potential of the individuals.

Objective Demonstrate whether the *tr* and *ts* genes, cumulated during 18 generations of bidirectional genetic selection, influence expression of two important immunobiological traits (IgE and mast cell) critical to allergic response.

Methods Mice with extreme phenotypes for oral tolerance to ovalbumin (OVA), produced by assortative mating (TS and TR Line), and their (TS × TR)F₂ segregating were used. Serum IgE levels assayed by ELISA, and mastocytes counted with toluidine blue staining were evaluated in naïve mice. Anaphylaxis was induced by intravenous injection of OVA, intestinal inflammation by oral administration of OVA 7 days after immunization, and pulmonary inflammation by intranasal and nebulization OVA challenges. Specific IgE was dosed by passive cutaneous anaphylaxis.

Results The naïve TS mice have a 20-fold lower serum IgE level and two- to threefold diminished mast cell numbers in mucosal sites, when compared with TR-mice, which were highly susceptible to allergic inflammation and anaphylactic shock. The associations of oral tolerance, serum IgE levels and mast cell numbers in naïve animals were confirmed analysing the simultaneous presence of these traits in individuals of a [TS × TR]F₂-segregating population.

Conclusion The results suggest that the complex of genes controlling TS and TR phenotypes play a main role in the regulation of the atopic potential of the individual. The studies of these traits in interline F₂ segregants demonstrated a co-segregation of TS and TR phenotypes with IgE responsiveness and mast cell numbers. Thus, the opposite capacity of the genetically modified mice may be involved in co-adaptative mechanisms reflecting a dynamic relation between gene frequencies in a natural population. These correlations give circumstantial evidence to support clinical applications of oral tolerance in allergic and autoimmune diseases.

Keywords allergic inflammation, co-segregation, genetic selected mice, IgE, mastocytes, oral tolerance, systemic anaphylaxis

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Introduction

Oral tolerance is a major biological process that has long been neglected by immunologists and has become an

active research area only more recently. It is probably involved in the control of hypersensitivity and deleterious inflammatory reactions to dietary proteins and potential reaction against normal intestinal flora [1, 2]. Oral

administration of an antigen can lead to peripheral immunological suppression, inhibiting both humoral and cellular immune responses, and is currently being considered as a therapeutic strategy for allergic desensitization and autoimmune disease treatments.

The mechanisms operating in generation and maintenance of oral tolerance are not well understood. Studies with genetically engineered knockout mice – genotype to phenotype approach – suggest a role for defined cytokines and their receptors, but the interpretation of these results has to be taken cautiously. As recently pointed out by Boyartchuk and Dietrich [3], the plasticity and redundancy of the immune system make difficult the experimental study on the genetic control of innate or adaptative components. The study of complex multi-loci systems, where genes cooperate to generate a quantitative spectrum of phenotypes, would benefit from a more appropriate experimental model, from phenotype to genotype, resulting in the selection of genes controlling the character. Moreover, this approach allows the general comprehension for the full genetic interaction that controls the phenotype.

To investigate the polygenic regulation of oral tolerance, we have generated, by bidirectional breeding, two lines of mice selected for susceptibility or resistance to oral tolerance to ovalbumin (OVA). From an original foundation population [F_0] consisting of a balanced intercross of eight different inbred mouse strains of distant origin (A, DBA/2, P, SWR, SJL, CBA, BALB/c, C57Bl/6), after 18 generations of consecutive assortative mating avoiding consanguinity, mice giving the best phenotypes for susceptibility (TS) or resistance (TR) to oral tolerance to OVA attained homozygosity for the relevant *ts* and *tr* loci controlling this quantitative trait. This character is under polygenic control and it was estimated that a minimum of eight or nine independent loci endowed with additive effects promoted the huge difference in the specific IgG response, 1000-fold higher titres, between susceptible [TS] and resistant [TR] oral tolerance phenotypes [4]. It is important to note that TS animals are not immunodeficient, as they respond vigorously to immunization in the absence of previous oral tolerance induction. On the other hand, TR mice are not totally resistant to tolerance, being able to down-modulate delayed type hypersensitivity responses [5]. Susceptibility to oral tolerance is not restricted to OVA, but is a general property of the TS strain, which is easily tolerizable for a series of distinct antigens [6]. Tolerance susceptibility for multiple immunogens suggests that the outcome of the genetic selection based on a single antigen could not be dissociated from a more profound alteration of the immune response. If the selected genes modify the immune function, it can affect other major immunobiological traits. Nevertheless, the goal of the genetic selection is to create mouse lines with extreme and easily differentiated phenotypes.

In the present study, we analysed the influence of the cumulated *tr* and *ts* genes on two important immunobiological traits that play a critical role in allergy: IgE serum levels and the anatomical and quantitative distribution of mast cells. The genetic correlation between susceptibility to oral tolerance to OVA, mucosal mast cell numbers and serum IgE levels, was studied in individuals of both parental TS and TR lines and their [TR $\times$ TS] F_2 hybrids expressing extreme oral tolerance phenotypes. The results clearly indicate that segregating genes controlling TS or TR phenotypes are the cause of correlation among these characters, showing, for the first time, a genetic correlation of oral tolerance, IgE serum levels and mastocyte numbers in naïve animals that has a profound impact on the atopic potential of the individual.

Materials and methods

Animals

Two- to 3-month-old mice of both sexes of the TS and TR strains were used [4]. Reciprocal crosses between the parental lines were mated for production of the F_2 segregant population. TR and TS mice were housed together in the animal facilities at the Cellular Biology and Genetics Department of Rio de Janeiro State University, under clean housing conditions. R/A rats (Leuvenhock) were utilized to dosage of specific IgE. Animals were caged and handled under ethical conditions according to international rules of animal care by the International Animal Welfare Recommendations, in accordance with the guidelines for the use of animals in biomedical research [7], and the Commission for Care and Use of Laboratory Animal of University of Rio de Janeiro, Brazil, approved the protocols of experiments.

Tissue fixation, staining and counting of mast cells

Mast cells were counted in stomach, small bowel submucosa, sub-epithelial bronchia, dorsal dermis, mesentery and peritoneal cavity cells. Mast cells from [TS $\times$ TR] F_2 -segregant mice were counted only on the mesentery. The stomach, intestine, lung and dermis tissues were fixed in Carnoy for staining in 0.5% toluidine blue in HCl 0.5 N for 30 min. The mesentery was fixed with a solution of 10% formaldehyde containing 1% toluidine blue and 0.1% acetic acid, and gently spread out on a glass slide. Each counting was made in 50 fields randomly chosen, and the results were expressed as mast cell numbers per mm² [8]. Peritoneal mast cells were obtained as follows: mice were killed and 2.0 mL of sterile, pyrogen-free 0.9% NaCl was injected into the peritoneal cavity. The abdomen was massaged gently and the peritoneal fluid was recovered. Cells were cytospun onto glass slides and stained with toluidine blue. Counting was performed under a light

microscope at a magnification of ($\times 400$). Each counting was made in 50 fields randomly chosen, and data were presented as the mean of mast cell numbers per mouse. Peritoneal mast cells were expressed as mean number $\times 10^4$ mast cells per mice. Data were analysed by the Image ProPlus program in an Olympus BX50 microscopy.

Immunization and oral tolerance induction

Mice were intraperitoneally (i.p.) immunized with 100 μ g of OVA (Sigma-Aldrich) in 1 mg alum, bled 28 days later and sera were maintained at -20°C until use. To induce oral tolerance, mice received 5 mg of soluble OVA in a single intragastric (i.g.) administration, 7 days before i.p. immunization; the control groups received saline.

Antibody titration

The individual IgG and IgE anti-OVA were measured by ELISA and purified preparations of IgG and IgE isotypes were used as standards (Southern Biotech., Birmingham, AL, USA). Microplates were coated with rabbit anti-mouse IgG or rat anti-mouse IgE (Southern Biotech.). After incubation with appropriate sera dilutions, goat anti-mouse IgG/horseradish peroxidase or rat anti-mouse IgE/horseradish peroxidase was added (Southern Biotech.). Tetra Methyl Benzidine (Zymed Lab. Inc, San Francisco, CA, USA) was the substrate for horseradish peroxidase and the reaction was read at 450 nm in a multiscan photometer. For antigen-specific IgG, the microplates (Polisorp, Nunc, Denmark) were previously coated with OVA. The anti-OVA agglutinin titres were determined by the passive haemagglutination test using sheep erythrocytes coupled with OVA [4]. OVA-specific IgE titres were determined by 48-h passive cutaneous anaphylaxis (PCA) as described previously [9]. Titres were expressed as $\log_2$ of the highest serum dilution giving a positive reaction.

Systemic anaphylaxis induction

Mice were i.p. immunized with 100 μ g of chicken egg OVA (grade V – Sigma-Aldrich, St Louis, MO, USA) in 5 mg alum. To induce anaphylactic shock, animals were further challenged with 100 μ g of soluble OVA by the intravenous route 21 days after immunization. The numbers of mice dead until 30 min after challenge were taken into account, and the results are given in percent of mortality.

Intestinal inflammation induction

Mice were immunized i.p. with 100 μ g of chicken egg OVA (grade V – Sigma-Aldrich) in 1 mg alum and were fed 7 days later with a 5.0 mg/mL solution of ovalbumin in

drinking bottles, 4 mL/mouse, for 1 week, corresponding approximately to 20 mg daily ovalbumin intake per mouse. This protocol is a modification of the murine model for the study of the onset of food allergy established by van Halteren et al. [10]. A second group of mice was fed 20 mg of soluble egg-white ovalbumin 7 days before i.p. immunization with 100 μ g of OVA/alum (oral tolerance protocol). Controls included normal animals, non-immunized mice fed with 20 mg of ovalbumin and mice only i.p. immunized with OVA/alum. Histological alterations and mast cell and eosinophil numbers were subsequently evaluated in the gastrointestinal tissues of the animals.

Pulmonary inflammation induction

The experimental asthma model was modified from Russo et al. [11]. Mice were sensitized on day 0 with an i.p. injection of 10 μ g chicken egg OVA (grade V – Sigma-Aldrich) and 2 mg alum in phosphate-buffered saline (PBS) to a total volume of 100 μ L and boosted on day 7 with OVA without adjuvant. This was followed by two intranasal OVA challenges on day 21 with 10 mg OVA/50 mL saline delivered into the nostrils under light ether anaesthesia with the aid of a micropipette, and administration of nebulized 5% OVA for 20 min on days 24 and 25. Nonsensitized control animals received only the PBS. The bronchoalveolar lavage (BAL) and lung collection for histology were obtained at 1 day after the last nebulization.

Bronchoalveolar lavage collection

The mice were killed with a lethal dose of pentobarbital. Tracheas were cannulated and lungs were washed twice with 0.5 and 1.0 mL PBS. A haemocytometer was used to count the total number of BAL cells. Cytospin preparations of BAL fluid (BALF) cells were stained with 'Panótico' Stain (for fast and differential count from Laborclin, Paraná, Brazil), and differential cell counts were performed on 200 cells on the basis of morphology and staining characteristics.

Histopathology

The intestines were removed, fixed with formaldehyde in PBS and prepared for staining with haematoxylin/eosin (H&E). Lungs were removed after BALF collection, perfused via the right ventricle with 1 mL of formaldehyde in PBS through the tracheal cannula and immersed in the same fixative 24 h and then in 70% ethanol. The intestines and lung tissues were embedded in paraffin and 5 μ m sections were cut and stained with H&E for light microscopy evaluation.

Statistical analysis

Differences between mean antibody levels and between mast cell numbers in different mouse groups were determined by Student's *t*-test and Mann-Whitney non-parametric test; groups were considered statistically different if $P < 0.05$. Statistical comparison of multiple groups was performed with ANOVA, Fischer's *F*-test; groups were considered statistically significant if $P < 0.05$.

Results

Mast cell numbers and serum Immunoglobulin E levels in TS and TR mouse strains

TS and TR lines show clear polarized phenotypes of susceptibility/resistance to oral tolerance [4, 5], as indicated by a 1000-fold difference in humoral response (IgG) to immunization with OVA, following oral tolerization protocol. This difference is not observed in non-tolerized and immunized animals that display equivalent antibody titres. Interestingly, we found that serum IgE levels differ markedly between non-immunized, naïve animals of both strains (Fig. 1a); approximately 20-fold higher titres of IgE are observed in TR mice when compared with TS mice. An inter-strain difference was not observed on total IgG [4, 5]. IgG1 is often associated with IgE, but we found that serum IgG1 concentrations were equivalent in naïve animals of both strains (Fig. 1b). Another striking dissimilarity between TR and TS lines was the magnitude of mast cell numbers in several tissues. Except for the skin, significantly lower mast cell counts were observed in TS mice for all other tissues, namely the stomach, intestine, lungs, peritoneal cavity and mesentery (Table 1).

Systemic anaphylaxis in TS and TR strains

To evaluate whether the strain differences in IgE level and mast cell numbers could have a biological impact on inflammatory responses, TS and TR mice were challenged

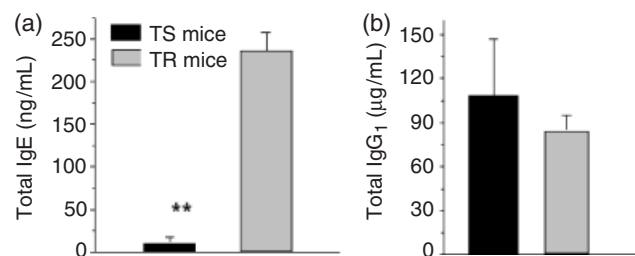


Fig. 1. IgE and IgG1 serum levels in normal TS and TR mice. (a) Naïve TS and TR mice were assayed for total serum IgE, and (b) for total serum IgG1 levels; a representative evaluation with five mice of each strain is shown. The mean values and standard deviations are shown in the figure. On average, TR mice showed an approximately 20-fold higher concentration of IgE when compared with TS mice.

Table 1. Mast cell numbers in TR and TS mice

Tissue	Mast cell numbers/mm ²	
	TR mice	TS mice
Stomach	50.0 (9.5)*	34.0 (3.1)
Jejunum	26.0 (5.2)*	11.0 (4.5)
Mesentery [†]	18.0 (1.5)***	8.6 (3.2)
Lung	4.5 (0.6)***	1.8 (0.4)
Peritoneum [‡]	2.3 (1.6)*	1.2 (0.6)
Dermis	44.6 (13.7)	41.1 (13.7)

Mast cell numbers were evaluated in different tissue sections of TR and TS mice/five animals per strain. The mean numbers are shown (numbers in parentheses indicate standard deviations). Statistical comparisons between TS and TR strains were performed with a Student's *t*-test;

* $0.01 < P < 0.05$, *** $P = < 0.001$.

[†]The experiments were performed four times on mesentery and twice in the other sites.

[‡]Mast cell numbers per mouse in the peritoneal cavity (means $\times 10^{-4} \pm$ SD).

in a model of antigen-driven systemic anaphylaxis, dependent on IgE and mast cells. Mice were i.p. immunized with OVA+Al(OH)₃, and further challenged with soluble OVA by an intravenous route 21 days after i.p. immunization. The results are shown in Fig. 2a. Both strains developed anaphylactic shock: mortality was 100% in TR and 60% in TS mice. Death in both strains occurred within 30 min after antigenic challenge. However, on receiving 5 mg of OVA i.g. 7 days before i.p. immunization, a full protection for the anaphylactic shock in TS mice was obtained, while the TR mice submitted to the same protocol died within 15 min after challenge (Fig. 2a). The IgE titres in immunized mice of both strains were scored by PCA, showing a strong reduction [30% of the control] in TS animals receiving OVA by the i.g. route; TR mice subjected to the same treatment showed a lesser but statistically significant inhibition of PCA (77% of the control; Fig. 2b).

Intestinal inflammation in TS and TR strains

Another model of inflammatory response was evaluated, directly associated with the gastrointestinal tract. Normal mice of both strains were divided into different groups and subjected to the following protocols of antigen administration: (i) Group I: mice fed with 20 mg/day of ovalbumin, for 7 consecutive days; (ii) Group II: mice fed with 20 mg/day of ovalbumin for 7 consecutive days and then immunized i.p. with OVA; (iii) Group III: mice immunized i.p. with OVA; and (iv) Group IV: immunized i.p. with OVA and then orally fed with 20 mg/day of ovalbumin. The protocol of Group IV is an established model of induction of intestinal inflammation [10]. Intestinal inflammation was only observed in animals of the TR strain of Group IV. Typical histological sections of

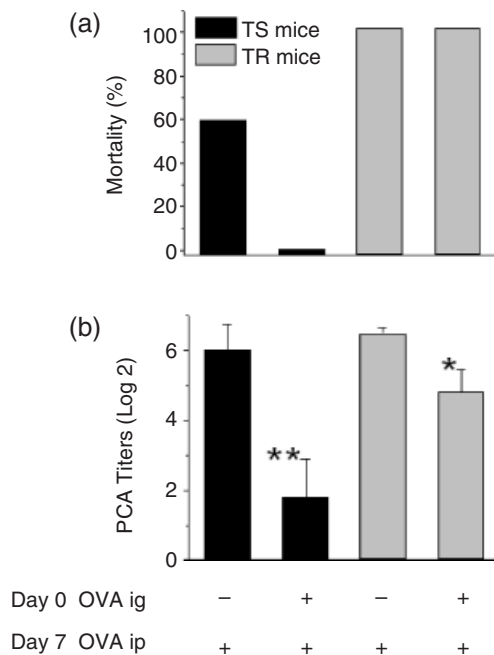


Fig. 2. Passive cutaneous anaphylaxis (PCA) titres and anaphylactic shock in TR and TS lines. Mice of both sexes ($n = 8$ mice per group) were subjected to an antigen-dependent (ovalbumin; OVA), systemic anaphylactic shock protocol; animals were intraperitoneally (i.p.) immunized with $100 \mu\text{g}$ of OVA in 5 mg Al(OH)_3 , and further challenged with $100 \mu\text{g}$ of soluble OVA by an intravenous route 21 days after i.p. immunization. Death was observed within 30 min of antigenic challenge in both strains. A group of TR, and another group of TS animals were orally pre-treated with 5 mg of soluble OVA in a single intragastric (i.g.) administration 7 days before i.p. immunization with OVA. (a) Percent survival of sensitized animals challenged i.p. with OVA. In orally pre-treated animals, only TR mice died; all TS animals that were orally pre-treated with OVA survived; (b) Specific IgE dosed by PCA. The mean values and standard deviations are shown in the figure. ($P < 0.001$, Student's t -test).

intestinal tissues, jejunum, are shown in Figs 3a and b. Cellular infiltrates with augmented number of eosinophils were obtained in Group IV where TR line expresses 3.8 ± 0.7 eosinophils/villous and TS mice 1.7 ± 0.6 eosinophils/villous. The total numbers of eosinophils/villous in control animals and Group IV mice are presented in Fig. 3e, indicating a $P < 0.02$ statistically significant difference between TR animals submitted to the intestinal inflammation protocol as established by Student's t -test. Eosinophil numbers are similar in naïve TS and TR mice and are within physiological limits.

The numbers of intestinal mast cells were also counted to investigate a possible relation between intestinal inflammation and the presence of augmented numbers of mucosal mastocytes. The results obtained are shown in Table 2. Mast cell numbers were twofold augmented in Group IV, whereas all the other groups did not differ significantly from the control group. Thus, previous immunization is necessary to augment mast cell numbers, showing that the exclusive mucosal contact with OVA, the

antigen used for the selection of TS and TR strains, is not sufficient to modulate mast cell population. Although mast cell numbers in TS mice of Group IV are higher than the TS control group, they are comparable to mast cell numbers in control TR animals, and much lower than mast cell numbers in TR mice in Group IV.

Pulmonary inflammation in TR and TS mice

An experimental asthma model was also evaluated in TR and TS mice. Bronchi with mucus and intense peribronchovascular inflammatory cell infiltration are more evident in TR when compared with TS mice (Figs 3c and d). Eosinophils were counted in cytospin preparations of BALF from TR and TS mice submitted to OVA-induced airway inflammation with huge differences between strains: $\text{TR} = 146.75 \pm 29.19 \times 10^4$ and $\text{TS} = 5.4 \pm 12.07 \times 10^4$ eosinophils/mL (Fig. 3f).

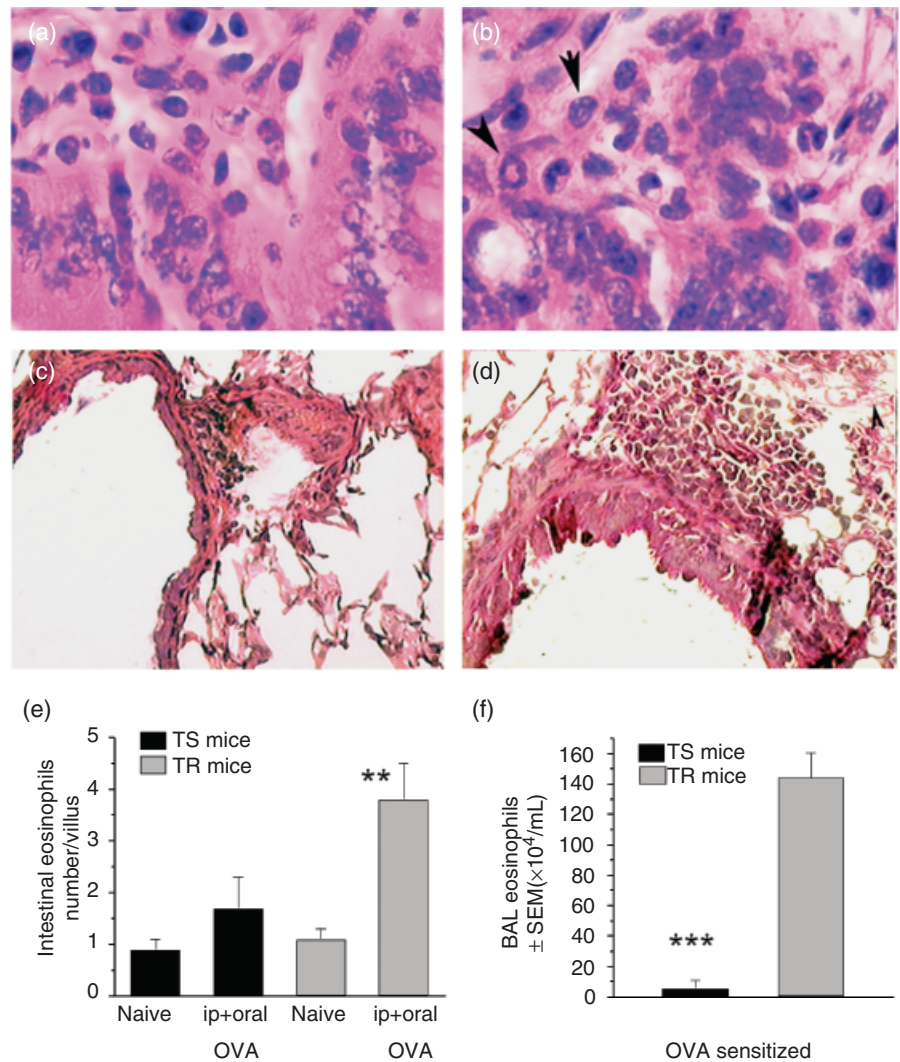
Genetic correlation between oral tolerance and mast cell numbers

As the TS/TR lines exhibited low/high IgE levels and low/high mucosal mast cell numbers, respectively, we investigated whether the presence of these associated traits was, at least in part, controlled by the same set of genes, or by genetically independent loci being fortuitously associated with susceptibility to oral tolerance by genetic drift during the selective process of TS and TR lines. We first investigated whether mast cell numbers correlate with oral tolerance phenotypes in a $[\text{TS} \times \text{TR}]F_2$ -segregating population. The F_2 mice were submitted to an oral tolerization protocol with OVA, and subsequently i.p. immunized with this immunogen. The IgG antibody titres of 92 F_2 mice were then determined by passive agglutination and confirmed by ELISA. The mean antibody titre in this F_2 population was $10.31 \pm 3.27 \log_2$, and the frequency distribution of the anti-OVA titres is shown in Fig. 4a. The F_2 animals with extreme phenotypes to oral tolerance were selected for mast cell counting. The eight mice chosen for their lower antibody titres had a mean agglutinin titre of $4.0 \pm 1.5 \log_2$ and a mean mast cell count of $11.3 \pm 4.0/\text{mm}^2$. The eight F_2 mice chosen as resistant expressed mean antibody titre of $16.2 \pm 2.8 \log_2$ and a mean mast cell count of $16.0 \pm 2.9/\text{mm}^2$. The correlation between both parameters is shown in a scatter plot in Fig. 4b. The difference in mast cell numbers between these two groups was statistically significant ($P < 0.04$, Mann-Whitney non-parametric test), strongly suggesting that physiological mast cell numbers are certainly correlated with susceptibility to oral tolerance.

Genetic correlation between total Immunoglobulin E level and mast cell numbers

Analogously, the simultaneous expression of low IgE levels/low mast cell numbers and high IgE levels/high

Fig. 3. Cellular infiltrate in gastrointestinal and pulmonary inflammation models. (a–d) Photomicrographs of representative haematoxylin/eosin-stained murine gastrointestinal tissue, jejunum (Group IV – see text) and lung sections from TR and TS animals submitted to protocols of inflammation. (a) TS mouse: no eosinophils were found in this section ($\times 1000$ magnification); the average number is 1.7 ± 0.6 eosinophils/villus (six animals were analysed). (b) TR mouse: eosinophils are indicated with arrows in the figure ($\times 1000$ magnification); the average number is 3.8 ± 0.7 eosinophils/villus (six animals were analysed). (c and d) Bronchi with mucus and intense peribronchovascular inflammatory cell infiltration are more evident in TR mice (d; $\times 400$ magnification). (e) Numbers of eosinophils/intestinal villus in control TS and TR animals, compared with Group IV TS and TR animals. (f) Number of eosinophils on bronchoalveolar lavage fluid (BALF) on the basis of morphology and staining characteristics from TR and TS mice sensitized with OVA. Results are expressed as means $\pm$ standard error of the mean (SEM). ANOVA, Fischer's *F*-test showed that the values obtained for TS mice are statistically different from TR mice. The statistical significance is indicated in the (e and f): $0.001 < P < 0.01$ (**); $P = < 0.001$ (***).



mastocyte numbers was investigated in the [TS $\times$ TR] F_2 -segregating population. Figure 5a shows the distribution of IgE levels in 51 naïve F_2 mice. Animals in both extremes of the curve were selected for mesentery mast cell counting, and Fig. 5b reveals a clear correlation between IgE levels and mastocyte numbers, the highest IgE producers being those that exhibit the highest mast cell numbers, and the lowest IgE responders being those with the lowest mast cell constitution. This difference was highly significant ($P < 0.01$, Mann–Whitney non-parametric test).

Discussion

In the present study, we clearly showed that the TS and TR mice, genetically selected for susceptibility/resistance to oral tolerance, also exhibited profound differences in serum IgE levels and mucosal mast cell numbers (Table 1, Fig. 1). These differences were already present in naïve animals, neither immunized nor tolerized. Analysing animals in both extremes of the distribution curve of

phenotype of the [TR $\times$ TS] F_2 population, we found that: (i) individuals with higher susceptibility to oral tolerance had reduced mucosal mast cell numbers, whereas individuals with higher resistance to oral tolerance had higher mucosal mast cell numbers; (ii) individuals with lower IgE had reduced mucosal mast cell numbers, whereas those with higher IgE levels had increased mucosal mast cell numbers (Figs 4 and 5). Together, these results rule out the explanation of character association by genetic drift of unlinked loci. This correlation of phenotypes can thus only be explained either by a close genetic linkage of the loci controlling the three characters, or by the same set of genes simultaneously influencing the three characters.

In artificial selection of quantitative characters, linkage is considered as a cause of transient correlation [12], and can be important when the loci controlling the quantitative trait are restricted to a few chromosomes or genome regions of low recombination. When the loci affecting the phenotype are spread on the genome, the genetic model of unlinked loci is a more valid assumption [13]. It has been

Table 2. Mast cell numbers in the intestinal inflammation model

Treatments	Mast cell numbers (SD $\pm$ mm ²) strains of mice	
	TR	TS
CTR-saline (5)	18.0 $\pm$ 1.5***	8.6 $\pm$ 3.2
Group I: oral OVA (10)	18.4 $\pm$ 3.7**	10.4 $\pm$ 3.2
Group II: oral OVA+i.p. OVA (10)	18.8 $\pm$ 7.9**	10.3 $\pm$ 3.6
Group III: i.p. OVA (10)	17.4 $\pm$ 3.4*	13.3 $\pm$ 3.4
Group IV: i.p. OVA+oral OVA (10)	29.6 $\pm$ 11.4**	16.1 $\pm$ 1.2

TR and TS mice were subjected to a protocol to induce antigen-dependent (OVA) intestinal inflammation (Group IV, see text). Control groups included animals injected with saline (CTR), OVA orally (Group I), OVA i.p (Group III), OVA orally followed by OVA i.p (Group II). The intraperitoneal immunization was performed with 100 μ g OVA + 1 mg Al (OH)₃. The oral total intake for 7 consecutive days was estimated as 20 mg/day of soluble OVA (5 mg/mL) in a drinking bottle. The time delay between treatments with OVA by different routes, oral followed by intraperitoneal or intraperitoneal followed by oral, was 7 days. The mast cells were always counted 1 day after the last treatment. The number of mice per group is indicated in parenthesis. Statistical analysis comparing groups within the same strain was performed with ANOVA, Fischer's *F*-test. For TR strain, Group IV was considered as different from all the other groups ($P < 0.0004$), which do not differ statistically among them. For the TS strain, Group IV was considered as different from all the Groups I, II and CTR ($P < 0.002$), which do not differ statistically among them. The inter-strain difference was evaluated with Student's *t*-test, and the statistical significance is indicated in the table.

*0.01 $< P < 0.05$, **0.001 $< P < 0.01$, *** $P = < 0.001$.

OVA, ovalbumin.

estimated that oral tolerance may be influenced by a minimum of eight or nine independent loci [4]. Therefore, it may be considered that the correlations of the phenotypes observed are due to the pleiotropic action of genes [12, 13], whereby the segregating gene causes simultaneous variation in multiple characters.

The analysis of the [TS $\times$ TR]F₂-segregant population showed that there was a strict association of phenotypes between high serum IgE/high mast cell numbers (H-IgE/HMC) and resistance to oral tolerance. In the reciprocal situation, only some individuals with low serum IgE/high mast cell numbers (L-IgE/HMC) were susceptible to oral tolerance. This is in agreement with the demonstration that resistance to oral tolerance is a dominant character in [TR $\times$ TS]F₁ hybrids, suggesting that tolerance susceptibility is a delicate assembling of genes that is easily disrupted by the introduction of different genes from the RT lineage, as in heterozygous F₁ and F₂. Moreover, it is known that experimental selection to metric characters almost always results in a reduction of one or more of the components of fitness [12]. Consequently, animals with extreme phenotypes exist at a very low frequency in natural populations and are difficult to produce by arti-

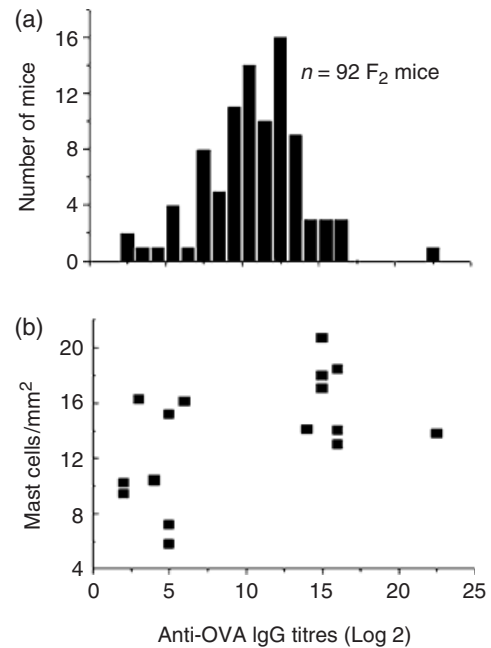


Fig. 4. Genetic association between mast cell numbers and susceptibility to oral tolerance. (a) Histogram of the distribution of anti-ovalbumin (anti-OVA) serum IgG titres in the (TS $\times$ TR)F₂ population, orally tolerized to OVA and subsequently immunized with that antigen. Serum anti-OVA IgG titres were determined by passive agglutination of OVA-coated erythrocytes. The mean antibody titre in the F₂ population was 10.31 ± 3.27 (log₂). The animals on both extremes of the distribution were selected for mast cell counting shown in Fig. 4b. (b) Scatter plot of the mast cell number vs. serum IgG anti-OVA. The eight mice chosen based on low IgG anti-OVA serum levels had a mean titre of 4.0 ± 1.5 (log₂) and a mast cell count of 11.3 ± 4.0 /mm². The eight mice with higher IgG anti-OVA serum levels had a mean titre of 16.2 ± 2.8 (log₂) and a mean mast cell count of 16.0 ± 2.9 /mm². Mast cell numbers in mice from Low IgG anti-OVA and High IgG anti-OVA differ significantly ($P < 0.04$, Mann-Whitney non-parametric test).

cial selection; interestingly, low fertility and infecundity have been observed in TS mice during the selective process. Therefore, mice with extreme tolerance susceptibility phenotypes may not be produced in sufficient numbers, such that the strict correlation observed for the high extreme of the phenotype curve cannot be observed for the low extreme, as for the data presented in Figs 4 and 5. The lower response to selection in TS [4] supports the notion that susceptibility to oral tolerance has a higher adaptive value and has been fixed in wild populations. Probably, oral tolerance, as a fitness component, was close to the optimal value in the foundation population used to derive TS and TR strains.

These results raise the question of the mechanisms leading to the association of the studied phenotypes. The correlation between low serum IgE and susceptibility to oral tolerance, observed in naïve TS animals, is consistent with previous data obtained with immunized mice [8, 9, 14]. Indeed, serum IgE in normal animals may represent the background allergic response to environmental antigens

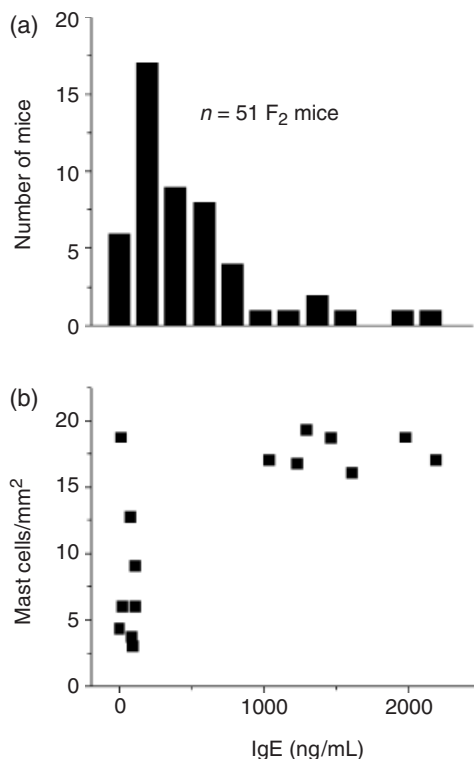


Fig. 5. Genetic association between mast cell numbers and serum IgE level. (a) Histogram of the distribution of serum IgE levels in naïve mice of the (TS × TR)_{F2} population. The IgE mean value and standard error of the F₂ population was 514 ± 497 ng/mL. Eight animals on the lower extreme and seven animals on the higher extreme of the distribution were selected for mast cell counting. (b) Scatter plot of the mast cell number vs. serum IgE levels. The eight mice chosen based on low IgE level had a mean of 6.36 ± 4.37 ng/mL and a mast cell count of 7.92 ± 5.38 /mm². The seven mice with a higher IgE level had a mean of 1564 ± 443 ng/mL and a mast cell count of 17.62 ± 1.24 /mm². Mast cell numbers in mice from Low IgE and High IgE differ significantly ($P < 0.01$, Mann–Whitney non-parametric test).

that come into contact with the immune system via the intestinal and respiratory mucosas. As it has been shown that synthesis of IgE is highly sensitive to negative modulation by oral tolerance (Fig. 2b) [8, 9, 14], the association of both characters in naïve animals of the TS line would be coherent with their general compliance to oral tolerance induction. TR mice show little suppression of IgE production upon oral administration of the antigen (Fig. 2b); thus, the inhibition of IgE production to common environmental antigens is very likely absent in naïve TR animals, possibly explaining their higher serum IgE levels.

A regulatory feedback between IgE levels and mast cell has recently been described. Asai *et al.* [15] and Kalesnikoff *et al.* [16] showed a role for IgE in the survival of mastocytes, protecting these cells from apoptosis *in vitro*. This effect does not depend on the cross-linking to the FcεRI [17], but only on the binding of IgE to mast cell surface IgE receptor. It is possible that an elevated level of serum

IgE would promote augmented mast cell half-life *in vivo*, resulting in a higher accumulation of mast cells in normal TR mice, compared with normal TS mice. Evidence, *in vivo*, has been produced that can support this interpretation [15]. The association of serum IgE levels with mastocytosis is well documented under pathological conditions, where inflammation is a hallmark of the disease. To our knowledge, the genetic association of these characters have not been reported in naïve animals; thus, our results extend this notion to healthy animals, showing that the association of both phenotypes is also present under physiological conditions.

Natural oral tolerance is thought to be fundamental in the control of hypersensitivity and deleterious inflammatory reactions to dietary proteins and normal intestinal flora, and the data presented here show that selection against this character can have biological consequences, which go well beyond food intolerance. TS strain was found to be fully protected from anaphylactic shock if the antigen is administered orally before i.p. immunization, whereas 100% death was observed in TR mice (Fig. 2a). The oral tolerization protocol is known to inhibit antigen-specific IgE production and can thus explain the protection against anaphylaxis in TS animals (Fig. 2b) [8, 9, 14]. In TR mice, the oral ingestion of OVA before i.p. immunization does not result in blockade of antigen-specific IgE production (Fig. 2b). The quantitative differences in mast cell numbers between normal TR and TS do not impair mast cell responses in the absence of oral tolerance induction, as both TS and TR mice exhibit anaphylactic shock in the absence of oral tolerization, showing that the dramatic difference in the inflammatory response in animals that received oral antigen before challenge cannot be ascribed only to differences in physiological mast cell numbers. The results described here strongly support the relevance of the physiological contacts with antigens entering by the oral route in defining the effector type of the response to subsequent encounters with that antigen. This process should have a critical impact on the immune response to infectious and non-infectious agents because of the large diversity of natural antigens reaching the mucosas, rendering cross-reactivity almost unavoidable.

The TR mouse strain can be an effective model for a better understanding of the reasons why the oral contact with antigen can result in inflammatory intestinal diseases [18]. Preliminary results indicate that the TH1/TH2 paradigm does not apply to TR/TS strains (not shown). Further genetic studies with TR and TS strains can contribute to the identification of new critical genes controlling oral tolerance and its relation to atopy [3].

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