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Stimulation by food proteins plays a critical role in the maturation of the immune system

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Abstract

The majority of contacts with foreign antigenic materials occur on the gut mucosa, and are represented by food proteins and the autochthonous microbiota. In the present study, we replaced intact dietary proteins by equivalent amounts of amino acids from weaning on and investigated its effects on the development of the immune system of mice. Adult animals reared on a balanced protein-free diet (Aa-mice) have a poorly developed gut-associated lymphoid tissue resembling suckling mice. They also display low numbers of lamina propria cells and TCRαβ intraepithelial lymphocytes, and low levels of secretory IgA. Levels of circulating IgG and IgA are also reduced in Aa-mice, whereas IgM levels are normal. *In vitro* cytokine production by cells from several lymphoid organs shows a predominant T_h2 profile with a high concentration of IL-10 and IL-4, and a low concentration of IFN-γ. These parameters also resemble the immunological patterns observed in pre-weaned mice. Thus, our data clearly show that exposure to food proteins after weaning has a physiological role in the maturation of the immune system both locally and systemically.

Introduction

Most contacts with foreign antigenic materials occur at the gut mucosa. The total area of the mucosa is 100-fold larger than that of the skin. In humans, the mucosa of the small intestine alone is estimated to be 300 m^2 (1). This large surface is constantly exposed to a large variety of antigenic materials. Approximately 30 kg of food proteins reaches the human intestine per year and 130–190 g of these proteins are absorbed daily in the gut (2). Although most dietary macromolecules are degraded by the time they reach the small intestine, both in humans and rodents, some undegraded or partially degraded proteins are absorbed to the blood (3,4). In addition, the number of bacteria colonizing the human large intestine can reach 10^{12} microorganisms/g of stool (5). Although smaller, the microbiota in the small intestine is an additional source of natural antigenic stimulation.

As a consequence of this constant and large amount of antigenic stimulation, the gut mucosa lodges the largest collection of lymphoid tissue in the body and the largest proportion of activated lymphocytes. There are 10^{12} Ig-producing cells per meter of human small intestine, and a very large number of T cells located both in the Peyer's patches and lamina propria of the gut (6,7). Most of these T cells bear activation markers ($CD45Rb^{lo}$, $CD44^{hi}$, $L\text{-}sel^{lo}$, $\alpha_4\beta_7{}^{hi}$) and secrete potent cytokines such as IFN-γ, IL-1, IL-6, IL-4, IL-13 and tumor necrosis factor-α (8). Significantly, in spite of the activated state of mucosal lymphoid cells, the most common result of antigenic exposure in the gut is oral tolerance (9–11) rather than pathological inflammatory immune responses.

The presence of a large gut-associated lymphoid tissue (GALT) has been generally attributed to stimulation by the antigens from the microbiota, because it is drastically reduced in germ-free animals. Germ-free mice bear smaller Peyer's patches, sparse lamina propria cells and half of the normal number of intraepithelial lymphocytes (IEL) (12,13). Local production of secretory IgA (sIgA), as well as serum levels of IgA and IgG, but not IgM, are also reduced in these animals (14). Moreover, changes in the bacterial colonization of the gut after weaning have been shown to influence the emergence of IgA-secreting cells in the gut lamina propria (15,16). Although

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this correlation has not been examined in detail, several reports in the literature have shown that neonates share an overall immunological pattern with germ-free animals. In neonates, the GALT as well as sIgA formation are barely developed. Neonates have relatively high levels of serum IgM, but low levels of IgG and IgA. This impairment in isotype switching associated to the immaturity of neonatal lymphoid tissues has been ascribed to inadequate antigen presentation to T cells (17–19).

Surprisingly little attention has been paid on the role played by natural sources of antigenic stimulation on the development of the immune system. Germ-free models have provided some important insights on the immunological role of physiological bacterial stimuli. However, most of the gut lymphoid tissue is not located in the large intestine, where the gut microbiota is prominent, but rather is concentrated in the proximal small intestine, where protein absorption predominantly takes place. In spite of that, the selective importance of stimulation by food proteins in the maturation and maintenance of the immune system has not been systematically addressed.

Herein, we evaluate the immunological influence of dietary proteins in the development of the immune system by analyzing mice in which dietary proteins were replaced by equivalent amounts of amino acids. C57BL/6 mice were reared from weaning on a whole-protein-deprived diet in open cage conventional conditions. These animals grow and look like normal mice, but show local and systemic abnormalities in their immune systems. The development of the GALT, and the production of sIgA and circulating IgG and IgA are impaired. IgM secretion is less affected. In addition, cytokine secretion by cells isolated from different lymphoid organs is skewed towards a T_h2 profile. All these features resemble those observed in suckling immature mice by other investigators. Thus, our data strongly indicate that food proteins play a major role in the maturation of the immune system after weaning.

Methods

Animals

C57BL/6 and BALB/c mice were bred and reared under conventional conditions in our facilities.

Diets

Mice at 3–4 weeks of age were introduced at experimental diets containing either 15% casein (Cas) or equivalent amounts of amino acids (Aa). All mice were kept on these diets from the weaning period up to 2.5 months of age (10 weeks). Diets were isocaloric and identical with respect to all other nutrients. The basic diet (Rhoster Indústria e Comércio LTDA, SP, Brazil) was according to AIN-93G (American Institute of Nutrition) and its detailed composition has been published (20). Diets, administered in solid form as pellets, and tap water were given *ad libitum*.

Cell preparations

Spleen and lymph nodes (inguinal, brachial, mesenteric and cecal) were removed, cell suspensions prepared using a tissue homogenizer, and gently centrifuged. Single-cell suspensions from bone marrow were prepared by injecting RPMI 1640 into femurs to flush out cells, followed by gentle pipeting and washing twice with medium.

Isolation of IEL

IEL were isolated by the method of Davies and Parrott (21) as previously described. Briefly, the small intestine was removed and placed in Petri dishes containing cold Ca^{2+} and Mg^{2+}-free HBSS solution with HEPES buffer and 2% FCS. Peyer's patches and connective tissues were removed before the small intestine was opened longitudinally, and washed twice to remove fecal contents. The small intestine was cut into 0.5-cm segments. These segments were transferred to conical tubes containing 20 ml cold Ca^{2+} and Mg^{2+}-free HBSS solution containing HEPES buffer, 10% FCS and 0.1 mM EDTA (Sigma, St Louis, MO), and incubated at 37°C for 30 min with gentle stirring. Tubes were then manually shaken vigorously for 15 s, and supernatant IEL and tissue fragments were separated by passage through stainless steel sieves. The tissue fragments were returned to conical tubes and the process repeated twice more. The IEL from the three incubations were pooled and washed. Cells were suspended in 24 ml 44% Percoll (Sigma) at room temperature. An 8-ml aliquot of cells in 44% Percoll was layered on top of 5 ml 67% Percoll in a 15-ml centrifuge tube (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) and the tubes centrifuged for 20 min at 600 *g* at room temperature. IEL were collected from above the 67% Percoll layer and resuspended in RPMI 1640 (Life Technologies, Grand Island, NY).

Intestinal lavage samples

For sIgA assays, intestinal contents of the small intestine were collected by carefully washing the intestinal lumen with 20 ml of cold PBS, transferred to a test tube, vigorously vortexed and centrifuged for 30 min at 850 *g* at 4°C. The resultant supernatant was transferred to a new test tube and freshly tested by ELISA for IgA concentration.

Analysis of Ig isotypes by ELISA

Levels of isotype-specific Ig in intestinal lavage samples and serum were determined by ELISA. Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with 0.1 μg/ml goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL) in coating buffer, pH 9.8. Wells were blocked with 200 μl of PBS contain 0.25% casein for 1 h at room temperature. After washing the plates 6 times serial dilutions of samples were added to wells and incubated for 1 h at 37°C. Plates were washed 6 times again and 0.5 μg/ml biotinylated goat anti-mouse μ, γ or α heavy chain-specific polyclonal antibodies (Southern Biotechnology) were added, and then incubated for 1 h at 37°C. After six washes, a detection solution containing a 1/2000 dilution of horseradish peroxidase-conjugated streptavidin (Southern Biotechnology Associates) was added and incubated for 30 min. After washing, the color reaction was developed at room temperature with 100 μl/well of orthophenylenediamine (1 mg/ml), 0.04% H_2O_2 substrate in sodium citrate buffer. The reaction was interrupted by the addition of 20 μl/well of 2 N H_2SO_2. Absorbance was measured at 450 nm by an ELISA reader

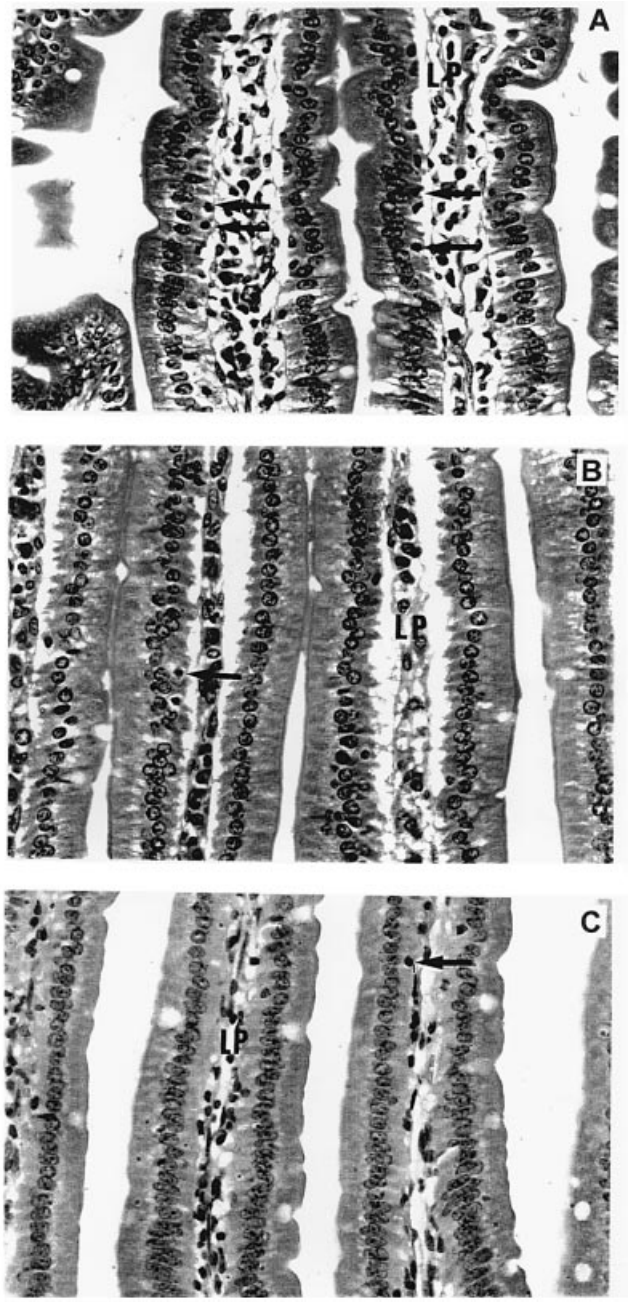

Fig. 1. Histological analyses of small intestinal mucosa. Visualization of morphology of lamina propria cells and IEL (arrows) of the villi of (A) C57BL/6 Cas-mice at 12 weeks of age, (B) C57BL/6 Aa-mice at 12 weeks of age and (C) C57BL/6 mice at weaning (4 weeks of age) presented at ×400 magnification.

(Bio-Rad, Hercules, CA) and Ig concentrations were determined using a standard curve.

Enumeration of Ig-producing cells by ELISPOT

To determine the number of IgM-, IgA- and IgG-producing cells in spleen and bone marrow, the ELISPOT assay was used. Briefly, 96-well plates (Nunc) were coated with 50 μl of 2 μg/ml of goat anti-mouse Ig (Southern Biotechnology Associates) in coating buffer, pH 9.8. Plates were blocked with 200 μl PBS containing 0.25% casein for 1 h at room temperature. Cells were resuspended in complete medium, added at varying concentrations and cultured at 37°C in 5% CO_2 for 4 h. After incubation, plates were washed with water containing 0.05% Tween 20 and then with PBS containing 0.05% Tween 20. For the detection of Ig-producing cells, 1 μg/ml of biotinylated goat anti-mouse μ, γ or α heavy chain-specific polyclonal antibodies (Southern Biotechnology Associates) was added and then a solution containing 1/2000 diluted horseradish peroxidase-conjugated streptavidin (Southern Biotechnology Associates). After overnight incubation at 4°C, the spots were developed with 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in AMP solution.

Analysis of cell subsets by flow cytometry

Cells were incubated with a combination of FITC-conjugated, phycoerythrin (PE)-conjugated, biotin-conjugated or purified antibodies in the presence of 10% heat-inactivated normal rat serum at 4°C for 30 min. Antibodies used for labeling were as follows: purified rat anti-mouse mAb 53-7.3 (anti-CD5) and G7 (anti-CD90, Thy-1); biotin-labeled hamster anti-mouse mAb H57-597 (anti-TCRβ chain) and GL3 (anti-TCRγδ); FITC-labeled rat anti-mouse mAb 145-2C11 (anti-CD3ε); PE-labeled hamster anti-mouse mAb 145-2C11 (anti-CD3ε); and FITC-labeled polyclonal goat anti-rat Ig specific polyclonal antibody (12114D) were purchased from PharMingen (San Diego, CA). PE-labeled rat anti-mouse mAb CT-CD4 (anti-CD4) and biotin-labeled rat anti-mouse mAb CT CD8α (anti-CD8) were purchased from Caltag (Burlingame, CA). Cell suspensions were washed twice with 1% BSA/PBS and then incubated for 15 min with either PE–streptavidin (PharMingen) to reveal the biotin reagent or anti-rat FITC-conjugated antibody for the purified antibodies and, finally, washed twice with 1% BSA/PBS. Stained cells were then applied to a FACScan (Becton Dickinson, Mountain View, CA). Lymphocytes were gated by light scatter and were analyzed. All data were analyzed using CellQuest software.

Histomorphometric analysis

Histological sections of small intestine were obtained from control, Cas- and Aa-mice. Intestinal morphometric analysis was performed by grouping regions designated as duodenum and proximal jejunum as the proximal region and distal jejunum, and ileum as the distal region of small intestine. Tissues were fixed in 10% PBS-buffered formalin, embedded in paraffin, and 3-μm thick sections were obtained, stained with hematoxylin & eosin and examined under a light microscope.

Ratios of lamina propria cells/villus and IEL/100 epithelial cells were calculated by manually counting cells in 10 villi.

Cytokine assays

Cells isolated from spleen (erythrocyte depleted), Peyer's patches, and peripheral, cecal and mesenteric lymph nodes were used for cytokine secretion assays. Cultures contained pooled cells from mice of each group. For cytokine secretion, 1×10^7 cells/ml (in 250 μl) were cultured in 96-well plates (Nunc, Naperville, IL) in complete RPMI with or without 2 μg/ml Concanavalin A (Con A). Supernatants were collected after

24, 48 and 72 h for a kinetic study of the secreted cytokines. Maximum cytokine levels were observed after 24 h for IL-2, and after 72 h for IFN-γ, IL-4 and IL-10. Therefore, these were the time points used to compare cytokine production by the different groups of mice.

Levels of IL-2, IL-4, IL-10 and IFN-γ in supernatants were determined by capture ELISA as described previously (22). Briefly, supernatants were added to microtiter plates (Nunc), previously coated with rat anti-mouse IL-2, IL-4, IL-10 or IFN-γ mAb (PharMingen, San Diego, CA) at 1–5 μg/ml and blocked with BSA-diluent/blocking solution. Standards and samples were added and incubated overnight at 4°C. Biotinylated rat anti-mouse IL-2, IL-4, IL-10 or IFN-γ mAb (PharMingen) were added and followed by peroxidase-labeled streptavidin (Sigma). Bound cytokine was detected by the addition of H_2O_2 and *o*-phenylenediamine, and by reading color development at 495 nm after addition of H_2SO_4 2 N. Cytokine levels were calculated from a standard curve obtained with recombinant cytokines (PharMingen) and results expressed in pg/ml or ng/ml. Threshold sensitivities of ELISA assays were 80, 320, 2 and 80 pg/ml for IL-2, IFN-γ, IL-4 and IL-10 respectively.

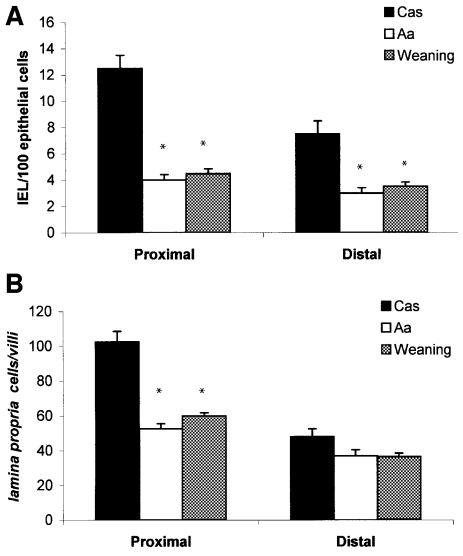

Fig. 2. Number of IEL (A) and lamina propria cells (B) in the proximal and distal regions of the small intestine of C57BL/6 Cas-mice (solid bar), Aa-mice (open bar) and C57BL/6 mice at weaning (gray bar). Cell counts were performed using an optical microscope. *$P < 0.05$ (two-tailed Student's *t*-test)

Statistical analysis

Results were expressed as the mean ± SEM and significance between groups was determined by Student's *t*-test.

Results

The presence of dietary proteins is important for the development of the GALT after weaning

Conventional mice were reared either in a balanced experimental diet containing casein as the sole protein source (Cas) or in a diet in which casein was replaced by equivalent amounts of amino acids (Aa) until they were adults. Examined when 10-week-old, Aa-mice presented normal numbers of Peyer's patches, although they were smaller than in Cas-mice (data not shown). As compared to Cas-mice, the small intestine of Aa-mice showed a significant decrease in the number of lamina propria cells and IEL (Figs 1 and 2). These morphological aspects are similar to those observed in the intestine of 4-week-old C57BL/6 mice analyzed just before weaning (Figs 1C and 2). Interestingly, proximal regions of the small intestine (duodenum and proximal jejunum), more involved in protein absorption, were more affected. Both in weaning mice and in young adult Aa-mice, the number of lamina propria cells and intraepithelial cells were underdeveloped, and the villi lost the typical pleated appearance observed in adult Cas-mice (Fig. 1). This close resemblance between Aa-mice and weaning animals suggests that the post-weaning exposure to dietary protein, regardless of the presence of the gut microbiota, may be critical for the development of the normal lymphoid tissue in the gut.

Exposure to food proteins is selectively important for the increase in TCRαβ⁺ intraepithelial lymphocytes

To ascertain which subpopulations of IEL were reduced, isolated IEL were analyzed for the expression of different phenotype markers: TCRαβ, TCRγδ, Thy-1 and CD5. As presented in Table 1, we observed a decrease in the proportion of the TCR αβ⁺ IEL and a parallel increase in the proportion of TCRγδ⁺ IEL subsets in Aa-mice as compared to Cas-mice. The frequency of TCRαβ⁺ cells expressing Thy-1 was also significantly reduced in Aa-mice. The proportion of TCR γδ⁺Thy-1⁺ in Aa-mice was higher than in the control group; CD5⁻ and CD5⁺ TCRαβ⁺ IEL were also reduced in Aa-mice. Of note, significance is higher for the difference in CD5⁺ IEL between groups. On the other hand, the percentages of TCRαβ⁺ and TCRγδ⁺ lymphocytes that do not express Thy-1 were similar in Cas- and Aa-mice. These results suggest that

Table 1. Phenotypic analyses of gut IEL

	TCRαβ	TCRγδ	αβ Thy-1⁺	αβ Thy-1⁻	γδ Thy-1⁺	γδ Thy-1⁻	αβ CD5⁺	αβ CD5⁻
Cas	41.3±3.5	35.7±2.9	26.3±1.5	12.9 ±2.4	10.6±1.8	25.9±1.8	23.7±1.2	16.3±2.8
Aa	22.8±3.2[a]	45.2±5.1[a]	9.9±3.3[a]	11.4±2.8	18.4±1.7[a]	26.8±6.1	10.0±2.4[a]	11.0±2.9[b]

IEL isolated from small intestine were stained with labeled specific antibodies and analyzed by two-color flow cytometry. At least 20,000 events were analyzed each time. Results represent the percentage (mean ± SEM) of a group ($n = 5$).

[a]$P < 0.001$ and [b]$P < 0.03$ (two-tailed Student's *t*-test).

antigenic stimulation in the gut provided by food proteins selectively affects the development of TCR$\alpha\beta^+$ Thy1$^+$ IEL.

Stimulation by food proteins plays a critical role in the production of intestinal sIgA

Our results show that exposure to dietary proteins influences the development of intestinal lamina propria cells. Intestinal sIgA derives from plasma cells housed at this site and our next step was to investigate the role of food proteins in sIgA production. Intestinal lavage samples were collected from Cas- and Aa-mice, and IgA levels were measured by isotype-specific ELISA. As shown in Fig. 3, IgA levels were significantly reduced in Aa-mice as compared to Cas-mice, suggesting that the activity of lamina propria cells was also underdeveloped in the former.

Stimulation by food proteins influences the production of circulating Ig

Total Ig, IgG, IgM and IgA levels were assayed in Cas- and Aa-mice. As shown in Fig. 4, Aa-mice display a drastic decrease in total serum Ig levels mostly due to the virtual absence of serum IgA and a great reduction in IgG production. Ig-secreting cells in the spleen (Fig. 5A) and bone marrow (Fig. 5B) were also enumerated by ELISPOT. In both organs, there was a significant reduction in the total numbers of Ig-producing cells as well as in the numbers of IgG- and IgA-secreting cells (Fig. 5A and B). This was consistent with the results obtained in the serum. In the bone marrow, an important site of IgA production, the number of IgA-producing cells was significantly reduced in Aa-mice as compared to Cas-mice. In spite of the reduction in total Ig-forming cells, the number of IgM-secreting cells in spleen and bone marrow was higher in Aa-mice than Cas-mice. Thus, ELISPOT results show that the majority of Ig-producing cells in Aa-mice were IgM-secreting cells, whereas only a small proportion secreted either IgG or IgA (Fig. 5A and B).

Exposure to food proteins is critical in the development of a mature profile of cytokine production

Cells were isolated from several lymphoid organs of Cas- and Aa-mice, including Peyer's patches, mesenteric and cecal lymph nodes, peripheral (inguinal and brachial) lymph nodes, and the spleen. When stimulated in culture with Con A (Fig. 6 and 7), cells from Aa-mice of both strains (C57BL/6 and BALB/c) produce higher levels of IL-2 than cells from control Cas-mice. On the other hand, IFN-γ production is lower in the majority of organs from Aa-mice as compared to Cas-fed mice (Fig. 6). Significantly higher levels of IL-4 and IL-10 were observed in all cell cultures from both strains of Aa-mice (Fig. 7), suggesting that Aa-mice present a general atypical T_h2 profile. Moreover, differences in cytokine production between Cas- and Aa-mice were similar regardless of the mouse strain tested.

Discussion

There are several reports in the literature on dramatic immunological changes in germ-free mice, intensified in antigen-free mice, which suggest that natural stimulation provided by the intestinal microbiota and dietary proteins influence the immune system. Data from germ-free animals clearly show that the microbiota, by itself, is important for the development of the immune system (13). Immunological alterations observed in antigen-free mice, which are deprived of both microbial and dietary stimulation, are more severe (23–26), showing that dietary proteins also participate in immunological maturation. However, antigen-free mice are difficult to maintain and do not allow a discrimination between the relative importance of bacterial versus food antigens. Surprisingly, to our knowledge, no systematic analysis was made of the immunological effects of food protein deprivation in animals maintained with nutritionally balanced diets and harboring an autochthonous microbiota.

Our model was designed to evaluate the immunological relevance of dietary proteins by replacing them by amino acids. It is important to notice that neither Cas- nor Aa-mice displayed nutritional or behavioral disorders. They had a healthy appearance and normal activity throughout the

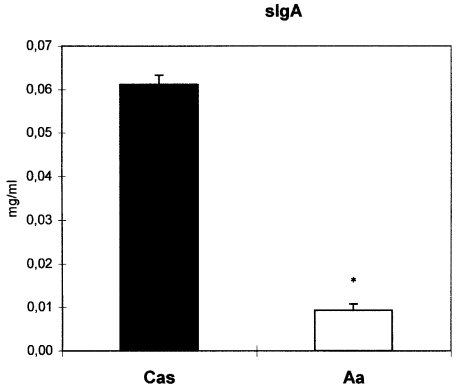

Fig. 3. Concentrations of intestinal sIgA in C57BL/6 Cas-mice (solid bar) and Aa-mice (open bar). IgA levels were determined by quantitative ELISA. Bars represent values (mean ± SEM) of a group (n = 5). *P < 0.05 (two-tailed Student's t-test).

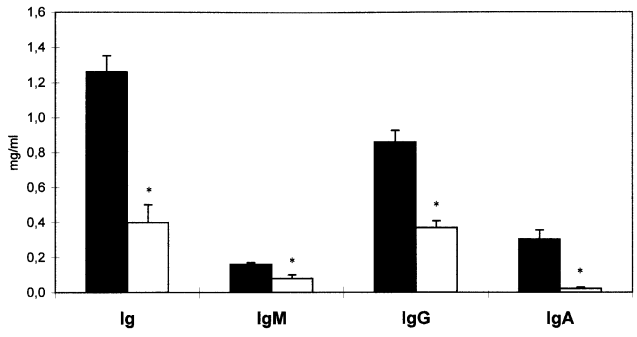

Fig. 4. Serum concentrations of Ig, IgM, IgG and IgA in C57BL/6 Cas-mice (solid bar) and Aa-mice (open bar). Ig levels were determined by quantitative ELISA. Bars represent values (mean ± SEM) of a group (n = 5). *P < 0.05 (two-tailed Student's t-test).

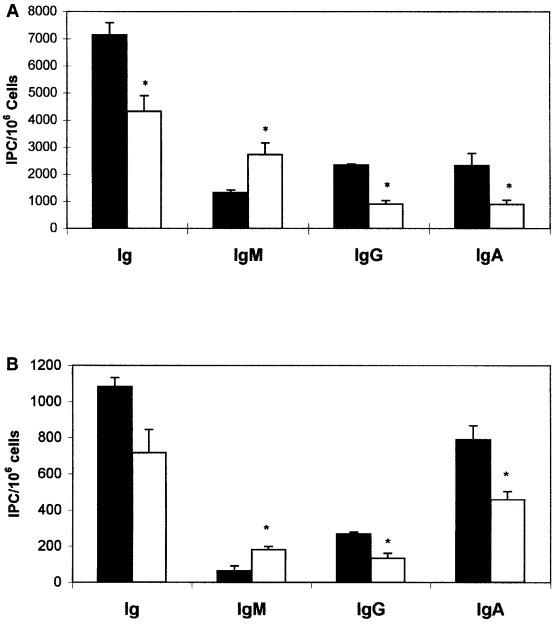

Fig. 5. Number of Ig-producing cells (IPC) in spleen (A) and bone marrow (B) of C57BL/6 Cas-mice (solid bar) and Aa-mice (open bar). IgM-, IgG- and IgA-producing cells were measured by ELISPOT assay. Bars represent values (mean ± SEM) of a group (n = 5). *P < 0.05 (two-tailed Student's t-test).

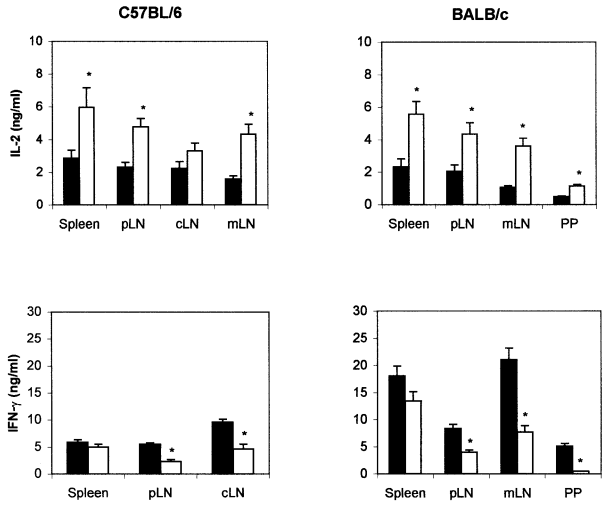

Fig. 6. Production of IL-2 and IFN-γ by cells from different organs of C57BL/6 and BALB/c mice. Cells were isolated from spleen, Peyer's patches (PP), and peripheral (pLN), cecal (cLN) and mesenteric (mLN) lymph nodes of Cas- and Aa-mice, and stimulated *in vitro* with Con A (2 μg/ml). Cell culture supernatants were harvested after 24 (for IL-2) and 72 h (for IFN-γ), and analyzed by quantitative ELISA. Bars represent values (mean ± SEM) of a group (n = 5). *P < 0.05 (two-tailed Student's t-test).

experimental period, had a normal growth curve, normal blood cell counts, and serum albumin and total serum protein concentration were within the normal range (data not shown).

As shown by our results, however, deprivation of dietary protein resulted in severe immunological changes. Aa-mice displayed a drastic underdevelopment of the GALT, a major reduction in the number of lamina propria cells and IEL, as compared to Cas-diet fed mice. They also presented a reduction in the size of Peyer's patches and their germinal centers as compared to Cas-mice (data not shown). It is known that neonatal animals present a barely developed GALT. In humans and mice, the period after weaning is associated with an increase in lamina propria cells and IEL, and the development of germinal centers in Peyer's patches and sIgA production concomitantly with rapid changes in the morphology of the intestine (27,28). Indeed, the morphological aspect of the intestinal mucosa at weaning resembles very closely that of 10-week-old Aa-mice, i.e. the villi are not as pleated as in Cas-mice, and there are few IEL and lamina propria cells. There are also low numbers of goblet cells both in suckling as well as in Aa-mice (data not shown). The immunological activity in the gut of neonatal mice is low. SIgA in the mucous appears at 3–4 weeks of age and adult levels of sIgA are reached only in 6-week-old mice. Normal levels of sIgA production are reached at weaning coincidentally with changes in bacterial colonization changes in the intestine (16). Thus, gut microbiota seem to exert an important effect since low levels of sIgA are observed in adult germ-free mice (16,29). However, our results show that levels of sIgA in Aa-mice are similar to those reported in neonates and germ-free mice. This indicates that probably both microbiota and dietary proteins are important for the development of lymphocytes committed to IgA production in Peyer's patches and in the lamina propria (29). A gross examination of the fecal contents of mice confirmed that Aa-fed mice have an amount of fecal bacteria comparable to Cas-fed mice (data not shown). A more detailed characterization of the bacterial composition in both groups of animals is currently being performed. Since proteins are degraded and absorbed in the proximal region of the small intestine, it is very unlikely that the simple replacement of dietary casein by its amino acid components would alter the bacterial colonization of the colon in Aa-mice as compared to Cas-mice (J. Nicoli, pers. commun.). However, changes may occur as a consequence of using a well-defined diet instead of regular chow. The presence of a microbiota in Aa-mice may explain why they have some sIgA production and a few germinal centers in their Peyer's patches (data not shown) as compared to neonates that have none of both (30). Similarly, chow-fed germ-free mice are not devoid of sIgA in the mucous (16,29). On the other hand, the decrease in lamina propria cells, in the overall size of Peyer's patches and in their germinal centers in Aa-mice is compatible with the minute amounts of sIgA that these animals produce as compared to Cas-mice.

Experiments comparing chow-, casein-diet- and Aa-diet-fed mice were performed, and we observed an increase in sIgA and serum Ig levels in chow-fed mice as compared to Cas-mice (data not shown). The presence of a high concentration of a protein mixture (>25%), lipopolysaccharide and non-defined amounts of other components in the regular mouse chow may explain these differences. The casein-containing diet used in our experiments is a balanced diet commercially available for mouse use and its choice lies

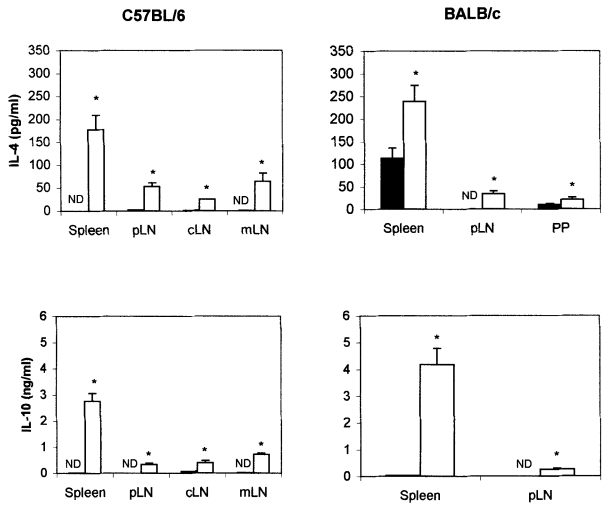

Fig. 7. Production of IL-4 and IL-10 by cells from different organs of C57BL/6 and BALB/c mice. Cells were isolated from spleen, Peyer's patches (PP), and peripheral (pLN), cecal (cLN) and mesenteric (mLN) lymph nodes of Cas- and Aa-mice, and stimulated *in vitro* with Con A (2 μg/ml). Cell culture supernatants were harvested after 72 h and analyzed by ELISA. Bars represent values (mean ± SEM) of a group (n = 5). ND = non-detectable values. *P < 0.05 (two-tailed Student's t-test).

exactly in the well-defined aspect of its components. This allows for the study of the exclusive effect of intact dietary protein in the immune' system. The effect of other components of the diet that may act as non-conventional antigens seem also to be relevant, but it was not within the scope of this study.

Phenotypic characterization of IEL subpopulations also show similarities between Aa-mice, and both germ-free and neonate mice. TCRαβ$^+$ T cells are selectively reduced in adult Aa-mice; TCRγδ$^+$ IEL cells are proportionally increased. Germ-free mice bear low numbers of TCRαβ$^+$ IEL, although they present normal numbers of TCRγδ$^+$ IEL (12). Expansion of IEL in humans begins at birth, but affects predominantly TCRαβ$^+$ cells, which increase 10-fold until adulthood. TCRγδ$^+$ IEL grow only 2–3 times after birth, suggesting that they are less affected by external stimulation (28). Our results show that introduction of food proteins at weaning is a potent stimulation for either recruitment or expansion of the TCRαβ$^+$ IEL subpopulation. Since TCRγδ$^+$ IEL do not bind conventional antigens, they seem not to be influenced by dietary changes at weaning.

There was also no alteration in the number of Thy-1$^-$ IEL in Aa-mice regardless of their TCR lineage. Thus, the Thy-1$^-$ IEL population does not seem to require antigenic stimulation either, as testified by its presence in germ-free and suckling mice (31,32). On the other hand, our experiments show that Thy-1$^+$ IEL subsets were significantly reduced in Aa-mice. Likewise, CD5$^-$ IEL were barely altered, whereas CD5$^+$ IEL were reduced by half. CD5 expression is up-regulated in conventional environments as opposed to specific pathogen-free conditions where it is down-modulated (33). In agreement with these previous reports, our results suggest that the observed low level of expression of both Thy-1 and CD5 in IEL is probably related to a reduction in activation of T cells in animals with reduced antigen load in the gut.

Thus, analysis of gut mucosa and its associated lymphoid tissue in Aa-mice clearly shows characteristics of immaturity that resemble those found in both germ-free and suckling animals.

Remarkably, dietary protein deprivation altered not only the GALT and mucosal immunological activity, but there were also systemic modifications. Serum levels of IgA and IgG levels are lower in Aa-mice. The total number of Ig-producing cells enumerated in spleen and bone marrow of Aa-mice was also significantly lower than that in control animals. The inhibited isotypes were IgG and IgA, but not IgM, suggesting an impairment in isotype switching in these animals. Our results show again a parallel between Aa-fed mice, and both neonates and germ-free mice. Neonates are known to be deficient in IgA, IgG and IgE production. Human cord blood B cells show little propensity to undergo isotype switching (34). After immunization, neonates usually produce polyreactive, low-affinity IgM, but not other isotypes (35). Studies on specific pathogen-free, germ-free and antigen-free mice also show a correlation between antigenic deprivation, and a reduction in the levels of IgG, IgA (14,25) and also IgE (36). In contrast, serum IgM concentrations and the numbers of IgM-producing cells in these animals are unaffected by the withdrawal of external stimulation (14,26). These animals also display a remarkable stability in the profile of reactivity of their serum IgM (24), suggesting the presence of an immunological activity which is independent of external antigens. Our results are in agreement with these reports, yet they add new information on the effects of external stimulation in IgM production. The number of IgM-secreting cells in the spleens of Aa-mice was actually significantly higher than in Cas-mice. However, levels of serum IgM in Aa-mice do not correlate with this increase. On the contrary, they were unaffected if not lower in Aa-mice. A likely explanation for this discrepancy is the relative undifferentiated state of B cells from Aa-mice. Stimulation by food proteins may be crucial not only for a basal physiological level of isotype switching, but also for driving IgM$^+$ B cells into plasma cell formation. In the absence of food protein stimulation, IgM-producing lymphoblasts may predominate over the more productive plasma cells. In this scenario, IgM$^+$ B cells secreting low levels of IgM would represent the major population in the spleen, as we observed in our results.

Production of high-affinity antibodies of IgG and IgA subclasses is clearly associated to antigenic experience as demonstrated by their low levels in neonates, germ-free mice and in our model. Appearance of these isotypes is highly dependent on germinal center formation, cytokine secretion by activated T cells and CD40–CD40 ligand interaction (18,37). Several authors have already shown that the impairment in isotype switching in neonates is related to the absence of a proper T–B cell interaction which is characteristic of an immature environment for antigen presentation (35).

In our model, cytokine secretion by lymphoid cells from Aa-mice stimulated with mitogen *in vitro* secreted less IFN-γ, and more IL-4 and IL-10, than control Cas-mice. This skewing towards a T_h2 profile occurs in different mouse strains. Lymphoid cells isolated from neonatal mice have also been

reported to produce a T_h2 profile of cytokine secretion when stimulated *in vitro* with immobilized anti-CD3 antibodies, a polyclonal stimulus. It is conceivable that the withdrawal of proteins from the diet hinders the progression of lymphoid cells towards the adult T_h1 profile and arrests the cells in a pre-weaning neonatal pattern. Many features of the neonatal environment seem to be responsible for this typical skewing in T cell activation. Neonatal mice show a reduced number of activated macrophages and dendritic cells. The major population of antigen-presenting cells in this period is represented by B cells which are known to induce a T_h2 profile of cytokines (18,19). *In vitro* addition of adult antigen-presenting cells to neonatal T lymphocytes and stimulation by immobilized anti-CD3 antibodies induce a high production of IL-2 and IFN-γ (38), suggesting that the absence of a proper set of co-stimulatory signals is a major factor driving the neonatal lymphocytes towards a T_h2 cytokine pattern of secretion. Similarly to neonatal cells, isolated cells from Aa-mice show the potential for an adult type of response, as shown by their increased levels of IL-2 production upon mitogen stimulation, yet they present an immature pattern of cytokine secretion. In concert with our data, a recent report by van den Brink *et al.* shows a preferential production of IL-4 in adult volunteers who were submitted to starvation, whereas an increase in IFN-γ production was detected shortly after meals (39).

Most of the reports on the impairment in T_h1 responses and in Ig production in neonates refers to the neonatal period as the early post-natal time in suckling animals. Our data clearly indicate that the developmental period during lactation is followed by profound immunological changes triggered by the food proteins introduced at weaning. Although a high concentration of intact proteins is provided by breast feeding, it has been reported that other milk components such as maternal hormones, cytokines and Ig play a role in maintaining a neonatal-like environment in suckling mice (40). Food protein intake after weaning seems to represent a major developmental step in the maturation of the immune system.

Consequently, our prediction is that protein malnutrition at an early age may have an unsuspected immunological impact in the infant. Several authors have previously reported states of relative immunodeficiency in malnourished mice (41–43) that are comparable to the deficit found in Aa-mice. The impairment in the immune function has been interpreted as a secondary outcome of malnutrition. However, malnourished animals usually ingest a diet containing 2–4% protein. In the light of our data, the reduction in immunological activity in these mice could be rather explained by a direct effect of the reduction of stimulation by dietary antigens. Indeed, when immunized with several antigens in different adjuvant preparations, Aa-mice presented a T_h2-biased immature immune response as compared to Cas-fed mice (data not shown).

In conclusion, we have shown for the first time that introduction of dietary proteins at weaning is important for a critical period in which both local and systemic aspects of the immune system undergo maturation. Thus, usual consequences of oral contact with antigens, such as sIgA production and oral tolerance induction, may be viewed as physiological keepers of an activation tonus for the immune system.

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Abbreviations

Con A	concanavalin A
GALT	gut-associated lymphoid tissue
IEL	intraepithelial lymphocyte
PE	phycoerythrin
sIgA	secretory IgA

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