

Diet selection in immunologically manipulated mice

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Abstract

Diet selection is a complex problem that animals in wildlife have to deal with daily. In their natural environment, these animals meet a great variety of foods some of which they are able and prepared to eat, yet, not all of it is eaten. In addition to the biological factors, some of which we shall discuss deeper in this paper, an important factor in food preference is social contact. Alterations in the physiology of mammals can have profound effects on the choice or preference for certain foods. On the other hand the decline of taste and smell perception in the elderly, the degree of food restriction, the sensorial properties of foods (such as presentation, taste, and smell) can be considered factors that influence feeding behavior leading to aversion. Many species, including man, learn to associate nausea with taste, and as a consequence avoid its specific intake, which has been shown to be persistent. Conditioned taste aversion is a form of associative learning in which animals display an aversion to the taste of a food that has previously been paired with illness. Our group has investigated the pattern of ingestion of foods that are frequently eaten by mice in wildlife and are potentially allergenic to humans in order to study the immunological consequences to these foods such as oral tolerance and inflammatory processes of the gut. We have chosen two seeds, peanuts (*Arachis hypogaea*) and cashew nuts (*Anacardium occidentale*), as our source of antigens as the first is considered to be one of the most potent food allergens and for the second there seems to be very little allergy in the human setting. We used male and female, normal, adult CBA/J, A/J, C57BL/6 and Balb/c mice 2–3 months old and hybrid (C57BL/6 × Balb/c) F1, (Balb/c × C57BL/6) F1, (C57BL/6 × DBA2) F1 mice. Food preference appeared to be strain-specific. Animals tolerized to a determined seed, then immunized with its protein extract and re-exposed to the seed in natura alter their feeding pattern. We suggest that diet selection, a multi-factorial event, is influenced by genetic factors such as the MHC and the immunological status of the animal.

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Keywords: Cashew nuts; Diet selection; Food allergy; Gut inflammation; Oral tolerance; Peanuts

Introduction

Diet selection is a complex problem that animals in wildlife have to deal with daily. In their natural

environment, these animals meet a great variety of foods some of which they are able and prepared to eat, yet, not all of it is eaten. In addition to the biological factors, some of which we shall discuss deeper in this paper, an important factor in food preference is social contacts or factors (Galeff and Whiskin, 1992). It has been documented that young rats quickly acquire the

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same food preferences of the adults in their social group (Bilkó et al., 1994). In humans, cultural factors, such as religious and familial factors, also have an important role in determining preferences and diet selection (Birch et al., 1982; Booth, 1985). One of the principles of this behavior is that animals select foods in a way which is most suitable for their well-being. From what is available, they select what is most adequate for their nutritional necessities and avoid the intake of foods that may be harmful.

Food preferences have been shown, in children, as of birth. Tastiness is associated with various factors, such as: the composition of the basic tastes, the necessity of ingesting salt because of the importance of sodium in physiological processes (Birch et al., 1982); the liking of *umami*, a taste associated with foods of animal origin (they are, thus, ingesting essential amino acids) (Nowlis and Kessen, 1976; Steiner, 1987); and the preference for sweet while rejecting bitter tastes. These differences in acceptability are considered to be adaptive characteristics (Glendinning, 1994; Rozin, 1976; Rozin and Vollmecke, 1986). Nevertheless, humans and other species have learned to appreciate bitter tastes, maybe because of their association with certain pharmacological effects, for example caffeine present in foods such as coffee, tea, chocolates, and others (Mattes, 1994). These primary tastes are maintained and distinguished even in complex foods giving each food its unique taste and influencing preference (Bartoshuk, 1993).

Alterations in the physiology of mammals can have profound effects on the preference for certain foods. On the other hand, the decline of taste and smell perception, the degree of food restriction, the sensorial properties of foods (such as presentation) can be considered factors that influence feeding behavior leading to aversion (Coward et al., 1994). Therefore, preference and aversion seem to be associated to hedonic alterations of taste.

Conditioned taste aversion is a form of associative learning in which animals of many species, including man, display an aversion to the taste of a food that has previously been paired with illness (Garcia et al., 1974). In contrast to other types of classical conditioning, where both stimuli must be concomitant. Although time dependent in food aversion, even intervals lasting several hours between nausea and the novel taste lead to aversion of a specific gustatory stimulus (Welzl et al., 2001). Cara et al. (1994) have shown that animals immunized to a specific food antigen will avoid its ingestion and suggest that this behavior is related to specific antibody concentration and to gut alterations due to local immunological alterations frequently observed in food allergies (Cara et al., 1994).

Wild mice in urban areas prefer cereals over foods containing high levels of proteins and fat such as meats and as a last choice they will select foods with high sugar content (Rozin, 1976). Our group has investigated the

pattern of ingestion of foods that are frequently eaten by mice in wildlife and are potentially allergenic to humans in order to study the immunological consequences of these foods, such as oral tolerance and inflammatory processes of the gut. We have chosen two seeds, peanuts and cashew nuts, as our source of antigens once the first is considered to be one of the most potent food allergens (Palmer and Burks, 2006) and for the second there seems to be very little allergy in the human setting.

Material and methods

Mice

Male and female, normal, 2–3- or 6-month-old adult CBA/J, A/J, C57BL/6J and Balb/c mice, bred and maintained at our facilities, were used. Hybrid (C57BL/6J × Balb/c) F1, (Balb/c × C57BL/6J) F1 and (C57BL/6J × DBA2) F1 mice were provided by the animal facility of the Federal Fluminense University – RJ (Núcleo de Animais de Laboratório – Departamento de Immunobiologia, Instituto de Biologia da Universidade Federal Fluminense).

Antigens and antigen extracts

The raw peanuts (*Arachis hypogaea*) and cashew nuts (*Anacardium occidentale*) were used as tolerogenic agents and their protein extracts as the immunizing antigens. To obtain the protein extracts the seeds were minced in an electrical coffee grinder sieved and defatted with ether. To the defatted seed preparation extraction buffer (sodium borate, 0.0125 M, SDS 1%, mercapto-ethanol 1% – pH 10) was added maintaining a 1:10 w/v ratio. This mixture was then placed on a rocker for 30 min at room temperature, and centrifuged at 3000 rpm for 30 min. The supernatant was collected and kept frozen at –20 °C until use.

Food intake

Purina Mouse chow, peanuts and cashew nuts *in natura* were mixed and offered to the animals *ad libitum* in weighed amounts daily for 1, 3 or 10 days. The animals were free to choose what to eat. An equal number of mice were fed mouse chow alone and used as controls.

After 24 h of free consumption the remains of each diet component was separated and weighed. The amount eaten was evaluated by dividing the difference of what was offered from what was eaten by the total weight of the animals in each group. The result was expressed as milligrams of food per gram of body weight (mg/gbw). All animals were weighed weekly.

Parenteral immunization protocol

Two days after the removal of the seeds from the diet, all groups of animals received parenteral immunizations. Primary immunization consisted of a dorsal subcutaneous (sc) injection of 0.2 ml of saline (0.15 M NaCl) containing 100 µg of protein extract plus 1 mg of Al(OH)₃. Secondary immunizations consisted of sc injection of 100 µg of soluble antigen in 0.2 ml of saline, given 21 days after the primary immunization. Blood samples were collected from the retro-orbital plexus 7 days after immunizations, diluted 1:10 v/v in saline, centrifuged 1500 rpm, serum was separated and stocked –20 °C until assayed.

Serum antibody assay

Antibodies were assayed by enzyme-linked immunosorbent assay (ELISA) using either peanut extract or cashew nut extract as the coating protein. Polystyrene microtiter plates were sensitized with 50 µl containing 2 µg of peanut or cashew nut extract diluted in 0.06 M sodium carbonate-bicarbonate buffer pH 9.6. The plates were incubated overnight at 4 °C and washed three times with phosphate-buffered physiological saline containing 0.05% Tween 20 (PBS/T). After washing coated plates were blocked with PBS containing 1% gelatin (PBS/G) for 2 h at room temperature (RT). After another single wash of 50 µl of serum diluted in PBS/G was added and incubated for 3 h at RT. Serial dilutions of the serum were performed in double dilution starting at 1/100. The plates were washed six times and goat mAb anti-mouse Ig (H+L) peroxidase-conjugated (Sigma) was added. After a 3 h incubation period at RT and six washes, 100 µl of 0.25 mg/ml *o*-phenylenediamine in 0.1 M citrate phosphate buffer, pH 5.0, containing 0.03% hydrogen peroxide was added. The plates were incubated at room temperature for 10 min. The reaction was stopped with 50 µl of 3 N sulphuric acid. Absorbance was read at 492 nm. The results are reported as mean ± SD of the ELISA (area under the curve of absorbance values read between 1:100 and 1:12,800 serum dilutions) of each individual serum (Faria et al., 1993).

Statistical analysis

The data were analyzed using ANOVA and Student's *t*-tests.

Results

Intake of peanuts or cashew nuts by normal adult mice

Groups of 5 or 6 male or female mice of the different strains were offered, separately, mouse chow, mixtures of

chow and peanuts or chow and cashew nuts. The intake was evaluated daily for 4 weeks. In each group, the daily variation of the amount of ingested food is not very big. Fig. 1A demonstrates an example of this behavior for two different strains (Balb/c and C57BL/6J). In all animals exposed to the mixtures of seeds and chow, the intake of chow dropped to approximately 20% of their regular intake ($p < 0.001$). There was also a significant reduction of the total amount of food intake in the groups exposed to the mixture of seed and chow. This was due to the higher caloric value of the seeds (Table 1), since the total caloric intake was kept constant (Fig. 1B).

Preference between peanuts and cashew nuts in normal mice

Mouse strains can be grouped into three categories: those that prefer peanuts, those that prefer cashew nuts, and those that eat equally both seeds. No strain preferred to eat the commercial mouse chow. C57BL/6J and C57BL/10J mice prefer peanuts to cashew nuts ($p < 0.001$) whereas Balb/c, DBA/2 and CBA mice prefer cashew nuts to peanuts ($p < 0.001$). In all animals studied the seeds substituted more than 80% of the chow (Fig. 2).

F1 generations of C57BL/6J × Balb/c (CB6F1 and B6CF1) and C57BL/6J × DBA/2 (B6D2F1) were generated to study any possible genetic inheritance or maternal influence in food preference. We tested both sexes of these F1 mice. None of these animals showed a significant preference for peanuts or cashews, although they significantly preferred the seed to mouse chow ($p < 0.001$, Fig. 3).

Preference between the seeds is not as clear-cut in younger mice when compared to older ones. When the daily intake of the seeds and chow of individual young (8 weeks) and older animals (24 weeks) was evaluated for 5 consecutive days, younger mice varied more significantly from day to day than the older ones. On the other hand, the variation of the intake of peanuts, the seed which this mouse strain prefers, was not as expressive (Fig. 4).

Immunogenicity of protein extracts

The immunogenicity of the protein extracts was tested in four inbred strains of mice (Balb/c, C57BL/6J, CBA/J, A/J) and three hybrid strains (CB6F1, B6CF1 and B6D2F1). As shown in Fig. 5, total protein extract from peanuts and cashew nuts are immunogenic in inbred strains tested, all responding with high levels of circulating antibodies ($p < 0.001$) as compared to saline controls. The same results were obtained in all F1 mice tested. No differences were observed when comparing female to male mice in any of the strains nor when comparing young adult (2–4 months) mice to older (12–16 months) mice (data not shown).

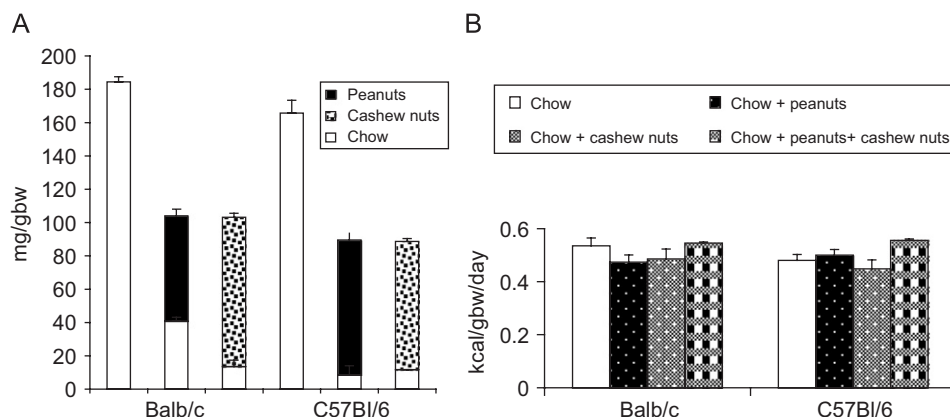


Fig. 1. (A) Typical result of the mean $\pm$ SD of daily consumption of peanuts, cashew nuts and mouse chow. Comparison between the offer of chow, and chow with peanuts or chow with cashew nuts over a 4-week period for groups of five adult (2–3 months) normal male mice. The results for other strains and for females are essentially the same. (B) Mean caloric consumption in groups of five adult (2–3 months) male mice of two different inbred strains exposed to chow, or simultaneously to chow and one seed or chow and two seeds over a period of 10 days. Identical results (not shown) were obtained for females and other strains (representative of three experiments).

Table 1. Nutritional composition of peanuts, cashew nuts and mouse chow

	Calories ^a	Proteins ^b	Lipids ^b	Carbohydrates ^b
Peanuts	579.7	29.31	48.75	5.94
Cashew nuts	556.2	17.89	37.00	37.92
Mouse chow	282.0	22.00	4.00	48.00

Franco (1982).

^aExpressed as cal/100 g and %.

^bExpressed as g% (w/w).

Induction of oral tolerance to peanuts and cashew nuts *in natura*

Mice which had free access to one of the seeds mixed with chow for 7–10 days and then immunized 2 days after removing the seeds showed significantly lower ($p < 0.001$) antibody titers than control mice which did not ingest the seeds (Fig. 6). Shorter periods of seed exposure (even 24 h) were sufficient for induction of oral tolerance in adult mice (data not shown).

Specificity of oral tolerance

Groups of mice were offered peanuts, cashew nuts or both seeds mixed with chow for 7 days; control mice received only chow. One group of animals was immunized with peanut protein extract, another with cashew nut protein extract. Animals that ate peanuts and were immunized with peanut extract had lower antibody levels (oral tolerance) but responded normally to cashew; the same happened to mice which ate cashew nuts: they became less responsive to cashew extract, but responded normally to peanut. Mice that ate both seeds became tolerant to both proteins when compared to animals that ate only mouse chow (Fig. 7).

Food preference of normal, tolerant and immune mice

We investigated further whether immunization or tolerance had influence on diet selection. To determine whether or not oral tolerance or systemic immunization interferes in diet selection, animals were offered a diet composed of peanuts, cashew nuts and chow after being

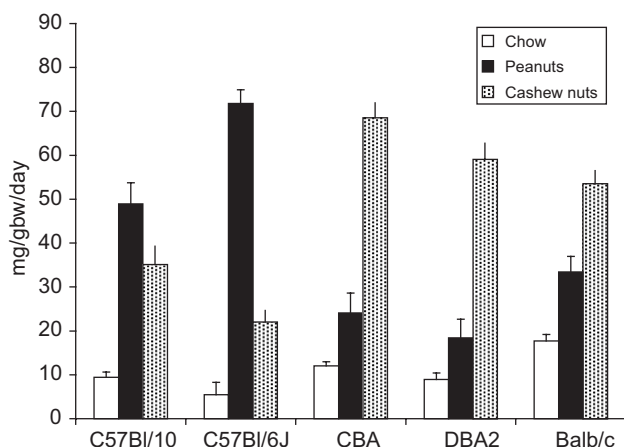


Fig. 2. Mean proportional consumption of chow, peanuts and cashew nuts in groups of five adult (2–3 months) male mice of six different inbred strains exposed simultaneously to chow, peanuts and cashew nuts over a period of 10 days. Identical results (not shown) were obtained for females (representative of three experiments).

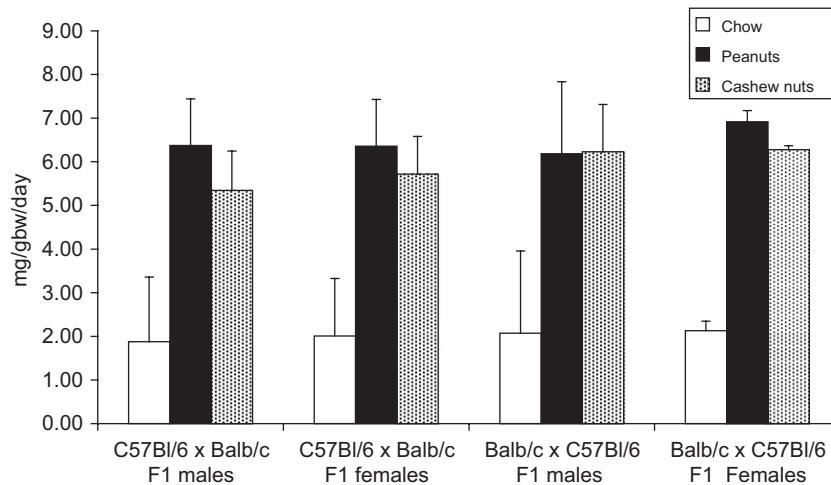


Fig. 3. Mean proportional consumption of chow, peanuts and cashew nuts in groups of five adult (2–3 months) C57Bl/6 × BALB/c F1 and BALB/c × C57Bl/6 F1 male and female exposed simultaneously to chow, peanuts and cashew nuts over a period of 10 days. Identical results (not shown) were obtained for B6D2F1 mice (representative of two experiments).

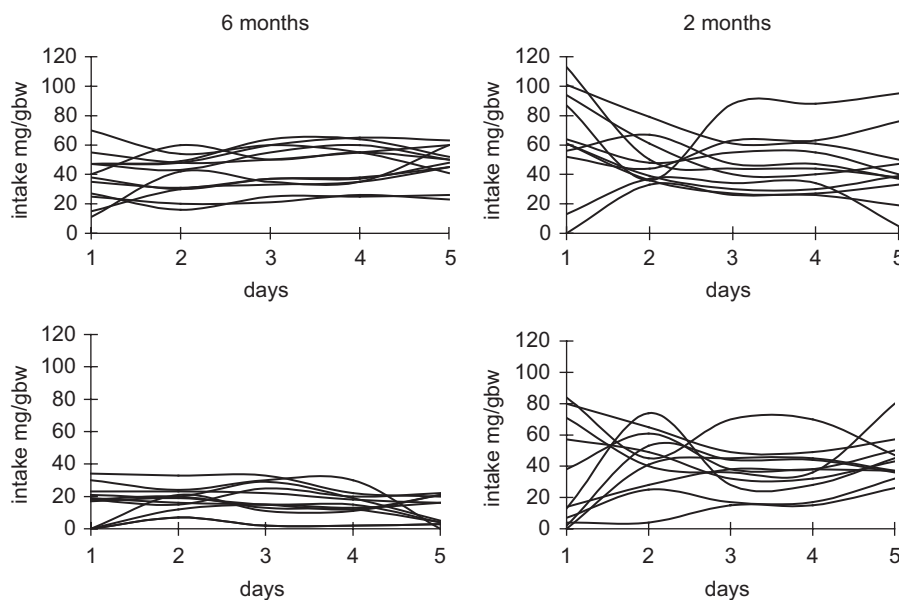


Fig. 4. Individual intake of peanuts (superior panels) and cashew nuts (lower panels) for male C57Bl/6 normal young adults (2 months) and older adult (6 months) over a 5 consecutive day period (representative of three experiments).

submitted to the tolerization or immunization protocols. Fig. 8 demonstrates that ingestion prior to immunization, which induces oral tolerance, does not interfere in the pattern of food preference. On the other hand, systemic immunization has profound effects on diet selection of these animals. Panel A demonstrates that C57BL/6J mice, which prefer peanuts, when systemically immunized to this seed show a clear shift in food preference, eating significantly less peanuts than cashew nuts. Panel E shows the same behavior of Balb/c mice

towards cashew nuts when the immunizing protein is derived from their seed of choice.

When the systemic immunization is towards the food which is not the preferred seed for the specific strain, then no significant alteration can be seen in the feeding behavior (Fig. 8 panels B–D).

F1 mice do not behave in a clear-cut manner although they present a significant reduction in the intake of the specific protein to which the animals are immunized, differently from the parental strains. These

animals do not compensate their ingestion with the other seed but with mouse chow as shown in Fig. 8 panels C and F.

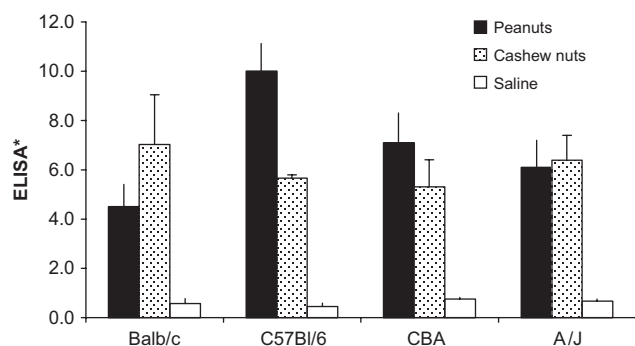


Fig. 5. Mean secondary antibody titers of different strains of mice ($n = 6$) have been immunized sc with 100 μ g of peanut protein extract, cashew nut protein extract or saline. Animals immunized with the seed extract show significantly ($p < 0.001$) higher antibody titers than those immunized with saline (representative of three experiments).

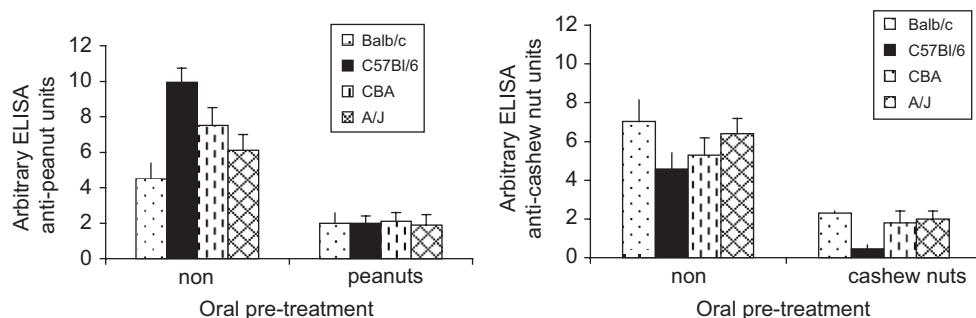


Fig. 6. Mean secondary antibody titers of different strains of six mice pre-treated or not with the seed to which they have been immunized sc with 100 μ g of the antigen. (Right panel peanuts, left cashew nuts.) Animals pre-treated with the seed show significantly ($p < 0.001$) lower antibody titers than those that did not eat the seed beforehand (representative of three experiments).

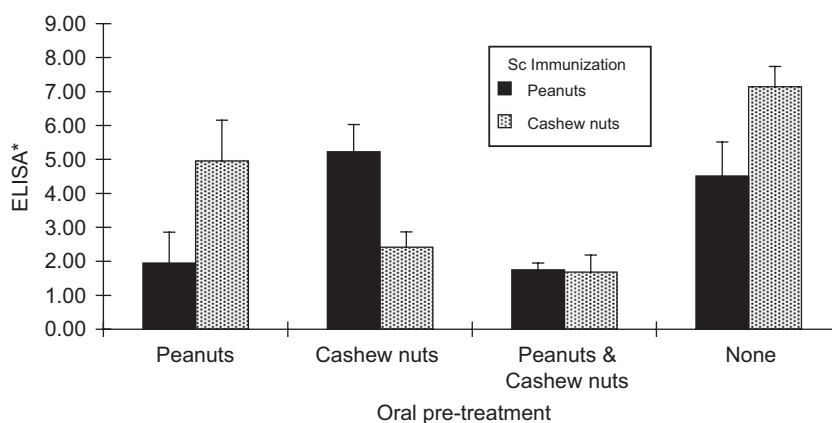


Fig. 7. Mean secondary antibody titers of six Balb/c mice pre-treated with the one, both or no seed and then immunized sc with 100 μ g of the peanut or cashew nut antigens. Animals which were fed the seed to which they were immunized showed significantly lower ($p < 0.05$) antibody titers compared to those animals which were fed the other seed or no seed at all (representative of two experiments).

Relation between antibody titers and food preference

After submitting a group of animals to the immunization protocol to peanut, these animals were housed individually so that their food intake could be scored individually. Fig. 9 shows a negative correlation between antibody titers and peanut intake where animals with the higher antibody titers show the lowest peanut intake ($p < 0.001$).

Relation between histopathological findings and food preference

To determine whether or not oral tolerance or systemic immunization interferes in the mucosal architecture of the gut after exposure to challenge diet composed of peanuts, cashew nuts and chow, all animals were submitted to necropsy for macroscopic and microscopic evaluation.

Macroscopic evaluation after the challenge diet showed that animals from the tolerant group do not

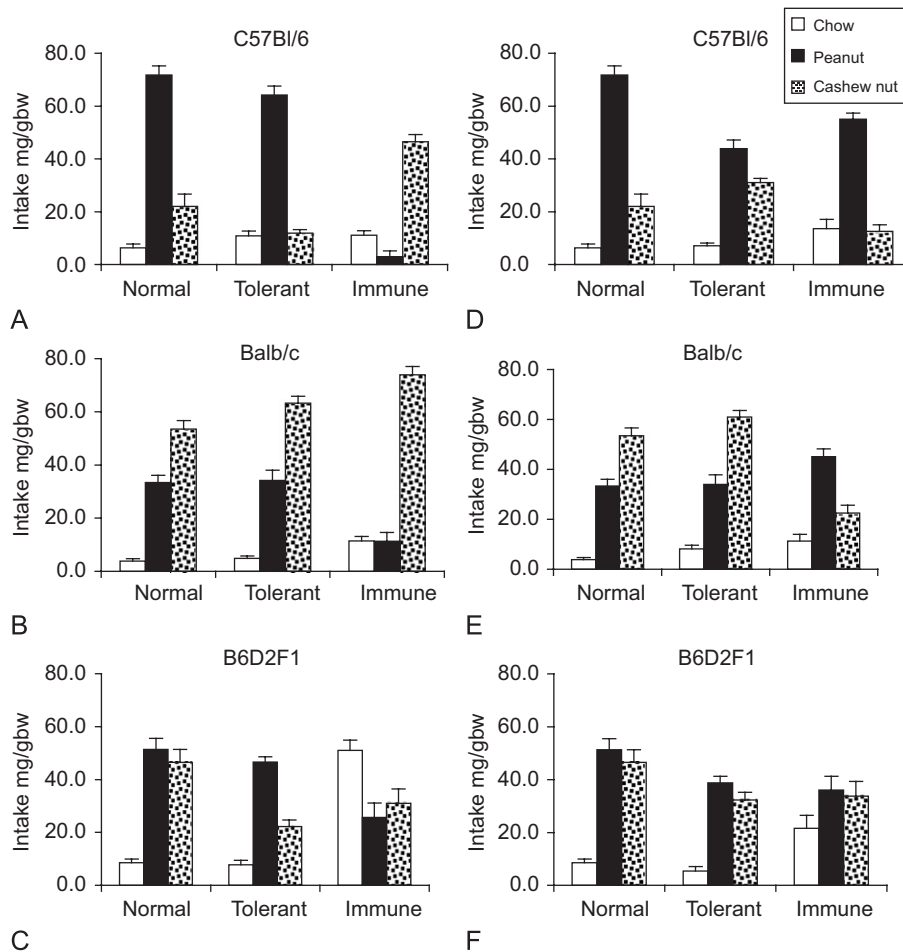


Fig. 8. Mean proportional consumption (milligram per gram of body weight) of chow, peanuts and cashew nuts in groups of five normal, tolerant and immune adult (2–3 months) male C57Bl/6, BALB/c and B6D2F1 mice exposed simultaneously to chow, peanuts and cashew nuts over a period of 10 days. Left panels (A, B, C) animals tolerant and immune to peanuts. Right panels (D, E, F) animals tolerant and immune to cashew nuts (representative of two experiments).

differ from normal mice. On the other hand, animals of the immune group present vascular congestion, enlargement of Peyer patches (that become easily visible) and frail intestinal tissue. Preliminary results of the microscopic architecture shows the same bias, where normal and tolerant animals do not differ significantly while immune animals present significant alterations of the mucosal structures, such as alterations in the length and width of the villi, inversion of the villi/crypt height ratio, increase in the number of intraepithelial inflammatory cells, edema and vascular congestion.

Discussion

Davis (1928) and Richter (1938) showed that children and rats, respectively, when offered an array of different foods, compose their diet in such a way as to satisfy their

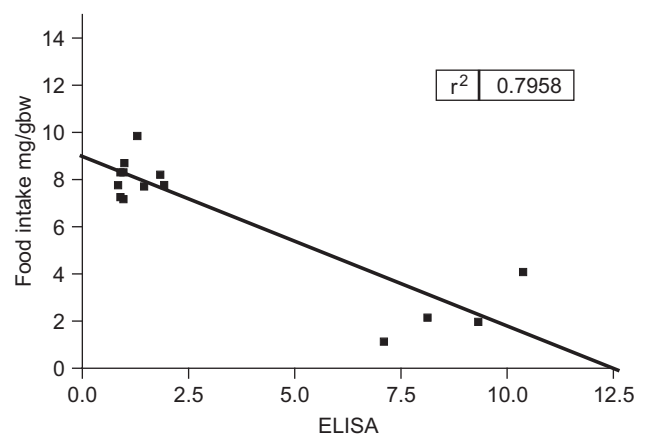


Fig. 9. Correlation between mean average food intake and antibody titers of 15 adult (2–3 months) male C57Bl/6 mice immune or tolerant to peanuts exposed to the seed for 10 days (representative of two experiments).

nutritional necessities. These papers led, to the idea that diet selection was a problem which individuals resolved instinctively. However, more recent reviews have suggested this is not a universal finding especially for mammals and in particular during weaning (Hall and Swithers-Mulvey, 1992).

Diet preferences are determined both by biological and social factors. Humans and animals eat what the body asks for – a physiological act. One also eats what is learned to be tasty, what one sees peers eating, and what brings pleasure (Yeomans et al., 1999). On the other hand, the association of a determined taste with an undesirable situation may cause aversion (Garcia et al., 1974). One also stops eating, for social and psychological reasons. Thus, eating is also a cultural act (Rappoport et al., 1993). So, could diet selection also be influenced by immunological factors? This important question has not been, to our knowledge, systematically studied.

Before we move forwards to our discussion, an important distinction in terminology between allergy, intolerance and aversion must be made. At the consensus meeting held in 1991 where clinical manifestations, mechanisms, diagnosis and treatments about food hypersensitivity were discussed, an agreement in the terminology defining alteration to food reaction was reached: food allergy is defined as immunologically mediated reaction, food intolerance as a non-immunological reaction and food aversion as a psychological reaction (Fig. 10) (de Monchy, 1991).

The gut is exposed to an enormous quantity of environmental antigens derived from various sources, such as food, intestinal flora and pathogenic micro-organisms. Walker and Isselbacher (1974) suggested that a requirement for the development of allergic diseases is the increased or inappropriate absorption of antigenic materials, which may be caused by various inflammatory pathological situations, including events such as gastroenteritis, surgery, malnutrition and ironically the use of anti-inflammatory drugs (Bienenstock, 1992). The true importance of the increased uptake of antigenic macromolecules is still not clear as in some cases it leads to allergic states and in others may suppress ongoing allergic reaction (Miller et al., 1991).

Two important factors determined our choice in using peanuts and cashew nuts. The first is that mouse chow does not contain them and the second is that peanut is considered as the food most frequently causing food allergy in human beings (Burks et al., 1994). On the other hand, we have not found many papers on food allergy (specifically on cashew nuts) in the last 10 years. In our first trials we also used Brazil nuts and raw soya beans but these seeds were not well accepted by the mice, maybe due to the large amount of oil in the first and the toxic effects of the second when it was ingested raw (data not shown).

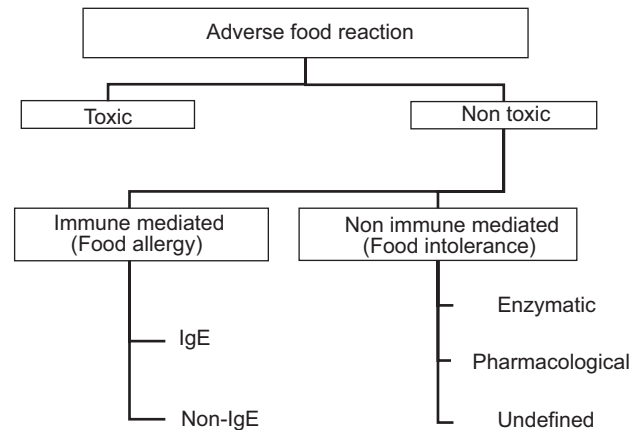


Fig. 10. Terminology defining alteration to food reaction: food allergy is defined as immunologically mediated reaction, food intolerance as a non-immunological reaction and food aversion as a psychological reaction.

Once shown that mice would eat seeds in sufficient quantities *in natura*, we showed that when peanuts or cashew nuts were offered together with the mouse chow, the animals prefer the seeds and consume them in larger quantities than chow. When the seeds are introduced in the diet a significant reduction ($p < 0.001$) of total food volume, without a significant change in calorie intake occurs. The seeds utilized have a higher lipid content (48.75 g% for peanuts and 37.00 g% for cashew nuts) than mouse chow (3.00 g%). This represents an intake of 5.79, 5.56 kcal/g of the seeds compared to 2.82 kcal/g of the mouse chow (Table 1) (Franco, 1982). Although the regulation of satiety is complex with the existence of multiple control systems, it is interesting to notice that nutrients in the small intestine such as hydrolysis products of fat stimulate the release of satiety peptides such as GLP-1 or PYY that serve as satiety signals (Beglinger and Degen, 2004, 2006).

We have shown that the behavior of the animal is different depending on which age it is exposed, for the first time, to the nuts. Having to choose between seeds or chow, all mice we studied had a clear preference to eating the nuts although they always ate some of the mouse chow, most probably due to the exploratory nature of young adults.

Given the choice of eating chow and peanuts as of weaning, these animals ate, of their total intake, approximately 95% seeds. When peanuts were reintroduced after a 4-week period of commercial chow, these animals ate more of the seed than those exposed to peanuts for the first time ($p < 0.001$) (data not shown). Animals that are weaned on peanuts and chow for a week and later are maintained on chow tend to eat more than those that are weaned exclusively on chow. It is possible that these animals weaned to energetically richer diets get used to eating more calories per day and

tend to eat more when exposed to calorically poorer diets. At least two separate learning systems, an appetitive (sensitive to the oral hedonic properties) and a consummatory response system (sensitive to nutrient properties) are involved in feeding behavior. Adult and weanling animals differ due to a set of important developmental changes in the neurobehavioral mechanisms such as learning competencies, prior experience with food, and ontogenetic constraints on behavior leading to differentiated flavor preference learning (Myers and Hall, 2000). To test this hypothesis, we increased the caloric value of mouse chow with corn oil. We observed, as for the seeds, a decrease in the intake of oil-added chow, but higher caloric intake per day. These animals gained more weight than control mice and once the enriched chow was removed, they tended to eat more of the normal chow when compared to the control ones (data not shown).

When a mixture of chow and two seeds were offered, different patterns of preferences are apparent. C57BL/6J and C57BL/10J mice prefer peanuts to cashew nuts, while Balb/c, DBA/2, CBA and A/J mice prefer cashew nuts to peanuts. B6D2F1, CB6F1, B6CF1 mice eat equal amounts of both seeds. In all tests involving seeds, the amount of chow eaten was relatively small. Older animals (6 months) eat in a more regular pattern compared to the younger ones (2 months). Younger mice varied more in their daily ingestion of each component of the diet. They seem to be more curious and to explore all components of the diet, although they maintain the pattern of the strain.

Powis and Geraghty (1995) asked “What is MHC?” puzzled by the many aspects of this gene complex. The original definition, as the region encoding cell-surface proteins primarily responsible for allograft rejection, is now unsatisfactory to accommodate non-classical MHC genes, peptide-presenting genes, olfactory-receptor genes and a host of other genes of unknown functions. The MHC is probably one of the most complex regions of the vertebrate genome. MHC products are associated with the olfaction involved in kin recognition, and may be related to the avoidance of inbreeding. It has been suggested that some olfactory receptor genes are located within the MHC class III region (Ziegler et al., 2000, 2002). May MHC heterozygosity be involved in immunological aspects of food preference? Our results indicate that inbred strains of mice show distinct patterns of food preference when two different nuts are introduced in their diet and their offspring show an intermediate pattern.

Balb/c and DBA/2, which prefer cashew nuts, share the same MHC haplotype (H-2^d), as do C57BL/6J and C57BL/10J mice (H-2^b), which prefer peanuts. The intermediate food selection behavior shown by the F1 mice generated from parents with distinct food preferences and MHC haplotypes shows a possible correlation

between these characteristics. Diet selection is a complex phenomenon and cannot be attributed to one or a few genes. One could speculate that the mothers' preference could influence their offspring but in our model we found no differences between the different F1 mice generated from normal mothers' bearing H-2^b or H-2^d haplotypes (Muller et al., 1994; Van Meerwijk et al., 1998).

The establishment of the fact that these animals eat the chosen seeds in quantities big enough to be measured daily and the fact that the different strains show distinct food preferences makes this a good model to study immunological oral tolerance and systemic immunization. The immunological status of the animals, dose, frequency, intervals between antigen exposure, age and probably other elements determine whether tolerance or immunization will result from antigen ingestion (Faria, 1989; Peng et al., 1989). The underlying immunoregulatory mechanisms of one or the other are still poorly understood and controversial. Although oral tolerance is usually defined as a state of systemic hyporesponsiveness it does not mean that it is necessarily a subtractive phenomenon (Bruce and Ferguson, 1986; Hanson et al., 1979). Furthermore, antigen-reactive T cells are not depleted following the development of oral tolerance to soluble antigen such as ovalbumin (Hoynes et al., 1995; Strobel and Mowat, 1998).

Although there are several methods to induce systemic immunological responses to food proteins such as the use of cholera toxin associated to the antigen and given through the oral route, we preferred the classical method-parenteral injections of protein extracts of peanuts or cashew nuts in normal mice. This procedure proved to be immunogenic for both proteins (Fig. 5). This is the routine procedure in our lab as we do not want any possible local residual reaction of cholera toxin upon challenge diet. Western blots analyzing peanut protein extracts demonstrate a variety of bands that react with the serum of immune mice and are comparable to data in the literature (Palmer and Burks, 2006). A similar variety of bands was observed in Western blots of cashew nut protein extract, as shown in our previous work (Paschoal et al., 2005). The prior ingestion of the seeds, on the other hand, resulted in a small increase in specific serum antibodies and, furthermore, resulted in oral tolerance (Figs. 6 and 7).

As was shown by Titus et al., it is possible to find precursors of anti-Ova secreting B cells in levels identical to those found in immunized animals a few days after the induction of oral tolerance to Ova (Carelli et al., 1978; Titus and Chiller, 1981). However, these cells do not expand and differentiate into antibody secreting cells. The injection of polyclonal B-cell mitogens, such as lipopolysaccharide (LPS) into tolerant mice results in the appearance of specific antibodies in the circulation in

amounts similar to those observed in immune mice. This situation is, therefore, similar to the physiological existence of auto-reactive lymphocytes, now broadly recognized (Coutinho et al., 1990, 1992). The vast majority of “natural immunoglobulin” is auto-reactive, but their concentrations are rigidly controlled and follow stable patterns throughout the life of the animal. Recent evidence suggests that a similar stability may be observed in specific antibody formation in oral tolerant mice (Verdolin et al., 2001).

Mice immunized with the peanut or cashew nut proteins and then exposed to the seed in their diet, will eat far less of it than normal or tolerant animals. Animals immune to one of the seeds and exposed to both of them simultaneously will reduce the ingestion of the seed to which it was immunized and will consume more of the other seed at a compensatory rate. During these experiments, there were no significant alterations in the consumption of chow, which was ingested in very small quantities. The patterns of preference shown by normal mice of the different strains are intensified when the animals are immunized with the seed to which they show less preference. On the other hand, when animals are immunized with the protein extract of the seed, which they prefer, an inversion of the pattern of the preference occurred.

There was a strong negative correlation between serum antibody titers to the protein extracts and patterns of preference; thus, animals with higher levels of circulating antibodies to peanut or cashew nut extract, consumed lower quantities of the respective seed. Mice avoid eating proteins to which they have been immunized, and in fact there are publications that have shown a negative correlation between specific antibody titers and preference (Cara et al., 1994). However, introduction of the seed in the diet prior to systemic immunization blocks the development of the specific aversion (Fig. 6). It was interesting to ask, therefore, whether immunological factors are involved in feeding behavior. Our findings show that the induction of oral tolerance results in no significant increase in the preference but differently from this, systemic immunization leads to specific aversion.

Sensitized mice re-exposed to the antigen through a challenge diet develop edema of the intestinal mucosa as well as an inflammatory profile as observed in our preliminary analysis. This can be related to an intestinal discomfort leading consequently to a reduced ingestion. Cara et al. (1994) verified that there is apparently no correlation between edema and aversion. A variety of anti-inflammatory drugs were unable to ameliorate aversion even though edema was significantly lower (Cara, 1995). During cancer chemotherapy, which causes nausea, corticosteroids exert an anti-emetic effect synergic to antagonists of serotonin receptor (Grunberg, 2007) and are able to inhibit the production of cytokines

such as IL-1 and TNF (Dinarello et al., 1982). Furthermore, corticosteroids are able to interfere with the affinity of receptors as is the case for TNF (Kull et al., 1985).

The histological preliminary findings in the present work demonstrate that immune animals present classical gut inflammation parameters, such as villous atrophy, crypt hyperplasia, prominent mononuclear leukocyte infiltrate and *lamina propria* edema in contrast to normal and tolerant mice exposed to the same seeds. These signs are typical of food-induced enteropathies such as milk allergies and celiac disease. In this model Teixeira demonstrated that mice with inflamed gut present a mild weight loss but no visible diarrhea (Teixeira, 2003). We suggest that more than edema, the global alterations of the gut are responsible for the observed aversion.

In conclusion, all mice accept peanuts and cashew nuts as foodstuff, substituting a major part of their diet with the offered seed. When two seeds are offered patterns of food selection can be observed for each strain. Protein extracts of both peanuts and cashew nuts are immunogenic with no significant difference in the titers each of them elicits. On the other hand, the ingestion of one of the seeds prior to immunization prevents sensitization, i.e., induces oral tolerance which is antigen specific. In other words, in our model both peanuts and cashew nuts are equally immunogenic or tolerogenic depending exclusively on the route of the first exposure. After being sensitized to a protein extract and being re-exposed to that seed in the diet, animals show an altered pattern of preference if the antigen belongs to the seed to which that animals show a preference. However, animals tolerated to a determined seed, then immunized with its protein extract and re-exposed to the raw seed do not alter the feeding pattern of their strain. Finally, we suggest that diet selection, a multi-factorial event, is influenced by genetic factors such as the MHC and the immunological status of the animal.

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